DEPARTMENT OF SAFETY ASSESSMENT

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A CHRONIC STUDY IN THE FATHEAD MINNOW (*Pimephales promelas*) WITH TECHNICAL CHLOROTHALONIL

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REPORT

A CHRONIC STUDY IN THE FATHEAD MINNOW (*Pimephales promelas*) WITH TECHNICAL CHLOROTHALONIL

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A CHRONIC STUDY IN THE FATHEAD MINNOW (Pimephales promelas) WITH TECHNICAL CHLOROTHALONIL

ABSTRACT

Fathead minnows (Pimephales promelas) were continuously exposed in duplicate aquaria to five nominal concentrations (25, 12.5, 6.3, 3.1, and 1.5 ug/liter) of technical chlorothalonil, a diluent water control, and a solvent (acetone) control throughout a complete (egg-egg) life cycle. The mean measured test concentrations of technical chlorothalonil as sampled from the five replicate aquaria were respectively 16, 6.5, 3.0, 1.4, and 0.60 ug/liter.

No significant effects were observed in either generation at mean measured concentrations $\leq$ 3.0 ug/liter.

The first generation (F_0) eggs exhibited a significantly reduced hatchability and survival of fry after 35 days when exposed to a mean measured concentration of 16 ug/liter. The reproductive success of F_0 fish was adversely affected (reduction in the number of eggs per spawn) by exposure to concentrations $\geq$ 6.5 ug/liter.

The second generation (F_1) eggs exhibited a significantly reduced hatchability when exposed to a mean measured concentration of 6.5 ug/liter. The survival of fry at this concentration was not affected.

Based on these data, the maximum acceptable toxicant concentration (mean measured) of technical chlorothalonil in water for Fathead minnows was estimated to be in the range of 3.0 to 6.5 ug/liter.

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STUDY OBJECTIVE

To assess the effects of chronic (egg-egg) exposure of technical chlorothalonil to the Fathead minnow (Pimephales promelas).

STUDY/DOCUMENT NUMBER

Diamond Shamrock Study Number: DTX-77-0127
Diamond Shamrock Document Number: 090-5TX-79-0049-003

TEST MATERIAL

Name:	chlorothalonil (technical)
Code:	T-117-2
Lot Number:	B1211 701
Source:	Diamond Shamrock Corporation
Active Ingredient:	chlorothalonil
Active Ingredient Purity:	96%
Synonym of Active Ingredient:	Daconil, DS-2787, DAC-2787

LABORATORY

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LABORATORY PROJECT NUMBER

J45-100

LABORATORY REPORT NUMBER

BW-79-6-443

(A complete copy of the laboratory report can be found in Appendix I).

LABORATORY REPORT DATE

January, 1980

INTRODUCTION

In order to evaluate the effects of chronic exposure of chlorothalonil to the Fathead minnow, data was compiled on egg hatchability, survival, growth, and reproductive success of first generation (F_0) fish and on the egg hatchability, survival, and growth of second generation (F_1) fry.

Based on the data compiled, a maximum acceptable toxicant concentration[1] (MATC) of chlorothalonil to the Fathead minnow was estimated. The MATC lies between the highest mean measured concentration producing no effects on exposed fish and the lowest mean measured concentration where toxicant related effects were observed.

METHODS AND MATERIALS

The chronic system included duplicate aquaria for five concentrations of technical chlorothalonil, a diluent water control, and a solvent (acetone) control. Technical chlorothalonil was delivered to appropriate aquaria by means of a proportional diluter system. A syringe operated by a mechanical injector delivered a precise volume of stock solution (test material) to the diluter. From the diluter, mixtures were delivered to a flow-splitting chamber which further mixed each dilution of test material. From the flow-splitting chamber, separate dilutions of test material were delivered to duplicate aquaria through separate glass delivery tubes.

A stock solution of technical chlorothalonil was prepared every two weeks in analytical grade acetone. Samples of the stock solution were taken for analysis of chlorothalonil on the day of preparation and two weeks after preparation.

The water in each individual aquarium was sampled on a weekly basis for analysis of chlorothalonil. The results were expressed as the actual measured concentrations of test material at each level. Analysis for a metabolite (DS-3701) was done at the end of each month.

Each glass aquarium was subdivided with stainless steel screen to provide a spawning area, an area for the egg cups, and two larval growth areas. Five spawning sites were prepared for each spawning chamber from cut sections of cement-asbestos drain tile. The egg cups were made from glass jars with stainless steel screen bottoms. The water level in each aquarium was maintained at 15 cm by means of a stand pipe. The flow rate to each aquarium was maintained at approximately eight times the tank volume every 24 hours. The proper flow rate insured an adequate concentration of dissolved oxygen in each aquaria. No aeration of test aquaria or controls was utilized.

The temperature of each aquarium was maintained at 25 $\pm$ 2°C by placing them in heated water baths. During the last four months of the study, a preheated diluent water was delivered into the system in addition to the water bath heaters which insured the proper aquaria temperature.

The photoperiod followed the EPA[2] recommended schedule using the day length of December 1 (Evansville, Indiana) for the first day of the test and adjusting the day length on the first and fifteenth of each month by means of an automatic timer.

Throughout the chronic study, aquaria were syphoned clean of debris twice weekly and brushed when fungi or algae growth began to build up. The diluter cells were brushed weekly and the amount of water being delivered through the system to each aquarium was measured monthly.

The temperature and dissolved oxygen levels were measured in each aquarium on a rotating basis so that each tank was analyzed at least once a week. Total hardness, alkalinity, pH, acidity, and specific conductivity were measured weekly in one control aquarium and one treatment aquarium on a rotating basis. The diluent water was characterized on test days 27 and 171 of the study for calcium, magnesium, sodium, potassium, chloride, sulfate, total solids, and total dissolved solids.

The diluent water used in the study was bedrock well water supplied at Bionomics and supplemented with well water supplied by the Town of Wareham, Massachusetts. Between test days 269-278, water was not drawn from the well at Bionomics due to reclamation operations. During this time, all water came from the town supply. Due to the softness of the water, it was passed through a Rainsoft® water hardening cartridge which was used from day 267 until the end of the study.

During the study, newly hatched fry were fed live brine shrimp nauplii three times daily for twenty days. After twenty days, the fish were fed frozen brine shrimp three times daily. Live Daphnia magna were substituted for one of the daily feedings of frozen brine shrimp when available.

A preliminary 35-day exposure of Fathead minnow fry to technical chlorothalonil was conducted in the chronic exposure system. The nominal concentrations used in the preliminary exposure were 50, 25, 12.5, 6.2, and 3.1 ug/liter. Twenty fry were tested in replicate aquaria at each concentration. Replicate solvent (acetone) and non-solvent controls were also tested. Water samples were collected from each aquarium on six days throughout the study for analysis of chlorothalonil. Data from this test was used for the final selection of test concentrations for the chronic study.

On May 31, 1978, the chronic study was initiated by incubating 100 eggs in each aquarium (50 eggs per egg cup). The eggs were obtained from E G & G, Bionomics brood stock which originated with National Water Quality Laboratory stock. Each day, prior to complete hatching, the eggs in each cup were counted and dead eggs were removed. A percentage hatching success was calculated for each duplicate aquarium. Immediately after completion of hatch, twenty fry were randomly selected from each egg cup and placed in their respective growth chambers. On days 35 and 64, each first generation (F_0) fry group was photographed over a millimeter grid to determine total lengths and percentage survival.

On test day 64, the total number of fish in each aquarium was reduced to fifteen by randomly selecting eight fish from one fry chamber and seven fish from the other fry chamber to continue exposure.

On test day 155, an attempt was made to determine the sex of each fish in order to establish a sex ratio of three males to six females in each aquarium. After examining the fish, however, a decision was made to wait

until sexual characteristics were more developed. On test day 172, the thinning process was performed. Exceptions to the desired ratio were where fewer than six females were present.

After day 155, the spawning tiles were checked daily for eggs. The number of eggs in each spawn was recorded and groups of fifty eggs from a maximum of eleven spawns in each aquarium were incubated to determine hatching success.

Groups of eggs spawned by control fish were incubated in aquaria in which first generation fish did not survive or where spawning was reduced or absent.

Twenty second generation (F_1) fry from each of the first two successfully hatched egg groups in each aquarium were randomly selected and placed in their respective growth chambers. After 34 days exposure, each fry group was photographed to determine percentage survival and total length. Each group was wet weighed and the average individual weight calculated. Each group was stored frozen for possible residue analysis.

The exposure of the first generation fish was terminated on day 283 when no spawning had occurred in any aquarium for a period of one week. Each fish was individually measured for total length, wet-weighed and internally examined to verify sex and gonadal condition. The fish were separated according to sex and frozen for possible residue analysis.

All tissue samples which were taken at all major life stages during the chronic study were shipped frozen and are stored at Diamond Shamrock Corporation by the Department of Safety Assessment.

The data generated for survival, growth, and reproduction success were subjected to a one-way analysis of variance ($P = 0.05$). If treatment effects were significant, the mean values for those parameters were then compared with both control values by Dunnet's procedure using one-sided comparison.[3]

RESULTS - DISCUSSION

During the preliminary 35-day exposure of Fathead minnow fry, the analysis of water samples from each aquarium resulted in mean measured concentrations of 49, 25, 8.9, 4.5, and 1.2 ug/liter for the respective nominal concentrations of 50, 25, 12.5, 6.2, and 3.1 ug/liter. The LC_{50} values for both 96-hour and 35-days were estimated to be in the range of 12.5 - 25 ug/liter (nominal concentration). Based on this acute toxicity data, the nominal concentrations selected for the chronic study were 25, 12.5, 6.2, 3.1, and 1.5 ug/liter chlorothalonil.

For reference purposes, the five test concentrations as well as both controls are identified according to the following groups (group numbers are the same as those used in the conduct of the study).

GROUP	REPLICATE	NOMINAL CONCENTRATION ug/liter	MEAN MEASURED CONCENTRATIONS OF EACH REPLICATE* ug/liter	MEAN MEASURED CONCENTRATIONS OF EACH GROUP* ug/liter
1	A	25	15	
	B		17	16
2	A	12.5	6.6	
	B		6.5	6.5
3	A	6.2	3.1	
	B		2.7	3.0
4	A	3.1	1.4	
	B		1.3	1.4
5	A	1.5	0.64	
	B		0.54	0.60
6	A	Solvent Control	-	
	B	(Acetone)	-	-
7	A	Non-Solvent Control	-	
	B		-	-

* The actual mean values listed for each group may vary from a calculated mean of group replicates. The group mean measured concentrations are an average of all values for both replicates in each group.

Throughout the chronic study, the weekly analysis of aquaria water for chlorothalonil resulted in mean measured concentrations of 16, 6.5, 3.0, 1.4, and 0.60 ug/liter for the respective nominal concentrations of 25, 12.5, 6.2, 3.1, and 1.5 ug/liter technical chlorothalonil.

The mean measured concentrations, based on the averaged values for the duration of the chronic study, ranged from 64 percent of nominal for Group 1 (high concentration) to 40 percent of nominal for Group 5 (low concentration). The difference between the nominal and measured concentrations of chlorothalonil in the aquaria was possibly due to adsorption of the compound to biomass (food, feces, fungi, algae, etc.), spawning tiles, or aquaria walls and also to the relatively short half-life of the compound in an aqueous solution. The short half-life was demonstrated in a fourteen day stability study with chlorothalonil which was conducted under static conditions in a heated (25°C) aquarium at a concentration of 25 ug/liter. Analysis of a water sample collected after one day revealed a concentration of 19.0 ug/liter (76% recovery) while a sample collected after seven days revealed a concentration of < 0.3 ug/liter (1% recovery).

The analyses of stock solutions on day 1 of preparation and after 14 days were within an average of 6 percent of the theoretical concentration.

In order to assure that the diluted test material delivered to each aquaria did contain the correct concentration of chlorothalonil, several analyses were performed. At three times during the study, samples of test solution were collected directly from the diluter system delivery tubes and subsequently analyzed. Aquaria water samples were collected at the same times for comparison with the tube samples. Results of the analyses showed the tube samples to be comparable to nominal concentrations. The tube samples averaged 108, 96, 86, 85, and 53 percent of nominal concentrations respectively for Groups 1 through 5. The results of the aquaria sample analyses were comparable with the mean measured averages presented for the duration of the study.

With respect to the tube sample analyses, it is evident that except for Group 5, relatively accurate (within 20 percent of nominal) concentrations of test material were being introduced to each aquaria. The mean measured concentrations, however, were considered to be more representative of the actual exposure of the eggs, fry, and adult fish to the test material.

The monthly analyses for the metabolite (DS-3701) in water did not show detectable amounts of this material.

No effect was observed in the F_0 generation on egg hatchability or fry survival at 35 or 64 days in groups 2-5. A significant reduction in the hatchability of first generation (F_0) eggs (TABLE A) was noted in Group 1 when compared to the non-solvent and solvent controls. The survival of the fry in Group 1 was also significantly reduced after 35 days when compared with both control groups. At 35 days, only 7 fish remained in Group 1 (3 in replicate A and 4 in replicate B). Due to the small number of fry remaining, these two replicates were photographed together for the purpose of measurement and subsequently combined in one tank.

The total length of (F_0) fry after 35 days was comparable in all test groups to both controls. After 64 days, there was no difference between the lengths of (F_0) fry from any test group and the solvent control fry. A statistically significant decrease in lengths, however, was found when the total lengths of fry in Group 2 and 4 were compared to Group 7 (non-solvent control). It was noted, however, that the differences were actually less than one millimeter for those two concentrations which was below the accuracy of the measuring technique. Since a decrease in length of fry in Group 1 was not observed, it was concluded that the differences observed in groups 2 and 4 were not compound related.

No abnormalities were noted in the fry at 35 days, and after 64 days, four cases of distended opercula were observed but not considered to be compound related.

All F_0 fry in the replicate A aquarium of the solvent control died between day 16 and 33. The mortality was attributed to a filamentous growth. On day 64, fifteen fish were transferred to that aquarium from the other replicate of solvent control.

A condition known as gas bubble disease was observed in a total of five male fish in four different aquaria on test day 168. The condition was evidently due to the fact that the incoming water temperature was low enough to cause a change of gas pressure in the aquaria water. At that time, a change to pre-heated diluent water was made and no additional cases of gas bubble disease occurred.

When fish were thinned to the final sex ratio on day 172, the male fish previously diagnosed as having gas bubble disease and still exhibiting symptoms were removed. Several other abnormalities were observed in the fish removed during the thinning process, but were not considered to be compound related. No abnormalities were noted in Group 1 fish. One fish was observed to have gas bubble disease and one with scoliosis in Group 2. One fish had gas bubble disease in Group 3. In Group 4, two fish had protruding eyes which may have been due to gas bubble disease and in Group 5 two fish were observed to have distended opercula. Gas bubble disease was also observed in two non-solvent control fish.

On test day 283, the termination day for all F_0 fish, the survival of all test and control groups remained relatively unchanged from that observed on day 35. The mortalities in the test groups which occurred from day 35 until termination were comparable with mortalities observed in Group 7 controls over the same period. No significant differences were noted in the comparison of individual lengths or wet weights of the male and female fish exposed in any test group with the non-solvent or solvent control groups. No external or internal abnormalities were noted in male or female fish in any group. The absence of abnormalities at termination indicated the conditions observed on day 172 were of a spontaneous nature and not compound related.

The reproductive success (number of eggs per spawn) of the F_0 fish in Groups 3 through 5 was comparable with both control groups. The number of total spawns observed in Group 3 was reduced in comparison to control groups, however, the number of eggs per spawn was unaffected. It was concluded that the number of eggs per spawn was the more reliable parameter of reproductive success because the commonly occurring variability of total spawns could be normalized.

In Groups 1 and 2, the number of eggs per spawn was significantly reduced when compared with both control groups. As mentioned previously, Group 1 consisted of only one replicate and the ratio was 3 males and 2 females rather than the desirable 3 males and 6 females. In this group, only one spawn was observed and due to the small number of eggs, no eggs were incubated. In addition, eggs did not hatch successfully when transferred from the control group to Group 1. The complete failure of F_1 control eggs to hatch in the Group 1 aquaria was significantly different than the 46 percent hatchability rate observed with F_0 eggs in the same group. However, the survival rate was low for F_0 fry and mortality occurred early after hatch. The difference observed in Group 1 between the F_0 and F_1 (control transfer) hatch could be due to biological variation.

The hatching success of F_1 eggs in Groups 3 through 5 was comparable with both control groups. The number of eggs per spawn and the hatching success of F_1 eggs (TABLE A) in Group 2 was significantly reduced when compared to the non-solvent control and replicate B of the solvent control. (The mean percentage hatch was greatly reduced in replicate A of the solvent control, in which several early and possibly infertile spawns were used in hatchability studies. The hatchability of eggs spawned in replicate B aquarium of the solvent control was comparable with the non-solvent control. For this reason, it is probable that the solvent was not responsible for reduced hatchability in this replicate. The use of replicate A was considered to be inappropriate for statistical comparison with test groups.)

After 34 days exposure, the survival, total length, and wet weight of second generation (F_1) fry Groups 2 - 5 were unaffected when compared with both control groups. No abnormalities were observed in any test group. One case of scoliosis was observed in a Group 7 fry (non-solvent control).

CONCLUSION

No compound related effects were observed in the F_0 and F_1 generation, Groups 2 through 7 with respect to survival, development, length and weight of fry, the length or weight of male and female fish at termination, and mortality.

No compound related effects were observed in the F_0 and F_1 generation, Groups 3 through 7 with respect to egg hatchability and numbers of eggs per spawn.

A significant reduction in the hatchability of eggs and survival of fry was observed in Group 1 of the F_0 generation. The reproductive success (eggs per spawn) of Group 1 was also reduced; and due to the insufficient number of eggs, a second generation was not hatched in this group. In Group 2, a significant reduction in the number of eggs per spawn and a significant reduction in the subsequent hatchability of those eggs was observed.

A comparison of first and second generation egg hatchability revealed a significant reduction of hatching success in Group 2 of the F_1 generation, when compared with the same group in the F_0 generation. A comparison of the percentage hatchability of F_1 eggs spawned in Group 2 with the hatchability of F_1 control eggs transferred to this group, revealed a significantly higher hatching success rate for eggs from unexposed (control) fish.

Based on the reduced number of eggs per spawn and the reduced hatchability of second generation (F_1) eggs exposed to a mean measured concentration of 6.5 ug/liter chlorothalonil (Group 2), the maximum acceptable toxicant concentration (mean measured) of technical chlorothalonil in water for Fathead minnows was estimated to be in the range of 3.0-6.5 ug/liter.

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REFERENCES

1. Mount, D. I. and C. E. Stephan, 1967. A method for establishing acceptable toxicant limits for fish - malathion and the butoxyethanol ester of 2,4-D. Transactions of the American Fisheries Society, 96 (2): 185-193.

2. U.S. EPA, 1971. Recommended bioassay procedures for fathead minnow (Pimephales promelas, Rafinesque) chronic tests.

3. Steel, R. G. D. and J. H. Torrie, 1960. Principles and Procedures of Statistics. McGraw-Hill, New York.

TABLE A

HATCHING SUCCESS AND SURVIVAL OF F_0 AND F_1 FATHEAD MINNOWS EXPOSED TO TECHNICAL CHLOROTHALONIL

Mean Measured Concentration ug/L		Hatching Success % F_0	Hatching Success % F_1 (N)	Control Transfers F_1 (N)	Survival % F_0 (35 Days)	Survival % F_1 (34 Days)
16	A*	42	- (0)	0 (3)	8	-
	B*	51	- (0)	0 (3)	10	-
	$\bar{X}$	47	-	0	9	-
6.5	A	74	- (0)	60 (4)	90	78
	B	85	40 (3)	88 (3)	98	80
	$\bar{X}$	80	40	74	94	79
3.0	A	82	95 (4)	98 (1)	93	92
	B	96	62 (2)	90 (3)	95	100
	$\bar{X}$	89	79	94	94	96
1.4	A	83	84 (10)		100	100
	B	98	74 (11)		100	95
	$\bar{X}$	91	79		100	98
0.6	A	96	84 (10)		100	88
	B	96	82 (2)		98	95
	$\bar{X}$	96	83		99	92
Sol Con	A	92	51 (11)		-	95
	B	93	85 (6)		98	98
	$\bar{X}$	93	68		98	97
Con	A	95	94 (10)		93	88
	B	85	92 (11)		95	90
	$\bar{X}$	90	93		94	89

*Replicates

APPENDIX I

THE CHRONIC TOXICITY OF T-117-2 TO THE FATHEAD MINNOW (*Pimephales promelas*).

SUBMITTED TO

DIAMOND SHAMROCK CORPORATION

PAINESVILLE, OHIO

REPORT #BW-79-6-443

SUBMITTED BY

E G & G, Bionomics
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Wareham, Massachusetts
January, 1980

ABSTRACT

Fathead minnows (Pimephales promelas) were continuously exposed to five concentrations of T-117-2, a diluent water control and a solvent control throughout a complete life cycle (egg to egg). The mean measured test concentrations of T-117-2 ranged from 0.60 to 16 µg/ℓ for the 297 day study.

Hatching success of eggs and survival of first generation (F_0) fry after 30 days exposure were significantly (P=0.05) reduced by exposure to a mean measured concentration of 16 µg/ℓ T-117-2. Reproductive success of F_0 fish was adversely affected by exposure to concentrations $\geq$6.5 µg/ℓ T-117-2, which significantly reduced the number of eggs per spawn. Hatching success of second generation (F_1) eggs was significantly reduced by exposure to 6.5 µg/ℓ T-117-2.

Based on these data, the maximum acceptable toxicant concentration of T-117-2 in water for fathead minnows was estimated to be >3.0<6.5 µg/ℓ. Using the 96-hour LC50 of 23 µg/ℓ derived from a preliminary flow-through exposure of fry, the limits on the application factor are 0.13-0.28.

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SECTION I

INTRODUCTION

The objective of this study was to evaluate the long-term effects of chronic exposure (egg to egg) to T-117-2 on the fathead minnow, Pimephales promelas. Exposure began on May 31, 1978 and ended on March 26, 1979. Data was compiled on the effects of exposure on hatching success, survival, growth and reproductive success of first generation (F_0) fish and on the hatching success, survival and growth of their progeny (F_1).

A maximum acceptable toxicant concentration (MATC) of T-117-2 in water for fathead minnows was to be estimated using the laboratory fish production index (Mount and Stephan, 1967). The MATC lies between the highest mean measured concentration producing no adverse effects on exposed fish and the lowest mean measured concentration where toxicant related effects are observed. In addition, an application factor was to be estimated dividing the MATC by the 96-hour LC50 value from a preliminary flow-through exposure of fathead minnows. The MATC for other fish species not chronically exposed to T-117-2 can be estimated by multiplying the acute LC50 value for that species by this application factor (Mount and Stephan, 1967).

The fathead minnow was used for this long-term study because of its adaptability to laboratory conditions and because toxicity information for other compounds and this species is available for comparing relative toxicity.

SECTION II

METHODS AND MATERIALS

Test Material

The test material used in this study was obtained from Diamond Shamrock Corporation, Painesville, Ohio. One kilogram of the gray powder was received in two opaque plastic containers labelled: T-117-2, 96% and dated 12-21-77. The containers of T-117-2 were covered with aluminum foil and stored in a closed cabinet at 20°C. Stock solutions were prepared on a weight: volume basis every two weeks by dissolving 0.7208 g of T-117-2 with analytical grade acetone in a 100 mℓ volumetric flask. The 100 mℓ stock solutions were stored in a foil-covered glass bottle at 20°C in a closed cabinet. Samples of the stock solutions were analyzed for T-117-2 when prepared and two weeks after preparation.

Test Water

The diluent water used in this study was drawn from a 125-meter deep bedrock well into a concrete reservoir where it was aerated and supplemented with well water supplied by the Town of Wareham, Massachusetts. No water was drawn from the E G & G, Bionomics well between test days 269-278 during well reclamation operations and 100% of the diluent water was from the town supply. Because of the softness of the water, diluent water was passed through a Rainsoft® water hardening cartridge, containing crushed oyster shells, which was attached to the chronic unit. The cartridge was used from day 267 until the end of the study.

Chronic Exposure System

A proportional diluter (Mount and Brungs, 1967) with a dilution factor of 0.50 was used to deliver five concentrations of T-117-2, a diluent water control and a solvent control to duplicate aquaria. The solvent control contained 4.5 µℓ/ℓ acetone which was approximately equal to the amount of acetone present in the highest test concentration (3.6 µℓ/ℓ). The difference in these solvent concentrations was due to the limitations of the diluter injector mechanism.

A 50-mℓ gas-tight syringe with a stainless steel needle was activated by a mechanical injector to deliver the toxicant to the glass mixing chamber of the diluter. Polyethylene tubing was attached to the end of the needle and extended below the surface of the water in the mixing chamber where a magnetic stirring bar continuously mixed the contents. The syringe was refilled with stock solution weekly and covered with black polyethylene to minimize potential light degradation of the test material.

A flow-splitting chamber was used to promote further mixing of each dilution of the toxicant prior to one liter of the mixture flowing to duplicate aquaria through separate glass delivery tubes. Each glass aquarium (90 x 30 x 30 cm) was subdivided by a stainless steel screen to provide a spawning area (50 x 30 x 30 cm) and an area where egg cups were suspended and two larval growth chambers (30 x 12 x 25 cm) were placed. The flow-splitting chamber over each aquarium directed 700 mℓ of

the test solutions to the spawning area and 150 mℓ to each of the growth chambers. The water level in each aquarium was maintained at 15 cm by a standpipe. The flow rate to each aquarium was equal to 8 times their volume (40 liters) per 24 hours.

Five spawning sites were made from 7.5 cm sections (halved) of cement-asbestos drain pipe 10 cm in diameter and were placed, concave surface down, in each spawning chamber. Egg groups were incubated in cups made from 5 cm diameter glass jars with 40 mesh stainless steel screen bottoms. Egg cups were oscillated a vertical travel distance of 1-1.5 inches in the test water by a rocker arm driven by a 2 rpm motor.

During the first six months of the study, the temperature in the aquaria was maintained at 25 $\pm$ 2°C by placing them in heated water baths. Circulating water in the baths was heated by immersion coil heaters connected to a relay and controlled by a mercury column thermoregulator.

During the last four months of the study, preheated diluent water was delivered to the test system. The diluent water was pumped through two gas-fired glass-lined water heaters before flowing through PVC piping to the test system. The heated water baths remained in use to provide insulation for the aquaria and insured a temperature of 25 $\pm$ 2°C.

The photoperiod followed the EPA recommended schedule using the daylength of Evansville, Indiana on December 1 for the first day of the test and adjusting the daylength on the first and fifteenth day of each month by means of an automatic timer. Illumination was provided by a combination of Durotest (Optima FS 50) and wide spectrum Grow-Lux fluorescent lights which were centrally located 64 cm above the aquaria water surface. The entire experimental unit was enclosed in black polyethylene curtains to prevent disturbance of the fish and to minimize the interference of laboratory lighting with the intended photoperiod.

Acute Toxicity Test

On March 23, 1978, a preliminary 35-day exposure of fathead minnow fry was initiated in the chronic exposure system. Twenty fry (4-8 days old), obtained from EG&G, Bionomics' brood stock, were randomly assigned to each duplicate aquarium containing nominal concentrations of 50, 25, 12, 6.2 and 3.1 µℓ/ℓ T-117-2, a control and a solvent control. The solvent control contained 9.0 µℓ/ℓ acetone which was approximately equal to the amount of acetone present in the highest test concentration (7.2 µℓ/ℓ). The difference in these solvent concentrations was due to the limitations of the diluter injection mechanism.

The observed fry mortality in this preliminary exposure was used

to calculate a 96-hour LC50 value and 95% confidence interval by the moving average method (Stephan, 1978, personal communication). In addition, these data were used to select nominal concentrations of T-117-2 for the chronic study.

Chronic Exposure Procedure

The methodology for the chronic exposure was modified from the "Recommended bioassay procedure for fathead minnows (Pimephales promelas, Rafinesque) chronic tests" (U.S. EPA, 1971) and is presented in Appendix I.

On May 31, 1978, the chronic study was initiated by incubating 100 eggs divided between two egg cups in each aquarium. Eggs were obtained from EG&G, Bionomics brood stock (originating from the National Water Quality Laboratory, Duluth, Minnesota) and were randomly assigned (by twos) to each of 28 egg cups until a total of 50 eggs per cup was reached. Each day, until hatching was completed (4 days), the eggs in each cup were counted and dead eggs removed. Percentage hatching success (number of live fry/100 eggs x 100) was calculated for each duplicate aquarium. Twenty newly-hatched fry were impartially selected from each egg cup and placed in their respective growth chambers. Each fry group was photographed over a millimeter grid on test days 35 and 64 to determine total lengths and percentage survival. On test day 64, eight fish from one fry chamber and seven fish from the other fry chamber in

each duplicate aquarium were impartially selected to continue exposure and were photographed as a group. Extra fish from each duplicate aquarium were photographed as a group, weighed in tared glass vials and frozen for possible residue analysis.

On test day 155, an attempt was made to determine the sex of each fish in order to establish a sex ratio of three males to six females in each aquarium. After examining the fish in three aquaria, it was decided to postpone the thinning process until sexual characteristics were more developed. On test day 172, the fish were again examined. Three males and six females were retained as spawners in all aquaria except where fewer than six females were present. Terminated fish from each aquarium were pooled and frozen as whole fish for possible residue analysis.

After day 155, the underside of the spawning tiles were examined daily for eggs. Spawning was very sparse until the sex ratio was adjusted on day 172. Spawns were removed from the tiles by gently rolling the egg mass with the fingertips. The number of eggs in each spawn was recorded and groups of fifty eggs from a maximum of eleven spawns in each aquarium were incubated to determine hatching success. Unincubated eggs were pooled by treatment levels, placed in separate glass vials and stored frozen for possible analysis. Eggs spawned by control fish were incubated in aquaria where no first generation fish survived or where spawning was reduced or absent. Twenty fry from each of the first two

successfully hatched egg groups in each aquarium were impartially selected and placed in their respective growth chambers. After 34 days exposure, each fry group was photographed to determine percentage survival and total length. Each group was wet weighed and the average individual weight calculated. Each group was stored frozen in a glass vial for possible analysis.

Exposure of first generation fish was terminated on test day 283 when no spawning had occurred in any aquarium for a period of one week. Each fish was individually measured with a centimeter ruler for total length, wet weight and internally examined to verify sex and gonadal condition. Males and females in each aquarium were separated and frozen as whole fish in glass jars for possible residue analysis.

Throughout the chronic study, aquaria were syphoned clean of debris twice weekly and brushed when fungus or algae growth began to build up. Diluter cells were brushed weekly and the amount of water being delivered through the system to each aquarium was measured monthly.

Temperature and dissolved oxygen were measured in each aquarium on a rotating basis, using a YSI Model #54 oxygen meter with a combination electrode polargraphic probe, so each aquarium was measured at least once weekly. Dissolved oxygen measurements remained >60% of saturation throughout the study. Total hardness,

alkalinity, pH, acidity and specific conductivity were measured weekly in one control aquarium and one treatment level aquarium on a rotating basis (APHA et al., 1975). The diluent water was analyzed at the beginning and near the middle of the study for calcium, magnesium, sodium, potassium, chloride, sulfate, total solids and total dissolved solids.

Water was sampled weekly from each duplicate aquarium and analyzed for T-117-2 concentrations. On the last sampling day of each month, water samples were also analyzed for the metabolite T-114-2. On test days 62, 141 and 167, a water sample was collected from the delivery tube in each aquarium in the A replicate, in addition to the aquarium sample, to determine if influent concentrations of T-117-2 were comparable to aquaria concentrations. Water samples collected on days 62 and 141 represented one diluter cycle, while the day 167 samples represent three diluter cycles.

To assess the stability of T-117-2 in an aqueous solution, a study was conducted under static conditions. A solution of 25 µg/ℓ T-117-2 was prepared in an aquarium (39 x 20 x 25 cm) which was placed inside a chronic unit aquarium with a temperature of 25°C. Water samples were taken 1, 3, 7 and 14 days after preparation and analyzed for T-117-2 and metabolite. Each stock solution of T-117-2 in acetone was analyzed when prepared and after fifteen days of storage, when it was discarded.

Feeding Regimen

Newly-hatched fry were fed live San Francisco Bay Brand brine shrimp nauplii three times daily for twenty days, after which fish were fed San Francisco Bay Brand frozen brine shrimp three times daily. Live Daphnia magna were substituted for one of the daily feedings of frozen brine shrimp when available. To demonstrate the absence of pesticide and PCB residues, a pesticide scan was performed on a sample of the brine shrimp eggs used during the study. Two samples of frozen brine shrimp, one from each of the two shipment lots used during the study, were also analyzed.

Statistical Methods

Data for survival, growth and reproduction were subjected to a one-way analysis of variance (P=0.05) (Steel and Torrie, 1968). Percentage survival and percentage hatching success were transformed to arc sin $\sqrt{\text{percentage}}$ prior to analysis of variance. If treatment effects was indicated, the treatment means for that parameter were compared to the control and to the solvent control mean by Dunnett's procedure using one-sided comparison (Steel and Torrie, 1960).

All data generated in the study are stored in the archives of EG&G, Bionomics, Wareham, Massachusetts.

Analytical Chemistry Methodology

All water analyses for the chronic study were performed at EG&G, Bionomics, Analytical Chemistry Laboratory, Wareham, Massachusetts. The analytical procedures for the determination of T-117-2 and its metabolite T-114-2 in water, are presented in Appendix II.

Brine shrimp tissue samples were analyzed for pesticide and PCB residues by the analytical procedure presented in Appendix III.

Characterization of test diluent water

Two 1-liter diluent water samples were collected in polyethylene bottles equipped with Teflon-lined screw caps. One sample was preserved by adjusting to pH 1 with nitric acid. The other sample was left unpreserved. Both samples were stored at $4^{o}C$ prior to analysis.

At the time of analysis, subsamples were removed from the acidified sample for the determination of calcium, magnesium, sodium, potassium, chloride and sulfate, and from the unpreserved sample for the determination of total dissolved solids. All analyses were performed according to the method references given in Table 1.

Water sample integrity study

A study was performed to determine the integrity of T-117-2 water samples refrigerated with and without solvent for one week. The samples were prepared by diluting 1.0 mℓ of a 55 µg/mℓ T-117-2 standard in acetone to 2000 mℓ with control aquaria water to produce a concentration of 28 µg/ℓ T-117-2. The sample was mixed and 14, 100 mℓ aliquots were transferred to 200 mℓ amber bottles. Two samples were extracted immediately. The pH of six samples was adjusted to 4.5 and 100 mℓ of diethyl ether added as preservative, according to our standard procedure for T-117-2 sample storage. These six preserved and the six unpreserved samples were refrigerated and two of each removed at intervals of 1, 4 and 7 days for extraction. At the end of the study, all of the samples were analyzed by gas chromatography.

Tissue Residue Analysis

Fish tissue samples were taken at all major life stages during the chronic study and shipped frozen to Diamond Shamrock Corporation, T.R. Evans Research Center, Painesville, Ohio for possible residue analysis.

SECTION III

RESULTS

Acute Toxicity Test

The 96-hour LC50 and 95% confidence interval for fathead minnow were calculated to be 23 (20-26) µg/ℓ T-117-2, using the mean measured concentrations for the 35-day preliminary exposure. Mortality of fry during this exposure occurred during the first 96 hours and remained relatively unchanged for the remainder of the 35 days (Table 2). The 35-day LC50 and 95% confidence interval were calculated to be 23 (19-26) µg/ℓ, virtually the same as the 96 hour information.

Based on the response of fry during the preliminary exposure, the nominal concentrations selected for the chronic study were 25, 12, 6.2, 3.1 and 1.5 µg/ℓ T-117-2.

Chronic Exposure

Water chemistry

Results of the analysis of water quality parameters during the chronic study indicated hardness, alkalinity, specific conductivity, acidity, dissolved oxygen and pH varied minimally between test aquaria (Table 3). A characterization of diluent water from water samples taken from the chronic system on test

days 27 and 171 is presented in Table 4.

Mean measured concentrations of T-117-2 in water during the 297 day chronic study are presented in Table 5. Concentrations of T-114-2 in water were below detectable limits throughout the study. The analytical results of solvent control and diluent water control samples on test day 138 revealed a significant concentration of "apparent" T-117-2. However, the profile for the entire chromatogram for these four samples was different from that of other samples for that day and other days, and that of analytical calibration standards. Figures 1-5 show the following:

(1) Analytical standards

(2) 3.1 and 1.5 µg/ℓ (nominal) samples for test day 138

(3) Control samples for test day 138

(4) Solvent control samples for test day 138

(5) Control samples for test day 145

All of the samples shown in the figures were analyzed on the same day. The analytical standards demonstrated a retention time for T-117-2 of 3.06 minutes to 3.13 minutes. The control samples, which contained peaks, have a measurable peak at 3.06 minutes. Although the retention times match, the marked difference in chromatograph profiles (i.e. extra peaks, etc.) and the lack of such profiles in standards or other samples, indicates a probable contamination, not necessarily attributable

to T-117-2. The use of these values in reporting the range of measured concentrations for control and solvent control water samples were considered inappropriate and they were omitted.

A comparison of measured concentrations of T-117-2 in water collected from chronic system delivery tubes and from aquaria in the A replicate on test days 62, 141 and 167 is presented in Table 6. Measured concentrations of T-117-2 were 25 to 78% lower in aquaria samples than in tube samples in all but three individual comparisons. Apparently, substantial loss of the test material was occurring in the test aquaria.

The results of the stability study of T-117-2 in a static aqueous solution which began on test day 110 are presented in Table 7. After seven days, only 1% of the nominal concentration of 25 µg/ℓ T-117-2 was measured, indicating the material is relatively unstable in aqueous solution. These data may explain the discrepancy between tube samples and aquaria samples reported above.

Analyses of the stock solutions indicated T-117-2 to be stable in solution with acetone. The mean measured concentration of 0 day old stocks was 6.8 mg/mℓ, while the 14 day old stock mean measured concentration was 7.0 mg/mℓ. The theoretical concentration of these stock solutions was 7.2 mg/mℓ.

Food analysis

The analytical results of a pesticide scan performed on the two forms of brine shrimp used during the chronic study revealed these food sources to be free of pesticide or PCB contamination with all results below detectable levels.

Water sample integrity

Results of the water sample integrity study are presented in Table 8. No appreciable differences were observed between preserved and unpreserved samples when stored refrigerated as long as seven days.

First generation (F_0)

Hatching success of eggs was significantly reduced by exposure to a mean measured concentration of 16 µg/ℓ T-117-2 when compared to the control and solvent control (Table 9). Thirty days after hatching, survival of F_0 fry exposed to 16 µg/ℓ T-117-2 was significantly reduced as compared to survival of both control groups. Only seven F_0 fry exposed to 16 µg/ℓ T-117-2 survived, with nearly all mortality occuring in the first week of exposure. Growth (total length) of fry was similar in all test concentrations $\leq$16 µg/ℓ T-117-2 and controls. After 64 days exposure, survival of F_0 fry remained unaffected by exposure to concentrations $\leq$6.5 µg/ℓ

T-117-2. Statistically significant differences were indicated when the mean total lengths of F_o fry, exposed to 1.4 and 6.5 µg/ℓ T-117-2 and acetone, were compared to control. However, these differences were actually less than one millimeter which was below the accuracy of the measuring technique. These responses did not appear to be toxicant related and on day 283 when F_o fish were terminated, there were no differences in total lengths between these fish and controls.

All F_o fry in the A replicate aquarium of solvent control died between day 16 and 33. This isolated incident of mortality among fish exposed to acetone was attributed to a filamentous growth that occurred in the aquarium during this time. Frequent brushing, syphoning and rinsing of the larval chambers failed to control this microbial growth. Fry appeared to be severely stressed and were observed to have filaments of the growth trailing from their gills and bodies. On day 64, fifteen fish were transferred to this aquarium from the B replicate of solvent control and provided data for the remainder of the study.

On test day 168, a total of five male fish in four different aquaria were diagnosed to have gas bubble disease. The temperature of the unheated water entering the aquaria was $10^{o}C$ lower than the temperature of the water in the aquaria. It was determined that the diluent water should be heated before entering the aquaria to eliminate the sudden decrease in temperature and gas pressure

in the aquaria water. For the remainder of the study, diluent water was delivered to the chronic unit through a heated water system, and no additional cases of gas bubble disease occurred.

On test day 172, fish in each aquarium were examined to determine their sex and establish a sex ratio of three males to six females wherever possible. In four aquaria, fewer than six females were present to select. The number of males and females retained as spawners are reported in Table 10. The male fish previously diagnosed to have gas bubble disease and still exhibiting symptoms were removed at this time as extra males.

The survival of fish exposed for 283 days to concentrations $\leq$16 µg/ℓ T-117-2 remained relatively unchanged from that observed on day 35 (Table 11). Analysis of variance of individual total lengths and wet weights of male and female fish indicated no differences in growth between fish exposed to test concentrations $\leq$16 µg/ℓ T-117-2 and control or solvent control fish.

Spawning

The reproductive success of F_0 fathead minnows chronically exposed to T-117-2 is presented in Table 12. The number of eggs per spawns was significantly less for fish exposed to 16 and 6.5 µg/ℓ T-117-2 when compared to control and solvent control spawns.

Second generation (F_1)

No eggs hatched successfully when transferred from control and incubated in 16 µg/ℓ T-117-2 (Table 13). Hatching success of eggs spawned by F_o fish exposed to 6.5 µg/ℓ T-117-2 was significantly reduced when compared to control. Statistical analysis also indicated reduced hatching success for eggs spawned by solvent control fish. However, the hatching failure of several early, and possibly infertile, spawns in the A replicate aquarium greatly reduce the mean percentage hatch for the treatment. Hatchability of eggs spawned in the B replicate aquarium of solvent control was comparable to control. After 34 days exposure, survival, total length and wet weight of F_1 fry was unaffected by test concentrations $\leq$6.5 µg/ℓ T-117-2.

SECTION IV

Summary and Discussion

Chronic exposure of fathead minnows to concentrations of 16 and 6.5 µg/ℓ T-117-2 produced adverse effects on egg production and on the survival of early life stages, as well as indicating a possible cumulative toxic effect from one generation to the next.

Second generation (F_1) fathead minnow eggs were apparently more sensitive to T-117-2 than were first generation (F_o) eggs. The percentage hatching success of F_1 eggs was significantly reduced, as compared to control, by exposure to a mean measured concentration of 6.5 µg/ℓ T-117-2, a concentration which did not significantly affect the hatching of F_o eggs. In fact, there was a statistically significant difference between the hatching success of F_o eggs and the hatching success of F_1 eggs exposed to 6.5 µg/ℓ T-117-2.

A comparison of the percentage hatching success of F_1 eggs spawned in 6.5 µg/ℓ T-117-2 with the hatching success of F_1 control eggs transferred to this treatment level revealed a significantly higher hatching success rate for eggs from unexposed (control) parents (Table 14). However, no F_1 control eggs transferred to 16 µg/ℓ T-117-2 hatched successfully, although 46% of the F_o eggs had hatched successfully at this concentration.

Based on an adverse effect on reproduction (reduced number of eggs per spawn) and the reduced hatchability of F_1 eggs exposed to a mean measured concentration of 6.5 µg/ℓ, the maximum acceptable toxicant concentration (MATC) of T-117-2 in water for fathead minnows was estimated to be >3.0<6.5 µg/ℓ. Using the 96 hour LC50 of 23 µg/ℓ from the preliminary flow-through exposure of fry, the limits on the application factor are 0.13-0.28.

SECTION V

REFERENCES

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Stephan, C.E. 1978. U.S. Environmental Research Laboratory, Duluth, Minnesota. Personal communication.

U.S. EPA. 1971. Recommended bioassay procedures for fathead minnow (Pimephales promelas, Rafinesque) chronic tests.

SECTION VI

TABLES

Table 1 -- Method references for water characterization.

Analyte	Method Reference
Calcium	EPA (1976), pp. 78-156
Magnesium	EPA (1976), pp. 78-156
Sodium	EPA (1976), pp. 78-156
Potassium	EPA (1976), pp. 78-156
Chloride	Standard Methods (1975), Sec. 408B
Sulfate	Standard Methods (1975), Sec. 427B
Total Solids	Standard Methods (1975), Sec. 208A
Total Dissolved Solids	Standard Methods (1975), Sec. 208B

"Manual of Methods for Chemical Analysis of Water and Wastewater" EPA-625/6-74-003a (1976).

"Standard Methods for the Examination of Water and Wastewater". 14th Edition, APHA, AWWA, WPCF (1975).

Table 2 -- Mortality (%) of fathead minnow fry during a 35-day preliminary flow-through exposure to T-117-2.

Mean measured[a] concentration (µg/ℓ)		% mortality # dead/20 x 100	
		96 hour	35 days
49	A	100	100
	B	100	100
25	A	75	75
	B	100	100
8.9	A	0	0
	B	0	0
4.5	A	0	5
	B	0	0
1.2	A	0	0
	B	5	5
solvent control	A	0	0
	B	0	0
control	A	0	0
	B	0	0

[a] Based on analysis of water samples taken on days 6, 8, 13, 14, 21 and 35.

Table 3 -- Measured water quality parameters during chronic exposure of fathead minnows to T-117-2.

$\bar{x}$ measured concentrations (µg/ℓ)	Mean concentrations (S.D.)					
	Hardness[a] (mg/ℓ $CaCO_3$)	Alkalinity[a] (mg/ℓ $CaCO_3$)	Specific conductivity[a] (µmhos/cm)	Acidity[b] (mg/ℓ $CaCO_3$)	Dissolved oxygen[c] (mg/ℓ)	pH range[d]
16	29(5)	26(3)	140(14)	5(2)	7.9(0.5)	6.8-7.4
6.5	30(4)	26(4)	136(18)	5(2)	7.8(0.5)	6.5-7.2
3.0	31(5)	27(4)	137(31)	4(1)	8.2(0.3)	7.0-7.4
1.4	31(4)	27(3)	135(29)	4	8.3(0.4)	7.0-7.3
0.60	31(5)	28(4)	141(13)	4(1)	8.3(0.4)	6.9-7.2
solvent control	30(4)	27(4)	140(21)	4(1)	7.2(0.7)	6.6-7.4
control	30(5)	27(4)	146(23)	6(1)	8.5(0.4)	6.6-7.3

a N=82.
b N=116.
c N=600.
d N=115.

Table 4 -- Characterization of diluent water used during T-117-2 chronic study.

Analyte	Analytical Concentrations (mg/ℓ) Sampled test day 27 (analyzed 9/12/78)	Sampled test day 171 (analyzed 1/25/79)
Calcium	9.4	4.4
Magnesium	2.6	1.9
Sodium	16	13
Potassium	1.0	1.0
Chloride	18	15[a]
Sulfate	12 (± 2)[a]	9.8 (± 0.3)[a]
Total solids	111 (± 1)[a]	85 (± 5)[a]
Total dissolved solids	28[a]	62 (± 4)[a]

[a] Average of two aliquots.

Table 5 -- Measured concentrations of T-117-2 in water during the 297 day exposure of fathead minnows.

Nominal concentration (µg/ℓ)		Mean ± S.D.	Range	N_1 [a]	N_2 [b]
25	A	15 ± 6	0.40-27	46	0
	B	17 ± 6	0.44-28	42	0
	$\bar{x}$	16 ± 6	0.40-28	88	0
12.5	A	6.6 ± 3.0	<0.2-12	45	1
	B	6.5 ± 3.3	<0.2-13	41	1
	$\bar{x}$	6.5 ± 3.2	<0.2-13	86	2
6.2	A	3.1 ± 1.5	0.60-6.2	46	0
	B	2.7 ± 1.4	0.40-6.3	44	0
	$\bar{x}$	3.0 ± 1.4	0.40-6.3	90	0
3.1	A	1.4 ± 0.8	<0.3-3.0	42	4
	B	1.3 ± 0.6	<0.2-3.0	40	4
	$\bar{x}$	1.4 ± 0.7	<0.2-3.0	82	8
1.5	A	0.64 ± 0.28	<0.2-1.2	40	6
	B	0.54 ± 0.22	<0.2-1.0	36	7
	$\bar{x}$	0.60 ± 0.25	<0.2-1.2	76	13
solvent control	A		<0.1-<0.4		
	B		<0.1-2.6 [c]		
control	A		<0.1-0.2 [c]		
			<0.1-<0.4		

[a] Number of samples used to calculate mean.

[b] Number of samples below detection level.

[c] Number represents one sample only.

Table 6 -- Comparison of measured concentrations of T-117-2 in water collected from chronic system delivery tubes and from aquaria on test days 62, 141 and 167.

Nominal concentration (µg/ℓ)	Test day	Measured concentrations (µg/ℓ)	
		Tube samples	Aquaria samples
25	62	23	17
	141	-[a]	13
	167	31	14
12.5	62	12	7.9
	141	11	4.2
	167	13	6.1
6.2	62	4.1	4.4
	141	3.9	0.99
	167	8.0	3.6
3.1	62	2.2	2.5
	141	1.7	0.91
	167	4.0	0.88
1.5	62	0.95	0.71
	141	0.44	0.2
	167	1.0	0.4

[a] No reliable data available.

Table 7 -- Stability of 25 µg/ℓ T-117-2 aged 1, 3, 7 and 14 days in a static aqueous solution.

Day [a]	Measured concentrations of T-117-2 (µg/ℓ)	Measured concentrations of T-114-2 (µg/ℓ)
1	19	<0.55
3	6.5	<0.55
7	0.40	<0.55
	<0.10	<0.55
	0.32	<0.55
14	0.24	<0.55
	0.32	<0.55
	0.32	<0.55

[a] Day 1 was 9/20/78 and day 14 was 10/3/78.

Table 8 -- Analytical results of preserved and unpreserved water samples refrigerated for 1, 4 and 7 days.

Storage day	Sample description	Analytical Results	
		concentration µg/ℓ T-117-2	% of day 0
0	-	27	-
0	-	26	-
1	preserved	27	100
1	preserved	27	100
1	unpreserved	21	79
1	unpreserved	30	110
4	preserved	26	98
4	preserved	30	110
4	unpreserved	25	94
4	unpreserved	25	94
7	preserved	22	83
7	preserved	27	100
7	unpreserved	22	83
7	unpreserved	21	79

Table 9 -- Hatching success, survival and total length of F_o fathead minnows after 35 and 64 days exposure to T-117-2.

Mean measured concentration (µg/ℓ)		Hatching success (%)	Day 35		Day 64	
			Survival (%)	Total length ± S.D. (mm)	Survival (%)	Total length ± S.D. (mm)
16	A	42[a]	8[a]	21 ± 3[b]	8	29 ± 5
	B	51[a]	10[a]			
6.5	A	74	90	20 ± 3	88	27 ± 3
	B	85	98	20 ± 3	95	27 ± 3
3.0	A	82	93	20 ± 3	88[c]	27 ± 3
	B	96	95	20 ± 3	95[c]	28 ± 4
1.4	A	83	100	20 ± 2	93	27 ± 3
	B	98	100	19 ± 3	100[d]	27 ± 4
0.60	A	96	100	20 ± 2	100	27 ± 3
	B	96	98	20 ± 2	98	28 ± 3
solvent control	A	92	0[e]	-	-	-
	B	93	98	20 ± 3	95	27 ± 5
control	A	95	93	20 ± 2	88	29 ± 2
	B	85	95	21 ± 3	95	28 ± 4

[a] Significantly (P=0.05) different from control.

[b] Fish from both replicates were combined.

[c] One fish with distended opercula.

[d] Two fish with distended opercula.

[e] All fish in replicate A of acetone control died between test days 16 and 33.

Table 10 -- Survival and sex ratio of F_o fathead minnows exposed 172 days to T-117-2.

Mean measured concentrations (µg/ℓ)		# of mortalities between days 64-172	Sex ratio Male/Female	Total # fish	Retained as spawners Male/Female	Total # fish
16	A	1	3/2	5	3/2	5
	B					
6.5	A	1	7[a]/7	14	3/6	9
	B	0	6/9	15	3/6	9
3.0	A	2	9[b]/4	13	3/4	7
	B	3	8/4	12	3/4	7
1.4	A	1	9[c]/5	14	3/5	8
	B	0	7/8	15	3/6	9
0.60	A	0	8[d]/7	15	3/6	9
	B	2	6[d]/7	13	3/6	9
solvent control	A	0[e]	9/6	15	3/6	9
	B	0	8/7	15	3/6	9
control	A	1	8[f]/6	14	3/6	9
	B	2	5/8	13	3/6	9

a
One fish with scoliosis, one with gas bubble disease.
b
One fish with gas bubble disease.
c
Two fish with protruding eyes probably due to gas bubble disease.
d
One fish with distended operculum.
e
On day 64, 15 fish were transferred to this aquarium from the B replicate aquarium of acetone control.
f
Two fish with gas bubble disease.

Table 11 -- Survival, growth and sexual maturity of F_0 fathead minnows after 283 days exposure to T-117-2.

Mean measured concentration (µg/ℓ)		Number of survivors		Number of mortalities (day 172-283)	Total length (mm) ± S.D.		Total weight (g) ± S.D.	
		male	female		male	female	male	female
16	A	3	2	0	82 ± 1	58 ± 1	5.15 ± 0.38	1.55 ± 0.06
	B	-	-	-	-	-	-	-
6.5	A	3	6	0	82 ± 10	56 ± 3	5.30 ± 2.68	1.45 ± 0.23
	B	3	6	0	81 ± 2	57 ± 3	5.27 ± 0.38	1.60 ± 0.33
3.0	A	3	4	0	79 ± 8	60 ± 4	4.88 ± 1.18	1.66 ± 0.43
	B	4	3	0	78 ± 3	63 ± 5	4.42 ± 0.77	1.94 ± 0.15
1.4	A	4	3	1	80 ± 2	55 ± 4	5.04 ± 0.72	1.35 ± 0.35
	B	3	6	0	80 ± 6	55 ± 5	5.32 ± 1.21	1.41 ± 0.44
0.60	A	3	6	0	78 ± 2	58 ± 4	4.80 ± 0.91	1.72 ± 0.35
	B	3	6	0	81 ± 7	54 ± 4	5.46 ± 1.26	1.34 ± 0.32
solvent control	A	3	6	0	81 ± 5	58 ± 5	5.46 ± 0.89	1.74 ± 0.57
	B	3	6	0	84 ± 5	56 ± 5	6.09 ± 1.46	1.58 ± 0.45
control	A	4	5	0	76 ± 6	55 ± 3	4.64 ± 1.09	1.42 ± 0.21
	B	3	6	0	77 ± 4	55 ± 6	4.55 ± 0.68	1.37 ± 0.40

Table 12 -- Reproductive success of fathead minnows chronically exposed to T-117-2.

Mean measured concentration (µg/ℓ)		Total spawns	Total eggs	Eggs/Spawn
16	A	1	18	18[a]
	B	-	-	-
6.5	A	1	12	12[a]
	B	14	676	48[a]
3.0	A	6	713	119
	B	2	111	56
1.4	A	18	1,868	104
	B	30	3,266	109
0.60	A	20	3,564	178
	B	5	301	60
solvent control	A	18	2,332	130
	B	14	1,339	96
control	A	31	2,265	73
	B	30	3,660	122

[a] Significantly different (P=0.05) from control.

Table 13 -- Hatching success, survival and growth of F_1 fathead minnows exposed to T-117-2 for 34 days.

Mean measured concentration (µg/ℓ)		Hatching success mean ± S.D. %	(N)[a]	Survival (%)	Total length (mm)	Average wet weight (g)
16	A	0	(3)[b]	-	-	-
	B	0	(3)[b]	-	-	-
6.5	A	60 ± 38	(4)[b]	78	24 ± 2	0.1095
	B	40[c] ± 26	(3)	80	22 ± 3	0.0847
3.0	A	95 ± 2	(4)	92	24 ± 2	0.0998
	B	62 ± 26	(2)	100	24 ± 3	0.1186
1.4	A	84 ± 13	(10)	100	24 ± 2	0.1188
	B	74 ± 37	(11)	95	21 ± 3	0.0772
0.60	A	84 ± 30	(10)	88	23 ± 2	0.0866
	B	82 ± 8	(2)	95	24 ± 2	0.1052
solvent control	A	51[c] ± 41	(11)	95	23 ± 3	0.1137
	B	85 ± 11	(6)	98	24 ± 3	0.1073
control	A	94 ± 3	(10)	88	25 ± 3	0.1218
	B	92 ± 4	(11)	90	23 ± 3	0.0973

[a] N=number of egg groups incubated.

[b] Eggs and subsequent fry transferred from control.

[c] Significantly different from control.

Table 14 -- Comparison of hatchability of F_1 eggs produced by parental fish exposed to T-117-2 with F1 eggs produced by unexposed parents (control fish).

Mean measured concentration (µg/ℓ)	Mean percentage hatching success			
	Eggs from exposed parents	(N)[a]	Eggs from unexposed parents[b]	(N)
6.5	40[c]	(3)	88	(3)
3.0	62	(2)	90	(3)

[a] N = number of egg groups incubated.

[b] Eggs transferred from control spawns to treatment levels.

[c] Significantly (P=0.05) lower than hatching success of eggs from unexposed parents.

FIGURES

Figure 1

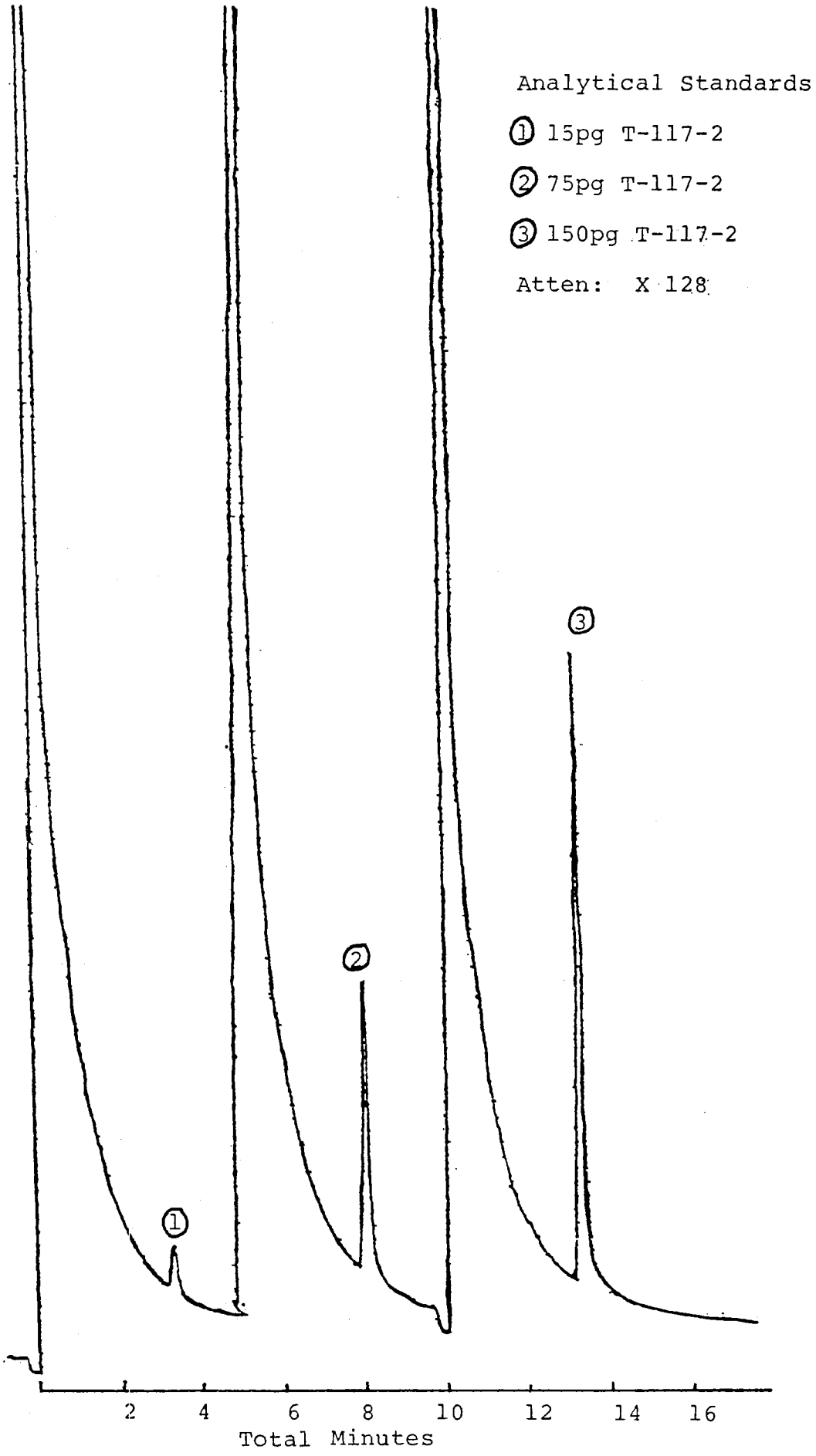

Analytical Standards
① 15pg T-117-2
② 75pg T-117-2
③ 150pg T-117-2
Atten: X 128
①
②
③
2
4
6
8
10
12
14
16
Total Minutes

Figure 2

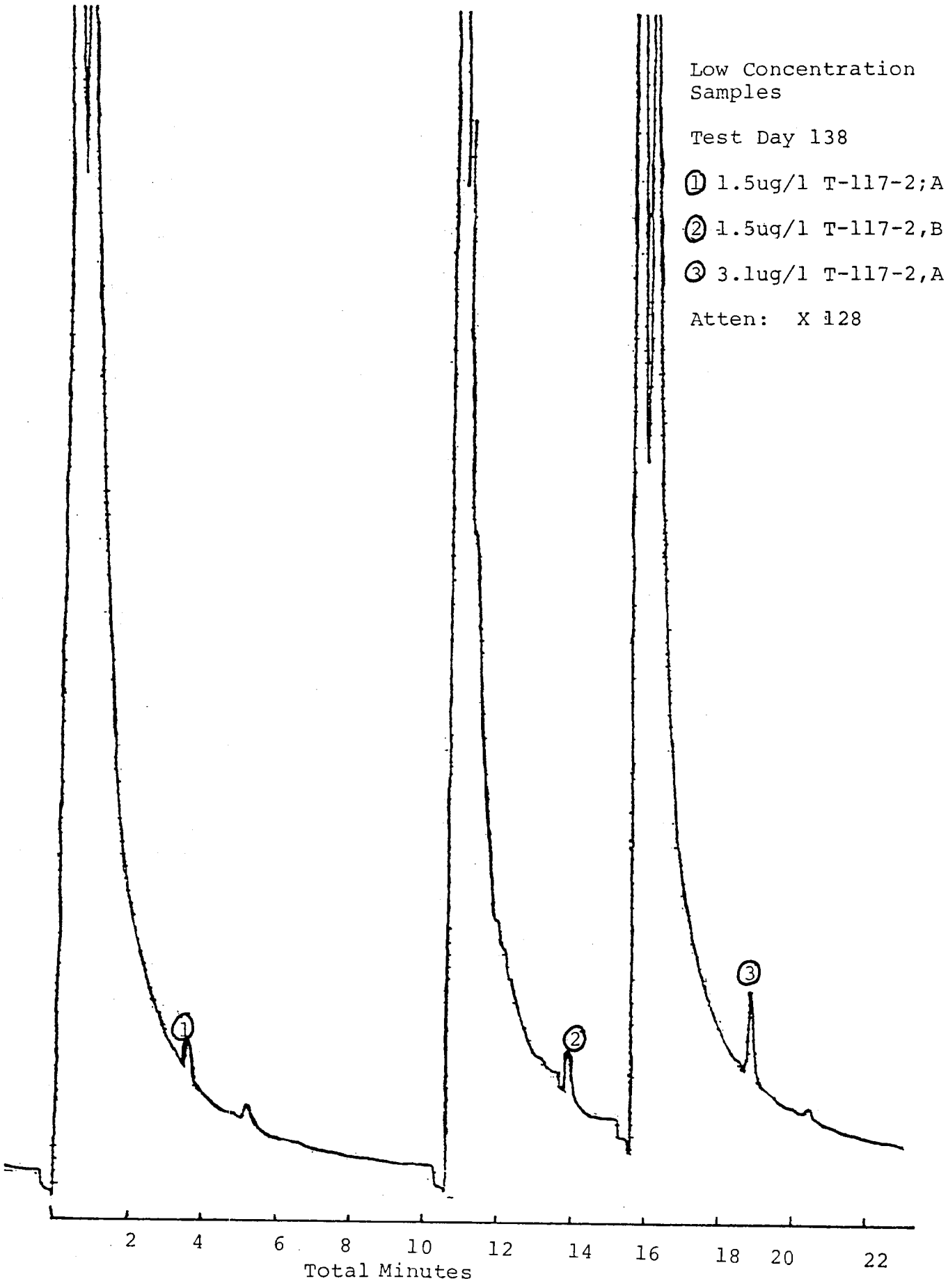

Low Concentration Samples
Test Day 138
1 1.5ug/l T-117-2;A
2 1.5ug/l T-117-2,B
3 3.1ug/l T-117-2,A
Atten: X 128
1
2
3
2
4
6
8
10
12
14
16
18
20
22
Total Minutes

Figure 3

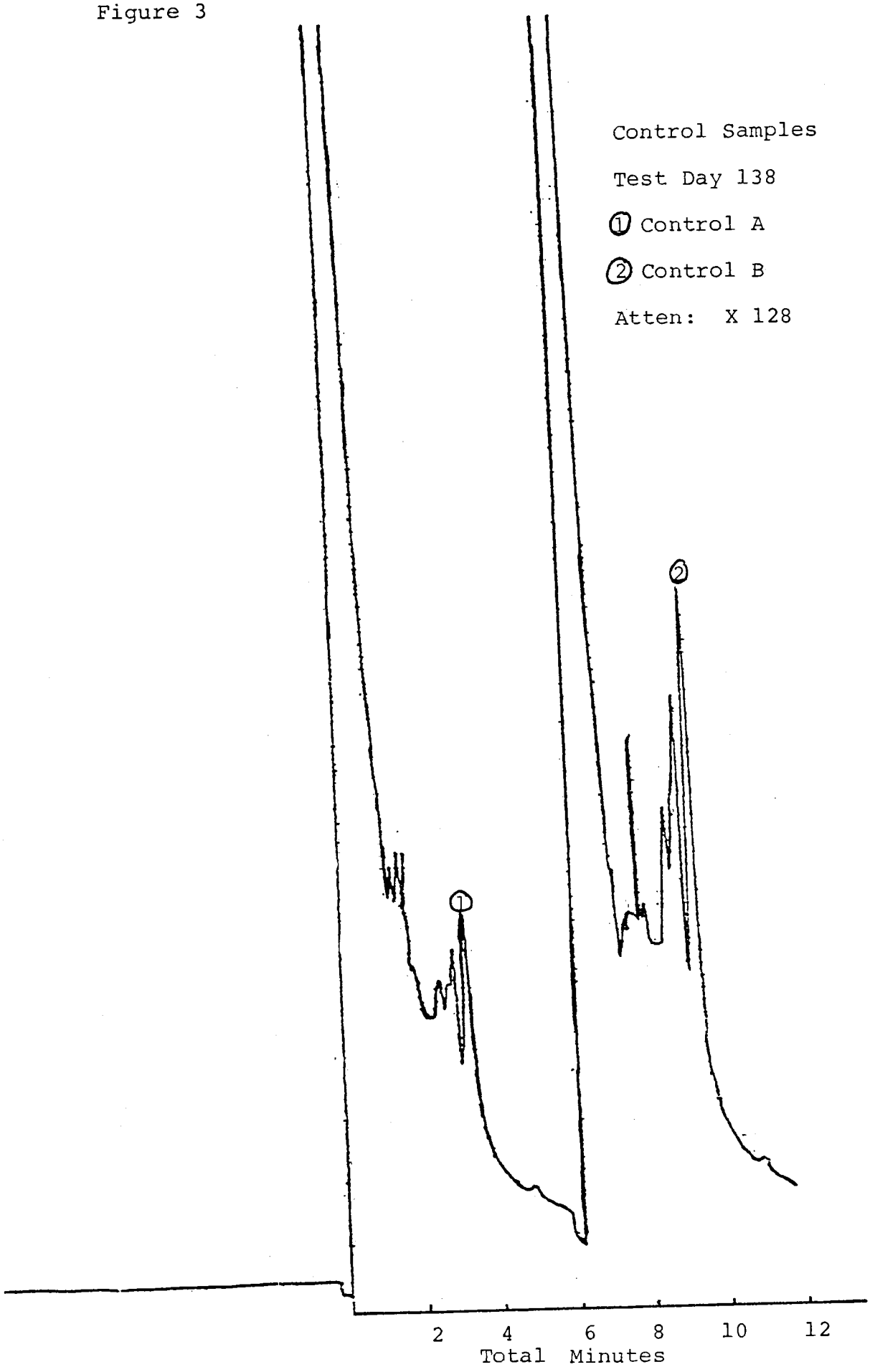

Control Samples
Test Day 138
① Control A
② Control B
Atten: X 128
2
4
6
8
10
12
Total Minutes

Figure 4

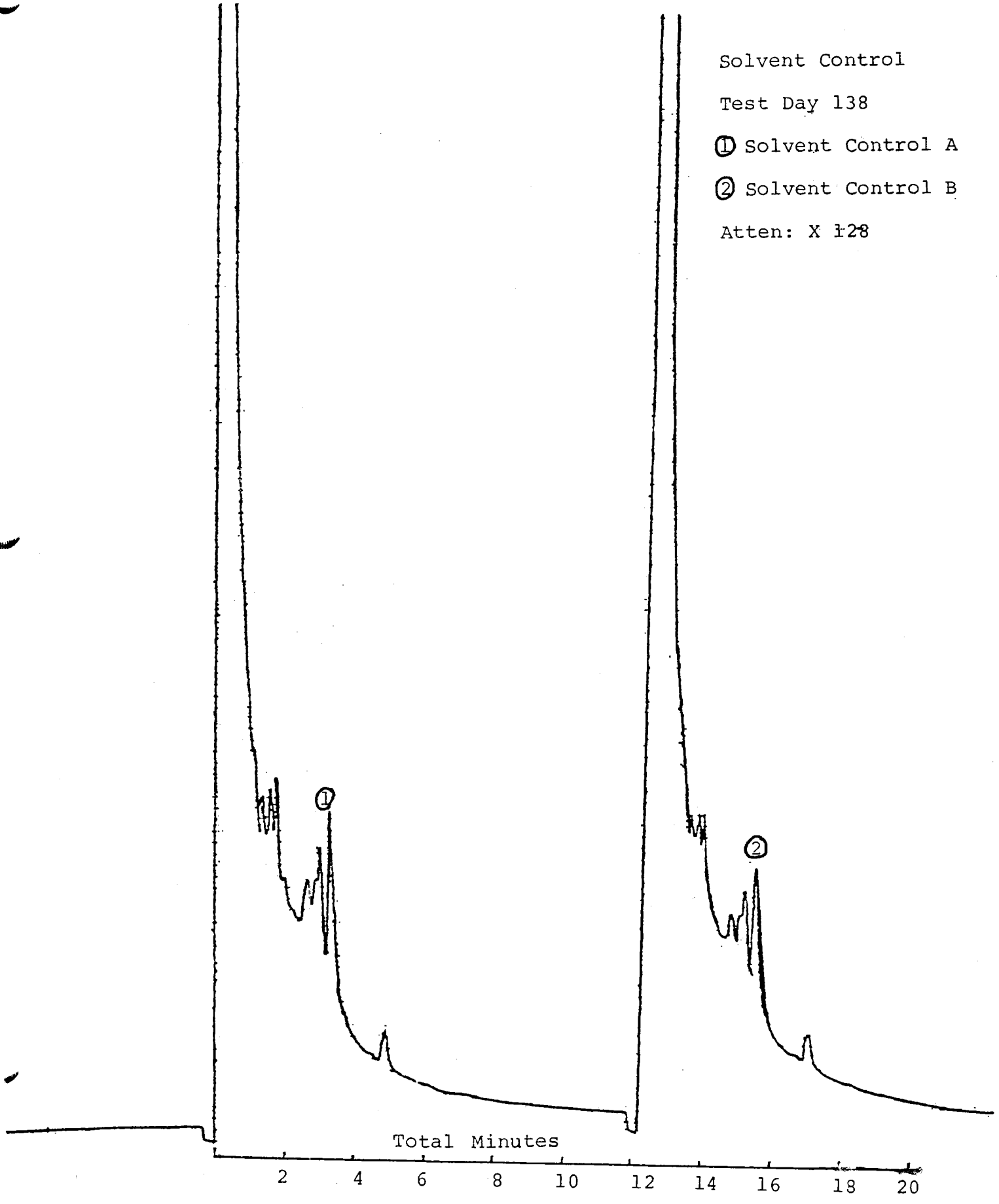
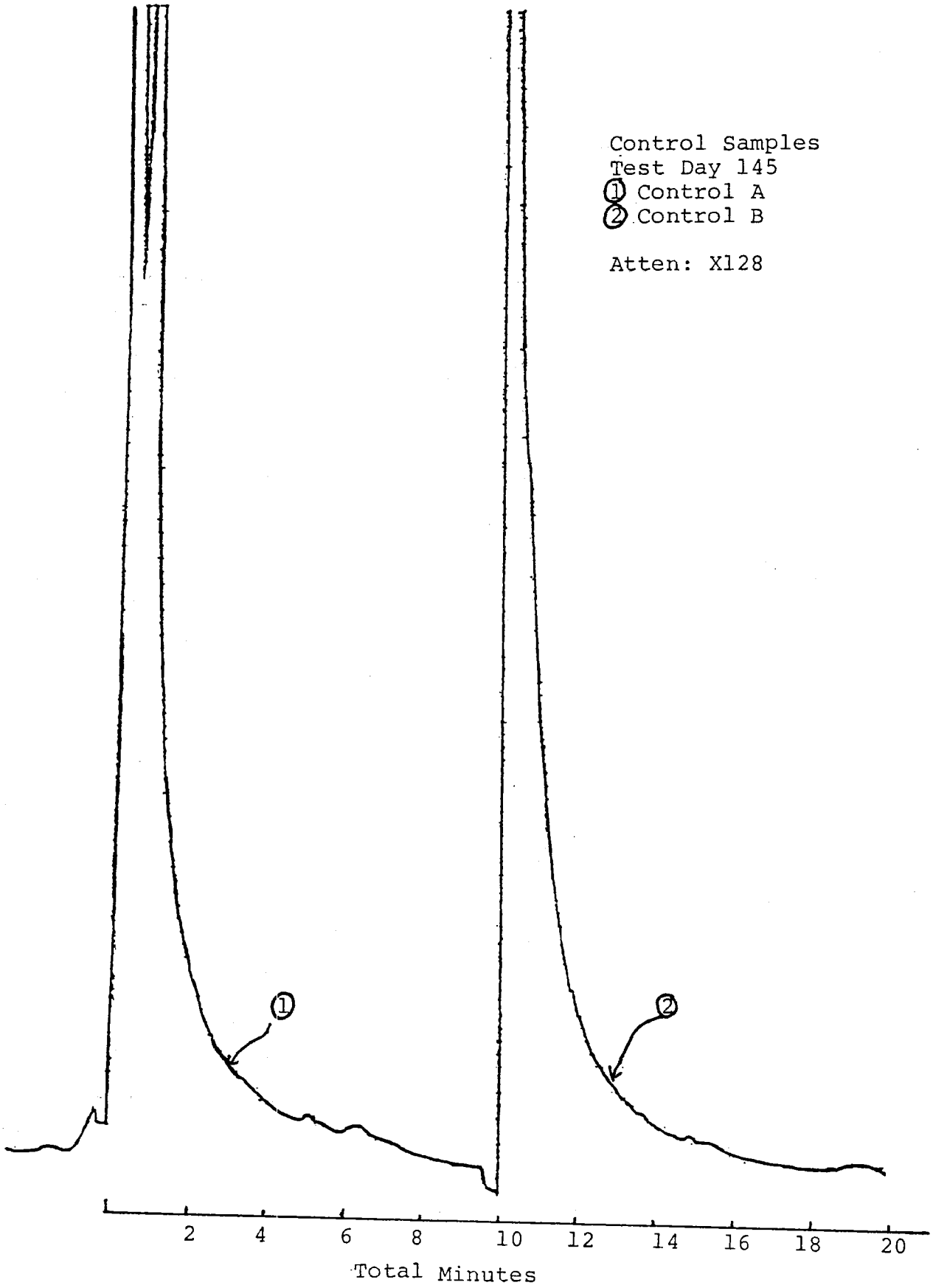

Solvent Control
Test Day 138
① Solvent Control A
② Solvent Control B
Atten: X 128
①
②
Total Minutes
2
4
6
8
10
12
14
16
18
20

Figure 5


Control Samples
Test Day 145
1 Control A
2 Control B
Atten: X128
1
2
2
4
6
8
10
12
14
16
18
20
Total Minutes

APPENDIX I

PROTOCOL

I STUDY:

A Chronic Study in the Fathead Minnow (*Pimephales promelas*).

II OBJECTIVE:

To assess the effects of chronic (egg to egg) exposure of T-117-2 to the Fathead minnow (*Pimephales promelas*).

III SPONSOR:

Diamond Shamrock Corporation
T. R. Evans Research Center
P. O. Box 348
Painesville, Ohio 44077
Phone: 216/352-9311

IV DIAMOND SHAMROCK STUDY DIRECTORS:

James C. Killeen, Jr., Ph.D.
Diamond Shamrock Corporation
P. O. Box 348
Painesville, Ohio 44077

John A. Budny, Ph.D.
Diamond Shamrock Corporation
P. O. Box 348
Painesville, Ohio 44077

CONTRACT LABORATORY STUDY DIRECTOR:

George A. Cary
EG&G Bionomics
790 Main Street
Wareham, Massachusetts 02571

V OWNERSHIP OF THE STUDY:

The sponsor owns the study. This includes the data, results, raw material, etc.

VI ON-SITE VISITS:

The Diamond Shamrock study director will visit the contract laboratory at pretest to set up the study and to discuss the protocol with the contract laboratory. The Diamond Shamrock study director and/or a person designated by the study director will visit the contract laboratory every two months to monitor the study.

VII SCHEDULE OF EVENTS:

Prior to receiving the final authorization to conduct the study, the contract laboratory will submit to the sponsor, in writing, the proposed starting date, date of completion of the study and the date for submission of the data to the sponsor.

VIII GLP STATEMENT:

It is the understanding that the study described herein will be conducted in accordance with the intent implied by the Proposed Regulations for Good Laboratory Practice as published in the Federal Register, 41(225), Friday November 19, 1976.

IX TEST MATERIAL: T-117-2

A. Storage: The test material will be stored in the dark in a tightly closed container at room temperature.

Purity: 96%

Source: Diamond Shamrock Corporation

B. Preparation: A stock solution of the test material will be prepared by dissolving the T-117-2 in acetone. The procedure should exclude light when possible and under no conditions should heat be applied to the stock solution. The stock solution will be used within 15 days of preparation. After 15 days it will be discarded.

Storage: The stock solution will be stored in a light excluding glass container at room temperature. Every attempt will be made to minimize the exposure of the stock solution to light.

Stability: The stock solution will be analyzed immediately after preparation and after 15 days of storage.

C. Aqueous Solutions: The solutions of test material to be used in the fish tanks will be prepared
diluting system.

Stability: To determine general stability of the test material in an aqueous system under water conditions at Bionomics, the following will be done:

1. An aqueous solution of the test material will be prepared at a concentration of 50 ppm and stored at 25°C for 14 days.

2. The solution will be analyzed for test material and metabolite on days 1, 3, 7 and 14 following preparation.

X TEST CONCENTRATIONS:

A preliminary study will be conducted for the purpose of obtaining data on which to base concentrations for the main chronic study. Initially the following concentrations are to be tested: 50, 25, 10, 5 and 1 ppb. Five concentrations will be selected for the main study. The selection of all test concentrations is the responsibility of the Diamond Shamrock study director (additional information on page 5).

XI MATERIALS AND METHODS:

A. Physical System:

1. Diluter: Proportional diluters (Mount and Brungs, 1967) will be employed for all long-term exposures. The operation of the diluter will be checked daily to ensure that it is functioning properly. For two weeks prior to the introduction of the eggs direct measurement of the test material will be made daily (except weekends and holidays) (by analysis) to insure that an equilibrium concentration has been established (Methods - Appendix B). During the remainder of the test, direct measurement of the test material will be made weekly. Five concentrations and two controls (one with solvent [acetone], one without solvent) will be used for each test. Each group will be run in duplicate. The levels for the chronic study will be selected from the data obtained in the preliminary study(B.2).

2. Test Material Mixing: A container to promote mixing of the test material and diluent water will be used between the diluter and tanks for each concentration. Separate delivery tubes will run from this container to each duplicate tank. This system will be checked at least once every month to assure that the correct amounts of water are going to each duplicate tank.

3. Tank: Spawning tanks will be used which have approximately one third of the volume screened off and divided in half for larval chambers. Test water from the test material mixer will be delivered separately to the larval and spawning chambers of each tank. One-third of the total water volume will flow to the larval chamber, the remaining two thirds will flow to the spawning chamber. Test water depth in tanks should be 6 inches.

4. Flow Rate: The flow rate to each chamber (larval or adult) will be equal to approximately 8 tank volumes/24 hours.

5. Aeration: Tank water should have total dissolved oxygen levels of greater than 60% saturation. The levels will be monitored daily except weekends and holidays. Should the saturation level fall below 60%, corrective steps will be taken (e.g. increase flow rate). Whenever the dissolved oxygen saturation falls below 60%, this fact will be documented as well as the steps taken to correct the situation. The tank water will be aerated (with oil free air) only as a last resort to maintain dissolved oxygen at 60% of saturation.

6. Cleaning: All adult tanks, larvae tanks and chambers after larvae swim up, must be cleaned a minimum of two times weekly and brushed or scraped when algal or fungus becomes excessive.

7. Spawning Substrates: Spawning substrates will be made from inverted cement and asbestos drain tile halves and will be 3 inches long.

8. Egg Cup: Egg incubation cups will be used which are made from 4 ounce, 2 inch OD round glass jars with the bottom cut off. One end of th jar will be covered with stainless steel or nylon screen (with a minimum of 40 meshes per inch). Cups will be oscillated in the test water by means of a rocker arm apparatus, (Mount, 1968). The vertical-travel distance of the cups will be 1 to 1-1/2 inches.

9. Light: The lights used will simulate sunlight as nearly as possible. A combination of Durotest (Optima FS) [1] [2] and wide spectrum Grow-Lux [3] (or equivalent) fluorescent tubes will be used.

10. Photoperiod: The photoperiod used (Appendix A) will simulate the dawn to dusk times at Evansville, Indiana. Adjustments in day-length are to be made on the first and fifteenth day of every Evansville test month. The table (Appendix A) is arranged so that adjustments need be made only in the dusk times. Regardless of the actual date that the experiment is started, the Evansville test photoperiod will be adjusted so that the mean length corresponds to the Evansville test day-length for December first. Also, the dawn and dusk times listed in the table need not correspond to the actual times where the experiment is being conducted.

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[1] Mention of trade names does not constitute endorsement.

[2] Duro-Test, Inc., Hammond, Indiana.

[3] Sylvania, Inc., New York, New York.

11. Temperature: Water temperature in the tanks will be 25° ± 2° C. Temperatures will be recorded daily except on weekends and holidays.

12. Disturbances: Adults and larvae will be shielded from any outside disturbances which might effect the results of the test or from extraneous light that might alter the intended photoperiod.

13. Construction Materials: Construction materials which contact the diluent water will not contain leachable substances and should not absorb amounts of substances from the water which will significantly alter the intended concentrations. Rubber will not be used. Plastic containing fillers, additives, stabilizers, plasticizers, etc., will not be used. Teflon, nylon and their equivalents should not contain leachable materials and should not absorb significant amounts of most substances. Unplasticized polyethylene and polypropylene should not contain leachable substances.

14. Water: The water used will be from a well. Data from the analysis of the diluent water will be maintained at Bionomics.

B. Biological System:

1. Test Animals: Stocks of Fathead minnows from the National Water Quality Laboratory in Duluth, Minnesota will be used. Each starting group will contain approximately equal numbers of eggs. The eggs will be derived from at least three different females.

2. Beginning Test and Selection of Test Concentrations: Acute mortality tests will be conducted on young fry at selected concentrations under dynamic conditions. The screening concentrations are to be based on the acute LC_{50} values with the test materials under static conditions for the bluegill (LC_{50} = 386 ppb). The screening levels are to be 50, 25, 10, 5 and 1 ppb. The duration of the acute mortality test will be a minimum of 2-3 weeks. Test concentrations for the main study will be selected based on the data obtained from the study described herein. Five concentrations of test material plus a control and solvent control will be used for the main study and will be approved by the Diamond Shamrock study director prior to initiation.

3. Food: Fish will be fed live brine shrimp for the first 20 days and frozen brine shrimp for the remainder of the test. The fish will be fed ad libitum at least once daily. Diets will be supplemented with live or frozen live food (e.g. Daphnia, chopped earthworms, etc). Each batch of food will be analyzed for the level of organo chlorine and the results of the analysis reported.

4. Disease: If a disease outbreak occurs, any treatment must be approved by the Diamond Shamrock study director. If it is decided that a treatment is appropriate, all tanks will receive the same treatments whether or not there are sick fish in all the tanks. The frequency of treatment will be held to a minimum. A complete record of all treatments will be maintained.

5. Measuring Fish: The individual lengths of all fish will be measured at 30 and 60 days post hatch by the photographic method of McKim and Benoit (1971). Larvae or juveniles will be transferred to a glass box containing 1 inch of test water. Fish will be moved to and from this box in a water-filled container, rather than by netting them. The glass box will be placed on a translucent millimeter grid over a fluorescent light platform to provide background illumination. Photos will then be taken of the fish over the millimeter grid. The length of each fish will be determined by comparing it to the grid. The lengths of discarded fish will be kept separate from those of fish that are maintained.

6. Thinning: When the starting fish are sixty (± 1 or 2) days old, the number of fish in each tank will be randomly reduced to 15 (if the sex of the fish can be determined a ratio of 3 or 4 males to 11 or 12 females will be maintained). Fish judged to be unfit may be discarded before the selection so long as the final number is not reduced below 15. A record of the number of fish judged unfit and discarded from each tank will be maintained. As a last resort in obtaining 15 fish per tank, 1 or 2 fish may be selected for transfer from one duplicate tank. At the initiation of the spawning period the population in each tank will be thinned to three males and six females. An effort should be made not to remove those males having well established territories under tiles where recent spawnings have occurred.

7. Removing Eggs: Eggs will be removed from spawning tiles starting at 1:00 p.m. Evansville test time (Appendix A) each day. Eggs will be loosened from the spawning tiles and at the same time separated from one another by lightly placing a finger on the egg mass and moving it in a circular pattern with increasing pressure until the eggs begin to roll. The groups of eggs will then be washed into separate, appropriately marked containers and subsequently counted, selected for incubation, or saved for possible residue analysis. As soon as possible after all eggs have been removed the spawning tiles will be put back into the test tanks.

8. Egg Incubation and Larval Selection: Fifty unbroken eggs from each spawning of 50 eggs or more will be randomly selected and placed in an egg incubator cup to determine viability and hatchability. The remaining eggs will be counted and saved for possible residue analys Viability and hatchability determinations will be made on each spawning (>49 eggs) until the number of spawnings (>49 eggs) in each tank equals the number of females in that tank. Subsequently, only eggs from every third spawning (>49 eggs) and none of those

obtained on weekends need be saved for hatchability; however, weekend spawns must still be removed from tiles and the eggs counted. If unforeseen problems are encountered in determining eggs viability and hatchability, additional spawnings will be sampled before switching to the every third spawning measurement of viability and hatchability. A total of 10 hatchability determinations from each tank will be considered adequate for computing a mean response for that group. Every day the number of live and dead eggs in the incubator cups will be recorded, the dead eggs removed and the cup screens cleaned. Total number of eggs accounted for should always add up to within two of 50 or the entire batch is to be discarded. When larvae begin to hatch, they should not be handled again or removed from the egg cups until all have hatched. Twenty larvae (or as many as possible if less than 20 have survived) will be transferred immediately to a larval test chamber. The 20 larvae should be selected randomly. Egg-cup-groups not used for survival and growth studies will be counted and saved for possible residue analysis.

9. Progeny Transfer: Control eggs will be transferred immediately after spawning to tanks where spawning is reduced or absent, or to where an effect is seen on survival of eggs or larvae. Eggs from treated tanks may also be transferred to a control tank. One larval chamber should always be reserved for eggs produced in that tank.

10. Larval Exposure: Larvae hatched from early spawnings in each tank will be used for 30 day growth and survival exposures in the larval chambers. Mortalities will be recorded and lengths of larvae measured at 30 days post-hatch. At the time the larval test is terminated they will also be weighed. Fish (larvae, juveniles or adults) will not be fed within 24 hours of when they are to be weighed.

11. Parental Termination: Parental fish testing will be terminated when a week passes in which no spawning occurs in any of the tanks. Total lengths and weights of parental fish will be measured. The sex and condition of gonads will be checked.

12. Necessary Data: Data that must be reported for each tank of a chronic test are:

    1. Number and individual lengths of normal and abnormal fish at 30 and 60 days; individual lengths, weights and numbers of either sex, both normal and abnormal, at end of test.

    2. Mortality during the test.

    3. Number of spawns and the number of eggs per spawn.

    4. Hatchability and viability of spawns when measured.

    5. Fry survival, growth and abnormalities.

C. Chemical System:

1. Preparing a Stock Solution: Because the test material has a low aqueous solubility (0.6 ppm), it is impractical to introduce it directly into the test water. A stock solution of the test material will be prepared by dissolving the test material in acetone. The amount of solvent (acetone) used will be kept to a minimum, but the calculated concentration of solvent to which any test organisms are exposed will never exceed one one-thousandth of the 96-hour LC_{50} for test species under the test conditions and will never exceed one gram per liter of water. Two sets of controls will be used, one exposed to no solvent and one exposed to the highest concentration of solvent to which any organism in the test is exposed.

2. Measurements of Test Material Concentrations: (See: Methods in Appendix B). At a minimum, the concentration of test material will be measured at each concentration level every week after an equilibrium has been established (See: A.1 and C.4). Water samples will be taken about midway between the top and bottom and the sides of the tank and will not include any surface scum or material stirred up from the bottom of the tank. At the end of each month water samples will be analyzed for the metabolite of the test material.

3. Measurement of Other Variables: Temperature and dissolved oxygen levels will be measured in the tanks daily, at least five days a week on alternating basis, so that each tank is analyzed once each week. However, if the test material or an additive causes a depression in dissolved oxygen, the test material concentration with the lowest dissolved oxygen concentration will be analyzed daily in addition to the above requirements.

   A control and one test concentration will be analyzed weekly for pH, alkalinity, hardness, acidity and conductance or more often, if necessary, to show the variability in the test water. However, if any of these characteristics are found to be affected by the test material, the tanks must be analyzed for the characteristics daily, at least five days a week, on an alternating basis so that each tank is analyzed once every other week.

   At minimum, the test water will be analyzed at the beginning and near the middle of the test for: calcium, magnesium, sodium, potassium, chloride, sulfate, total solids and total dissolved solids.

4. Residue Analysis:

   A. Water:

      i. Preliminary Study: During the first two weeks of the preliminary study the test material concentrations will be measured three times a week. During the remainder of the preliminary test, analysis for the test material will be made in each tank weekly.

ii Main Study: Water from each tank will be analyzed for test material daily for approximately two weeks prior to introduction of the eggs in the test or until an equilibrium concentration has been established, whichever is longer. Once the system is equilibrated, analysis for the test material will be made in each tank weekly.

B. Fish: Composite samples by group of eggs, juvenile and mature fish will be collected where possible. All attempts will be made to maximize the size of the samples. When possible the samples will be segregated by sex. All samples should be clearly identified.

These samples will be kept frozen at -20°F until shipment. The samples will be shipped to the sponsor for analyses within two weeks of collection. The analyses will be performed by the Metabolism and Residue Group of Diamond Shamrock Corporation.

C. Depuration: The half-life of the test material in fish has been determined using radio-tracer methods in another study. This data (submitted to the Environmental Protection Agency, November 15, 1972) indicates that the half-life is 7-10 days. Because of the availability of this data, a measurement of depuration will not be included in this study.

D. Statistics:

1. Duplicates: True duplicates will be used for each level of test material, i.e., no water connections between duplicate tanks.

2. Distribution of Tanks: The tanks will be assigned to locations by stratified random assignment (random assignment of one tank for each level of test material in a row followed by random assignment of the second tank for each level of test material in another or an extension of the same row).

E. Miscellaneous:

1. Additional Information: All routine bioassay flow-through methods not covered in this procedure will be followed as described in Methods for Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians (EPA-660/3-75-009, April, 1975).

2. Acknowledgements: This procedure was adapted from the procedures for the Pathead minnow compiled by John Eaton for the Committee on Aquatic Bioassays. The participating members of this committee are: Robert Andrew, John Arthur, Duane Benoit, Gerald Bouck, William Brungs, Gary Chapman, John Eaton, John Hale, Kenneth Hokanson, James McKim, Quentin Pickering, Wesley Smith, Charles Stephen and James Tucker.

XII REPORTING:

The final report will be the responsibility of the Diamond Shamrock study director. The contract laboratory will be responsible for submitting to Diamond Shamrock Corporation the following information and data:

1. Name and address of the facility performing the study and the dates on which the study was initiated and completed.

2. Objectives and procedures stated in the approved protocol, including any changes to the original protocol.

3. Data generated while conducting the study and any transformations, calculations or operations performed on the data.

4. Statistical methods employed for analysis of the data.

5. The test substances identified by name and/or code number and the date each batch (if more than one) was used.

6. Methods used.

7. Test system used. When animals are used, include the number in the study, sex, body weight range, source of supply, species, strain or substrain, age and procedure used for the identification of the test system.

8. Concentrations of the test material.

9. Deviations from the laboratory standard operating procedure.

10. The name of the Diamond Shamrock study director and the contract laboratory study director.

11. The results of the test submitted in a format which is in accordance with the discretion of the Diamond Shamrock study director.

12. The location where all raw data on the final report are to be stored.

13. The individuals responsible for the technical conduct and supervision of the study.

Any discussions of the results, summaries or conclusions will be the responsibility of the Diamond Shamrock study director.

Verified copies of all raw data will be submitted to Diamond Shamrock within 45 days of the termination of the study.

XIII CHANGING OF THE PROTOCOL:

A written statement will be submitted to the other party in the event either sponsor or contract laboratory desires to change the protocol. The contact laboratory must obtain approval from the sponsor before any changes in the protocol can be made. All verbal changes will be followed by a written change within 7 days.

XIV SAFETY AND HEALTH:

A. Laboratory personnel will practice good sanitation and health habits.

B. Any health or other condition that may be considered to adversely affect the study will be reported to the Diamond Shamrock study director.

C. Any injury obtained during this study which may be considered related to the test material will be reported to the Diamond Shamrock study director immediately.

XV SAFETY STANDARDS FOR RESEARCH INVOLVING RESEARCH CHEMICALS:

Every precaution should be taken to prevent inadvertent exposure of personnel and the environment to the test material. Applicable Federal, state and local regulations must be adhered to. The National Cancer Institute's Safety Standard for Research involving chemical carcinogens will be used as a guideline throughout the study (See: NCI-CG-TR-1, pp. 37-49).

XVI INTERIM PROGRESS REPORT:

At three month intervals the contract laboratory will submit a brief progress report to the sponsor, summarizing the study to date. Immediate contact with the Diamond Shamrock study director will be made when any event has occurred which has impact on the scientific interpretation or creditability of the study.

XVII SIGNATURES:

James C. Killeen, Jr., Ph.D.
Diamond Shamrock Corporation
Painesville, Ohio 44077

James C. Killeen Jr. DATE Feb 1 1978

George A. Cary
EG&G Bionomics
Wareham, Massachusetts 02571

George A Cary DATE 24 February, 1978

CIRCULATE XEROX

ARROWS	☐	☐
ENTLEY	☐	☐
UCCAFUSCO	☐	☐
UXTON	☑	☑
ARY	☐	☑
AVISON	☐	☐
LLS	☐	☐
ENOS	☐	☐
OSTER	☐	☐
HARTLEY	☐	☐
KRASNY	☐	☑
LeBLANC	☐	☐
LINDSAY	☐	☐
MACEK	☐	☐
PARRISH	☐	☐
PETROCELLI	☐	☐
SAUTER	☐	☑
SLEIGHT	☐	☐
WILSON	☐	☐
FILE	☑	☑
LIBRARY	☐	☐

T. R. Evans Research Corp.

Diamond Shamrock

January 17, 1979

George Cary
EG&G Bionomics
Aquatic Toxicology Laboratory
790 Main Street
Wareham, Massachusetts 02571

Dear George,

This letter will confirm our desire to make two protocol changes on the Chronic Fish Study with T-117-2. These changes are as follows:

1. The protocol (p. 7) calls for saving larvae not used in survival studies. The initial groups of larvae were not saved and there are significant questions about how they could be stored and what, if any, residue determinations could be made? After reviewing this section of the protocol I wish to delete the saving of larvae for future analysis because of the problems associated with sampling.

2. On page 10 of the protocol, the section describing reporting is somewhat unclear. It is my desire that Bionomics submit a report to include items one through thirteen and a discussion of the results. In my judgment the scientists at Bionomics are better qualified than I to interpret the data from this test.

If you have any questions please do not hesitate to contact me.

Since the above involves a change or clarification of the original protocol, I would like you to sign this letter on the appropriate lines below, returning a copy to me and maintaining the original with your protocol.

Best personal regards.

Sincerely,

James C. Killeen, Jr., Ph. D.

George Cary, EG&G Bionomics — DATE 23 Jan '79

jd

E G & G, BIONOMICS

NO. _____

REVISION _____

QUALITY ASSURANCE FORM

SUBJECT: RECORD OF TELEPHONE COMMUNICATION

TO: Distribution

FROM: Lewis A. Sleight III

DATE: 11/17/78

CLIENT: Diamond Shamrock

CLIENT REPRESENTATIVE: Dr. Killeen

JOB REFERENCE: J45 Phm Chronic

☐ Requested Client to send letter to document conversation

☒ Will send ~~this memo~~ ~~letter~~ to client to document conversation

☐ No documentation required

I called Jim Killeen and told him that we have observed what is probably gas bubble disease in several (4-5) individuals in the chronic — it is not concentration dependent.

Since our ΔT is now 10°C it seems logical that if we could pre heat the water we might eliminate the problem. So we shall switch to the heated water loop and document the change etc.

Jim concurs in our judgement.

(Continued on back)

cc: Cory Keasey ◇ Shamrock — Jim Killeen
Scutter
Dionne

Diamond Shamrock

	CIRCULATE	XEROX
BARROWS	☐	☐
BENTLEY	☐	☐
BUXTON	☐	☐
CARY	☐	☐
DAVISON	☐	☐
ELLS	☐	☐
ENOS	☐	☐
JUDGE	☐	☐
KRASNY	☐	☐
LeBLANC	☐	☐
MACEK	☐	☐
PARRISH	☐	☐
PETRUCELLI	☐	☐
SAUTER	☐	☐
SLEIGHT	☐	☐
WILSON	☐	☐
FILE	☐	☐
LIBRARY	☐	☐

T. R. Evans Research Center

July 25, 1978

George Cary
EG&G Bionomics
Aquatic Toxicology Laboratory
790 Main Street
Wareham, Mass. 02571

Dear George,

I would like to make the following changes in the protocol for the study entitled, "A Chronic Study in the Fathead Minnow" that is being conducted with test material T-117-2.

1. The stability tests described on page 3 of the protocol will be done at a concentration of 25 ppb instead of 50 ppm.

2. During the month of July water samples will be collected from a delivery tube of each group (total of seven samples) and analyzed.

3. Page 2, Part IX-C: Aqueous Solutions should read, "The solutions of the test material to be used in the fish tanks will be prepared with an automatic diluting system." In the original protocol the words, "with an automatic" were somehow omitted.

Since the above involves a change in the protocol, I would like you to sign this letter in the appropriate place below and return a copy to me.

Best personal regards.

Sincerely,

James C. Killeen, Jr., Ph.D.

PROTOCOL CHANGES NOTED AND ACCEPTED:

George Cary, Bionomics — 7 August '78 Date

jd

Diamond Shamrock

Environmental Labs

June 8, 1978

George Cary
EG&G Bionomics
Aquatic Toxicology Laboratory
790 Main Street
Wareham, Massachusetts 02571

Dear George,

This letter is to confirm the discussions I had with you and Ken Buxton of your laboratory during my visit on June 1, 1978. Frozen water samples were taken for the equilibration period for the chronic fish study with T-117-2 from May 15 to May 22 and non-frozen water samples were taken from May 23 to May 31. As we decided on June 1 it is felt that we have demonstrated that freezing the water samples affects the analysis of the test material at levels of less than 6.2 ppb. Since we will have complete analysis of all non-frozen samples during the equilibration period it has been decided that only the frozen samples from May 15 to May 18 and the 12.5 ppb level on May 22 are to be analyzed.

Water samples from June 1 and June 2, the days following the exposure of the eggs, will also be analyzed.

Since the above involves a change in the original protocol I would like you to sign this letter in the appropriate place below and return a copy to me.

Best personal regards.

Sincerely,

James C. Killeen, Jr., Ph.D.

George Cary

22 June, 1978
Date

jd

APPENDIX A

TEST (Evansville, Indiana) PHOTOPERIOD

FOR FATHEAD MINNOW FULL CHRONIC

Dawn to Dusk Times	Date	Day-Length (hour/minutes)	
6:00 - 4:45	Dec. 1	10:45	
6:00 - 4:30	15	10:30	
6:00 - 4:30	Jan. 1	10:30	
6:00 - 4:45	15	10:45	
6:00 - 5:15	Feb. 1	11:15	5-month pre-spawning
6:00 - 5:45	15	11:45	frowth period
6:00 - 6:15	Mar. 1	12:15	
6:00 - 7:00	15	13:00	
6:00 - 7:30	Apr. 1	13:30	
6:00 - 8:15	15	14:15	
6:00 - 8:45	May 1	14:45	
6:00 - 9:15	15	15:15	
6:00 - 9:30	June 1	15:30	4-month spawning
6:00 - 9:45	15	15:45	period
6:00 - 9:45	July 1	15:45	
6:00 - 9:30	15	15:30	
6:00 - 9:00	Aug. 1	15:00	
6:00 - 8:30	15	14:30	
6:00 - 8:00	Sep. 1	14:00	
6:00 - 7:30	15	13:30	
6:00 - 6:45	Oct. 1	12:45	Post spawning period
6:00 - 6:15	15	12:15	
6:00 - 5:30	Nov. 1	11:30	
6:00 - 5:00	15	11:00	

APPENDIX B

ANALYTICAL PROCEDURE FOR DETERMINATION OF T-117-2(TEST MATERIAL) AND ITS METABOLITE(T-114-1) IN WATER

The procedure presented here is specifically for determining the concentration of T-117-2 and its metabolite (T-114-1) in water.

A. T-117-2 (Test Material)

1. Transfer 50 ml of the water sample to a 150 ml beaker.
2. Add 2 ml of 0.4M $NaHCO_3$.
3. Adjust solution to pH 4.5 with the aid of a pH meter using 1N H_2SO_4.
4. Transfer the aqueous solution to a 125 ml separatory funnel with the aid of 50 ml isopropyl ether.
5. Shake thoroughly for 2 minutes. Allow the phases to separate.
6. Drain aqueous layer into a second 125 separatory funnel.
7. Transfer the isopropyl ether to a 125 ml Erlenmeyer flask which has been rinsed with acetone. Care must be taken so that no water is transferred with the ether.
8. Rinse the first separatory funnel with an additional 50 ml of isopropyl ether.
9. Transfer to the second separatory funnel and repeat the extraction.
10. Drain the aqueous phase into a 150 ml beaker and reserve for T-114-1 analysis if required.
11. Combine the isopropyl ether extracts.
12. Add 0.2 ml of 2% paraffin oil in toluene to the combined ether extracts.
13. Place the flask in a water bath at 35°C and concentrate to 0.5 ml with a gentle stream of dry air directed on the surface of the solvent to aid removal.
14. Remove flask from water bath and evaporate to dryness using air only.
15. Dissolve the remaining residue, containing T-117-2 in either benzene or toluene, and reserve for quantitation by electron capture gas chromatography.

    a. The final volume will vary upon the sensitivity of the instrument used for quantitation. This volume should be sufficient to permit a method detectability of 0.01 ppm.

B. T-114-1 (Metabolite).

16. Transfer the water remaining from step 10 to a 125 ml separatory funnel using 2 ml water and 50 ml isopropyl ether.

17. Add 2 ml of 1:1 concentrated H_2SO_4:water.

18. Shake thoroughly for 2 minutes. Allow the phases to separate

19. Drain aqueous layer into a 150 ml beaker.

20. Transfer the isopropyl ether to a 125 ml Erlenmeyer flask which has been rinsed with acidic water (<pH 1), deionized water, acetone and allowed to dry before use. Care must be taken so that no water is transferred with the ether.

21. Transfer the water back to the separatory funnel and repeat the extraction with another 50 ml isopropyl ether.

22. Combine the isopropyl ether extracts in the flask.

23. Place the flask in the water bath as previously described and evaporate to dryness.

24. Convert T-114-1 to its methyl ether by adding 3 ml of the diazomethane solution to the residue remaining in the flask.

    a. The diazomethane solution is prepared as published in Stanley, C.W., "Derivatization of Pesticides - Related Acids and Phenols for Gas Chromatographic Determination", J. Agric. Food Chem., 14(3), 321-323 (1966). The precursor needed for this reaction can be obtained from Aldrich Chemical Company as Cat. #12, 944-1.

25. Place the flask in a fume hood for 15 minutes.

26. Add 0.2 ml of 2% paraffin oil in toluene to the combined ether.

27. Evaporate to dryness using only a dry air stream.

28. Dissolve the residue in an appropriate volume (as described for T-117-2) of either benzene or toluene. T-114-1, as its methyl ether is quantitated by electron capture gas chromatography.

C. Quantitation of T-112-2 and T-114-1.

1. Instrumentation: Quantitate T-117-2 and T-114-1 on a gas chromatograph equipped with a ^{63}Ni electron capture detector. Temperature should be: injection-235°C, column: 200°C and detector: 300°C. Use a glass column (6' x 1/4" O.D.) equipped for on-column injection and packed with 6% DC-200 (12,500 cs) on 80/100 Gas Chrom Q with nitrogen carrier gas at 45 ml/min.

2. T-117-2:

a. Prepare a serial dilution of the analytical standard (T-119-1 is the analytical standard for T-117-2) in either benzene or toluene to a final concentration of 0.1 µg/ml.

b. Inject 1 µg to 4 µg of the 0.1 µg/ml standard solution.

c. Prepare a standard curve by plotting peak height versus concentration.

d. Dilute the sample until the portion injected into the gas chromatograph is within the linear range defined by the standard curve.

e. Determine the sample concentration from the standard curve.

3. T-114-1:

a. Prepare a serial dilution of analytical standard (T-114-1) in acetone to a final concentration of 1 µg/ml.

b. Add 1 ml of the 1 µg/ml standard to a flask and evaporate the solvent as described in steps 22 and 23.

c. Methylate the T-114-1 using the method as described for the samples in steps 24-28.

d. Bring to final volume of 10 ml in either benzene or toluene.

e. Use this solution (0.1 µg/ml) as the standard solution for quantitation.

f. Quantitate the T-11401 concentration in the samples exactly as previously described for T-117-2.

D. Determine the presence of any interference in final chromatograms by processing untreated water samples through entire analytical procedure.

E. Generate recovery values from untreated water fortified with both T-117-2 and T-114-1 at the level expected in treated samples and carried through analytical procedures.

APPENDIX II

Analytical Procedure for the Determination of T-117-2 in Water

A 4 mℓ volume of 0.4 M sodium bicarbonate solution was added to 100 mℓ water samples in a 250 mℓ beaker. The pH was adjusted to 4.5 with 1N H_2SO_4. The sample was then transferred with ethyl ether to a 250 mℓ separatory funnel and extracted for 2 minutes with a 100 mℓ volume of ethyl ether. The aqueous layer was drained into a second separatory funnel and extracted with a fresh 100 mℓ portion of ether. The water layer was transferred to a 250 mℓ beaker for analysis of T-117-2 as necessary, or discarded. The ether extracts were passed through a column containing 5 inches of anhydrous sodium sulfate and collected in a 400 mℓ beaker. A 50 mℓ rinse was passed through the column and added to the sample. A 0.2 mℓ portion of 1% ethylene glycol in benzene solution was added to the beaker which was then placed over a steam bath at 50°C and concentrated to ca. 5 mℓ using a gentle stream of clean, dry air. The sample was then evaporated to dryness using air only, and immediately transferred to a 10 mℓ glass-stoppered, graduated cylinder with 10.0 mℓ of Burdick & Jackson toluene.

The extract was then analyzed by gas/liquid chromatography using the following instrumental conditions:

Instrment: Perkin-Elmer Model 3920 gas chromatograph equipped with a 15 mCi NI-63 electron capture detector

Column: 6' x 2 mm (ID) Pyrex, packed with 10% SP2100 on 100/120 mesh Supelcoport

Temperatures (^{o}C): Column: 250
Inlet: 260
Interface: 275
Detector: 325

Gas Flows: Carrier - 50 cc/min. of 5% methane in Argon

Recorder: Perkin-Elmer Model 3920, 0-1 mV full-scale

Chart speed: 40 cm/hour

Attenuation: 512

Retention time: 3.0 min.

Response: Half-scale chart deflection for 0.15 ng T-117-2

Quality control samples were produced by adding 1.0 mℓ of a 0.091 µg/mℓ, a 1.0 µg/mℓ and a 4.7 µg/mℓ T-117-2 in acetone standards to 100 mℓ of glass-distilled water to produce concentrations of 0.91 µg/ℓ, 10 µg/ℓ and 47 µg/ℓ, respectively. Samples of glass-distilled water were also taken and left unfortified to be used as blanks. All quality control samples were analyzed by the above method with the results summarized in Table II-1.

The minimum detectable concentration of T-117-2 in water was 0.1 µg/ℓ for a 100 mℓ sample.

Analytical Procedure for the Determination of T-114-2 in Water

The aqueous sample containing T-114-2 was transferred to a 250 mℓ separatory funnel with a 4 mℓ rinse of glass-distilled water and acidified with 4 mℓ of 1:1 H_2SO_4. The sample was then extracted for 2 minutes with a fresh 100 mℓ portion of ethyl ether. The ether extracts were collected in a 400 mℓ beaker. The sample was cencentrated to ca. 5 mℓ over a 100°C steam bath after the addition of 0.2 mℓ of 1% ethylene glycol in benzene. The extract was transferred with ether to a 15 mℓ graduated centrifuge tube and evaporated to dryness using a 45°C water bath. The volume was adjusted to 5.0 mℓ with Burdick and Jackson methanol and an aliquot removed for analysis by HPLC using the following instrumental conditions:

Instrument: Waters Associates Model ALC/GPC - 204 liquid chromatograph equipped with a 440 UV absorbance detector

Column: 30 cm x 1/4" (OD) x 3.9 mm (ID) stainless steel, packed with Bondapak C^{18} (Waters Associates)

Recorder: Houston Instruments, Omniscribe^R, 0-10 mV full scale

Chart Speed: 0.5 cm/minute

Temperatures: Reservoir: ambient
Column: ambient
Detector: ambient

Mobile Phase: methanol/water 80/20 (v/v)

Pressure: 1000 psi

Flow Rate: 1.5 mℓ/minute

Sensitivity: 0.01 absorbance units full scale

Wavelength: 280 nm

Retention Time: 3.8 minute

Response: Half scale chart deflection for 25 ng T-114-2

Quality control samples were produced by adding 1 mℓ of a 1.9 µg/mℓ and 2 mℓ of 48 µg/mℓ T-114-2 in methanol standard to 100 mℓ glass distilled water to produce concentrations of 19 µg/ℓ and 960 µg/ℓ T-114-2. Samples of glass distilled water were also taken and left unfortified to be used as blanks. All quality control samples were analyzed by the above method with the results summarized in Table II-2.

The analytical results of all samples were corrected for the 91 percentage recovery of the method. The minimum detectable concentation of T-114-2 in water was 0.5 µg/ℓ for a 100 mℓ sample.

Table II-1 -- Recovery of T-117-2 from water.

Date: 4/25/78

Sample ID	T-117-2 Added (µg)	T-117-2 Recovered (µg)	% Recovery
Blank - A	-	<0.01	-
B	-	<0.01	-
C	-	<0.01	-
0.91 ppb - A	0.091	0.075	82
B	0.091	0.071	78
C	0.091	0.068	75
10 ppb - A	1.0	0.88	88
B	1.0	0.91	91
C	1.0	0.94	94
47 ppb - A	4.7	3.7	79
B	4.7	3.9	83
C	4.7	4.1	87

Average Recovery = 84.1 ($\pm$ 9.2)%

Table II-2 -- Recovery of T-114-2 from water.

Date: 9/10/78

Sample ID	T-114-2 Added (µg)	T-114-2 Recovered (µg)	% Recovery
Blank - A	-	<0.05	-
B	-	<0.05	-
19 µg/ℓ - A	1.9	1.9	100
C	1.9	1.6	84
D	1.9	1.9	100
960 µg/ℓ - A	96	82	85
B	96	86	90
C	96	82	85

Average Recovery - 90.7 ($\pm$ 7.5)%

APPENDIX III

Analytical procedure for the determination of pesticides and PCB in brine shrimp tissue

Brine shrimp samples (ca. 10 g) were prepared for gas chromatographic analysis by extracting the sample twice with 30 mℓ portions of 1:1 diethyl ether/hexane for one minute using a Polytron PT 20 Homogenizer. The sample was centrifuged between extractions and the extracts filtered through anhydrous sodium sulfate into a Kuderna-Danish concentrator equipped with a 10 mℓ graduated evaporator tube. The extract was concentrated over a steam bath and the volume adjusted to exactly 5.0 mℓ.

A 3.0 mℓ portion of the concentrate was transferred to a 0.9 x 25 cm Pyrex chromatographic column containing 2.3 g of activated (130°C) Florisil 60/100 mesh with a 1 cm layer of anhydrous sodium sulfate above it. The column was pre-rinsed with 50 mℓ of hexane before sample application.

The column was eluted with a 50 mℓ volume of 6% diethyl ether in hexane to remove PCB and pesticides, except Endrin and Kepone, which are then stripped from the column with a 50 mℓ portion of 1% methanol in benzene. The 6% diethyl ether in hexane fraction was concentration to ca. 2 mℓ for silica gel chromatography. The 1% methanol in benzene fraction was concentrated to 5.0 mℓ for gas chromatographic analysis. Both volume reductions were carried out over a steam bath using a gentle stream of clean dry air.

The concentrated 6% diethyl ether in hexane fraction was transferred to a 0.9 x 25 cm pyrex chromatographic column containing 3.0 g of activated (150°C) grade 922 Silica Gel. The column was pre-rinsed with a 50 m volume of pentane before sample application.

The column was eluted with a 50 mℓ volume of pentane followed by a 50 mℓ volume of 1% methanol in benzene using 2-3 psi nitrogen gas pressure. The fractions were collected separately, concentrated to 5.0 mℓ using a gentle stream of clean dry air, and analyzed by gas-liquid chromatography with the fraction pattern listed in Table III-1 and retention time and response in Table III-2.

Gas chromatographic analyses were performed using the following instrumental conditions:

Instrument: Perkin-Elmer Model 3920 gas chromatograph equipped with 15 mCi NI-63 Electron Capture detector

Recorder: Perkin-Elmer Model 023; 0-1 mV full scale

Column: 6' x 2 mm (ID) Pyrex packed with 3% OV-10, 80/100 mesh Supelcoport.

Temperatures (°C): Column: 200
Inlet: 250
Interface: 250
Detector: 350

Gas flows: Carrier: 50 cc/min. 55 methane/95% argon

Chart Speed: 40 cm/hr.

Attenuation: x 32

Calibration curves were produced by plotting peak height (mm) versus weight (ng) of standard injected. Analytical standards were prepared by dilution of analytical pesticide and PCB standards with hexane to yield working standards of the required concentrations. A mixed standard was used for all the pesticides quantitated except chlordane. Separate analytical standards were used for chlordane and Arochlor 1254. Arochlor 1254 and chlordane were each quantitated based on a single isomer peak.

Table III-1 -- Silica gel fractionation for pesticide analysis of San Francisco Bay Brand frozen brine shrimp and eggs.

Compound	Pentane	1% Methanol in Benzene
Aldrin	x	
Heptachlor	ca. 5%	ca. 95%
Chlordane	ca. 5%	ca. 95%
Arochlor 1254	x	
Mirex	x	
Lindane		x
o,p'DDE		x
Dieldrin		x
pp' DDD		x
pp' DDT		x
Methoxychlor		x

Table III-2 -- Retention time and responses for pesticide analysis of San Francisco Bay Brand frozen brine shrimp and eggs.

Compound	Retention time, min.	Half-scale chart-response (pg)
Lindane	1.0	160
Heptachlor	1.6	240
Aldrin	2.2	220
o,p' DDE	3.3	500
Dieldrin	4.2	500
p,p' DDD	5.4	500
Endrin	8.2	1,500
Methoxychlor	10.9	3,500
p,p' DDT	7.2	1,500
Mirex	13.4	1,600
Arochlor 1254	6.1[a]	250
Chlordane	1.5[a]	200

[a] Isomer used for quantitation.

SUBMITTED BY:

E G & G, Bionomics
Aquatic Toxicology Laboratory
790 Main Street
Wareham, Massachusetts
January, 1980

PREPARED BY: Emily Dionne

Emily Dionne

Assistant Biologist

STUDY DIRECTOR: George A. Cary

George A. Cary Jr.

Director, Aquatic Biology

APPROVED BY: Kenneth J. Macek, Ph.D.

Kenneth J. Macek

Manager

Kenneth Buxton

Kenneth Buxton Jr.

Director of Analytical Chemistry

AMENDMENT I

Report #BW-79-6-443

A. Reference to test concentrations refers to mean measured concentrations for the entire study, unless otherwise specified.

B. Abnormalities referred to in Tables 9 and 10 gave no indication of being toxicant-related.

C. Live Daphnia magna used as a food supplement were not analyzed as they were cultured under controlled conditions at EG&G, Bionomics.

D. Amendment I has been signed by the Manager, Aquatic Toxicology Laboratories, who has taken over the responsibilities of the Director of Aquatic Biology.

Emily Dionne Date 1-29-80

Assistant Biologist

Bruce A. Sleight III Date 1-29-80

Manager, Aquatic Toxicology Laboratories