A BIOCONCENTRATION TEST

STEADY-STATE CONCENTRATIONS OF 2,2',4,4'-TETRACHLOROBIPHENYL IN TROUT

By

D. R. Branson*, G. E. Blau, H. C. Alexander, D. R. Thielen and W. B. Neely

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All correspondence should be sent to The Dow Chemical Company, 628 Building, Midland, Michigan 48640.

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INTRODUCTION

The objective of this investigation is to develop an accelerated test procedure for measuring the potential of a chemical to bioconcentrate in fish. The potential to bioconcentrate (bioconcentration factor) is expressed as the ratio of the concentration in the fish to the concentration of the chemical in the exposure water at steady-state. The need for a formalized accelerated test procedure is partly due to the many unevaluated commercially important chemicals, or chemicals being developed as new products that may enter the aquatic environment. The results of such an accelerated test are intended to help classify chemicals. A direct extrapolation of the bioconcentration factor to the environmental significance of a particular chemical is not the intended interpretation. A single number, bioconcentration factor at steady-state, is desired for each chemical because it simplifies the comparison of several compounds.

A variety of test procedures have been used to evaluate the potential of organic compounds to bioconcentrate in fish. Holden 1962, studied the absorption of DDT in brown trout using static water conditions. Murphy 1971, and 1970, illustrated the importance of fish size and water salinity on direct uptake of DDT using mosquito fish. Macek, et al 1970a and 1970b, Reinert 1972, and Chadwick and Brocksen 1969, compared dietary and direct accumulation of DDT and/or dieldrin in rainbow trout, guppy and sculpin. The concepts of steady-states and clearance rates have been reported by Cakstatter and Weiss 1967, for DDT, dieldrin and lindane with bluegills; Hamelink et al 1971, used DDT with green sunfish and pumpkinseed sunfish; and Hansen et al 1972, used Aroclor 1254 with spot fish. The variety of test procedures must be minimized to a formalized test procedure so the results can be compared.

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The proposed accelerated test is based on the concept that the uptake and clearance phenomena can be described kinetically. Then it is possible to calculate the biological steady-state concentrations from kinetic rate constants. In particular, assume that the uptake and clearance of a chemical in fish from water can be described by the following reversible reaction model:

$$C_W \underset{k_2}{\overset{k_1}{\rightleftharpoons}} C_F \qquad \text{Equation 1}$$

where C_W is the concentration in the water, C_F is the concentration in the fish and k_1 and k_2 are kinetic rate constants for the movement of the chemical into and out of the fish respectively.

While a fish is continually exposed to a concentration of a chemical in water, C_W, the change with time in the concentration of the chemical in fish, C_F, is:

$$\frac{dC_F}{dt} = k_1 C_W - k_2 C_F \qquad \text{Equation 2}$$

Since C_W is a constant, this equation can be readily solved for the concentration in the fish to give the following:

$$C_F = k_1/k_2 \, C_W(1-e^{-k_2 t}) \qquad \text{Equation 3}$$

As the exposure time, t, approaches infinity, the equation for the steady-state condition becomes:

$$\frac{C_F}{C_W} = \frac{k_1}{k_2} = K_b \qquad \text{Equation 4}$$

which defines the bioconcentration factor at steady-state K_b.

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The following biological assumptions are made to describe the uptake and clearance phenomenon kinetically:

1. The uptake of a chemical is assumed to be a matter of movement through the gills, penetration through the skin, and whatever was adsorbed from the gut as a result of consuming food with adsorbed chemicals.

2. By convention, muscle samples are emphasized in this procedure because it is usually the part that is eaten and easily analyzed. Other portions require additional techniques especially for radioanalysis and the results are more variable due to skin adsorption and fat content. The distribution of a chemical into various fish tissues is assumed to be mainly a matter of partitioning between hydrophilic and the hydrophobic natural products (e.g. fats, oils, lipids, proteins, etc.)

3. Clearance is assumed to be a combination of movement back through the gills and skin plus excretion in the urine and feces. The clearance of a metabolite or addition of significant storage compartments could be handled as separate equilibria when necessary by modifying the reversible reaction model, Equation 1. These modifications should follow the principal of "parsimony". That is, keep the model as simple as possible unless the data demands an increase in complexity. (Blau and Branson, 1973)

An indication of the validity of the accelerated test procedure is to predict concentrations in fish from long term exposures and verify these predictions experimentally. The demonstration chemical, 2,2',4,4'-tetrachlorobiphenyl (48.5% chlorine), was selected because of its identify as a confirmed (GC-MS) isomer of polychlorinated biphenyls.

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EXPERIMENTAL METHODS

Laboratory Maintenance of Fish

Rainbow trout (Salmo gairdneri Richardson) were adapted to laboratory food and water for two weeks prior to testing. They were 4-5 inches length and 8-10 gm weight. The trout were fed Purina #2 Trout Chow three times each day (8 a.m., 5 p.m., and 8 p.m.) at a rate to insure vigorous feeding; about 0.2 - 0.4 gm Trout Chow/trout/day. A photoperiod of 16/8 hours: daylight/night was routinely maintained in the laboratory. Lake Huron water was dechlorinated by passage through activated carbon and cooled by refrigeration to 53-55°C. The following list describes the chemical composition of the Lake Huron Water.

1.	pH	8
2.	Total dissolved solids	150 ppm
3.	Chloride	16 ppm
4.	Calcium	27 ppm
5.	Magnesium	7 ppm
6.	Phosphate	30 ppb
7.	Organic nitrogen	nil
8.	Ammonia nitrogen	0.1 ppm

The chemical composition of this water was analyzed before filtration according to USPH drinking water standard criteria (Standard Methods 1971).

Equipment

The bioconcentration test equipment consisted of five aquaria, a constant temperature water bath, and two proportional dilutors. A

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chemical recovery filter was used when radioactive chemicals were evaluated. Each dilutor, as described by Mount and Brungs, 1967, was constructed for delivery of two chemical concentrations, each an order of magnitude apart. During each cycle, about 1.1 ml acetone solution of the chemical was introduced into the chemical mixing cell of the dilutor.

Each 5 minute cycle delivered one liter of water to each of three aquaria. Since 1.0 and 10.0 micrograms chemical/liter water were usually sublethal and above the analytical detection limits, an attempt was made to set these concentrations during each exposure. The actual concentrations were measured daily. Two liters of acetone containing 20 mg chemical in a Mariotte bottle would last for a 96 hour exposure.

Experimental

Two exposure durations were evaluated. Bioconcentration values calculated from 5 day exposures were compared to actual values from a 42 day exposure.

Forty fish in each of three 12 liter aquaria were acclimated to flow through conditions for two days before chemical exposure. The dissolved oxygen was greater than 5 ppm and was controlled by the dilutor cycle time. The fish were fed during the experiment.

Each chemical exposure was initiated (time t = 0 hr.) by transferring the acclimated fish to three other aquaria containing approximately 0, 1.0 and 10 ppb 2,2',4,4'-tetrachlorobiphenyl (purity >99 percent, purchased from Analabs, North Haven, Conn.). The actual concentration of chemical in the exposure water was determined by gas chromatographic analyses daily during the 120 hour exposure experiments and every other day for the 42 day exposure experiments. Duplicate

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500 ml water samples were taken from each aquaria and extracted immediately in glass stoppered volumetric flasks.

Four fish were removed from each aquaria at 6, 12, 24, 48 and 120 hour intervals during 5 day uptake exposures. The remaining fish were placed in clean aquaria with water from a similar dilutor only without chemical. These fish, in the clearance phase of the experiment, were removed in sets of four at 2, 4, 8, 16, and 32 day intervals.

Seven fish from the long term exposure experiments were removed at each of 14, 28 and 42 day intervals.

The fish were skinned and the muscle removed with clean surgical tools. The weighted muscle samples from each fish were frozen in glass vials with caps lined with aluminum foil. The skin and remaining other portions of each fish were combined and frozen. The concentration of chemical in the whole fish was calculated from the amount of chemical in the muscle plus other portions, and divided by the combined weight.

Chemical Analysis

Extracts of the fish and water samples were analyzed with aid of a Hewlett Packard gas chromatograph Model 5750, that was equipped with a 72 x 0.25 inch column (3 percent Dexsil 300 packing) and an electron capture detector. The gas chromatographic detector response that corresponds to 2,2',4,4'-tetrachlorobiphenyl was confirmed by GC mass spectrometry.

Each 500 ml water sample was extracted by shaking with 5 ml hexane for about 16 hours. Each fish tissue sample was digested with 10 ml 20% KOH for one hour at 50°C, or until the sample was liquidified. It was cooled, and then extracted with 10 ml diethyl ether. The

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extraction efficiency of 2,2',4,4'-tetrachlorobiphenyl from water was greater than 90 percent, the reproducibility ± 5 percent and the lower limit of detection was 10 ppb for flesh and 0.1 ppb for water for the procedures described. The extraction efficiency of 2,2',4,4'-tetrachlorobiphenyl from flesh was presumed to be 95-100% based on ^{14}C-hexachlorobenzene analysis of fish (Branson et al 1973).

Rate Constant Values: Parameter Estimations

Several techniques have recently been developed for estimating parameters in kinetic models from experimental concentration-time data. These methods require the use of high speed digital computers and non-linear statistical theory. Bard et al (1968), Blau et al 1972.

Here the approach used was to find the values of k_1 and k_2 for which the differential equation, Equation 2, best described the data collected. Computer programs to perform this calculation are available on the IBM 370-155 computer at Dow's Computations Research Laboratory. The bioconcentration factor at steady-state K_b is obtained directly from Equation 4.

Since the rate constants are estimated from experimental data, a measure of the "variability" of these estimates is necessary. The variability of a single parameter is called its variance while the variability between different parameters is called covariance. These variabilities are calculated by a technique described by Draper and Smith (1973). The square root of this variance is the familiar standard deviation.

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RESULTS AND DISCUSSION

Acute Exposures - Uptake and Clearance

Trout were first exposed to 2,2',4,4'-tetrachlorobiphenyl in water for 120 hours and then placed in fresh water. The concentration of chemical in fish muscle which describes the uptake and clearance for each experiment are summarized in Figure 1. Values of k_1 and k_2 were calculated from the data and used to predict the concentrations at various time periods. These predicted values agreed quite well with experimental measurements lending credence to the postulated reversible reactions model, Equation 1.

The bioconcentration factor at steady-state, K_b was calculated to be 12,400 ± 2,290 from the rate constants for the combined data (Table I). The K_b calculated from the rate constants of the individual exposure were 11,900 ± 3,200 and 13,400 ± 3,400. The disagreement between these results may have been an effect of the acetone solvent on the partitioning between water and fish during uptake. The 26-30 day half-life of clearance for each exposure was indistinguishable at the 0.01 level according to a t-test for parallelism of two regression lines (Draper and Smith 1966).

TABLE I

Acute Exposure (C_w) ppb	Uptake Rate Constant (k_1)	Clearance Rate Constant (k_2)	Bioconcentration Factor at Steady-State $\frac{k_1}{k_2} = K_b$
1.6 ± 0.2[A]	10.3 ± 0.7 hr^{-1}	0.00087 ± 0.0002 hr^{-1}	11,900 ± 3,200
9.0 ± 2.0	12.7 ± 0.9 hr^{-1}	0.00095 ± 0.0002 hr^{-1}	13,400 ± 3,400
Combined	12.2 ± 0.65 hr^{-1}	0.00099 ± 0.0002 hr^{-1}	12,400 ± 2,290

[A]The standard deviation of the time weighed average concentration of chemical in water was determined by pooling the variance of each set of duplicate analysis. Actually, the water concentration gradually increased during the exposure as the fish approached steady-state. In the future C_w will be considered a variable during the exposure.

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Chronic Exposures - Approaching Apparent Steady-State

To evaluate the feasibility of predicting the apparent steady-state concentration of a chemical in fish muscle from a given exposure in water, trout were exposed to 1.5 and 14 ppb of 2,2',4,4'-tetrachlorobiphenyl for 14, 28 and 42 days. The predicted concentrations were calculated from Equation 3 using the rate constants from the combined exposure data. The agreement between the predicted and actual concentrations is illustrated in Figure 1. This agreement demonstrates that the bioconcentration of 2,2',4,4'-tetrachloro biphenyl in chronically exposed trout can be predicted from data generated from an acute exposure. This same agreement between observed and predicted was further collaborated with another compound, diphenyl oxide (Branson et al, 1973a).

The concentration of 2,2',4,4'-tetrachlorobiphenyl in trout muscle was 43-64 percent of steady-state after 42 days exposure. Using Equation 3, 90 percent of the steady-state concentration would be reached after 97 days exposure.

The average concentration of 2,2',4,4'-tetrachlorobiphenyl in the muscle of seven trout exposed to 14 ppb for 42 days was 82 $\pm$ 20 ppm. The large variation associated with this average is probably due to the noticeable weight depletion of some fish. Although 82 $\pm$ 20 ppm 2,2',4,4'-tetrachlorobiphenyl in trout muscle was sublethal and below a level of histopathological damage, the fish exhibited a slower rate of feeding than the fish exposed at 1.5 ppb or the controls. The livers of the exposed fish further indicated this depletion phenomenon. The average weights per liver were 157 mg, 153 mg and 113 mg after 42 days exposure at 0, 1.5 and 14 ppb. The concentrations of 2,2',4,4'-tetrachlorobiphenyl in the livers were higher than the muscle following 42 days exposure; 21 ppm in the liver versus 9.6 ppm in the muscle from 1.5 ppb and 103 ppm in the

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liver versus 82 ppm in muscle from 14 ppb in the water. Metabolites of 2,2',4,4'-tetrachlorobiphenyl were not detected in concentrated diethyl ether extracts of fish containing 100 ppm 2,2',4,4'-tetrachlorobiphenyl. For this reason, the uptake, storage and clearance are considered to be the parent compound.

Discussion of the Test Method

Important aspects of this test method are as follows:

1. The duration of continual exposure should be 2 to 5 days. More water insoluble chemicals require longer exposure times to observe even a minimal decrease in the rate of uptake.

2. At least two exposure concentrations should be evaluated.

   a. Each exposure concentration should be below levels that are acutely toxic. Sick fish have been shown not to bioconcentrate as much DDT as healthy fish (Hamelink et al, 1971).

   b. The concentrations should be above the limit of analytical detection for both water and fish flesh and should be about one order of magnitude from the other. This may not be possible because of toxicity or a limit on the analysis.

   c. Each aquaria should be analyzed daily in duplicate.

3. At least five sample intervals should be selected during uptake and five during clearance phase. The timing of each sample interval should increase logrithmatically from the beginning of each phase.

4. Sufficient numbers of fish should be sampled at each interval to permit meaningful calculations of the standard deviation for each interval.

5. Rate constants for uptake and clearance should be estimated and statistical tests made to access the suitability of the

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model. If necessary, the model can be modified to accommodate other biological phenomenon (Blau and Branson 1973).

6. The bioconcentration factor at steady-state and the time to reach 90 percent of the apparent steady-state should be calculated.

7. The significance of the bioconcentration factor to the environmental characteristics of a chemical should be by comparison with the bioconcentration factor of other chemicals, "bench marks". When possible, a bioconcentration factor plus fish residues of significance should be linked. For example, the temporary fish tolerance on PCBs of 5 ppm (Fine, 1972) linked with a bioconcentration factor of 12,400 for 2,2',4,4'-tetrachlorobiphenyl, suggests that 0.6 ppb in water may be hazardous. For a commercial PCB, the bioconcentration factor of 70,000 (kinetically calculated from uptake and clearance data, Hansen, et al, 1971) suggests 0.07 ppb in water may be hazardous.

   Further generalizations about the relationship between a bioconcentration factor and hazardous concentrations in water is premature at this time. Perhaps in time the definition of "hazardous in water" will include those concentrations of chemicals in fish that may be toxic to fish, may taint fish flavor and may not be safely consumed by man, animals or birds.

8. Several interesting aspects of this proposed test are currently being investigated to add depth to the interpretation of results. There appears to be a correlation between partition coefficient and steady-state bioconcentration factors. This observation is based, so far, on six organic chemicals that range from 10^2 to 10^5 partitioning between n-octanol and water. The clearance of some of these chemicals is controlled by more than one limiting

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processes, which suggests the development of more complicated mathematical models. The range of exposures over which a single bioconcentration factor is valid may be limited by solubility of the chemical in storage tissues or a toxic effect. Variations of this test procedure, like the size of fish, species, age, sex, and fat content are being investigated. Other variations, like the effect of temperature, the effect of water quality and the effect of chemical mixtures on bioconcentration are also interesting aspects and should be investigated in time. However, it is restressed, that to compare results between chemicals, the variables must be minimized whenever possible.

CONCLUSIONS

1. It is feasible to estimate the rate constants of uptake and clearance from an accelerated test procedure. This will save the expense of chronic exposures for routine evaluations.

2. The concentration of 2,2',4,4'-tetrachlorobiphenyl in trout muscle after 14, 28 and 42 days exposure to 14 and 1.5 ppb in water was predicted within experimental error. Therefore, the kinetic approach to predicting steady-state is valid for determining the potential of 2,2',4,4'-tetrachlorobiphenyl to bioconcentrate in trout under the defined conditions.

3. For 2,2',4,4'-tetrachlorobiphenyl the optimized rate constants were: uptake (k_1) = 12.2 $\pm$ 0.65 $hr.^{-1}$, clearance (k_2) = 0.00099 $\pm$ 0.0002 $hr.^{-1}$, and the bioconcentration factor at steady-state, K_b = 12,400 $\pm$ 2,290.

4. The important aspects of the test are: Continual exposure for a few days at two carefully selected water concentrations, five uptake and five clearance sample intervals, estimations of the uptake and clearance rate constants, selection of a mathematical model which fits the biological data and calculation of the steady-state bioconcentration factor.

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FIGURE 1

BIOCONCENTRATION OF A PCB ISOMER IN TROUT

The results of four experiments of trout exposed to 2,2',4,4'-tetrachlorobiphenyl were illustrated. Each data point from trout exposed to 1.7 ± 0.2 and 9.0 ± 2.0 ppb for five days and then freshwater represents the mean concentration in four fish muscles. Each data point from trout exposed to 1.5 ± 0.2 and 14 ± 2 ppb for 14, 28 and 42 days were the mean concentration in seven fish muscles. The steady-state concentration in fish muscle from 14 ppb exposure would be 170 ± 33 ppm, and from 1.5 ppb exposure, 19 ± 4.0 ppm, which corresponds to a bioconcentration factor at steady-state of 12,400 ± 2,290.

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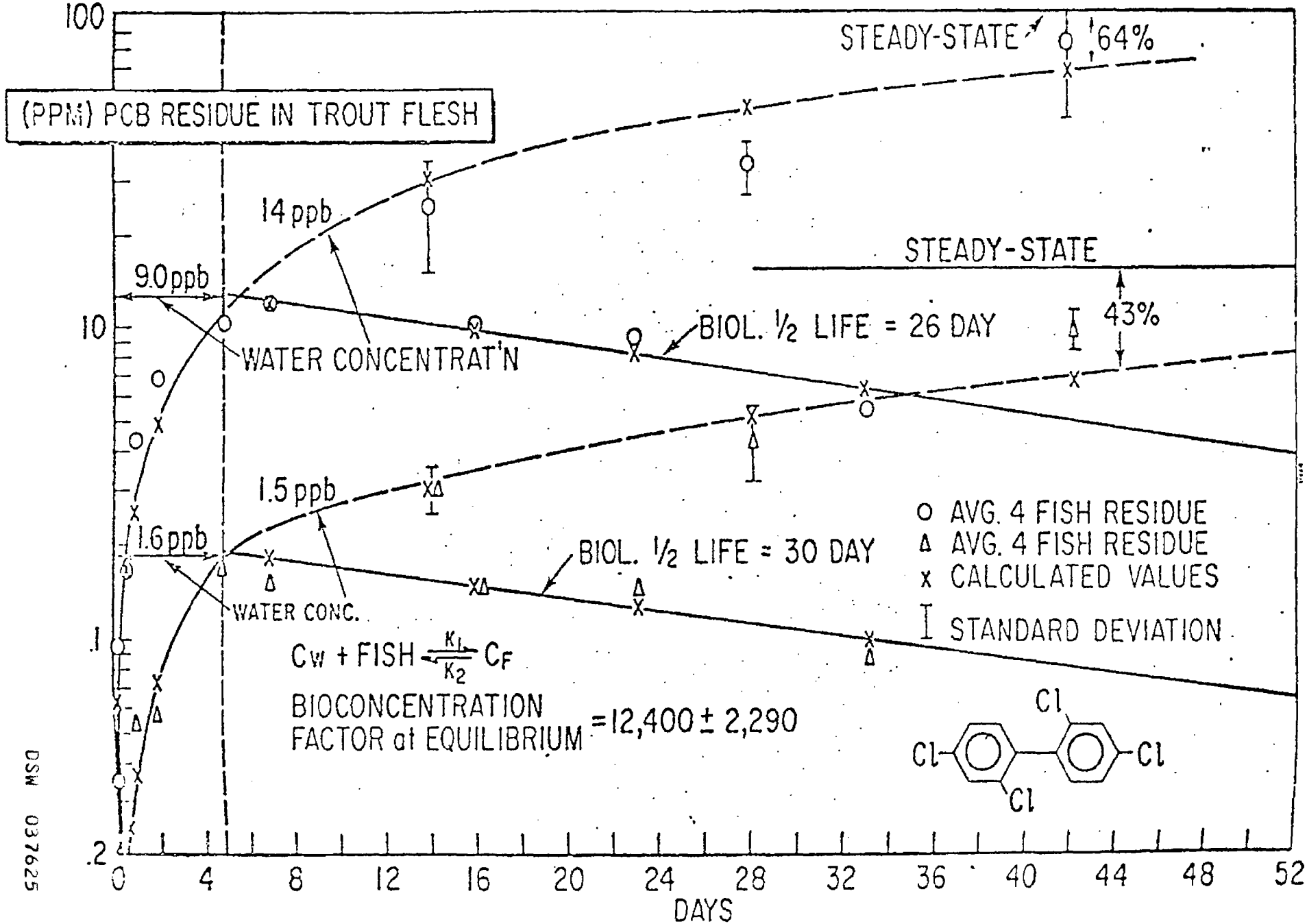
FIGURE 1

BIOCONCENTRATION OF A PCB ISOMER IN TROUT



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