WRC RESEARCH REPORT NO. 95

COMPARATIVE BIOCHEMISTRY, BIODEGRADABILITY, AND TOXICITY OF DDT AND CARBOFURAN ANALOGUES

Robert L. Metcalf, Project Director; Keturah A. Reinbold,
James R. Sanborn, W. F. Childers,
W. N. Bruce, and Joel Coats

Illinois Natural History Survey, Urbana-Champaign, Illinois

FINAL REPORT

Project No. B-070-ILL

The work upon which this publication is based was supported by funds provided by the U. S. Department of the Interior as authorized under the Water Resources Research Act of 1964, P.L. 88-379
Agreement No. 14-31-0001-3879

UNIVERSITY OF ILLINOIS
WATER RESOURCES CENTER
2535 Hydrosystems Laboratory
Urbana, Illinois 61801

December 1974

ABSTRACT

COMPARATIVE BIOCHEMISTRY, BIODEGRADABILITY, AND

TOXICITY OF DDT AND CARBOFURAN ANALOGUES

The aquatic persistence, toxicity, and biodegradability of DDT, methoxychlor, and 28 analogues were evaluated using the green sunfish Lepomis cyanellus. Study of DDT analogues with degradophores located in aryl or alkyl moieties of the DDT-type molecule showed that the toxicity to and persistence of these compounds in the green sunfish is correlated with the environmental temperature of exposure and ability of multi-function oxidase enzymes of the fish to attack specific chemical groupings on various parts of the molecule. The green sunfish can readily oxidize alkyl and methylthio groups of the aryl portion of the molecule to water partitioning moieties but alkoxy groups are not as readily attacked. Persistent and highly insecticidal DDT molecules can be developed which have greatly reduced toxicity to fish.

The biochemical role of the multi-function oxidase enzymes in biodegradation of pesticides in the green sunfish was explored in detail using the oxidase inhibitor piperonyl butoxide together with radiolabeled methoxychlor, aldrin, and trifluralin. The reactions which were inhibited were O-demethylation, N-dealkylation, and epoxidation. Where piperonyl butoxide was present, the fish accumulated from 15 to 45 times more of the parent compound, over a 16 day period.

Metcalf, Robert L.
COMPARATIVE BIOCHEMISTRY, BIODEGRADABILITY, AND TOXICITY OF DDT AND CARBOFURAN ANALOGUES
Matching Grant Project B-070-ILL (14-31-0001-3879)
KEYWORDS: DDT Analogues*/ methoxychlor/ green sunfish/ biodegradation/ aldrin/ trifluralin/carbofuran/pesticides

ACKNOWLEDGEMENTS

The writers are grateful for additional support from The Rockefeller Foundation "Development of Novel, Selective, and Non-Persistent Pesticidal Chemicals", and the U.S. Environmental Protection Agency Research Grant R-802022. The writers are indebted to Mrs. Jean Wilson for expert gas chromatography.

CONTENTS

I. INTRODUCTION

A. Objectives. This project was developed to evaluate the physiological and biochemical factors affecting the uptake and storage, i.e. bioconcentration, of DDT and other persistent pesticides in fish and to determine if suitable chemical modifications of the DDT-type molecule could be devised which would incorporate suitable insecticidal activity together with appropriate biodegradability and elimination from fish rather than concentration and storage in tissue lipids.

Specifically the work has devolved around two major projects I, the evaluation of a number of new DDT type analogues for toxicity and degradation in the green sunfish Lepomis cyanellus, carried out in 1300 liter aluminum tanks in a polyethylene-covered greenhouse, and II, laboratory study of the biochemical mechanisms and degradative pathways of radiolabeled pesticides as absorbed by green sunfish in 5 liter cylindrical pyrex jars. Comparisons were made between the fate of the pesticide alone and in combination with the synergist piperonyl butoxide, in order to evaluate the role of the multifunction oxidases in the biodegradation processes.

B. Background. DDT has been used and liberated into the environment in total amount approximating 2×10^9 lbs. since its introduction as an insecticide in 1946. As a result of its stability, water insolubility (ca. 0.0012 ppm) and lipid solubility (ca. 100,000 ppm), DDT has become the classical example of a ubiquitous environmental micropollutant, appearing everywhere in living tissues of both terrestrial and aquatic organisms. Moreover, DDT although highly stable as an organic compound is not totally non-degradable and is biologically converted by dehydrochlorination to DDE or 2,2-bis-(p-chlorophenyl)-1,1-dichloroethylene,

and by reductive dechlorination to DDD or 2,2-bis-(p-chlorophenyl)-1,1-dichloroethane. These environmentally produced degradation products closely resemble DDT in water insolubility and lipid solubility, and DDE is substantially more stable environmentally than DDT. Therefore DDT, DDE, and DDD are found in varying amounts in the lipids of organisms at various trophic levels with those in higher positions in the food chain showing increasing amounts of total DDT-type residues as well as increased percentages of DDE (Woodwell et al 1967, Metcalf et al 1971a).

The presence of DDT, DDE, and DDD in the aquatic environment has resulted in their bioconcentration or biomagnification to levels undreamed of a few years ago. Thus the average level of DDT in Lake Michigan is about 0.000002 ppm and this is concentrated by amphipods to 0.410 ppm, by fish 3-6 ppm, and by herring gulls to 99 ppm or to overall levels of 10^6 to 10^7 (Harrison et al 1970).

Fish are particularly ineffective in degrading and excreting DDT and its principal degradation products (DDT-T) and continue to accumulate these compounds throughout their lives (Reinbold et al 1971, Youngs et al 1972). This aquatic bioaccumulation has resulted in a major pollution problem with fish in Lake Michigan as shown by the following average DDT-T levels in lake trout Salvelinus namaycush of various ages (EPA 1972):

trout - length in inches	DDT-T in ppm
2.0 - 5.9	0.89
6.0 - 9.9	2.24
10.0 - 15.9	6.00
16.0 - 21.9	8.00
22.0 - 26.9	14.62
27.0 - 32.9	19.23

These residues in mature fish exceed all FDA tolerances for DDT in raw food commodities and in most cases the FDA action level for seizure as "unfit for human consumption". A similar condition exists for most other fish in the lake.

There is therefore an important need to develop pesticides having some of the persistent characteristics of DDT on inert surfaces but possessing appropriate biodegradability when ingested by living organisms. Studies with methoxychlor or 2,2-bis-(p-methoxyphenyl) 1,1,1-trichloroethane (H_2O solubility 0.62 ppm) have shown that it is readily degraded in most living organisms by O-demethylation forming first the monophenol 2-(p-hydroxyphenyl)-2-(p-methoxyphenyl)-1,1,1-trichloroethane (H_2O solubility 0.80 ppm) and then the bis-phenol 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (H_2O solubility 76 ppm). These metabolic changes in polarity from predominately lipid partitioning to more water partitioning products promote the elimination of the products from the body rather than storage and accumulation (Kapoor et al 1970). The comparative behavior of DDT and methoxychlor in the fish Lepomis cyanellus and Tilapia mossambica was investigated by Reinbold et al (1971), who found the bioconcentrations shown in Table 1. Thus Tilapia exposed to DDT at 0.01 ppm for 31 days accumulated 10,600 X the water concentration in its body and Lepomis exposed in the same way accumulated 4020 X the water concentration. With exposure to methoxychlor at 0.01 ppm for 31 days, Tilapia accumulated only 200 X and Lepomis only 270 X and the methoxychlor concentrations decreased very rapidly upon transfer to clean water.

From these and other studies it seemed clear that methoxychlor which is a persistent pesticide resembling DDT in residual action is biodegradable in living organisms because of the presence of the CH_3O-groups which act as

degradophores. These degradophores are subject to attack by the multi-function oxidase enzymes or "drug metabolizing enzymes" which convert xenobiotics to readily excretable more polar entities and thus protect the organism from the toxic effects of these xenobiotic compounds (Kapoor et al 1971). Therefore this laboratory began studies using the DDT-type compound as a model, to determine the biological effects of incorporation of a variety of degradophore groupings, i.e. CH_3O, C_2H_5O, C_3H_7O, CH_3, C_2H_5, CH_3S, $-OCH_2O-$, etc. in various parts of the DDT molecule upon both insecticidal action (Metcalf et al 1971b) and upon environmental persistence (Kapoor et al 1973).

These investigations have led to a considerable number of new DDT-type analogues which are both insecticidal and biodegradable. The new asymmetrical diaryltrichloroethanes are described in U.S. Patent 3, 787, 505 (Metcalf et al 1974). Other DDT-like α-trichloromethylbenzyl phenyl ethers and α-trichloromethylbenzylanilines were described by Hirwe et al (1972) and in patents applied for (Metcalf et al 1972).

A newer series of DDT analogues having altered aliphatic moieties have been studied for insecticidal activity and biodegradability by Coats (1974) and are covered in patent applied for (Metcalf and Coats 1974). These interesting DDT analogues provided the major thrust for the evaluation of their toxicity and biodegradability in fish as detailed in this report.

II. METHODOLOGY

Synthesis of compounds was carried out by a variety of chemical techniques described by Metcalf et al (1971b), Hirwe et al (1972) and Coats (1974). In all cases, the compounds were recrystallized to 98+% purity and constant melting point and their assigned chemical structures were verified by NMR spectrometry.

Fish toxicity tests were performed in circular aluminum tanks 1.68 m. in diameter (Figure 1) containing 1300 liters of water. The compounds at 130 mg. each were dissolved in approximately 5 ml. of acetone and added dropwise to the water with forceful stirring, to give a final concentration of 0.1 ppm compound. After approximately 4 hours, five green sunfish Leponis cyanellus 6-8 inches long were added to the treated water and observed for mortality. The fish were collected from a farm pond in Vermillion County, Illinois. Whenever fish were killed, they were removed and examined for tissue levels of compound and degradation products using GLC. Tanks in which fish were killed were restocked on the third and seventh days and thereafter so that the duration of lethal effect could be determined as shown in the Tables. These experiments were conducted in a polyethylene covered greenhouse gas-heated during winter at 13-20°C. During the summer the temperature was that of the out-of-doors, 23-32°C.

Water samples were strained through glass wool and extracted successively in 2-liter separatory funnels with three 100-ml. portions of methylene chloride. The combined extracts were dried over anhydrous sodium sulfate and concentrated through a Snyder column. Hexane was added to remove the methylene chloride and volume adjusted for either GLC or radioassay. In some studies, water was hydrolyzed by adding concentrated HCl to 0.25 N and heating for 1 hour in a steam bath. After hydrolysis, the water samples were reextracted with three 100-ml portions of methylene chloride and analyzed by GLC or radioassay.

Fish were homogenized with methylene chloride and anhydrous sodium sulfate, except those in the trifluralin study which were homogenized in methanol. Extracts were filtered through Whatman 2V filter paper. One part methanol extract was placed in a separatory funnel with 2 parts of 5 per cent sodium chloride in water and extracted twice with 1 part methylene chloride. The combined extracts were dried over anhydrous sodium sulfate reduced in volume and exchanged with hexane through a Snyder column for cleanup by column chromatography on activated Florisil. The compounds were eluted by hexane or 10% ether in hexane and the eluates concentrated in Snyder column of GLC.

Gas-Liquid Chromatography was performed with a Varian series 1400 chromatograph with ^{3}H election capture detector or with a Tracor model 550 chromatograph with a ^{63}Ni election capture detector. Various columns and temperatures were used: methoxychlor and DDT analogues with 2% QF-1 and 1.5% OV-17 on 100/120 mesh Supelcoport at 200°C, aldrin, phorate, and trifluralin with 5% QF-1 on 100/120 mesh Varaport 30; aldrin at 200°C, phorate 180°C, and trifluralin 180°C.

Carbofuran was analyzed by converting the water and fish extracts to the 2,4-dinitrophenyl ether derivative by treatment with 1-fluoro-2,4-dinitrobenzene as described by Holden (1973). GLC was on 2% QF-1 and 1% OV-17 on 100/120 mesh Supelcoport at 220°C.

Thin-Layer Chromatography was carried out on glass plates coated with 0.25 mm. fluorescent silica gel (E. Merck GF-254). Extracts were cochromatographed with model degradation products and the plates were evaluated by radioautography on Kodak Blue-Brand X-ray film. The radioactive areas were scraped from the plates into scintillation vials for radioassay.

Radiotracer studies were conducted on green sunfish, reared in the laboratory from parents collected from a Vermillion County farm pond. The fish were placed in Pyrex jars 20 cm in diameter and 20 cm high, containing

5 l. of synthetic hard water (Marking and Daws 1973), containing $NaHCO_3$ 192 mg, $CaSO_4$ $2H_2O$ 120 mg., $MgSO_4$ 120 mg. and KCl 8 mg., per liter. Water was aerated before and during the experiment. The radiolabeled pesticides as described in Section III, were assayed in triplicate at 0.01 ppm alone and with 0.1 ppm piperonyl butoxide, added to the water in 1 ml. acetone. The temperature was maintained at 20.5 - 22.0 $\pm$ 1°C at pH 7.5 - 8.0 and at 500 - 550 reciprocal megohms conductivity, with 12 hours diurnal cycle.

Five fish weighing 0.30 - 0.95 g. each were placed in each jar. They had not been fed for 48 hours, but were fed live daphnia seven times during the experiment between days 3 and 15. Feeding was limited to daphnia consumed immediately to eliminate uptake of radiolabeled compounds through the daphnia.

On days 1, 2, 4, 8, and 16 duplicate 1 ml. water samples were removed for radioassay and one fish from each jar was removed, rinsed, blotted dry, weighed and measured and frozen for radioassay. On day 16, one liter of water was removed from each jar for extraction and radioassay.

For the metabolism studies, the fish were homogenized in methylene chloride and sodium sulfate, or with trifluralin in 90% acetone - 10% water. An aliquot of each extract was removed for radioassay and the remainder was concentrated in Kontes small-scale evaporative concentrators with modified micro Snyder columns and used for TLC and radioautography. For best results it was necessary to treat the acetone-water extracts by heating to 60° to eliminate acetone and then by adding 5% sodium chloride in distilled water and extracting twice with methylene chloride.

After extraction, fish tissues were solubilized with 0.5 ml Protocol (New England Nuclear) per 0.5 g. tissue at 50°C for two hours with shaking. Then 0.2 ml of 20% benzoyl peroxide in toluene was added per 0.5 g of tissue

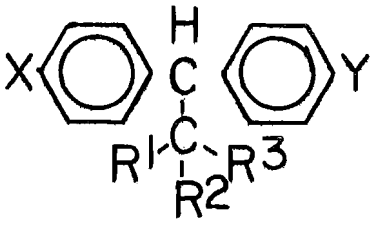
to bleach the color. After one hour the samples were treated with Aquasol and radioassayed.

Radioassay was carried out in a Packard 3320 Tri-Carb liquid scintillation spectrometer with correction for quenching by external standard and quench curve. Water, extracts and silica gel were counted in 10 ml. of a cocktail of 120 g. naphthalene, 7 g. of 2,5-diphenyloxazole, 0.05 g. of 1,4-bis-{2-(4-methyl-5-phenyloxazolyl)}-benzene in one 1 dioxane. Solubilized fish tissues were counted in 10 ml. Aquasol (New England Nuclear).

III. MODIFICATIONS OF THE DDT-TYPE MOLECULE [1/]

As discussed in Section I, DDT or 2,2-bis-(p-chlorophenyl)- 1,1,1-trichloroethane is highly persistent in the aquatic environment, exhibits pronounced bioconcentration into fish, and is converted biologically to DDE or 2,2-bis-(p-chlorophenyl)-1,1-dichloroethylene and DDD or 2,2-bis-(p-chlorophenyl)-1,1-dichloroethane both of which are bioconcentrated and stored in fish (Metcalf et al 1971a, Reinbold et al 1971). The common property of DDT and its biodegradation products which make these compounds important environmental micropollutants is the great stability of the carbon chlorine bonds, especially those of the two aryl rings. This is clearly demonstrated by the large improvement in biodegradability found with methoxychlor or 2,2-bis-(p-methoxyphenyl)-1,1,1-trichloroethane (Kapoor et al 1970, Reinbold et al 1971). However, as shown in the fish toxicity tests of Table 2 methoxychlor still displays considerable initial toxicity to the green sunfish Lepomis cyanellus and this has been a barrier to its practical use in some areas.

The DDT-type molecule is particularly well suited for investigation of the basic principles of biodegradability because of the variety of ways in which substitutions of the five chlorine atoms can be made to incorporate other small molecular moieties, e.g. CH_3, CH_3O, C_2H_5, C_2H_5O, CH_3S, H, NO_2, while still preserving insecticidal activity (Metcalf et al 1971a, Coats 1974):



[1/] Some of the material in this Section was taken from the Ph.D Thesis of Joel R. Coats, University of Illinois, June 1974.

A. Effects of structural modifications on fish toxicity.

Effects of altered aryl substituents. These are shown in Table 2. Toxicity to the green sunfish is decreased by substitution of CH_3O or CH_3 for Cl, but not with C_2H_5O, or C_3H_4O. The introduction of a single aryl CH_3 to provide an asymmetrical molecule e.g. VII, VIII, IV, V,XI, greatly decreased fish toxicity suggesting that microsomal oxidation of CH_3 to COOH takes place readily in the fish (Kapoor et al 1973). Fish microsomal enzymes have little ability to O-dealkylate C_2H_5O and C_3H_7O as shown by the high toxicity of III and VI which are equal to or more persistent than DDT.

Effects of altered aliphatic substituents. As shown in Table 2, the nature of the aryl substituents on the DDT-type molecule has a critical affect on toxicity to the green sunfish. Data in Table 3 shows the effects of keeping the aryl-substituents constant with p,p'-di C_2H_5O, and replacing the aliphatic CCl_3 moiety with a variety of other groups. Many of these combinations were highly toxic even when CCl_3 was replaced with $C(CH_3)_3$ (XII). This appears rather surprising but Coats et al (1974) have shown that 1,1-bis-(p-methoxyphenyl)2,2,2 trimethylethane (neopentane) is little more biodegradable than its isostere methoxychlor. From the data in Table 3, it is apparent that highly toxic and persistent compounds to fish result from nearly all combinations of Cl, CH_3 NO_2, and H. The only combination of low toxicity was XIX. This compound is also a poor insecticide for reasons not well understood.

B. Persistence of DDT Analogues in Water and Green Sunfish

The effects of varying the aromatic and aliphatic substituents of DDT analogues upon persistence in water and in the tissues of fish as determined by GLC, are shown in Table 5. The values for water indicate

the rapidity with which the parent compound is destroyed environmentally.

The persistence and accumulation of DDT(I) is shown clearly. The GLC data indicated the conversion of DDT, 2,2-bis-(p-chlorophenyl) -1,1,1-trichloroethane to DDE, 2,2-bis-(p-chlorophenyl)-1,1-dichloro-ethylene, and to DDD 2,2-bis-(p-chlorophenyl)-1,1-dichloroethane:

		ppm		
		DDT	DDE	DDD
water	1 week	.0076	.0008	.011
	2 weeks	.0060	.0008	.0011
	3 weeks	.0027	.0002	.0004
	4 weeks	.0026	.0002	.0003
	8 weeks	.000061	.000016	.000015
fish	1 week	3.82		2.59
	4 weeks	7.17		3.50
	8 weeks	2.20		0.64

This interconversion of DDT is well known (Reinbold et al 1971) and accounts for a large portion of DDT-T residues in fish tissues.

Effects of alkoxy and alkyl aryl substituents. The properties of methoxychlor in which CH_3O is substituted for Cl in the aromatic portion of DDT, are well known to favor O-dealkylation and loss of insecticide residues rather than storage as with DDT (Kapoor et al 1970, Reinbold et al 1971). This effect is shown in Table 5 where methoxychlor (II) although initially present in water and in fish tissues is lost much more rapidly than DDT(I).

O-demethylation evidently is more efficient in the green sunfish than O-dealkylation of longer chains as compound VI with C_2H_5O and C_3H_7O is clearly much more persistent than II.

The introduction of a single aryl CH_3 or C_2H_5 group in place of Cl, CH_3O, or C_2H_5O substantially decreases persistence (compare I and VII, V and VI, I and VIII, IV and XI in Table 5). The effect results from the facile oxidation of CH_3 or C_2H_5 to COOH which seems to occur much more readily in fish than O-dealkylation. This effect is an important reason for the very low fish toxicity of compounds IV and XI as shown in Table 2, and for XXVII and XXVIII as shown in Table 4. The incorporation of alkyl groups on aromatic rings is a key to production of low toxicity compounds for fish.

Effects of methylenedioxy. The methylenedioxy group $-OCH_2O$ has been shown to be an autosynergist or synergaphore. This group attached to the DDT-type molecule prevents its own detoxication by the mixed function oxidases by inhibiting these enzymes (Metcalf et al 1971a). The GLC data for the methoxychlor type DDT molecule incorporating the 3,4-methylenedioxyphenyl group (X, Table 5) demonstrates this effect by showing extreme persistence in fish (compare II and X). Although this grouping increases the insecticidal potency it appears to have undesirable environmental effects.

Oxidation of methylthio. The CH_3S group as in compound IX is very rapidly oxidized to sulfoxide and sulfone. This dramatically changes the polarity of the molecule and the oxidation products are non-insecticidal. GLC data indicate the rapidity of the changes in the green sunfish with compound IX:

	% total extractives		
	CH_3S	CH_3SO	CH_3SO_2
initial	1.36	63	36
1 week	2.8	62	35.2
2 weeks	0	58	42
6 weeks	+	47	53

This compound has been found to be rapidly degradable on surfaces and does not accumulate in tissues of mice and other organisms (Kapoor et al 1972).

Effect of piperonyl butoxide synergist. As discussed in Section IV of this report, piperonyl butoxide is an effective inhibitor of microsomal oxidases. The incorporation of 1.0 ppm of this synergist with 0.1 ppm methoxychlor in the aluminum tank experiments with the green sunfish extended the length of toxic action of methoxychlor from 2 weeks to 13 weeks. This increased toxic effect was not due to any protective effect of piperonyl butoxide on methoxychlor in the water phase but rather was because of a substantial in vivo protective effect on the amount of intact methoxychlor remaining in the green sunfish.

		ppm methoxychlor alone	ppm piperonyl butoxide (10X)
water	initial	.057	.046
	1 week	.029	.019
	2 weeks	.016	.012
	3 weeks	.011	.009
	4 weeks	.0030	.0030
fish	1 week	13.5	24.0
	2 weeks	--	11.0
	4 weeks	1.75	1.78

This data together with the much longer duration of toxicity to fish of methoxychlor at lower temperatures, demonstrates the importance of O-dealkylation as a detoxication mechanism in the green sunfish. The effect will be explored in detail in Section IV.

Effects of Altered Aliphatic Substituents. As shown in Table 4, changes in the aliphatic moiety of DDT, e.g. substitution of CH_3 for Cl do not necessarily have the same biological consequences as corresponding changes in the aryl moiety. In the latter CH_3 proved to be an exceptionally effective degradaphore in reducing residue accumulations in the green sunfish. (Table 5 compare I and VII and II and IV). However, as shown in Table 5, the replacement of the $-CCl_3$ group with its methyl isostere $C(CH_3)_3$ (compare VI and XIII) had an unfavorable effect on persistence in the green sunfish. Coats et al (1974) have compared the environmental degradation of methoxychlor ($-CCl_3$) and dianisylneopentane ($-CMe_3$) and found essentially no difference in persistence. Thus the neopentyl group appears to be environmentally highly stable and we can conclude that aliphatic CH_3 groups are not as labile to mixed function oxidase attack as are aromatic CH_3 groups. This notion is confirmed by data in Table 5 for compound XV or 1,1-bis-(p-ethoxyphenyl)-iso-butane. This compound killed the green sunfish for long periods (Table 3) and is resistant to degradation in fish. There is not much difference in degradation between XV and XIV which is the corresponding dichloroethane 2,2-bis-(p-ethoxyphenyl)-1,1-dichloroethane.

In remarkable contrast however, is compound XVI with one Cl and one CH_3 in the aliphatic moiety, i.e. 3,3-bis-(p-ethoxyphenyl)-2-chloropropane. This compound exhibited much lower duration of toxicity to the green sunfish (Table 3) and, as shown in Table 5, did not accumulate in fish. Compounds XIX, 3,3-bis-(p-ethoxyphenyl)-2,2-dichloropropane and XX 3,3-bis-(p-ethoxyphenyl)-2-chloro-2-methylpropane are also isosteres which differs astonishingly in their fish toxicity as XX was non-toxic and XIX was extremely persistent and toxic. The data in Table 5 shows persistent residues of XIX, but as

none of the fish from treatment XX died, they were not analyzed,

We are unable to account for these puzzling differences but clearly, the susceptibility of aliphatic CH_3 groups to detoxication depends in the neighboring groups and their overall electronic character. This point is amplified by the nitropropane compounds. Compound XVII (Table 3) was highly persistent and toxic to fish and is closely related to XV and XVI. The presence of the 2-nitropane group does not appear to greatly affect detoxication. However, XXIII (Table IV) is 1,1-bis-(p-chorophenyl)-2-nitropropane or Prolan^R which is less persistently toxic than DDT (Table II). As shown in Table 5, (compare I with XXIII), Prolan^R is much more readily degraded in the green sunfish than DDT. Hirwe et al (1974) have studied the environmental degradation of Prolan^R and have shown that it is converted initially to an ethylene 1,1-bis-(p-chlorophenyl)-1-propene which is further oxidized to 1,1-bis-p-chlorophenyl)-acetic acid and thus is not bioaccumulated as in DDE.

Phenyl-benzyl ethers and benzylanilines. These interesting compounds resemble DDT in insecticidal action (Hirwe et al 1972) and are sterically similar. The α-trichloromethyl p-ethoxybenzyl p-chlorophenyl ether (XXX Tables 4 and 5 is persistently toxic to the green sunfish and of very low degradability. In contrast, α-trichloromethyl p-chlorobenzyl p-methoxyaniline (XXIX) was of very low persistence and toxicity. This latter compound has been shown by Hirwe et al (1972) to be readily dechlorinated, rearranged, and cleaved to p-chlorobenzoic acid and p-methoxyaniline. These two compounds represent DDT analogues with altered bridging structures between the aromatic rings and XXIX illustrates environmental degradation by cleavage of

phenyl benzyl aniline while XXX is an example of the highly persistent and stable phenyl benzyl ether.

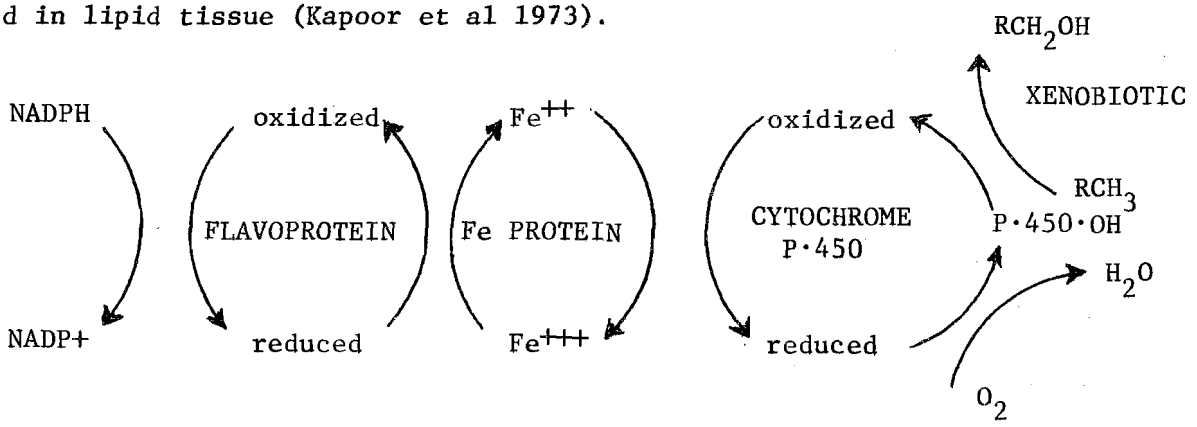
C. Comparison of Toxicity to Insects and Degradability in Fish.

The ideal insecticide should be persistent on inert surfaces, as for the control of malaria mosquitoes in houses or elm bark beetles on bark, yet biodegradable in the tissues of living animals to prevent bioaccumulation and toxicity. An important purpose of this study has been to determine if modification of the DDT-type structure could promote suitable biodegradability while retaining adequate insecticidal performance. To demonstrate that this is possible, we present in Table 6, the residual persistence of a number of the modified DDT analogues, as measured by the number of weeks they killed Culex fatigans mosquitoes resting for 1 hour on plywood treated at 2 g. per m^2, and compare this with the toxic effects on the green sunfish as shown in Tables 2, 3, and 4. Perhaps the most interesting effects here are the marked reduction in fish toxicity resulting from replacement of C_2H_5O (in XV and XVI) by CH_3 (in XXVII and XXVIII).

It is evident that adequate residual toxicity on plywood to adult mosquitoes Culex fatigans can be combined with low toxicity to fish and persistence in water. Several of the compounds investigated here appear to be useful persistent biodegradable insecticides. Compounds III and IV are covered by U.S. Patent 3,787,505 (Metcalf et al 1974). Compounds XV, XVI, XVII, XIX, XXX, XXVII and XXVIII are covered in a recent patent application (Metcalf and Coats 1974).

IV. EFFECTS OF THE SYNERGIST PIPERONYL BUTOXIDE ON THE TOXICITY AND METABOLISM OF PESTICIDES IN GREEN SUNFISH LEPOMIS CYANELLUS[1/]

As shown in Part I, the toxicity and persistence of a variety of DDT analogues to the green sunfish Lepomis cyanellus are related to the presence of degradophores on the various parts of the DDT molecule. These degradophores are molecular groupings such as CH_3, CH_3O, CH_3S, C_2H_5O etc. which act as substrates for the mixed function oxidase (MFO) enzymes. These enzymes are believed to have evolved evolutionarily to protect organisms against xenobiotic compounds and convert molecular oxygen to a hydroxylating intermediate associated with cytochrome P.450 (Conney and Burns 1972). By a variety of hydroxylating mechanisms such as ring hydroxylation, O-dealkylation, side chain oxidation, sulfoxidation, and N-dealkylation; the MFO attack results in conversion of lipid soluble, water insoluble xenobiotics, e.g. methoxychlor, into more water soluble moieties which can be excreted by the organism rather than stored in lipid tissue (Kapoor et al 1973).



Piperonyl butoxide or 3,4-methylenedioxy-6-propylbenzyl butyldiethylene glycol ether has been used for 25 years as a synergist for the botanical pyrethrins insecticides. After a great deal of study it has

1/ Material in this Section was taken from the Ph.D. Thesis of Keturah Ann Reinbold, University of Illinois, October 1974.

been shown to inhibit the in vivo detoxication of the pyrethrins esters in insects by combining with cytochrome P_{450} and interfering with the production of the $P_{450}{}^{\bullet}OH$ activated oxygen complex (Casida 1970). Thus piperonyl butoxide inhibits the action of the MFO enzymes and retards the destruction of the xenobiotic pyrethrins.

Piperonyl butoxide has been shown to have a substantial synergistic effect in preventing the detoxication of methoxychlor and other DDT type compounds having degradophores (Kapoor et al 1970, Metcalf et al 1971b). Therefore it was chosen as a general inhibitor for use with several ^{14}C radiolabeled insecticides including methoxychlor, aldrin, trifluralin, and phorate, all attacked by MFO detoxication systems, to study the degradation pathways and role of MFO enzymes in protecting the green sunfish against xenobiotics.

A. STUDIES WITH ^{14}C-METHOXYCHLOR

^{14}C ring labeled methoxychlor or 2,2-bis-(p-methoxyphenyl) 1,1,1-trichloroethane, specific activity 4.03 m Ci per m mole, radiopurity >98%, was added in triplicate to cylindrical pyrex jars containing 5 l. of synthetic hard water, with $NaHCO_3$, $CaSO_4$ $2H_2O$, $MgSO_4$, and KCl at 192,120,120, and 8 mg/l. The methoxychlor was added at 0.01 ppm alone and together with 0.1 ppm piperonyl butoxide, at 22°C with 12 hour diurnal cycle. Five green sunfish, 0.20-0.95 g. were placed in each jar. The jars were aerated and the fish were fed Daphnia 7 times between days 3 and 15. On days 1, 2, 4, 8, and 16 duplicate water samples of 1 ml. each were removed from each jar, and 1 fish was removed from each jar. The water samples were counted directly for total radioactivity and extracted with methylene chloride and concentrated for TLC and radioautography. The fish were homogenized, with methylene chloride and sodium sulfate. An aliquot of the extract was assayed

for total radioactivity and the remainder was concentrated in a small Kuderna-Danish evaporative concentrator on a water bath and evaluated with TLC and radioautography. The nature of the degradation products on the TLC plates was determined by cochromatography with model compounds (Kapoor et al 1970) and the areas of the TLC plates containing ^{14}C were scraped into liquid scintillation vials for the quantitative determination of ^{14}C, using 10 ml. of a cocktail of 120 g. naphthalene, 7 g. of 2,5-diphenyloxazole. 0.05 g. of 1,4-bis-{2-(4-methyl-5-phenyloxazoyl)}-benzene in one l. dioxane.

Results. The data obtained with methoxychlor alone and methoxychlor plus 10 x piperonyl butoxide (P.B.) shown in Figure 2 and in Tables 7 and 11. The presence of P.B. dramatically affected the rates of O-demethylation of methoxychlor to the mono-hydroxy derivative 2-(p-methoxyphenyl)-2-(p-hydroxyphenyl)-1,1,1-trichloroethane (R_f 0.53) and to the di-hydroxy derivative 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (R_f 0.40). These are the principal degradation products of methoxychlor (Kapoor et al 1970). Thus with methoxychlor alone, the green sunfish contained 0.04 ppm parent compound after 16 days as compared to 0.605 ppm when P.B. was also present at 0.1 ppm, an increase of 15 x. Similarly, with methoxychlor alone, the di-hydroxy degradation product was present after 16 days at 0.216 ppm but with the addition of 0.1 ppm P.B., the concentration of this degradation product was only 0.055 ppm, a decrease of 4.3 X. It is evident that inhibition of MFO detoxication in the green sunfish with P.B. has substantially decreased the rate of O-demethylation and excretion of methoxychlor. The inhibition of the MFO detoxication has substantially shifted degradative pathways to dehydrochlorination and methoxychlor ethylene (R_f 0.80) or 2,2-bis-(p-methoxyphenyl) 1,1-dichloro-ethylene which was present after

treatment with methoxychlor alone in trace amounts, 0.008 ppm after 16 days; was found in the P.B. treatment at 0.133 ppm, an increase of 16.6 X. The decisive effects of P.B. on methoxychlor degradation are shown in Figure 3, where the percentage of total radioactivity remaining as parent compound after 16 days, was 8 X higher following cotreatment with piperonyl butoxide than with methoxychlor alone.

B. STUDIES WITH ^{14}C-ALDRIN

^{14}C ring labeled aldrin or 1,2,3,4,10,10-hexachloro-1,4,4a,5,8, 8a-hexahydro-1,4-endo,exo-5,8-dimethanonaphthalene, specific activity 3.63 m Ci per m mole, radiopurity >98%, was evaluated for detoxication in the green sunfish Lepomis cyanellus at 0.01 ppm alone and with 0.1 ppm piperonyl butoxide, exactly as described with methoxychlor (Section II-A).

Results. Aldrin is well known to be oxidized by MFO enzymes to the 3,4-epoxide or dieldrin. It was expected that the rate of this reaction would be decreased in the experiment with aldrin plus 10 X piperonyl butoxide (P.B.). As is shown in Table 8 and in Figure 3, the presence of P.B. substantially reduced the rate of dieldrin formation (R_f 0.56) and storage in the green sunfish. Thus with aldrin alone, the dieldrin/aldrin ratio ranged from 1.6 X at day 1 to 65 X at day 16, reaching 3.29 ppm (Table 8). However after cotreatment with P.B. the dieldrin/aldrin ratio ranged from 0.30 at day 1 to 2.8 at day 16, reaching 3.021 ppm dieldrin. The inhibitory effect of P.B. was also evident in the production of further metabolic products such as 3-OH dieldrin (R_f 0.31) and 3-C=0 dieldrin (R_f 0.23) both of which formed considerably more slowly in the presence of P.B. The decisive effects of P.B. on aldrin metabolism are shown in Figure 3, which indicates that the percentage of total radioactivity remaining as parent compound after 16 days was 17 X higher after cotreatment with P.B. than with aldrin alone.

C. STUDIES WITH ^{14}C-TRIFLURALIN

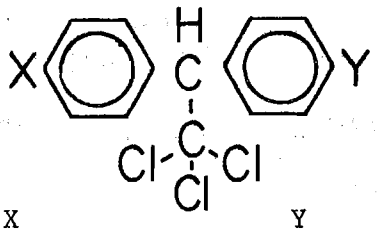
^{14}C-ring labeled trifluralin or α,α,α-trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine, specific activity 3.02 m Ci per m mole, radiopurity >99%, was evaluated for detoxication in the green sunfish Lepomis cyanellus at 0.01 ppm alone and with 0.1 ppm piperonyl butoxide, exactly as described with methoxychlor (Section IV-A).

Results. Trifluralin contains the $N(C_3H_7)$ group which should be particularly susceptible to N-dealkylation by the microsomal enzyme (MFO) system. As shown in Table 9 trifluralin disappeared from the green sunfish much more rapidly when applied to water alone than in the presence of 10 X P.B. Thus with trifluralin alone the green sunfish contained only 0.005 ppm parent compound after 16 days as compared to 0.225 ppm in the presence of P.B. The difference in concentrations was 45 X. As shown in Table 9, there was a decrease in the amounts of the NHC_3H_7 metabolite R_f 0.84 produced by N-dealkylation, in the fish exposed to P.B. However, the P.B. treatment produced no significant effect on the rate of reduction of the 6-NO_2 group of trifluralin to 6-NH_2 (R_f 0.63). Figure 3 provides a striking illustration of the effect of P.B. in decreasing the degradation of trifluralin, showing that the percentage of total radioactivity remaining as parent compound after 16 days, was 15 X higher after cotreatment with P.B. than with trifluralin alone.

Table 1. Comparative Uptake from Water of ^{14}C DDT and ^{3}H Methoxychlor by *Tilapia* and *Lepomis* (Reinbold et al 1971)

H_2O - ppm	days exposure	*Tilapia* - ppm		*Lepomis* - ppm	
		DDT	methoxychlor	DDT	methoxychlor
0.001	3	1.3	0.8	0.5	0.8
	10	0.7	0.2	0.6	0.3
	31	6.8	0.2	3.9	0.2
0.003	3	4.4	1.8	2.2	--
	10	2.5	0.3	1.7	0.6
	31	12.0	0.6	10.2	0.6
0.01	3	16.4	9.0	--	7.4
	10	13.9	1.0	8.6	1.9
	31	166.0	2.0	40.2	2.7

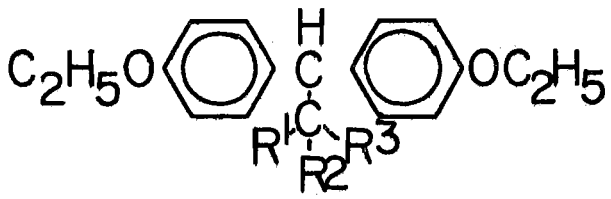
Table 2 . Toxicity of Disubstituted Diaryl Trichloroethanes to the Green Sunfish



	X	Y	0.1 ppm in water duration of toxicity in days 1/ summer	winter
I	Cl	Cl (DDT)	18	
II	CH_3O	CH_3O (methoxychlor)	2	41
III	C_2H_5O	C_2H_5O	22	
IV	C_2H_5O	CH_3	0	15
V	C_2H_5O	C_2H_5		5
VI	C_2H_5O	C_3H_7O		41
VII	CH_3	Cl	0	
VIII	C_2H_5	Cl		0
IX	CH_3O	CH_3S	0	
X	CH_3O	OCH_2O (3,4)		28
XI	CH_3O	CH_3		14
XII	CH_3	C_2H_5	0	

1/ 5 fish used per tank, when killed restocked at weekly intervals.

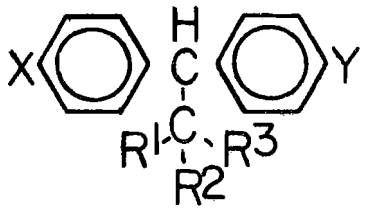
Table 3. Toxicity of Diethoxy Diaryl Substituted Ethanes to the Green Sunfish.



				0.1 ppm in water duration of toxicity in days 1/	
	R^1	R^2	R^3	summer	winter
III	Cl	Cl	Cl	22	
XIII	CH_3	CH_3	CH_3		76
XIV	Cl	H	Cl		62
XV	CH_3	H	CH_3	>42	52
XVI	Cl	H	CH_3	4	60
XVII	CH_3	H	NO_2		64
XVIII	C_2H_5	H	NO_2		77
XIX	CH_3	Cl	Cl	34	80
XX	Cl	CH_3	CH_3		0
XXI	cyclopropyl-Cl_2			23	

1/ 5 fish used per tank, when killed restocked at weekly intervals.

Table 4. Toxicity of Various Disubstituted DDT Analogues to Green Sunfish.



	X	Y	R^1	R^2	R^3	0.1 ppm in water duration of toxicity in days summer	winter
XXII	Cl	Cl	Cl	H	CH_3		99
XXIII	Cl	Cl	CH_3	H	NO_2	3	
XXIV	Cl	Cl	cyclopropyl-Cl_2			5	
XXV	CH_3O	CH_3O	Cl	Cl	CH_3		34
XXVI	CH_3O	CH_3O	Cl	H	Cl		0
XXVII	CH_3	C_2H_5O	CH_3	H	CH_3		0
XXVIII	CH_3	C_2H_5O	Cl	H	CH_3		0
XXIX	$CH_3OC_6H_4NHCH(CCl_3)C_6H_4Cl$						0
XXX	$ClC_6H_4OCH(CCl_3)C_6H_4OC_2H_5$						17

Table 5. Residues in Water and Green Sunfish from DDT Analogues with Altered Substituents at 0.1 ppm.

		Water Residue – ppm weeks after treatment						Fish Residue – ppm weeks after treatment			
		0	1	2	3	4	6–7	1	2	4	6–8
I.	$ClC_6H_4CH(CCl_3)C_6H_4Cl$	–	.0076	.0059	.0027	.0026	.000045 (DDT-T .0002)	3.82	—	7.17	2.20 (DDT-T 2.84)
II.	$CH_3OC_6H_4CH(CCl_3)C_6H_4OCH_3$	.0575	.029	.0165	.0112	.0030	—	13.5	—	1.75	—
IV.	$CH_3C_6G_4CH(CCl_3)C_6H_4OC_2H_5$	.125	.022	.0043	—	—	.0023	1.96	—	—	.41
V.	$C_2H_5OC_6H_4CH(CCl_3)C_6H_4C_2H_5$	.033	.0054	.0023	—	.0019	—	2.03	—	—	.70
VI.	$C_2H_5OC_6H_4CH(CCl_3(C_6H_4OC_3H_7$	.044	—	.0093	.0034	.0017	.0010	3.57	3.76	2.49	2.07
VII.	$CH_3C_6H_4CH(CCl_3)C_6H_4Cl$	.060	.0049	.0014	—	—	.0005	13.07	—	—	.12
VIII.	$C_2H_5C_6H_4CH(CCl_3)C_6H_4Cl$	.060	—	.0021	.0008	.0004	—	29.52	—	4.57	—
IX.	$CH_3OC_6H_4CH(CCl_3)C_6H_4SCH_3$	.0008	.0002	—	—	—	—	—	—	—	0.0
X.	$CH_3OC_6H_4CH(CCl_3)C_6H_4OCH_2$9(3,4)	.025	—	.0064	.0034	.0029	.0018	17.53	17.77	18.01	6.66

Table 5. (Cont.) Residues in Water and Green Sunfish from DDT Analogues with Altered Substituents at 0.1 ppm.

		Water Residue - ppm weeks after treatment						Fish Residue - ppm weeks after treatment			
		0	1	2	3	4	6-7	1	2	4	6-8
XIII.	$C_2H_5OC_6H_4CH(CMe_3)C_6H_4OC_2H_5$	.0038	--	.0068	.0007	.0005	.0003	10.25	13.41	6.17	7.50
XIV.	$C_2H_5OC_6H_4CH(CHCl_2)C_6H_4OC_2H_5$	--	.066	.083	.047	.059	.046	--	3.30	8.62 (5 wks)	8.55
XV	$C_2H_5OC_6H_4CH(CHMe_2)C_6H_4OC_2H_5$	.039	0.28	.015	.030	.011	.009 (5 wks)	3.75	3.76 (3 wks)	2.29	4.94
XVI.	$C_2H_5OC_6H_4CH(CHMeCl)C_6H_4OC_2H_5$	--	.15	.029	.030	.026	.023	0	0	0	0
XIX.	$C_2H_5OC_6H_4CH(CCl_2Me)C_6H_4OC_2H_5$	.26	.028	.011	.010	.006	.006 (5 wks)	1.30	--	1.29	.94
XX.	$C_2H_5OC_6H_4CH(CClMe_2)C_6H_4OC_2H_5$	.042	.011	.012	.010	.013					
XXI	$C_2H_5OC_6H_4CH(CH_2CCl_2)C_6H_4OC_2H_5$	.018	.033	.0064	.0047	.0022	.0006 (8 wks)	2.09	--	--	.59
XXIII.	$ClC_6H_4CH(CHMeNO_2)C_6H_4Cl$	--	.0072	.0036	.0009	.0004	.00002	12.44	--	--	.041
XXIX.	$CH_3OC_6H_4NHCH(CCl_3)C_6H_4Cl$	0	0	0	0	0	0	--	--	.80	--
XXX	$ClC_6H_4OCH(CCl_3)C_6H_4OC_2H_5$	.077	--	.013	.012	.005		17.94	18.4	18.2	11.2

Table 6. Comparison of Insecticidal Toxicity and Persistence with Fish Toxicity for DDT Analogues

	compound	topical LD_{50} *Musca domestica*	weeks for kill *Culex fatigans*	weeks for kill *Lepomis cyanellus* Summer	weeks for kill *Lepomis cyanellus* Winter
I.	$ClC_6H_4CH(CCl_3)C_6H_4Cl$ (DDT)	14	52	18	
II.	$CH_3OC_6H_4CH(CCl_3)C_6H_4Cl$ (methoxychlor)	45	66	2	41
III.	$C_2H_5OC_6H_4CH(CCl_3C_6H_4CH_3$	9	80	0	15
IV.	$CH_3OC_6H_4CH(CCl_3)C_6H_4CH_3$	23.5	35		14
IX.	$CH_3OC_6H_4CH(CCl_3)C_6H_4SCH_3$	32	1	0	
XIII.	$C_2H_5OC_6H_4CH(CMe_3)C_6H_4OC_2H_5$	37.5	20		76
XV.	$C_2H_5OC_6H_4CH(CHMe_2)C_6H_4OC_2H_5$	21.5	50		52
XVI.	$C_2H_5OC_6H_4CH(CHClMe)C_6H_4OC_2H_5$	9.5	>76	4	60
XIX.	$C_2H_5OC_6H_4CH(CCl_2Me)C_6H_4OC_2H_5$	17.5	--		80
XX.	$C_2H_5OC_6H_4CH(CClMe_2)C_6H_4OC_2H_5$	115	--		0

Table 7. Concentration of ^{14}C-Methoxychlor and Degradation Products in Green Sunfish and Water from Treatment at 0.01 ppm.

Methoxychlor

	ppm-methoxychlor equivalents						
	Fish					Water	Hydrolyzed Water
	Day 1	Day 2	Day 4	Day 8	Day 16	Day 16	Day 16
Total ^{14}C	5.496	3.729	4.036	1.781	2.055	0.0037	
Methoxychlor ethylene (R_f 0.80[a];0.29[b])	0.098	0.049	0.027	0.008	0.008	0.000011	0.000001
Methoxychlor (R_f 0.80;0.22)	0.805	0.867	0.331	0.073	0.040	0.000127	
Unknown I (R_f 0.65;0.17)	0.015	0.021	0.020	0.008	0.019	0.000040	0.000002
Mono-OH (R_f 053;0.13)	0.357	0.255	0.791	0.310	0.417	0.000352	0.000032
DiOH + (R_f 0.40;0.00)	0.041	0.042	0.122	0.126	0.216	0.000276	0.000132
DiOh ethylene							
Unknown II (R_f 0.25;0.00)						0.000017	0.000022
DiOH COOH (R_f 0.07:0.00)	0.040	0.049	0.051	0.039	0.047	0.000003	0.000006
Polar (R_f 0.0)	0.063	0.079	0.067	0.041	0.039	0.000003	0.000005
Unextractable	4.077	2.367	2.628	1.176	1.269	0.002670	

Table 7. Continued

Methoxychlor + PB (0.1 ppm)

	(ppm - methoxychlor equivalents						
	Fish					Water	Hydrolyzed Water
	Day 1	Day 2	Day 4	Day 8	Day 16	Day 16	Day 16
Total ^{14}C	4.593	7.272	7.512	4.696	2.753	0.0037	
Methoxychlor ethylene (R_f 0.80;0.29)	0.237	0.347	0.472	0.259	0.133	0.000075	0.000004
Methoxychlor (R_f 0.80;022)	2.986	4.276	4.652	2.474	0.605	0.000764	
Unknown I (R_f 0.65;0.17)	0.006	0.011	0.020	0.034	0.010	0.000057	0.000004
Mono-OH (R_f 0.53;0.13)	0.102	0.219	0.320	0.251	0.522	0.000408	0.000043
DiOH + (R_f 0.4-;0.00)	0.009	0.013	0.041	0.023	0.055	0.000168	0.000155
DiOH ethylene							
Unknown II (R_f 0.25;0.00)						0.000016	0.000028
DiOH COOH (R_f 0.07;0.00)	0.005	0.012	0.022	0.024	0.023	0.000002	0.000013
Polar (R_f 0.0)	0.028	0.026	0.071	0.028	0.063		0.000003
Unextractable	1.220	2.367	1.913	1.603	1.341	0.001960	

[a] TLC with petroleum ether (b.p. 60-68°C) - chloroform - methanol, 3:2:1.

[b] TLC with diethyl ether - petroleum ether, 1:9.

Table 8. Concentrations of ^{14}C-Aldrin and Degradation Products in Green Sunfish and Water from Treatment at 0.01 ppm.

	Aldrin						
	ppm-aldrin equivalents						
	Fish					Water	Hydrolyzed Water
	Day 1	Day 2	Day 4	Day 8	Day 16	Day 16	Day 16
Total ^{14}C	5.632[a]	5.694[a]	4.934	5.537	3.800	0.0013	
Aldrin (R_f 0.68[b])	2.015	1.293	0.403	0.179	0.051	0.000022	0.0000008
Dieldrin (R_f 0.56)	3.180	3.971	3.907	4.938	3.289	0.000867	0.0000027
Unknown I (R_f 0.44)	0.009	0.004	0.072	0.044	0.010	0.000008	0.0000001
9-OH dieldrin (R_f 0.31)	0.017	0.025	0.110	0.063	0.062	0.000013	—
9-C=O dieldrin (R_f 0.23)	0.023	0.018	0.009	0.021	0.016	0.000011	—
Unknown II (R_f 0.15)	0.007	0.003	—	0.0008	--	0.000011	0.0000021
DiOH trans aldrin (R_f 0.05)	0.0045	0.003	0.005	0.007	0.007	0.000016	0.0000045
Polar (R_f 0.0)	0.006	0.007	0.0165	0.009	0.006	0.000032	0.0000794
Unextractable	na[c]	na[c]	0.412	0.275	0.357	0.000230	

Table 8. Continued

Aldrin + PB (0.1 ppm)

	ppm aldrin equivalents						
	Fish					Water	Hydrolyzed Water
	Day 1	Day 2	Day 4	Day 8	Day 16	Day 16	Day 16
Total ^{14}C	5.532	7.611	7.688	6.700	4.738	0.0013	
Aldrin (R_f 0.68)	3.9599	5.179	4.572	3.455	1.076	0.000043	0.0000032
Dieldrin (R_f 0.56)	1.1881	1.572	2.422	2.693	3.021	0.000583	0.0000062
Unknown I (R_f 0.44)	0.00085	0.045	0.018	0.011	0.002	0.000008	0.0000003
9-OH dieldrin (R_f 0.31)	0.0112	0.017	0.0225	0.017	0.014	0.000005	—
9-C=O dieldrin (R_f 023)	0.0015	0.020	0.015	0.008	0.006	0.000003	—
Unknown II (R_f 0.15)	0.0060	0.018	0.017	0.007	0.001	0.000010	0.0000028
DiOH trans aldrin (R_f 0.05)	0.0056	0.005	0.004	0.003	0.009	0.000020	0.0000025
Polar (R_f 0.0)	0.0078	0.012	0.0185	0.013	0.019	0.000028	0.0001250
Unextractable	0.351	0.743	0.599	0.493	0.590	0.000460	

[a] Including estimated ppm unextractable.

[b] TLC with hexane - diethyl ether, 1:1.

[c] Not analyzed.

Table 9. Concentration of ^{14}C-Trifluralin and Degradation Products in Green Sunfish and Water from Treatment at 0.01 ppm.

	Trifluralin						
	ppm-trifluralin equivalents						
	Fish					Water	Hydrolyzed Water
	Day 1	Day 2	Day 4	Day 8	Day 16	Day 16	Day 16
Total ^{14}C	3.031	2.709	2.761	0.650	0.234	0.0042	
Trifluralin ($2,6\text{-}NO_2\text{-}4\text{-}CF_3\text{-}C_6H_2N(C_3H_7)_2$) ($R_f$ 0.93[a])	1.979	1.611	1.058	0.137	0.005	0.0000068	0.0000005
$2,6,NO_2\text{-}4\text{-}CF_3\text{-}C_6H_2NHC_3H_7$ (R_f 0.84)	0.111	0.084	0.091	0.022	0.002	0.0000194	0.0000143
$2\text{-}NO_2\text{-}4\text{-}CF_3\text{-}6NH_2\text{-}C_6H_2N(C_3H_7)_2$ (R_f 0.63)	0.018	0.009	0.010	0.004	0.001	0.0000042	0.0000036
$1,2\text{-}(N\text{-}CH(C_2H_5)N\text{-}C_3H_7)\text{-}3\text{-}NO_2\text{-}5\text{-}CF_3$ (R_f 0.53)	0.002	0.001	0.001	0.002	0.003	0.0000048	0.0000028
$2,6\text{-}NO_2\text{-}4\text{-}CF_3\text{-}C_6H_2NH_2$ (R_f 0.47)	0.017	0.005	0.011	0.002	—	0.0000211	0.0000159
Unknown I (R_f 0.39)	0.075	0.095	0.082	0.050	0.009	0.0002000	0.0000816
Unknown II (R_f 0.31)	0.027	0.012	0.020	0.005	0.005	0.0000709	0.0000167
$1,2\text{-}(N\text{-}CH(C_2H_5)\text{-}NH)\text{-}3\text{-}NO_2\text{-}5\text{-}CF_3\text{-}C_6H_2$ (R_f 0.19)	0.020	0.028	0.048	0.017	0.021	0.0002063	0.0000204
Unknown III (R_f 0.17)	0.044	0.028	0.055	0.015	0.017	0.0000578	0.0000245
Unknown IV (R_f 0.13)						0.0001529	0.0000265
Unknown V (R_f 0.10)	0.068	0.081	0.116	0.066	0.042	0.0001334	0.0000530
Unknown VI (R_f 0.07)						0.0001751	0.0000611
Polar (R_f 0.0)	0.374	0.492	1.010	0.263	0.086	0.0000767	0.0000685
Unextractable	0.295	0.263	0.259	0.067	0.044	0.00268	

Table 9. Continued

	Trifluralin + PB (0.1 ppm)						
	ppm-trifluralin equivalents						
	Fish					Water	Hydrolyzed Water
	Day 1	Day 2	Day 4	Day 8	Day 16	Day 16	Day 16
Total ^{14}C	4.014	4.215	5.233	3.461	0.580	0.0013	
Trifluralin ($2,6\text{-}NO_2\text{-}4\text{-}CF_3\text{-}C_6H_2N(C_3H_7)_2$) ($R_f$ 0.93[a])	3.634	3.557	4.683	3.101	0.225	0.0000679	0.0000007
$2,6\text{-}NO_2\text{-}4\text{-}CF_3\text{-}C_6H_2NHC_3H_7$ (R_f 0.84)	0.023	0.030	0.048	0.027	0.006	0.0000312	0.0000158
$2\text{-}NO_2\text{-}4\text{-}CF_3\text{-}6NH_2\text{-}C_6H_2N(C_3H_7)_2$ (R_f 0.63)	0.033	0.018	0.025	0.006	0.0015	0.0000031	0.0000013
$1,2\text{-}(N\text{-}CH(C_2H_5)N\text{-}C_3H_7)\text{-}3\text{-}NO_2\text{-}5\text{-}CF_3$ (R_f 0.53)	0.001	—	0.0025	—	0.001	0.0000070	0.0000019
$2,6\text{-}NO_2\text{-}4\text{-}CF_3\text{-}C_6H_2NH_2$ (R_f 0.47)	0.015	0.016	0.011	0.008	0.001	0.0000147	0.0000062
Unknown I (R_f 0.39)	0.015	0.018	0.008	0.010	0.013	0.0000785	0.0000224
Unknown II (R_f 0.31)	0.004	0.009	0.007	0.004	0.004	0.0000069	0.0000062
$1,2\text{-}(N\text{-}CH(C_2H_5)\text{-}NH)\text{-}3\text{-}NO_2\text{-}5\text{-}CF_3\text{-}C_6H_2$ (R_f 0.19)	0.0115	0.007	0.011	0.006	0.016	0.0000935	0.0000064
Unknown III (R_f 0.17)	0.008	0.002	0.007	0.007	0.008	0.0000295	0.0000080
Unknown IV (R_f 0.13)						0.0000227	0.0000225
Unknown V (R_f 0.10)	0.007	0.025	0.011	0.013	0.022	0.0000363	0.0000116
Unknown VI (R_f 0.07)						0.0000396	0.0000090
Polar (R_f 0.0)	0.074	0.292	0.105	0.099	0.2125	0.0000284	0.0000172
Unextractable	0.188	0.241	0.313	0.180	0.070	0.00071	

[a] TLC with hexane - acetone - methanol, 90:10:2 in unsaturated tank.

Table 10. Carbofuran Residues in Water and Green Sunfish after Treatment with 0.1 ppm alone and with 1.0 ppm Piperonyl Butoxide

Days After Treatment	13-20°C carbofuran-ppm alone		13-20°C carbofuran-ppm P.B.[a/]		23-32°C carbofuran-ppm alone		23-32°C carbofuran-ppm P.B.	
	water	fish	water	fish	water	fish	water	fish
0	.081		.094		.076		.073	
3	.072		.067		.001		.001	
7	.056		.058		ND[b/]		.0007	
14	.016		.031		ND		ND	
15						<.00001		<.00001
21	.011		.011					
24		<.00001		<.00001				

a/ piperonyl butoxide

b/ none detectable

Table 11. Methoxychlor Residues in Water and Green Sunfish after Treatment with 0.1 ppm alone and with 1.0 ppm Piperonyl Butoxide

Days After Treatment	13-20°C methoxychlor-ppm				23-32°C methoxychlor-ppm			
	alone		P.B.[a/]		alone		P.B.	
	water	fish	water	fish	water	fish	water	fish
0	.0497		.0288		.055		.061	
1		11.38		6.57		8.95(2d)		19.37
3	.0542		.0447		.040		.029	
7	.0455		.0474		.026	14.6(5d)	.027	6.29(5d)
9		6.83		12.68				
14	.0455		.0364		.013		.014	
17		6.27		10.30(16d)				2.72(16d)
21	.0248		.0277		.006		.011	
28	.0339		.0647		.0638		.006	
30		trace		11.58		1.88(29d)		1.21(31d)

[a/] piperonyl butoxide

V. DEGRADABILITY OF CARBOFURAN IN WATER AND GREEN SUNFISH

Carbofuran or 2,2-dimethyl-2,3-dihydrobenzo-furanyl-7 N-methyl-carbamate is widely used as a soil insecticide in replacement for the organochlorine insecticides aldrin and heptachlor. Carbofuran is believed to be a substantially biodegradable insecticide. Therefore it was of interest to compare its degradability in water and in the green sunfish under identical conditions to those used for the evaluation of DDT and analogues (Section III). The data on carbofuran obtained by GLC (Section II) are shown in Table 10 and are to be compared with those for methoxychlor, a moderately biodegradable compound as shown in Table 11. The comparison shows very clearly the rapid degradation of carbofuran in both water and the tissues of the green sunfish as compared with methoxychlor which is itself much more degradable than DDT (Table 5). Piperonyl butoxide had no appreciable effect on the rate of degradation of carbofuran (Table 10) but had a marked effect on the degradation of methoxychlor (Table 11).

VI. CONCLUSIONS

The persistence in the aquatic environment and bioaccumulation in fish has been a major reason for the unsuitability of DDT as an insecticide and its consequent ban from use. There is, however, a need for insecticides which have residual persistence on inert surfaces yet are free from these objectionable properties in the aquatic environment. Incorporation of groups attacked by the multifunction oxidases (degradophores)into the DDT-type molecule affords a practical solution to this problem. The experiments reported here demonstrate that methoxy and ethoxy groups on the aryl rings of the DDT-type molecule do not serve as highly effective degradophores in regard to fish toxicity, e.g. methoxychlor. However, methyl and ethyl groups in the aryl position are far more effective as degradophores in the DDT-type molecule. DDT analogues with combinations of methyl and alkoxy groups, e.g. 2-(p-methylphenyl)-2-(p-ethoxyphenyl) -1,1,1-trichloroethane combine low toxicity and bioaccumulation in fish with adequate insecticidal activity.

Alterations in the aliphatic portion of the DDT-type molecule were also investigated for their role in promoting biodegradability. Isosteric replacement of Cl atoms of the CCl_3 moiety with methyl groups produced surprisingly little improvement in fish toxicity, i.e. $C(CH_3)_3$ was as persistently toxic as CCl_3. The most useful combination was the chloroethyl group, $ClCHCH_3$, which combined substantially reduced fish toxicity with adequate insecticidal activity. When this aliphatic grouping was combined with the optimum aryl substitution, e.g. 2-(p-methylphenyl)-2-(p-ethoxyphenyl)-2-chloropropane a highly insecticidal compound was produced which was of very low fish toxicity. This sort of methodology in incorporating suitable degradophores into pesticides and other chemicals liberated into the environment should be very useful in eliminating severe problems of fish toxicity and bioaccumulation.

The critical role of the multifunction oxidase enzymes in determining bioaccumulation in fish was evaluated using ^{14}C-radiolabeled pesticides in combination with the multifunction oxidase inhibitor piperonyl butoxide. Fish exposed to piperonyl butoxide together with methoxychlor accumulated 15 times as much methoxychlor over a 16 day period as with methoxychlor alone. With piperonyl butoxide plus aldrin accumulation of aldrin and dieldrin was increased 21 times and with piperonyl butoxide plus trifluralin, accumulation of trifluralin was increased 45 times. These studies not only demonstrate the critical role of the multifunction oxidases of fish in detoxifying and eliminating xenobiotics but also emphasize the potential problems resulting from combinations of pollutants in the aquatic environment.

VII. SIGNIFICANCE OF RESEARCH

A major problem resulting from the widespread usage of pesticides has been fish toxicity and bioaccumulation in fish tissues. Data has been presented in this report to demonstrate that pesticides can be designed by the incorporation of degradophores to produce compounds that are relatively persistent on inert surfaces yet biodegradable and non-accumulative in fish tissues. Substitution of intentionally designed biodegradable compounds for persistent non-biodegradable pesticides could be a major factor in preventing disasterous pollution of water and aquatic organisms.

The biochemical studies have shown the astonishing effects of the synergist piperonyl butoxide in the aquatic environment in preventing the detoxication of a variety of pesticides which accumulated to tissue residues of 8 to 17 fold higher in its presence. These exceptional and undesirable environmental effects resulting from combinations of pesticides illustrate the potential hazards of mixtures of compounds in the environment. Clearly there is a need for additional investigation of the biological effects of mixtures of chemicals entering the aquatic environment.

REFERENCES

Casida, J. E. J. Agr. Food Chem. 18, 753 (1970).

Coats, Joel R. Toxicity and Metabolism of Three Methoxychlor Analogues with Altered Aliphatic Moieties, and the Development of Some New DDT Analogues. Thesis for the Ph.D. Degree, University of Illinois, June 1974.

Coats, J. R., R. L. Metcalf, and I. P. Kapoor. Pesticide Biochem. Physiol. 4, 201 (1974).

Conney, A. H. and J. J. Burns. Science 178, 576 (1972).

Harrison, H. L., O. L. Loucks, J. W. Mitchell, D. F. Parkhurst, C. R. Tracy, D. G. Watts, and V. J. Yannacone, jr. Science 170, 503 (1970).

Hirwe, A. S., R. L. Metcalf, and I. P. Kapoor. J. Agr. Food Chem. 20, 818 (1972).

Hirwe, A. S., R. L. Metcalf, Po-Yung Lu, and Li-Chun Chio. In Press. Pesticide Biochem. Physiol. (1974).

Holden, E. R. J. Ass. Office Anal. Chem. 56, 713 (1973).

Kapoor, I. P., R. L. Metcalf, A. S. Hirwe, J. R. Coats, and M. S. Khalsa. J. Agr. Food Chem. 21, 310 (1973).

Kapoor, I. P., R. L. Metcalf, A. S. Hirwe, and G. K. Sangha. J. Agr. Food Chem. 18, 1145 (1970).

Metcalf, R. L. and Joel Coats. U. S. Pat. Applied For (1974).

Metcalf, R. L., I. P. Kapoor, and A. S. Hirwe. Bull. W.H.O. 44, 363 (1971b).

Metcalf, R. L., I. P. Kapoor, and A. S. Hirwe. Insecticidal Biodegradable Analogues of DDT. U. S. Pat. 3,787,505 (1974).

Metcalf, R. L., G. K. Sangha, and I. P. Kapoor. Environ. Sci. Technol. 5, 709 (1971a).

Reinbold, Keturah A. Effects of the Synergist Piperonyl Butoxide on Toxicity and Metabolism of Pesticides in Green Sunfish (Lepomis cyanellus Rafmesque) Thesis for the Ph.D Degree, University of Illinois, October 1974.

Reinbold, K. A., I. P. Kapoor, W. F. Childers, W. N. Bruce, and R. L. Metcalf. Bull. Ill. Nat. Hist. Survey Art. 6, June (1971).

U. S. Environmental Protection Agency "An Evaluation of DDT and Dieldrin in Lake Michigan" EPA-R3-72-003 August, 1972.

Woodwell, G. M., C. F. Wurster, jr. and P. A. Isaacson. Science 156, 821 (1967).

Youngs, W. G., W. H. Gutenmann, and D. J. Lisk. Environ. Sci. Technol. 6, 451 (1972).

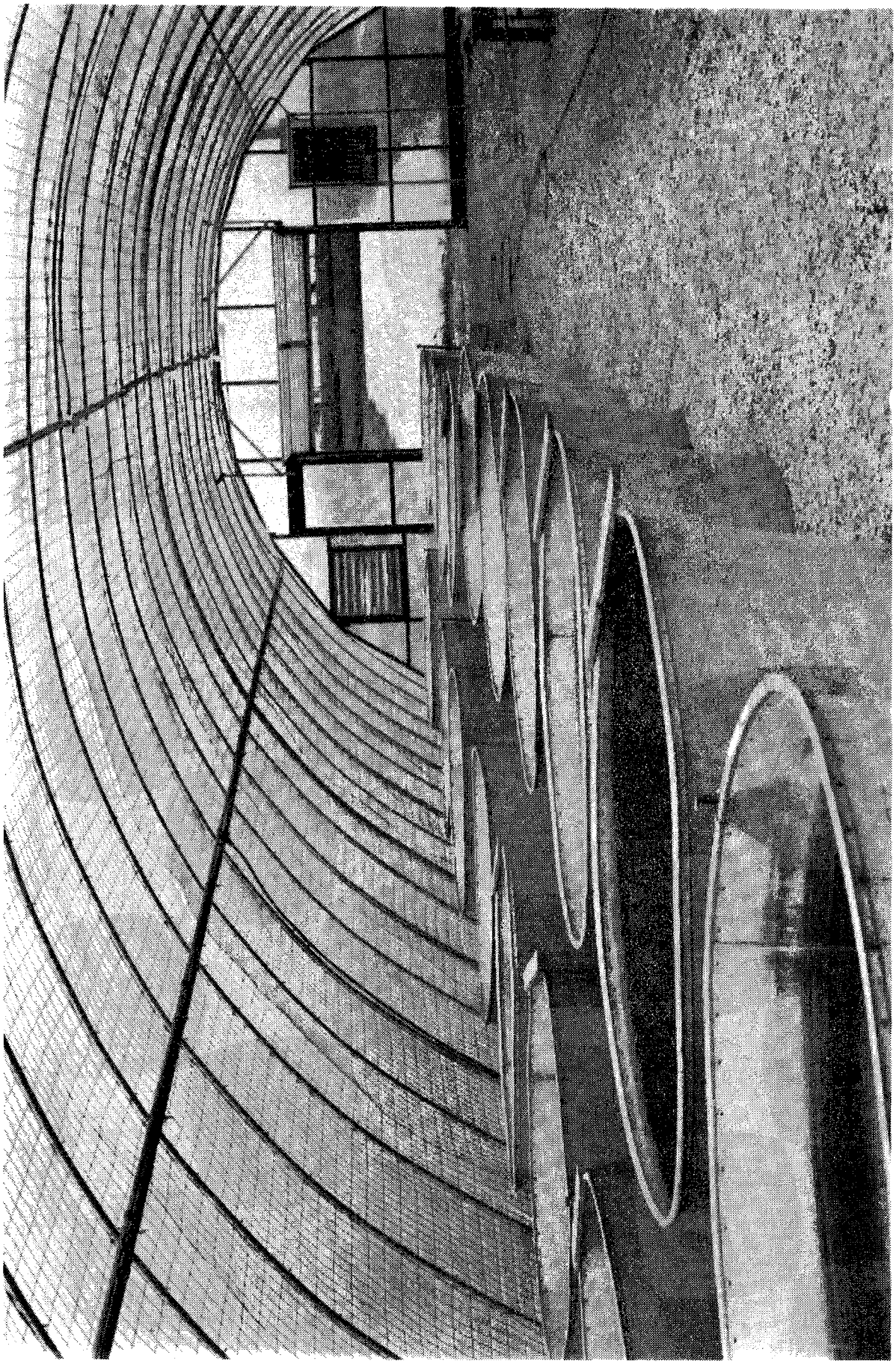
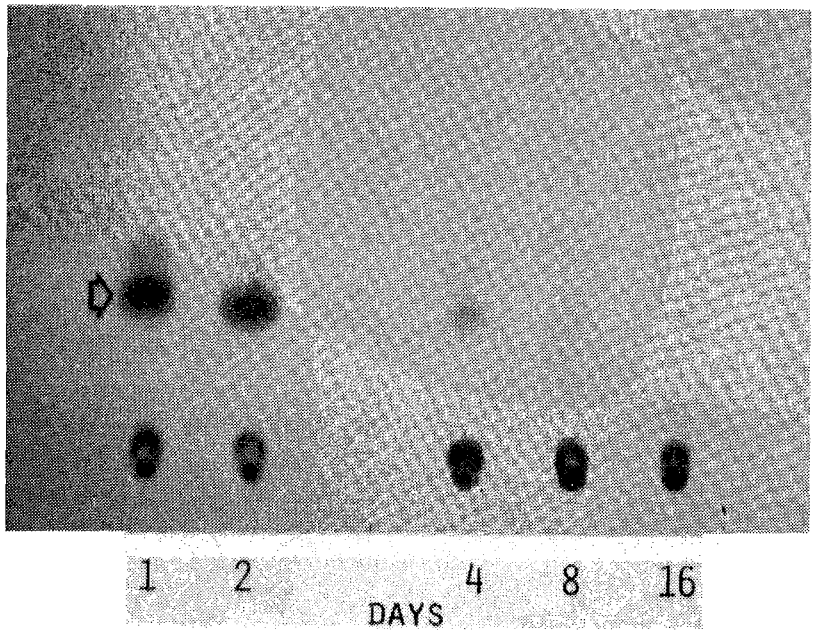


Figure 1. Aluminum pools in which fish toxicity experiments were conducted.



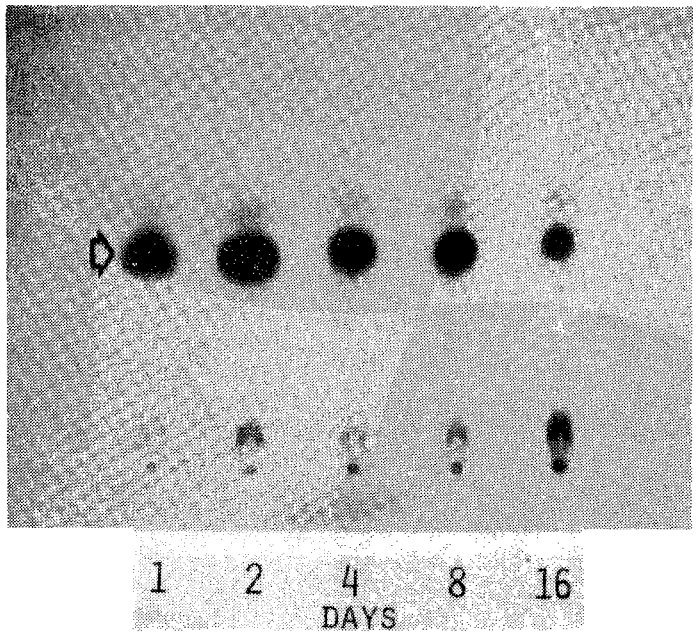


Figure 2. Radioautograms of thin-layer chromatograms of extracts of green sunfish exposed to methoxychlor (above) and methoxychlor + PB (below). The thin-layer chromatograms were developed in diethyl ether--hexane, 1:9. The arrows indicate the locations of methoxychlor.

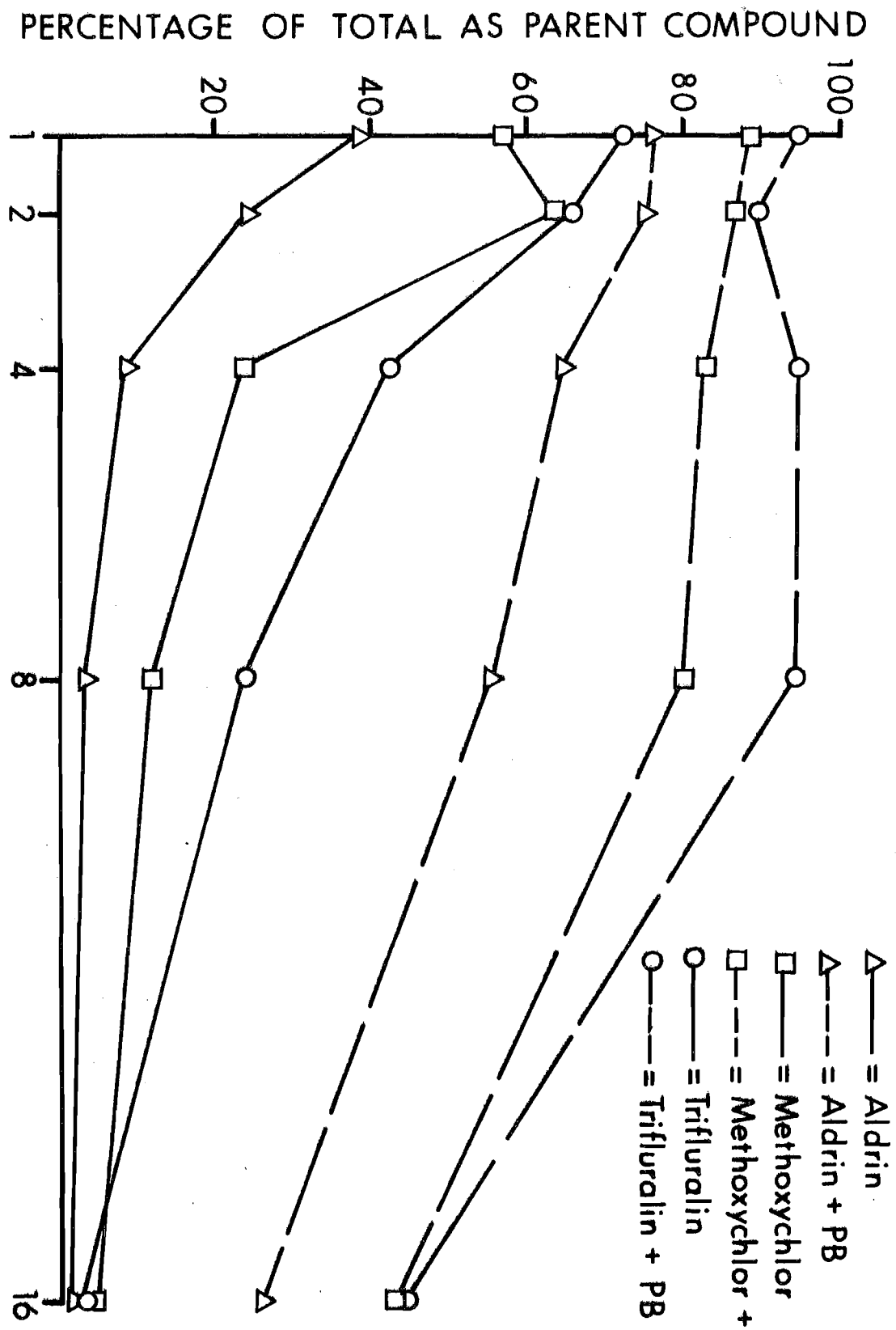


Figure 3. Comparison of percentage of total radioactivity in the form of parent compound in extracts of green sunfish exposed to pesticides alone versus those exposed to pesticides plus PB.