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Five species of aquatic organisms were exposed to TCDD. Lethal effects of initial exposure to coho salmon of 23.4 nanogram TCDD/gram fish wet weight (23.4 ng/g), or equivalent to 100 nanogram per liter (100 ng/l) TCDD in water, for 24 hours were irreversible; 95% mortality occurred in 60 days. Exposure to 5.4 ng/g, 5.6 ng/l, resulted in 55% mortality. The no response level for 100% of the salmon is below 0.054 ng/g, 0.056 ng/l. Initial exposure of 14-21 day old guppies to 420 ng/g, 56 ng/l, resulted in 93% mortality in 20 days. Length of exposure affected survival time less than either level of exposure or fish size. A positive relationship exists between body size and survival time. TCDD exposure of worms, snails and mosquito larva to 200 ng/l TCDD in water affected reproduction of worms, the survival of juvenile snails and had no effect on the rate or success of pupation in mosquito larva.

Analysis of TCDD in water, with and without fish, at concentrations as low as 10 ng/l TCDD was performed; 60% of the TCDD was recovered 4 hours after addition to water only. 48%, 39%, and 22% was recovered 24 hours, 48 hours, and 96 hours respectively in water containing fish. The fate of the unrecovered portion of the chemical is unknown.

The Chronic Toxicity of 2, 3, 7, 8-Tetrachloro-dibenzo-p-dioxin (TCDD) to Five Selected Aquatic Organisms

by

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Typed by Opal Grossnicklaus for Richard Arlen Miller

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I often think help people give to one another is like a long chain, where links are made of hands. As the chain is moved forward, the position of one link is continuously replaced by the link behind it, moving the chain of people through infinite levels of enlightment. I like to see myself as a link in this chain and wish to give my gratitude to those people who have helped bring me through these different levels.

I thank Dr. Carl E. Bond for being my major professor and for giving me a gentle pull and timely direction for the ultimate completion of this work.

I feel honored to have been initially encouraged to take on the TCDD research by Dr. Logan A. Norris, of the Forestry Sciences Laboratory. But more important to me has been his total commitment to this work and his willingness to lead me to scientific research. Through his patient guidance I have discovered a new level of awareness.

I am indebted to Bobby Loper for his experience and unlimited kindness and for his help with the analytical phase of the experiments. Appreciation is extended to Carol Burkett for many helpful hours freely given and to Erland Juntunen for helping me get started in this project.

I especially thank my wife, Dottie, for her encouragement during the dark days of failed experiments and for her typing and proofreading used and unused thesis pages.

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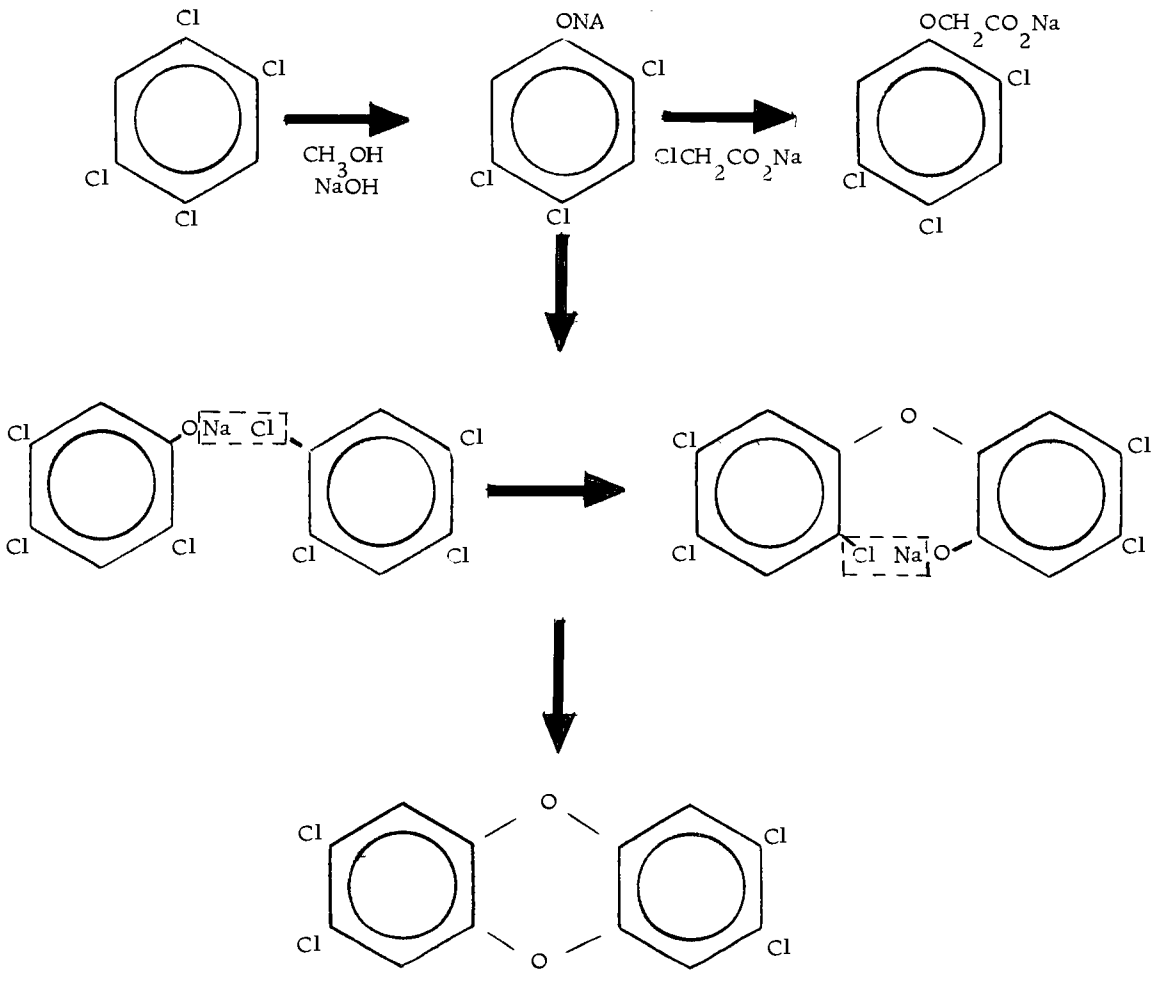
INTRODUCTION

A recent concern has developed over the toxic properties of a little-known family of chemicals, the polychlorinated dibenzo-p-dioxins (dioxins). The dioxins are unwanted by-products of the manufacture of some chlorinated pesticides from polychlorinated phenols (Fig. 1). In the production of the herbicide 2, 4, 5-trichlorophenoxyacetic acid, 2, 4, 5-T, the starting material is tetrachlorobenzene. Tetrachlorobenzene is hydrolized to trichlorophenol which is subsequently reacted with chloroacetic acid to form 2, 4, 5-T. In the production of the trichlorophenol temperatures exceeding 160 C cause the formation of the dioxins (Fig. 1). Commercial grades of pentachlorophenol have been found to contain up to 2500 milligram/kilogram (mg/kg) of certain dioxins (Johnson, 1973). Fats and oils used in poultry feeds have been contaminated by dioxins (Firestone, 1971). Greater than 0.1 mg/kg of dioxins have been found in 34 out of 55 chlorophenols analyzed (Kearney et al., 1970).

Of all the dioxins, 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) possesses the greatest toxic potency to animals. A single oral dose of 0.6 microgram per kilogram (µg/kg) of body weight to guinea pigs has caused death (Schwetz et al., 1973). Chemical workers exposed



(By-product)

Figure 1. The formation of the byproduct 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) from the commercial manufacture of the herbicide 2, 4, 5-T.

to TCDD during the manufacture of certain pesticides have been inflicted with serious skin diseases (Bauer, Schutlz, and Spiezelberg, 1961). Kearney (1970) analyzed 103 pesticides for TCDD contamination, 23 contained levels greater than 0.1 mg/kg. The greatest concern about TCDD contamination in pesticides has been in the herbicide 2, 4, 5-trichlorophenoxyacetic acid (2, 4, 5-T). Amounts from 0.1 mg/kg to 40.0 mg/kg have been found in formulations of different manufacturers prior to 1969. Recent government regulations require that formulations of 2, 4, 5-T have no more than 0.5 mg/kg and recommendations are for future production to contain less than 0.1 mg/kg; therefore, there will remain a potential for environmental contamination from TCDD whenever 2, 4, 5-T is used. Low levels of the contaminant will continue to be found in the herbicide. The best manufacturing methods allow small quantities to be formed and removal from the herbicide is not possible.

2, 4, 5-T is a useful tool for forest and range management. In the forest it is used to control undesirable broadleaf hardwood species that compete for light, space, nutrients, and moisture with the economically desirable timber producing conifers. 2, 4, 5-T is often applied aerially as low volatile esters dissolved or emulsified in diesel oil or water (Montgomery and Norris, 1970). When spraying operations are adjacent to lakes or forest streams there is a high probability that some of the herbicide, and small amounts of

accompanying TCDD, will enter the water by direct application or wind drift. Norris (1971) has found 2, 4, 5-T in forest streams adjacent to spray sites. Concentrations as high as 0.1 mg/l and 0.01 mg/l at 1 and 24 hours from application have been observed; 2, 4, 5-T residues rapidly disappear and have not been detected after long periods of heavy rain. Although TCDD concentrations in natural waters have not been reported, it is possible that using the quantities of 2, 4, 5-T reported above, as high as 0.05 nanogram per liter (ng/l) could be found in the water provided the maximum allowable TCDD was in the herbicide (Table 1). This calculated level is not known to be safe to stream organisms. TCDD toxicity to mammals and fowl has been vigorously studied; however, there have been no toxicity studies, to date, on aquatic organisms.

Table 1. TCDD in stream water after aerial application of 2, 4, 5-T to forest land

2, 4, 5-T in stream water, mg/l	Anticipated TCDD in stream water, ng/l	
	level 1[a]	level 2[b]
1.0	0.5	0.1
0.1	0.05	0.01
0.05	0.025	0.005
0.01	0.005	0.001
0.005	0.0025	0.0005

[a] Level 1: 2, 4, 5-T contains 0.5 mg/kg TCDD

[b] Level 2: 2, 4, 5-T contains 0.1 mg/kg TCDD

The purpose of this study is to determine the effects of TCDD on five selected aquatic organisms using the static water toxicity tests. The animals used were two fish species, guppies (Poecilia reticulatus) and coho salmon (Oncorhynchus kisutch); a snail (Physa sp.); a segmented worm (Paranais sp.); and a mosquito larva (Aedes aegypti). Coho salmon, Physa and Paranais are common inhabitants to fresh water streams in the Pacific Northwest and are excellent animals for use in laboratory conditions. Although the guppy and the yellow fever mosquito are not common to this region they are very useful bioassay organisms. They have been used extensively in research with other environmental toxicants; results obtained from TCDD exposure to them can be easily compared to these toxicants.

In all of the tests, animals were treated in TCDD solutions for various time periods and then removed to clean containers for additional observation. Fish were measured for growth and survival, snails for reproduction and survival of the progeny of exposed adults, worms for reproduction and growth and mosquito larva for survival, success and rates of pupation.

TCDD loss in water was studied. Known amounts were added to water-filled test containers with and without salmon. Losses were determined analytically using gas liquid chromatography. Extensive water cleanup methods for the isolation of TCDD were employed.

The study is of limited scope, measuring only the single dose

response to each of the five organisms. Once an order of magnitude threshold response level is established then more elaborate investigations can follow. Running water toxicity tests, feeding studies, food chain transfer and reproductive effects of TCDD exposure should be studied.

Background

During the past 20 years a number of chemical manufacturing plants have been involved in accidents that have caused highly specific illnesses to the inplant personnel. The accidents occurred while polychlorinated phenols were either being manufactured or used to manufacture more complex compounds. The illnesses resulting from the accidents were a highly specific skin disease, chloroacne, and, in some cases, severe damage to internal organs.

In 1949 an accident occurred at a 2, 4, 5-T chemical manufacturing plant owned by the Monsanto Chemical Company. The resultant release of chemicals and exposure to workers near the accident site was responsible for 117 cases of a severe skin disease known as chloroacne. In addition, persons who were not near the accident site, but were in some way connected to the plant or employees of the plant were also affected. The causative agent of the disease was later found to be the highly toxic chemical, 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) (Report of the Panel on Herbicides of the

President's Science Advisory Committee, 1971).

An explosion of the Coalite and Chemical Products 2, 4, 5-T manufacturing plant in England discharged a white crystalline solid that was later analyzed to be TCDD (Milnes, 1971).

Bauer (1961) reports three separate instances involving chemical plants in Germany during the 1950's when 108 cases of severe chloroacne developed. The first case was of 17 workers involved in the production of pentachlorophenol; the second reported instance was of 60 workers who had been engaged for long periods, generally several years, in the saponification of 1, 2, 4, 5-tetrachlorobenzene to 2, 4, 5-trichlorophenol by treatment with methanolic caustic soda solution. The third report was that of 31 workers in the trichlorophenol department of their company, in which 2, 4, 5-T acid was manufactured from technical 2, 4, 5-trichlorophenol.

In addition to chloroacne, damage to digestive organs, liver, heart, kidney and nervous system was observed. These anomalies were again believed to be a result of the formation of TCDD and resultant exposure to the workers in the plants.

In this 20 year period some chicken raisers throughout the United States were experiencing severe mortality in their flocks. In 1957 millions of broiler chickens died in the eastern and midwestern U. S. These animals showed symptoms of unusually high amounts of fluid in the paracardial and abdominal cavities and death began

three weeks from hatching. In 1969 another outbreak of the disease occurred in North Carolina.

The disease producing factor in both events was isolated and later identified as a family of polychlorodibenzo-p-dioxins. The 1957 outbreak was traced to tallows and greases obtained from animal hides preserved with chlorophenol. The 1969 incident was caused by chlorophenol contamination of soapstock used as feed fats (Firestone, 1973; Verrett, 1970). Later analysis of the toxic fats isolated 4.3 mg/kg TCDD among other dioxins present (Firestone et al., 1971). Fats and oils are fractionated under high temperatures in the presence of strong alkalies before processing into product components. Dioxins are produced when chlorophenols and strong alkalies are subjected to elevated temperatures.

In 1964 the National Cancer Institute contracted with Bionetics Research Laboratories to perform screening studies for carcinogenicity and teratogenicity on a number of pesticides. The results, released in October 1969, indicated that of the 53 compounds examined, 2, 4, 5-T in particular showed embryo toxicity and abnormal fetuses in mice when dosed daily with 113 and 46.4 mg/kg and daily doses to rats at 10 and 4.6 mg/kg when given for several days during organogenesis. This disclosure had serious implications due to the quantity of 2, 4, 5-T manufactured and used in the U. S. each year. The samples of 2, 4, 5-T used in these studies were contaminated with

30 mg/kg TCDD (Courtney et al., 1970).

The U. S. production of major formulation of 2, 4, 5-T for the year 1967 was 67 million pounds. 2, 4, 5-T has been in domestic use for the past 20 years. Its use in 1964 alone was about 9 million pounds, covering nearly 8 million acres. Estimates indicate these values may be approximately correct through 1969. The absence of 2, 4, 5-T as a tool for vegetation control could cost an additional $52 million in land and water management each year (Report of the Advisory Committee on 2, 4, 5-T, 1971).

Large areas of the Pacific Northwest forests have been sprayed to control undesirable broadleaf vegetation, releasing the commercially important conifers. Usually 0.5 to 4 pounds per acre are applied. A serious concern is the subsequent TCDD contamination of surface waters. Present formulations of 2, 4, 5-T contain 0.5 to 0.1 mg/kg TCDD. Montgomery and Norris (1970) report that 2, 4, 5-T (and TCDD) enter the streams through direct application or accidental drift directly to the water surface. They have found concentrations of 2, 4, 5-T in streams never exceeded 0.1 mg/l or 0.01 mg/l for more than 1 day after application. With these concentrations it can be expected that accompanying TCDD concentrations will be 0.05 to 0.005 ng/l respectively, assuming the maximum allowable TCDD concentration of 0.5 mg/l is in the herbicide formulation.

Except for short term bioaccumulation experiments by

Matsumura and Benezet (1973) using very high levels of TCDD, 81 to 162 times its water solubility of 0.2 μg/l, there has been no work on the rate of uptake or the toxicity of TCDD to aquatic organisms. The above stream concentrations are not known to be either safe or toxic levels.

Aside from the civilian use of 2, 4, 5-T the military has found it to be useful in warfare. Defoliating large areas of broadleaf cover over guerrilla forces in Vietnam was a common practice. Defoliating began in 1962 and was finally discontinued in April 1970, after a concern was raised because of the teratogenic properties of the herbicide. A total of 25×10^6 pounds of 2, 4, 5-T, covering 1.9×10^6 acres is estimated to have been sprayed through 1968, the formulations used at that time contained up to 40 mg/kg TCDD (Orians and Pfiffer, 1970.

In Vietnam there have been reports of unusually high human birth defects in heavily sprayed provinces. Boffey (1971) reports increases in the birth defects pure cleft palate and spina bifida in 1967 and 1968. In addition to this there have been reports of increased fish death in and around areas sprayed. Evacuees, 98, of a previously sprayed area were interviewed; 48% of the group stated that fish in ponds and rivers died following spraying. Some singled out fish in still water suffering the heaviest casualties (Rose and Rose, 1972). The director of the Vietnam Institute of Fisheries has

received reports of the increase in the occurrence and intensity of a seasonal disease since spraying began (Orians et al., 1970).

The introduction of TCDD into the environment is wholly a result of man's activities. As already shown, there are some cases of accidental release of TCDD and the possibilities of others in the future are not without consideration. The intentional introduction into the ecosystem of this chemical is a reality.

TCDD in the Ecosystem

TCDD can be extremely persistent in nature; its breakdown is sluggish in water and fails entirely in thin-pure films on dry irradiated soil surfaces. However, in the presence of oils of pesticide formulations or natural plant oils and waxes a hydrogen donor is provided and the molecule is decomposed in the presence of sunlight in a matter of a few hours (Crosby, Moilanen, and Wong, 1973).

Soil microbes have little effect in decomposition. Kearney et al. (1972) analyzed five soils one year after TCDD treatment and found that of the total applied, 54-71% was recovered. No metabolites of decomposition were found. By using approximately 100 microbial strains which have previously shown the ability to degrade persistent pesticides only five strains were found to have some ability to degrade TCDD (Matsumura and Benezet, 1973).

TCDD can possibly be concentrated through food chains. In

laboratory experiments Matsumura et al. (1973) found in a two-step food chain using mosquito larva and fish that the body burdens of the fish were increased 350 times that of fish exposed to TCDD without the food organisms present.

Miller, Hawkes, and Norris (1973) found that young rainbow trout fed 0.63 μg TCDD each per week in the regular ration began to lose weight after seven days of initial feeding and deaths occurred in 33 days.

Zitko (1972) was unable to detect TCDD at detection limits of 40 μg/kg on a wet tissue basis of the white shark, eggs of the double crested cormorant and herring gulls, the muscle of an eel and chain pickerel, and in commercial samples of herring oil and ground herring fish meal. However, the detection limits of TCDD in Zitko's work are much too high to warn of ecological hazards. Recent work of Baughman and Meselson (1973) indicates that a method is now available to detect TCDD at limits below those known to be toxic. They report being able to detect 1 ng/kg in animal tissue by an extensive cleanup method and gas liquid chromatography with high resolution mass spectroscopy. From analysis of fish and shrimp, collected from a river flowing through a 2, 4, 5-T treated watershed in South Vietnam, they detected from 18 to 814 ng/kg TCDD in the tissues. This evidence shows that bioaccumulation is possible and does occur in the environment.

TCDD Toxicity in Mammals

All of the dioxins exhibit varying degrees of toxicity to animals. TCDD has been shown to be by far the most toxic member of the family and probably the most toxic small molecule known. The LD 50 for a single oral dose of TCDD is 200-400 μg/kg for rats, 600-1300 μg/kg for mice and 0.62 μg/kg for guinea pigs (Schuetz et al., 1973).

As has already been pointed out, the effects of TCDD on animals are unpredictable and vary widely among animal species. There has been recent research on the organs affected of animals exposed to TCDD. Rats, mice and guinea pigs, when given sublethal doses of TCDD, exhibited atrophy of the lymphoid organs, thymus, spleen and lymph nodes; suppression of cell-mediated immunity in guinea pigs and mice was observed (Vos et al., 1973; Gupta et al., 1973). Female rats were given 10 μg/kg per day of TCDD; some were killed ten days after treatment. Nonspecific alterations of hematopoietic function and derangements in blood coagulation occurred (Weissberg and Zinkl, 1973).

Tissue from livers of TCDD treated rats were examined. Sections of treated rat livers were histologically indistinguishable from control; however, ultrastructural changes were observed. Liver parenchymal cells were seen to have proliferation of the smooth

and rough endoplasmic reticulum three days after treatment (Fowler et al., 1973).

Poland and Glover (1973) suggest that the chemically inert TCDD is not the toxic moiety, but cell damage is dependent on metabolism to a high reactive intermediate. In one experiment using rats microsomal and mitochondrial enzymes were induced after one 5 μg/kg oral dose of TCDD as early as one day after dosage; microsomal protein contents were increased by 15% (Lucier et al., 1973).

In mice, maternal exposure to TCDD during pregnancy caused variations in fetal kidney maturation. The thymus and spleen weights were reduced in pups of exposed mothers during pregnancy or exposed lactating mothers (Moore et al., 1973). Hepatic changes in guinea pigs receiving a lethal dose were quite mild. Gupta et al. (1973) hypothesized that liver damage is the cause of death in rats and immunity suppression for guinea pigs and mice.

MATERIALS AND METHODS

Toxicant

The 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) used in the toxicity tests was supplied by the Dow Chemical Company, Midland, Michigan. The chemical received was 98.7% pure, the balance made up of 0.8% trichloro- and 0.7% pentachlorodibenzo-p-dioxin. A stock solution was made by dissolving 24.65 mg of dioxin crystals into 100 ml of reagent grade chloroform and was used to prepare further dilutions using acetone as the solvent.

The treatment regimes used in the toxicity tests are summarized in Table 2 for each experiment. In these experiments exposure levels are expressed in two ways, in concentration of TCDD in water, ng/l, and nanograms (ng) TCDD originally added to the test containers divided by the total fish biomass in the container at the beginning of the experiment, ng/g. The last relationship has a greater meaning than the concentration value. It allows comparisons of results to be made between experiments on the basis of a per unit organism weight to amounts of toxicant in the test containers. Care must be taken not to confuse the exposure value, ng/g, with specific body burdens of TCDD; body burden analysis was not made in this study.

TCDD concentration values are given in Table 2. They are to be used as a reference point only. They have a very limited value in

Table 2. Summary of test procedures to determine the toxicity of TCDD in aquatic organisms.

Experiment number	Organism	Container[1]	Water volume l	Water temperature °C	Exposure regime: Level ng/l	Exposure regime: Level ng/g BW[2]	Exposure regime: Duration hr	Total observ. period days	Feeding regime	Experimental design[3]	Number replications[4]
1[5]	Guppies 10-40 mm	1 ga WMJ	3	20	0 100 1,000 10,000		120	37	Tubifex worms, Ad Lib. Post Exposure Period	CRD	3 (n=20)
2	Guppies 8-12 mm	1 ga WMJ	3	20	56 100 320 1,000	447 800 2,560 8,000	24 48 96	25	Powdered Comm. Preparation	SPF	4 (n=20)
3	Guppies 20-35 mm	1 ga WMJ	3	20	100		10 70	40	Tubifix worms, Ad Lib. Post Exposure Period	CRD	3 (n=20)
4	Guppies 8-12 mm	1 ga WMJ	3	20	0.01 0.1 1.0 10.0	0.08 0.8 8.0 80.0	24	140	Powdered Comm. Preparation	CRD	4 (n=15)
5	Snails Adult and Juvenile	1 ga WMJ	3	23-27	0 200		1152	48	Elodea, OMP[6] Ad. Lib. during Exposure Period	CRD	4 (n=7 adults)
6	Worms 40 mm	8 in culture dish	1	23-27	0 200		1176	55	OMP[6] 1/week, during Exposure Period	CRD	4 (n=20)
7	Mosquito Larvae	8 in culture dish	1	23-27	0 200		408	39	Yeast 2/week during Exposure Period	CRD	4 (n=20)

Table 2. (Continued)

Experiment number	Organism	Container[1]	Water volume l.	Water temperature °C	Exposure regime: Level ng/l	Exposure regime: Level ng/g BW[2]	Exposure regime: Duration hr	Total observ. period days	Feeding regime	Experimental design[3]	Number replications[4]
8[7]	Salmon 7.25 g Wet Weight	5 ga WMJ	17	12-18	56 100 560 1,000	13.1 23.4 131.3 234.0	24 48 96	76	OMP[6] 3/week, Post Exposure Period	SPF	4 (n=10)
9[7]	Salmon 1.33 g Wet Weight	5 ga WMJ	17	12-18	5.6 11.5 28.0 56.0	7.1 14.1 35.7 71.0	24 48 96	33	OMP[6] 3/week, Post Exposure Period	SPF	4 (n=10)
10[7]	Salmon 3.51 g Wet Weight	5 ga WMJ	17	12-18	0.056 0.56 5.6 56.0	0.054 0.54 5.4 54.0	24 48 96	59	OMP[6] 3/week, Post Exposure Period	SPF	4 (n=5)
11	Salmon (TCDD Recovery from Water) 2.9 g Wet Weight	5 ga WMJ	17	12-16	0 50		24 48 96		None	CRD	4 (n=10)

1 WMJ - Wide mouth jar

2 Nanograms TCDD per gram wet body weight

3 SPF - Split plot factorial, CRD-completely randomized

4 n = Beginning number of organisms per treatment per replication

5 Norris and Miller (10)

6 OMP - Oregon moist pellet

7 Exposed to all possible combinations of levels and durations of exposure listed.

interpreting static water toxicity tests. Concentrations do not remain constant during the exposure period. As soon as TCDD is added to the water the concentrations of the toxicant decline rapidly. The binding of the chemical to the container walls, precipitating out with organic matter and uptake by the fish all account for this loss. Therefore, TCDD concentrations used in these tests cannot be used to interpolate from concentrations of TCDD found in natural waters.

Water Quality

Laboratory water was pumped from a well located nearby and stored for use in a 500 ga wooden tank. In the early stages of the work it was noticed that some of the salmon were suffering from gas bubble disease. The water was immediately analyzed and found to be 130% gas saturated at 11 C. A system of baffles was installed in the inlet side of the reservoir to remove all excess gas. Table 3 lists the chemical properties of the test water.

Table 3. Characteristics of test water.

Constituent	Concn. level, mg/l	Constituent	Concn. level, mg/l
Calcium	12.0	Chloride	4.3
Silica	6.9	Nitrate	6.1
Magnesium	7.8	Iron	0.03
Sodium	7.7	Dissolved solids	132.0
Potassium	1.45	Hardness	64.0
Bicarbonate	79.0	Specific conductance umko	164.0
Carbonate	0.0	pH	6.9
Sulfate	5.1		

Experimental Animals

Salmon

There were three toxicity tests using juvenile coho salmon, all from the Oregon State Fish Commission Hatcheries. The first salmon experiment was performed in August 1971, with fish hatched early that year. These animals were received in June and held in a 500 ga wooden tank at the Fairplay laboratory. Forty-eight hours prior to testing they were anesthetized with tricane methanesulfonate and individually weighed and marked using the cold branding technique of Edmundson and Everest (1967). Sixty groups of ten identically marked fish were used. In the last two experiments the fish were from 1972 hatches. They were received in April 1972, and held in the manner described. These animals were not anesthetized or marked but were weighed only 48 hours prior to testing. Sixty groups of ten fish each were used in experiment nine and 60 groups of five fish each were used in experiment ten. Mortality of fish being held in preparation of the experiments was insignificant, $<1\%$; any fish that looked unhealthy or had visible imperfections was discarded.

In the salmon experiments the animals were exposed to TCDD in 5 ga glass jars (Fig. 2). Four groups of 15 jars per group were placed into four wooden troughs 96 inches by 24 inches by 15 inches deep. Each group was a complete experimental replicate of 12

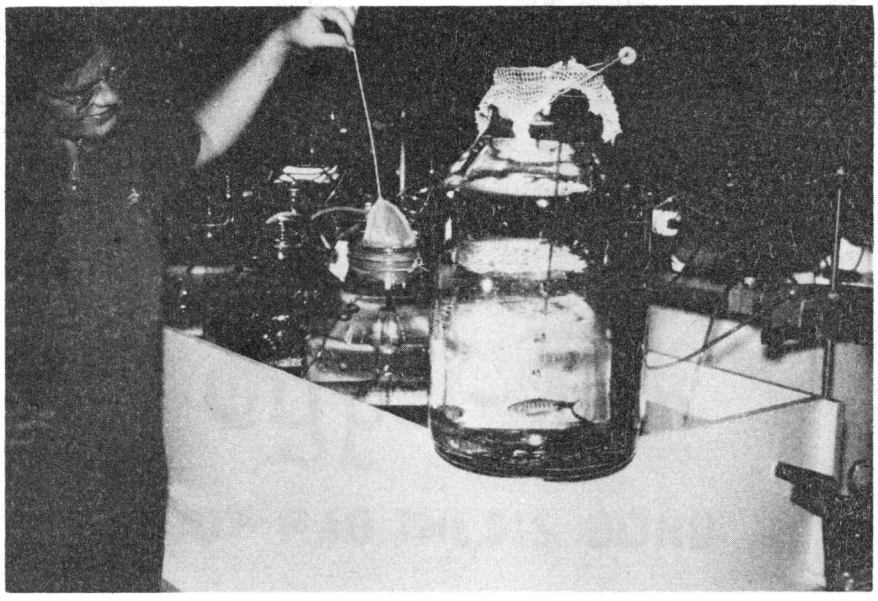


Figure 2. TCDD-salmon toxicity test exposure unit, illustrating glass exposure jars, constant temperature water bath and air-water manifold.

treatments and three controls. Flowing well water of 12 C was circulated through the troughs to maintain uniform temperature in the test containers. Each jar was provided an individual air and water supply from overhead manifolds. Test water was dosed with TCDD in acetone and was pipetted slowly into the water of each treatment jar and mixed simultaneously by stirring. Treatments and controls received an equivalent amount of acetone.

At the end of each of the three exposure periods, 24, 48, and 96 hours, water was siphoned from the jars and fresh water added continuously at the rate of 5 liters per hour washing away residual and excreted TCDD. The water was shut off after 12 hours and the fish remained in the static aerated water until the completion of all exposure regimes.

After exposure to TCDD in experiment eight the fish were placed in two 4 ft by 4 ft by 2 ft black Polyvinylchloride aquaria and remained here during the post-exposure observation period. The aquaria were supplied with fresh running well water and were continuously aerated. Observations were made regularly and records on dead fish were kept indicating wet weight at death and individual identification marks.

The post-exposure conditions in experiments nine and ten were modified from conditions in experiment eight. Fish remained in the glass jars throughout the observation periods. Water was

allowed to exchange in each jar at a rate not less than 1 1/2 liters per hour and was also aerated.

In all the salmon experiments, Oregon Moist Pellets were fed on a regular basis. In experiments eight and nine the animals were fed freely until appetites were satisfied. In experiment ten measured amounts of pelleted food were fed equally to all treatments; uneaten food was determined by pellet counts 20 minutes after placement into the jars.

The aquaria and glass jars in these experiments were siphon-cleaned weekly.

Guppies

Guppies used in experiments one, two, three, and four were from progeny of female brood stock obtained from the Oak Creek laboratory, Oregon State University. The breeding animals were maintained under conditions for maximum productivity at the Fairplay laboratory. The progeny were periodically collected and used in the TCDD static water toxicity tests. In all but experiment one the fish were selected for uniformity of size.

Guppy experiments were performed in 1 ga glass jars. The animals were acclimatized for 48 hours before treating. They were dosed with TCDD acetone by pipetting the solution slowly and mixing with a glass rod. Experiments one, three, and four were performed

by placing the treatment jars into a constant temperature water bath. Jars used in experiment two were placed on a counter top in the laboratory; temperatures were maintained by conditioning the laboratory atmosphere. Because of the size of experiment two and the numbers of fish required, each of the four replicates of 12 treatments and three controls were performed at different times. This in no way affected the statistical analysis of the experiment. Water used in the guppy experiments was replaced at least every 14 days after completion of the exposure period. There was no aeration at any time.

Snails

The snails used in experiment five were local species collected from the University's Soap Creek ponds in October 1972. They were held at Fairplay laboratory in a 10 ga aerated aquarium at a constant temperature of 25 C for a month. Several days after bringing the animals into the laboratory egg cases began appearing on the glass walls and water plants within the aquarium. The largest adult snails (Fig. 3) were selected and placed into eight 1 ga glass jars, four containing TCDD and four containing pure well water. The jars were immersed in a constant temperature water bath during the period of egg deposition. Egg cases were deposited on the walls of the jars and on the elodea for 37 days; the adults were then removed from

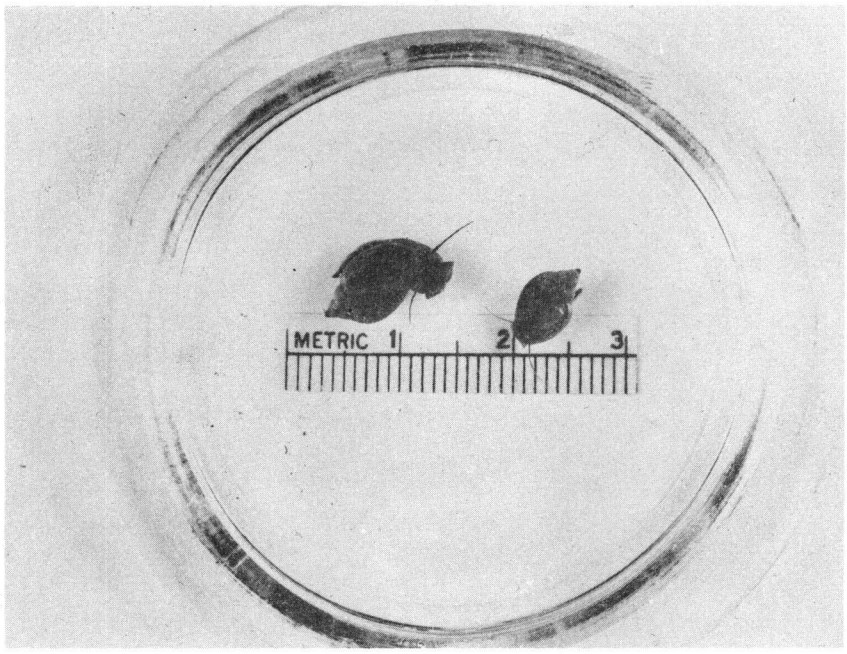


Figure 3. Adult snails, Physa, used in TCDD toxicity tests.

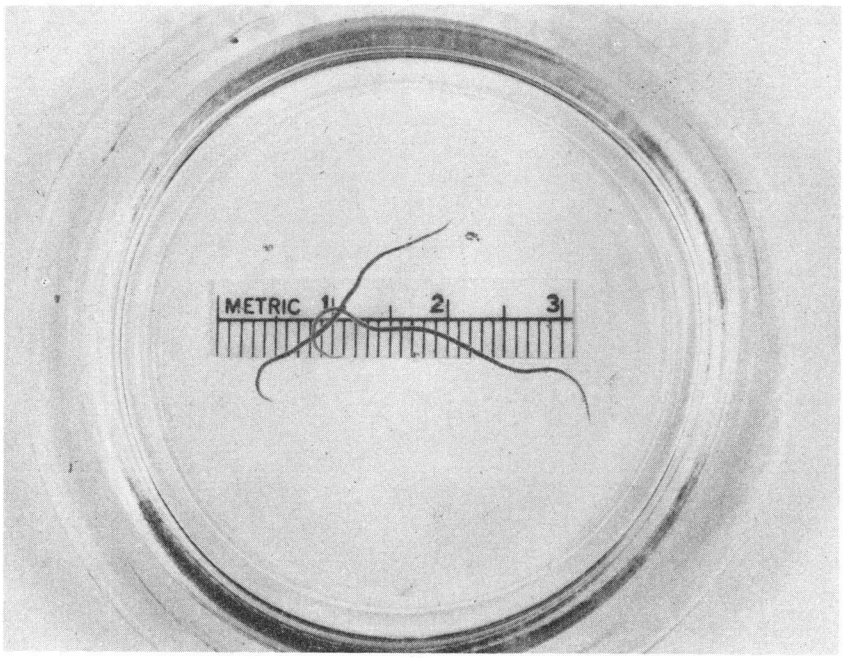


Figure 4. Adult worms, Paranais, used in TCDD toxicity tests.

the containers. The entire experiment was moved to a greenhouse behind the Forestry Sciences Laboratory on Jefferson Way, Corvallis, where the eggs were allowed to develop in the jars for an additional 11 days. Juveniles were then carefully brushed away from the glass walls and plant material. The live snails and empty shells were retained by filtering through fine nitex screen. The animals and empty shells were immediately counted under a dissecting microscope at 20X.

Worms

The worms were collected from the North Fork of the Alsea River and maintained in a flowing water trough outside the laboratory building. Three weeks prior to the toxicity test the animals were placed into 1 liter culture dishes and allowed to acclimatize to the experimental conditions.

Twenty adult worms (Fig. 4) were placed into each of the eight ℄ in culture dishes, four containing TCDD and four containing water only. The dishes were inside a greenhouse that was kept at temperatures of 23-27 C. The water was constantly aerated, very slowly, to maintain a slight water flow. Ten days after initial exposure brown paper toweling was placed on the bottom of the dishes and used as a substrate for the worms. The worms were fed dry dog food ad lib. At the end of the 49 day exposure period the animals were counted,

vacuum dried at 29 in Hg at 30 C for 24 hours and weighed.

Mosquitoes

Mosquito eggs were supplied by Dr. Robert Goulding of the Oregon State University Entomology Department. Larva for experiment six were obtained from eggs produced by second generation adults (Fig. 5). To insure uniform egg development, eggs of less than two weeks of age were placed into a vacuum incubator, 10 inches of mercury at 27 C. Larva hatching during the first 12 hours of incubation were collected and incubated further for two days, without vacuum. The test animals used were second instar larva. The larva were then placed into eight 8 in culture dishes by glass transfer pipettes. Each culture dish contained one liter of water; TCDD was added to water in four of the dishes. The experiment was performed in the temperature-regulated greenhouse described. The climatic conditions at the time of this bioassay were severely cold and the greenhouse temperatures were more often on the lowest side of the desired range. This condition unquestionably accounts for the longer-than-normal time taken for the animals to pupate during the experiment.

Throughout the experiment the larva were fed small quantities of a yeast-water slurry several times weekly. As the animals pupated they were transferred out of the culture dishes and placed into

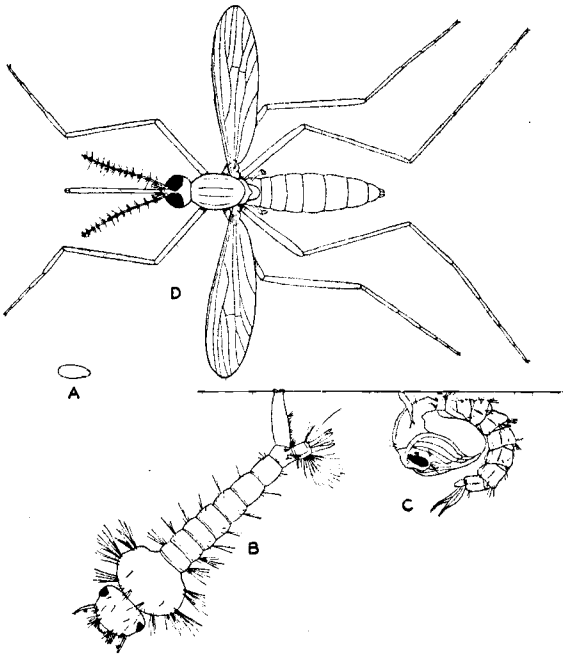


Figure 5. Life stages of Aedes mosquito. A. egg, B. mature larva, C. pupa, D. adult female. Pennak (1953).

sealed jars and allowed to emerge as adults.

Analysis for TCDD in Water

Gas Liquid Chromatography (GLC) Apparatus and Conditions

Instrument	Varion Aerograph Series 1200
Column	1.5 m total length, 2 mm inside diameter, glass coil packed with 3% OV 17 on chromasorb W, 100/120 mesh solid support
Column temperature	180 C
Inlet temperature	200 C
Detector temperature	205 C
TCDD retention time	12.3 min
Recorder	Westronics LS11A

Decontamination of Glassware

A rigid glassware cleanup procedure was found to be necessary in the gas liquid chromatography analysis of TCDD. If this procedure was not followed exactly, background levels of contaminants invalidated the analysis. All glassware used was first thoroughly washed in a laboratory detergent, rinsed and then soaked in a saturated sodium dichromate-sulfuric acid solution at 60 C to 80 C for one

hour. The glass was rinsed with tap water, followed by double distilled water and then dried upside down. After drying, the glassware was stored in closed drawers until use.

Reagents

Hexane and benzene solvents were used extensively in the TCDD extraction and analysis. The use of dirty solvent obscures the reading of the desired molecule due to background contamination (Fig. 6); therefore, great care was taken in the distillation and handling of these. Both solvents were double distilled in glass only. Twenty liter of drum material was distilled; the first eight liter to come off were discarded and the remaining 12 liter redistilled. Again the first 8 liter were discarded and the remaining 4 liter were retained for use. Before the distillate was used, samples were first analyzed by GLC for interfering chemicals.

Reagents used in the water cleanup were concentrated sulfuric acid, technical grade; 0.1 N sodium hydroxide prepared from reagent grade pellets and double distilled water.

Analytical Procedures

Water to be analyzed was siphoned from the test containers into one-half gallon glass jugs. The TCDD was extracted immediately. 1.8 liter of the toxicant-containing water were then placed into

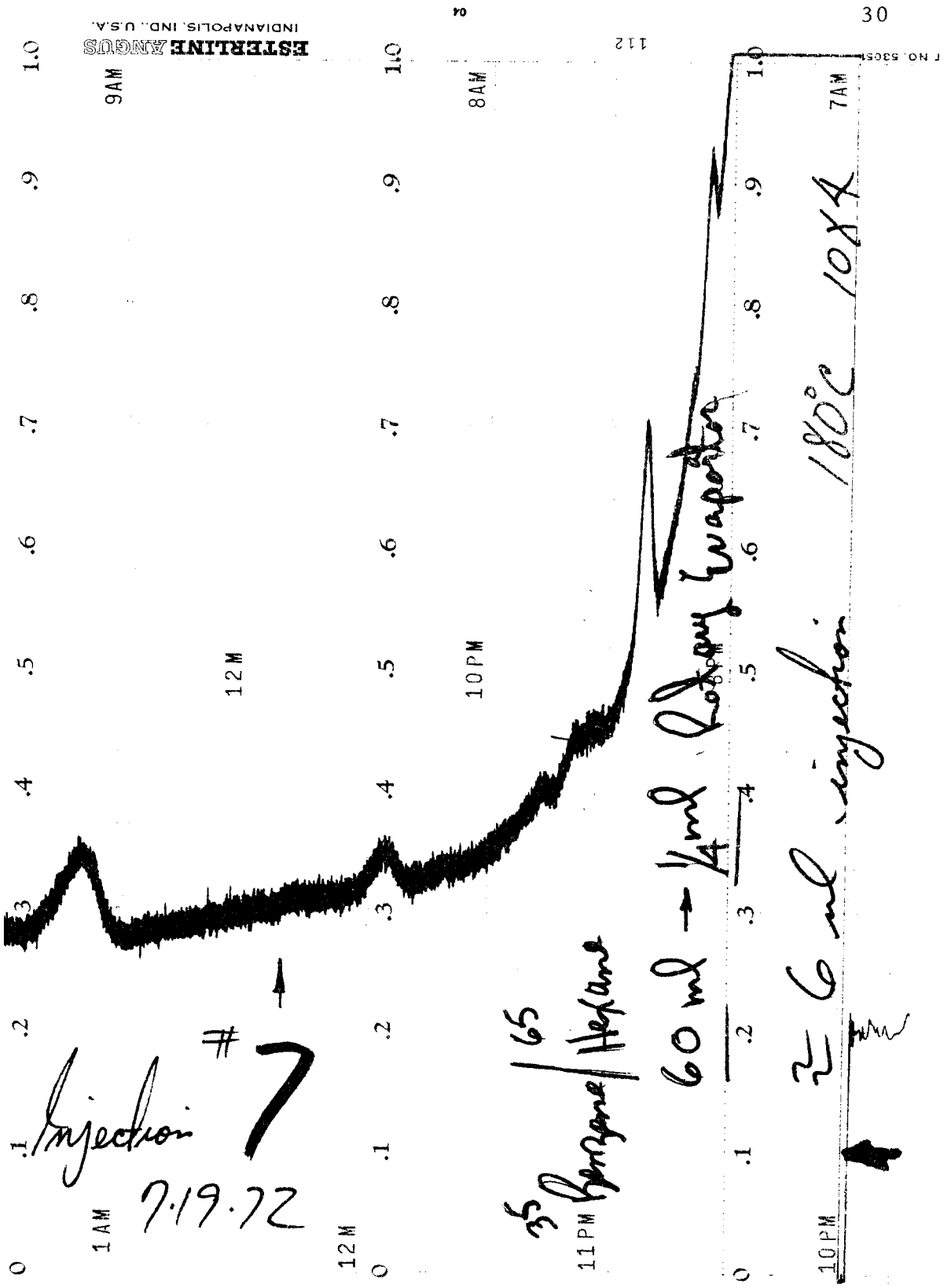


Figure 6. Electron-capture gas chromatogram of 6.0 μl injected sample of eluting solvent used in TCDD analysis.

two liter separatory funnels and 80 ml hexane added. The mixture was shaken on a mechanical shaker for four hours. After standing for one hour, the aqueous layer was discarded, 10 ml of 0.1 N NaOH was added to the hexane and vigorously shaken for two minutes. This caustic layer was drawn off and 25 ml distilled water added to shake for one minute. The water was removed, 5 ml concentrated sulphuric acid added and shaken for two minutes. Three one-minute water washes of 25, 200 and 200 ml followed.

The remaining hexane was transferred to evaporation flasks and the volume reduced on a vacuum rotary evaporator to 4 ml; it was then pipetted into 10 ml Kurdana-Danish collection flasks and 5 ml of hexane was used as a rinse and added to the collection flasks. This solution was reduced to 5 ml by nitrogen evaporation and transferred to alumina cleanup columns.

Cleanup columns were prepared by pouring 3 g of activity grade one aluminum oxide (Woelm basic) into 50 ml, K4201OD chromaflex glass columns packed with a small amount of glass wool and filled with benzene.

As the last 1/2 cm of benzene was passing through the column, 25 ml hexane was carefully added and also allowed to pass through. Before the hexane emptied from the column the TCDD hexane solution was added and washed with 100 ml of additional hexane by passing it through the column. The TCDD was eluted with 80 ml of 35% benzene

and 65% hexane mixture. This was collected in 100 ml beakers, the volume reduced to 1 ml by nitrogen evaporation and analyzed by GLC for TCDD.

The analysis of TCDD in these solutions was made by comparing the peak height produced by the recorder with the peak height from a standard solution of known quantity (Figs. 7 and 8).

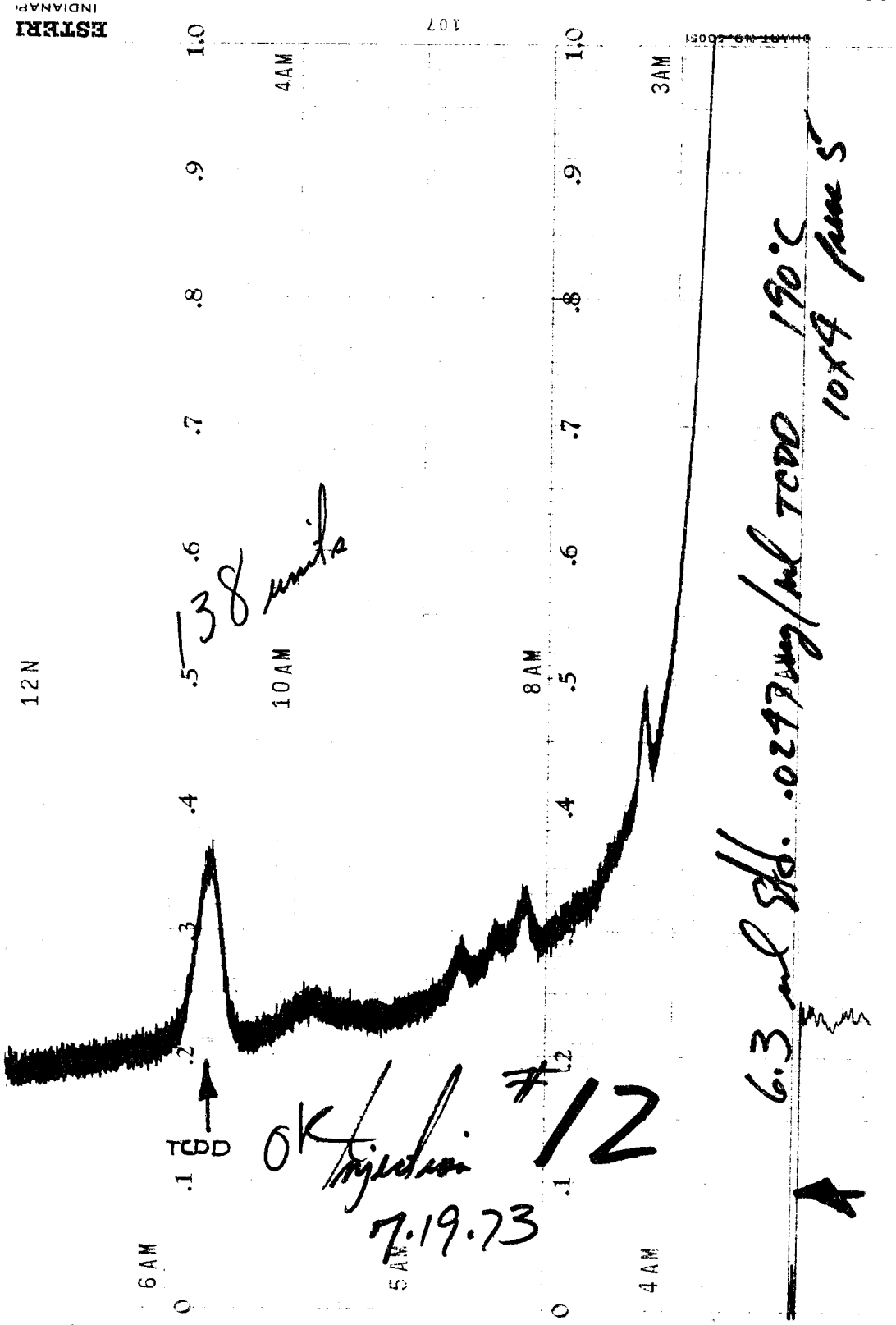


Figure 7. Electron-capture gas chromatogram of 6.3 µl injected sample of 0.0247 µg/ml TCDD-benzene standard solution, retention time 4.5 min.

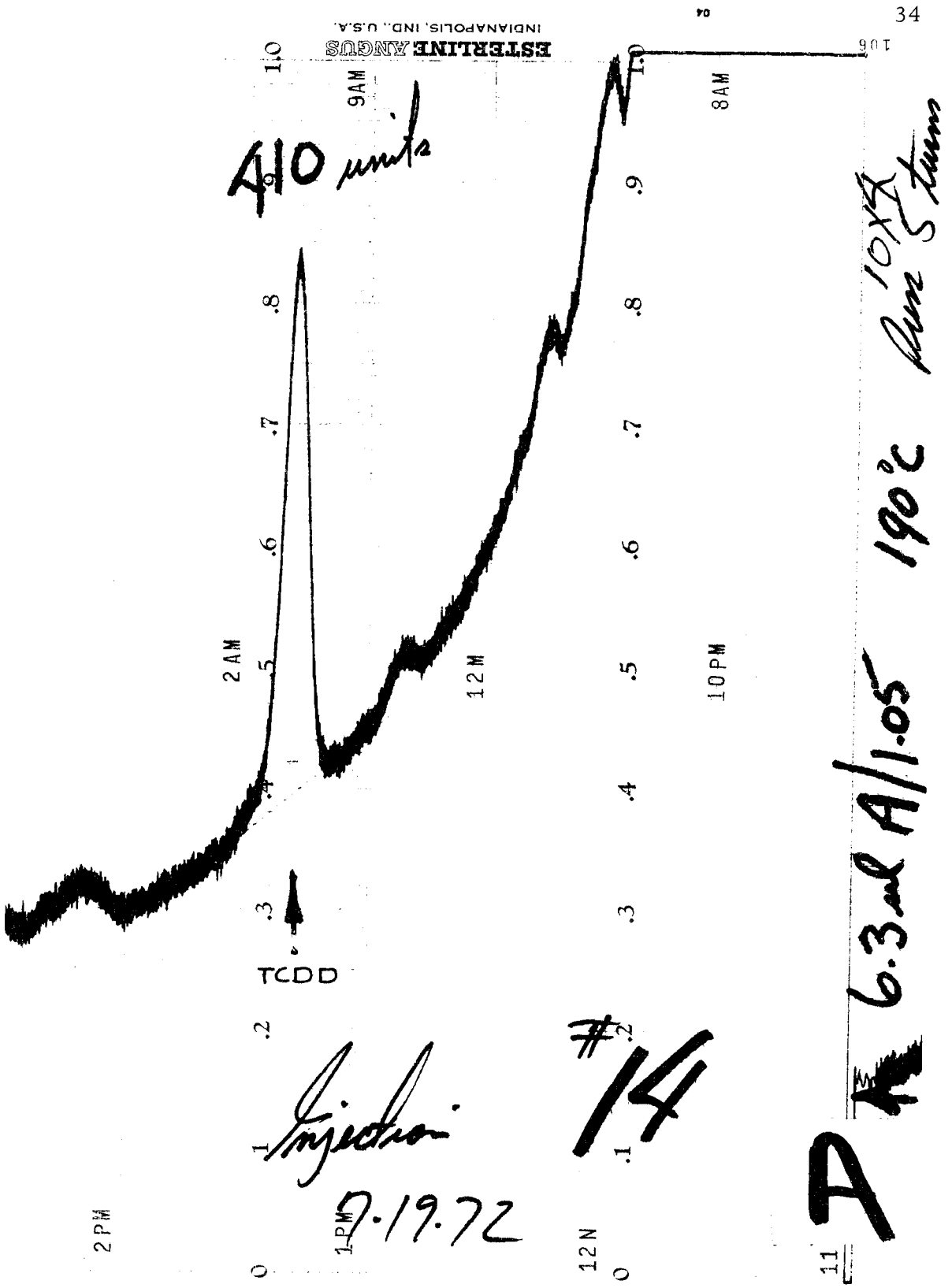


Figure 8. Electron-capture gas chromatogram of 6.3 µl injected sample of TCDD extracted from water, retention time 4.5 min.

RESULTS AND DISCUSSION

Recovery of TCDD in Water

The TCDD level in water with young salmon declined significantly with time (Fig. 9). Regression analysis indicated recovery between 24 and 96 hours was linear with time (Draper and Smith, 1966).

$$Y = 57.2 - 0.377X$$

Where Y is percentage recovery of TCDD, X is time in hours after addition of TCDD to water containing coho salmon; $r^2 = 0.90$.

TCDD concentration decreased more rapidly between 0 and 24 hours than between 24 and 96 hours which may suggest more than one mechanism of loss was operative. The rapid loss of TCDD during the first 24 hours may largely be the result of sorption phenomena which rapidly attained equilibrium. This hypothesis is supported by results from a similar test in which fish were not included and TCDD recovery was 60.0% four hours after addition of the chemical.

The fate of TCDD in the system is not known. I suspect that some of the chemical is taken up by the fish, adsorbed on the glass and suspended organic matter, and possibly lost due to aeration. Organisms in the static water toxicity tests were exposed to rapidly declining levels of TCDD, because exposure solutions were not

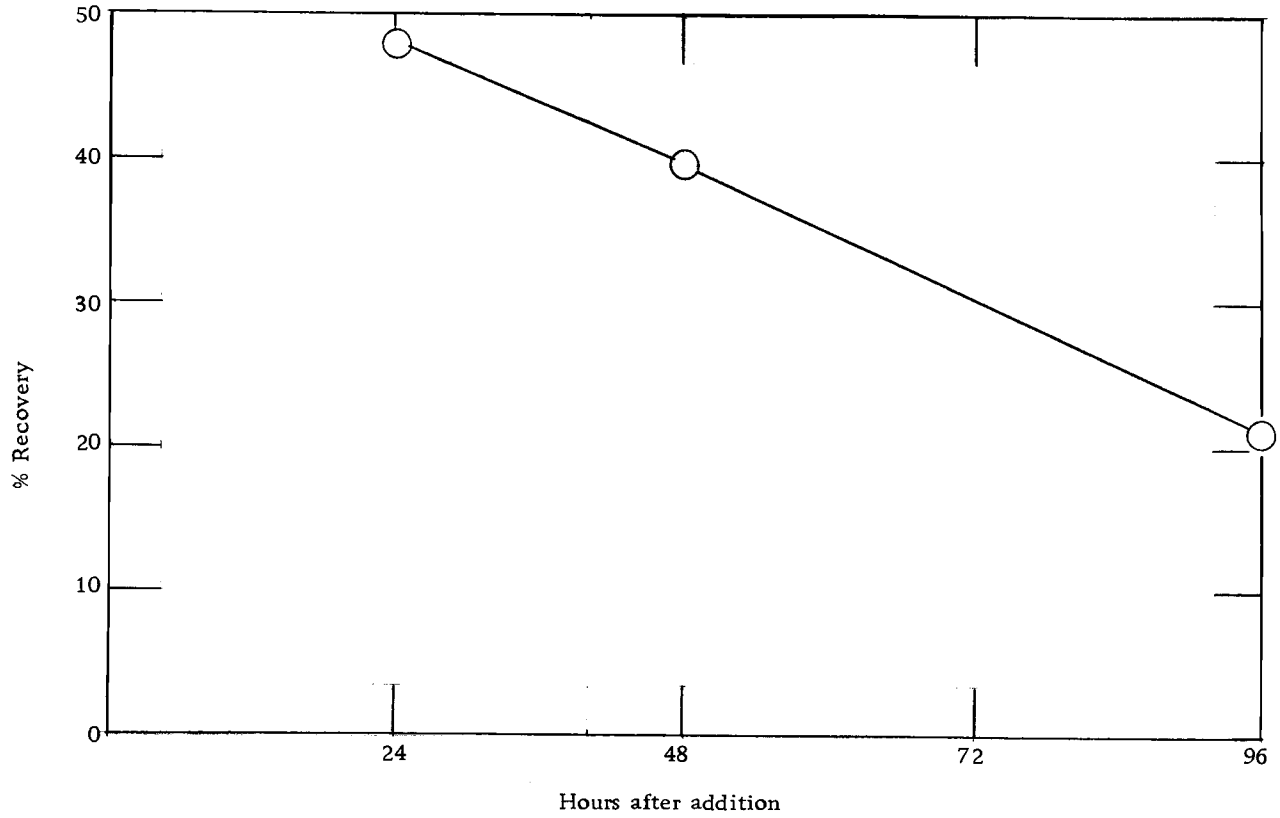


Figure 9. Average recovery of TCDD from water with 50 ng/l TCDD added and containing 10 young coho salmon (four replications).

replenished. The exposure levels in Table 1 are the initial levels, no adjustment being made for possible changes in TCDD concentration with time.

Toxic Effects of TCDD to Some Aquatic Animals

A difficulty in studying the toxicity of TCDD to fish is that the response to the chemical is not immediate. In most static water toxicity texts, observations would have been terminated after 96 hours. In these tests, initial response to the chemical did not occur for five to ten days after the beginning of the exposure period and mortality often extended over the following two to three months.

Fish exposed to toxic levels of TCDD first showed a declining interest in feeding. Salmon reduced their food intake as early as eight days after TCDD exposure (Table 4); guppies declined food in five days. Affected salmon often rejected the food shortly after taking it in their mouths. Growth of exposed salmon was inhibited (Fig. 10). There was also a general body deterioration. Coho exposed to as low as 5.4 ng/g had an average increase in weight of only 8% and 32% of of the weight of controls when exposed for 96 and 24 hours respectively. The control animals gained an average of 3.8 g each during the 60 day period, an average increase of 105% (Table 5).

Skin discoloration and fin necrosis began to appear 15 and 30 days after initial exposure of guppies and salmon, respectively.

Table 4. Fffect of level and duration of exposure on food intake (experiment 10).

		Days since exposure				
		7[b]	15[c]	22[d]	30[d]	40[d]
Duration (hours)	Level (ng/l)	% available food consumed in 20 min. (adjusted to surviving fish)				
	0[a]	91	92	98	96	100
24	.056	98	96	100	93	100
	.56	92	95	98	99	100
	5.6	97	88	79	83	84
	56.0	91	71	19	0	0
48	.056	83	97	90	79	88
	.56	100	96	100	98	99
	5.6	97	83	55	26	31
	56.0	85	50	20	0	0
96	.056	90	91	100	100	100
	.56	98	94	100	100	92
	5.6	87	76	65	54	27
	56.0	49	9	23	0	0

[a] $\frac{60 \text{ fish}}{\text{wt. of food fed per fish, g.}}$

[b] .117

[c] .18

[d] .21

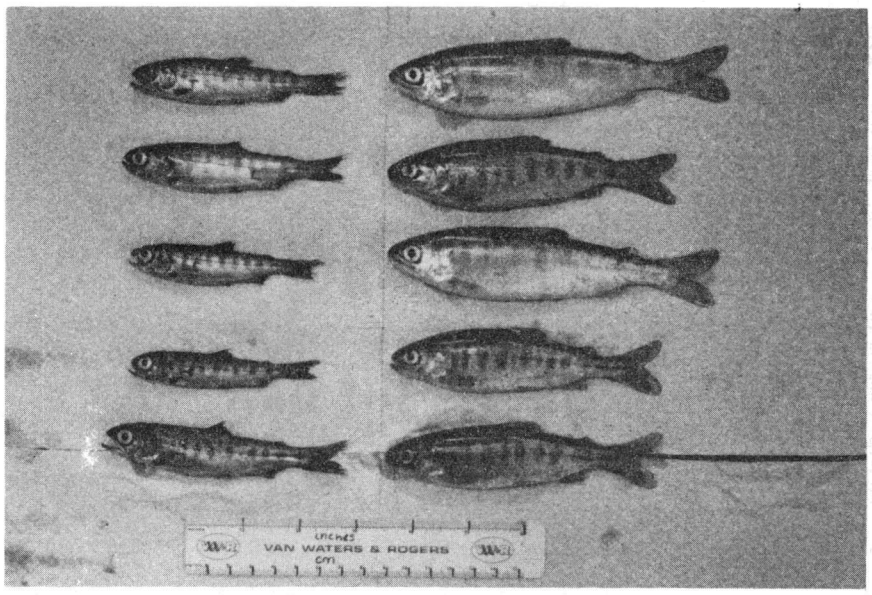


Figure 10. TCDD-exposed (13.1 ng/g, 96 hours) and control young coho salmon 80 days after beginning of exposure period.

Table 5. Mean change in body weight for young coho salmon exposed to TCDD, 60 day observation period (experiment 10).

Level of exposure, ng/g BW[1]												
0.054			0.54			5.4			54.0			
Duration of exposure, hr												
0	24	48	96	24	48	96	24	48	96	24	48	96
Avg. fish gain or loss, g												
3.8	3.7	2.8	3.8	3.4	4.0	3.2	1.2	.4	.3	-.1	0	-.1
% change in 60 days												
106	105	80	106	97	114	91	34	11	9	-3	0	-3

[1] ng TCDD/g wet body weight

Complete loss of the caudal fin occurred in both guppies and salmon. Areas showing skin discoloration often became the site of attack for disease organisms. In salmon, large fungal growths completely encircled some animals and inhibited swimming. Erosion of the upper jaw was seen in guppies surviving one to two months after exposure, but not in salmon. Prior to death, fish often remained close to the bottom of the test containers and showed very little movement. There was no definite pattern; however, some fish that appeared perfectly healthy one day were dead the next day, while other apparently diseased individuals remained alive for weeks. Detectable differences were not observed in behavior or body conditions of the invertebrate organisms.

Toxicity of TCDD to Fish

Effect of Level and Duration of Exposure on Survival Time

The deaths among exposed salmon frequently did not occur for ten days after the beginning of the exposure period, regardless of exposure level. In experiment seven the effects of exposure to more than 23 ng TCDD/fish wet weight (23 ng/g) for 24 hours was irreversible, and most fish died within 60 days (Fig. 11). The effects of level of exposure were quite marked while the effects of duration of exposure were less prominent.

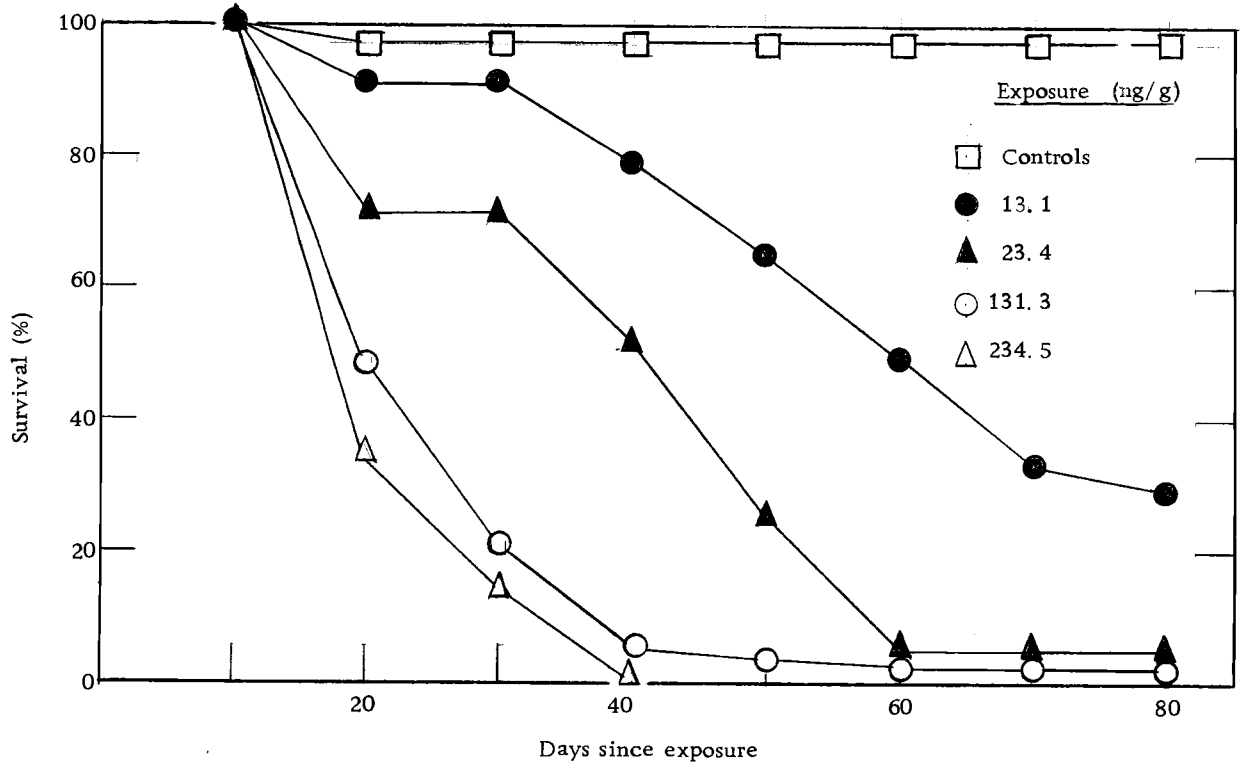


Figure 11. Survival of young coho salmon (experiment 8) after exposure to TCDD in water. Values are means for 24, 48, and 96 hours exposure (four replications).

In experiments nine and ten an attempt was made to identify the minimum threshold response level (Table 2). The pattern of delayed mortality observed in experiment eight was also prominent in these two experiments (Figs. 12 and 13). In experiment nine an exposure level of 54 ng/g for 24 hours or longer was irreversible and caused complete death within 40 days. Exposure to 5.4 ng/g resulted in 55% mortality during the 60-day observation period. Levels of TCDD as low as 0.054 ng/g caused 12% mortality in the 60-day exposure period compared to 2% mortality in controls. These lower levels may approach the minimum threshold-response. This is supported by the high rates of weight increase for the fish exposed to 0.054 ng/g in experiment seven, Table 5.

Homelink (1971) proposed that DDT is rapidly accumulated in aquatic organisms through an exchange equilibrium with a partition coefficient of 1×10^6 for DDT in water and fat; fish of 2% body fat would concentrate DDT by a factor of 2×10^4 over the concentration of water. TCDD has a water solubility of 0.2 μg/l or at least six times lower than DDT; it, like DDT, is lipophylic. Therefore, fish and other aquatic organisms can be a sink for the collection of TCDD. This, in part, explains the rapid reduction of TCDD from water and the lack of a strong duration of exposure effect in bioassays.

The duration of exposure appears less important than levels of exposure in determining mean survival time (Fig. 14). In

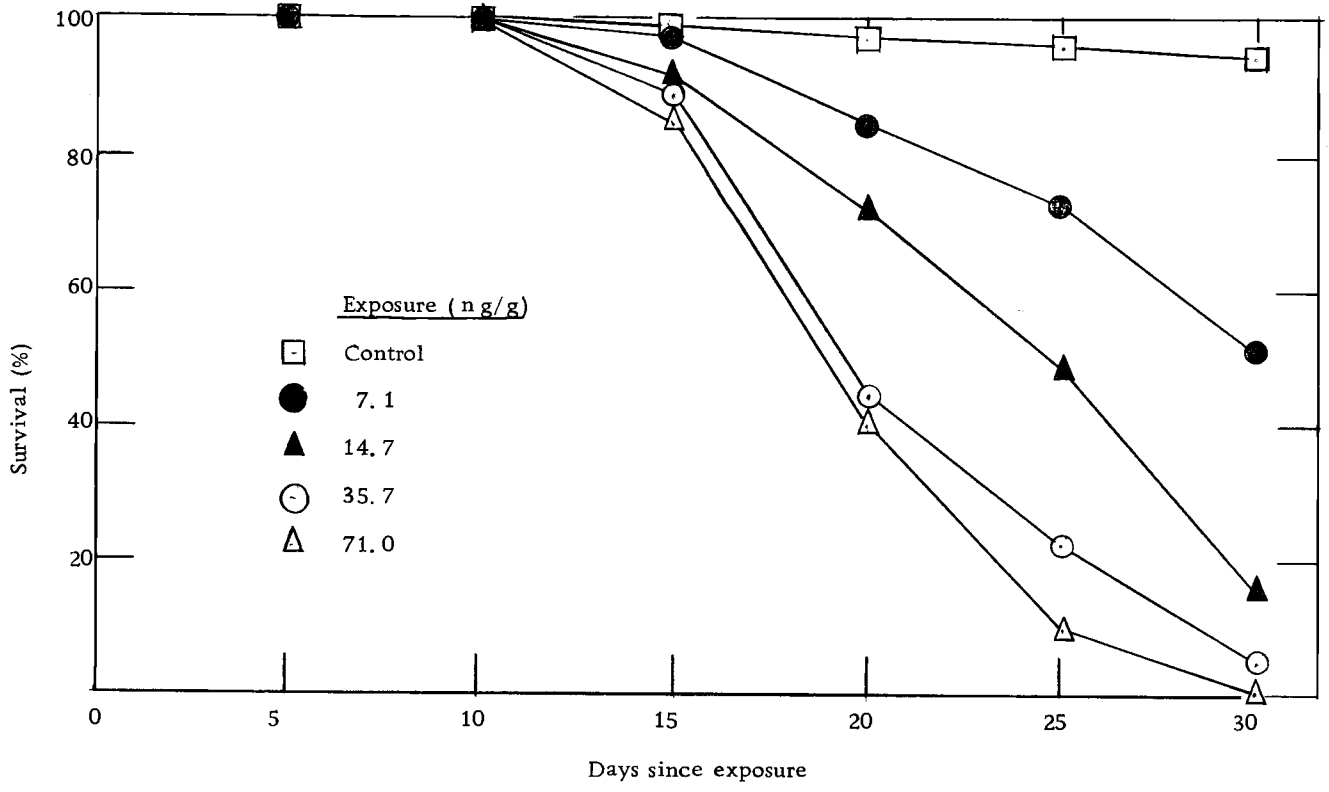


Figure 12. Survival of young coho salmon (experiment 9) after exposure to TCDD in water. Values are means for 24, 48, and 96 hours exposure (four replications).

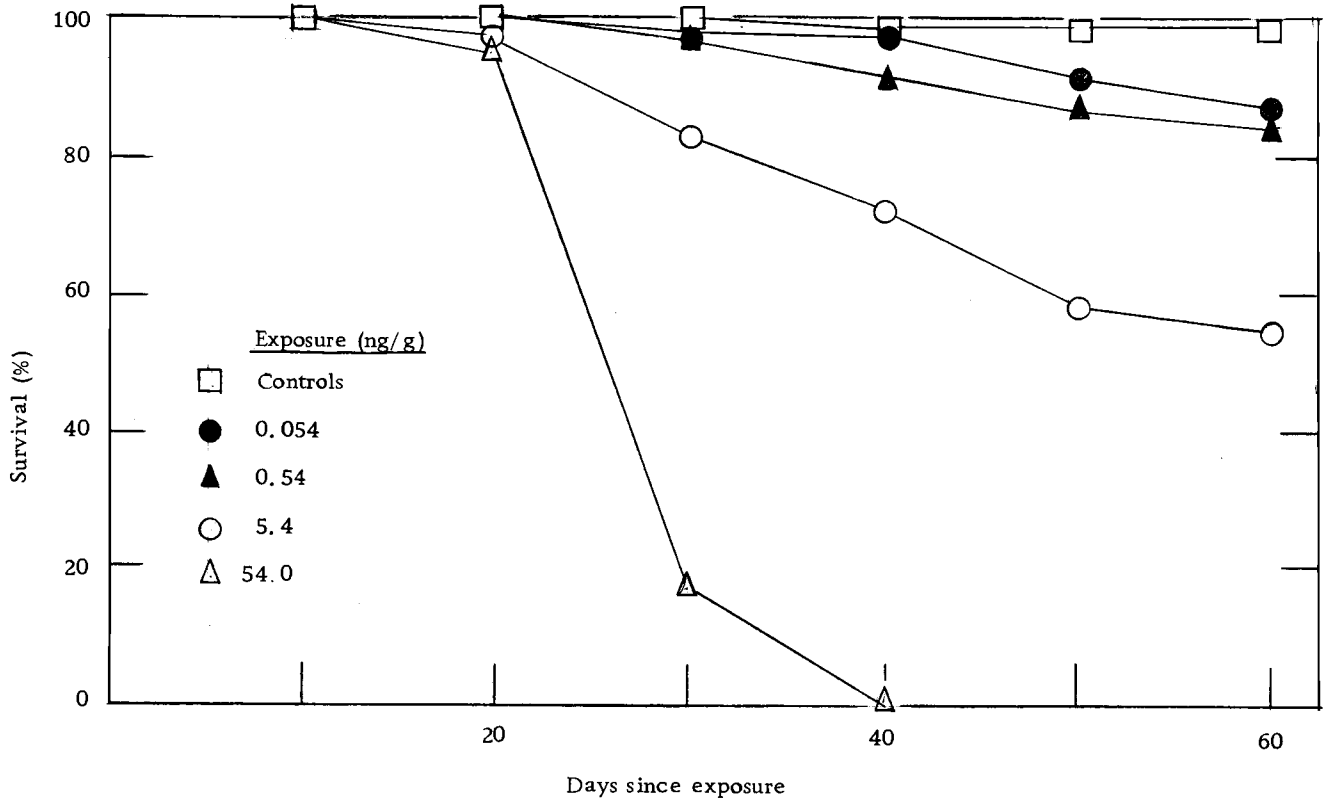


Figure 13. Survival of young coho salmon (experiment 10) after exposure to T CDD in water. Values are means for 24, 48, and 96 hours exposure (four replications).

experiment two, guppies two to three weeks old were used. As observed in the salmon experiments, death occurred during the post-exposure period, beginning five days from initial exposure (Fig. 15). Only 4% of the treated animals remained alive 20 days after initial exposure whereas there was 92% survival in the controls. The effect of increasing concentration accounted for small differences in mean survival between the treatment groups. This is obviously due to exposure levels that are at least 40 times greater than an estimated threshold level, using salmon experiment nine as a reference point.

For statistical analysis, data were expressed as days to death and subjected to multivariate analysis of variance. Mean survival time was significantly reduced with increasing TCDD exposure levels in experiments eight, nine, and ten ($P < 0.01$) (Figs. 11, 12, and 13). The duration of exposure effect was less marked, but was significant in these experiments ($P < 0.05$). The duration of exposure-concentration interaction was not significant in any experiment. The duration of exposure effect in tests with salmon may be more pronounced as the minimum threshold-response level is approached and as the duration of exposure is reduced. A duration of exposure effect was not observed in guppies. In experiment three, 10 and 70 hour exposure periods at one exposure level were compared (Table 6). The levels of exposure in this experiment were 56 times greater, on a TCDD weight per fish wet weight basis, than the 5.4 ng/g exposure

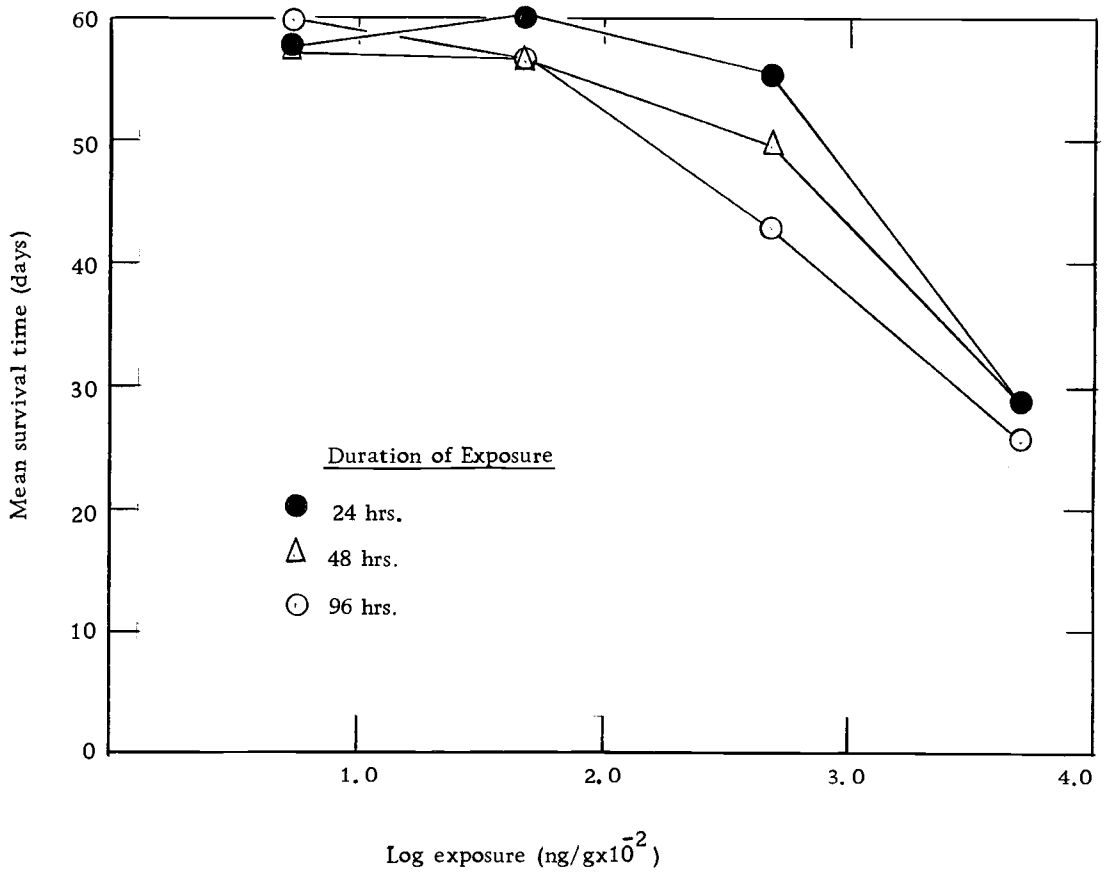


Figure 14. Influence of duration of exposure to TCDD on mean survival time of young coho salmon (experiment 10) (four replications).

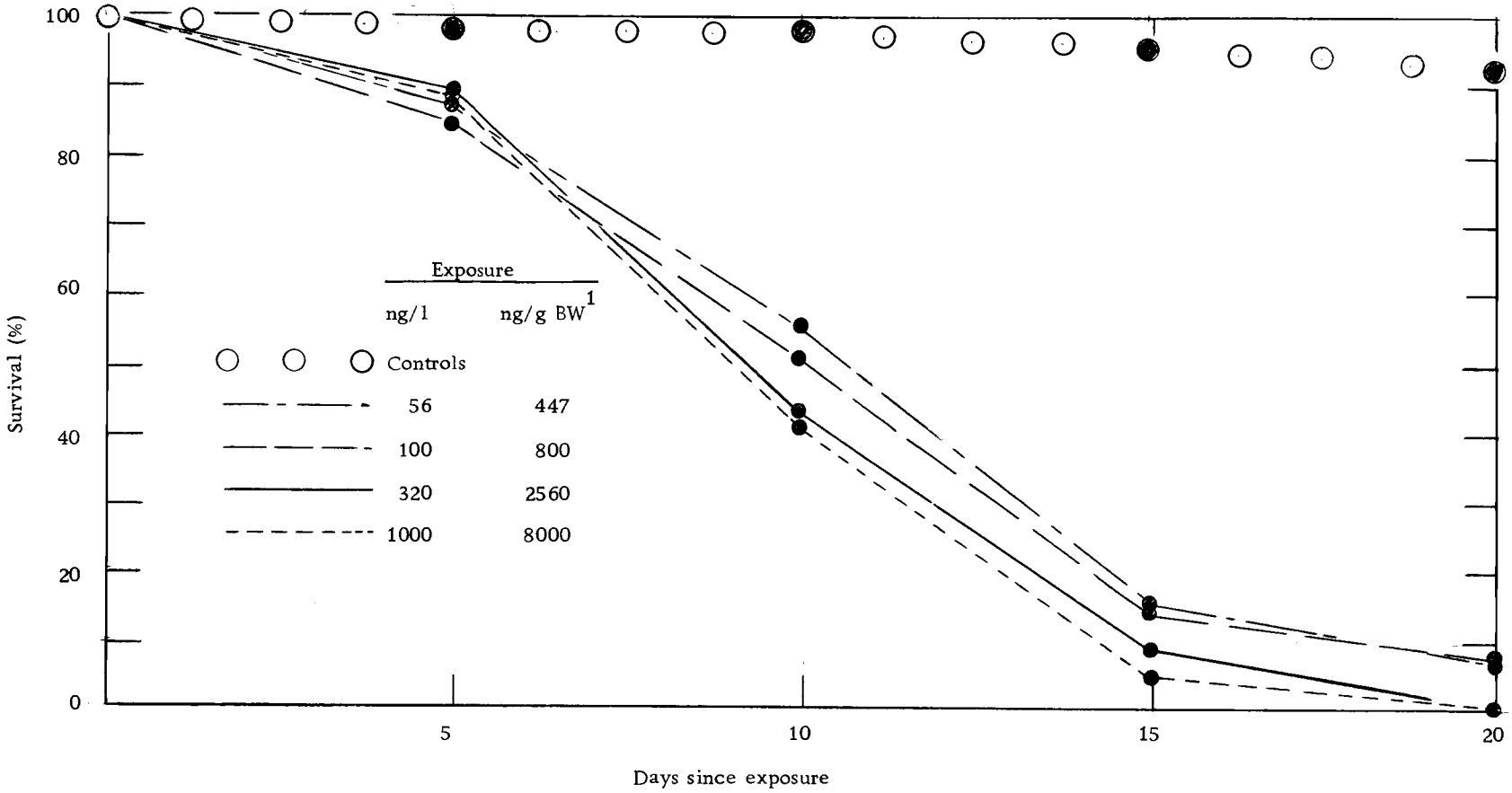


Figure 15. Survival of young guppies (experiment 2) after exposure to TCDD in water.

[1]BW = body weight

level of experiment ten with salmon (Fig. 13). As the threshold response level is neared a duration-of-exposure effect will no doubt also be seen.

Table 6. Survival (in percent) for guppies exposed to 100 mg/l TCDD for 10 and 70 hours (experiment 3).

	Duration of Exposure	
Days since exposure	10 hr	70 hr
10	100	100
20	93	87
30	3	5
40	3	0

Effect of Size of Fish on Survival Time

In both salmon and guppy experiments, larger fish survived for longer periods than smaller fish after TCDD exposure. In some earlier work, experiment one, mean survival time was plotted as a function of body length for TCDD exposed guppies ranging from 10 to 40 mm in length (Fig. 16). The regression equation was linear and highly significant ($P < 0.01$). Body length accounted for 93% of the variation of the dependent variable. The same effect was observed in salmon when data from experiments eight, nine, and ten were

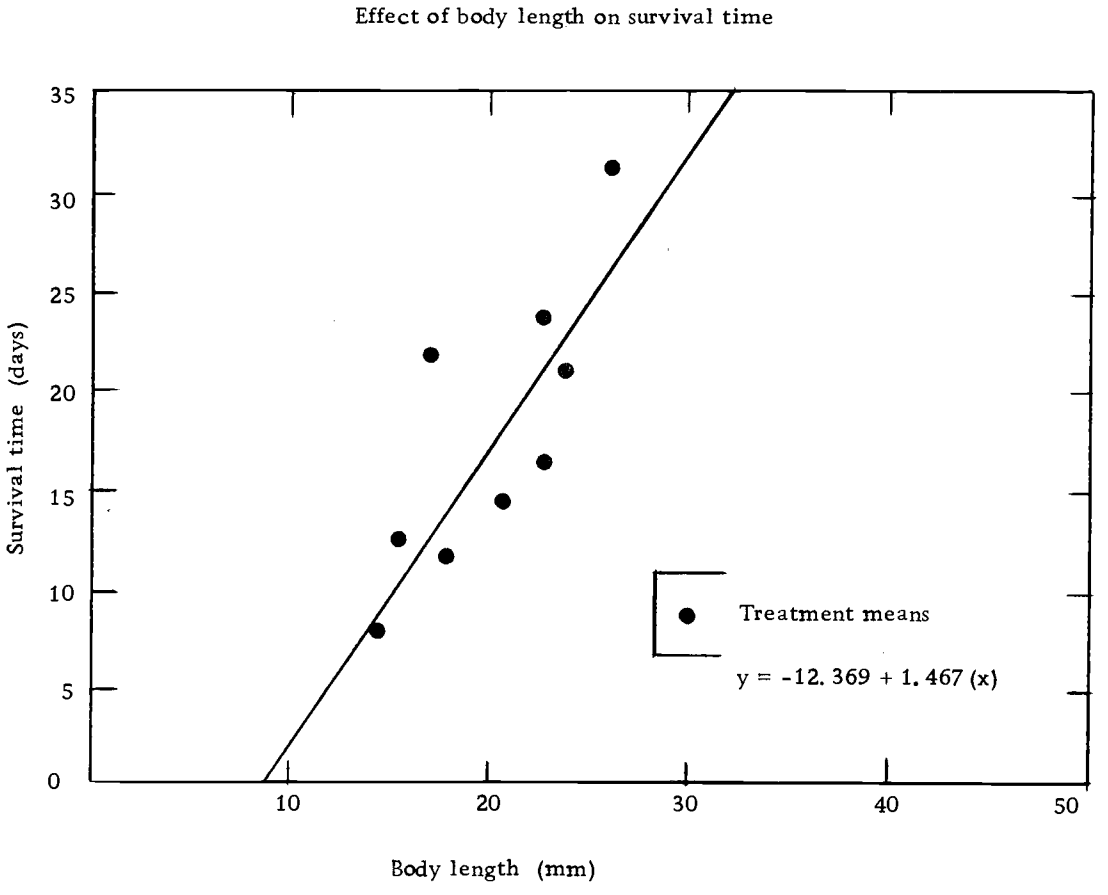


Figure 16. Effect of body length on mean survival time of guppies exposed to 100; 1,000; and 10,000 ng/l TCDD for 120 hours (Norris and Miller, in press) (three replications).

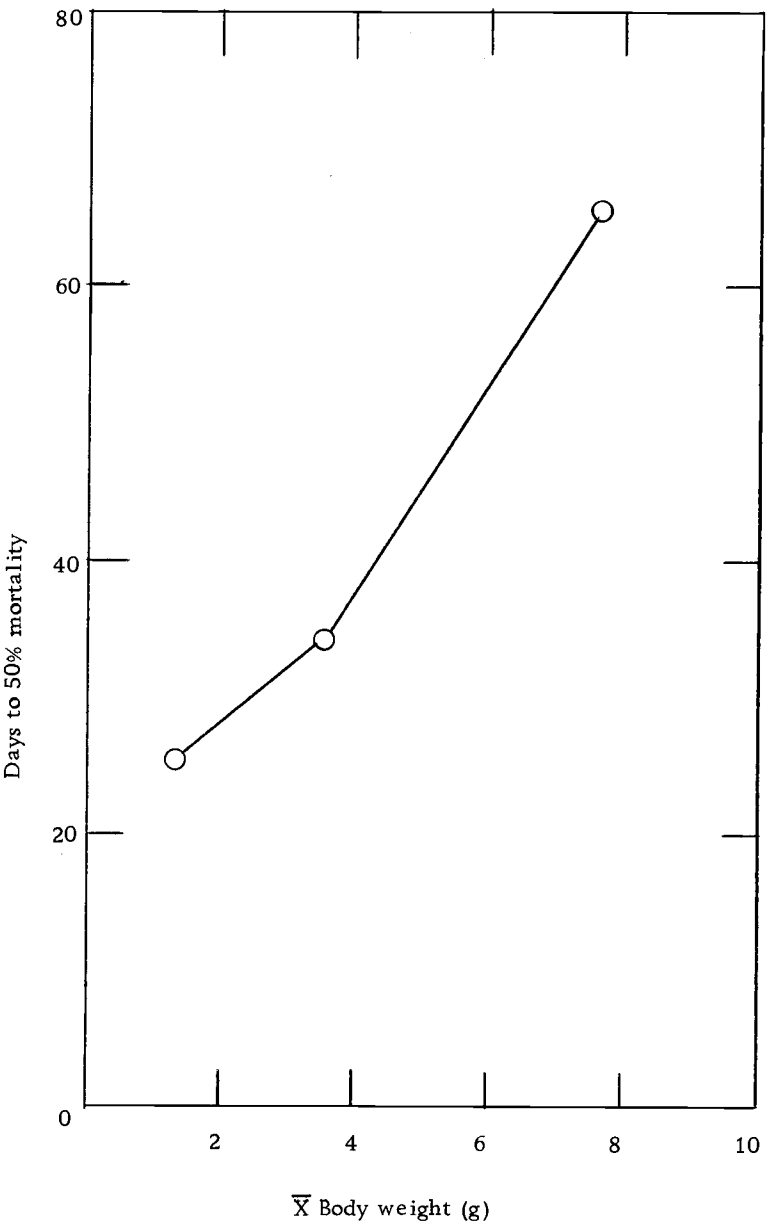


Figure 17. Effect of body weight on time to 50% mortality in young coho salmon exposed to 10 ng TCDD per gram wet body weight. Values are means for 24, 48, and 96 hours exposure (four replications).

combined (Fig. 17). Time to 50% mortality for salmon exposed to 10 ng/g for 96 hours was determined graphically for each experiment. Regression analysis showed that the effect of body weight on survival time was linear and significant ($P < 0.01$):

$$Y = 13.8 + 7.7X$$

where Y is time, in days, to 50% mortality, and X is body wet weight, in grams; $r^2 = 0.87$.

Similar responses have been reported for other toxicants (Post and Schroeder, 1971). The ability to tolerate environmental stresses increases up to a point with increasing age and body mass in many organisms (Warren, 1971). Toxicant uptake, storage, and detoxification probably change with fish age, lipid levels and gill surface area to body mass ratios, as reported by Cope (1971), and Murphy and Murphy (1971).

Sublethal Effects of TCDD Exposure to Young Guppies

In all of the fish experiments a general fin deterioration has been observed. In experiment four, young guppies were exposed to 0.08 to 80.0 ng/g TCDD. There was no significant survival difference between control and exposed fish. A diseased fin condition was observed, however, and the difference between control and exposed fish was significant, ($P < 0.01$) (Fig. 18). TCDD exposure of 0.8 ng/g was the lowest level to cause a diseased condition. Observations of all fins were made at two time intervals

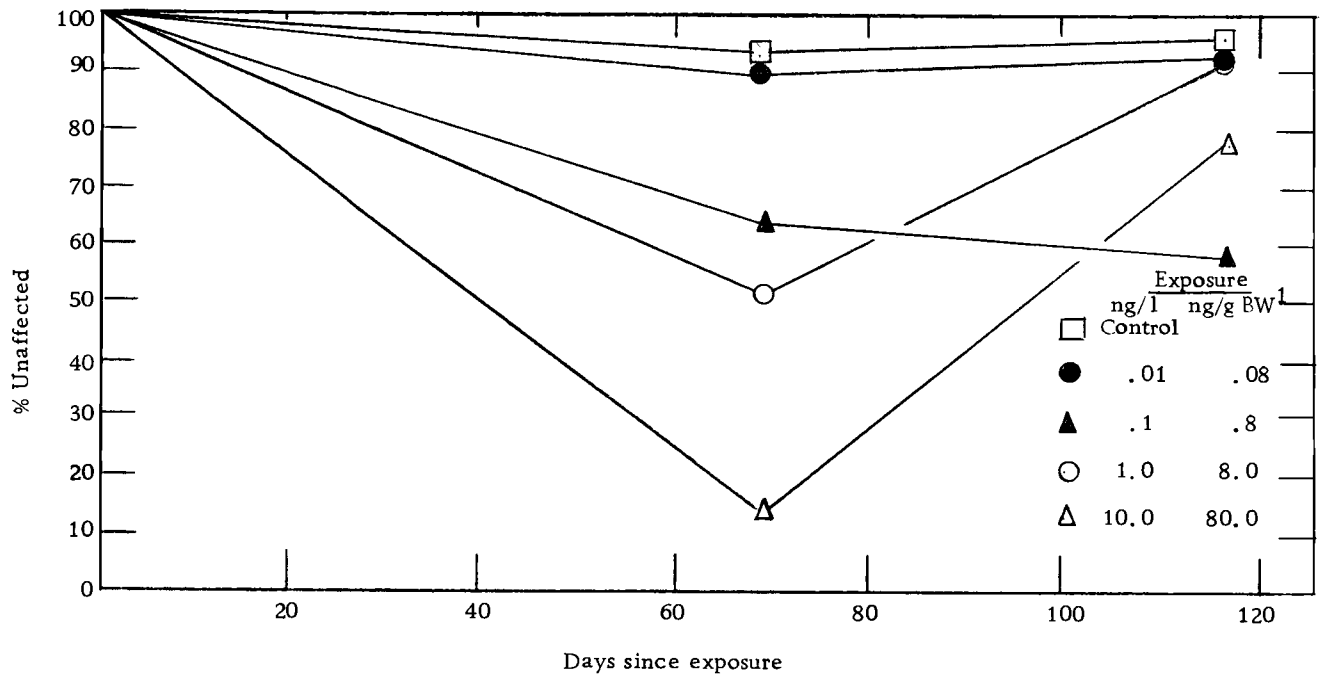


Figure 18. The effects of TCDD exposure on producing fin disease in young guppies.

[1] BW = Body Weight

after initial exposure, with the greatest effect on fins 69 days after exposure. In all but the 0.8 ng/g exposure level the percentage of fish affected decreased at the 117 day observation. This suggests that fish are able to overcome the effects of sublethal levels of TCDD exposure.

Toxicity of TCDD to Invertebrate Aquatic Organisms

Toxicity to Aquatic Worms

Adult worms were exposed to 0 or 0.2 μg/l TCDD in water for 55 days. Animals were counted at 30, 48, and 55 days after the beginning of the exposure period. At 55 days, total and mean dry weights were determined.

Exposure of worms to TCDD resulted in a decrease in the total number of worms present at the end of the 55-day exposure period ($P < 0.05$) (Table 7). Reductions in total worm biomass between treated and control organisms occurred in each replication, but variation among replications reduced the statistical sensitivity of this test ($P = 0.057$). TCDD exerted its principal effect on reproduction rather than growth of individual worms.

Toxicity to Mosquito Larva

In tests with mosquitoes, observations were made on the maturation of larva from the second instar through pupation during and

after 17-day exposure in water which originally contained 0 or 0.2 μg/l TCDD. There were no significant differences in total pupation or the rate of pupation among treated and control mosquitoes during the 30-day test period (Fig. 19).

Table 7. Toxicity of TCDD to aquatic worms, exposure level .2 μg/l in water.

Days since initial treatment	Numbers of worms	
	control	treated
0	80	80
30	233	195
48	409	310
55	414	266
	Total dry weight, mg	
55	374	193
	Mean dry weight, mg	
55	0.99	0.73

Toxicity to Aquatic Snails

Adult snails deposited numerous egg cases in containers of well water which originally contained 0 or 0.2 μg/l TCDD during a 36-day exposure period. Snail eggs completed development in the original exposure solution and live juvenile snails and empty juvenile snail shells were counted 48 days after the beginning of the experiment. There was no significant difference between the survival of treated and control adult snails, Figure 11.

Differences in the total snail hatch between treated and control organisms were observed in each replication, but variation among replications reduced the statistical sensitivity of these tests ($P = 0.056$). Differences in the percentage survival of young snails were not significant. TCDD had its major impact on the reproductive success of snails rather than on survival of either adult or juvenile forms.

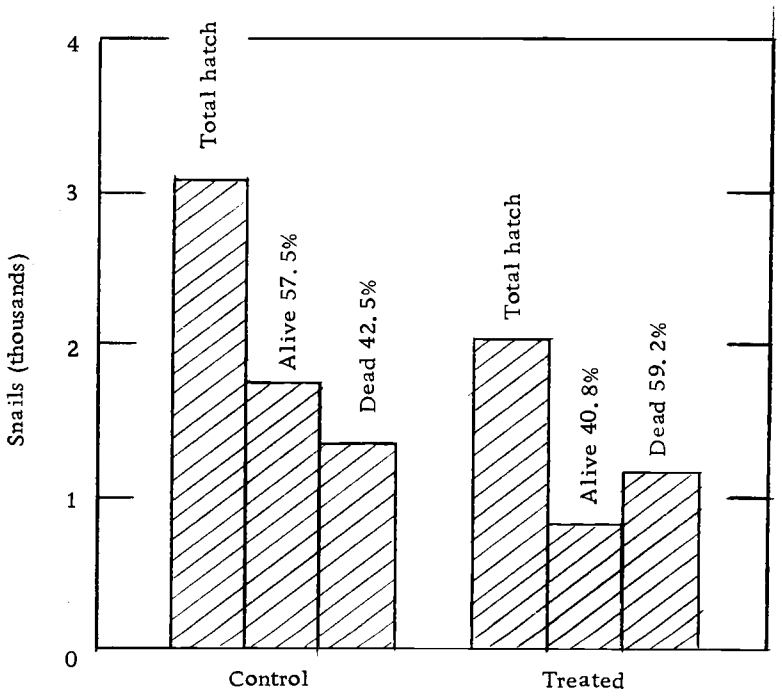


Figure 19. Total hatch and survival of juvenile snails from egg masses deposited in 0 or 200 ng/l TCDD in water during a 36-day adult snail exposure period. Counts were made 48 days after the beginning of the exposure period.

CONCLUSIONS

TCDD in water or food is toxic to fish. The effects of exposure for 24 to 96 hours for young salmon to TCDD in water at levels greater than 23 ng/g is irreversible and death results in 10 to 80 days. Duration of exposure is less important than level of exposure except as threshold response levels are approached. The critical exposure period may be somewhat less than 24 hours in static water toxicity tests in which TCDD concentration may change markedly with time. Small fish are more sensitive than large fish on an equivalent exposure level basis. TCDD at 0.2 μg/kg had no effect on pupation of mosquito larva, but reduced the reproductive success of an aquatic pulmonate snail and an Oligochaete worm.

This research established some toxicity characteristics of TCDD in fish, but considerable work remains to be done. Establishment of minimum threshold response levels during long- and short-term growth and reproduction of fish needs attention. Information on the movement, persistence, and fate of TCDD in aquatic systems will be required to adequately assess the impact of TCDD in streams. Serious attempts to determine TCDD residues in various parts of the natural aquatic ecosystem are badly needed. The most sensitive analytical techniques and positive means of residue identification will be necessary.

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