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Environmental Toxicology and Chemistry, Vol. 7, pp. 47–62, 1988
Printed in the USA. Pergamon Journals Ltd.

0730-7268/88 $3.00 + .00
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TOXICITY AND BIOCONCENTRATION OF 2,3,7,8-TETRACHLORODIBENZODIOXIN AND 2,3,7,8-TETRACHLORODIBENZOFURAN IN RAINBOW TROUT

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(*Received* 25 *March* 1987; *Accepted* 14 *July* 1987)

Abstract—Among the most toxic isomers of polychlorinated dibenzodioxins and polychlorinated dibenzofurans, two groups of toxic aromatic compounds, are 2,3,7,8-tetrachlorodibenzodioxin (TCDD) and 2,3,7,8-tetrachlorodibenzofuran (TCDF). We examined the chronic toxicity of these compounds to rainbow trout (*Salmo gairdneri*). The fish (0.38 ± 0.09 g) were continuously exposed in an intermittent-flow proportional diluter for 28 d to 0, 38, 79, 176, 382, and 789 pg TCDD/L (parts per quadrillion) or to 0, 0.41, 0.90, 1.79, 3.93, and 8.78 ng TCDF/L (parts per trillion); exposures to each chemical were followed by a 28-d depuration phase. TCDD had significant effects on survival, growth, and behavior during the exposure and depuration phases. The no observed effect concentration was lower than the lowest exposure concentration of 38 pg/L. The average measured BCF at 28 days was 26,707. The estimated bioconcentration factor at steady-state equilibrium was 39,000 in the lowest exposure concentration where fish were least affected. TCDF, like TCDD, induced similar effects on survival, growth and behavior. The no observed effect concentration, based on survival, was 1.79 ng/L; that based on growth was 0.41 ng/L. The measured bioconcentration factor was 6,049 in fish exposed to 0.41 ng/L, and 2,455 in fish exposed to 3.93 ng/L for 28 d.

Keywords—Dioxin Furan 2,3,7,8-tetrachlorodibenzodioxin (TCDD) Rainbow trout 2,3,7,8-tetrachlorodibenzofuran (TCDF)

INTRODUCTION

Polychlorinated dibenzofurans (PCDFs) and polychlorinated dibenzo-*p*-dioxins (PCDDs) are two groups of toxic compounds composed of 135 and 75 individual isomers, respectively. Certain of these isomers are extremely toxic, particularly those with chlorine substituents in the 2,3,7,8-positions of the aromatic rings. PCDFs occur as trace contaminants in polychlorinated biphenyls (PCBs) and are sometimes formed in significant quantities from pyrolysis or incomplete combustion of PCBs [1]. Isomer specific PCDFs and PCDDs also occur as contaminants in the manufacture and pyrolysis of certain chlorinated phenols [2]. During combustion of these formulations, PCDDs are formed primarily from thermal dimerization and conversion of chlorinated phenoxyphenols, whereas PCDFs are formed from chlorinated diphenyl ethers. PCDDs and PCDFs have also been found in fly ash of municipal waste incinerators [3].

The isomers 2,3,7,8-tetrachlorodibenzodioxin (TCDD) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) have been reported as contaminants in fish and sediment. Both have been detected in fish from the Great Lakes [4–6], and residues have been found in resident and migratory fish, crustaceans and sediment in the Chesapeake Bay area [7] and in industrialized and heavily populated areas of the northeastern United States [8]. The concentrations of these compounds in fish vary widely from low pg/g to ng/g quantities, and those of

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TCDF are usually higher than those of TCDD. In certain areas of the Great Lakes and the northeastern United States (Newark Bay, Passaic River), TCDD residues in fish and crustaceans exceed the U.S. Food and Drug Administration (FDA) "levels of concern" of 25 pg/g and 50 pg/g, respectively [8,9].

The chronic toxicity and bioconcentration of TCDD and TCDF in aquatic species have not been elucidated. Helder [10,11] reported that exposing fertilized eggs of rainbow trout (*Salmo gairdneri*) for 96 h to TCDD concentrations of 0.1 ng/L significantly decreased the growth of the resulting fry, and that exposing rainbow trout fry for 96 h to 10 and 100 ng/L TCDD retarded growth, caused histological changes in tissues and delayed mortality. Miller et al. [12] reported the toxicity and pathologic changes induced by short-term exposures of guppies (*Poecilia reticulata*) and coho salmon (*Oncorhyncus kisutch*) to TCDD. Coho salmon exposed to 56 pg/L and 1,000 ng/L for 24 h exhibited delayed mortality. Cooper et al. [13] observed delayed development and decreased survival in Japanese medaka (*Oryzias latipes*) exposed to TCDD concentrations of 6 to 500 ng/L. The oral toxicity and metabolism of TCDD in rainbow trout and yellow perch (*Perca flavescens*) were recently reported by Kleeman et al. [14,15]. In rainbow trout exposed for 6 h to 107 ng/L TCDD, followed by a 139-d depuration period, Branson et al. [16] estimated the bioconcentration factor (BCF) to be 9,270 and the elimination half-life to be 58 d. Significant delayed effects were similar to those reported by Miller et al. [12]. No similar studies have been conducted to characterize the toxicity and bioconcentration of TCDF in aquatic species.

Because of the lack of chronic toxicity data involving continuous low-level exposures of fish to TCDD and TCDF, we attempted to measure the chronic toxicity of these two compounds to rainbow trout. Their effects on survival, growth, and behavior were evaluated during a 28-d continuous exposure followed by a 28-d depuration phase. Uptake and depuration kinetics and BCFs for TCDD and TCDF were also evaluated.

METHODS

Test organisms

Eyed eggs of rainbow trout obtained from the Erwin (Tennessee) National Fish Hatchery came from two-year-old spawners of the "Fish Lake" strain; they were transferred to the National Fisheries Contaminant Research Center (NFCRC), Columbia, Missouri, where they hatched on 11 April 1985. About 2,000 swim-up fry produced from the eggs were shipped by air to Battelle Laboratories, Columbus, Ohio, on 2 May 1985. Mortality associated with shipping was less than 5%.

The fish were maintained in reconstituted water in 1,200-liter fiberglass tanks until the study was begun. The fish were held at a temperature of 11°C (±1°C), and were fed Tetramin floating flake food ad libitum. Analysis of the food showed no detectable quantities of TCDD (detection limit, less than 0.06 ng/g), TCDF (detection limit, less than 0.04 ng/g) or other organochlorine compounds.

Experimental approach

A flow-through diluter was used to continuously expose rainbow trout for 28 d to five duplicated concentrations each of [^{3}H]TCDD and TCDF plus duplicated controls. After the exposure period, toxicant input to the exposure chambers was terminated and the fish were held in laboratory water under flow-through conditions in the same test chambers during the 28-d depuration period. The fish were fed Tetramin floating flake food ad libitum throughout the study.

Fifty fish (0.38 ± 0.09 g each) were stocked in each aquarium. Samples of fish for residue analyses were taken on days 7, 14, 21, and 28 of the exposure phase and on day 28 of the depuration phase. To determine initial background concentrations of TCDD and TCDF, 30 fry with no previous TCDD and TCDF exposure history were weighed, measured, frozen, and analyzed for TCDD and TCDF. Fish collected for residue analyses were frozen until the time of analysis.

Daily survival records were maintained throughout the study. In addition, we recorded daily observations of swimming behavior, feeding behavior, location and position in the exposure tank, external lesions, and deformities.

Diluter and toxicant exposure system

The diluter system used in the study was constructed at NFCRC and installed in the West Jefferson Environmental Research Laboratory, Battelle Laboratories, Columbus, Ohio. The system consisted of two separate proportional flow-through diluters in a temperature-controlled waterbath. Both the diluter and waterbath were enclosed in a vented Plexiglas structure to reduce environmental exposures resulting from volatilization of the compounds. Each diluter delivered five concentrations (50% dilutions) of each compound (plus water for controls) into duplicate tanks containing

15 liters of water. Over the course of the study the diluter cycle rate varied between 2.4 and 3.0 cycles per hour; the replacement volume was 500 ml per replicate tank per cycle. The approximate water turnover rate in the exposure tanks was 2.4 times per day. The maximum fish loading in each test tank throughout the study was about 1.3 g/L and the maximum fish loading was 0.5 g/L of water passing through the tank in 24 h. Excess food and fecal matter were removed daily. Daily records of diluter operations were maintained throughout the studies. Nominal exposure concentrations (ng/L) were 0 (control), 0.115, 0.231, 0.463, 0.925, and 1.85 for TCDD; and 0 (control), 1.3, 2.7, 5.3, 10.6, and 21.3 for TCDF. Water temperature in the exposure tanks was maintained at 12 ± 1°C.

The combined effluents from the diluter system were recycled through two columns containing activated charcoal to remove TCDD and TCDF from solution. GC-MS and radiometric analyses were used to monitor the effluent for TCDD and TCDF.

Toxicants

Monsanto Company (St. Louis, MO) supplied the TCDD and TCDF used in the studies. The [^{3}H]TCDD (99+% pure; 22% unlabeled, 42% monotritiated and 36% ditritiated) used had a specific activity of 2.81×10^5 dpm/ng (0.128 μCi/ng) as determined by radiometric and GC-MS analyses. The TCDF provided by Monsanto was originally obtained from KOR, Inc. (Cambridge, MA), and was 98+% pure as determined by GC-MS.

Preparation of stock solutions

All glassware used to prepare stock solutions was rinsed several times with reagent-grade solvents. Carrier solvent for the compounds was acetone (Baker-analyzed). The [^{3}H]TCDD was diluted with acetone to a concentration of 36 ng/L. The stock solution was analyzed by GC-MS and by liquid scintillation radiometric analysis. Toxicants were delivered by an automatic pipetting system (Micromedic) that provided 0.05 ml/L or less of acetone to each exposure concentration. The TCDF was diluted with acetone to a measured concentration of 407 ng/L. This stock solution was used throughout the study and was delivered to exposure tanks by Micromedic pipetting systems. The acetone concentration delivered to each tank was 0.05 ml/L or less.

Water chemistry

In an effort to reduce the number of instruments coming in contact with the toxicants, we performed routine water chemistry only on the control chambers of both compounds, and only once during the exposure phase and once during the depuration phase. Alkalinity was measured by potentiometric titration with 0.02 N H_2SO_4 to pH 4.5, and hardness was titrated with EDTA according to standard methods [17]. We used an Orion

Table 1. Concentration of 2,3,7,8-tetrachlorodibenzodioxin (TCDD) in exposure water as measured by radiometric and GC-MS analyses

		TCDD nominal concentration (pg/L)					
Day	Measurement	0	115	231	463	925	1,850
1	pg/L (^{3}H)[a]	1.2	31	62	130	280	527
	pg/L (GC-MS)	—[b]					—[b]
7	pg/L (^{3}H)[a]	1.4	41	78	169	359	705
	pg/L (GC-MS)	<25[c]					840
14	pg/L (^{3}H)[a]	1.1	34	69	146	298	606
	pg/L (GC-MS)	<15[c]					730
21	dpg/L (^{3}H)[a]	0.7	41	87	200	466	970
	pg/L (GC-MS)	<15[c]					1,220
28	pg/L (^{3}H)[a]	1.3	44	99	234	507	1,135
	pg/L (GC-MS)	<20[c]					1,400
$\bar{x}$ pg/L (^{3}H) ± SD		1.1	38 ± 5	79 ± 15	176 ± 42	382 ± 101	789 ± 256
$\bar{x}$ pg/L (GC-MS) ± SD		<15[c]					1,048 ± 315

[a]Measured by radiometric analyses for [^{3}H]TCDD. Conversion of dpm/L to pg/L (^{3}H) based on specific activity of 2.81×10^5 dpm ^{3}H/ng TCDD.
[b]Not determined.
[c]None detected (less than minimal detectable limits).

digital pH meter to measure pH, a Sybron/Barnstead Model pM-70CB conductivity bridge to measure conductivity and a Varian Model 3700 gas chromatograph to measure ammonia. Water chemistry determinations were as follows: hardness, 153 ppm; alkalinity, 88 ppm; pH, 7.7; conductivity, 215 μohms; un-ionized ammonia, 0.0013 mg/L; and dissolved oxygen, 65 to 85% saturation.

Analyses of exposure water

During the exposure phase of the study, samples for GC-MS analysis were extracted from the TCDD control and highest exposure concentrations and from all TCDF exposure concentrations on days 0, 7, 14, 21, and 28. On each day immediately following the date of sample collection for GC-MS, we took samples for radiometric TCDD analyses from all exposure chambers. Radiometric analyses of all water extracts were conducted at Battelle Laboratories. Water from replicate *A* was sampled on days 0, 7 and 21, and water from replicate *B* on days 1, 14, and 28. On day 7 of the depuration period, the TCDD control and highest concentrations were measured radiometrically, and the TCDF control and highest concentrations were sampled for GC-MS analysis. On day 7 of the depuration phase, only 92 pg/L TCDD was measured in water from the highest TCDD exposure chamber, and 0.56 ng/L TCDF in the highest TCDF exposure chamber. The TCDD and TCDF exposure concentrations measured throughout the exposures are shown in Tables 1 and 2.

Water samples of a volume necessary to provide an adequate amount of analyte were collected from the diluter tanks with solvent-washed glassware and transferred directly to a glass separatory funnel. The water sample was then spiked with the appropriate internal standard solution containing [$^{13}C_{12}$]2,3,7,8-TCDD and [$^{13}C_{12}$]2,3,7,8-TCDF at 4.0 pg/μl in acetonitrile. The water sample was extracted three times with 50-ml portions of methylene chloride (CH_2Cl_2) and the extracts were passed through a column (about 2 × 6 cm) of anhydrous, granular sodium sulfate to break emulsions and remove suspended water. The extract was then rotary-evaporated to a low volume and transferred with three or four portions of CH_2Cl_2 to a glass ampoule, blown to dryness with nitrogen and flame-sealed.

The sample was removed from the opened ampoule with four 1.5-ml portions of 20% CH_2Cl_2 in hexane onto a dual column arrangement of 2 × 0.5 cm 40% H_2SO_4 on silica gel (SA-SG) in the first column and 15 mg Amoco PX-21 activated carbon dispersed in 150-mg glass fibers (CGF) [18]. The efficiency of transfer of [^{3}H]TCDD from these ampoules in the presence of solid residues was determined to exceed 99%. The SA-SG column was then discarded and the CGF column slightly pressurized to move the sample entirely onto the carbon adsorbent. We applied 15 ml CH_2Cl_2 to the CGF column at about 2 ml/min under pressure, and discarded the eluate.

The analyte, either [^{3}H]TCDD or TCDF, was recovered from the CGF by back-flushing with 15 ml toluene. The toluene was removed by rotary evaporation in a waterbath at 65 to 70°C under a 9.8-cm vacuum (sample taken just to dryness).

At this point, we added 2-(4-biphenyl)-6-phenylbenzoxazole (PBBO) to perform radiometric analyses on each sample or aliquots thereof containing [^{3}H]TCDD. The quench curve for counting efficiency was determined by the sealed tritium standard (HAV3612), corrected for decay, as the reference point, and replicate analyses of samples of [^{3}H]TCDD at various quench values. We used the equation, dpm = cpm/0.85 × *S*, where dpm is disintegrations per minute, cpm is counts per minute and *S* is the quench value.

Table 2. Concentration (ng/L) of 2,3,7,8-tetrachlorodibenzofuran (TCDF) as measured by GC-MS in exposure water during a 28-d chronic toxicity study with rainbow trout

	TCDF nominal concentration (ng/L)					
Day	0	1.3	2.7	5.3	10.6	21.3
1	0.02	0.38	0.70	1.40	3.20	6.60
7	<0.06	0.33	0.91	1.98	3.84	9.04
14	<0.029	0.44	0.86	1.56	3.82	7.97
21	<0.025	0.37	0.93	1.93	4.19	10.4
28	0.017	0.52	1.10	2.10	4.60	9.9
$\bar{x}$ ± SD	<0.02	0.41 ± 0.07	0.90 ± 0.14	1.79 ± 0.30	3.93 ± 0.52	8.78 ± 1.53

We applied the sample to alumina (Bio-Rad AG4 acid alumina, 3.5 ml = 3.65 g activated at 190°C) packed in a 5-ml graduated pipet with solvent reservoir using multiple washings of hexane totaling 5.0 ml. The column was then washed with 10 ml 5% CH_2Cl_2 in hexane (discarded) and the analyte recovered with 10 ml 20% CH_2Cl_2/hexane. The sample was evaporated just to dryness by rotary evaporation and transferred with three 1-ml portions of CH_2Cl_2 to a conical vial. The solvent was gently removed under a stream of nitrogen. The sample was then dissolved in a minimum of 5 μl *o*-xylene in preparation for GC-MS analysis.

We carried out the GC-MS analysis on a Finnigan 4023 quadrupole mass spectrometer (EI mode at 35 eV), using a 30 m × 0.25 mm DB-5 (0.25 μm) column (J&W Scientific, Inc., Rancho Cordova, CA) and helium carrier gas at about 35 cm/s. The temperature program was 120°C, hold 1 min, increase 20°C/min to 210°C, 5°C/min to 270°C and 4.5°C/min to 300°C. Selected ions monitored were m/z 304, 306, and 308 summed for 2,3,7,8-TCDF; m/z 316, 318 and 320 summed for [$^{13}C_{12}$]2,3,7,8-TCDF; m/z 320, 322, 324 and 326 summed for [^{3}H]2,3,7,8-TCDD; and m/z 332, 334, and 336 summed for [$^{13}C_{12}$]2,3,7,8-TCDD. We calibrated the internal standard solutions by preparing calibration mixtures of these standards with quantitative standards of native 2,3,7,8-TCDD and 2,3,7,8-TCDF prepared at the NFCRC and 2,3,7,8-TCDD solution as a U.S. Environmental Protection Agency (EPA) quality assurance material (Ref. No. 20603; EPA, Las Vegas, NV). We assumed equal integrated GC-MS responses for the molecular ions of native and [^{3}H]2,3,7,8-TCDD. The level of tritiation of the [^{3}H]2,3,7,8-TCDD computed from the molecular ion abundances measured by GC-MS gave a mole fraction of tritium of 27.3% and a specific activity of 2.15×10^5 dpm/ng. We calculated the specific activity, using the GC-MS-determined concentration and measured activity, to be $2.81 \pm 0.07 \times 10^5$ dpm/ng (triplicate analyses).

Collection of fish for residue analyses

Fish for whole-body TCDD and TCDF residue analyses were collected during the exposure period on days 0 (prior to exposure), 7, 14, 21, and 28, and on day 56 (after 28 d of depuration). When we removed fish from the exposure tanks for residue analyses on day 7, we removed unequal numbers from different tanks to reduce the number of fish remaining in all tanks to 42, and thus reduce the biomass and avoid potential overloading in the exposure tanks.

Fish for residue analyses were collected randomly from the exposure tanks for each toxicant. Individual weights and lengths were measured for fish collected on day 7 of the exposure and on day 28 of the depuration phase. Fish collected on other sampling days were weighed but not measured for length. All fish were blotted dry before they were weighed and were then wrapped in hexane-rinsed aluminum foil, placed in labeled screw-topped glass vials and stored at −10°C until residue analyses were begun.

GC-MS determinations of TCDD and TCDF in fish

Analyses of fish samples were performed by the method of Smith et al. [19]. The GC-MS conditions and spiking procedures were as described above for the analysis of the water samples.

Sample extracts that required radiometric analysis for [^{3}H]TCDD were rotary-evaporated and brought to 10.0-ml volumes; an appropriate aliquot (usually 1.00 ml) was then taken for scintillation counting. The quench values for the aliquots of the fish extracts were uniformly near the minimum (*S* values of 0.65), as observed for analytical standards. Negative and positive control samples were routinely included in the radiometric determinations of [^{3}H]TCDD and established so that there was no procedural background contribution in these determinations.

The internal standard procedure for GC-MS determinations of both [^{3}H]TCDD and TCDF provided internal quality control for overall accuracy of quantitation. In all reported determinations of these analytes, the criteria attained were relative GC retention time (±1 scan number in 1,160 or ±0.001 relative retention units) and correct ion abundances of the three or four molecular ion cluster members (±10% of theoretical value). The limit of quantitation was five times the signal-to-noise ratio and the limit of detection was three times the signal-to-noise ratio. The molecular ion cluster for [^{3}H]TCDD was significantly distorted from that produced by the native populations of ^{35}Cl and ^{37}Cl. Relative ion abundances of m/z 320, 324, and 326 were 24, 75, 100 and 70%, respectively. This pattern remained constant throughout the study, indicating no significant exchange of hydrogen for tritium in TCDD during the exposure. This observation also demonstrated no significant background of native 2,3,7,8-TCDD in any of the samples, because the presence of

native dioxin would have had an easily discernible effect on this pattern. Procedural background controls showed no 2,3,7,8-TCDD (limit of quantitation, less than 0.006 ng/g) by radiometric analysis and no TCDF (limit of quantitation, less than 0.06 ng/g) by GC-MS. The limit of quantitation for [^{3}H]TCDD was also less than 0.06 ng/g by GC-MS.

Analyses of fish food were carried out by the same procedure used for fish samples, and analyses of [^{3}H]TCDD and TCDF stock solutions were performed by direct dilution before analysis.

We computed percent recoveries of [^{13}C]TCDD and [^{13}C]TCDF internal standards by the less precise external standard technique, using the responses of the [^{13}C]TCDD and [^{13}C]TCDF internal standards; the recoveries of [^{13}C]TCDF and [^{13}C]TCDD, respectively, are listed here according to the various matrices: stock solutions, 71 ± 30% and 71 ± 33%; exposure water, 134 ± 55% and 109 ± 52%; fish, 101 ± 37% and 117 ± 46%; all matrices combined, 112 ± 51% and 105 ± 47%.

Determination of total concentration of [^{3}H]TCDD species in fish by biological material oxidation procedure

Determinations of total body burden of [^{3}H]TCDD residues in fish, as opposed to extractable residue, were made on homogenate aliquots of individual fish by the method of total burn, followed by liquid scintillation radiometric analysis of the combustion products. A Harvey Biological Materials Oxidizer (Model OX-100, R. J. Harvey Instrument Corp., Hillsdale, NJ) and a Harvey tritium cocktail (lot No. DC02) were used in the procedure. The combustion/trapping efficiency was 84% with triplicate analyses of a [^{14}C]PCB standard. Cryogenic traps and dry ice and methanol were used to trap the tritiated water produced in the combustion. The combustion/trapping efficiency observed for a standard of [^{3}H]TCDD was 89 ± 3% for spiked fish tissue. The scintillation counting efficiency when the tritium cocktail was used was 37%, and radioactivity was calculated from scintillation analysis using the equation, dpm = cpm/0.64 × S, after subtraction of 50 cpm background.

Samples that had previously been weighed, wrapped in filter paper and aluminum foil and stored in the freezer were transferred along with the approximately 1-cm^2 pieces of filter paper to the quartz combustion boats. Before combustion of samples, we ran a series of blanks and spikes to ensure that performance was satisfactory. Each sample was combusted twice into the cryogenic trap, which contained about 0.5 ml residual methanol. The glass elbow connecting the trap and oxidation chamber was heated with a hot air gun during the procedure to prevent loss by condensation. The condensed residue was transferred from the trap to a scintillation vial with three 5-ml portions of the cocktail. We then washed the trap thoroughly three times with methanol, leaving about 0.5 ml to aid in the next trapping. Because previous tests had indicated that carryover between sample combustions was a potential problem, blank combustions were performed after each sample and control. Scintillation analysis of the blanks showed that carryover was negligible.

Observation of fish for behavioral responses

The behavioral responses of rainbow trout were assessed daily during the TCDD and TCDF exposures. A checklist of behavioral reactions modified from Drummond et al. [20] was used to systematically document and characterize abnormal responses. The responses included coloration, activity (hyperactive, lethargic), excitability by external stimuli (hyperactive, unresponsive), location in aquaria, mode of swimming (head-up, frequent sinking and rising, swimming on side, swimming on back, free swimming), feeding, and morphological observations (bent spine, fin erosion). Observations were made each day by the same observer at the time of feeding.

An aberrant behavioral reaction was recorded when at least one fish in a given treatment responded in a manner that obviously differed from that of controls. Although no attempt was made to quantify the number of fish responding abnormally, an overall measure of the onset, duration and sequence of behavioral changes was made from the systematic daily observations.

Statistical analyses

Daily mortality was analyzed by one-way analysis of variance on the arc-sin transformed values. Differences among means were determined using Fisher's least significant difference (LSD) procedure [21].

Growth as measured by weight or length was analyzed by analysis of variance, including the effects of treatment, replicate within treatment, day, treatment × day, and replicate (treatment × day). Since the replicates, not the individual fish, were the experimental unit, replicate within treatments was used as the error term for testing the effect of treatment, and replicate (treatment × day) was used as the error term for testing the effects of day and treatment × day. We deter-

mined differences among means by calculating a t statistic, using the standard error of the difference for a split-plot design. For growth of TCDD-exposed fish during the depuration phase, we tested the control and lowest exposure concentration groups for equal population means, using a two-sample t test adjusted for unequal variance where appropriate [21].

The cumulative number of days on which fish showed abnormal behavior, from the time of induction to the day of depuration, was analyzed by simple regression against concentration, to provide an estimate of the behavioral responses to chemical exposure.

The BIOFAC computer program [22] was used to estimate the bioconcentration kinetics for TCDD and TCDF. Data from only the exposure phase in each study were used to estimate the kinetics because the number of fish residue samples available during the depuration phase was not adequate. In addition, the fish were held in their original exposure test tanks during the depuration phase, which resulted in the presence of the toxicants in the water because they desorbed from the glass aquaria. Because water concentration measurements and sufficient fish to sample during the depuration phase were not available, we were unable to use data from the depuration phase to estimate rate constants for the toxicants.

To estimate the 56-d LC50 value for TCDD, we computed a multiple-regression model to determine the relationship between percent mortality (arc-sin transformation) to concentration and time of exposure. The linear statistical model contained the effects of linear concentration (CL), days of exposure linear (DL), concentration quadratic (CQ), and day of exposure quadratic (DQ): CL * DL, CL * DQ, CQ * DL and CQ * DQ [21]. We used a quadratic function relationship to estimate the concentration of TCDD at a constant mortality (50%) and period of exposure (56 d).

RESULTS AND DISCUSSION

Mortality

TCDD induced significant mortality in rainbow trout within 14 d of exposure in the highest exposure concentration (789 pg/L), and there was a trend toward increased mortality in fish exposed to 176 and 382 pg/L (Table 3). After 28 d of exposure, significant mortality was evident in the three highest exposure concentrations; the no observed effect concentration (NOEC) was 79 pg/L. Although no mortality was observed, fish in the 38 and 79 pg/L exposure groups were obviously stressed, as judged by reduced growth and behavioral responses. Only rainbow trout in the control group and the three lowest exposure concentrations were observed during the 28-d depuration phase of the study; fish in the two highest exposure concentrations were excluded because the survivors were few and obviously stressed. Significant mortality continued to occur throughout the depuration period in fish previously exposed to 38, 79, and 176 pg/L. There was no apparent recovery in the fish during the 28-d depuration period in clean

Table 3. Cumulative mortality (%) in rainbow trout continuously exposed to 2,3,7,8-tetrachlorodibenzodioxin (TCDD) for 28 d followed by a 28-d depuration period

	Mean TCDD exposure concentration (pg/L)						
Phase and day	0	38	79	176	382	789	F value
Exposure							
7	5	0	1	4	6	10	1.79
14	5	1	1	13	17	33[a]	5.48[b]
21	5	3	9	36[a]	46[a]	74[a]	28.02[b]
28	5	6	18	50[a]	73[a]	85[a]	27.51[b]
Depuration							
7	5	12	64[a]	85[a]	—[c]	—[c]	9.33[b]
14	5	22	78[a]	95[a]	—	—	30.49[b]
21	7	33	83[a]	95[a]	—	—	28.63[b]
28	7	45[a]	83[a]	95	—	—	27.72[b]

[a]Significantly different from controls by least-significant-difference multiple means comparison test ($p < 0.05$).
[b]Significant treatment effect (one-way analysis of variance; $p < 0.05$).
[c]Exposure groups not part of depuration phase.

water. The NOEC of TCDD, based on mortality throughout the exposure and depuration phases, was less than the lowest exposure concentration of 38 pg/L (parts per quadrillion).

Further insight into the NOEC was inferred from the background concentration of 1.1 pg/L of TCDD detected by radiometric analyses in the control group throughout the study. This low background was probably due to volatilization of TCDD and translocation within the diluter system. Mortality in the control group was 5% during the exposure phase and most of the depuration phase. We suggest from these observations that the NOEC was between 1.1 and 38 pg/L. However, the minimal detectable limits for TCDD in water by GC-MS were not adequate to confirm the 1.1 pg/L detected by radiometric analyses.

A 56-d LC50 of 46 pg/L was calculated from the combined mortality data for the exposure and depuration phases. The surface response curve describing the relation among daily mortality, time and exposure concentrations is shown in Figure 1. The quadratic equation describing this relation was used to derive the 56-d LC50.

Significant mortality was induced by TCDF in rainbow trout within 14 d at exposure concentrations of 3.93 and 8.78 ng/L (Table 4). No additional significant mortality occurred throughout the 28-d exposure phase. During the depuration phase, additional mortality occurred only in fish exposed to 8.78 ng/L. The NOEC throughout the exposure and depuration phases was 1.79 ng/L.

Growth

Growth as measured by the weight of the fish was significantly decreased by all TCDD concentrations after 28 d of exposure (Table 5). There were trends of decreased growth within 14 d of exposure, but significant effects in all concentrations were not observed until 28 d of exposure. During the 28-d depuration phase, growth was measured in fish from only the control and the lowest exposure concentration because of the excessive mortality in the higher TCDD exposure concentrations. There was a significant decrease in growth in the fish exposed to 38 pg/L after the 28-d depuration phase. Fish exposed to 38 pg/L TCDD did not grow during the depuration phase, whereas the weight of fish in the control group exhibited an 80% increase. The NOEC of TCDD on growth during the exposure and depuration phases was less than the lowest exposure concentration of 38 pg/L.

TCDF exposure concentrations of 1.79, 3.93 and 8.78 ng/L significantly decreased the growth of rainbow trout within 28 d of exposure (Table 6). There were trends toward decreased growth

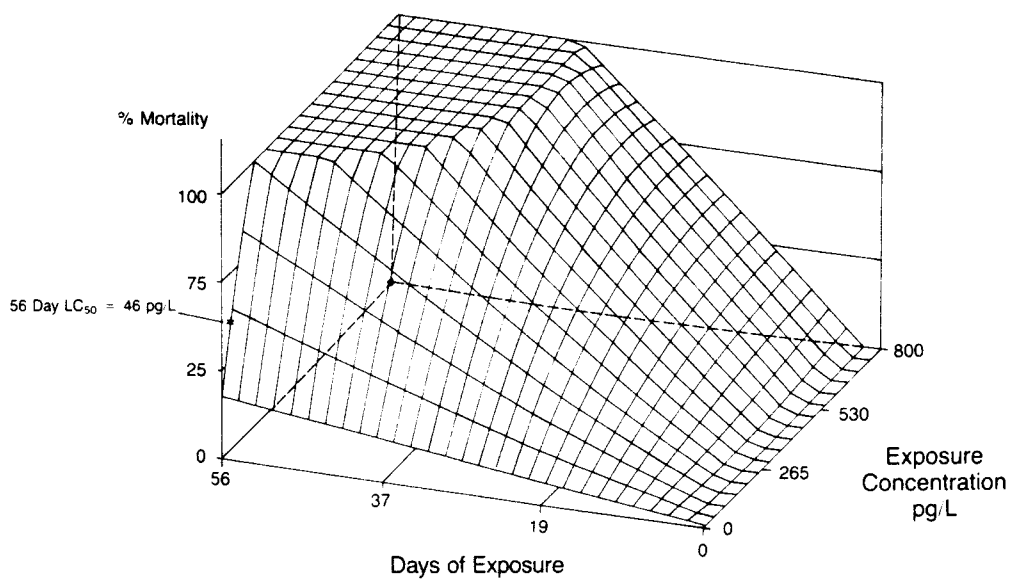

Fig. 1. Surface response describing the relation among daily mortality, time of exposure during the 28-d exposure and 28-d depuration phases, and TCDD exposure concentrations. The quadratic relation was used to derive a 56-d LC50 value of 46 pg/L TCDD for rainbow trout.

Table 4. Cumulative mortality (%) in rainbow trout continuously exposed to 2,3,7,8-tetrachlorodibenzofuran (TCDF) for 28 d followed by a 28-d depuration period

Phase and day	Mean TCDF exposure concentration (ng/L)						*F* value
	0	0.41	0.90	1.79	3.93	8.78	
Exposure							
7	0	1	1	2	2	12	2.54
14	0	1	3	3	16[a]	22[a]	4.51[b]
21	0	2	5	3	18[a]	23[a]	3.73[b]
28	0	2	6	3	18[a]	28[a]	4.49[b]
Depuration							
7	0	2	6	3	20[a]	37[a]	6.53[b]
14	0	2	6	3	22[a]	46[a]	8.56[b]
21	0	2	6	3	22[a]	46[a]	8.56[b]
28	0	2	6	3	22[a]	46[a]	8.56[b]

[a]Significantly different from controls by least-significant-difference multiple means comparison test ($p < 0.05$).
[b]Significant treatment effect (one-way analysis of variance; $p < 0.05$).

Table 5. Weight (g) of rainbow trout continuously exposed to 2,3,7,8-tetrachlorodibenzodioxin (TCDD) for 28 d followed by a 28-d depuration period

Phase and day	Mean TCDD exposure concentration (pg/L)					
	0	38	79	176	382	789
Exposure[a]						
7	0.37	0.36	0.38	0.33	0.36	0.33
14	0.41	0.39	0.42	0.33	0.35	0.40
21	0.48	0.35[b]	0.40	0.39	0.39	0.44
28	0.61	0.53[b]	0.47[b]	0.49[b]	0.45[b]	0.42[b]
Depuration[c]						
28	1.1	0.54[b]	—[d]	—[d]	—[d]	—[d]

Weights are expressed as the mean of 7 to 22 observations.
[a]Analysis of variance used for testing the effects of exposure concentration and time; $F = 2.43$ (time × exposure), $p < 0.03$.
[b]Significantly different from control group (t test; $p < 0.05$).
[c]Fish weight in depuration phase analyzed by t test adjusted for unequal variances.
[d]No measurements made.

after 21 d of exposure but the decrease observed was significant only in the group exposed to 3.93 ng/L. Decreased growth was evident in fish exposed to 0.90 ng/L or more after the 28-d depuration phase. The NOEC for TCDF based on growth during the exposure and depuration phases was 0.41 ng/L. This was the most sensitive response to TCDF.

Behavioral responses

Exposure to TCDD and TCDF induced behavioral impairments that became progressively worse over time and with increasing concentration. The two highest concentrations of TCDD caused behavioral changes within two weeks of exposure that included lethargic swimming, feeding inhibition, and lack of response to external stimuli, for example, waving of hand above aquaria (Fig. 2). Similar changes were evident in all groups exposed to TCDD by the end of the 28-d exposure, whereas the behavior of the controls remained normal. Although significant mortality did not occur in the two lowest exposure concentrations during 28 d of exposure, the fish were seriously stressed, as evidenced by an abnormal head-up swimming posture and confinement to the bottom of the aquar-

Table 6. Weight (g) of rainbow trout continuously exposed to 2,3,7,8-tetrachlorodibenzofuran (TCDF) for 28 d followed by a 28-d depuration period

	Mean TCDF exposure concentration (ng/L)					
Phase and day	0	0.41	0.90	1.79	3.93	8.78
Exposure[a]						
7	0.33	0.35	0.37	0.36	0.35	0.32
14	0.39	0.40	0.43	0.42	0.31	0.41
21	0.55	0.47	0.45	0.50	0.39[b]	0.44
28	0.59	0.59	0.53	0.48[b]	0.50[b]	0.46[b]
Depuration[c]						
28	1.1	0.91	0.85[b]	0.80[b]	0.79[b]	0.71[b]

Weights represent the mean of 8 to 24 observations.
[a]Analysis of variance used for testing the effects of exposure concentration and time; $F = 4.37$ (time × exposure), $p < 0.05$.
[b]Significantly different from controls (t test; $p < 0.05$).
[c]Analysis of variance used for testing the effect of exposure concentration; $F = 5.73$ (exposure), $p < 0.03$.

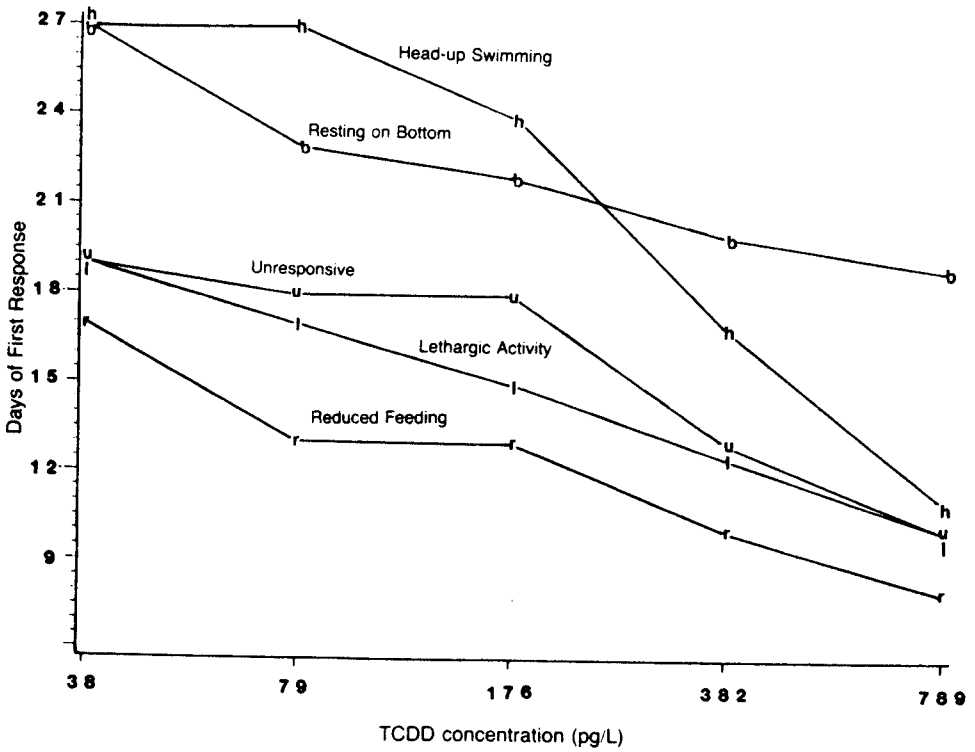

Fig. 2. Days of TCDD exposure required to induce behavioral changes in rainbow trout during a 28-d exposure.

ia. The feeding inhibition and other behavioral changes were not reversed during the 28-d depuration period.

Behavioral reactions similar to those observed in the TCDD exposure were observed in fish exposed to TCDF; however, the responses were of lesser magnitude (Fig. 3). Lethargy, unresponsiveness to external stimuli and diminished feeding

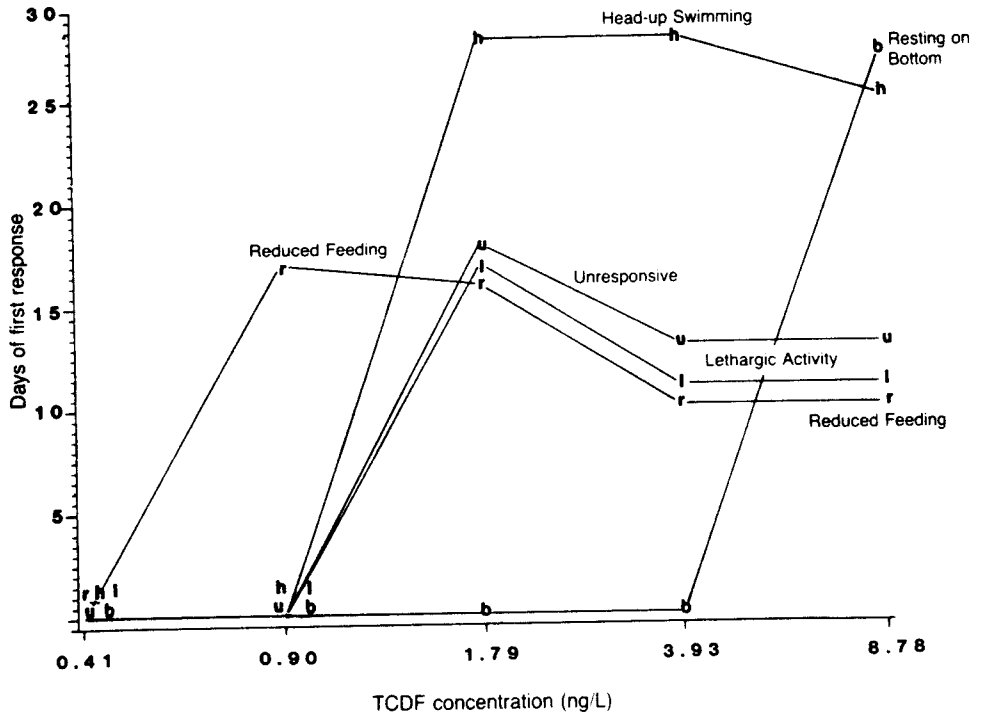

Fig. 3. Days of TCDF exposure required to induce behavioral changes in rainbow trout during a 28-d exposure.

reactions increased significantly in the three highest exposure groups. Recovery of behavioral function was evident in all but the two highest treatment groups by the end of the 28-d depuration period.

Neither TCDD nor TCDF induced observable responses in coloration or morphological characteristics such as scoliosis or lordosis; however, fin erosion was observed in fish in the lowest TCDD exposure concentration at the end of the depuration phase. In addition, exposure to both TCDD and TCDF induced observable, unique characteristics in fecal appearance. The two highest exposure concentrations of each toxicant induced long, stringy faces within the last several days of the 28-d exposure phase.

Bioconcentration

The BCFs for TCDD and TCDF differed greatly during the 28 d of continuous exposure. Whole-body residues throughout the exposure phase were in the low end of a 0.41 to 15.41 ng/g range for TCDD (Table 7). The greater the exposure concentration, the higher were the whole-body residues of TCDD during the 28-d exposures. The measured BCF for TCDD ranged from 8,558 to 28,664 during the exposure and did not appear to reach steady-state equilibrium in any of the exposure concentrations during the 28-d exposure (Table 8). The GC-MS analyses for whole-body TCDD levels agreed closely with the whole-body radiometric determinations for [^{3}H]TCDD. This similarity suggests that the ^{3}H label on the TCDD molecule was not being exchanged, and that the ^{3}H detected in the fish tissue was associated with the parent TCDD molecule. This similarity also indicates that organic extracted [^{3}H]TCDD was not being appreciably metabolized during the exposure and depuration phases. However, as judged by the results of total combustion of fish samples, it appears that about 30% of the ^{3}H label was associated with polar compounds that could have been TCDD metabolites.

Since it was apparent that a steady-state equilibrium for TCDD bioconcentration had not been reached after 28 d of exposure, we used the BIOFAC computer program [22] to estimate the bioconcentration kinetics for TCDD based only on data from the exposure phase. The estimated BCF at steady-state equilibrium was relatively consistent in fish from different exposure concentrations; the

Table 7. Whole-body residues of 2,3,7,8-tetrachlorodibenzodioxin (TCDD) in rainbow trout continuously exposed for 28 d followed by a 28-d depuration phase

Phase and day	Mean TCDD exposure concentration (pg/L)				
	0	38	176	382	789
Exposure					
0	[<0.02][a]				
7	0.012[a]	0.41[b] (0.05) [0.38]	1.68[b] (0.15)	3.44[b] (0.20)	6.75[b] (0.37) [6.78]
14	0.022[c]	0.77[b] (0.06) [0.71]	2.81[b] (0.18)	6.22[b] (0.67)	11.67[b] (0.68) [12.3]
21	0.023[d]	0.99[c] (0.03) [0.96]	3.87[b] (0.14)	10.10[c] (1.42) [11.3]	15.41[c] (0.86) [17.6]
28	0.027[a]	0.98[b] (0.05) [0.93]	4.52 (0.41)	10.95[b] (0.87) [10.8]	ND
	[<0.02]				
Depuration					
28	0.22[a]	0.74[b] (0.11) [0.78]	ND	ND	ND

Values (ng/g) represent the mean (with standard deviation in parentheses) of individual fish analyzed radiometrically for [^{3}H]TCDD. Values in brackets represent GC-MS analyses performed on a pooled sample of fish, expressed as ng/g. ND, not determined.
[a]One observation.
[b]Six observations.
[c]Two observations.
[d]Four observations.
[e]Eight observations.

Table 8. Measured bioconcentration factor (BCF)[a] for 2,3,7,8-tetrachlorodibenzodioxin (TCDD) in rainbow trout exposed continuously for 28 d

Days of exposure	Measured TCDD exposure concentration (pg/L)			
	38	176	382	789
7	10,736	9,551	9,005	8,558
14	20,131	15,966	16,282	14,790
21	25,947	21,977	26,439	19,510
28	25,789	25,670	28,664	ND

[a]BCF = $(C_f/C_w) \times 1{,}000$. ND, not determined.

estimated BCF at 90% steady-state equilibrium ranged from about 37,000 to 86,000 (Table 9). Fish exposed to 382 pg/L showed somewhat different kinetics in that the estimated BCF, time to reach steady-state equilibrium and half-life were greater than in the other exposure concentrations. The relatively low K_2 value, compared with K_2 values from other exposure groups, suggested that metabolic effects may have been reducing the elimination of TCDD.

Ideally, the BCF should be estimated in fish not showing toxicity-induced responses. Inasmuch as the fish exposed to the lowest TCDD concentration of 38 pg/L showed the least toxic responses during the 28-d exposure, we suggest that the predicted BCF of 39,000 is probably the most reliable

Table 9. Estimated bioconcentration kinetics[a] of 2,3,7,8-tetrachlorodibenzodioxin (TCDD) in rainbow trout exposed to TCDD for 28 d

Kinetic parameter	TCDD exposure concentrations (pg/L)			
	38	176	382	702[b]
K_1, uptake rate constant (d^{-1})	1,852 (132)[c]	1,543 (69)	1,337 (61)	1,591 (53)
K_2, depuration rate constant (d^{-1})	0.047 (0.01)	0.041 (0.005)	0.015 (0.005)	0.043 (0.005)
BCF-K_B	39,000 (9,400)	37,560 (5,032)	86,000 (25,000)	36,637 (4,290)
Time to reach 90% steady state (d)	49 (11)	56 (7)	149 (43)	53 (6)
Elimination half-life, $t_{1/2}$ (d)	15 (3)	17 (2)	48 (13)	16 (2)

[a]Estimated kinetics using BIOFAC [22].
[b]Mean of TCDD measurements at days 1, 7, 14 and 21.
[c]Values in parentheses represent standard deviations.

estimate. The range in BCF we observed was substantially greater than the BCF of 7,000 to 9,270 previously reported in the literature [16,23,24]. Results from our study were perhaps better estimates of the equilibrium BCF because we used a continuous exposure in flowing water for a longer period at lower exposure concentrations. Based on the water solubility of 7.9 ng/L for TCDD [25], the predicted BCF would be about 467,000 if the regression equation, $\log BCF = 2.791 - 0.564 \log S$ [26], were used; it would be about 1,000,000 if the regression equation, $\log BCF = 3.41 - 0.508 \log S$ [27], were used.

We suggest from our experimental data that the overall bioconcentration from water to fish is probably much less than the theoretical estimation. The obvious toxicity-induced effects of TCDD, as well as potential influences on membrane transport and other metabolic functions, could account for the observed BCF being less than the theoretical predictions.

The estimated elimination half-life ($t_{1/2}$) from the BIOFAC ranged from 15 to 17 d among exposure concentrations, except for the estimated half-life of 48 d in fish exposed to 382 pg/L. Adams et al. [24] reported an elimination half-life of 15 d, and Branson et al. [16] reported a half-life of 58 d. In the fish exposed to 38 pg/L for 28 d and then held during the 28-d depuration phase, the whole-body residues did not decrease sufficiently to support an estimated half-life in the range of 15 to 17 d (Table 7). The whole-body residues decreased from 0.93 (±0.05) to 0.74 (±0.11) ng/g during the 28-d depuration phase. Excessive mortality in the other TCDD exposure concentrations precluded our obtaining experimental data on elimination in fish exposed to higher concentrations.

The uptake and depuration of TCDF were measured in fish exposed to 0.41 and 3.93 ng/L. In contrast to TCDD kinetics, TCDF uptake reached an apparent steady-state equilibrium after only 7 d of exposure (Table 10). Whole-body residues of TCDF did not increase after 7 d of exposure in fish exposed to 0.41 and 3.93 ng/L. In fish exposed for 28 d, the measured BCF was 6,049 at 0.41 ng/L and 2,455 at 3.93 ng/L (Table 11). The estimated bioconcentration kinetics of TCDF are shown in Table 12. Rainbow trout apparently were able to readily eliminate or metabolize TCDF. The whole-body residues in fish held during the 28-d depuration phase suggested a very short elimination half-life for this compound. Although TCDD and TCDF are structurally very similar, their bioconcentration kinetics and toxicities were found to be very different.

Table 10. Whole-body residues of 2,3,7,8-tetrachlorodibenzofuran (TCDF) in rainbow trout continuously exposed for 28 d followed by a 28-d depuration phase

Phase and day	Mean TCDF exposure concentration (ng/L)		
	0	0.41	3.93
Exposure			
0	<0.06		
7	0.17	1.63 (0.89)	11.9 (2.88)
14	0.12	1.80 (0.62)	9.30 (2.26)
21	0.19	1.05 (0.44)	10.7 (2.24)
28	0.22	2.48 (1.32)	9.65 (1.30)
Depuration			
28	<0.06	0.09 (0.06)	0.54 (0.08)

Values represent the mean (with standard deviation in parentheses) of four observations performed on individual fish, expressed as ng/g wet weight.

Table 11. Measured bioconcentration factors (BCF)[a] for 2,3,7,8-tetrachlorodibenzofuran (TCDF) in rainbow trout exposed continuously for 28 d

Days of exposure	TCDF exposure concentration (ng/L)	
	0.41	3.93
7	3,976	3,028
14	4,390	2,366
21	2,561	2,730
28	6,049	2,455

[a]BCF = $(C_f/C_w) \times 1{,}000$.

CONCLUSIONS

We conclude that TCDD and TCDF—especially TCDD—are extremely toxic to rainbow trout. A relative comparison of TCDD and TCDF chronic toxicities with those of several other organochlorine compounds demonstrated that TCDD is more than 10,000 times as toxic to fish as either endrin or toxaphene, and that TCDF is about 1,000 times more toxic than either of these insecticides (Table 13). Results from previous toxicity studies with fish by Helder [10,11], Miller et al. [12] and Adams et al. [24] demonstrated the toxicity of TCDD to be in the low ng/L range. However, we have shown that our lowest TCDD exposure concentration of 38 pg/L induced significant adverse effects on survival, growth, and behavioral responses. Results from our studies are perhaps more adequate estimates of TCDD toxicity because we used continuous exposure techniques for a longer time than had been used in previous studies. For similar reasons, we believe the BCF for TCDD derived from our studies is a more accurate estimate of the bioconcentration potential than are the estimates reported by Branson et al. [16] and Adams et al. [24]. Although we showed that TCDD was ex-

Table 12. Estimated bioconcentration kinetics[a] for TCDF in rainbow trout exposed to 2,3,7,8-tetrachlorodibenzofuran (TCDF) for 28 d

Kinetic parameter	TCDF exposure concentration (ng/L)	
	0.41	3.93
K_1 uptake rate constant (d^{-1})	1,228 (1,191)	6,852 (8,037)
K_2 depuration rate constant (d^{-1})	0.28 (0.30)	2.60 (3.04)
BCF-K_B	4,449 (6,481)	2,640 (4,379)
Time to reach 90% steady state (d)	8 (9)	0.90 (1.04)
Elimination half-life, $t_{1/2}$ (d)	3 (3)	0.27 (3.1)

Values in parentheses represent standard deviations.
[a]Estimated kinetics using BIOFAC [22].

Table 13. Chronic no effect concentrations (μg/L) for growth and survival of freshwater fish exposed to various organochlorine chemicals

Chemical and fish species	Days of exposure	Survival	Growth[a]	Source
Aroclor 1254, brook trout	118	9.0	9.0	[28]
Chlorodecone, fathead minnows	120	>0.31	>0.31	[29]
Pentachlorophenol (ultrapure), fathead minnows	90	>139	>139	[30]
Toxaphene, brook trout	90	>0.50	0.38	[31]
Toxaphene, channel catfish	90	0.096	0.20	[32]
Endrin, bluntnose minnows	30	0.1	0.1	[33]
TCDD, rainbow trout	56	<0.000038	<0.000038	This study
TCDF, rainbow trout	56	0.00179	0.00041	This study

[a]Change in weight of fish.

tremely toxic to rainbow trout, even our lowest exposure concentration was too high to derive a NOEC.

Acknowledgement – We are grateful to the many people from the Fish and Wildlife Service (FWS), Battelle and Monsanto who assisted in conducting these studies. We especially thank Mr. Dave Zumwalt for his efforts in designing the specialized diluter exposure system, and Mr. Zumwalt and Mr. George Holroyd for their outstanding efforts in constructing and calibrating the system. We appreciate the excellent advice and assistance of Dr. Mark Ellersieck and Ms. Linda Sappington in analyzing the data, and the technical assistance of Mr. Rick Archeski in analyzing the behavioral data. We give special thanks to Dr. Paul Eschmeyer, Editorial Section, Office of Information Transfer, FWS, Fort Collins, Colorado, for his review of the manuscript and for editorial advice.

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