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TOXICITY OF DIAZINON TO BROOK TROUT AND FATHEAD MINNOWS

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TOXICITY OF DIAZINON TO BROOK TROUT AND FATHEAD MINNOWS

by

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FOREWORD

Our nation's fresh waters are vital for all animals and plants, yet our diverse uses of water---for recreation, food, energy, transportation, and industry---physically and chemically alter lakes, rivers, and streams. Such alterations threaten terrestrial organisms, as well as those living in water. The Environmental Research Laboratory in Duluth, Minnesota, develops methods, conducts laboratory and field studies, and extrapolates research findings

--to determine how physical and chemical pollution affects aquatic life;

--to assess the effects of ecosystems on pollutants;

--to predict effects of pollutants on large lakes through use of models; and

--to measure bioaccumulation of pollutants in aquatic organisms that are consumed by other animals, including man.

This report describes the acute and chronic effects of the organophosphate insecticide diazinon on two species of freshwater fishes.

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ABSTRACT

Average 96-hr LC50's for diazinon under flow-through conditions were 7.8, 1.6, 0.77, and 0.46 mg/l, respectively, for fathead minnows, flagfish, brook trout, and bluegills.

The chronic effects of diazinon on fathead minnows and brook trout were determined in flow-through systems with constant toxicant concentrations. Fathead minnows exposed to the lowest concentration tested (3.2 µg/l) from 5 days after hatch through spawning had a significantly higher incidence of scoliosis than the control ($P = 0.05$). Hatch of their progeny was reduced by 30% at this concentration. Yearling brook trout exposed to 4.8 µg/l and above began developing scoliosis and lordosis within a few weeks. Growth of brook trout was substantially inhibited during the first 3 months at 4.8 µg/l and above. Neurological symptoms were evident in brook trout at 2.4 µg/l and above early in the tests, but were rarely observed after 4 or 5 months of exposure. Exposure of mature brook trout for 6 - 8 months to concentrations ranging from 9.6 µg/l to the lowest tested (0.55 µg/l) resulted in equally reduced growth rates for their progeny. Transfer of progeny between concentrations indicated that effects noted for progeny of both species at lower concentrations were the result of parental exposure alone and not the exposure of progeny following fertilization.

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ACKNOWLEDGMENTS

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SECTION 1

INTRODUCTION

The organophosphate insecticide diazinon [0,0-diethyl-0-(2-isoprophyl-6 methyl-4-pyrimidinyl) phosphorothioate] has been used on agricultural crops since 1954 (Bartsch, 1974). Diazinon usage has increased in recent years because of the world-wide trend towards intensified agricultural production. Records for a representative corn-producing region in Kansas where irrigation was introduced in 1963 indicate that diazinon was first used in 1964. Three years later 12 times as much diazinon was being applied annually (Knutson et al., 1971). Actual or potential restriction on the use of organochlorine pesticides has increased interest in the use of organophosphates. Diazinon has been considered as one of two logical substitutes for chlordane, which was applied at an annual rate of 600,000 pounds circa 1972 (Anonymous, 1972).

Diazinon is used throughout the world on rice crops where application every 20 days has proved effective (Sethunathan and Pathak, 1972). Currently, in the United States diazinon is one of the insecticides most frequently applied to onions and sweet potatoes (Bartsch, 1974). It is recommended for pest control on most of the major vegetable, fruit, and nut crops, for grasshopper control on rangeland, for control of lawn and household pests, and as a livestock spray (Farm Chemicals Handbook, 1974).

The probability of water contamination by diazinon and the subsequent persistence of the chemical appear to be influenced by a complex of physical and biological factors which are poorly understood at present. Munson (1970) observed that field-applied diazinon remained in the top few inches of soil until degraded or removed by runoff. This study indicates a low probability for direct contamination of ground water. Suett (1971) observed that field-applied diazinon persisted longer in peaty loam than in sandy loam but he notes that laboratory studies by other workers have produced conflicting results. Rate of hydrolysis is greatly influenced by pH, but diazinon has an apparent potential for persisting many months in non-acid bodies of water. Cowart et al. (1971) recorded a half-life of less than 2 weeks at pH 6.0. Gomaa et al. (1969) reported half-life values of about 6 and 4 months at pH 7.4 and 9.0, respectively. Aerobic conditions accelerate degradation, and suspended soil particles inhibit degradation in water (Sethunathan and Pathak, 1972). Sethunathan and co-workers have found that at least two species of naturally occurring bacteria are able to rapidly degrade diazinon in water and flooded soil. Large populations of these bacteria develop following repeated application of diazinon. These

bacterial populations do not develop in the presence of structurally related organophosphates or affect their rates of degradation (Sethunathan and Pathak, 1972). No reports were found of general monitoring of natural waters for the presence of diazinon.

Studies of the toxicity of diazinon to fishes have been limited to a few acute exposures (Weiss, 1961; Coppage, 1972). The present study was undertaken to discover the effects of prolonged exposure to diazinon on freshwater fishes and to estimate the highest concentration which would not be detrimental to their populations. We used the laboratory fish-production index (LFPI) defined by Mount and Stephan (1967) and their concept that the highest concentration of a toxicant producing no measurable effects on production rates of adults and progeny in a laboratory environment is termed the maximum acceptable toxicant concentration (MATC). An "application factor" is then derived for a given toxicant and a given species by dividing the MATC by the acute toxicity (LC50) value for that combination. Mount and Stephan (1967) proposed that, if a number of application factors for several species subjected to one toxicant fell within a narrow range, the "safe" chronic concentrations for similar species could be estimated by multiplying their acute toxicity LC50's by this application factor. An ancillary purpose of the present test was to determine if the application-factor concept could be applied to diazinon and freshwater fishes. Fathead minnows (Pimephales promelas) and brook trout (Salvelinus fontinalis) were selected for the chronic exposures as representatives of warm- and cold-water fishes that could be reared under laboratory conditions. Acute toxicity values (96-hr LC50) were obtained for these species as well as bluegills (Lepomis macrochirus) and flagfish (Jordanella floridae).

SECTION 2

CONCLUSIONS

Although a long-term no-effect concentration was not determined with fathead minnows, 3.2 µg/l appeared to be close to a maximum acceptable toxicant concentration (MATC) as defined by Mount and Stephan (1967). The application factor (MATC/96-hr LC50) for diazinon with this fish is probably 10^{-4}.

Most chronic effects noted for brook trout were minimal or no longer apparent at the lower concentrations. However growth inhibition of progeny was the same at all tested concentrations (9.6 - 0.55 µg/l). Therefore, it is probable that the MATC was considerably less than 0.55 µg/l, which indicates an application factor for brook trout of 10^{-4} or lower.

Diazinon-related effects observed at lower concentrations for progeny of both species appear to have been caused by the exposure of their parents and not by exposure following fertilization (indicated by transfer of progeny between concentrations). Progeny exposed to diazinon throughout embryonic, larval, and juvenile stages did not develop the neurological symptoms and spinal deformities observed in their parents. Results following exposure of control progeny to diazinon indicate that this phenomenon was not due to selection of resistant parental stock.

An initial exposure of brook trout to diazinon coinciding with the spawning period might cause a greater reduction in reproductive potential than that observed in the present test.

Whole-body levels of diazinon in fishes will probably not exceed 100 times the ambient water concentration even after exposure for many months.

SECTION 3

RECOMMENDATIONS

The estimated application factors for diazinon reported in this paper should not be used to extrapolate MATC's for other species of fishes from their 96-hr LC50's. A just-completed chronic exposure of another fish indicates that diazinon may elicit a wide range of species-related application factors.

The variance in application factors plus the occurrence at low concentrations of effects potentially harmful to population survival suggest that chronic-exposure studies should be conducted with other species of fishes.

Definitive studies should be made to determine if progeny of diazinon-exposed fish are more resistant than their parents or if young fish in general are more resistant.

The effects of diazinon on survival-related behavior should be studied.

The chronic toxicity of diazinon to invertebrates should be investigated.

SECTION 4

MATERIALS AND METHODS

The acute toxicity tests met the requirements outlined by the American Public Health Association (1971). The designs of the chronic and partial-chronic toxicity tests followed the procedures recommended by the committee on aquatic bioassays, Environmental Research Laboratory-Duluth, Minnesota (Appendixes A and B), except as noted below.

PHYSICAL SYSTEMS

All of the tests described in this paper were conducted under flow-through conditions. Proportional diluters (Mount and Brungs, 1967) delivered five toxicant concentrations plus control water to duplicate exposure chambers in all tests. Lake Superior water was delivered at a rate of 10 to 14 test-chamber volumes per day except as noted in Appendix C, Table 1, during the fathead minnow acute tests. Water quality values for the acute tests, other than temperature and dissolved oxygen, fell within the ranges given for the chronic and partial chronic tests in Appendix C, Tables 2 and 3. The physical characteristics of the test chambers are given in Appendix C, Table 4.

The normal Duluth, Minnesota, photoperiod was used during the brook trout partial-chronic test instead of the Evansville, Indiana, photoperiod given in Appendix B. Modified spawning substrates described by Benoit (1974) were substituted for pans of loose gravel.

CHEMICAL SYSTEMS

Technical grade diazinon (92.5% purity) was introduced by diluter-operated syringe injectors in all tests except the brook trout partial chronic. The brook trout partial chronic utilized a Mariotte bottle and metering device similar to that described by Mount and Brungs (1967). Stock solutions of diazinon were dissolved in acetone. In all tests except those with bluegills, Triton X-100 was added as a surfactant at 3% of the diazinon concentration. Therefore, within individual test systems the nominal concentrations of Triton X-100 and acetone were directly proportional to nominal diazinon concentrations in that system. The maximum nominal amount of acetone concurrent with the highest concentration of diazinon was 24 mg/l in the acute tests, less than 2 mg/l in the fathead minnow chronic test, and less than 0.1 mg/l in the brook trout partial-chronic test.

Water concentrations of diazinon were measured three to six times during each 96-hr acute toxicity test (Appendix C, Table 1). During the chronic, preliminary, and partial-chronic tests alternate chambers from each

treatment level were measured at least once a week whenever fish were present. The concentrations in larval chambers were measured separately (Appendix C, Tables 5 and 6).

All determinations for diazinon concentration were made by gas chromatography (GC) and electron capture detector (sensitivity limit = 50 picograms). At least one water sample in each set was run in duplicate, and spiked control water was used to monitor recovery rate.

The methods used to determine diazinon residues in brook trout tissues are outlined in Appendix D.

BIOLOGICAL SYSTEMS

Acute Toxicity Tests

Species, sources, ages, and numbers of fishes used are presented in Appendix C, Table 1.

Fathead Minnow Chronic Toxicity Tests

Fifty 4-day-old fathead minnows from laboratory stock were randomly assigned to each test chamber on September 14, 1971. Survivors were counted and measured after 30 and 60 days by the photographic method of McKim and Benoit (1971). All chambers were randomly thinned to 15 fish at 61 days. After 91 days of exposure it was decided that all diazinon concentrations (1,100 - 69 µg/l) were too high, and the test was terminated.

A second test was begun on January 5, 1972, with 50 5-day-old larvae per chamber and average measured concentrations of diazinon between 60.3 and 3.2 µg/l. Counts and lengths of survivors were determined by photography at 31, 64, and 97 days. Random thinning of each test chamber to 15 fish was delayed until the 167th day of exposure because larger numbers were desired to measure the development of scoliosis. Five spawning substrates were placed in each test chamber at thinning. As there appeared to be no subsequent competition between males for spawning territories, no additional fish were removed. Adult exposures were terminated after 274 days when no spawning was observed in any group for one week. Larvae from the initial hatches were exposed 30 days and then replaced by newly hatched larvae which were exposed for 60 days.

Brook Trout Preliminary Test

Beginning, January 2, 1973, duplicate 2-month exposures of six yearling brook trout were conducted at average measured concentrations of 56, 11, 2.6 and 0 µg/l. These fish were taken from the same stock reserved for the partial-chronic test, and results from the preliminary exposures were used to determine concentrations to be used in the partial chronic.

Brook Trout Partial-Chronic Toxicity Test

Yearling brook trout were obtained from a commercial hatchery and accliminated 4 months to laboratory conditions. Twelve fish were randomly

assigned to each adult exposure chamber on April 3, 1973. Lengths and weights of individual fish anethetized with Ethyl-m-Aminobenzoate Methanosulfonate were taken at the start of the test and after 91 and 173 days. At 173 days each exposure chamber was thinned to a nominal of two males and four females. Only fish that appeared healthy, undeformed, and mature were kept. Two spawning substrates were placed in each adult exposure chamber at this time (September 24, 1973).

All spawnings of 10 or more eggs were incubated at least 11 days to determine viability. Samples of 50 eggs from spawnings of this size or larger were incubated until completion of hatch or the death of all eggs. Larvae were inventoried by photograph 2 days after completion of hatch, randomly thinned to 25, and returned to their hatching containers. Feeding was initiated 25 days after the median hatch date. Thirty days after the median hatch date larvae were inventoried by photograph and transferred to available larval chambers. Excess groups of larvae were killed and inventoried at this age. Fish reared in the larval chambers were photographed at 60 and 90 days. Survivors were killed and inventoried 122 days after hatch.

STATISTICAL ANALYSIS

The 96-hr LC50's for the acute toxicity tests were derived by the method described by Litchfield and Wilcoxon (1949).

Data from the chronic and partial-chronic toxicity tests were subjected to one-way analysis of variance ($P = 0.05$) and Dunnett's procedure for comparison of treatment means to control mean, $P = 0.05$ (Steel and Torrie, 1960). Percentage data (e.g. survival) were transformed to arc sin $\sqrt{\%}$ prior to analysis. Other statistical analyses were used as noted in the section on results.

SECTION 5

RESULTS

ACUTE TOXICITY TESTS

Bluegills were most sensitive to acutely toxic concentrations of diazinon. They were followed in order by brook trout, flagfish, and fathead minnows. Results of individual tests and average 96-hr LC50's are presented in Table 1.

FATHEAD MINNOW CHRONIC TOXICITY TESTS

Except for two apparently random occurrences, fathead minnows exposed to diazinon at average concentrations ranging from 69 to 1,100 µg/l had higher survival than either control during the first 30 days. During the next 31 days there was some evidence of toxicant-related deaths, but the differences were not statistically significant. Growth measurements at 30 and 61 days showed a non-significant concentration-related decrease in average length of survivors. However, analysis of variance of the instantaneous growth rates ($[\ln (length_2/length_1)]/days$) between 30 and 61 days demonstrated a significant decrease for fish exposed to 229 µg/l. and above (Table 2). After 13 weeks the incidence of scoliosis in diazinon-exposed groups ranged from 60 to 88% versus 7% in the controls (Table 3). The first test was terminated at this time.

During the second fathead minnow chronic test the fish were exposed to a series of average concentrations between 3.2 and 60.3 µg/l. Survival was lower in the controls than in treated groups during the first 167 days of the test. There was a non-significant increase in mortality rate for fish exposed to 60.3 µg/l. between 97 and 167 days (Table 4). During the remainder of the test (107 days), which encompassed spawning, adult fathead minnows exposed to 60.3 µg/l. suffered 50% mortality compared to 7% in the control. Mortality in lower concentrations did not differ significantly from that in the control during this period.

There were indications of diazinon-related inhibition of growth between 64 and 97 days (Table 4), but none of the growth data for exposed parental fish were significantly different from that of the control throughout the entire second test.

The incidence of scoliosis followed the same pattern observed in the first test and generally declined with decreasing concentrations (Table 3). Although the incidence of deformed fish in the controls during the second test was higher, analysis of variance indicated significant differences between the controls and parental fish exposed to all concentrations except 6.9 µg/l.

TABLE 1. ACUTE TOXICITIES OF DIAZINON TO FATHEAD MINNOWS (*PIMEPHALES PROMELAS*), BLUEGILLS (*LEPOMIS MACROCHIRUS*), BROOK TROUT (*SALVELINUS FONTINALIS*), AND FLAGFISH (*JORDANELLA FLORIDAE*)

Species	Test	96-hr LC50[a] (mg/l.)	Slope	Average 96-hr LC50 (mg/l.)
Fathead minnows	1	6.8 (5.4 - 8.5)	1.8	
	2	6.6 (5.1 - 8.6)	1.7	
	3	10.0 (6.7 - 15.0)	2.4	7.8
Bluegills	1	0.48 (0.34 - 0.67)	2.2	
	2	0.44 (0.31 - 0.62)	1.9	0.46
Brook trout	1	0.80 (0.44 - 1.14)	1.8	
	2	0.45 (0.32 - 0.63)	2.1	
	3	1.05 (0.72 - 1.52)	2.5	0.77
Flagfish	1	1.5 (1.2 - 1.9)	2.2	
	2	1.8 (1.6 - 2.0)	1.4	1.6

[a]Calculated by the method of Litchfield and Wilcoxon (1949). The 95% confidence interval is given in parenthesis.

TABLE 2. SURVIVAL AND GROWTH OF PARENTAL STOCK[a] OF FATHEAD MINNOWS (PIMEPHALES PROMELAS) CONTINUOUSLY EXPOSED TO DIAZINON AFTER 30 AND 61 DAYS (TEST #1)

Item	Measured diazinon concentration in water (µg/l.)											
	1,100		511		229		118		69		Control	
	A	B	A	B	A	B	A	B	A	B	A	B
					30 days							
Survival (%)[b]	70	78	84	80	46	82	74	0[c]	82	70	58	36
Average total length (mm)	8.6	8.7	10.4	10.7	10.9	11.9	11.2	-	11.5	11.3	11.9	10.3
(Standard deviation)	(1.6)	(1.3)	(2.1)	(1.9)	(1.9)	(2.0)	(1.6)	-	(2.0)	(2.0)	(2.0)	(2.0)
					61 days							
Survival (%)[b]	38	58	68	66	44	70	62	0[c]	60	42	48	28
Average total length (mm)	11.9	12.6	14.9	14.8	16.1	16.5	17.7	-	19.4	19.7	19.9	19.7
(Standard deviation)	(1.8)	(1.9)	(2.6)	(2.4)	(2.4)	(3.2)	(2.6)	-	(3.4)	(3.4)	(3.7)	(3.4)
Instantaneous growth rate[d] X 10,000	105	119	116	105	126	105	147		169	179	166	209
	112*		110*		116*		-		174		188	

[a] This test discontinued before spawning because of high incidence of scoliosis at all diazinon concentrations.

[b] Each group started with fifty 4-day-old larvae.

[c] All of the larvae in this test chamber died suddenly 2 weeks after start of test from unexplained cause.

[d] $\frac{\ln (\text{length}_2/\text{length}_1)}{\text{days}}$

*Significantly different from control (P = 0.05).

TABLE 3. INCIDENCE OF SCOLIOSIS IN PARENTAL FATHEAD MINNOWS (PIMEPHALES PROMELAS) CONTINUOUSLY EXPOSED TO DIAZINON AFTER HATCH (TESTS #1 AND #2)

Item	Average measured diazinon concentration in water (µg/l.)											
	1,100	511	229	118	69	Control	60.3	28.0	13.5	6.9	3.2	Control
Total number of fish	23	24	29	27	30	30	63	71	81	54	65	48
Scoliosis at 13 weeks (%)[a]	83*	88*	86*	78*	60*	7						
Scoliosis at 19 weeks (%)[b]							67*	44*	40*	26	29	19
Scoliosis at 24 weeks (%)[c]							70*	51*	41*	26	34*	21

[a]Results at termination. Each group had previously been thinned randomly to 30 fish.

[b]Results for survivors of 100 fish per group at start of test.

[c]Results include deaths between 19 and 24 weeks.

*Significantly different from control (P = 0.05).

TABLE 4. SURVIVAL AND GROWTH OF PARENTAL STOCK OF FATHEAD MINNOWS (PIMEPHALES PROMELAS) CONTINUOUSLY EXPOSED TO DIAZINON AFTER 31, 64, 97, 135, 167, AND 274 DAYS (TEST #2)

Item	Average measured diazinon concentration in water (µg/l.)											
	60.3		28.0		13.5		6.9		3.2		Control	
	A	B	A	B	A	B	A	B	A	B	A	B
	31 days											
Survival (%)[a]	88	88	84	84	88	92	66	86	80	76	70	72
Average total length (mm)	13.9	14.2	14.6	14.5	15.2	14.5	14.4	14.0	13.9	14.2	13.7	12.9
(Standard deviation)	(1.7)	(1.7)	(1.8)	(1.7)	(1.4)	(1.8)	(2.2)	(2.3)	(2.7)	(2.2)	(2.6)	(2.8)
	64 days											
Survival (%)[a]	78	82	80	80	84	90	60	74	72	68	58	60
Average total length (mm)	19.6	20.1	22.7	22.0	22.2	21.1	21.5	18.6	20.1	19.7	19.0	19.0
(Standard deviation)	(2.9)	(3.6)	(4.1)	(3.2)	(3.5)	(4.2)	(5.1)	(4.8)	(4.9)	(3.5)	(4.3)	(6.4)
Instantaneous growth rate[b] X 10,000	104	105	134	126	115	114	121	86	112	99	99	117

Continued on page 13

TABLE 4 (Continued). SURVIVAL AND GROWTH OF PARENTAL STOCK OF FATHEAD MINNOWS (PIMEPHALES PROMELAS) CONTINUOUSLY EXPOSED TO DIAZINON AFTER 31, 64, 97, 135, 167, AND 274 DAYS (TEST #2)

Item	Average measured diazinon concentration in water (µg/l.)											
	60.3		28.0		13.5		6.9		3.2		Control	
	A	B	A	B	A	B	A	B	A	B	A	B
	97 days											
Survival (%)[a]	72	78	78	72	82	90	58	66	68	68	52	52
Average total length (mm)	24.2	25.2	28.8	27.1	27.4	25.6	26.6	24.1	26.2	24.5	25.7	24.8
(Standard deviation)	(5.5)	(6.4)	(6.9)	(6.1)	(5.3)	(6.9)	(7.6)	(7.5)	(7.1)	(5.8)	(6.2)	(8.6)
Instantaneous growth rate[b] X 10,000	64	69	72	63	64	59	65	79	80	66	92	81
	135 days											
Survival (%)[a]	60	76	72	70	76	86	56	52	64	66	50	46
	167 days											
Survival (%)[a]	50	64	70	62	74	82	56	50	60	66	50	46
	167 days-274 days											
Survival (%)[c]	40	60	80	80	93	93	87	80	100	100	93	93
	50*		80		93		83		100		93	

[a]Each group started with fifty 5-day-old larvae.

[b] $\frac{\ln (length_2/length_1)}{days}$

[c]Each group randomly thinned to 15 fish at 167 days.

*Significantly different from control (P = 0.05).

No spawning was observed in 60.3 µg/l, and spawning was very limited in 28.0, 13.5, and 6.9 µg/l. However, observed spawning in one of the duplicate control groups was limited to 11 eggs, although egg production from the other control duplicate was roughly equivalent to the total egg production from both groups exposed to 3.2 µg/l (Table 5). More than 500 eggs from the 13.5 µg/l concentration were incubated, but all died within 2 days. Three egg samples (50 eggs each) from 6.9 µg/l had a hatch success of 76%. Thirty egg samples from 3.2 µg/l had an average hatch success of 65% versus 92% for 20 samples from the one control group that spawned regularly. It was observed that hatch success was very variable in 3.2 µg/l and quite uniform in the control. Bartlett's test for homogeneity of variance indicated a significant difference (Snedecor, 1956; Steel and Torrie, 1960). Application of Cochran and Cox's approximate test for unpaired observations and unequal sample variances indicated that the average hatch success in 3.2 µg/l was significantly lower than that in the control (Snedecor, 1956; Steel and Torrie, 1960). Hatch success of paired egg samples from parents exposed to 3.2 µg/l was not improved by incubation in control water.

Hatch success of control eggs was not affected by being transferred to either 60.3 or 28.0 µg/l shortly after fertilization (Table 5). Diazinon had no apparent effect on incubation time between fertilization and hatch.

The growth and survival of progeny up to 60 days after hatch were not affected by 3.3 µg/l (3.2 µg/l as eggs incubated in adult chambers). The one group of progeny reared at 6.8 µg/l had lower survival but greater growth than the control (Table 6). Control progeny transferred to 62.6 and 28.0 µg/l at spawning and reared at these concentrations for 60 days after hatch weighed less than controls and had an average survival rate only two-thirds that of the control. There was no evidence of scoliosis in the progeny after 2 months of exposure to diazinon.

BROOK TROUT PRELIMINARY TEST

In 56 µg/l diazinon brook trout showed signs of distress (lethargy and loss of equilibrium) within 1 week and ceased feeding by the second week. The same symptoms were observed after 3 weeks in 11 µg/l. Both groups began eating in the second month of exposure, but tetanic convulsions were common when the fish were disturbed. One fish died in 56 µg/l during the 2-month exposure. None of the trout developed permanent deformities, but temporary flexing of the spine resembling scoliosis and lordosis was observed. No distress was observed in 2.6 µg/l.

BROOK TROUT PARTIAL-CHRONIC TOXICITY TEST

Reduced feeding, lethargy when undistrubed, and hyperactivity followed by tetanic convulsions when disturbed were common symptoms at exposures of 9.6 and 4.5 µg/l after 1 and 4 weeks, respectively. Some tetany was observed in 2.4 µg/l during the second and third month. Fish from these three concentrations were inactivated very rapidly by Ethyl-m-Aminobenzoate Methanesulfonate used as an anesthetic during inventory at 91 days. Between the third and sixth month none of these symptoms were observed, and even those fish with severe spinal deformities began to eat normally.

TABLE 5. SPAWNING AND HATCHABILITY OF EGGS FROM FATHEAD MINNOWS (PIMEPHALES PROMELAS) CONTINUOUSLY EXPOSED TO DIAZINON (TEST #2)

Item	Average measured diazinon concentration in water (µg/l.)											
	60.3		28.0		13.5		6.9		3.2		Control	
	A	B	A	B	A	B	A	B	A	B	A	B
Number of mature females at termination	1	6	6	7	5	7	2	3	5	7	8	6
Number of spawnings	0	0	1	0	5	0	5	0	42	24	53	1
Total number of eggs	0	0	7	0	767	0	551	0	3,630	2,000	5,767	11
Eggs/spawning	-	-	7	-	153	-	110	-	86	83	109	11
Eggs/female	0	0	1	0	153	0	275	0	726	285	720	2
Hatchability[a] %	93[b]	93[b]	94[b]	88[b]	0	-	76	-	68	61	92	-
									65*		92	
(Standard deviation)	(5.0)	(4.0)	(4.3)	(6.6)	-	-	(8.0)	-	(23.5)	(19.5)	(7.0)	-
(N)	(10)	(9)	(4)	(4)	-	-	(3)	-	(18)	(12)	(22)	-

Continued on page 16.

TABLE 5 (Continued). SPAWNING AND HATCHABILITY OF EGGS FROM FATHEAD MINNOWS (PIMEPHALES PROMELAS) CONTINUOUSLY EXPOSED TO DIAZINON (TEST #2)

Item	Average measured diazinon concentration in water (µg/l.)											
	60.3		28.0		13.5		6.9		3.2		Control	
	A	B	A	B	A	B	A	B	A	B	A	B
	Overall reproductive potential											
Estimated larvae/ female	0		1		0		84		304		380	
	Gonadal development [c]											
Mature males	3	2	7	4	5	4	5	5	7	3	4	6
Mature females	1	7	7	7	5	7	2	3	5	7	8	6
Mature males and females (%)	27	60	93	73	67	73	47	53	80	67	80	80

[a] Hatchability samples contained 50 eggs.

[b] Hatchability of eggs transferred from control A at spawning.

[c] Includes deaths during spawning period. Each group started with 15 fish.

* Significantly different from control (P = 0.05) using approximate test for unequal sample variance.

TABLE 6. SURVIVAL AND GROWTH AFTER 30 AND 60 DAYS FOR PROGENY OF FATHEAD MINNOWS (PIMEPHALES PROMELAS) CONTINUOUSLY EXPOSED TO DIAZINON (TEST #2)

Item	Average measured diazinon concentration in water (µg/L.)									
	62.6		28.0		6.8		3.3		Control	
	A	B	A	B	A	B	A	B	A	B
	30 Days									
Survival (%)	73[a]	79[a]	51[a]	78[a]	55	-	78	70	60	73[a]
(Standard deviation)	(13)	(8)	(42)	(9)	-	-	(10)	(18)	(19)	(14)
Average total length (mm)	13.3[a]	13.6[a]	13.2[a]	11.0[a]	16.0	-	13.0	13.8	13.7	15.4[a]
(Standard error of mean)	(0.5)	(1.2)	(1.0)	(0.4)	-		(0.4)	(0.6)	(0.2)	(0.4)
(N)[b]	(4)	(4)	(3)	(4)	(1)		(4)	(4)	(6)	(4)
Average weight (mg)	28[a]	40[a]	14[a]	13[a]	-	-	22	18	28	44[a]
(Standard error of mean)	(3)	(1)	-	(1)			(5)	(2)	(2)	(6)
(N)[b]	(2)	(2)	(1)	(2)			(2)	(2)	(4)	(2)

continued on page 18.

TABLE 6 (Continued). SURVIVAL AND GROWTH AFTER 30 AND 60 DAYS FOR PROGENY OF FATHEAD MINNOWS (PIMEPHALES PROMELAS) CONTINUOUSLY EXPOSED TO DIAZINON (TEST #2)

Item	Average measured diazinon concentration in water (µg/l.)									
	62.6		28.0		6.8		3.3		Control	
	A	B	A	B	A	B	A	B	A	B
				60 Days						
Survival (%)	41[a]	46[a]	35[a]	44[a]	52	-	59	74	61	72[a]
(Standard deviation)	(1)	(6)	-	(6)	-		(1)	(23)	(16)	(15)
Average total length (mm)	19.1[a]	18.0[a]	21.9[a]	17.3[a]	26.7	-	19.4	21.9	20.8	22.1[a]
(Standard error of mean)	(0.1)	(1.9)	-	(1.3)	-		(0.2)	(0.6)	(1.5)	(0.3)
Average weight (mg)	92[a]	80[a]	136[a]	63[a]	259	-	102	131	119	134[a]
(Standard error of mean)	(8)	(21)	-	(17)	-		(4)	(4)	(18)	(2)
(N)[b]	(2)	(2)	(1)	(2)	(1)		(2)	(2)	(2)	(2)

[a]Eggs transferred from control A at spawning.

[b]Number of larval groups initially composed of 40 larvae from a single hatch of eggs.

During the first 173 days mortality in 9.6, 4.8, and 2.4 µg/l was 25%, 4%, and 4%, respectively (Table 7). The first death was observed (in 9.6 µg/l) after 24 days, but more than half of the deaths occurred between 163 and 173 days. Although all fish retained at thinning were apparently healthy, there was an additional 8% mortality during the spawning period in both 9.6 and 4.8 µg/l.

During the first 91 days brook trout exposed to 9.6 µg/l lost 4% of their initial weight. Fish in 4.8 µg/l gained 12%. Weight gain in lower concentrations and the control ranged from 39% to 44% (Table 7). Reduced growth in the higher concentrations was a temporary phenomenon. During the next 82 days percentage gain in weight was equivalent in all concentrations except one sublot in 9.6 µg/l. This latter group also gained weight but at about one-half the general rate. Analysis of variance of instantaneous growth rates indicated significant inhibition of growth in 9.6 and 4.8 µg/l only during the first 91 days. Irregular feeding and shedding of eggs during spawning precluded growth-rate analyses after 173 days.

Some of the brook trout exposed to 9.6 and 4.8 µg/l developed incipient scoliosis and lordosis within a few weeks. After 173 days the incidence of spinal deformities was 33% and 12%, respectively, at these concentrations (Table 7). Although all deformed fish were discarded at thinning, one fish in 4.8 µg/l subsequently developed lordosis.

Incorrect sex ratios and high within-treatment variance in egg viability prevented any accurate analysis of egg production, egg viability, or hatch. There was no indication that diazinon adversely affected these indices of reproduction (Table 8). The incubation time of eggs was not affected by diazinon. All females in the control and lower concentrations spawned, but both the 9.6 and 4.8 µg/l concentrations had one female with immature ovaries 20 days after all other females had ceased spawning and another female in 9.6 µg/l had not spawned by this time although her body cavity was filled with loose eggs.

There was no correlation between diazinon concentration and incidence of embryonic deformity or survival of progeny from hatch to 122 days (Table 9).

The average total length of larvae from parents exposed to 9.6 µg/l (eggs exposed to 11.1 µg/l) was significantly less than that of control larvae two days after hatch. After 122 days all progeny groups from parents exposed to diazinon were significantly smaller than the controls. Analysis of variance of instantaneous growth rate to 122 days indicated that growth was significantly inhibited in all treated groups except those reared in 2.7 µg/l (Table 9). Cross transfers of paired egg samples between the control and 11.1 µg/l indicated that the growth of progeny during the larval stage was not affected by the presence or absence of diazinon. Progeny from parents exposed to diazinon showed no increase in growth when reared to 30 days after hatch in control water. Exposure of progeny from control parents to 11.1 µg/l for 122 days after hatch caused no decrease in growth.

TABLE 7. SURVIVAL, GROWTH, AND INCIDENCE OF SCOLIOSIS AND LORDOSIS FOR PARENTAL BROOK TROUT (SALVELINUS FONTINALIS) CONTINUOUSLY EXPOSED TO DIAZINON FOR 91 AND 173 DAYS

Item	Average measured diazinon concentration in water (µg/l.)											
	9.6		4.8		2.4		1.1		0.55		Control	
	A	B	A	B	A	B	A	B	A	B	A	B
Start												
Average total length (mm)	225	227	232	232	231	230	230	224	237	234	230	228
(Standard deviation)	(17)	(13)	(17)	(18)	(21)	(17)	(21)	(15)	(23)	(21)	(19)	(15)
Average weight (g)	112	121	126	128	124	123	121	112	135	129	120	118
(Standard deviation)	(21)	(31)	(34)	(36)	(37)	(29)	(32)	(28)	(41)	(39)	(33)	(23)
91 Days												
Survival (%)[a]	92	92	100	100	100	100	100	100	100	100	100	100
	92*		100		100		100		100		100	
Average total length (mm)	224	223	234	236	250	249	253	247	258	258	251	254
(Standard deviation)	(17)	(15)	(17)	(15)	(24)	(17)	(20)	(19)	(19)	(16)	(23)	(17)
Average weight (g)	108	116	138	144	180	173	175	168	192	189	177	180
(Standard deviation)[a]	(40)	(34)	(43)	(29)	(51)	(33)	(36)	(46)	(45)	(37)	(43)	(35)
Instantaneous growth rate[b] X 10,000	-4	-5	10	13	41	37	41	45	39	42	43	46
	-4*		12*		39		43		40		44	
Scoliosis and/or lordosis (%)	17	17	17	0	0	0	0	0	0	0	0	0

Continued on page 21.

TABLE 7 (Continued). SURVIVAL, GROWTH, AND INCIDENCE OF SCOLIOSIS AND LORDOSIS FOR PARENTAL BROOK TROUT (SALVELINUS FONTINALIS) CONTINUOUSLY EXPOSED TO DIAZINON FOR 91 AND 173 DAYS

Item	Average measured diazinon concentration in water (µg/l.)											
	9.6		4.8		2.4		1.1		0.55		Control	
	A	B	A	B	A	B	A	B	A	B	A	B
					173 Days							
Survival (%)[b]	83	67	92	100	92	100	100	100	100	100	100	100
	75*		96		96		100		100		100	
Average total length (mm)	236	-[c]	-[c]	262	289	272	293	283	288	296	287	284
(Standard deviation)	(24)	-	-	(17)	(28)	(42)	(22)	(30)	(23)	(18)	(32)	(26)
Average weight (g)	158	137	202	220	270	262	280	264	269	291	272	260
(Standard deviation)	(85)	(70)	(81)	(49)	(89)	(70)	(59)	(98)	(73)	(54)	(109)	(79)
Instantaneous growth rate[b] X 10,000	46	20	46	52	49	51	57	55	41	53	52	45
Scoliosis and/or lordosis (%)	17	50	25	0	0	0	0	0	0	0	0	0

[a] Each group started with 12 yearling fish.

[b] $\frac{\ln (\text{weight}_2/\text{weight}_1)}{\text{days}}$

[c] Lengths unavailable for some fish because of extreme deformities.

*Significantly different from control (P = 0.05).

TABLE 8. SPAWNING, VIABILITY, AND HATCHABILITY OF EGGS FROM BROOK TROUT (SALVELINUS FONTINALIS) CONTINUOUSLY EXPOSED TO DIAZINON FOR 6 - 8 MONTHS

Item	Average measured diazinon concentration in water (µg/l.)											
	2.6(11.1)[a]		1.8(5.6)[a]		2.4(2.7)[a]		1.1(1.4)[a]		0.55(0.80)[a]		Control	
	A	B	A	B	A	B	A	B	A	B	A	B
Number of females spawning	1	1	1	2	2	2	3	3	4	1	2	4
Total number of eggs spawned	667	215	549	1,060	642	1,730	2,947	1,893	2,678	1,004	1,037	1,905
Number eggs/female	667	215	549	530	321	865	982	631	670	1,004	518	476
Viable spawns[b]												
Number	5	0	0	1	1	3	7	3	3	3	4	1
Total number eggs	647	-	-	253	268	868	2,378	728	855	987	983	387
Number viable eggs	620	-	-	142	137	817	1,849	634	763	972	730	342
Percent viability	96	-	-	56	51	94	78	87	89	98	74	88
(Standard deviation)	(5)	-	-	-	-	(2)	(29)	(7)	(12)	(1)	(8)	-
Percent hatch[c]	94	-	-	49	35	70	68	78	87	94	67	47
(Standard deviation)	(5)	-	-	-	-	(25)	(30)	(13)	(10)	(10)	(6)	-
Gonadal development												
Total number males	5	3	4	4	4	4	3	3	2	5	4	2
Total percent mature males	60	66	50	50	50	25	66	100	100	40	25	100
Total number females	1	3	2	2	2	2	3	3	4	1	2	4
Total percent mature females	100	66[d]	50	100	100	100	100	100	100	100	100	100

[a]Values in parenthesis are concentrations at which eggs were incubated following spawning.

[b]Spawns which contained some viable eggs.

[c]Hatchability samples contained 50 eggs from each viable spawn.

[d]One ripe female did not spawn.

TABLE 9. SURVIVAL AND GROWTH TO 122 DAYS FOR PROGENY OF BROOK TROUT (SALVELINUS FONTINALIS) CONTINUOUSLY EXPOSED TO DIAZINON

Item	Average measured diazinon concentration in water (µg/l.)											
	11.1		5.6		2.7		1.4		0.80		Control	
	A	B	A	B	A	B	A	B	A	B	A	B
2 days												
Average total length (mm)	14.9*		15.1		15.2		15.5		15.2		15.8	
(Standard error of mean)	(0.1)		--		(0.2)		(0.3)		(0.1)		(0.3)	
(N)[a]	(3)		(1)		(4)		(4)		(4)		(4)	
30 days												
Survival (%)	100	--	--	90	94	100	99	100	100	100	99	100
(Standard deviation)	(0.0)	--	--	--	--	(0.0)	(1.9)	(0.0)	(0.0)	(0.0)	(2.3)	--
(N)[a]	(3)	(0)	(0)	(1)	(1)	(3)	(7)	(3)	(2)	(3)	(3)	(1)
Average total length (mm)	20.2*		20.6		21.8		21.8		21.2*		22.5	
(Standard error of mean)	(0.1)		--		(0.2)		(0.5)		(0.1)		(0.2)	
(N)[a]	(3)		(1)		(4)		(4)		(4)		(4)	
Instantaneous growth rate[b] X 10,000	109*		111		129		122		119		126	

Continued on page 24.

TABLE 9 (Continued). SURVIVAL AND GROWTH TO 122 DAYS FOR PROGENY OF BROOK TROUT (SALVELINUS FONTINALIS) CONTINUOUSLY EXPOSED TO DIAZINON

Item	Average measured diazinon concentration in water (µg/l.)											
	11.1		5.6		2.7		1.4		0.8		Control	
	A	B	A	B	A	B	A	B	A	B	A	B
	122 days											
Survival (%)	83	--	--	65	94	96	60	94	88	67	96	81
(Standard deviation)	(9.2)	--	--	--	--	(4.0)	(5.7)	(2.8)	--	(15.1)	(4.0)	--
(N) [a]	(3)	(0)	(0)	(1)	(1)	(3)	(2)	(2)	(1)	(3)	(3)	(1)
Average total length (mm)	55.5*		51.2		59.0*		55.2*		55.1*		65.8	
(Standard error of mean)	(0.4)		--		(0.2)		(3.5)		(1.3)		(0.8)	
(N)[a]	(3)		(1)		(4)		(4)		(4)		(4)	
Instantaneous growth rate [b] X10,000	143*		133		147		138*		140*		155	
Average weight (g)	1.69*		1.19		2.18*		1.63*		1.66*		2.76	
(Standard error of mean)	(0.05)		--		(0.04)		(0.29)		(0.11)		(0.17)	

[a] Random samples of 25 larvae (less when hatch was less than 50%) taken 2 days after hatch from individual hatchability samples of 50 eggs.

[b] $\frac{\ln (length_2/length_1)}{days}$

*Significantly different from control (P = 0.05).

There was no evidence of scoliosis or lordosis among surviving progeny 122 days after hatch.

DIAZINON ACCUMULATION IN BROOK TROUT TISSUES

Accumulation data are presented in Appendix D, Table 1.

SECTION 6

DISCUSSION

The purpose of this study was the determination of chronic "no-effect" concentrations and subsequent calculation of "application factors" by comparison with acute data.

Reductions in production rates were statistically significant for both fathead minnows and brook trout at the lowest diazinon concentrations tested (3.2 and 0.55 µg/l, respectively). Therefore, maximum acceptable toxicant concentrations (MATC), as defined by Mount and Stephan (1967) cannot be postulated for these species. However, subjective examination of the data suggests that rough approximations of no-effect levels may be determined. In the fathead minnow chronic test it appeared that effects that could influence population success became progressively less as concentrations decreased. Probably 3.2 µg/l is not too much higher than the concentration at which measurable effects would not be observed. The brook trout chronic test produced mixed results. Concentrations of 4.8 µg/l and above were obviously detrimental to the fish, but the apparent toxicant-related stunting of progeny was just as pronounced at 0.55 µg/l as at concentrations as high as 9.6 µg/l. Therefore, it was not possible to estimate a concentration at which all detrimental effects could no longer be measured, but overall effects on population survival would probably be minimal below 0.55 µg/l. In summation, these chronic exposures indicated application factors (MATC/96-hr LC50) in the order of magnitude of 10^{-4} or lower for both brook trout and fathead minnows. These values are about 2 to 3 orders of magnitude lower than the actual or tentative application factors reported for other pesticides tested against one or both of these species (Mount and Stephan, 1967; Carlson, 1972; Hermanutz *et al.*, 1973; several unpublished studies performed by or supervised by the Environmental Research Laboratory-Duluth). On the other hand, a recent chronic diazinon exposure of flagfish (*Jordanella floridae*) by Allison (unpublished data) produced an application factor with this species of the order of magnitude of 10^{-2}. At this time we cannot justify the application of a single factor to acute toxicity results to estimate the chronic toxicity of diazinon to other species of fishes.

The generally higher early survival of fathead minnow larvae in the presence of diazinon may have been an artifact resulting from the use of acetone in the diazinon stock solutions. Increased growth of microorganisms in the test chambers was correlated with the amounts of acetone introduced as well as diazinon concentrations present. These organisms may have contributed substantially to the food of larval fish during a period when a continuous supply was needed.

The negative results reported during brook trout spawning should be viewed with some reservation. Inaccurate determination of sex at thinning resulted in undesirable sex ratios in most groups during spawning. Reproductive success in some concentrations was based on the productivity of only four females rather than eight as planned. In addition, most of the spawnings throughout the system were apparently not fertilized as no embryonic development was observed in these eggs. A few spawnings with high viability rates occurred in all concentrations usually early in the spawning period. It is believed that some defect in the design of the modified spawning substrates was responsible for this phenomenon as it was concurrently observed in other tests in which this equipment was used in this laboratory and elsewhere.

Mortality and inhibition of ovarian development or spawning response of female brook trout indicate that, under similar conditions approximately one-quarter and one-half of the females in a population would make no contribution to reproduction in 4.8 and 9.6 µg/l, respectively. However, the small number of fish used in this test should be taken into consideration if extrapolation to larger populations is attempted. The neurological symptoms prevalent early in the test were not observed during the reproductive period. If the initial exposure to diazinon had occurred shortly before the spawning period, reproduction might have been inhibited to a greater extent in some or all of the concentrations tested.

A number of investigators have reported spinal deformities in fishes exposed to pesticides. MaCann and Jasper (1972) made an extensive study of crippling in juvenile bluegills following acute exposure to several pesticides. Fractures of the caudal vertebrae and localized hemorrhaging often occurred within a few hours. Dislocation of the spine was permanent after the fish were removed from the toxicant. Fathead minnows and brook trout in our chronic exposures developed scoliosis and lordosis over periods of weeks or months. Individual response was variable and ranged from small kinks in the caudal peduncle to extensive spinal displacement approaching 180^{o}. The acutal effects observed in fathead minnows were greater than the reported data indicate. In diazinon-exposed fish the majority of these deformities were gross, whereas most deformities in the controls were relatively minor and did not appear to affect swimming ability. No spinal deformities were observed in progeny of either species although these fish were exposed for relatively long periods to concentrations that produced scoliosis and lordosis in the parental stock. It seemed possible that genetic adaptation was involved as abnormal brook trout were culled before spawning, perhaps leaving more resistant fish. However, progeny from unexposed parents in the control groups also failed to develop spinal deformities when reared in the presence of diazinon. This appears to preclude selection of resistant progeny as the reason for this phenomenon. Brook trout used in the preliminary test did not develop permanent deformities although the highest concentration was almost six times the maximum used in the chronic test. Fish used in the preliminary test were approximately 4 months younger, which may indicate that age has some bearing on this syndrome.

Freshwater fish populations could be directly damaged by prolonged exposure to diazinon at concentrations several thousand times lower than those causing acute mortality. Diazinon may persist many months in some freshwater environments although comprehensive field data are not available. There is evidence that the introduction of diazinon into the aquatic environment and its subsequent persistence are affected by a great number of poorly understood physical, chemical, and biological factors. Uniform concentrations of an organophosphate insecticide, as employed in these tests, could probably occur only in exceptional circumstances (such as point discharge from a manufacturing operation). Further studies investigating the chronic effects of fluctuating and intermittent exposures of fishes and invertebrates to diazinon and other organophosphates are needed to aid in the establishment of environmentally safe concentrations for these "non-persistent" pesticides.

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APPENDICES

APPENDIX A

RECOMMENDED BIOASSAY PROCEDURE FOR

FATHEAD MINNOW PIMEPHALES PROMELAS RAFINESQUE CHRONIC TESTS

by

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RECOMMENDED BIOASSAY PROCEDURES

Preface

Recommended Bioassay Procedures are established by the approval of both the Committee on Aquatic Bioassays and the Director of the National Water Quality Laboratory. The main reasons for establishing them are: (1) to permit direct comparison of test results, (2) to encourage the use of the best procedures available, and (3) to encourage uniformity. These procedures should be used by National Water Quality Laboratory personnel whenever possible, unless there is a good reason for using some other procedure.

Recommended Bioassay Procedures consider the basic elements that are believed to be important in obtaining reliable and reproducible results in laboratory bioassays. An attempt has been made to adopt the best acceptable procedures based on current evidence and opinion, although it is recognized that alternative procedures may be adequate. Improvements in the procedures are being considered and tested, and revisions will be made when necessary. Comments and suggestions are encouraged.

Director, National Water Quality Lab, (NWQL)

Committee on Aquatic Bioassays, NWQL

Recommended Bioassay Procedure for

Fathead Minnow Pimephales promelas Rafinesque Chronic Tests

April, 1971

(Revised January, 1972)

A. Physical system

1. Diluter: Proportional diluters (Mount and Brungs, 1967) should be employed for all long-term exposures. Check the operation of the diluter daily, either directly or through measurement of toxicant concentrations. A minimum of five toxicant concentrations and one control should be used for each test with a dilution factor of not less than 0.30. An automatically triggered emergency aeration and alarm system must be installed to alert staff in case of diluter, temperature control or water supply failure.

2. Toxicant mixing: A container to promote mixing of toxicant bearing and w-cell water should be used between diluter and tanks for each concentration. Separate delivery tubes should run from this container to each duplicate tank. Check at least once every month to see that the intended amounts of water are going to each duplicate tank or chamber.

3. Tank: Two arrangements of test tanks (glass, or stainless steel with glass ends) can be utilized:

   a. Duplicate spawning tanks measuring 1 x 1 x 3 ft. long with a one sq. ft. portion at one end screened off and divided in half for the progeny. Test water is to be delivered separately to the larval and spawning chambers of each tank, with about one-third the water volume going to the former chamber as to the latter.

   b. Duplicate spawning tanks measuring 1 x 1 x 2 ft. long with a separate duplicate progeny tank for each spawning tank. The larval tank for each spawning tank should be a minimum of 1 cu. ft. dimensionally and divided to form two separate larval chambers with separate standpipes, or separate 1/2 sq. ft. tanks may be used. Test water is to be supplied by delivery tubes from the mixing cells described in Step 2 above.

   Test water depth in tanks and chambers for both a & b above should be 6 inches.

4. Flow rate: The flow rate to each chamber (larval or adult) should be equal to 6 to 10 tank volumes/24 hr.

5. Aeration: Total dissolved oxygen levels should never be allowed to drop below 60% of saturation, and flow rates must be increased if oxygen levels do drop below 60%. As a first alternative flow rates can be increased above those specified in A.4. Only aerate (with oil free air) if testing a non-volatile toxic agent, and then as a last resort to maintain dissolved oxygen at 60% of saturation.

6. Cleaning: All adult tanks, and larvae tanks and chambers after larvae swim-up, must be siphoned a minimum of 2 times weekly and brushed or scraped when algal or fungus growth becomes excessive.

7. Spawning substrate: Use spawning substrates made from inverted cement and asbestos halved, 3-inch ID drain tile, or the equivalent, each of these being 3 inches long.

8. Egg cup: Egg incubation cups are made from either 3-inch sections of 2-inch OD (1 1/2-inch ID) polyethylene water hose or 4-oz., 2-inch OD round glass jars with the bottoms cut off. One end of the jar or hose sections is covered with stainless steel or nylon screen (with a minimum of 40 meshes per inch). Cups are oscillated in the test water by means of a rocker arm apparatus driven by a 2 r.p.m. electric motor (Mount, 1968). The vertical-travel distance of the cups should be 1 to 1 1/2 inches.

9. Light: The lights used should simulate sunlight as nearly as possible. A combination of Durotest (Optima FS)[1,2] and wide spectrum Grow-lux[3] fluorescent tubes has proved satisfactory at the NWQL.

10. Photoperiod: The photoperiods to be used (Appendix A) simulate the dawn to dusk times of Evansville, Indiana. Adjustments in day-length are to be made on the first and fifteenth day of every Evansville test month. The table 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 should be adjusted so that the mean or estimated hatching date of the fish used to start the experiment 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. To illustrate these points, an experiment started with 5-day-old larvae in Duluth, Minnesota, on August 28 (actual date), would require use of a December 5 Evansville test photoperiod, and the lights could go on anytime on that day just so long as they remained on for 10 hours and 45 minutes. Ten days later (Sept. 7 actual date, Dec. 15 Evansville test date) the day-length

[1]Mention of trade names does not constitute endorsement
[2]Duro-Test, Inc., Hammond, Ind.
[3]Sylvania, Inc., New York, NY

would be changed to 10 hours and 30 minutes. Gradual changes in light intensity at dawn and dusk (Drummond and Dawson, 1970), if desired, should be included within the day-lengths shown, and should not last for more than 1/2 hour from full on to full off and vice versa.

11. Temperature: Temperature should not deviate instantaneously from 25° C by more than 2° C and should not remain outside the range of 24 to 26° C for more than 48 hours at a time. Temperature should be recorded continuously.

12. Disturbance: Adults and larvae should be shielded from disturbances such as people continually walking past the chambers, or from extraneous lights that might alter the intended photoperiod.

13. Construction materials: Construction materials which contact the diluent water should not contain leachable substances and should not sorb significant amounts of substances from the water. Stainless steel is probably the preferred construction material. Glass absorbs some trace organics significantly. Rubber should not be used. Plastic containing fillers, additives, stabilizers, plasticizers, etc., should not be used. Teflon, nylon, and their equivalents should not contain leachable materials and should not sorb significant amounts of most substances. Unplasticized polyethylene and polypropylene should not contain leachable substances, but may sorb very significant amounts of trace organic compounds.

14. Water: The water used should be from a well or spring if at all possible, or alternatively from a surface water source. Only as a last resort should water from a chlorinated municipal water supply be used. If it is thought that the water supply could be conceivably contaminated with fish pathogens, the water should be passed through an ultraviolet or similar sterilizer immediately before it enters the test system.

B. Biological system

1. Test animals: If possible, use stocks of fathead minnows from the National Water Quality Laboratory in Duluth, Minnesota or the Fish Toxicology Laboratory in Newtown, Ohio. Groups of starting fish should contain a mixture of approximately equal numbers of eggs or larvae from at least three different females. Set aside enough eggs or larvae at the start of the test to supply an adequate number of fish for the acute mortality bioassays used in determining application factors.

2. Beginning test: In beginning the test, distribute 40 to 50 eggs or 1 to 5-day-old larvae per duplicate tank using a stratified random assignment (see D.3). All acute mortality tests should be conducted when the fish are 2 to 3 months old. If eggs or 1 to 5-day-old larvae are not available, fish up to 30 days of age may be used to start the test. If fish between 20 and 60 days old are used, the exposure should be designated a partial chronic test. Extra test animals may be added at the beginning so that fish can be removed periodically for special examinations (see B.12.) or for residue analysis (see C.4.).

3. Food: Feed the fish a frozen trout food (e.g., Oregon Moist). A minimum of once daily fish should be fed ad libitum the largest pellet they will take. Diets should be supplemented weekly with live or frozen-live food (e.g., Daphnia, chopped earthworms, fresh or frozen brine shrimp, etc.). Larvae should be fed a fine trout starter a minimum of 2 times daily, ad libitum; one feeding each day of live young zooplankton from mixed cultures of small copepods, rotifers, and protozoans is highly recommended. Live food is especially important when larvae are just beginning to feed, or about 8 to 10 days after egg deposition. Each batch of food should be checked for pesticides (including DDT, TDE, dieldrin, lindane, methoxychlor, endrin, aldrin, BHC, chlordane, toxaphene, 2,4-D, and PCBs), and the kinds and amounts should be reported to the project officer or recorded.

4. Disease: Handle disease outbreaks according to their nature, with all tanks receiving the same treatment whether there seems to be sick fish in all of them or not. The frequency of treatment should be held to a minimum.

5. Measuring fish: Measure total lengths of all starting fish at 30 and 60 days by the photographic method used by McKim and Benoit (1971). Larvae or juveniles are transferred to a glass box containing 1 inch of test water. Fish should be moved to and from this box in a water-filled container, rather than by netting them. The glass box is placed on a translucent millimeter grid over a fluorescent light platform to provide background illumination. Photos are then taken of the fish over

the millimeter grid and are enlarged into 8 by 10 inch prints. The length of each fish is subsequently determined by comparing it to the grid. Keep lengths of discarded fish separate from those of fish that are to be kept.

6. Thinning: When the starting fish are sixty (± 1 or 2) days old, impartially reduce the number of surviving fish in each tank to 15. Obviously injured or crippled individuals may be discarded before the selection so long as the number is not reduced below 15; be sure to record the number of deformed fish discarded from each tank. As a last resort in obtaining 15 fish per tank, 1 or 2 fish may be selected for transfer from one duplicate to the other. Place five spawning tiles in each duplicate tank, separated fairly widely to reduce interactions between male fish guarding them. One should also be able to look under tiles from the end of the tanks. During the spawning period, sexually maturing males must be removed at weekly intervals so there are no more than four per tank. An effort should be made not to remove those males having well established territories under tiles where recent spawnings have occurred.

7. Removing eggs: Remove eggs from spawning tiles starting at 12:00 noon Evansville test time (Appendix A) each day. As indicated in Step A.9., the test time need not correspond to the actual time where the test is being conducted. Eggs are 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 being to roll. The groups of eggs should then be washed into separate, appropriately marked containers and subsequently handled (counted, selected for incubation, or discarded) as soon as possible after all eggs have been removed and the spawning tiles put back into the test tanks. All egg batches must be checked initially for different stages of development. If it is determined that there is more than one distinct stage of development present, then each stage must be considered as one spawning and handled separately as described in Step B.8.

8. Egg incubation and larval selection: Impartially select 50 unbroken eggs from spawnings of 50 eggs or more and place them in an egg incubator cup for determining viability and hatchability. Count the remaining eggs and discard them. Viability and hatchability determinations must be made on each spawning (>49 eggs) until the number of spawnings (>49 eggs) in each duplicate 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 set up to determine hatchability; however, weekend spawns must still be removed from tiles and the eggs counted. If unforseen problems are encountered in determining egg viability and hatchability, additional spawnings should be sampled before switching to the setting up of eggs from every third spawning. Every day record the live and dead eggs in the incubator cups, remove the dead ones, and clean the cup screens. Total numbers 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, generally after 4 to 6 days, they should not be handled again or removed from the egg-cups until all have hatched. Then, if enough are still alive, 40 of these are eligible to be transferred immediately to a larval test chamber. Those individuals selected out to bring the number kept to 40 should be chosen impartially. Entire egg-cup-groups not used for survival and growth studies should be counted and discarded.

9. Progeny transfer: Additional important information on hatchability and larval survival is to be gained by transferring control eggs immediately after spawning to concentrations where spawning is reduced or absent, or to where an affect is seen on survival of eggs or larvae, and by transferring eggs from these concentrations to the control tanks. One larval chamber in, or corresponding to, each adult tank should always be reserved for eggs produced in that tank.

10. Larval exposure: From early spawnings in each duplicate tank, use the larvae hatched in the egg incubator cups (Step B.8. above) for 30 or 60 day growth and survival exposures in the larval chambers. Plan ahead in setting up eggs for hatchability so that a new group of larvae is ready to be tested for 30 or 60 days as soon as possible after the previously tested group comes out of the larval chambers. Record mortalities, and measure total lengths of larvae at 30 and, if they are kept, 60 days post-hatch. At the time the larval test is terminated they should also be weighed. No fish (larvae, juveniles, or adults) should be fed within 24 hr's. of when they are to be weighed.

11. Parental termination: Parental fish testing should be terminated when, during the receding day-length photoperiod, a one week period passes in which no spawning occurs in any of the tanks. Measure total lengths and weights of parental fish; check sex and condition of gonads. The gonads of most parental fish will have begun to regress from the spawning condition, and thus the differences between the sexes will be less distinct now than previously. Males and females that are readily distinguishable from one another because of their external characteristics should be selected initially for determining how to differentiate between testes and ovaries. One of the more obvious external characteristics of females that have spawned is an extended, transparent anal canal (urogenital papilla). The gonads of both sexes will be located just ventral to the kidneys. The ovaries of the females at this time will appear transparent, but perhaps containing some yellow pigment, coarsely granular, and larger than testes. The testes of males will appear as slender, slightly milkly, and very finely granular strands. Fish must not be frozen before making these examinations.

12. Special examinations: Fish and eggs obtained from the test should be considered for physiological, biochemical, histological and other examinations which may indicate certain toxicant related effects.

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

    a. Number and individual total length of normal and deformed fish at 30 and 60 days; total length, weight and number of either sex, both normal and deformed, at end of test.

    b. Mortality during the test.

    c. Number of spawns and eggs.

    d. Hatchability.

    e. Fry survival, growth, and deformities.

C. Chemical system

1. Preparing a stock solution: If a toxicant cannot be introduced into the test water as is, a stock solution should be prepared by dissolving the toxicant in water or an organic solvent. Acetone has been the most widely used solvent, but dimethylformanide (DMF) and triethylene glycol may be preferred in many cases. If none of these solvents are acceptable, other water-miscible solvents such as methanol, ethanol, isopropanol, acetonitrile, dimethylacetamide (DMAC), 2-ethoxyethanol, glyme (dimethylether of ethylene glycol, diglyme (dimethyl ether of diethylene glycol) and propylene glycol should be considered. However, dimethyl sulfoxide (DMSO) should not be used if at all possible because of its biological properties.

   Problems of rate of solubilization or solubility limit should be solved by mechanical means if at all possible. Solvents, or as a last resort, surfactants, can be used for this purpose, only after they have been proven to be necessary in the actual test system. The suggested surfactant is p-tert-octylphenoxynonaethoxyethanol (p-1, 1, 3, 3-tetramethylbutylphenoxynonaethoxyethanol, OPE_{10}) (Triton X-100, a product of the Rohm and Haas Company, or equivalent).

   The use of solvents, surfactants, or other additives should be avoided whenever possible. If an additive is necessary, reagent grade or better should be used. The amount of an additive used should be kept to a minimum, but the calculated concentration of a solvent to which any test organisms are exposed must never exceed one one-thousandth of the 96-hr. TL50 for test species under the test conditions and must never exceed one gram per liter of water. The calculated concentration of surfactant or other additive to which any test organisms are exposed must never exceed one-twentieth of the concentration of the toxicant and must never exceed one-tenth gram per liter of water. If any additive is used, two sets of controls must be used, one exposed to no additives and one exposed to the highest level of additives to which any other organisms in the test are exposed.

2. Measurement of toxicant concentration: As a minimum the concentration of toxicant must be measured in one tank at each toxicant concentration every week for each set of duplicate tanks, alternating tanks at each concentration from week to week. Water samples should be taken about midway between the top and bottom and the sides of the tank and should not include any surface scum or material stirred up from the bottom or sides of the tank. Equivolume daily grab samples can be composited for a week if it has been shown that the results of the analysis are not affected by storage of the sample.

Enough grouped grab samples should be analyzed periodically throughout the test to determine whether or not the concentration of toxicant is reasonably constant from day to day in one tank and from one tank to its duplicate. If not, enough samples must be analyzed weekly throughout the test to show the variability of the toxicant concentration.

3. Measurement of other variables: Temperature must be recorded continuously (see A.10.).

   Dissolved oxygen must be measured in the tanks daily, at least five days a week on an alternating basis, so that each tank is analyzed once each week. However, if the toxicant or an additive causes a depression in dissolved oxygen, the toxicant concentration with the lowest dissolved oxygen concentration must be analyzed daily in addition to the above requirement.

   A control and one test concentration must 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 affected by the toxicant the tanks must be analyzed for that characteristic daily, at least five days a week, on an alternating basis so that each tank is analyzed once every other week.

   At a minimum, the test water must 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: When possible and deemed necessary, mature fish, and possibly eggs, larvae, and juveniles, obtained from the test, should be analyzed for toxicant residues. For fish, muscle should be analyzed, and gill, blood, brain, liver, bone, kidney, GI tract, gonad, and skin should be considered for analysis. Analyses of whole organisms may be done in addition to, but should not be done in place of, analyses of individual tissues, especially muscle.

5. Methods: When they will provide the desired information with acceptable precision and accuracy, methods described in Methods for Chemical Analysis of Water and Wastes (EPA, 1971) should be used unless there is another method which requires much less time and can provide the desired information with the same or better precision and accuracy. At a minimum, accuracy should be measured using the method of known additions for all analytical methods for toxicants. If available, reference samples should be analyzed periodically for each analytical method.

D. Statistics

1. Duplicates: Use true duplicates for each level of toxic agent, i.e., no water connections between duplicate tanks.

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

3. Distribution of test organisms: The test organisms should be assigned to tanks by stratified random assignment (random assignment of one test organism to each tank, random assignment of a second test organism to each tank, etc.).

E. Miscellaneous

1. Additional information: All routine bioassay flow through methods not covered in this procedure (e.g., physical and chemical determinations, handling of fish) should be followed as described in Standard Methods for the Examination of Water and Wastewater, (American Public Health Association, 1971), or information requested from appropriate persons at Duluth or Newtown.

2. Acknowledgments: These procedures for the fathead minnow were 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 Stephan, and James Tucker.

3. References: For additional information concerning flow through bioassays with fathead minnows, the following references are listed:

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Approved by the Committee
on Aquatic Bioassays, NWQL

Approved by the Director, NWQL

Appendix A

Test (Evansville, Indiana) Photoperiod

For Fathead Minnow Chronic

Dawn to Dusk Time	Date	Day-length (hour and minute)	
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-
6:00 - 5:45)	15	11:45)	spawning growth
		)	period
6:00 - 6:15)	MAR. 1	12:15)	
6:00 - 7:00)	15	13:00)	
		)	
6:00 - 7:30)	PR. 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)	
6:00 - 9:45)	15	15:45)	4-month spawning
		)	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)	SEPT. 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

RECOMMENDED BIOASSAY PROCEDURE FOR

BROOK TROUT SALVELINUS FONTINALIS (MITCHILL) PARTIAL CHRONIC TESTS

RECOMMENDED BIOASSAY PROCEDURES

Preface

Recommended Bioassay Procedures are established by the approval of both the Committee on Aquatic Bioassays and the Director of the National Water Quality Laboratory. The main reasons for establishing them are: (1) to permit direct comparison of test results, (2) to encourage the use of the best procedures available, and (3) to encourage uniformity. These procedures should be used by National Water Quality Laboratory personnel whenever possible, unless there is a good reason for using some other procedure.

Recommended Bioassay Procedures consider the basic elements that are believed to be important in obtaining reliable and reproducible results in laboratory bioassays. An attempt has been made to adopt the best acceptable procedures based on current evidence and opinion, although it is recognized that alternative procedures may be adequate. Improvements in the procedures are being considered and tested, and revisions will be made when necessary. Comments and suggestions are encouraged.

Director, National Water Quality Lab (NWQL)

Committee on Aquatic Bioassays, NWQL

Recommended Bioassay Procedure for

Brook Trout Salvelinus fontinales (Mitchill) Partial Chronic Tests

April, 1971

(Revised January, 1972)

A. Physical system

1. Diluter: Proportional diluters (Mount and Brungs, 1967) should be employed for all long-term exposures. Check the operation of the diluter daily, either directly or through the measurement of toxicant concentrations. A minimum of five toxicant concentrations and one control should be used for each test with a dilution factor of not less than 0.30. An automatically triggered emergency aeration and alarm system must be installed to alert staff in case of diluter, temperature control or water supply failure.

2. Toxicant mixing: A container to promote mixing of toxicant bearing and w-cell water should be used between diluter and tanks for each concentration. Separate delivery tubes should run from this container to each duplicate tank. Check to see that the same amount of water goes to duplicate tanks and that the toxicant concentration is the same in both.

3. Tank: Each duplicate spawning tank (preferably stainless steel) should measure 1.3 X 3 X 1 ft. wide with a water depth of 1 foot and alevin-juvenile growth chambers (glass or stainless steel with glass bottom) 7 X 15 X 5 in. wide with a water depth of 5 inches. Growth chambers can be supplied test water by either separate delivery tubes from the mixing cells described in Step 2 above or from test water delivered from the mixing cell to each duplicate spawning tank. In the second choice, test water must always flow through growth chambers before entering the spawning tank. Each growth chamber should be designed so that the test water can be drained down to 1 inch and the chamber transferred over a fluorescent light box for photographing the fish (see B.10).

4. Flow rate: Flow rates for each duplicate spawning tank and growth chamber should be 6-10 tank volumes/24 hr.

5. Aeration: Brook trout tanks and growth chambers must be aerated with oil free air unless there are no flow limitations and 60% of saturation can be maintained. Total dissolved oxygen levels should never be allowed to drop below 60% of saturation.

6. Cleaning: All tanks and chambers must be siphoned daily and brushed at least once per week. When spawning commences, gravel baskets must be removed and cleaned daily.

7. Spawning substrates: Use two spawning substrates per duplicate made of plastic or stainless steel which measure at least 6 X 10 X 12 in. with 2 inches of .25 to .50 inch stream gravel covering the bottom and 20 mesh stainless steel or nylon screen attached to the ends for circulation of water.

8. Egg cup: Egg incubation cups are made from 4-oz. 2-inch OD round glass jars with the bottoms cut off and replaced with stainless steel or nylon screen (40 meshes per inch). Cups are oscillated in the test water by means of a rocker arm apparatus driven by a 2 r.p.m. electric motor (Mount, 1968).

9. **Light**: The lights used should simulate sunlight as nearly as possible. A combination of Duro-Test (Optima FS)[1,2] and wide spectrum Gro-lux[3] fluorescent tubes has proved satisfactory at the NWQL.

10. Photoperiod: The photoperiods to be used (Appendix A) simulate the dawn to dusk times of Evansville, Indiana. Evansville dates must correspond to actual dates in order to avoid putting natural reproductive cycles out of phase. Adjustments in photoperiod are to be made on the first and fifteenth of every Evansville test month. The table is arranged so that adjustments need be made only in the dusk times. The dawn and dusk times listed in the table (Evansville test time) need not correspond to the actual test times where the test is being conducted. To illustrate this point, a test started on March first would require the use of the photoperiod for Evansville test date March first, and the lights could go on any time on that day just so long as they remained on for twelve hours and fifteen minutes. Fifteen days later the photoperiod would be changed to thirteen hours. Gradual changes in light intensity at dawn and dusk (Drummond and Dawson, 1970), may be included within the photoperiods shown, and should not last for more than 1/2 hour from full on to full off and vice versa.

11. Temperature: Utilize the attached temperature regime (see Appendix B). Temperatures should not deviate instantaneously from the specified test temperature by more than 2° C and should not remain outside the specified temperature ±1° C for more than 48 hours at a time.

12. Disturbance: Spawning tanks and growth chambers must be covered with a screen to confine the fish and concealed in such a way that the fish will not be disturbed by persons continually walking

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[1] Mention of trade names does not constitute endorsement.
[2] Duro-Test, Inc., Hammond, Ind.
[3] Sylvania, Inc., New York, N. Y.

past the system. Tanks and chambers must also be shielded from extraneous light which can affect the intended photoperiod or damage light sensitive eggs and alevins.

13. Construction materials: Construction materials which contact the diluent water should not contain leachable substances and should not sorb significant amounts of substances from the water. Stainless steel is probably the preferred construction material. Glass absorbs some trace organics significantly. Rubber should not be used. Plastic containing fillers, additives, stabilizers, plasticizers, etc., should not be used. Teflon, nylon, and their equivalents should not contain leachable materials and should not sorb significant amounts of most substances. Unplasticized polyethylene and polypropylene should not contain leachable substances, but may sorb very significant amounts of trace organic compounds.

14. Water: The water used should be from a well or spring if at all possible, or alternatively from a surface water source. Only as a last resort should water from a chlorinated municipal water supply be used. If it is thought that the water supply could be conceivably contaminated with fish pathogens, the water should be passed through an ultraviolet or similar sterilizer immediately before it enters the test system.

B. Biological system

1. Test animals: Yearling fish should be collected no later than March 1 and acclimated in the laboratory to test temperature and water quality for at least one month before the test is initiated. Suitability of fish for testing should be judged on the basis of acceptance of food, apparent lack of diseases, and 2% or less mortality during acclimation with no mortality two weeks prior to test. Set aside enough fish to supply an adequate number for short-term bioassay exposures used in determining application factors.

2. Beginning test: Begin exposure no later than April 1 by distributing 12 acclimated yearling brook trout per duplicate using a stratified random assignment (see D.3). This allows about a four month exposure to the toxicant before the onset of secondary or rapid growth phase of the gonads.

   Extra test animals may be added at the beginning so that fish can be removed periodically for special examinations (see B.12), or for residue analysis (see C.4).

3. Food: Use a good frozen trout food (e.g., Oregon Moist). Fish should be fed the largest pellet they will take a minimum of two times daily. The amount should be based on a reliable hatchery feeding schedule. Alevins and early juveniles should be fed trout starter a minimum of five times daily. Each batch of prepared food should be checked for pesticides (including DDT, TDE, dieldrin, endrin, aldrin,

BHC, chlordane, toxaphene, 2,4-D, and PCBs), and the kinds and amounts should be reported to the project officer or recorded.

4. Disease: Handle disease outbreaks according to their nature, with all tanks receiving the same treatment whether there seems to be sick fish in all of them or not. The frequency of treatment should be held to a minimum.

5. Measuring fish: Record mortalities daily, and measure fish directly at initiation of test, after three months and at thinning (see B.6) (total length and weight). Fish should not be fed 24 hours before weighing and lightly anesthetized with MS-222 to facilitate measuring (100 mg MS-222/liter water).

6. Thinning: When secondary sexual characteristics are well developed (approximately two weeks prior to expected spawning), separate males, females and undeveloped fish in each duplicate and randomly reduce sexually mature fish (see D.4) to the desired number of 2 males and 4 females, and discard undeveloped fish after examination. Place two spawning substrates (described earlier) in each duplicate. Record the number of mature, immature, deformed and injured males and females in each tank and the number from each category discarded. Measure total length and weight of all fish in each category before any are discarded and note which ones were discarded (see C.4).

7. Removing eggs: Remove eggs from the redd at a fixed time each day (preferably after 1:00 p.m. Evansville time, so the fish are not disturbed during the morning).

8. Egg incubation and viability: Impartially select 50 eggs from the first eight spawnings of 50 eggs or more in each duplicate and place them in an egg incubator cup for hatch. The remaining eggs from the first eight spawnings (>50 eggs) and all subsequent eggs from spawnings should be counted and placed in separate egg incubator cups for determining viability (formation of neural keel after 11-12 days at 9° C). The number of dead eggs from each spawn removed from the nest should be recorded and discarded. Never place more than 250 eggs in one egg incubator cup. All eggs incubated for viability are discarded after 12 days. Discarded eggs can be used for residue analysis and physiological measurements of toxicant related effects.

9. Progeny transfer: Additional important information on hatchability and alevin survival can be gained by transferring control eggs immediately after spawning to concentrations where spawning is reduced or absent, or to where an affect is seen on survival of eggs or alevin, and by transferring eggs from these concentrations to the control tanks. Two growth chambers for each duplicate spawning tank should always be reserved for eggs produced in that tank.

10. Hatch and alevin thinning: Remove dead eggs daily from the hatchability cups described in Step 8 above. When hatching commences, record the number hatched daily in each cup. Upon completion of hatch in any cup, randomly (see D.4) select 25 alevins from that cup. Dead or deformed alevins must not be included in the random selection but should be counted as being dead or deformed upon hatch. Measure total lengths of the 25 selected and discarded alevins. Total lengths are measured by the photographic method used by McKim and Benoit (1971). The fish are transferred to a glass box containing 1 inch of test water. They should be moved to and from this box in a water filled container, rather than by netting them. The glass box is placed on a translucent millimeter grid over a fluorescent light box which provides background illumination. Photos are then taken of the fish over the millimeter grid and are enlarged into 8 X 10 inch prints. The length of each fish is subsequnetly determined by comparing it to the grid. Keep lengths of discarded alevins separate from those which are kept. Place the 25 selected alevins back into the incubator cup and preserve the discarded ones for initial weights.

11. Alevin-juvenile exposure: Randomly (see D.4) select from the incubation cups two groups of 25 alevins each per duplicate for 90-day growth and survival exposures in the growth chambers. Hatching from one spawn may be spread out over a 3 to 6 day period; therefore, the median-hatch date should be used to establish the 90-day growth and survival period for each of the two groups of alevin. If it is determined that the median-hatch dates for the five groups per duplicate will be more than three weeks apart, then the two groups of 25 alevin must be selected from those which are less than three weeks old. The remaining groups in the duplicate which do not hatch during the three week period are used only for hatchability results and then photographed for lengths and preserved for initial weights. In order to equalize the effects of the incubation cups on growth, all groups selected for the 90-day exposure must remain in the incubation cups three weeks before they are released into the growth chambers. Each of the two groups selected per duplicate must be kept separate during the 90-day period. Record mortalities daily, along with total lengths 30 and 60 days post-hatch and total length and weight at 90 days post-hatch. Alevins and early juveniles should not be fed 24 hours before weighing. Total lengths are measured by transferring the growth chambers described earlier to a translucent millimeter grid over a fluorescent light box for photographing as described in Step 10 above. Survival and growth studies should be terminated after three months. Terminated fish can be used for tissue residue analysis and physiological measurements of toxicant related effects.

12. Parental termination: All parental fish should be terminated when a three week period passes in which no spawning occurs in any of the spawning tanks. Record mortality and weigh and measure total length of parental fish, check sex and condition of gonads (e.g., reabsorption, degree of maturation, spent ovaries, etc.).(see C.4).

13. Special examinations: Fish and eggs obtained from the test should be considered for physiological, biochemical, and histological investigations which may indicate certain toxicant related effects.

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

    a. Number and individual weights and total lengths of normal, deformed, and injured mature and immature males and females at initiation of test, three months after test commences, at thinning and at the end of test.

    b. Mortality during the test.

    c. Number of spawns and eggs. A mean incubation time should be calculated using date of spawning and the median hatch dates.

    d. Hatchability.

    e. Fry survival, growth and deformities.

C. Chemical system

1. Preparing a stock solution: If a toxicant cannot be introduced into the test water as is, a stock solution should be prepared by dissolving the toxicant in water or an organic solvent. Acetone has been the most widely used solvent, but dimethylformanide (DMF) and triethylene glycol may be preferred in many cases. If none of these solvents are acceptable, other water-miscible solvents such as methanol, ethanol, isopropanol, acetonitrile, dimethyl-acetamide (DMAC), 2-ethoxyethanol, glyme (dimethylether of ethylene glycol, diglyme (dimethyl ether of diethylene glycol) and propylene glycol should be considered. However, dimethyl sulfoxide (DMSO) should not be used if at all possible because of its biological properties.

   Problems of rate of solubilization or solubility limit should be solved by mechanical means if at all possible. Solvents, or as a last resort, surfactants, can be used for this purpose, only after they have been proven to be necessary in the actual test

system. The suggested surfactant is p-tert-octylphenoxynonaethoxyethanol (p-1, 1, 3, 3-tetramethylbutylphenoxynonaethoxyethanol, OPE_{10}) (Triton X-100, a product of the Rohm and Haas Company, or equivalent).

The use of solvents, surfactants, or other additives should be avoided whenever possible. If an additive is necessary, reagent grade or better should be used. The amount of an additive used should be kept to a minimum, but the calculated concentration of a solvent to which any test organisms are exposed must never exceed one one-thousandth of the 96-hr. TL50 for test species under the test conditions and must never exceed one gram per liter of water. The calculated concentration of surfactant or other additive to which any test organisms are exposed must never exceed one-twentieth of the concentration of the toxicant and must never exceed one-tenth gram per liter of water. If any additive is used, two sets of controls must be used, one exposed to no additives and one exposed to the highest level of additives to which any other organisms in the test are exposed.

2. Measurement of toxicant concentration: As a minimum the concentration of toxicant must be measured in one tank at each toxicant concentration every week for each set of duplicate tanks, alternating tanks at each concentration from week to week. Water samples should be taken about midway between the top and bottom and the sides of the tank and should not include any surface scum or material stirred up from the bottom or sides of the tank. Equivolume daily grab samples can be composited for a week if it has been shown that the results of the analysis are not affected by storage of the sample.

Enough grouped grab samples should be analyzed periodically throughout the test to determine whether or not the concentation of toxicant is reasonably constant from day to day in one tank and from one tank to its duplicate. If not, enough samples must be analyzed weekly throughout the test to show the variability of the toxicant concentration.

3. Measurement of other variables: Temperature must be recorded continuously (see A.11).

Dissolved oxygen must be measured in the tanks daily at least five days a week on an alternating basis, so that each tank is analyzed once each week. However, if the toxicant or an additive causes a depression in dissolved oxygen, the toxicant concentration with the lowest dissolved oxygen concentration must be analyzed daily in addition to the above requirement.

A control and one test concentration must 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 affected by the toxicant, the tanks must be analyzed for that characteristic daily, at least five days a week, on an alternating basis, so that each tank is analyzed once every other week.

At a minimum, the test water must be analyzed at the beginning and near the middle of the chronic test for calcium, magnesium, sodium, potassium, chloride, sulfate, conductance, total solid, and total dissolved solids.

4. Residue analysis: When possible and deemed necessary, mature fish, and possibly eggs, larvae, and juveniles, obtained from the test, should be analyzed for toxicant residues. For fish, muscle should be analyzed, and gill, blood, brain, liver, bone kidney, GI tract, gonad, and skin should be considered for analysis. Analyses of whole organisms may be done in addition to, but should not be done in place of, analyses of individual tissues, especially muscle.

5. Methods: When they will provide the desired information with acceptable precision and accuracy, methods described in Methods for Chemical Analysis of Water and Wastes (EPA, 1971) should be used unless there is another method which requires much less time and can provide the desired information with the same or better precision and accuracy. At a minimum, accuracy should be measured using the method of known additions for all analytical methods for toxicants. If available, reference samples should be analyzed periodically for each analytical method.

D. Statistics

1. Duplicates: Use true duplicates for each level of the toxic agent, i.e., no water connections between duplicate tanks.

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

3. Distribution of test organisms: The test organisms should be assigned to tanks by stratified random assignment (random assignment of one test organism to each tank, random assignment of a second test organism to each tank, etc.).

4. Selection and thinning test organisms: At time of selection or thinning of test organisms the choice must be random (random, as defined statistically).

E. Miscellaneous

1. Additional information: All routine bioassay flow through methods not covered in this procedure (e.g., physical and chemical determinations, handling of fish) should be followed as described in Standard Methods for the Examination of Water and Wastewater (American Public Health Association, 1971).

2. Acknowledgments: These procedures for the brook trout were compiled by J. M. McKim and D. A. Benoit 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 Stephan, and James Tucker.

3. References: For additional information concerning flow through bioassay tests with brook trout, the following references are listed:

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Approved by the Committee on
Aquatic Bioassays, NWQL

Approved by the Director, NWQL

Appendix A

Test (Evansville, Indiana) Photoperiod

For Brook Trout Partial Chronic

Dawn to Dusk Time	Date		Day-length (hour and minute)	
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)	Juvenile-
6:00 - 9:45)		15	15:45)	adult exposure
			)	
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)	SEPT.	1	14:00)	
6:00 - 7:30)		15	13:30)	
6:00 - 6:45)	OCT.	1	12:45)	
6:00 - 6:15)		15	12:15)	
			)	Spawning and
6:00 - 5:30)	NOV.	1	11:30)	egg incubation
6:00 - 5:00		15	11:00)	
6:00 - 4:45)	DEC.	1	10:45)	
6:00 - 4:30)		15	10:30)	
			)	
6:00 - 4:30)	JAN.	1	10:30)	Alevin-juvenile
6:00 - 4:45)		15	10:45)	exposure
			)	
6:00 - 5:15)	FEB.	1	11:15)	
6:00 - 5:45)		15	11:45)	

Appendix B

Temperature Regime for Brook Trout Partial Chronic

Months		Temperature ° C
Mar.	Juvenile-adult exposure	9
Apr.		12
May		14
June		15
July		15
Aug.		15
Sept.		12
Oct.	Spawning and egg incubation	9
Nov.		9
Dec.	Alevin-juvenile exposure	9
Jan.		9
Feb.		9
Mar.		9

A constant temperature must be established just prior to spawning and egg incubation, and maintained throughout the 3-month alevin-juvenile exposure.

APPENDIX C

TABLE 1. TEST CONDITIONS DURING 96-HR ACUTE EXPOSURES OF FATHEAD MINNOWS (*PIMEPHALES PROMELAS*), BLUEGILLS (*LEPOMIS MACROCHIRUS*), BROOK TROUT (*SALVELINUS FONTINALIS*) AND FLAGFISH (*JORDANELLA FLORIDAE*) TO DIAZINON

	Fathead minnow			Bluegill		Brook trout			Flagfish	
	1	2	3	1	2	1	2	3	1	2
Source	ERL-D[a] culture	ERL-D[a] culture	ERL-D[a] culture	Federal hatchery	Federal hatchery	Commercial hatchery	Commercial hatchery	Commercial hatchery	ERL-D[a] culture	ERL-D[a] culture
Age	15-week	20-week	13-week	1-year	1-year	1-year	1-year	1-year	6-week	7-week
Average length (mm)	~30	~30	~35	~50	56.6	~190	~220	~170	18.1	17.8
Number fish/ concentration	20	20	20	10	20	20	20	20	40	40
Water volume (liters)	19	19	57	19	19	57	57	57	27	27
Flow (tank volume/day)	5	10	8	10	10	10	10	10	11	11
Temperature (°C)	25±1	25±1	25±1	25±0.5	25±0.5	12±0.5	12±0.5	12±0.5	25±0.5	25±0.5
Dissolved oxygen (% saturation)[b]	105 (95-115)	96 (87-101)	104 (100-108)	100 (93-103)	98 (88-103)	65 (43-106)	75 (58-107)	86 (78-95)	105 (103-107)	103 (102-105)
Measured concentration (mg/l.)[b]	11.7 (11.0-12.6)	10.6 (8.6-12.3)	7.9 (7.4-8.6)	0.89 (0.86-0.93)	0.80 (0.69-0.88)	0.92 (0.76-1.2)	0.76 (0.68-0.82)	2.3 (1.9-2.6)	3.1 (2.9-3.3)	3.0 (2.9-3.2)
	6.0 (5.6-6.5)	4.9 (4.3-5.9)	4.1 (3.6-4.7)	0.44 (0.43-0.45)	0.44 (0.38-0.47)	0.39 (0.34-0.47)	0.35 (0.28-0.39)	0.93 (0.88-1.0)	1.6 (1.5-1.8)	2.1 (1.8-2.2)
	3.4 (3.2-3.7)	3.4 (2.9-3.8)	3.0 (2.6-3.4)	0.22 (0.21-0.23)	0.22 (0.21-0.24)	0.16 (0.14-0.18)	0.14 (0.12-0.16)	0.51 (0.46-0.57)	0.82 (0.76-0.85)	1.3 (1.2-1.4)
	2.1 (1.9-2.3)	1.9 (1.7-2.1)	2.3 (2.1-2.6)	0.08 (0.07-0.09)	0.10 (0.09-0.11)	0.08 (0.07-0.10)	0.06 (0.05-0.07)	0.23 (0.20-0.26)	0.36 (0.35-0.38)	0.92 (0.91-0.95)
	1.1 (1.0-1.1)	1.1 (0.9-1.2)	1.7 (1.4-1.9)	0.04 (0.02-0.06)	0.04 (0.04-0.05)	0.04 (0.03-0.06)	0.03 (0.03-0.04)		0.20 (0.17-0.22)	0.68 (0.60-0.77)
	0	0	0	0	0	0	0	0	0	0
(N)/concentration	(3)	(3)	(5)	(3)	(3)	(3)	(3)	(4)	(6)	(6)

[a] Environmental Research Laboratory-Duluth

[b] Range in parentheses.

TABLE 2. QUALITY OF TEST WATER DURING CHRONIC EXPOSURES OF FATHEAD MINNOWS (PIMEPHALES PROMELAS) TO DIAZINON (TESTS #1 AND #2)

Item	Average	Range
Temperature (°C)		
Adult chambers	25.0	24.0-26.0
Larval chambers	25.5	24.5-26.5
Dissolved oxygen (% saturation)	85	74-107
pH	7.5[a]	7.2-7.8
Hardness	44	42-47
Alkalinity	42	39-44
Acidity	2.9	1.2-4.8

[a]Mode.

TABLE 3. QUALITY OF TEST WATER DURING PARTIAL-CHRONIC EXPOSURES OF BROOK TROUT (SALVELINUS FONTINALIS) TO DIAZINON

Item	Average	Range
Temperature (°C)	-	± 1° from recommended temperature for date
Dissolved oxygen (% saturation)		
Adult chambers	86	54-103
Larval chambers	101	88-109
pH	7.3[a]	7.0-7.6
Hardness	45	42-47
Alkalinity	42	40-47
Acidity	3.4	1.2-11.3

[a]Mode.

TABLE 4. PHYSICAL CHARACTERISTICS OF TEST CHAMBERS USED FOR LONG-TERM EXPOSURES OF FATHEAD MINNOWS (PIMEPHALES PROMELAS) AND BROOK TROUT (SALVELINUS FONTINALIS) TO DIAZINON

Species	Exposure	Material	Dimensions (cm) (hxlxw)	Water depth (cm)	Volume (liters)
Fathead minnows	adult	glass	30x53x30	18	28.6
	larval	glass	32x30x13	18	7.0
Brook trout	preliminary	glass	30x90x30	15	40.5
	adult	stainless steel	40x90x30	30	81.0
	larval	glass	18x40x12	13	6.2

TABLE 5. NOMINAL AND MEASURED DIAZINON CONCENTRATIONS (µg/l.) IN WATER DURING CHRONIC EXPOSURES OF FATHEAD MINNOWS (PIMEPHALES PROMELAS)

Item	Adult chambers (test #1)						Adult chambers (test #2)						Larval chambers (test #2)					
Nominal concentration	1,000	500	250	125	62.5	0	62.5	31.2	15.6	7.8	3.9	0	62.5	31.2	15.6	7.8	3.9	0
Average measured concentration	1,099	511	229	118	69	0	60.3	28.6	13.5	6.9	3.2	0	62.6	28.0	-[b]	6.8	3.3	0
(Standard deviation)	(98)	(52)	(28)	(17)	(5)	-	(9.6)	(5.5)	(2.4)	(1.4)	(0.7)	-	(11.4)	(3.0)	-	(1.6)	(0.6)	-
(N)[a]	(15)	(16)	(16)	(14)	(15)	(16)	(42)	(42)	(41)	(42)	(43)	(42)	(18)	(27)	(0)	(13)	(21)	(15)

[a]Number of observations.

[b]No larvae reared at this concentration.

TABLE 6. NOMINAL AND MEASURED DIAZINON CONCENTRATIONS (µg/1.) IN WATER DURING PARTIAL-CHRONIC EXPOSURES OF BROOK TROUT (SALVELINUS FONTINALIS)

Item	Adult chambers						Larval chambers					
Nominal concentration	12.0	6.0	3.0	1.5	0.75	0	12.0	6.0	3.0	1.5	0.75	0
Average measured concentration	9.6	4.8	2.4	1.1	0.55	0	11.1	5.6	2.7	1.4	0.80	0
(Standard deviation)	(2.2)	(1.2)	(0.6)	(0.4)	(0.19)	-	(1.2)	(0.6)	(0.3)	(0.3)	(0.18)	-
(N) [a]	(29)	(29)	(29)	(29)	(29)	(29)	(34)	(36)	(34)	(35)	(35)	(35)

[a]Number of observations.

APPENDIX D

ACCUMULATION OF DIAZINON IN TISSUES OF ADULT BROOK TROUT

It was expected that diazinon would not be concentrated in fishes to the extent common for the organochlorine pesticides. The exposure of brook trout for extended periods of time during this study provided material to test this hypothesis. Brook trout removed for thinning and at termination were analyzed for diazinon.

Tissue concentrations were determined by gas chromatography with the same equipment used to monitor water concentrations. Most of the tissue-residue data were rejected because it was subsequently found that diazinon decomposed rapidly even in frozen tissues. A few determinations made by the methods outlined below were deemed acceptable.

Extraction from muscle tissue and eggs was begun within 30 min. of death. Three extractions were made with hexane in a stainless steel blender; anhydrous sodium sulphate was used as a drying agent. Samples were cleaned on 20-g activated florisil columns.

Blood was drawn in heparinized syringes and placed in glass vials with Teflon-lined lids. Three milliliters of hexane and glass beads were added immediately, and the samples were shaken vigorously. Blood samples were extracted four times with hexane and dried with anhydrous sodium sulphate.

Results are presented in Table 1 (Appendix D). Although the data are limited, the accumulation factor for this organophosphate apparently is low compared to that of most organochlorine pesticides. Tissue concentration was directly proportional to water concentration.

TABLE 1. ACCUMULATION FACTORS[a] FOR DIAZINON IN TISSUES OF ADULT BROOK TROUT (SALVELINUS FONTINALIS)

Item	Adult exposed 6 months		Item	Adults exposed 8 months (spawning completed)						
Exposure (water concentration)	4.8 µg/l.	1.1 µg/l.	Exposure (water concentration	9.6 µg/l.	4.8 µg/l.			2.4 µg/l.	1.1 µg/l.	0.55 µg/l.
					Mature males	Immature males	Spawned females			
Blood	13x	17x	Muscle	34x	24x	51x	19x	35x	25x	25x
			(Standard deviation)[c]		(9)	(26)	(6)			
(N)[b]	(11)	(12)	(N)[b]	(11)	(4)[c]	(4)[c]	(3)[c]	(12)	(12)	(12)
			Eggs	151x						
			(N)[b]	(1)						

[a]Tissue concentration (ng/g)/water concentration (µg/l.)

[b]Number of tissue samples. Pooled samples unless otherwise noted.

[c]Analyzed individually.

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

1. REPORT NO.	2.	3. RECIPIENT'S ACCESSION NO.
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4. TITLE AND SUBTITLE	5. REPORT DATE
TOXICITY OF DIAZINON TO BROOK TROUT AND FATHEAD MINNOWS	May 1977 issuing date
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15. SUPPLEMENTARY NOTES

16. ABSTRACT

Fathead minnows exposed to diazinon from 5 days through 24 weeks post hatch developed severe scoliosis. The incidence and degree of spinal deformity correlated to exposure level. Fish in 3.2 µg/l (the lowest concentration tested) had 60% more deformities than controls (P=0.05). Hatch of eggs from fathead minnows exposed to 3.2 µg/l was 30% lower than the controls.

Yearling brook trout exposed to 4.8 µg/l and above developed scoliosis and lordosis within a few weeks. Growth was substantially inhibited (P=0.05) during the first 3 months of exoosure at 4.8 µg/l and above. Exposure to 2.4 µg/l and above caused frequently observed neurological symptoms for the first 4 to 5 months. Progeny of parents exposed 6 to 8 months to all levels tested (0.55 to 9.6 µg/l) were smaller than controls at 122 days post hatch (P=0.05).

Acute toxicity tests with diazinon yielded 96-hr LC50's of 7.8, 1.6, 0.77 and 0.46 mg/l respectively for fathead minnows, flagfish, brook trout and bluegills.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Pesticides Organic phosphates Diazinon Toxicity Bioassay Fresh water fishes Trout Minnows	Chronic toxicity Acute toxicity	6F 7C

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