EPA-600/3-75-013
November 1975

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TOXAPHENE EFFECTS ON REPRODUCTION, GROWTH, AND MORTALITY OF BROOK TROUT

Environmental Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
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TOXAPHENE EFFECTS ON REPRODUCTION, GROWTH, AND MORTALITY OF BROOK TROUT

by

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Contract No. EPA-IAG-0153 (D)

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ABSTRACT

Yearling brook trout (Salvelinus fontinalis) were continuously exposed to toxaphene (0, 39, 68, 139, 288, and 502 ng/l) in a flow-through diluter system. Day length and water temperature were altered monthly to correspond to natural conditions. Adult growth was reduced in the 288 and 502 ng/l toxaphene exposures, and the added stress of spawning activities caused extensive mortalities in these concentrations. The numbers of eggs spawned and percent viability were inversely related to increasing toxaphene concentrations. All groups of fry exposed to toxaphene had reduced rates of growth and survival. Biochemical investigations on fry backbones demonstrated that bone collagen may be a sensitive indicator of normal and abnormal growth and development prior to being observed in the whole fish. Toxaphene was accumulated by brook trout 5,000 to 76,000 times that in the water and the more chlorinated isomers of toxaphene were preferentially stored.

This report was submitted in fulfillment of Contract Number EPA-IAG-0153 (D) by the Fish-Pesticide Research Laboratory, Fish and Wildlife Service (USDI) under the sponsorship of the Environmental Protection Agency. Work was completed March 1974.

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ACKNOWLEDGMENTS

Assistance in the diluter construction and maintenance by W. A. McAllister, D. D. Holz, and L. McLain was greatly appreciated. We thank Ms. M. E. DeClue, O. Yarbrough, and S. Cummins for their technical assistance in performing the collagen, calcium, and phosphorous assays. Also, special thanks to P. L. Crutcher, Pathologist, for conducting the histopathological examinations. Toxaphene residue analyses were conducted by J. N. Huckins with assistance from J. L. Johnson. Portions of the study were funded by the Fish and Wildlife Service, United States Department of the Interior.

SECTION I

CONCLUSIONS

1. Growth of yearling brook trout is reduced through continuous exposure to 288 and 502 ng/l of toxaphene for six months.

2. Increased stress due to spawning activities in the presence of toxaphene results in high rates of mortality at toxaphene concentrations of 288 and 502 ng/l.

3. Toxaphene concentrations of 68 ng/l and higher reduce egg viability.

4. Toxaphene increases the ratio of minerals to organic content in the vertebral column of brook trout fry during a 90-day exposure. The no-effect concentration of toxaphene is less than 39 ng/l.

5. The effects of chronic toxaphene exposure on brook trout fry growth and development are predictable by collagen measurements on fry after only seven days.

6. The no-effect concentration of toxaphene on growth of brook trout fry is below 39 ng/l after 90 days of exposure.

7. Toxaphene was accumulated by brook trout 5,000 to 76,000 times that in water.

8. The more chlorinated toxaphene isomers are preferentially stored by brook trout while the less chlorinated ones are more rapidly eliminated.

9. The maximum acceptable toxicant concentration of toxaphene in water is below 39 ng/l for brook trout.

SECTION II

RECOMMENDATIONS

1. Additional research on other aquatic organisms is recommended to determine more accurately the no-effect concentration of toxaphene.

2. Consideration should be given to using hydroxyproline and collagen measurements as early biochemical indicators of growth and developmental changes in fishes.

3. Utilizing the data in this study, water concentrations of toxaphene should be below 39 ng/l to protect aquatic life.

SECTION III

INTRODUCTION

The extensive use and persistence of organochlorine insecticides have resulted in their widespread occurrence in the environment. Their presence in aquatic habitats as well as their adverse effects on aquatic organisms have been reported[1,2]. Although much of the use of organochlorine insecticides has been reduced in recent years, some are still extensively used; 30 to 40 million pounds of toxaphene are applied annually on crops and livestock in the United States[3]. Toxaphene is the technical grade of chlorinated camphene, containing 67-69% chlorine. The empirical formula is $C_{10}H_{10}Cl_8$. Its primary use is as an insecticide on cotton, but it is also registered for use on certain grains, alfalfa, fruit, and vegetables. Since the use of DDT was restricted in 1969, toxaphene has often been used to replace it, both by itself and in combination with other insecticides[4].

Previously, there have been few reports of toxaphene residues in fish, water, or food products. However, toxaphene has been identified in analysis of water and tissue samples[5-7]. Due to the numerous toxaphene isomers ($\simeq$175)[8], it is unlikely that most routine gas chromatographic analyses would be sensitive to less than 1-5 µg/l of toxaphene in water or 0.5 µg/g in whole fish. The presence of polychlorinated biphenyls, other organochlorine insecticides, and phthalic acid esters in environmental samples further complicate analytical results unless special separation and cleanup or other special procedures are used. For example, Nicholson, et al.[9] measured the annual cycle of toxaphene residues in a river and found them to range from 7 to 410 ng/l. However, this level of sensitivity was possible only by collection of toxaphene from 19 m^3 of water on carbon.

The persistence of toxaphene in aquatic environments was demonstrated in past years when it was evaluated as a piscicide[10-20]. Detoxification times for lakes that had been treated with toxaphene ranged from four weeks to over six years based on fish mortality and residue studies. The time required for detoxification of lentic waters was dependent on physical and chemical characteristics of the water. Detoxification rates of toxaphene were apparently greater in more eutrophic situations.

Several investigators have observed acute effects of toxaphene on aquatic organisms[4,21-38]. However, little data are available from which the chronic effects of toxaphene on fish and aquatic invertebrates or its biological significance in the aquatic environment can be assessed. We therefore undertook the following study to assist the U.S. Environmental Protection Agency in establishing water quality criteria and standards for toxaphene. The objectives of the study were: 1) to determine the effect of continuous exposures of toxaphene on brook trout (Salvelinus fontinalis) growth, reproduction, and mortality; 2) to investigate potential physiological and biochemical "predicators" of abnormal growth and development; 3) to determine the accumulation of toxaphene in several life stages of brook trout; 4) to determine the residual isomer changes and elimination rates of toxaphene.

SECTION IV

MATERIALS AND METHODS

GROWTH, REPRODUCTION, AND MORTALITY

This portion of the study basically was conducted according to the recommended procedures for partial chronic tests with brook trout by EPA[39]. Yearling brook trout were obtained from the Manchester National Fish Hatchery, Iowa, on April 3, 1972. The fish were treated with 2 mg/l of Hyamine 3500® continuously for one hour daily for three consecutive days. On April 10, 26 fish, 12 for growth and 14 for residue studies, were placed in each of 12 stainless steel tanks measuring 51 cm deep x 137 cm long x 36 cm wide and having a water depth of 30 cm. Well water (Table 1) was delivered to the tanks at a rate of 800 ml/min/tank through a proportional diluter system modeled after Mount and Brungs[40]. Each tank was aerated with filtered air to maintain oxygen concentrations above 70% of saturation. The adult fish were fed the Modified Oregon Test Diet[41] ad libitum throughout the study. The young fry were fed a commercial trout starter (EWOS).

All fish were weighed and measured (total length) on April 22, and 12 fish from each tank were tagged with surgical wound clips attached to the anterior base of the dorsal fin to follow growth on the same group of fish throughout the study. Growth was determined on the twelve tagged fish on August 11 and again on October 5. After the last growth determination, the fish were thinned to two males and four females per tank and two spawning substrates were placed in each tank. Fifty eggs from each spawning were placed in incubator cups for hatchability and subsequent growth determinations on the surviving fry. After spawning ceased, four adults from each tank were preserved for pathological evaluation. Most of the remaining eggs from each spawning (≠250) were placed in separate incubator cups for determining viability (formation of neural keel after 11-12 days). All eggs died in the eyed stage and the mortality appeared to be caused by a covering of a clear slime bacteria. Additional eggs were obtained from White Sulphur Springs National Fish Hatchery, West Virginia on January 12, 1973. One hundred eggs were placed in each of six incubator cups per duplicate tank. The eggs were exposed to toxaphene for 22 days before the median hatch date. When hatching

Table 1. CHEMICAL CHARACTERISTICS OF WELL WATER AT THE FISH-PESTICIDE RESEARCH LABORATORY

Analyte	Specified sensitivity limits, mg/l	Concentration, mg/l
Ca	0.1	70
Mg	0.1	27
K	0.5	3.9
SO_4	0.01	4.4
NO_3	0.05	< 0.05
NO_2	0.05	< 0.036
NH_4/N	0.01	0.066
Phenol	0.001	<0.001
Cl_2	0.001	<0.001
Cl	0.01	29
F	0.01	0.34
CN	0.005	0.006
Fe	0.01	0.014
Cu	0.001	0.0045
Zn	0.001	< 0.001
Cd	0.001	< 0.0005
Cr	0.01	< 0.01
Pb	0.001	0.0015
Alkalinity	1.0	237
Hardness (EDTA)	1.0	272
pH	0.1	7.4
Temperature	± 0.5 C	16 C

was complete, four groups of 25 fry each per duplicate tank were selected from the incubation cups and placed in growth chambers measuring 14 cm deep x 38 cm x 15 cm wide and having a water depth of 10 cm. The remaining fry were placed in the adult tank for the physiological and residue dynamics portions of the study. Total lengths of the fry were determined by the photographic method of McKim and Benoit[42] immediately after completion of hatching, and at 30, 60, and 90 days thereafter. The weight of the fry was measured at 90 days. Mortalities were recorded daily.

A diluter system with the modification of McAllister, Mauck, and Mayer[43] and a dilution factor of 0.5 between the concentrations was used to deliver five concentrations of toxaphene and a control for the chronic test. The nominal toxaphene concentrations were 0, 41, 75, 125, 270, and 500 ng/l. The concentrations were selected to include an estimated no-effect concentration (108 ng/l) which was determined by multiplying the 96 hr LC50 value (10.8 µg/l) by 0.01 as recommended in establishing water quality criteria[44]. Toxaphene concentrations in the water of each exposure tank were measured weekly, and the average measured concentrations were 0, 39, 68, 139, 288, and 502 ng/l with an analytical sensitivity of 10 ng/l. Acetone was used as the carrier solvent for toxaphene and did not exceed 0.28 ml/liter. An experimental-use sample of toxaphene (X-16189-49) was furnished by Hercules Inc. and was used throughout the study. Flow-splitting chambers designed by Benoit and Puglisi[45] were utilized to thoroughly mix and divide each toxaphene concentration for delivery to the duplicate adult exposure tanks. The water temperature in the tanks was controlled by Min-O-Cool® refrigeration units suspended in a circulating water bath. Artificial daylight was provided by the method of Drummond and Dawson[46] . The water temperature regime and photoperiod were those recommended by EPA for brook trout tests[40].

An acute toxicity test of toxaphene on yearling brook trout was also conducted. The lethal threshold concentration (determined when the rate of death was 10% or less of the original number of fish in any concentration during the preceding 24 hour period) was determined following recommendations by Eaton[47]. A proportional diluter delivering five concentrations and a control and with a dilution factor of 0.75 between the concentrations was utilized. The test was conducted at 10 C in the same size exposure tanks as were used in the chronic study. Twenty fish were exposed to each concentration, and mortalities were recorded daily.

The design of the chronic study was a randomized block design. Growth data of both the adults and fry were analyzed by analysis of variance to determine if significant differences existed. A

multiple means comparison test (least significant difference) was used to compare treatments. The effects of toxaphene on egg viability were determined by conducting an analysis of variance on the arcsin transformation for proportions (angle = arcsin $\sqrt{\text{percentage}}$)[48] followed by a least significant difference test. Mortalities occurring in adults and fry were analyzed by the binomial chi-square analysis for data arranged in two classes[49]. LC50s for the acute toxicity test were calculated by the method of Litchfield and Wilcoxon[50].

PHYSIOLOGY AND BIOCHEMISTRY

Eight brook trout fry from each concentration were sampled at 7, 15, 30, 60 and 90 days after the median hatch date. The fry from the 7 and 15 day samples were blotted to remove excess water, then frozen on dry ice. Individual fry were weighed, homogenized in 2 ml 10% trichloroacetic acid, and centrifuged for 5 minutes (1500 rpm). The supernatant was discarded, and the protein precipitate was washed twice with distilled water. Hydrolysis of the protein precipitate was performed at 120 C in 3 ml of 6N HCl for 24 hours. The hydrolysate was diluted to 25 ml with distilled water. Hydroxyproline (HyP) was determined in a 2 ml aliquot according to the method of Woessner[51]. Detection of HyP in the protein hydrolysate was used as the indicator for collagen synthesis, because HyP is restricted to collagen or elastin in animal tissues. Since the total amount of elastin is very small in comparison with that of collagen, and since the hydroxyproline content is only about one-tenth as high in elastin, its contribution to the total hydroxyproline content is negligible compared with that of collagen.[53] Fry from the 30, 60, and 90 day samples were blotted and placed on dry ice. After the fry were frozen, the backbone was removed. The backbone of each fry was dried at 110 C in a forced air oven for 2 hr, then weighed. The dried bone was subjected to hydrolysis at 120 C in 3 ml 6N HClovernight. The hydrolysate was diluted to 10 ml with distilled water. HyP was determined in the hydrolysate as previously mentioned. Phosphorous was determined on the hydrolysate by a modification of the Fiske and Subbarow[54] spectrophotometric method. Calcium was assayed in the hydrolysate using atomic absorption spectrophotometry. The percision of each of the methods varied by less than 3%, and the recovery from spiked samples was 95-99%.

The amount of HyP found in the bone hydrolysates was used as a direct measurement for collagen in the bone. The percent collagen in the backbone was also estimated by an indirect method. Pooled

samples of backbones from approximately 20 fry at each of the 30, 60, and 90 day sampling periods were assayed for pure collagen according to the method of Flanagan and Nichols[55]. The concentration of HyP in pure collagen was measured and used to derive a factor for converting the concentration of HyP in backbone hydrolysates to concentration of collagen in the backbone. The following formula was used to estimate the amount of collagen in the backbone:

$$\frac{\text{g HyP}}{\text{g bone}} \times \frac{1}{\frac{\text{g HyP}}{\text{g collagen}}} = \frac{\text{g collagen}}{\text{g bone}}$$

The percent of HyP in the pure collagen extracts was 6.51, 6.56, and 6.61 in the 30, 60, and 90 day samples, respectively. The average of the three values, 6.56%, was used to derive the conversion factor for each group. The data were analyzed by analysis of variance and the least significant difference multiple means comparison test.

RESIDUE DYNAMICS

The sampling schedule for yearling brook trout during the 161 day uptake phase of the study is presented in Table 2. The fish sampled from the 39, 139, and 502 ng/l toxpahene exposures were reserved to determine which toxaphene isomers are accumulated or eliminated by brook trout. However, total toxaphene residues were determined on all fish analyzed. Toxaphene residues were determined on both fillet and offal of four adult fish from each toxaphene concentration after 161 days of exposure. Upon completion of the uptake phase, twelve adult fish from each concentration were placed in uncontaminated water at 9 C to determine the elimination rate of toxaphene. Three fish were sampled from each exposure after 7, 14, 28, and 56 days in the fresh water. Yearling and adult fish were analyzed individually. Thirty, 25, 20, 15, and 10 brook trout fry were sampled from each duplicate tank after 7, 15, 30, 60, and 90 days of exposure, respectively. The fry sampled from each duplicate were pooled for residue analysis, since at least two grams of tissue were required for analysis. All fish were frozen immediately after sampling.

Various sources of polychlorinated biphenyls (PCBs) and phthalic acid ester (PAE) contaminants were major obstacles in the measurement of low concentrations of toxaphene in water and fish. A major source of PCB contamination was found to be in the air used

Table 2. SAMPLING SCHEDULE FOR TOXAPHENE RESIDUES IN YEARLING BROOK TROUT
(number/concentration)

Days of Exposure	Water concentration, ng/l					
	0	39	68	139	288	502
1		4		4		4
3		4		4		4
7	4		4		4	
10		4		4		4
18	4		4		4	
24		4		4		4
60	6	4	6	4	6	4
140	6	4	6	4	6	4
161	8	4	8	4	8	4

to aerate water in the test tanks. Activated carbon filters were installed in the air lines which eliminated that source of contamination. Another source of contamination by PCBs and PAEs was avoided by using redistilled acetone in preparing stock solutions of toxaphene. Well water used in the study was checked for the presence of PCB and PAEs, but none were detected at a sensitivity of 10 ng/l.

Physical reduction by grinding followed by extraction of the fish tissues were accomplished by the procedures of Benville and Tindle[56] and Hesselberg and Johnson[57]. Initial sample cleanup was automated gel permeation chromatography[58] followed by modified silicic acid chromatography[59]. Quantitation of toxaphene residues was done by gas liquid chromatography (GLC) with ^{63}Ni-electron capture detection. A 2.1 m long x 2 mm i.d. coiled glass column packed with 3% (w/w) OV-7 on chromosorb W-hp was utilized with a nitrogen flow rate of 40 ml/min. A column temperature of 200 C was used for total toxaphene residues to reduce the long retention time, and a column temperature of 180 C was optimum for toxaphene isomer resolution. The minimum detection limit of toxaphene in fish tissue was 0.05 µg/g, but below 0.1 µg/g was difficult to quantitate. Complete details on the toxaphene residue technology used in this study are described by Stalling and Huckins[60]. Recovery of toxaphene from spiked tissue samples was 97% & 100%, from water spiked at 100 ng/l was 97% & 104%, at 50 ng/l was 75.6% & 84.7% and at 25 ng/l was 44% & 56%.

SECTION V

RESULTS AND DISCUSSION

GROWTH, REPRODUCTION, AND MORTALITY

After three months of exposure to toxaphene, the yearling brook trout were easily excitable and of lighter coloration in the 288 and 502 ng/l concentrations. The presence or absence of fin erosion appeared to be an index to the excitability of the fish. While conducting the three month growth measurements, examination of the fish revealed an incidence of fin erosion of 3, 7, 3, 10, 31, and 50% in the 0, 39, 68, 139, 288, and 502 ng/l concentrations, respectively. By late August, the nervousness had subsided and most of the eroded fins had healed. Warner, Peterson, and Borgman[61] also reported an increased responsiveness to external stimuli in goldfish (Carassius auratus) continuously exposed to 1.8 µg/l of toxaphene for 96 hours.

The last measurements to determine the effect of toxaphene on growth were completed October 5. The growth of the fish was significantly decreased ($P<0.05$) in the 288 and 502 ng/l toxaphene concentrations only after six months of continuous exposure (Table 3). All fish visually appeared to be in good health and no mortality had occurred. However, just prior to spawning, the additional stress of physiological changes occurring before and during spawning caused a 50 and 100% mortality in the 288 and 502 ng/l toxaphene concentrations, respectively (Fig. 1). Eight percent of the fish died in each of the 39, 68, and 139 ng/l concentrations, but no fish died in the controls. Histopathological analyses of selected tissues indicated atrophy of liver cells, degeneration of pancreatic acinar tissue, and proliferation of interrenal tissues of the kidney in all fish exposed to toxaphene.

The 96-hr LC50 of toxaphene for 16-month-old brook trout was 10.8 µg/l (Table 4) and was approximately 38 times that amount of toxaphene killing 50% of the fish in the chronic study.

Egg viability was reduced at a much lower concentration of toxaphene than was parental growth (Table 5). Concentrations of toxaphene of 68 ng/l and higher significantly reduced viability ($P<0.05$). No relationship between the number of females spawning or numbers of spawns and toxaphene exposure was evident except in the highest concentration. The reduction in the number of females spawning and numbers of spawns in the high concentration was due to the extensive mortality prior to spawning. The number of eggs per spawn tended to be less in the higher toxaphene concentrations, but the difference was not statistically significant.

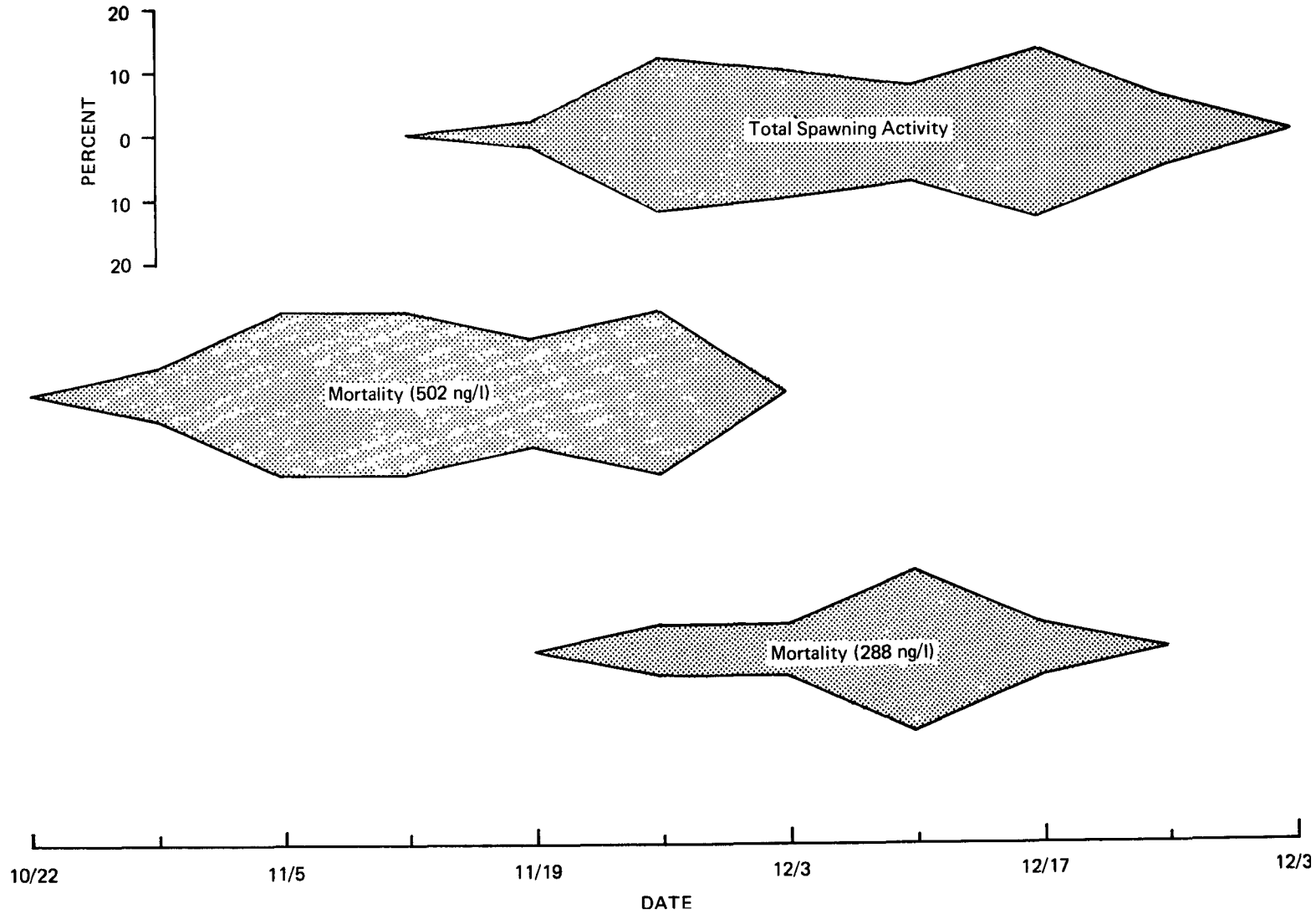

Figure 1. Relationship between mortality and spawning activity of adult brook trout exposed to 288 and 502 ng/l of toxaphene. Spawning activity was based on the number of spawns in time of all groups of fish expressed as a percentage.

Table 3. THE EFFECT OF TOXAPHENE ON YEARLING BROOK TROUT GROWTH

Exposure, ng/l	Statistic	April 22, 1972		July 11, 1972		Gain (4/22-7/11)	
		Length, mm	Weight, g	Length, mm	Weight, g	Length, mm	Weight, g
Control[a] (0)[b]	Mean	229	141	247	170	17	29
	S.D.[c]	14	32	17	37		
41 (39±3)	Mean	233	134	247	165	14	31
	S.D.	10	15	14	27		
75 (68±4)	Mean	228	131	245	161	17	30
	S.D.	9	20	9	20		
125 (139±10)	Mean	227	137	246	169	19	32
	S.D.	14	30	16	32		
270 (288±22)	Mean	234	145	248	167	13	22
	S.D.	14	32	15	27		
500 (502±40)	Mean	223	126	238	144	15	18
	S.D.	13	22	11	29		

Table 3. (continued)

Exposure, ng/l	Statistic	October 5, 1972		Gain (4/22-10/5)	
		Length, mm	Weight, g	Length, mm	Weight, g
Control[a] (0)[b]	Mean	290	281	60	140
	S.D.	16	51		
41 (39±3)	Mean	288	276	55	142
	S.D.	13	43		
75 (68±4)	Mean	279[d]	264	52	133
	S.D.	11	38		
125 (139±10)	Mean	283	272	56	135
	S.D.	17	58		
270 (288±22)	Mean	280[d]	252[d]	46[d]	107[d]
	S.D.	16	46		
500 (502±40)	Mean	268[d]	206[d]	45[d]	80[d]
	S.D.	17	45		

[a] Nominal concentration.

[b] Measured concentration ± the standard error.

[c] Standard deviation.

[d] Significantly different from controls ($P<0.05$).

Table 4. LC50's (AND 95% CONFIDENCE LIMITS) OF TOXAPHENE TO YEARLING BROOK TROUT[a] DETERMINED DAILY

(µg/1)

3 days	4 days	5 days	6 days	7 days	8 days
10.4 (9.7-11.2)	10.8 (9.1-12.8)	7.5 (6.8-8.3)	5.8 (5.5-6.1)	5.2 (4.9-5.5)	4.9 (4.6-5.3)

9 days	10 days	11 days	12 days	13 days	14 days
4.4 (4.1-4.8)	4.2 (3.9-4.7)	4.1[b] (3.8-4.4)	3.8 (3.4-4.2)	3.7 (3.3-4.1)	3.6 (3.2-4.0)

[a] Weight = 133 g ± standard deviation of 33, length = 231 mm ± 19.

[b] Lethal threshold concentration according to requirements described by Eaton[47].

Table 5. THE EFFECT OF TOXAPHENE ON BROOK TROUT REPRODUCTION

Exposure, ng/l	Number of females spawning	Number of spawns	Eggs per spawn	Eggs per female	% viability
Control[a] (0)[b]	4	9	380	855	69
41 (39±3)	6	8	406	541	60
75 (68±4)	6	9	344	516	22[c]
125 (139±10)	6	12	271	542	15[c]
270 (288±22)	4	7	264	462	6[c]
500 (502±40)	1	1	617	617	0[c]

[a] Nominal concentration.

[b] Measured concentration ± the standard error.

[c] Significantly different from controls (P 0.05).

Due to the loss of the eggs in the first part of the study, additional eyed eggs were obtained and exposed to toxaphene for 22 days before hatching. No effect on hatching was observed. The resulting fry were continuously exposed to toxaphene for an additional 90 days for growth determination.

Growth of the fry as measured by length, was significantly affected ($P<0.05$) after 30 days of toxaphene exposure in the 139 and 288 ng/l concentrations (Table 6). All fry in the 502 ng/l treatments were dead by the end of the 30 day period. After 60 days exposure, growth was significantly reduced in all groups of fish exposed to toxaphene (39-502 ng/l). The effects on the weight of fry were the same as on length at 90 days.

Mortality of the brook trout fry in all toxaphene concentrations was higher than that in the control fish at 30 days and thereafter (Table 7). The mortality rate of fry in the controls was also high during the first 60 days of exposure and all fish fed poorly. However, no disease organisms or other problems could be found. The rate of mortality in the control fish decreased after 60 days of exposure and the fish began feeding normally.

In our 12 month study, the no-effect concentration of toxaphene to brook trout under continuous exposure conditions was below 39 ng/l based on fry growth and mortality. Nicholson, et al.[9] reported toxaphene concentrations ranging from 7-410 ng/l in water from Flint Creek, Alabama. Concomitant studies by Grzenda, et al.[62] were conducted on zooplankton, bottom fauna, and fish populations of Flint Creek. There was no convincing evidence that continuous toxaphene contamination resulted in any gross damage to the aquatic organisms. The fish that Grzenda, et al. studied were warm water fish and may have been more tolerant to toxaphene. Also, the fish in Flint Creek may have developed resistance to toxaphene as described by Ferguson, et al.[63] in Mississippi.

Mount and Stephan[64] proposed that an application factor, calculated by dividing the maximum acceptable toxicant concentration (MATC), the highest continuous toxicant concentration that has no adverse effect on growth, reproduction, and survival, by the 96-hr LC50 value, be used to determine safe concentrations of toxic pollutants. However, Eaton[47] states that the lethal threshold concentration might provide better acute values for calculating application factors than 96-hr LC50 values. Further, Mount and Stephan suggest that the application factor for a given toxicant,

Table 6. BROOK TROUT FRY GROWTH AS AFFECTED BY TOXAPHENE

Exposure, ng/l	Statistic	Fry length, mm				Fry weight at 90 days, g
		0 day	30 days	60 days	90 days	
Control[a] (0)[b]	Mean	16.5	23.4	34.3	42.0	0.81
	S.D.[c]	0.8	1.2	4.4	9.2	0.60
41 (39±3)	Mean	16.8	23.3	31.4[d]	35.8[d]	0.44[d]
	S.D.	1.1	2.2	3.3	6.2	0.30
75 (68±4)	Mean	16.8	23.6	31.3[d]	38.3[d]	0.59[d]
	S.D.	1.1	2.0	3.4	8.2	0.44
125 (139±10)	Mean	16.8	22.0[d]	30.8[d]	35.1[d]	0.43[d]
	S.D.	1.2	2.1	4.3	7.7	0.37
270 (288±22)	Mean	16.3	19.4[d]	-[e]	-	-
	S.D.	1.0	1.6	-	-	-
500 (502±40)	Mean	16.4	-	-	-	-
	S.D.	1.0	-	-	-	-

[a]Nominal concentration.

[b]Measured concentration ± the standard error.

[c]Standard deviation.

[d]Significantly different from controls ($P<0.05$).

[e]All fish were dead.

Table 7. MORTALITY IN BROOK TROUT FRY CONTINUOUSLY EXPOSED TO TOXAPHENE (%)

Exposure, ng/l	15 days	30 days	45 days	60 days	75 days	90 days
Control[a] (0)[b]	1	21	39	52	62	64
41 (39±3)	11[c]	40[c]	60[c]	67[c]	77[c]	83[c]
75 (68±4)	14[c]	50[c]	62[c]	69[c]	75[c]	78[c]
125 (139±10)	6[c]	47[c]	66[c]	71[c]	79[c]	82[c]
270 (288±22)	4	66[c]	100[c]	-	-	-
500 (502±40)	8[c]	100[c]	-	-	-	-

[a] Nominal concentration.

[b] Measured concentration ± the standard error.

[c] Significantly different from controls ($P<0.05$).

experimentally determined for one species of fish in one type of water, might be applicable to other waters and other aquatic species. The MATC of a given toxicant could be estimated thereafter for other species, by determining the lethal threshold concentration for the species of concern in the appropriate water quality and multiplying by the previously determined application factor for the toxicant.

The MATC of toxaphene for brook trout was not established in this study. However, the effects of toxaphene on fry growth were supported by physiological and biochemical determinations (see PHYSIOLOGY AND BIOCHEMISTRY) and a MATC of toxaphene for brook trout would have to be below 39 ng/l. An application factor of 0.0095 was derived by using the 39 ng/l concentration from the chronic study and the lethal threshold concentration of 4.1 µg/l (Table 4). The application factor closely approximates the 0.01 factor recommended in establishing water quality criteria for organochlorine insecticides[44].

PHYSIOLOGY AND BIOCHEMISTRY

Development of trout fry is usually evaluated by making length and weight measurements. In the present study we evaluated the effects of toxaphene on differentiation and development of brook trout fry by the conventional method as well as by biochemical techniques to ascertain if a method for evaluating toxicant effects on development prior to discernible effects on weight and length gain could be established. The biochemical technique applied was measuring the synthesis of the amino acid hydroxyproline and the protein collagen. The use of collagen as a representative "differentiated" protein for study of embryonic development has been reported in amphibian embryological investigations[52,53]. During the first cleavage stages of frog embryos (Xenopus laevis) collagen synthesis is repressed, but during gastrulation collagen synthesis begins and increases 500-fold through neurulation, hatching, and posthatching stages.

Collagen is the major fibrous protein of all vertebrates and most of the invertebrate phyla[65]. Its most important function in vertebrates is serving as the major component of the organic matrix of connective tissues and bones. It is around and within the collagen fibrils that calcification and mineralization takes place and as development proceeds, more calcium and phosphate salts are deposited resulting in mature bone.

Hydroxyproline and hydroxylysine in the collagen molecule are derived from the hydroxylation of their respective precursors, proline and lysine, after their incorporation into the polypeptide (protocollagen). The enzyme collagen hydroxylase or peptidyl proline hydroxylase, which commences its activity during gastrulation, catalyzes the hydroxylation[66]. Ascorbic acid, ketoglutarate, and ferrous iron are cofactors for the enzyme.

Toxaphene significantly decreased growth in brook trout fry after 30, 60, and 90 days of exposure as determined by both length-weight measurements and biochemical analyses. Only biochemical analyses were performed on whole fry after the 7 and 15 day samplings, since the fry were too small to be handled for length-weight measurements; however, significant decreases in growth, as determined biochemically, were observed. In the 7 and 15 day samplings, toxaphene concentrations of 68, 139, and 288 ng/l significantly decreased ($P<0.05$) the concentration of hydroxy-proline in the sac fry (Table 8). The no-effect toxaphene concentration was between 39 and 68 ng/l. These results suggest that toxaphene significantly decreased collagen synthesis and consequently decreased the precursor molecules of the organic framework of skin, scale, and bone. The use of whole-fry hydroxyproline analyses as an indicator or predictor of future growth and development appears to be quite adequate based upon length-weight and biochemical analyses performed on fry from the 30, 60, and 90 day samplings. Instead of whole-fry analyses from these latter sampling periods, we performed collagen, as well as mineral analyses on the backbones.

All toxaphene concentrations significantly altered ($P< 0.05$) the composition of backbones after 30, 60, and 90 days of exposure (Table 9). More pronounced effects were observed after 90 days of exposure than after 30 and 60 days of exposure. These results demonstrate changes similar to those thought to be indicated by the hydroxyproline measurements from the 7 and 15 day samplings, i.e., collagen content was significantly decreased by toxaphene, and the effect of toxaphene on collagen synthesis prior to the 30 day sample had a significant impact on bone composition and development. Concomitantly, toxaphene decreased the growth of the trout fry as determined by length measurements (Table 6). However, only the 139 and 288 ng/l exposures decreased growth during the first 30 days, whereas all toxaphene concentrations decreased growth as determined biochemically. These results illustrate that the actual growth (length)

Table 8. COLLAGEN SYNTHESIS[a] IN BROOK TROUT SAC FRY AS AFFECTED BY TOXAPHENE (expressed as μg hydroxyproline/g fry)

Days after hatch	Toxaphene concentration, ng/l					
	0	39	68	139	288	502
7	649	607	463[a]	498[a]	459[a]	481[a]
15	783	765	683[a]	588[a]	650[a]	532[a]

[a] Significantly different from controls ($p<0.05$), n = 8.

Table 9. BACKBONE COMPOSITION OF BROOK TROUT FRY AS AFFECTED BY TOXAPHENE

Days after hatch	Backbone constituent	Toxaphene concentration, ng/l					
		0	39	68	139	288	502
30	Hydroxyproline, mg/g	51	34[b]	26[b]	20[b]	25[b]	-[c]
	% collagen (estimated)[a]	78	51[b]	40[b]	30[b]	38[b]	-
	% phosphorous	5.7	7.3	9.9	8.8	11	-
	% calcium	1.2	1.0	1.3	1.0	1.4	-
60	Hydroxyproline, mg/g	20	14[b]	14[b]	11[b]	-	-
	% collagen (estimated)	29	20[b]	20[b]	16[b]	-	-
	% phosphorous	12	11	9.9	7.7[b]	-	-
	% calcium	2.6	2.8	2.5	1.3	-	-
90	Hydroxyproline, mg/g	19	16[b]	16[b]	16[b]	-	-
	% collagen (estimated)	30	25[b]	25[b]	25[b]	-	-
	% phosphorous	11	16	20[b]	20[b]	-	-
	% calcium	10	15	21[b]	21[b]	-	-

[a] Estimated collagen based upon pure collagen containing 6.56% hydroxyproline.

[b] Significantly different from controls ($P<0.05$), n = 6 to 8.

[c] All fish were dead.

of the fry was altered by a toxaphene concentration of 139 ng/l or greater, but the development or composition of bone which will ultimately reflect the structure and size of the fry is altered by a concentration less than 39 ng/l.

After 60 days of exposure, toxaphene had a similar biochemical effect as after 30 days of exposure (Table 9). The collagen concentration was significantly decreased ($P<0.05$) by toxaphene, the phosphorous concentration in the bone from the 139 ng/l group was significantly decreased ($P<0.05$), and the calcium concentration was unchanged by all toxaphene concentrations. The significance of the phosphorous decrease was not completely understood. The main point to note, however, was the decrease in the percentage of collagen between the 30 and 60 day sampling periods. This decrease was apparently due to the increase in calcium and phosphorous salts which occurred as the backbone matured. It appeared that the process of mineralization began most extensively between the 60 and 90 day period, as judged by the increase in phosphorous and calcium concentrations. The mineralization process did not appear to be affected by toxaphene as was the collagen content. The length measurements in all groups exposed to toxaphene were significantly decreased (Table 6), which support the collagen analyses. Thus, the biochemical analyses and length measurements reflected the same effects after 60 days of toxaphene exposure.

Similar results as in the 30 and 60 day samples were observed after 90 days of toxaphene exposure, except that the mineral contents were significantly altered ($P<0.05$). The length and weight measurements were also decreased by all toxaphene exposures which reflect the same effects as the biochemical analyses. The no-effect concentration of toxaphene on growth and development as determined by both length-weight and biochemical measurements was below 39 ng/l. However, evaluation of collagen and mineral concentrations after 90 days of exposure gave an indication of not only status of growth and development, but also the quality of bone. The mineralization of bone is accomplished by a complex mechanism which involves the immature bone tissue accumulating phosphorous salts and then calcium salts[67]. The data presented in Table 9 supports this fact. Also, the mineralization process can take place independently of the collagen matrix, i.e., the organic substrate is not believed to be necessary for initiation of mineralization[67].

The data presented in our study suggested that toxaphene decreased collagen synthesis which resulted in whole-body collagen and backbone collagen concentrations being decreased. Concomitantly, the rate of mineralization appeared to proceed normally and was not affected by toxaphene. The increase in calcium and phosphorous in the backbone of fry exposed to 68 and 139 ng/l of toxaphene after 90 days of exposure, was hypothesizedto be due to the decrease in collagen content. The resulting effect of toxaphene on bone composition was an increase in the ratio of minerals: collagen which is depicted in Fig. 2. The implication of this type of bone composition on trout fry development beyond 90 days remains to be elucidated, as does the mode of action of toxaphene causing this condition.

RESIDUE DYNAMICS

Toxaphene in water was concentrated by yearling brook trout from 5,000 times in the low concentration to 16,000 times in the high concentration (Table 10). Equilibria between water concentrations of toxaphene and residues in brook trout were reached after approximately 140 days of continuous exposure. After 161 days, whole body residues of toxaphene in fish from the 288 and 502 ng/l concentrations were 2.4 and 8.0 µg/g, respectively. Toxaphene concentrations in adult brook trout of this magnitude would be considered detrimental since extensive mortality occurred in the 288 and 502 ng/l toxaphene exposures just prior to and during spawning activities (Fig. 1). Kallman, Cope, and Navarre[15] also reported symptoms of poisoning associated with toxaphene concentrations of 8 to 15 µg/g in bullheads (Ictalurus natalis and I. melas). Fish from all toxaphene exposures were analyzed for the distribution of toxaphene residues between the fillet and the remaining tissue (offal) after 161 days. Toxaphene residues in the offal were 2.8 to 4.2 times that found in the fillet (Table 11). The highest residue detected in the fillets was 4.9 µg/g, whereas, residues in offal were 13 µg/g. All toxaphene residues in fillets were below the allowable FDA limit of 5 ug/g for toxaphene in foods consumed by man[68].

Residues of toxaphene in the eggs immediately after spawning were 0, 0.4, 0.9, 1.8, 2.9, and 5.2 µg/g for the 0, 39, 68, 139, 288, and 502 ng/l exposure concentrations, respectively. Egg viability was significantly reduced ($P<0.05$) when egg concentrations of toxaphene were equal to or greater than 0.9 µg/g (Table 5). Since the original eggs died in the eyed stage, no relationship between toxaphene residues in eggs and egg hatchability could be determined.

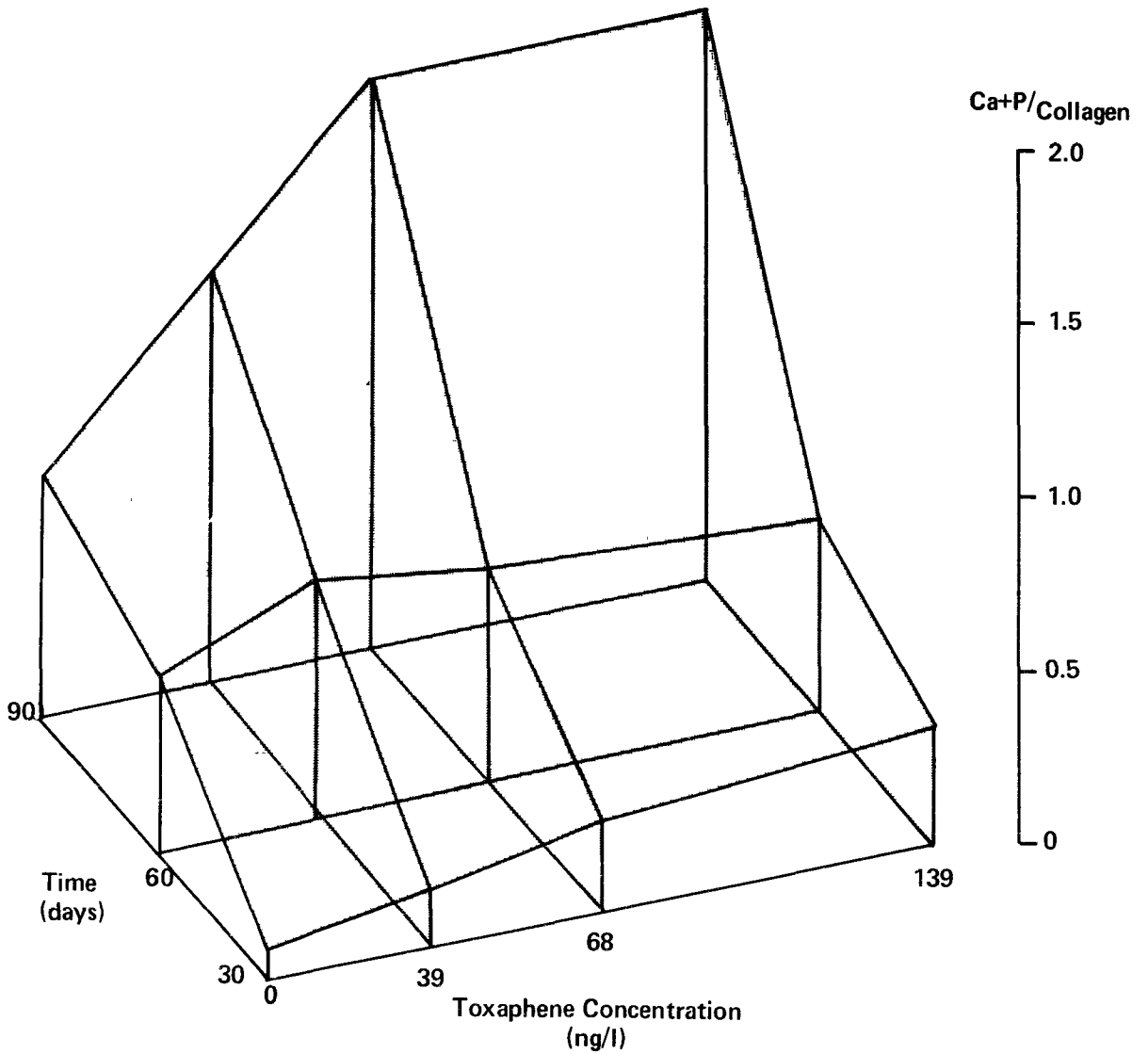

Figure 2. Effect of toxaphene on backbone composition of brook trout fry after 30, 60, and 90 days of exposure.

Table 10. WHOLE-BODY RESIDUES OF TOXAPHENE IN YEARLING BROOK TROUT CONTINUOUSLY EXPOSED TO TOXAPHENE

(µg/g)

Exposure, ng/l	Statistic	Days of exposure							
		3	7	10	18	24	60	140	161
41[a] (39±3)[b]	Mean ± S.E.[c]	N.D.[d]		N.D.		<0.1	<0.1	0.3±0.05	0.2±0.03
	Number	4		4		4	4	4	4
75 (68±4)	Mean ± S.E.		<0.1		<0.1		0.1±0.02	0.8±0.08	0.4±0.04
	Number		4		4		6	6	7
125 (139±10)	Mean ± S.E.	N.D.		<0.1		0.3±0.11	0.5±0.03	0.6±0.06	0.4±0.06
	Number	4		4		4	4	4	4
270 (288±22)	Mean ± S.E.		<0.1		0.3±0.06		1.8±0.12	3.5±0.30	2.4±0.19
	Number		4		4		6	5	8
500 (502±40)	Mean ± S.E.	<0.1		0.5±0.04		2.0±0.12	4.5±0.46	7.7±0.10	8.0±0.55
	Number	4		4		4	4	4	3

[a] Nominal concentration.

[b] Measured concentration ± the standard error.

[c] Standard error.

[d] None detected, minimum detection limit 0.05 µg/g.

Table 11. TOXAPHENE RESIDUES IN ADULT BROOK TROUT FILLET AND OFFAL AFTER 161 DAYS OF EXPOSURE

(µg/g)

Exposure, ng/l	Statistic	Fillet	Offal
41[a] (39±3)[b]	Mean ± S.E.[c]	<0.10	0.42±0.05
	Number	4	4
75 (68±4)	Mean ± S.E.	0.17±0.04	0.62±0.07
	Number	4	4
125 (139±10)	Mean ± S.E.	0.21±0.3	0.66±0.11
	Number	4	4
270 (288±22)	Mean ± S.E.	0.87±0.13	3.0±0.2
	Number	4	4
500 (502±40)	Mean ± S.E.	4.0 ±0.72	11±1.2
	Number	3	3

[a] Nominal concentration.

[b] Measured concentration ± the standard error.

[c] Standard error.

Brook trout fry from the second batch of eggs (adults not exposed) concentrated toxaphene up to 76,000 times that in the exposure water in only 15 days (Table 12). Uptake in all toxaphene exposures passed through a maximum after 15 days, declined through 60 days, and then tended to increase again between 60 and 90 days of exposure. Accumulation factors of toxaphene in the fry exposed for 90 days was similar to that found in the adults. The reason for the greater uptake of toxaphene during the first 15 days may have been due to anatomical differences present during this stage of life. Gills are generally not functional until the yolk sac is absorbed and most respiration occurs through a vascular network enveloping the yolk[69]. Also, the yolk contains glyceride fat droplets in which toxaphene would be readily stored and these nutrients are utilized towards the end of the yolk sac phase of development[69]. Shortly after the yolk sac is absorbed and just prior to the time that the fry begin to feed (30-34 days post-hatch), fat content is probably lowest and therefore a lower equilibrium between toxaphene in the water and that in the fish occurs. As the fry begin to feed actively (35-36 days post-hatch) and lipid content increases, the accumulation of toxaphene again increases.

The decline in whole body residues in adult brook trout 56 days after transfer to uncontaminated water varied with toxaphene concentration and ranged from 0 to 54% (Table 13). Toxaphene residues in fish from the 68 and 139 ng/l exposures did not decline. However, residues in fish from the 288 and 502 ng/l exposures were reduced by 32 and 51%, respectively. The elimination of toxaphene was reflected by a marked decrease in toxaphene isomers with GLC retention times less than 1.48 relative to p,p'-DDE (Table 14). These isomers are generally less chlorinated, more hydrophilic, and therefore can be more easily degraded and/or excreted than higher chlorinated components. However, the abrupt change that occurred in the elimination of toxaphene isomers up to a relative retention time of 1.48 and the accumulation of later eluting ones is not fully understood. Differences of isomer storage in toxaphene residues from fish during the uptake portion of the study were not as dramatic as those changes observed in the elimination study (Table 15). However, with few exceptions the more chlorinated toxaphene isomers were stored to a greater extent by brook trout than were the less chlorinated isomers. Preferential storage of the more chlorinated isomers was probably due to their being more lipophilic and less easily degraded than the less chlorinated isomers. Differences in storage and elimination of

Table 12. WHOLE-BODY RESIDUES OF TOXAPHENE IN A BROOK TROUT FRY[a] CONTINUOUSLY EXPOSED TO TOXAPHENE

(μg/g)

Exposure, ng/l	Statistic	Days of exposure 7	15	60	90
41[b] (39±3)[c]	Mean	0.2	2.6	0.4	0.6
	number	2	2	2	2
75 (68±4)	Mean	1.0	3.7	0.9	1.4
	number	2	2	2	2
125 (139±10)	Mean	2.2	8.3	1.8	2.6
	number	2	2	2	2
270 (288±22)	Mean	4.5	18	-[d]	-
	number	2	2		
500 (502±40)	Mean	9.2	38	-	-
	number	2	2		

[a] Composite sample of several fry (2 g).

[b] Nominal concentration.

[c] Measured concentration ± the standard error.

[d] All fish were dead.

Table 13. TOXAPHENE RESIDUES IN ADULT BROOK TROUT DURING ELIMINATION STUDY (μg/g)

Exposure[a] ng/l	Statistic	Days after cessation of toxaphene exposure 7	14	28	56
41[b] (39±3)[c]	Mean ± S.E.[d]	0.1±0.03	0.1	N.D.[e]	N.D.
	Number	3	2	2	2
75 (68±4)	Mean ± S.E.	0.3±0.00	0.2±0.13	0.4±0.10	0.3±0.06
	Number	3	3	3	3
125 (139±10)	Mean ± S.E.	0.7	0.8	0.9	0.7±0.03
	Number	2	2	2	3
270 (288±22)	Mean ± S.E.	2.2±0.17	1.6±0.10	1.4±0.35	1.5±0.07
	Number	3	3	3	3
500 (502±40)	Mean ± S.E.	7.5±0.62	5.8±0.59	5.8±0.58	3.7
	Number	3	3	3	2

[a] Water concentration before cessation of toxaphene exposure.

[b] Nominal concentration.

[c] Measured concentration ± the standard error.

[d] Standard error.

[e] None detected, minimum detection limit 0.05 μg/g.

Table 14. MEAN PERCENT CHANGE[a] OF SELECTED GLC PEAKS FROM TOXAPHENE RESIDUES IN ADULT BROOK TROUT AFTER CESSATION OF 502 ng/l TOXAPHENE EXPOSURE (%)

Peak #	Rt[b]	P.H.%[c]	Days after cessation of toxaphene exposure			
			7	14	28	56
1	1.05	10	-23	+10	+17	- 2
2	1.18	16	-18	- 8	- 7	-16
3	1.39	13	-24	-24	-23	-42
4	1.48	16	-27	-35	-30	-44
5	N.R.[d]					
6	1.90	18	+14	+26	+ 6	+14
7	2.16	10	+33	+25	+28	+58
8	2.66	12	+18	+ 4	+ 5	+20
9	3.40	5	+49	+29	+45	+86

[a] Based on average change of individual toxaphene peak heights (expressed as percent of sum of eight peak heights) when compared to a standard.

[b] Retention time relative to p,p'-DDE

[c] Percent of total peak height (8 peaks) for selected toxaphene standard components.

[d] Not resolved by GLC into measurable component.

Table 15. MEAN PERCENT CHANGE[a] OF SELECTED GLC PEAKS FROM RESIDUES IN YEARLING BROOK TROUT CONTINUOUSLY EXPOSED TO TOXAPHENE

(%)

Exposure, ng/l	Peak #	Rt[b]	P.H.%[c]	Days of exposure			
				24	60	140	161
125[d] (139±10)[e]	1	N.R.[f]					
	2	1.18	17	-[g]	- 2	- 4	- 9
	3	1.39	14	-	-10	- 9	-11
	4	1.48	16	-	-17	-17	-23
	5	1.76	11	-	- 8	-12	-12
	6	1.90	17	-	+10	+12	+13
	7	2.16	11	-	+21	+23	+25
	8	2.66	10	-	- 2	+ 5	+12
	9	3.40	4	-	+17	+26	+58
500 (502±40)	1	1.05	12	- 2	- 2	+ 1	+ 2
	2	1.18	15	+ 5	+ 3	+ 2	+ 5
	3	1.39	14	- 7	- 9	-14	-11
	4	1.48	17	- 9	-16	-18	-18
	5	N.R.					
	6	1.90	16	+ 4	+ 7	+ 6	+ 6
	7	2.16	10	+ 4	+ 8	+12	+11
	8	2.66	12	+ 2	+ 6	+ 6	+ 1
	9	3.40	4	+ 7	+21	+23	+31

[a] Based on average change of individual toxaphene peak heights (expressed as percent of sum of eight peak heights) when compared to standard.

[b] Retention time relative to p,p'-DDE

[c] Percent of total peak height (8 peaks used) for selected toxaphene standard components.

[d] Nominal concentration.

[e] Measured concentration ± standard error.

[f] Not resolved by GLC into measurable component.

[g] No determinations due to low toxaphene residues.

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different toxaphene isomers in brook trout were not due to any alterations in the toxaphene that the fish were exposed to. Water samples from the 502 ng/l concentration were analyzed using difference chromatography[60] and the resulting curves demonstrated that no major changes in early or late eluting toxaphene isomers occurred in water prior to uptake by brook trout.

SECTION VI

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SECTION VII

LIST OF PUBLICATIONS

Mayer, F. L., Jr., P. M. Mehrle, and W. P. Dwyer. Brook Trout Development as Affected by Toxaphene. 35th Midwest Fish & Wildlife Conference Abstracts. St. Louis, Missouri. December 1973. p. 58-59.

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO	2.	3. RECIPIENT'S ACCESSION NO.
EPA-600/3-75-013		

4. TITLE AND SUBTITLE	5. REPORT DATE
TOXAPHENE EFFECTS ON REPRODUCTION, GROWTH, AND MORTALITY OF BROOK TROUT	November 1975 (Issuing Date) 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) Foster L. Mayer, Jr., Paul M. Mehrle, Jr., and William P. Dwyer	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS Fish-Pesticide Research Laboratory Fish and Wildlife Service United States Department of the Interior Columbia, Missouri 65201	10. PROGRAM ELEMENT NO. 1BA021 (ROAP/Task 16AAK) 11. CONTRACT/GRANT NO. EPA-IAG-0153(D)
12. SPONSORING AGENCY NAME AND ADDRESS Environmental Research Laboratory Office of Research and Development U.S. Environmental Protection Agency Duluth, Minnesota 55804	13. TYPE OF REPORT AND PERIOD COVERED Final (4/72-3/74) 14. SPONSORING AGENCY CODE EPA-ORD
15. SUPPLEMENTARY NOTES	

16. ABSTRACT

Yearling brook trout (Salvelinus fontinalis) were continuously exposed to toxaphene (0, 39, 68, 139, 288, and 502 ng/l) in a flow-through diluter system. Day length and water temperature were altered monthly to correspond to natural conditions. Adult growth was reduced in the 288 and 502 ng/l toxaphene exposures, and the added stress of spawning activities caused extensive mortalities in these concentrations. The numbers of eggs spawned and percent viability were inversely related to increasing toxaphene concentrations. All groups of fry exposed to toxaphene had reduced rates of growth and survival. Biochemical investigations on fry backbones demonstrated that bone collagen may be a sensitive indicator of normal and abnormal growth and development prior to being observed in the whole fish. Toxaphene was accumulated by brook trout 5,000 to 76,000 times that in the water and the more chlorinated isomers of toxaphene were preferentially stored.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Growth Collagens Reproduction (biology) Mortality Pesticides Trout	Brook trout Toxaphene Chronic effects Continuous exposure Residue dynamics Salmonids Partial life cycle	6C 6F 6T

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)*	21. NO. OF PAGES
RELEASE TO PUBLIC	UNCLASSIFIED	51
	20. SECURITY CLASS *(This page)*	22. PRICE
	UNCLASSIFIED	

EPA Form 2220-1 (9-73)