# UPTAKE OF $^{36}Cl$ TOXAPHENE IN MOSQUITO FISH, *GAMBUSIA AFFINIS* (BAIRD AND GIRARD)

by

Robert Allen Schaper

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SIGNED: Robert A. Schaper

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

Larry A. Crowder

LARRY A. CROWDER
Assistant Professor of Entomology

Jan. 13, 1977
Date

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ABSTRACT

A study was undertaken to quantify the uptake of $^{36}Cl$-Toxaphene (polychlorocamphene, $C_{10}H_{10}Cl_8$) by the mosquito fish, Gambusia affinis (Baird and Girard). The $LC_{50}$ for fish selected for this study was 860 ppb for 20 hours of exposure. Experiments carried out at toxaphene concentrations of 2000 ppb demonstrated 12 hours to be the $LT_{50}$ of the population.

Using radioassay, uptake of $^{36}Cl$-Toxaphene and toxaphene related residues by G. affinis was demonstrated to be linear with respect to time. Regression analysis resulted in a statistically significant uptake curve.

A five stage series was identified in the toxicity syndrome, and fish were analyzed at each stage. Death occurred when residue levels reached 0.62 micrograms of $^{36}Cl$-toxaphene. By the time the second stage of poisoning was reached, fish had sorbed 90.3% of the mean fatal residue level.

# INTRODUCTION

The mode of action of cyclodiene insecticides is at present poorly understood. The high susceptibility of fish to certain cyclodienes and the discovery of resistant strains has encouraged research in this area. Unfortunately, most work has been in the direction of assaying different populations of fish for different susceptibilities to different pesticides. The fact of high susceptibility and the fact that aquatic organisms are difficult to dose directly and individually has hindered quantitative research. Although there is a great deal of information on $LD_{50}$s and residues, there has been little work done to quantify actual uptake of toxicant from the aquatic medium. Ferguson, Ludke, and Murphy (1966) studied uptake and excretion of endrin by using bioassay techniques. These techniques demonstrated the existence of processes of uptake and excretion, but did not quantify them in such a way as to help elucidate the mode of action. Since the dosage is the most critical factor in toxicological studies, quantification of the amount of toxicant actually absorbed is a prerequisite for meaningful in vitro investigations into the mode of action of these insecticides. The present research proposes to quantify the uptake and excretion of a cyclodiene related insecticide, toxaphene, by mosquito

fish, Gambusia affinis (Baird and Girard). These data may provide the information necessary to interpret in vitro investigations and provide additional insights into the mechanism of insecticide resistance.

## REVIEW OF LITERATURE

Early toxicity studies for representative fish demonstrated lethal concentrations (LC) of toxaphene in the water medium to be 0.032 to 10.0 ppm. The higher concentration is an estimate of 1-10 ppm (Negherbon, 1959). The discovery of Gambusia affinis (Baird and Girard), mosquito fish, that were resistant to DDT by Vinson, Boyd, and Ferguson (1963) revealed that populations of fish living in waters heavily contaminated with insecticides had developed resistance. Work began immediately to demonstrate resistance in several species of fish to different organochlorine pesticides (Culley and Ferguson, 1969; Ferguson et al., 1965; Boyde and Ferguson, 1964). Ferguson et al. (1964) demonstrated very high resistance in three species of fish to DDT, toxaphene, aldrin, dieldrin, and endrin. For toxaphene the normal median tolerated limit for 36 hours of exposure ($LM_{36}$) was 30 ppb, 23 ppb, and 38 ppb for golden shiners, blue gills, and green sunfish respectively. $LM_{36}$'s for resistant populations of these same species were 1200 ppb, 1600 ppb, and 1500 ppb. In this paper, as in most others dealing with resistant populations, resistant fish were obtained from irrigation canals and streams containing high insecticide residues due to agricultural runoff. Most

of this work has been done in the lower Mississippi River basin where insecticides have been heavily used in cotton producing areas.

Since very little is known about the mode of action of toxaphene and other cyclodienes, the discovery of resistant populations provided an excellent tool for exploration into the effects of insecticides on aquatic vertebrates.

Uptake, penetration, and storage of insecticides has been studied most extensively in mosquito fish, G. affinis. Ferguson et al. (1966) studied the dynamics of endrin uptake and release in mosquito fish. In this series of well-planned experiments, they demonstrated that the major source of endrin uptake was contaminated water rather than accumulation through the food chain. Absorption of endrin into the fish was mainly through penetration in the head region, presumably the gills. Uptake was faster in live rather than dead fish, indicating that active processes were involved. Rates of absorption for susceptible and resistant fish were about the same.

Respiration of resistant and susceptible fish was also equal until the onset of toxic symptoms of endrin poisoning. A rapid increase in oxygen consumption accompanied erratic darting behavior of the toxic symptoms. This increase in oxygen consumption appeared to be related

to the behavior of toxic symptoms rather than the simple effect of endrin on the system.

Endrin was also released by contaminated fish into the water which indicates some type of excretion mechanism. The rate of excretion was, however, similar in resistant and susceptible fish. This excretion mechanism was efficient enough that resistant fish which contain high concentrations of endrin could release enough toxic material into pure water to kill susceptible fish. The chemical identity of the excreted toxin was not determined but was presumed to be endrin. The ability of G. affinis to excrete endrin correlated well with the fact that field collected fish from a given population showed large differences in body residues when collected at different times of the year. These results indicated that tolerance to endrin in G. affinis is dependent not on differences in uptake or excretion but on the ability of resistant fish to tolerate high body levels of endrin.

Fabacher and Chambers (1971) correlated resistance to higher lipid content in resistant fish. It has been shown that livers of resistant fish contain more lipids and are larger than livers in susceptible fish. This would also help explain seasonal variations of $LD_{50}$ since varying availabilities of food could determine lipid content. However, the reported 1.7 fold increase in total lipids in resistant fish over susceptible fish does not adequately

account for 40 to 50 fold differences in $LD_{50}$'s. Boyde and Ferguson (1964) suggested the terminology of "resistance" for populations having $LD_{50}$'s at least ten times that of susceptible strains. Any value less than this would be referred to as "tolerance." These same workers demonstrated the genetic character of DDT resistance in G. affinis by rearing several generations of resistant fish in pesticide free ponds. After 3-4 generations there was no change in $LD_{50}$ from the original stock.

Wells and Yarbrough (1972a, 1972b) and Yarbrough and Wells (1971) studied retention of DDT, aldrin, dieldrin, and endrin in resistant and susceptible G. affinis. Using radioactive tagging, they demonstrated that cell membranes of resistant fish bind more insecticide than membranes of susceptible fish. In addition, brains of resistant fish contain a lower proportion of endrin in relation to liver than do susceptible fish. These results suggested that resistance is in part the result of a membrane barrier in resistant fish. There also appeared to be a blood/brain barrier in resistant fish, possibly due to differences in the form or structure of myelin.

Wells, Ludke, and Yarbrough (1973) reported that G. affinis is capable of metabolizing aldrin to form tieldrin or other water soluble metabolites and that this process occurs more rapidly in resistant fish. Thus in the case of aldrin, resistance may be due to differences in the

rates of detoxication. Ludke, Gibson, and Lusk (1972) demonstrated the existence of mixed function oxidase which catalyzes this epoxidation as well as the activation of parathion.

Prather and Ferguson (1966) demonstrated the conversion of DDT to DDD and DDE in G. affinis and indicated that there may be a difference in rate between susceptible and resistant populations.

Relatively little work has been done on the effect of insecticides at the level of cellular respiration in fish. Yarbrough and Wells (1971) demonstrated that endrin inhibits the activity of succinic dehydrogenase in G. affinis. In resistant G. affinis, succinic dehydrogenase activity was inhibited only after disrupting the mitochondria membrane. With an intact mitochondrial membrane, endrin had no effect, or slightly stimulated enzymatic activity. Moffet (1971) and Moffet and Yarbrough (1972) repeated this work with DDT, toxaphene, and dieldrin. DDT and dieldrin experiments paralleled the endrin study suggesting the resistance in G. affinis is due to a mitochondrial membrane barrier excluding insecticides from the mitochondria and thereby protecting cellular respiration. The effect of these insecticides on fish may be an overall inhibition of cellular respiration rather than a purely central nervous system phenomenon. The results with

toxaphene, however, were unclear since toxaphene inhibited succinic dehydrogenase activity in intact resistant mitochondria.

Despite the increased usage of toxaphene in the last few years, little work has been done on the uptake, storage, and excretion of toxaphene. The work of Ferguson et al. (1966) represents one of the best studies of the dynamics of cyclodiene uptake and excretion.

## METHODS AND MATERIALS

### Selection of a G. affinis Population

Selection of a population of G. affinis to employ in this investigation depended on several qualifications. First, since G. affinis is normally very susceptible to toxaphene, it was important to select fish that could withstand high body burdens of toxaphene in order that the dynamics of absorption and excretion could be investigated. Secondly, the population from which the fish were collected had to be stable. That is, they should have relatively homogenous genetic make-up with regard to insecticide tolerance and should not fluctuate in size due to seasonal changes.

G. affinis were collected from a total of five locations in Pima and Pinal County, Arizona. Four were eliminated due to their high susceptibility to toxaphene or the seasonable instability of their environment. Fish for this research were obtained from a sewage oxidation pond located approximately 100 yards east of Oracle Road, 0.6 miles north of River Road, Tucson, Pima County, Arizona. The fish used for experiments were collected by seining. Fish were transported and stored in 115 l poly-trash cans and maintained in the laboratory in aged tap water for 24 hours prior to testing. Fish used in experiments were

selected by randomly dip-netting fish from the stock container. The sex of individual fish was not recorded. A total of 492 fish were used experimentally excluding those used for preliminary trials and standards. The mean weight was 1.082 g with a standard deviation of 0.627 g.

A total of 100 fish was used to determine the $LC_{50}$ for the oxidation pond fish. Since future experiments were to be conducted over short time periods, the purpose of this testing was to determine appropriate experimental concentrations rather than to define the lethal concentration of a particular population of fish. Thus the $LC_{50}$ (Fig. A.1, Appendix A) represents a 20 hour time period. The $LC_{50}$ as determined by a best fit line is approximately 860 ppb. Extrapolating from this curve, 2000 ppb was selected as the appropriate experimental concentration for testing fish. Figure A.2 (Appendix A) illustrates the $LT_{50}$ for oxidation pond G. affinis at 2000 ppb of toxaphene to be 12 hours. Since both uptake and excretion were to be studied, eight hours was selected as the optimum period for sorption of this concentration level. Figure A.3 (Appendix A) illustrates that at periods longer than eight hours mortality increases rapidly making excretion results impossible to interpret in moribund fish.

## Preparation of Solutions

Toxaphene (polychlorocamphene, $C_{10}H_{10}Cl_8$) prepared with radioactive chlorine ($^{36}Cl$) was used so that absorption and excretion could be assayed indirectly by the absorption and excretion of radioactive materials. Generally labelled $^{36}Cl$-Toxaphene was obtained from Hercules Corporation (batch # XI 6189-49, 1/25/71) and had a specific activity of 0.042 mCi/mg.

Toxaphene solutions used for preliminary experiments were prepared by the appropriate dilution of a 1% acetone stock solution of $^{36}Cl$-Toxaphene in a method described by Boyde and Ferguson (1964). An aqueous stock solution of 10 parts per million (ppm) was prepared from the 1% solution. This was used for preliminary tests of $LC_{50}$s to screen populations of G. affinis. It was found to be impractical because its toxicity declined rapidly perhaps due to toxaphene adhering to the glass and thereby reducing the effective concentration.

All other experimental solutions were prepared by appropriate dilution of a $1 \times 10^{-2}$ M solution of $^{36}Cl$-Toxaphene in redistilled acetone. This solution contained 4.09 mg $^{36}Cl$-Toxaphene per ml. Thus 1.22 cc added to 5 l of water resulted in an approximate 1000 parts per billion (ppb) test solution. Test solutions of other concentrations were similarly prepared.

Experimental solutions were prepared using tap water which was aged for at least 24 hours at room temperature prior to use. A solvent control test was conducted at the rate of 2 ml redistilled acetone per liter of water and resulted in 0% mortality after 24 hours. This concentration of solvent was roughly five times as high as the highest experimental solution.

All experiments were conducted at room temperature in glass or plastic jars which were lined with disposable polyethylene liners. These liners permitted the reuse of jars without the possibility of glassware contamination due to toxaphene adhering to test chambers.

## Extraction Techniques

Analysis of fish for absorbed toxaphene and toxaphene related (TR) residues involved extraction of all radioactive material from the fish and quantifying this material using liquid scintillation techniques. Several techniques were examined before selecting one of them for this investigation. Since large numbers of fish were to be analyzed, the technique had to be as simple and efficient as possible while extracting as high a percentage of material as possible.

Attempts at homogenizing fish in a 1:1, hexane: acetone solution were abandoned due to the formation of a very stable colloid in combination with the fish oils. A

second technique involved homogenizing fish in acetone and filtering the extract into a separatory funnel. The toxaphene and TR residues were then partitioned with 30 ml of hexane by addition of water and hexane to the funnel. This hexane fraction was finally evaporated to smaller volumes and placed in scintillation vials. This technique was considered too complex for large numbers of fish and also eliminated any possible water soluble metabolites.

Attempts at solubilizing entire fish using NCS® tissue solublizer were frustrated by the formation of a very dark product. Acidification and bleaching with 30% hydrogen peroxide were ineffective in clearing the extracts.

The extraction technique finally selected for this study was to, first, simply grind the fish in 40 ml of redistilled acetone in a ground glass tissue grinder. The acetone theoretically removed not only the non-polar toxaphene from the tissue but also removed most of the free water and presumably water soluble metabolites. After grinding, the extract was filtered through filter paper and evaporated to approximately 10 ml before being placed in scintillation vials. The samples were then standardized for solvents by evaporating over heat to dryness and then redissolving them in 0.25 ml of distilled water and 0.25 ml of hexane. The sample was shaken vigorously. Scintillation fluor was added to the samples. To quantify the recovery rate of the extraction technique, fish were topically dosed

with 0.1 ml of $^{36}$Cl-Toxaphene solution just prior to extraction. Extraction of ten fish resulted in an average recovery rate of 96.01 ± 5.28%. A recovery of 96% was used in the calculation of all experimental data.

## Radioassay

Data from the samples were collected by analysis in low sodium glass scintillation vials. The fluor used in preliminary experiments was toluene based containing 0.06 g POPOP (p-bis- 2-(5-Phenyloxazolyl) -benzene) and 5.0 g PPO (2, 5-Diphenyloxazole) (Research Products International Corp.) in 1 liter of reagent grade toluene. This fluor was added to samples at the rate of 10 ml per sample and the samples were dark adapted prior to counting at room temperature. Bray's solution (Liquid Scintillation Counting, 1966) a dioxane based fluor, was employed in all other experiments. Bray's solution contains 60 g Naphthalene, 10 ml methanol, 20 ml ethylene glycol, 4 g PPO, 0.2 g POPOP, and 1 liter reagent grade dioxane. This fluor was stored in darkness until use and due to the instability of dioxane, added to samples immediately before counting.

Samples were counted on a Nuclear Chicago Liquid Scintillation Counter, model #6833. A standard of $^{36}$Cl-Toxaphene was used to run a Voltage Attenuation profile (Figure A.4, Appendix A). A series of samples was prepared to establish quench curves by extracting freshly collected

fish. Nitro-methane was used to provide artificial quenching. The series was counted and the results provided a curve necessary for determining quench of experimental samples (Figure A.5, Appendix A).

Data from each sample were key-punched for analysis on the University of Arizona's CDC-6400 computer. The program calculated the number of mg of toxaphene represented by the scintillation counts per sample and also per gram of fish. This value is misleading as it represents not only toxaphene present but also any metabolites of toxaphene which contain radioactive chlorine. Because of the long half-life of $^{36}Cl$, no calculation was made to counteract error due to nuclear disintegration.

## Toxic Symptom Experiments

On the basis of preliminary tests, it was found that fish exposed to lethal concentrations of toxaphene exhibited a particular chain of toxic symptoms. This chain was divided into 5 characteristic stages as follows:

1. Fish apparently healthy.
2. Fish swimming at surface, often swimming perpendicularly into the side of the aquarium.
3. Fish losing equilibrium; no longer swimming in horizontal attitude. Tail end down, swimming against sides of container; sometimes rolling over as they swim.

4. Fish prostrate on bottom of aquarium; gills ventilating rapidly; occasional darting behavior.
5. Death as indicated by the cessation of gill movements.

To correlate the amount of toxaphene residues to these symptoms, the following was performed. Approximately 60 G. affinis were placed in a 5 l aquarium containing 2 ppm of $^{36}$Cl-Toxaphene. Samples were taken during the next 10 hours so that there were 10 fish sampled at each of the 5 toxic stages. The samples were rinsed in tap water and frozen for subsequent analysis.

## Uptake Experiments

Two 5 l aquaria were set up with aged tap water 24 hours prior to experimentation. Just before adding fish, the appropriate amount of acetone based $^{36}$Cl-Toxaphene solution was added and stirred into the water to produce suspensions containing 2 ppm of toxaphene. Approximately 50 randomly selected G. affinis were introduced to each aquarium at 0800 and sampled hourly for 8 hours. Each sample consisted of 10 fish which were rinsed with fresh tap water and frozen individually in glass vials for subsequent extraction. At the time of sampling, the toxic stage of each fish was recorded. This experiment was duplicated.

## Excretion Experiment

Two 5 l aquaria were set up as in the uptake experiments and fish introduced at 0800. After 8 hours of exposure to 2 ppm of $^{36}$Cl-Toxaphene, all fish were transferred to 5 l aquaria containing untreated aged tap water. At the time of this transfer, all dead fish were removed, rinsed in tap water, and frozen. Also at the time of transfer, a sample of 10 fish was collected, rinsed, and frozen. Samples were taken every hour until all fish were removed. At the time of sampling, the toxic symptoms of each fish were recorded. This experiment was duplicated.

## Controls

During these investigations, a total of 50 fish was used for controls. Fish were placed in 2 aquaria containing 0 ppm toxaphene and sampled hourly for 5 hours. The samples were analyzed via liquid scintillation.

## Partitioning of Metabolites

A total of 34 fish killed by an eight hour exposure to 2000 ppb $^{36}$Cl-Toxaphene was used to characterize the composition of radioactive fish extract. Fish were ground together in 500 ml of acetone. Five 10 ml samples of this extract were prepared for radioassay. Two hundred ml of extract were evaporated to dryness and then partitioned in 100 ml distilled water and 100 ml of hexane added to the nonpolar fraction. Both fractions were concentrated by

evaporation to 100 ml and transferred to scintillation vials in 10 ml aliquots. They were evaporated to dryness, redissolved in 0.25 ml distilled water and 0.25 ml hexane, and prepared for radioassay.

## RESULTS

The cpm of each sample were computed to calculate the equivalent mg of toxaphene per sample and per gram of fish. Fish from the control experiment resulted in a mean of 58.8 cpm. Three blank vials were counted with each experiment. These were averaged to provide a blank value which was deducted from each experimental count. The average cpm for 8 blanks was 57.2. This value falls within the standard deviation of the controls and was therefore considered identical with them and by definition 0.

Total recovery was calculated on the basis of the uptake experiments. In each case, 9.67 mg of $^{36}$Cl-Toxaphene was added to the test solutions. For the 2 uptake experiments, the average total recovery was 0.046 mg, or 0.47%. Presumably the remainder was left in the water or adsorbed on the test containers.

Figure 1 represents the results of the uptake experiments with the mean concentration of $^{36}$Cl-Toxaphene and TR residues for each trial plotted over time. Regression analysis of each trial resulted in linear uptake equations (Table 1). These were significant at the .01 level.

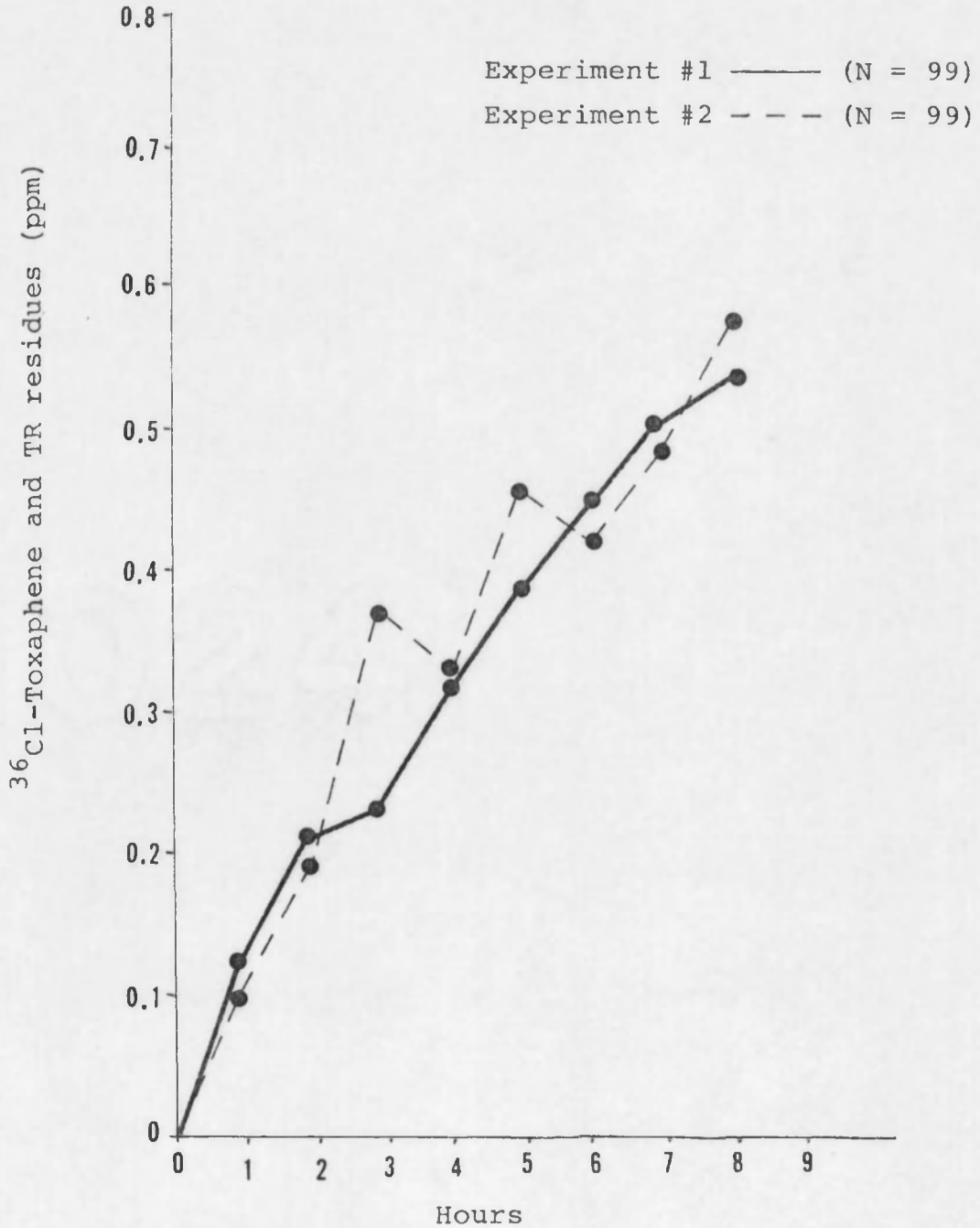


Fig. 1. The mean uptake of $^{36}$Cl-Toxaphene for G. affinis plotted as a function of exposure to 2000 ppb $^{36}$Cl-Toxaphene.

Table 1. Results of regression analysis for the uptake and excretion of $^{36}$Cl-Toxaphene by G. affinis exposed to 2000 ppb of $^{36}$Cl-Toxaphene for 8 hours.

| Experimental Group | Regression Equation | r |
|---|---|---|
| Uptake A (Exp. 1) | Y = 0.00068 + 0.00058 (X) | 0.76266* |
| Uptake B (Exp. 2) | Y = 0.00071 + 0.00052 (X) | 0.86208* |
| Excretion A (Exp. 1) | Y = 0.00601 + 0.00004 (X) | 0.04808 |
| Excretion B (Exp. 2) | Y = 0.00471 + 0.00002 (X) | 0.04089 |

*Significant at the .01 level, student t test.

On the basis of these results, several observations can be made concerning the dynamics of toxaphene uptake by G. affinis. Uptake is a linear function and is directly proportional to the length of exposure. Figure 2 plots uptake of two different weight groups of fish, less than 800 mg and more than 1200 mg. The graph shows that at every point, small fish contain more $^{36}$Cl-Toxaphene per gram tissue and TR than large fish. Mean body load of fish weighing between 800 and 1200 mg in every case fell between the plots shown in Fig. 2. Comparison between paired points reveals that fish weighing less than 800 mg acquired between 10-42% more residue than fish weighing more than 1200 mg.

Mean concentration of $^{36}$Cl-Toxaphene and TR residue for each trial of the excretion experiment was plotted over

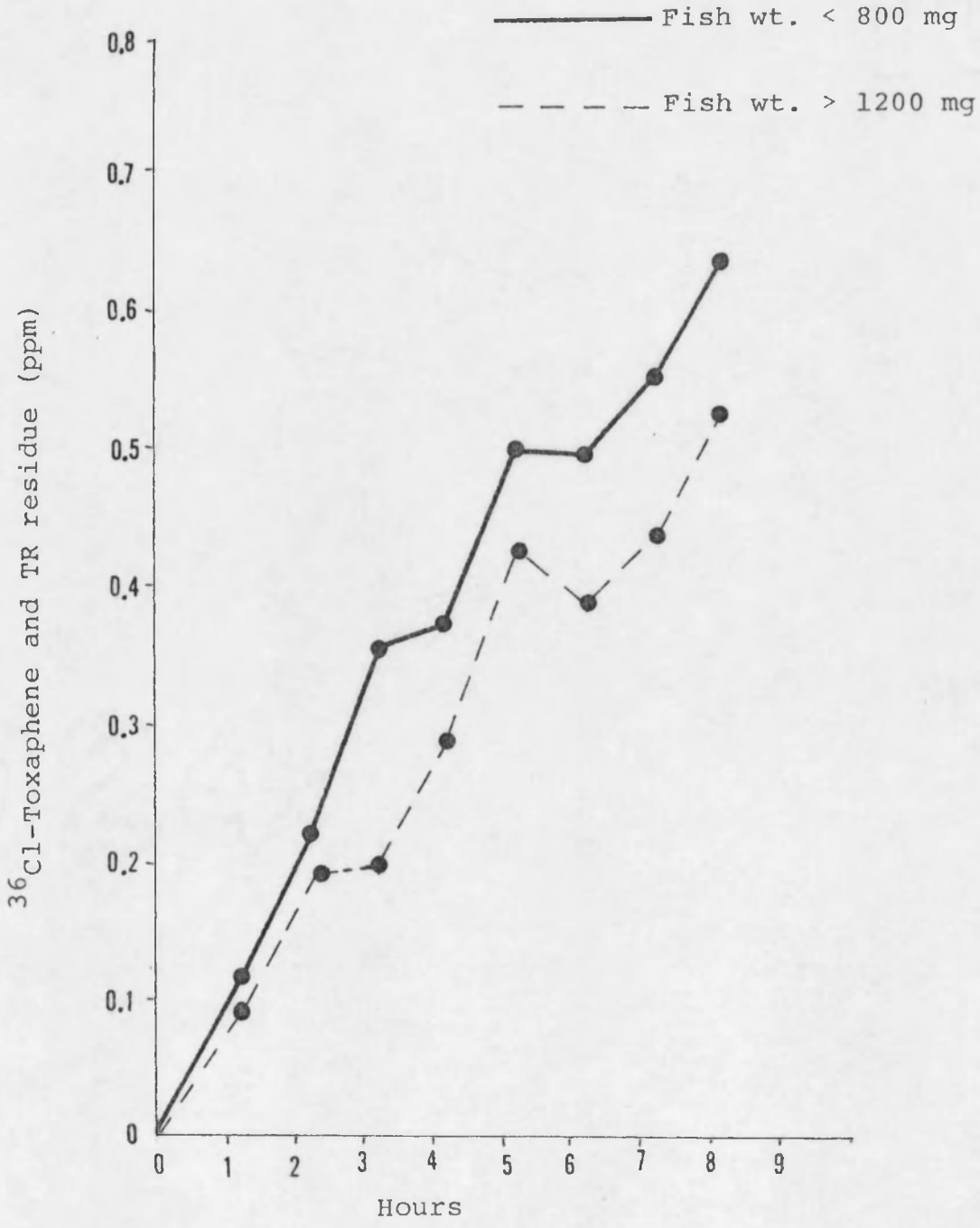


Fig. 2. The mean uptake of $^{36}$Cl-Toxaphene for large and small fish as a function of time.

time (Fig. 3). Regression analysis of data resulted in statistically nonsignificant excretion equations (Table 1). Calculation of confidence intervals with individual points indicated that at the 0.05 level, there is no significant difference in body load over time in either trial. Therefore from these data, there was no indication of excretion after six hours of exposure.

Table 2 represents partitioning of fish extracts into water and hexane fractions. This reveals that 88.7% of the radioactive chlorine was soluble in the nonpolar phase.

Observation of the toxic syndrome and characterization of a graded series into 5 stages are a subjective process. Interpretation of toxic symptoms without a knowledge of the mode of action of toxaphene is impossible although certain behavior may suggest physiological correlates. The first stage of the toxic syndrome, and the most difficult to assess, was when fish began to swim at the surface against the side of test containers. Swimming at the surface is normal in water with low oxygen content and is also the normal feeding position for G. affinis. Normal fish, however, retreated from the surface when the aquarium is approached whereas toxic fish remained at the surface. Subsequent toxic stages were all marked by rapid gill ventilation which further suggested respiratory involvement. The third toxic stage was characterized by fish swimming against the side of aquaria, but with some

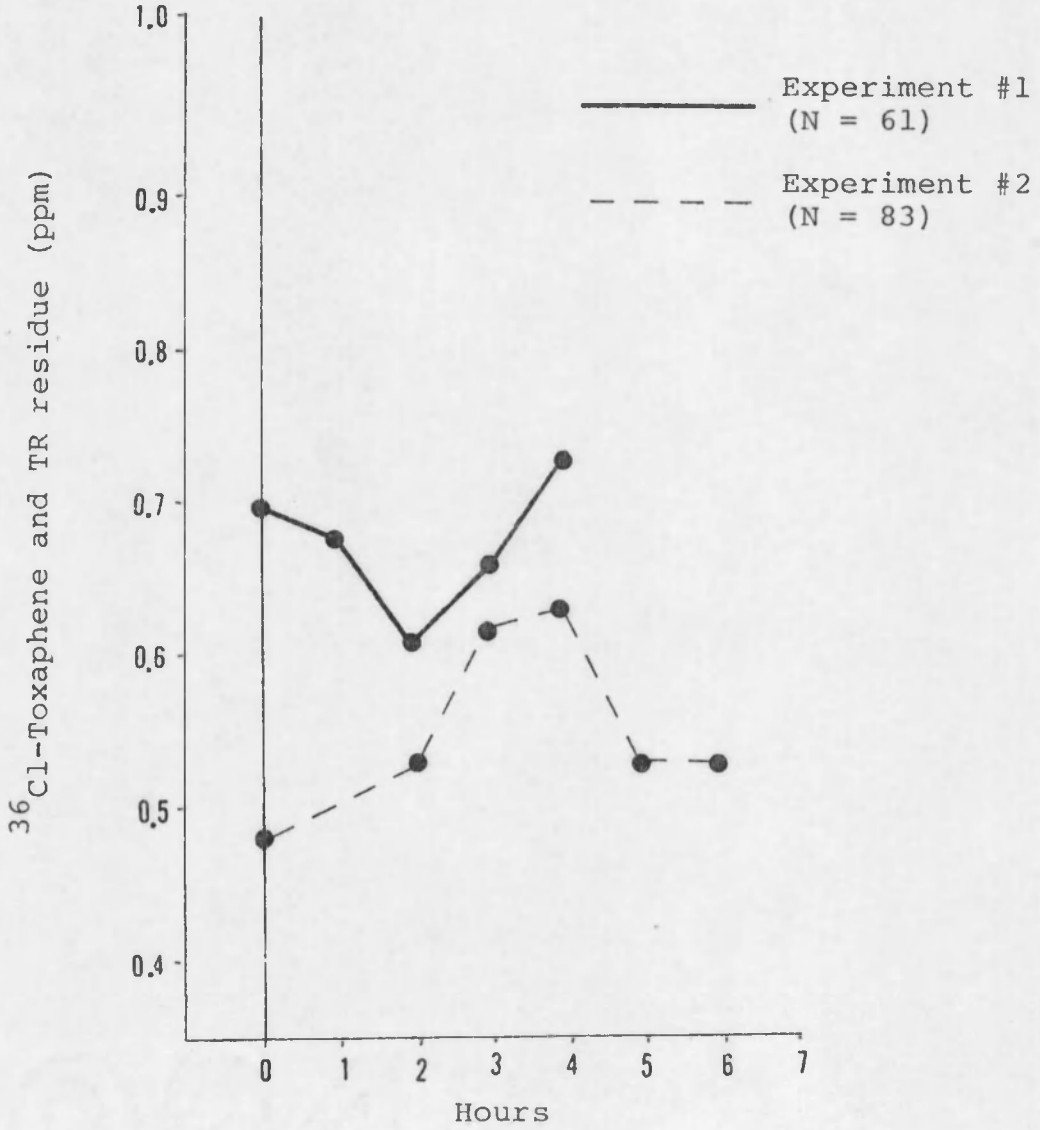


Fig. 3. Body load of $^{36}$Cl-Toxaphene and TR residue as a function of time in fresh water following 8 hours of exposure in 2000 ppb $^{36}$Cl-Toxaphene in water.

Table 2. Averaged results of metabolite partitioning into hexane and water soluble fractions -- The average $^{36}$Cl-Toxaphene and TR residue for controls was 0.68 ± .01 micrograms/gm of fish.

| | Hexane Fraction | Water Fraction | Total |
|---|---|---|---|
| $^{36}$Cl-Toxaphene and TR residue per sample (in micrograms) | .676 | .084 | .760 |
| Per cent of total | 88.7 | 11.3 | 100 |
| $^{36}$Cl-Toxaphene and TR residue/ gm of fish (in micrograms) | .586 | .072 | .658 |

loss of equilibrium which was evidenced by sinking of the posterior end so that fish attempted to swim up toward the surface. Finally, the fish lost its ability to maintain its normal dorsal-ventral orientation and rolled to the side. At stage 4 fish sink to the bottom and were prostrate with rapidly ventilating gills. At this stage, there was occasional darting behavior, until death was identified by the cessation of gill movements.

Figure 4 indicates the average bodyload of $^{36}$Cl-Toxaphene and TR residues at each of the toxic symptoms. By the time fish exhibited the first toxic response to toxaphene, rising to the surface of the water, they had already absorbed 90.3% of the average fatal residue. Fish which were characterized as normal had accumulated 35% of

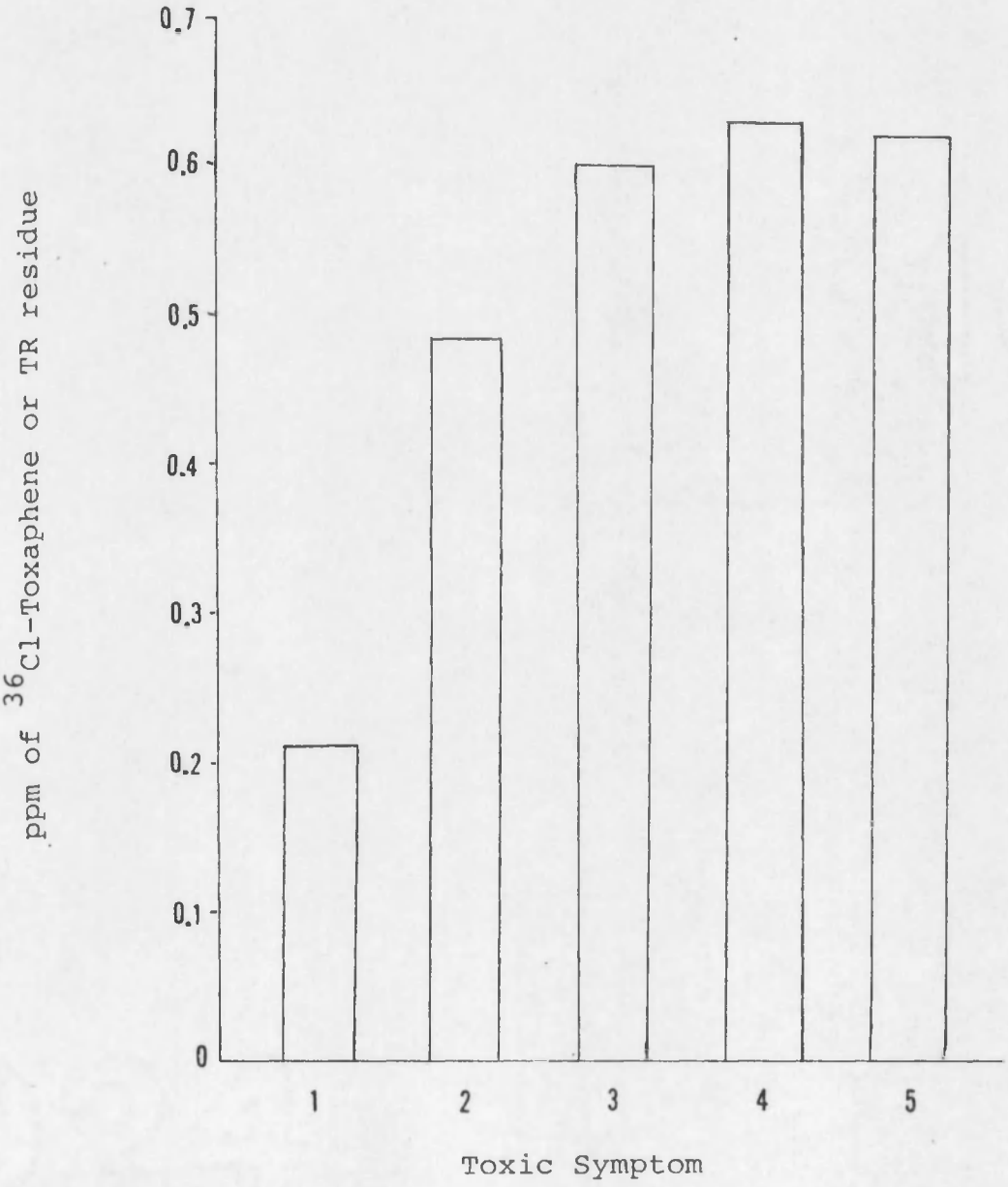


Fig. 4. Average body load of $^{36}$Cl-Toxaphene at each stage of the toxicity syndrome for G. affinis during exposure to 2000 ppb $^{36}$Cl-Toxaphene in water.

of the fatal residue. In stages 3, 4, and 5, fish showed obvious signs of toxicity.

Average residue per gram of fish and toxic symptoms exhibited at each hour is shown in Table 3. The fish progressed through toxic symptoms at approximately the same rate until the eighth hour. This is the point on the mortality curve (Figure A.3, Appendix A) where mortality increases rapidly. At this point it is possible to examine differences in body load between fish with identical exposure times, but which exhibit different toxic symptoms. The onset of a particular toxic stage is directly proportional to body load.

Since fish were processed whole in these experiments, it is impossible to determine what portion of these residues had been absorbed into particular organs and what portion was simply absorbed to the scales and fins. Information concerning toxic symptoms is perhaps most important when considering in vitro studies into the mode of action of toxaphene in fish. Data show that earliest toxic symptoms are visible when the toxaphene content is as low as 0.2 ppm and this is in a toxaphene resistant population. In nonresistant populations, where the $LC_{50}$ is around 30 ppb, toxic symptoms would presumably be apparent with even lower body loads.

Table 3. The mean $^{36}Cl$-Toxaphene and TR residue for G. affinis at each time and toxic symptom during uptake and excretion experiments -- Results expressed in ppm).

| Toxic Symptom | Uptake | | | | | | | | Excretion | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Hours | | | | | | | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 1 | 2 | 3 | 4 | 5 | 6 |
| 5 | | | | | | | | .680 | | | | | | |
| 4 | | | | | | | | | | | | .750 | | |
| 3 | | | | | | | | .610 | | | .660 | .705 | .527 | .523 |
| 2 | | | | | .420 | .435 | .497 | .537 | .605 | .569 | .631 | .649 | | |
| 1 | .109 | .202 | .254 | | | | | | | | | | | |

## DISCUSSION

Compared to the 36 hour $LD_{50}$s reported by Boyde and Ferguson (1964) G. affinis used in these experiments were highly resistant. The highest resistance reported by them was 480 ppb while nonresistant populations have $LD_{50}$s around 10 ppb.

Due to virtual insolubility of toxaphene in water, its presence in any aquatic medium is either as a suspension or else is carried by particles to which it adheres in the water. Thus under experimental conditions, uptake by fish may be due to (1) the adsorption of toxaphene to the body of the fish, (2) simple diffusion of toxaphene into the fish, and (3) active processes of absorption into the fish. On the basis of absorption alone, it would be expected that toxaphene residues of whole fish would increase as a function of time. All fish of the same weight or body surface would theoretically have the same body load at any given point. Table 1 shows, however, that at any time where more than one toxic symptom was exhibited, the mean body load was different for fish showing different symptoms, and that the amount of residue present was consistent with the severity of the symptom. Since exposure time was equal for all of the fish, different body loads at various toxic stages appear to reflect differences in rates of uptake. If

absorption or diffusion were solely responsible for uptake, there should be no differences in body load at any given time regardless of toxic symptom; thus, active processes of absorption appear to be implicated in the uptake process. This agrees with the conclusions of Ferguson et al. (1966) concerning endrin uptake in G. affinis.

Partitioning of fish extracts revealed that 88.7% of the radioactive chlorine was recovered from the nonpolar fraction. Recovery rates, however, for the hexane/water partitioning procedure using extracts of fish topically dosed with a $^{36}Cl$-Toxaphene standard also averaged 87.62%. Thus, there was apparently minimal metabolic alteration in the toxicant after a period of eight hours. Dehydrochlorination has been reported as a method of toxaphene metabolism in rats (Ohsawa et al., 1975). If this were the case in fish, one would expect $^{36}Cl$ to appear in the water fraction. One would also expect the fish to excrete chloride. The excretion data show no evidence of excretion within eight hours which correlates with the lack of toxaphene metabolism during that period. Ferguson et al. (1966) found no evidence of metabolic or chemical alteration of endrin in their studies with G. affinis.

Insecticide resistance in fish are not completely understood at this time. Fabacher and Chambers (1971) have correlated resistance with fish lipid content and suggest that resistance is due in part to binding of lipophilic

insecticides in body lipids which protects the organisms from their effect. Ferguson et al. (1966) attribute resistance to increased physiological tolerance rather than differences in the rates of uptake. Data presented here show that the onset of toxic symptoms varies within any given population, and that the onset of particular toxic symptoms is directly proportional to body load. Thus within this population, differences in toxic response was due to different body loads rather than to different tolerances of a particular toxicant level.

Insecticide resistance in organisms can be the result of differences in the ability to tolerate high body loads of toxicant or differences in the rates of uptake and/or excretion. Yarbrough and Wells (1971), Moffet (1971), and Moffet and Yarbrough (1972) demonstrated that resistance in G. affinis to endrin, DDT, and dieldrin may be due to a mitochondrial membrane barrier. This did not, however, appear to be the case with toxaphene in which disruption of the mitochondrial membrane had no consistent effect on enzyme inhibition. This suggests that resistance to endrin, DDT, and dieldrin may be the result of increased physiological tolerance due to the barrier effect of the mitochondrial membrane while resistance to toxaphene may depend on a different mechanism.

The mode of action of toxaphene is poorly understood, and fish being particularly susceptible, may provide

clues to this problem. Observations of the progression of toxic symptoms in G. affinis suggest that intoxication involves an increased oxygen requirement. The first stage of toxic symptoms is marked by fish swimming at the surface of the water which is typical for fish in water of low oxygen content. Swimming toward the surface, coupled with decreased activity before finally sinking to the bottom, suggests a depletion of energy. This is in spite of an apparent increasing need for oxygen which was evidenced by rapid gill ventilation which continues until death. Ferguson et al. (1966) noted increased oxygen requirements for endrin poisoned fish, but related them to the increased activity that characterized endrin poisoning. With toxaphene, activity decreased with the onset of toxic symptoms while the requirement for oxygen appeared to increase.

The very sharp rise in mortality at 8 hours observed in the mortality curve (Figure A.3, Appendix A) coupled with the similarity of body loads at toxic stages 3-5 suggested that mortality was due to a very critical level of toxaphene at the site of action. If several different sites of action were involved, one would expect each to be affected at slightly different concentrations which would tend to flattenthe mortality curve. This suggests, therefore, that the toxic response of G. affinis

to toxaphene is due primarily to toxic mechanisms acting at one site.

## SUMMARY AND CONCLUSIONS

In order to quantify the absorption and excretion of toxaphene by G. affinis, a partially resistant population of fish was examined using radioassay techniques. After determination of $LC_{50}$ and $LT_{50}$ (200 ppb), fish were sampled following exposure to 2000 ppb of toxaphene in an aquatic medium. Excretion was studied by sampling fish following an 8 hour exposure to toxaphene.

Analysis of fish was accomplished by acetone extraction. Toxaphene and TR residues were not identified by partitioning in hexane and water demonstrated that 88.7% of the recovered $^{36}Cl$ was present in the hexane fraction.

On the basis of observations, the toxicity syndrome was divided into 5 stages, and the residue level at each stage was determined. By the time fish were exhibiting the second toxic response, 90.3% of the mean fatal residue level had been sorbed. Regression analysis indicated that absorption of toxaphene is a linear function with respect to time.

The data suggest that sorption of toxaphene is an active process, and that differences in individual mortality is due to differences in rates of uptake rather than differences in ability to tolerate particular body loads of toxaphene.

# APPENDIX A

## CHARACTERIZATION OF *GAMBUSIA AFFINIS* POPULATION, VOLTAGE/ATTENUATION PROFILE, AND QUENCH CURVE FOR *GAMBUSIA AFFINIS*

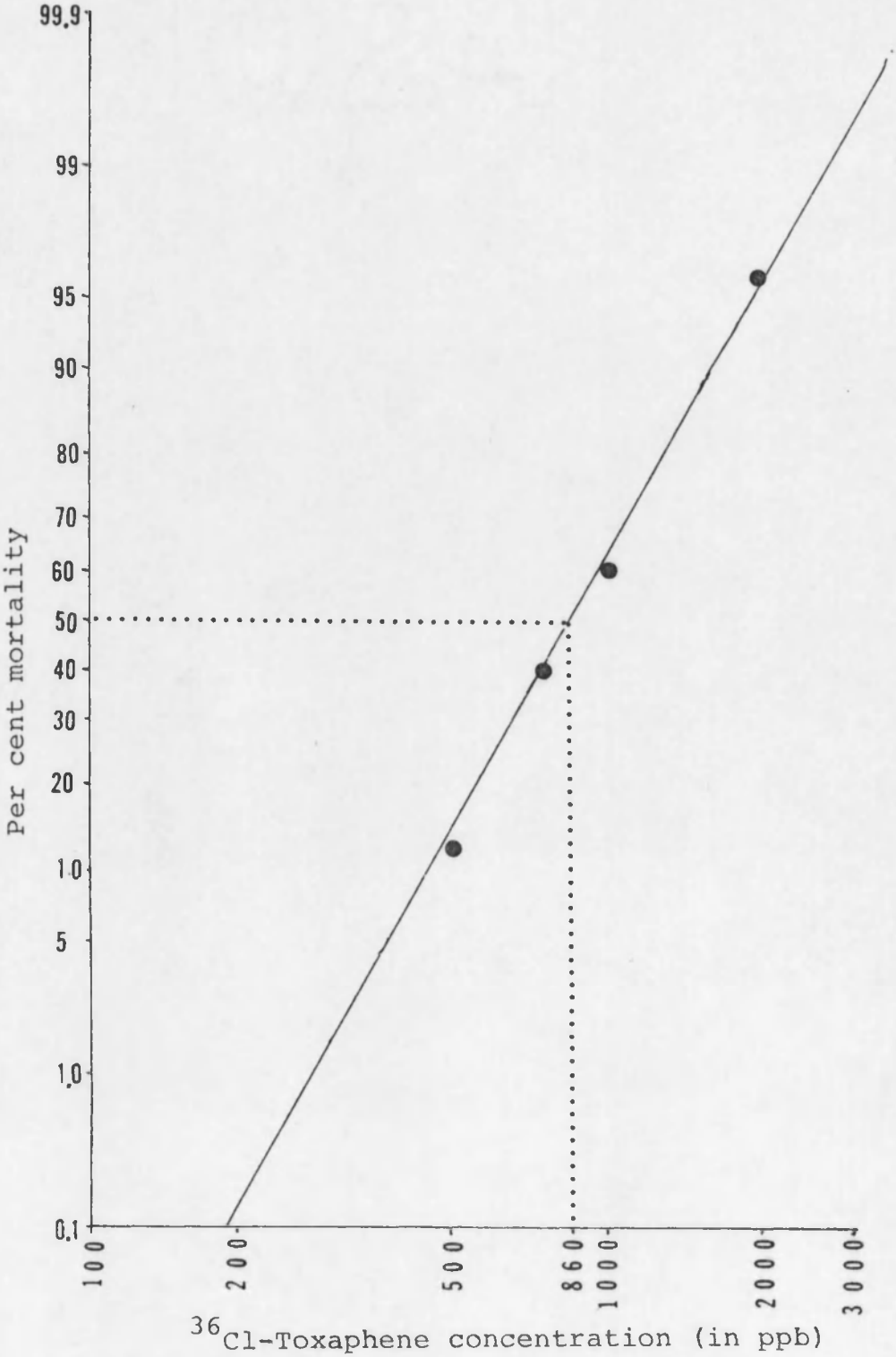


Fig. A.1. Determination for G. affinis exposed to various $^{36}$Cl-Toxaphene concentrations for 20 hours.

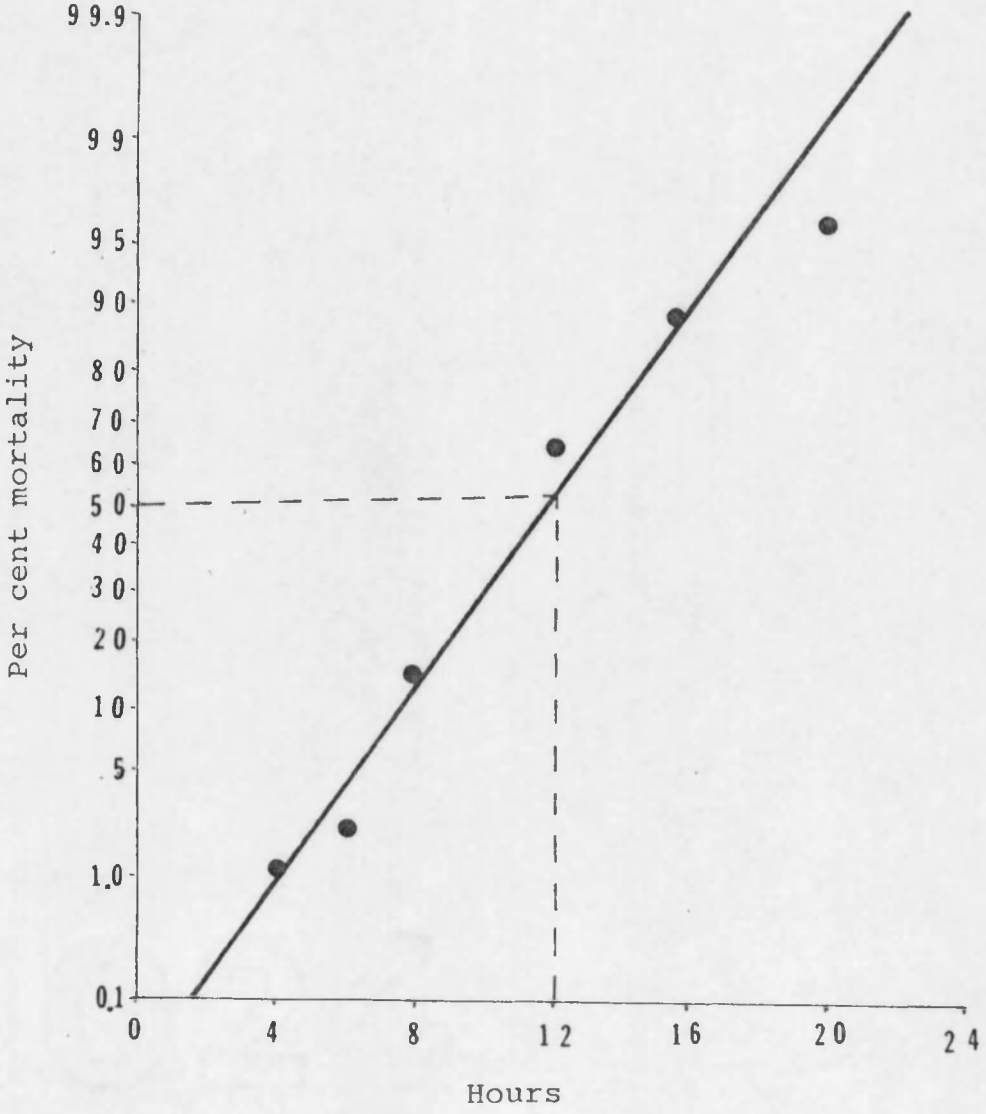


Fig. A.2. $LT_{50}$ determination for G. affinis exposed to 2000 ppb $^{36}Cl$-Toxaphene.

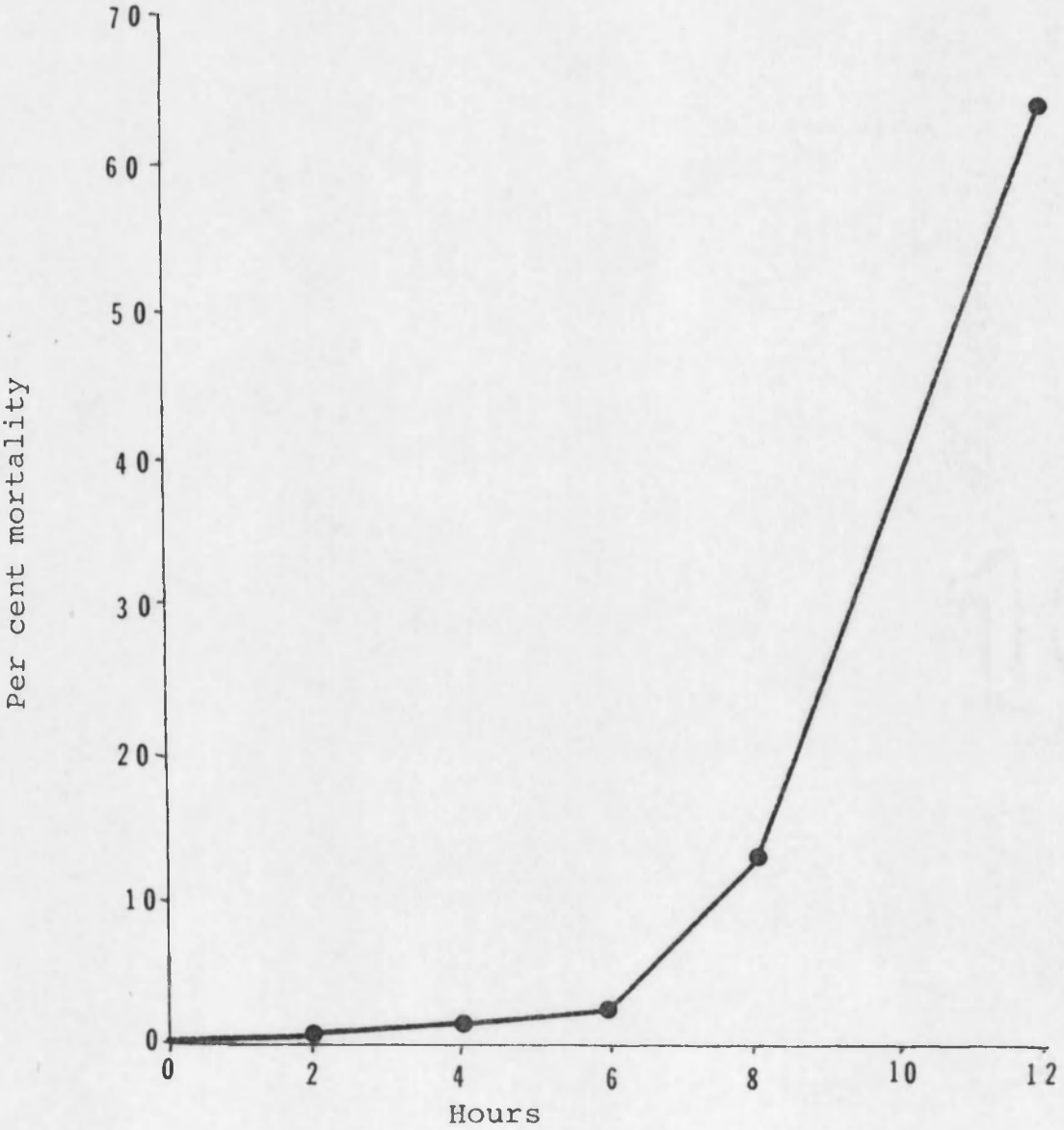


Fig. A.3. Per cent mortality as a function of exposure to 2000 ppb toxaphene showing rapid increase in mortality beginning at 8 hours.

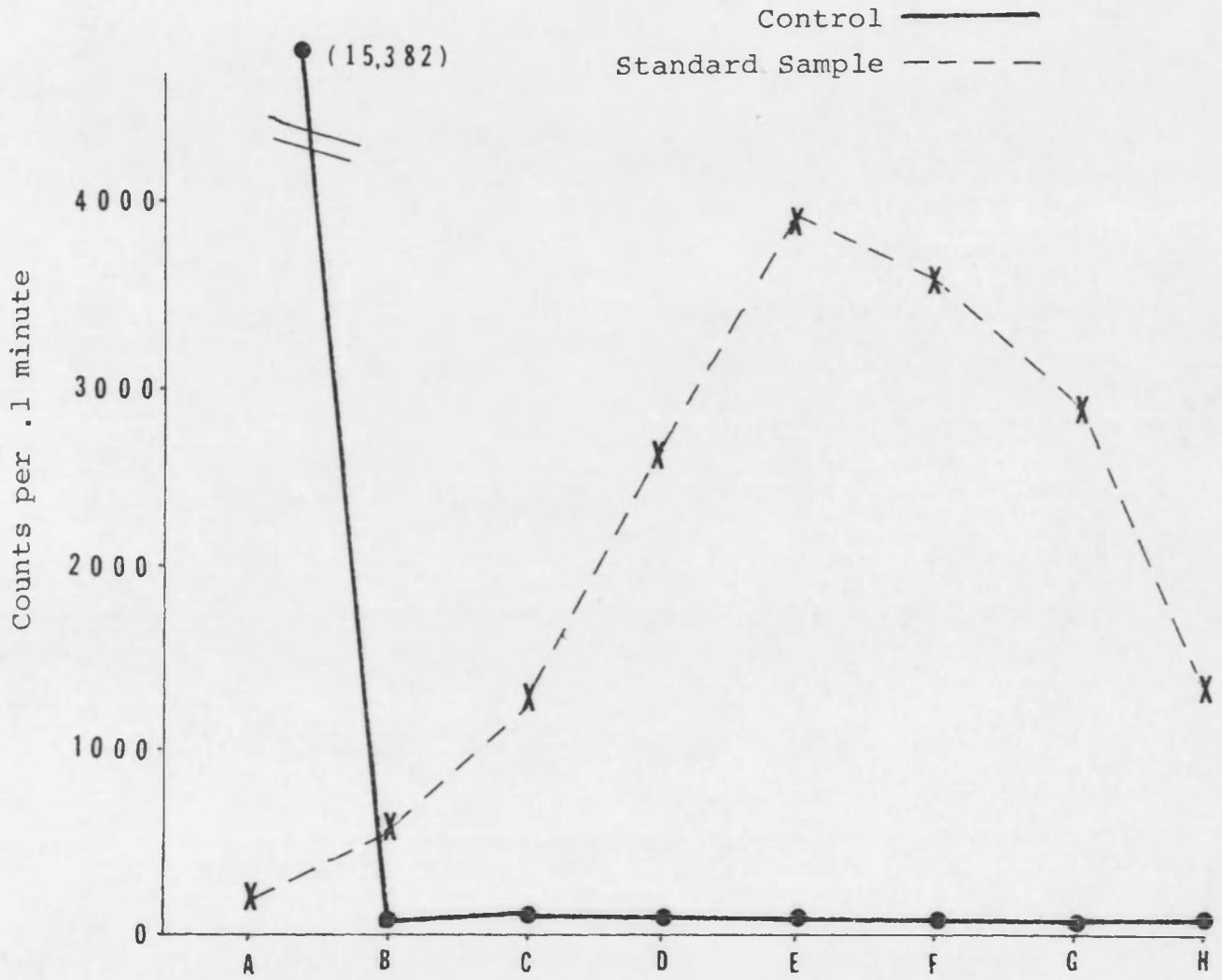


Fig. A.4. Voltage/attenuation profile for G. affinis extracts in Bray's solution.

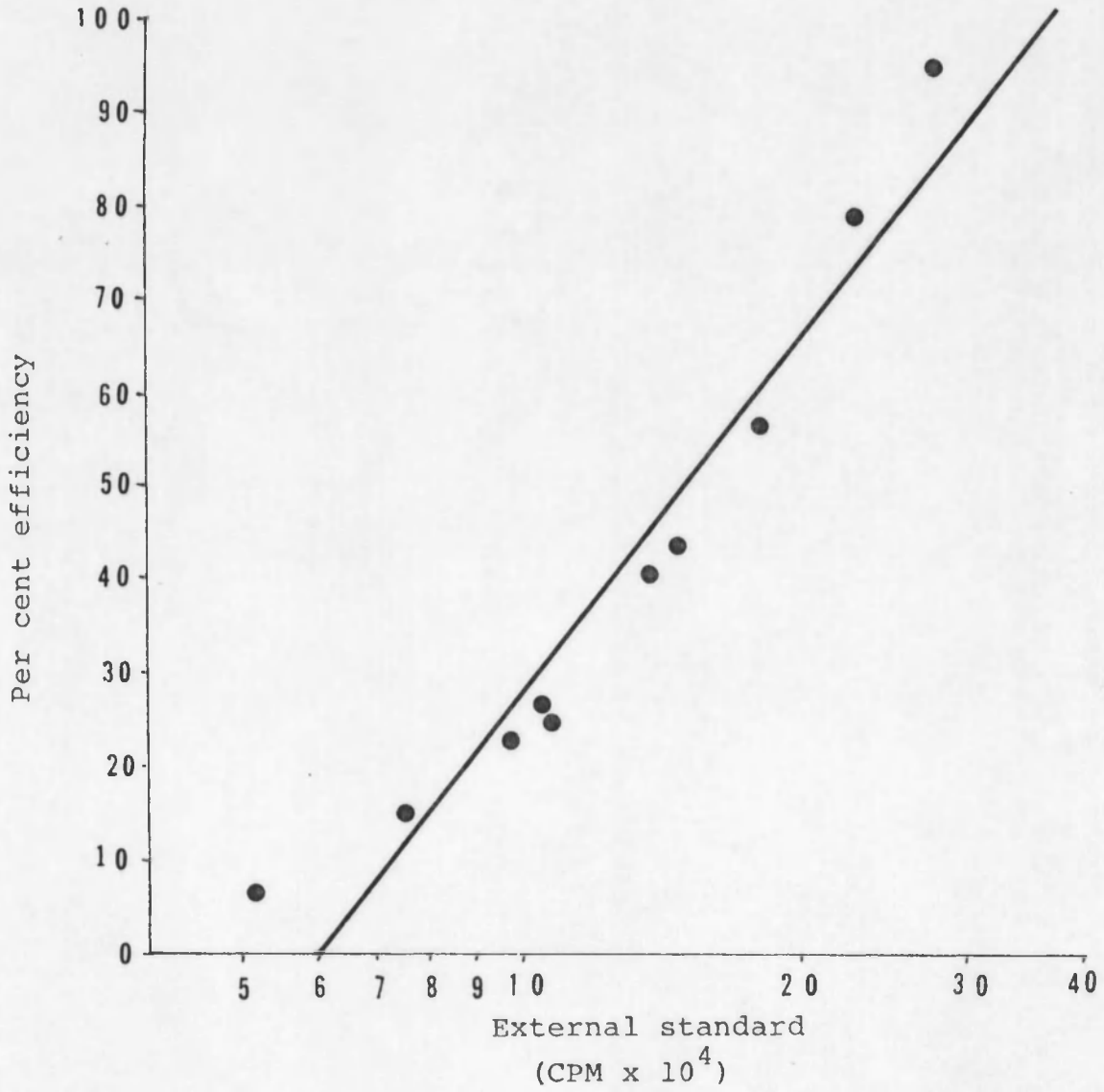


Fig. A.5. Quench curve for acetone based extract of G. affinis in Bray's solution.

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