To the Graduate Council:

I am submitting herewith a thesis written by Nabil B. Shamiyeh entitled "Interactions of Heptachlor (A Chlorinated Hydrocarbon Insecticide) and Soil Microflora." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Agricultural Biology.

Leander F. Johnson, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

4

Ag-VetMed
Thesis
77
.S435
cop. 2

INTERACTIONS OF HEPTACHLOR (A CHLORINATED HYDROCARBON INSECTICIDE) AND SOIL MICROFLORA

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Nabil B. Shamiyeh

March 1977

1321076

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to the following: Dr. Leander F. Johnson, Professor, for his constant encouragement and valuable advice; Dr. Carroll J. Southards, Professor and Department Head, for allowing me to pursue and achieve my goals; Dr. Charles D. Pless, Associate Professor, for serving on my committee and for his valuable advice; the Agricultural Biology staff for their patience and encouragement; and my wife, Anna Jean, and my two children, Paul and Kimberly, for their understanding and encouragement.

ABSTRACT

The effect of heptachlor on soil microorganisms was studied under field, greenhouse, and laboratory conditions. Growth of many soil microorganisms was inhibited on culture media containing heptachlor. At a concentration of 25 ppm, heptachlor was bactericidal to 63% of the bacteria tested. Heptachlor, at 100 ppm in agar media used for isolating microorganisms from soil, prevented the development of 89% of the bacteria, 81% of the actinomycetes, and 50% of the fungi that appeared on isolation plates without heptachlor. After heptachlor was added to soil, fungal populations declined and bacterial populations increased. Numbers of bacteria were related to amount of heptachlor added; higher concentrations in soil resulted in larger populations. A selective increase in numbers of fungi which would grow on media containing heptachlor at 100 ppm occurred in soils amended with heptachlor in amounts ordinarily used in field practices (1 lb./A.), but a similar increase of heptachlor-resistant bacteria occurred only in soils amended with much higher amounts of heptachlor. One bacterium, Bacillus cereus, isolated from soil was found to degrade heptachlor to its parent compound, chlordene.

TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION	1
II.	LITERATURE REVIEW	4
III.	MATERIALS AND METHODS	8
	Effect of Heptachlor on Growth of Soil Microorganisms in Pure Culture	8
	Effect of Heptachlor on Microbial Populations in Soil	9
	Microbial Degradation of Heptachlor	10
	Isolation of heptachlor resistant soil microorganisms	10
	Effect of crop residues on heptachlor degradation	11
	Volatilization of heptachlor	12
	Gas chromatographic conditions	12
IV.	RESULTS	13
	Effect of Heptachlor on Growth of Soil Microorganisms in Pure Culture	13
	Effect of Heptachlor on Soil Microbial Populations	15
	Fungi	15
	Actinomycetes	15
	Bacteria	20
	Change in Populations of Soil Microorganisms Resistant to Heptachlor	22
	Heptachlor Degradation	27
V.	DISCUSSION	34
VI.	SUMMARY AND CONCLUSIONS	36
LITERATURE CITED		38
VITA		42

LIST OF TABLES

TABLE		PAGE
1.	Growth Inhibition of Microorganisms Isolated from Soil by Heptachlor on Agar Media	14
2.	Percentage of Microorganisms Completely Inhibited from Developing on Soil Dilution Plates Containing Heptachlor	16
3.	Effect of Heptachlor (Applied in June) on Populations of Fungi in Soil	17
4.	Effect of Heptachlor (Applied in June) on Populations of Actinomycetes in Soil	18
5.	Effect of Heptachlor (Applied in June) on Populations of Bacteria in Soil	19
6.	Changes in Populations of Bacteria in Soils Treated with Heptachlor (49 lb./A.) in the Greenhouse. Data Are Expressed as Percent Deviation from the Untreated Control Soil	21
7.	Changes in Populations of Actinomycetes in Soils Treated with Heptachlor (49 lb./A.) in the Greenhouse. Data Are Expressed as Percent Deviation from the Untreated Control Soil	21
8.	Percent of Fungi from Heptachlor-Treated Soil Resistant to Heptachlor Incorporated in Isolation Media	23
9.	Percent of Actinomycetes from Heptachlor-Treated Soil Resistant to Heptachlor Incorporated in Isolation Media	24
10.	Percent of Bacteria from Heptachlor-Treated Soil Resistant to Heptachlor Incorporated in Isolation Media	25
11.	Percent of Bacteria from Heptachlor-Treated Soil Resistant to Heptachlor Incorporated into the Isolation Medium	26
12.	Percent of Actinomycetes from Heptachlor-Treated Soil Resistant to Heptachlor Incorporated into the Isolation Medium	26
13.	Numbers of Bacteria Isolated from Soil Amended with High Concentrations of Heptachlor after 13 Weeks of Incubation	28

TABLE PAGE

14. Effect of Oat Straw and Alfalfa Amendments to Soil on Degradation of Heptachlor after Eight Months of Incubation 29

15. Effect of Alfalfa and Oat Straw Amendments to Soil on Degradation of Heptachlor after Eight Months of Incubation 29

16. Effect of Heptachlor Incorporated in Isolation Media on Development of Microorganisms on Soil Dilution Plates 30

17. Effect of Four Microorganisms on Degradation of Heptachlor (10 ppm) in Liquid Culture after Four Weeks of Incubation 31

18. Volatilization of Heptachlor from Water-Soil Systems 33

CHAPTER I

INTRODUCTION

Two major disposal systems exist in the world, the soil and the sea. Each year, the soil and the sea inherit an increasing load of residues which ultimately must be degraded to their elemental compounds to prevent contamination beyond levels tolerated by living organisms. In the past, agricultural scientists were primarily concerned with research directed toward increasing soil productivity and disregarding environmental pollution. Soils and waters were contaminated with pesticides, detergents, industrial wastes, manure, and sewage. In several instances, soils and streams have been rendered unfit for agricultural or wildlife use by one or more of these contaminants.

Most contaminants are readily retained in the soil, but considerable variation exists in the rate at which they are dissipated. Topography, soil type, climate, and the nature of the underlying geologic strata are among the most important factors in determining the disposal capacity of a given area. Generally, it has been assumed that the soil will retain, dilute, inactivate, and ultimately dissipate these toxic compounds and that continued use, therefore, would not result in a toxic accumulation. However, the persistence of phytotoxic levels of herbicides in soil over a year or more, the movement of insecticides in surface runoff into streams and lakes, and the leaching of various pesticides to considerable depths in soils are evidence that the disposition of these compounds in the soil cannot be taken for

granted. The fact that the soil is the site of biochemical activity through which natural products and water, as well as pesticides, must pass make it imperative to determine the ultimate disposition of these materials in soil. There is a great need for information on the major sources of pesticides in soils, the amount of pesticides that persist in soil under various level management systems, the mechanism of movement through soils, and the processes involved in their inactivation or degradation. In addition, the facts concerning pesticides in soil need to be related to the larger systems which include meterological factors, plant ecological factors, environmental factors, and watershed characteristics, all of which have an effect on the impact of pesticides on the soil and closely-related water.

The chlorinated hydrocarbon insecticides are of special interest, since they are not readily degraded and have been shown to enter biological systems and accumulate in food chains and fatty tissues of higher mammals.

Heptachlor is one of the synthetic chlorinated hydrocarbons which quickly gained acceptance as a soil insecticide. In pure form, heptachlor is a white crystalline substance which has a melting point of 95-96°C. The total chlorine content is 66.5%. Heptachlor is long residual and has an acute oral LD_{50} (rat) of 90 mg/kg. Heptachlor was chosen in the present study, since it was an insecticide commonly added to soil alone or in combination with fertilizers. The study was designed to: (1) determine the effect of heptachlor on viability and growth of soil microorganisms in pure culture, (2) determine the

effect on microbial populations when heptachlor is added to soil, and (3) isolate and study soil microorganisms capable of breaking down heptachlor to other products.

CRANES CREST

CHAPTER II

LITERATURE REVIEW

Although extensive research has been done with chlorinated hydrocarbons, not much is known of the effect and degradation of heptachlor on and by soil microorganisms. Wilson and Choudri (1946) found that DDT and BHC in amounts considerably exceeding practical field application had no significant effect on development of bacteria and molds. Smith and Wenzel (1947) found that 5 to 200 ppm DDT were not bactericidal and in some cases were stimulative to heterotrophic bacteria.

Bollen et al. (1954) observed that numbers of fungi were reduced by DDT and benzene hexachloride and increased by toxaphene. Chlorinated hydrocarbon insecticides were found by Gray and Rogers (1955) to be toxic to soil microorganisms in pure culture. Benzene hexachloride at 125 ppm in agar plates used for estimating bacteria and actinomycetes in soil prevented the development of more than 90% of these organisms appearing as colonies on controls; only gram-negative short rods grew on treated plates. Martin et al. (1959) found no measurable effects on numbers of fungi and bacteria when aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, lindane, or toxaphene were added to soil in amounts ordinarily used in field practice.

Richardson and Miller (1960) studied inhibition of Rhizoctonia solani by chlorinated hydrocarbon insecticides in culture and found a correlation with insecticide vapor pressure or water solubility.

Heptachlor, aldrin, and chlordane were highly toxic because of their high vapor pressure and lindane because of its relatively high water solubility. DDT, methoxychlor, dieldrin, and endrin were less toxic because of their low vapor pressure and low water solubility. When chlorinated hydrocarbon insecticides were added to soil in amounts higher than ordinarily used in field practices, numbers of soil microorganisms increased (Pathak et al., 1961; Roberts and Bollen, 1955; Jones, 1956; Horn, 1952). In other studies, however, no increase in numbers was obtained at the higher rates (Eno and Everett, 1958; Ko and Lockwood, 1968). Aldrin, at a concentration of 20 ppm in culture, was found by Cowley and Lichenstein (1970) to inhibit the growth of Fusarium oxysporum by 37-44%, but there was little inhibition when yeast extract or nitrogenous nutrients were added to the culture medium.

The dynamics of chlorinated hydrocarbon insecticide behavior in plants and soils are closely associated with the subject of "insecticide residues" after all the processes of "weathering" and biotransformation have occurred. The metabolism of chlorinated hydrocarbon insecticides is a complex matter because of the stereochemical possibilities afforded by the three-dimensional molecules (Brooks, 1975).

Chacko and Lockwood (1966) demonstrated that several actinomycetes can degrade DDT. In culture, most of the actinomycetes and filamentous fungi tested degraded PCNB (pentachloronitrobenzene), but none of them degraded dieldrin. Guenzi and Beard (1967) incubated DDT anaerobically for four weeks after which they separated seven possible decomposition

products by thin layer chromatography. Matsumura and Boush (1967) examined 600 soil microbial isolates and found 10 which had degrative ability towards dieldrin. All the isolates found to degrade dieldrin also converted endrin. Tu et al. (1968) examined the ability of 92 pure cultures of soil microorganisms to metabolize aldrin and showed that fungi could be classified according to their ability to convert aldrin to dieldrin.

The formation of heptachlor epoxide as a residue following the application of heptachlor to plants was first observed many years ago, and later work showed that heptachlor is absorbed through the roots and can be epoxidized in the plant. Miles (1969) showed that out of 92 microorganisms isolated from soil which were examined for their ability to transform heptachlor, 35 out of 47 fungi and 26 out of 45 bacteria and actinomycetes affected epoxidization. In 1971, Miles et al. showed that heptachlor epoxide was degraded to the less toxic 1-hydroxychlordene with a mixed culture of soil microorganisms. The conversion rate was about 1% per week during the 12-week test periods. The same mixed culture reduced heptachlor to chlordene but was inactive when incubated with 1-hydroxychlordene or 1-hydroxy-2,3-epoxychlordene. This degradation of heptachlor epoxide may explain the occurrence of high levels of 1-hydroxychlordene and low levels of heptachlor epoxide found in heptachlor-treated soils.

The increasingly stringent requirements for the location and identification of minute amounts of pesticides in biological materials has led to remarkable advances in analytical techniques during the last 15 years. The modern development began with the introduction in

1960 of gas chromatographic analysis of chlorinated hydrocarbons with electron capture detection (Goodwin et al., 1960, 1961). Lovelock and Lipsky's (1960) electron capture method for chlorinated hydrocarbon detection achieved great popularity in a very short time, partly due to the ease with which detectors having excellent sensitivity can be made inexpensively in the laboratory.

CHAPTER III

MATERIALS AND METHODS

I. EFFECT OF HEPTACHLOR ON GROWTH OF SOIL MICROORGANISMS IN PURE CULTURE

Microorganisms were isolated on dilution-plates (Johnson and Curl, 1972) from surface samples of an Etowah silt loam. Bacteria and actinomycetes were isolated on James' soil extract agar; final dilution of soil to water was $1:10^6$. Fungi were isolated on peptone-dextrose-rose bengal agar containing 2 ppm chlortetracycline, and final dilutions were $1:10^4$ (Johnson and Curl, 1972). Numbers of microorganisms appearing on the dilution plates were determined after five days (fungi) and eight days (bacteria and actinomycetes) of incubation at 24°C. For further studies, isolates were selected at random and transferred to pure culture.

The effect of heptachlor (1,4,5,6,7,8,8-heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoidene) on growth of soil microorganisms in pure culture was determined by mixing samples of a sterile emulsifible concentrate of heptachlor (2 lb./gal) with sterile agar media. Final concentrations of heptachlor in the media were 1, 10, and 25 ppm. Media used were nutrient agar for bacteria and actinomycetes and potato-dextrose agar for fungi (Johnson and Curl, 1972). The media were dispensed in Petri dishes and organisms isolated previously in pure culture were streaked on the surface of the agar. Amount of linear growth was recorded after five days of incubation at 24°C.

Isolates that did not grow were transferred to media without heptachlor to determine whether the insecticide was bactericidal/fungicidal or bacteriostatic/fungistatic. These isolates were also tested on media containing the inert ingredients in the emulsifible concentrate of heptachlor (heptachlor, diluent, and emulsifiers supplied by Velsicol Chemical Corporation, Chicago, Illinois).

II. EFFECT OF HEPTACHLOR ON MICROBIAL POPULATIONS IN SOIL

A field plot experiment was designed to determine if heptachlor applied to soil at normal and high rates induces a population change in the microflora. The field plot, 60 x 180 ft, was divided into 12 equal subplots, each 30 x 30 ft. Heptachlor, in the form of 5% granules, was applied uniformly with a fertilizer spreader in June on each of nine subplots with three remaining untreated. The granules were then incorporated into the top six inches of soil with a disc harrow. Heptachlor treatments were 0, 1, 7, and 49 lb./A. with three plot replications of each. After heptachlor incorporation into the soil, soybeans were planted over the experimental area. Three months later, the soybeans were plowed under and alfalfa was planted. Soil samples were collected for microbial analysis at three month intervals (3, 6, 9, and 12 months).

At each sampling interval, 10 core samples (6 x .6 inches diameter) from each subplot were bulked and standard soil dilution plates were made. For isolation of bacteria and actinomycetes, final dilutions on James' soil extract agar of $1:10^6$ were prepared. A final dilution

of $1:10^4$ on modified rose bengal agar was used for isolation of fungi. Microbial populations were determined by standard plate counts (Johnson and Curl, 1972).

In order to determine if there was a build-up of organisms resistant to heptachlor in treated soils, final dilutions of all soils were also incorporated in media containing heptachlor at concentrations of 25, 50, and 100 ppm for the field experiment and 10, 25, 50, and 100 ppm for the greenhouse experiment.

A greenhouse experiment was performed to determine the effect of particle size of heptachlor granules on populations of bacteria and actinomycetes in soil. Treatments were: (1) heptachlor granules (10%) mixed with soil in a concrete mixer at a concentration of 25 ppm, (2) heptachlor granules (10%) crushed to a powder and mixed with soil in a concrete mixer at a concentration of 25 ppm, and (3) soil without heptachlor. Treated soils were placed in two-gallon crocks, six for each treatment, and incubated in the greenhouse. The soils were watered when necessary to prevent drying out. Initially, and at intervals of approximately one month thereafter for one year, six core samples were bulked from each crock, and standard soil dilution plates were made. Populations of bacteria and actinomycetes were determined on James' soil extract agar containing 0, 25, 50, and 100 ppm heptachlor.

III. MICROBIAL DEGRADATION OF HEPTACHLOR

Isolation of Heptachlor Resistant Soil Microorganisms

Clay granules containing 20% heptachlor were crushed into a powder with a mortar and pestle. Samples of field soil were diluted to $1:10^5$

and $1:10^6$ and aliquots were incorporated into Thorntons Standardized Medium (Johnson and Curl, 1972) containing heptachlor at concentrations of 200 and 2000 ppm; 1% sodium hexametaphosphate was used as a dispersing agent. Organisms isolated were transferred to tubes of nutrient agar to be used in heptachlor degradation experiments.

Technical heptachlor was incorporated in 50 ml of liquid media (1.0 g K_2HPO_4, 1.0 g $(NH_4)_2NO_3$, 0.5 g NaCl, 0.2 g $MgSO_4 \cdot 7\ H_2O$, 0.01 g $Fe_2(SO_4)_3$, 20.0 g dextrose, 1000 ml H_2O) at the rate of 10 ppm in 250 ml flasks. Technical heptachlor was first dissolved in ethanol and then incorporated into the media. The heptachlor media were then inoculated with the previously isolated soil microorganisms from media containing 200 and 2000 ppm heptachlor and incubated for four weeks at room temperature. The flasks were shaken every two or three days. Heptachlor was extracted from the liquid cultures by the addition of 50 ml of hexane to each flask and shaken briefly at 15 minute intervals for four hours; then, 0.5 µl samples were withdrawn from the heptachlor-hexane solution and injected into the gas chromatograph.

Effect of Crop Residues on Heptachlor Degradation

Alfalfa hay and oat straw were added to soil at rates of 1 and 4% in the form of mature, dried plants, chopped to one-eighth inch particle size in a Wiley mill. Heptachlor granules, ground into a powder, were added to the residue-amended soil in 250 ml flasks at rates of 20 and 200 ppm. There were six replicates per treatment. The soil was brought to approximately 60% m.h.c. by spraying water onto the soil while rotating the flasks. They were covered with polyethylene film

and incubated in the laboratory for eight months. Soil in each flask was extracted with xylene; concentrations of heptachlor were determined with a gas chromatograph.

Volatilization of Heptachlor

Technical heptachlor was incubated in flasks containing different proportions of field soil and water at the rate of 10 ppm. Technical heptachlor in liquid media at the same rate was also incubated in screw-cap bottles and cotton-plugged 250 ml flasks. They were incubated for four weeks and then extracted with hexane for gas chromatographic determinations of heptachlor concentrations.

Gas Chromatographic Conditions

All gas chromatographic determinations were performed on the Perkin-Elmer Model 900 gas chromatograph with parameters chosen from previous determinations. The parameters were:

Column loading - DC 200 (10% loading) - Chromosorb W (80-100 mesh)

Column type - 6 ft stainless steel

Column temperature - 170°C

Injector temperature - 180°C

Manifold temperature - 180°C

Electron capture detector temperature - 200°C

Carrier gas - 95% argon-5% methane

Gas flow - 80 ml/min

Sensitivity - 128 x 10

Regulator pressure - 75 lb./sq. in.

Pulse mode - 50 μ sec.

CHAPTER IV

RESULTS

I. EFFECT OF HEPTACHLOR ON GROWTH OF SOIL MICROORGANISMS IN PURE CULTURE

A total of 1097 cultures of bacteria, actinomycetes, and fungi isolated at random from soil dilution plates were tested individually for growth inhibition by heptachlor at two concentrations in culture media. When growth on heptachlor-treated media was compared to that on media without heptachlor, it was found that bacteria were most sensitive; some growth retardation occurred with 87% of the isolates tested on media containing 25 ppm (Table 1). Sixty-three percent of the bacteria did not grow at all on this medium. Growth of many of the actinomycetes and fungi were inhibited, but they were generally less sensitive to heptachlor than the bacteria. None of the organisms tested were completely inhibited by heptachlor at 1 ppm, but growth of 9% of the bacteria was retarded at this concentration. Cells of bacteria whose growth was completely inhibited at 25 ppm were scraped from the test medium after five days of incubation and transferred to fresh medium without heptachlor. None of the 55 isolates selected at random and tested for viability in this manner grew on the fresh medium. The diluent and emulsifiers in the heptachlor formulation were incorporated into media at equivalent concentrations. Sensitive bacteria were not inhibited at 100 ppm, and only slight growth inhibition occurred at 1000 ppm.

TABLE 1

GROWTH INHIBITION OF MICROORGANISMS ISOLATED FROM SOIL BY HEPTACHLOR ON AGAR MEDIA

Microorganism	Number Tested	Percent Inhibition		Percent Completely Inhibited	
		10 ppm	25 ppm	10 ppm	25 ppm
Bacteria	500	54	87	15	63
Actinomycetes	287	16	38	5	16
Fungi	310	26	35	0	4

Heptachlor incorporated into the isolation medium prevented the development of many colonies of microorganisms on soil dilution plates when compared to plates without heptachlor (Table 2). At 100 ppm, 89% of the bacteria, 81% of the actinomycetes, and 50% of the fungi were prevented from developing.

II. EFFECT OF HEPTACHLOR ON SOIL MICROBIAL POPULATIONS

The data obtained are listed in Tables 3, 4, and 5. There was some variation in population numbers of microorganisms during the experimental period in the plots without heptachlor. This was expected since microbial activity varies with changing environment. There was a general increase in bacterial numbers after six months of heptachlor application when the soybean residue was plowed under three months earlier.

Fungi

Heptachlor at all levels had an initial depressing effect on fungal populations (Table 3). At six months, there was a population increase at 1 and 7 lb./A. over the untreated plots. There was no difference in populations at 9 months, but at 12 months there was a decrease in population (when compared to the control), especially at the 7 lb./A. level.

Actinomycetes

There appeared to be no major change in actinomycete populations after heptachlor treatments (Table 4). Considerable variation among replications occurred, thus most differences between treatments were not significant.

TABLE 2

PERCENTAGE OF MICROORGANISMS COMPLETELY INHIBITED FROM DEVELOPING ON SOIL DILUTION PLATES CONTAINING HEPTACHLOR

	Concentration of Heptachlor (ppm)		
Microorganism	25	50	100
Bacteria	60	77	89
Actinomycetes	55	68	81
Fungi	21	38	50

TABLE 3

EFFECT OF HEPTACHLOR (APPLIED IN JUNE) ON POPULATIONS OF FUNGI IN SOIL

Heptachlor Field Treatment (Lb./A.)	Heptachlor in Isolation Medium (ppm)	Months after Application/Number Per Gram of Soil x 10^3							
		3	$\overline{sx}$	6	$\overline{sx}$	9	$\overline{sx}$	12	$\overline{sx}$
0	0	579.6	39	451.0	20	221.0	30	309.9	34
	25	360.6	26	381.0	17	216.6	36	225.8	27
	50	292.9	32	220.0	14	193.2	40	186.7	23
	100	257.6	45	208.0	18	145.2	33	132.8	18
1	0	409.3	21	616.0	20	205.7	28	259.5	52
	25	292.6	39	538.0	4	226.6	19	193.7	6
	50	266.3	18	427.0	20	191.9	26	194.3	6
	100	203.0	33	298.0	17	180.0	18	110.9	7
7	0	371.0	42	550.0	6	218.1	15	217.6	34
	25	259.6	43	461.0	10	215.6	52	169.5	26
	50	205.6	39	366.0	15	194.7	27	152.3	18
	100	140.0	37	270.0	26	155.7	23	69.5	13
49	0	314.0	46	457.0	27	202.3	13	259.9	18
	25	199.0	37	349.0	23	185.2	12	275.7	58
	50	137.0	32	274.0	15	204.6	28	209.5	68
	100	92.6	22	197.0	9	212.8	31	122.8	21

TABLE 4

EFFECT OF HEPTACHLOR (APPLIED IN JUNE) ON POPULATIONS OF ACTINOMYCETES IN SOIL

Heptachlor Field Treatment (Lb./A.)	Heptachlor in Isolation Medium (ppm)	Months after Application/Number Per Gram of Soil x 10^5							
		3	$\overline{sx}$	6	$\overline{sx}$	9	$\overline{sx}$	12	$\overline{sx}$
0	0	452.0	107	501.0	115	334.0	143	352.8	83
	25	192.6	31	193.0	31	124.8	62	211.7	70
	50	151.7	29	141.0	33	114.0	47	118.1	48
	100	79.0	10	75.0	29	62.1	39	79.5	34
1	0	512.0	215	514.0	130	338.0	48	344.4	71
	25	246.0	90	166.0	29	142.8	8	185.0	57
	50	188.0	70	147.0	32	107.8	8	122.3	21
	100	126.0	51	96.0	15	55.7	10	78.4	13
7	0	259.3	57	624.0	77	387.5	37	410.6	21
	25	101.3	23	256.0	40	141.4	41	261.8	51
	50	74.7	9	214.0	54	107.1	28	182.6	40
	100	34.0	9	149.0	48	51.4	23	131.9	32
49	0	354.0	138	715.0	96	288.5	8	356.3	18
	25	147.3	30	290.0	68	93.4	35	145.9	19
	50	112.0	21	230.0	64	60.0	5	66.1	15
	100	70.0	14	167.0	50	40.7	5	63.1	15

TABLE 5

EFFECT OF HEPTACHLOR (APPLIED IN JUNE) ON POPULATIONS OF BACTERIA IN SOIL

Heptachlor Field Treatment (Lb./A.)	Heptachlor in Isolation Medium (ppm)	Months after Application/Number Per Gram of Soil x 10^5							
		3	$\overline{sx}$	6	$\overline{sx}$	9	$\overline{sx}$	12	$\overline{sx}$
0	0	260.0	81	958.0	181	549.8	118	1647.7	347
	25	134.6	27	279.0	30	129.4	32	533.7	154
	50	76.6	20	208.0	19	102.6	26	336.7	107
	100	41.3	10	93.0	13	41.4	28	133.3	53
1	0	358.0	143	1407.0	85	753.9	3	1008.3	85
	25	150.0	68	234.0	22	200.7	17	306.8	74
	50	107.3	46	191.1	17	135.7	12	155.2	20
	100	58.0	24	97.0	27	74.2	4	50.5	5
7	0	165.3	26	1647.0	68	951.5	64	1200.0	192
	25	59.5	14	374.0	20	259.2	35	358.0	26
	50	38.0	6	258.0	17	181.4	59	222.2	48
	100	14.6	3	142.0	7	104.2	37	68.1	24
49	0	273.3	90	2613.0	322	1267.1	76	1612.9	190
	25	103.3	18	606.0	145	350.0	130	482.8	120
	50	55.7	6	414.0	95	246.5	77	325.9	72
	100	30.7	3	201.1	40	136.3	40	126.1	43

Bacteria

In previous laboratory tests it was shown that 65% of the soil bacteria would not survive in media containing 25 ppm of heptachlor. This lethal effect was not demonstrated in soil containing an equivalent amount (49 lb./A.) of heptachlor (Table 5). When compared to the control, there was little change in bacterial populations at three months. Significant increases in bacterial populations occurred at all levels of heptachlor treatments at six and nine months, the highest increase being in plots treated with 49 lb./A. At 49 lb./A. bacterial populations were 175 and 135% higher than the control at six and nine months, respectively. At 12 months there was little difference in bacterial numbers when compared to the control.

In the greenhouse, heptachlor induced a 335% increase in bacterial populations during the first three months after treatment (Table 6). There was a greater increase in soils containing powdered heptachlor than in soils containing granular particles. At four months, bacterial populations had dropped to a point where there was no significant difference between populations in treated and control soils. At six months, bacterial populations increased to about 50% higher in treated than in control soil and were relatively stable during the remaining six months of the experiment. Heptachlor appeared to have no effect on total populations of actinomycetes throughout the experimental period (Table 7).

TABLE 6

CHANGES IN POPULATIONS OF BACTERIA IN SOILS TREATED WITH HEPTACHLOR (49 LB./A.) IN THE GREENHOUSE. DATA ARE EXPRESSED AS PERCENT DEVIATION FROM THE UNTREATED CONTROL SOIL

	Days after Application						
Treatment	1	35	77	129	172	247	300
Granular	0	+65	+202	-7	+40	+60	+28
Powder	+37	+127	+335	-15	+65	+56	+133

TABLE 7

CHANGES IN POPULATIONS OF ACTINOMYCETES IN SOILS TREATED WITH HEPTACHLOR (49 LB./A.) IN THE GREENHOUSE. DATA ARE EXPRESSED AS PERCENT DEVIATION FROM THE UNTREATED CONTROL SOIL

	Days after Application						
Treatment	1	35	77	129	172	247	300
Granular	+14	+3	+36	-25	+52	-7	+16
Powder	+51	+10	+32	-32	+45	-15	+28

III. CHANGE IN POPULATIONS OF SOIL MICROORGANISMS RESISTANT TO HEPTACHLOR

Numbers of colonies obtained on heptachlor-treated media from each heptachlor soil treatment (field and greenhouse) were compared to those obtained on media without heptachlor. The percent resistant organisms for each soil treatment was obtained as follows:

$$\frac{\text{Population on heptachlor medium} \times 100}{\text{Population on medium without heptachlor}}$$

In the field, the percentage of resistant fungi, actinomycetes, and bacteria at three concentrations of heptachlor are listed in Tables 8, 9, and 10, respectively. The total population of fungi after nine months of treatment was low compared to that determined at other sampling periods, but the percent of resistant fungi at this time was quite high. For example, numbers obtained on plates containing 100 ppm heptachlor were as high at 49 lb./A. as on media containing no heptachlor. A few samples contained actinomycetes in which a build-up of resistant organisms occurred. No such stimulation of heptachlor-resistant bacteria was demonstrated.

In the greenhouse, the percentages of resistant organisms to four concentrations of heptachlor are recorded in Tables 11 and 12 for bacteria and actinomycetes, respectively. Apparently, populations of bacteria resistant to heptachlor were not selectively increased in heptachlor-treated soils. Resistant actinomycetes, however, tended to be increased selectively over nonresistant ones.

Similar results were obtained on isolation media containing heptachlor at 25 and 100 ppm. Numbers of heptachlor-resistant bacteria

TABLE 8

PERCENT OF FUNGI FROM HEPTACHLOR-TREATED SOIL RESISTANT TO HEPTACHLOR INCORPORATED IN ISOLATION MEDIA

Soil-Heptachlor Treatment (Lb./A.)	Months after Application	Concentration of Heptachlor in Medium (ppm)		
		25	50	100
0	3	62	51	44
1		71[a]	65[a]	50[a]
7		70[a]	55	38
49		63	44	29
0	6	84	49	46
1		87	69[a]	48
7		84	67[a]	49
49		76	60[a]	43
0	9	98	87	66
1		100	94[a]	88[a]
7		99	89	71[a]
49		92	100[a]	100[a]
0	12	73	60	43
1		75	78[a]	43
7		78[a]	70[a]	32
49		100[a]	81[a]	47

[a]Increased resistance by 5% or more.

TABLE 9

PERCENT OF ACTINOMYCETES FROM HEPTACHLOR-TREATED SOIL RESISTANT TO HEPTACHLOR INCORPORATED IN ISOLATION MEDIA

Soil-Heptachlor Treatment (Lb./A.)	Months after Application	Concentration of Heptachlor in Medium (ppm)		
		25	50	100
0	3	43	34	17
1		48[a]	37	25[a]
7		39	29	13
49		42	32	20
0	6	39	28	15
1		32	29	19
7		41	34[a]	24[a]
49		41	32	23[a]
0	9	37	34	19
1		42[a]	32	16
7		36	28	13
49		32	21	14
0	12	60	33	23
1		54	36	23
7		64	44[a]	32[a]
49		41	19	18

[a]Increased resistance by 5% or more.

TABLE 10

PERCENT OF BACTERIA FROM HEPTACHLOR-TREATED SOIL RESISTANT TO HEPTACHLOR INCORPORATED IN ISOLATION MEDIA

Soil-Heptachlor Treatment (Lb./A.)	Months after Application	Concentration of Heptachlor in Medium (ppm)		
		25	50	100
0	3	52	29	16
1		42	30	16
7		36	23	9
49		38	20	11
0	6	29	22	10
1		17	14	7
7		23	16	7
49		23	16	8
0	9	24	19	8
1		27	18	10
7		27	19	11
49		28	19	11
0	12	32	20	8
1		30	15	5
7		30	18	6
49		30	20	8

TABLE 11

PERCENT OF BACTERIA FROM HEPTACHLOR-TREATED SOIL RESISTANT TO HEPTACHLOR INCORPORATED INTO THE ISOLATION MEDIUM

Months of Incubation	Heptachlor Soil Treatment (25 ppm)	Concentration of Heptachlor in Medium (ppm)			
		10	25	50	100
4	No heptachlor	48	38	32	13
	Granules	53	40	31	16
	Powder	47	47	27	13
10	No heptachlor	33	23	20	10
	Granules	42	23	21	23[a]
	Powder	29	17	12	15

[a]Increased resistance by 10% or more.

TABLE 12

PERCENT OF ACTINOMYCETES FROM HEPTACHLOR-TREATED SOIL RESISTANT TO HEPTACHLOR INCORPORATED INTO THE ISOLATION MEDIUM

Months of Incubation	Heptachlor Soil Treatment (25 ppm)	Concentration of Heptachlor in Medium (ppm)			
		10	25	50	100
4	No heptachlor	72	55	45	34
	Granules	68	63[a]	53	45[a]
	Powder	86[a]	69[a]	39	33
10	No heptachlor	48	28	12	4
	Granules	62[a]	41[a]	21	7
	Powder	69[a]	31	22[a]	3

[a]Increased resistance by 10% or more.

increased, however, in soil amended with very high concentrations of heptachlor and stored in flasks in the laboratory (Table 13). Numbers of colonies from soil containing 12,800 ppm heptachlor on heptachlor media equaled the number on media without heptachlor.

IV. HEPTACHLOR DEGRADATION

It was determined that crop residues in the form of alfalfa and oat straw affected the degradation of heptachlor. More heptachlor was changed over to heptachlor epoxide in residue amended soil than in nonamended soil (Tables 14 and 15). The amount of epoxide present correlated positively with concentrations of residue amendment. Heptachlor concentration was considerably less in extracts of soils containing alfalfa.

It is probable that if microorganisms exist which can degrade heptachlor, they would also be resistant to growth inhibition by heptachlor. The 34 isolates that grew on media containing 200 and 2000 ppm heptachlor were transferred to pure culture for further study (Table 16). All of the 30 bacterial isolates examined were gram-negative, short rods. Fungi transferred were species of Penicillium, Aspergillus, Fusarium, and yeast. No actinomycetes were found at these concentrations.

All 34 heptachlor resistant organisms were tested separately for their ability to metabolize heptachlor. Only three microorganisms were found to change heptachlor back to its parent compound, chlordene (Table 17). Since it is a hydrolysis product of heptachlor, 1-hydroxychlordene was present in all cultures. Bacterium Number 5

TABLE 13

NUMBERS OF BACTERIA ISOLATED FROM SOIL AMENDED WITH HIGH CONCENTRATIONS OF HEPTACHLOR AFTER 13 WEEKS OF INCUBATION

Heptachlor in Soil (ppm)	Average Number Per Plate		
	Isolation Medium without Heptachlor	Isolation Medium with Heptachlor (100 ppm)	Resistant
0	44	8	18
800	184	119	65
12,800	1310	1315	100

TABLE 14

EFFECT OF OAT STRAW AND ALFALFA AMENDMENTS
TO SOIL ON DEGRADATION OF HEPTACHLOR
AFTER EIGHT MONTHS OF INCUBATION

Soil with 20 ppm Heptachlor	Heptachlor (ppm)	Heptachlor Epoxide (ppm)	Total (H + HE)
No amendment	3.3	0.6	3.9
1% alfalfa	2.7	2.1	4.8
4% alfalfa	2.9	3.3	6.2
1% oat straw	3.3	1.8	5.1
4% oat straw	5.9	2.1	7.0

TABLE 15

EFFECT OF ALFALFA AND OAT STRAW AMENDMENTS
TO SOIL ON DEGRADATION OF HEPTACHLOR
AFTER EIGHT MONTHS OF INCUBATION

Soil with 200 ppm Heptachlor	Heptachlor (ppm)	Heptachlor Epoxide (ppm)	Total (H + HE)
No amendment	82	3	85
1% alfalfa	68	5	73
4% alfalfa	43	13	56
1% oat straw	61	4	65
4% oat straw	78	11	89

TABLE 16

EFFECT OF HEPTACHLOR INCORPORATED IN ISOLATION MEDIA ON DEVELOPMENT OF MICROORGANISMS ON SOIL DILUTION PLATES

Concentration of Heptachlor in Medium	Dilution	Number Per Petri Dishes				Average Per Dish
		1	2	3	4	
None	$1:10^5$	128	112	140	116	124
	$1:10^6$	36	38	48	34	39
200 ppm	$1:10^5$	3	5	8	6	6
	$1:10^6$	2	2	1	0	1
2000 ppm	$1:10^5$	1	1	0	0	1
	$1:10^6$	3	1	0	1	1

TABLE 17

EFFECT OF FOUR MICROORGANISMS ON DEGRADATION OF HEPTACHLOR (10 PPM) IN LIQUID CULTURE AFTER FOUR WEEKS OF INCUBATION

Isolate	Concentration in Hexane Extract (ppm)		
	Heptachlor	1-Hydroxychlordene	Chlordene
Bacterium, No. 2	4.9	Trace	0.0
Bacterium, No. 5	1.7	1.9	1.0
Fungus, No. 14	1.0	Trace	0.4
Bacterium, No. 15	4.0	Trace	0.4
Control	4.6	1.7	0.0

was identified as Bacillus cereus which seemed to have the best degradative ability.

It was observed that percent extraction recovery of heptachlor from liquid culture media was low. In order to determine if the material was lost from the media by volatilization, heptachlor was added to flasks containing soil and water in different proportions. It was determined that the greater the soil to water ratio, the less volatilization of heptachlor (Table 18). Dry soil and the 5:1 soil to water ratio adsorbed the greatest concentration of heptachlor. In other tests, heptachlor was added to liquid culture media in cotton-plugged flasks and screw cap medicine bottles and extracted after four weeks of incubation. Heptachlor was completely volatilized from the flasks while very little volatilization occurred in the screw cap medicine bottles. Most of the heptachlor was found to be retained in the cotton plugs.

TABLE 18

VOLATILIZATION OF HEPTACHLOR FROM WATER-SOIL SYSTEMS

Volume:Weight	Concentration in Hexane Extract (ppm)	
	Heptachlor	1-Hydroxychlordene
Water	Trace	Trace
Water:Soil (2:1)	1.2	0
Water:Soil (1:2)	3.7	0
Water:Soil (1:5)	7.3	0
Soil	7.4	0

CHAPTER V

DISCUSSION

The mechanism of increase of microbial populations in soil after application of heptachlor has not clearly been defined. Pathak et al. (1961) concluded that increase of microbial populations by other chlorinated hydrocarbon insecticides was associated with mineralization of organic phosphorus. It is doubtful that in the present study more available phosphorus could have caused such large increases in bacterial numbers, especially in soils containing higher concentrations of heptachlor. Also, bacteria resistant to heptachlor were increased selectively in these soils. It is possible that multiplication of these resistant bacteria was caused by the release of nutrients by the death of other more susceptible soil organisms. Since powdered heptachlor induced a greater increase in bacterial populations than granular heptachlor, it is assumed that powdered heptachlor had better soil coverage, thus coming in contact with and killing more soil organisms.

During the fifth month of heptachlor incubation in the greenhouse, there was a very sharp decline in numbers of bacteria and actinomycetes followed by a gradual increase of bacterial numbers and a fluctuation in numbers of actinomycetes. Drastic changes in environmental conditions such as low soil moisture and fluctuating temperatures are suspected as the cause for this population decline. Considerable variation existed in numbers of microorganisms isolated at different

sampling periods from control replicates. These were responses to environmental conditions and not related to heptachlor application; therefore, the data were expressed as percent deviation in numbers isolated from heptachlor-treated plots as compared to numbers isolated from plots without heptachlor.

Miles et al. (1969, 1971) reported the degradation of heptachlor to chlordene by soil microorganisms. They suspected that volatilization of heptachlor from the liquid medium was responsible for their inability to obtain quantitative duplication. In the present study, volatilization of heptachlor from liquid media was confirmed. Incorporation of 10 ppm heptachlor in liquid culture media in cotton-plugged flasks resulted in volatilization of the heptachlor and its absorption by the cotton plug. Volatilization seems to be rapid, thus leaving only small quantities of heptachlor to be metabolized by soil microorganisms. Although heptachlor is slightly soluble in water, its volatilization is hard to explain. Heptachlor's high vapor pressure of 4×10^{-4} mm at 25°C might be a possible explanation of its high volatility in aqueous solutions. Richardson and Miller (1960) reported that heptachlor was highly toxic to Rhizoctonia solani because of its high vapor pressure.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Results of the present study revealed the following:

1. Heptachlor incorporated in media was more toxic to bacteria than fungi and actinomycetes in pure culture.

2. There was a significant increase in bacterial populations in heptachlor-treated soils in the field and greenhouse studies.

3. There was a significant increase in numbers of heptachlor-resistant fungi in the heptachlor-treated soils.

4. There was a direct positive correlation between increase in bacterial numbers and concentration of heptachlor.

5. Numbers of heptachlor-resistant bacteria increased in soils amended with very high concentrations of heptachlor.

6. Heptachlor degradation was facilitated by incorporation of alfalfa hay to heptachlor-treated soil.

7. Heptachlor volatilized rapidly in liquid culture media and in soils highly saturated with water.

8. Heptachlor was metabolized to chlordene by a soil bacterium, Bacillus cereus.

Since heptachlor is highly toxic and long residual in soil, its ban from use in this modern age of highly concerned environmentalists was inevitable. Its metabolism by soil microorganisms to its parent compound, chlordene, is not sufficient since chlordene is just as toxic and long residual as heptachlor. If chlorinated hydrocarbons are to

be brought back to general use, ways to metabolize them to nontoxic and short-residual compounds would have to be found.

LITERATURE CITED

LITERATURE CITED

Bollen, W. B., H. E. Morrison, and H. H. Crowell. 1954. Effect of field treatments of insecticides on numbers of bacteria, streptomycetes and molds. J. Econ. Entomol. 47:302-306.

Bowman, M. C., F. Acre, Jr., C. S. Lofgren, and M. Beroza. 1964. Chlorinated insecticides: fate in aqueous suspensions containing mosquito larvae. Science 146:1480.

Brooks, G. T. Chlorinated Insecticides. I. Technology and Application. (CRC Press, Cleveland, Ohio, 1975.)

Brooks, G. T. Chlorinated Insecticides. II. Biological and Environmental Aspects. (CRC Press, Cleveland, Ohio, 1975.)

Chacko, C. E., and J. L. Lockwood. 1966. Chlorinated hydrocarbon pesticides: degradation by microbes. Science 156:959-961.

Chandra, P. 1967. Effect of two chlorinated insecticides on soil microflora and nitrification process as influenced by different soil temperature and texture. In Progress In Soil Biology (Otto Graff and J. E. Satchell, eds.), pp. 320-330. North Holland, Amsterdam.

Cowley, G. T., and E. P. Lichtenstein. 1970. Growth inhibition of soil fungi by insecticides and annulment of inhibition by yeast extract of nitrogenous nutrients. J. Gen. Microbiol. 62:27-34.

de Faubert Maunder, M. J., H. Egan, and J. Roburn. 1964. Some practical aspects of the determination of chlorinated pesticides by electron-capture gas chromatography. Analyst 89:157.

Eno, C. F., and P. H. Everett. 1958. Effect of soil applications of ten chlorinated hydrocarbon insecticides on soil microorganisms and the growth of stringless Black Valentine beans. Soil Sci. Soc. Am. Proc. 22:235-238.

Forrest, J., O. Stephenson, and W. A. Waters. 1946. Chemical investigations of the insecticide 'DDT' and its analogues. J. Chem. Soc. (Lond.) 333.

Goodwin, E. S., R. Goulden, A. Richardson, and J. G. Reynolds. 1960. The analysis of crop extracts for traces of chlorinated pesticides by gas-liquid partition chromatography. Chem. Ind. (Lond.) 1220.

Goodwin, E. S., R. Goulden, and J. G. Reynolds. 1961. Rapid identification and determination of residues of chlorinated pesticides in crops by gas-liquid chromatography. Analyst 86:697.

Gray, P. H. H., and C. G. Rogers. 1955. Effect of benzene hexachloride on soil microorganisms. Can. J. Microbiol. 1:312-318.

Guenzi, W. D., and W. E. Beard. 1967. Anaerobic biodegradation of DDT to DDD in soil. Science 156:1116-1117.

Horn, G. C. 1952. The effect of certain insecticides on the flora of Arrendondo fine sand. Soil Sci. Soc. Florida Proc. 12:62-67.

Johnson, L. F., and E. A. Curl. Methods for Research on the Ecology of Soil-Borne Plant Pathogens. (Burgess Publ. Co., Minneapolis, Minnesota, 1972.)

Jones, L. W. 1956. Effect of some pesticides on microbial activities of the soil. Utah Agricultural Experiment Station Bulletin No. 390.

Ko, W. H., and J. L. Lockwood. 1968. Conversion of DDT to DDD in soil and the effect of these compounds on soil microorganisms. Can. J. Microbiol. 14:1069-1073.

Lovelock, J. E., and S. R. Lipsky. 1960. Electron affinity spectroscopy—a new method for the identification of functional groups in chemical compounds separated by gas chromatography. J. Am. Chem. Soc. 82:431.

Martin, J. P., R. B. Harding, G. H. Connel, and L. D. Anderson. 1959. Influence of five annual applications of organic insecticides on soil biological and physical properties. Soil Sci. 87:334-338.

Matsumura, F., and G. M. Boush. 1967. Dieldrin: degradation by soil microorganisms. Science 156:959-961.

Miles, J. R. W., C. M. Tu, and C. R. Harris. 1969. Metabolism of heptachlor and its degradation products by soil microorganisms. J. Econ. Entomol. 62:1334-1338.

Miles, J. R. W., C. M. Tu, and C. R. Harris. 1971. Degradation of heptachlor epoxide and heptachlor by a mixed culture of soil microorganisms. J. Econ. Entomol. 61:839-841.

Pathak, A. H., H. Sharkar, and K. W. Awasthi. 1961. Effect of some pesticides on available nutrients and soil microflora. Indian Soc. Soil Sci. J. 9:197-200.

Richardson, L. T., and D. M. Miller. 1960. Fungitoxicity of chlorinated hydrocarbon insecticides in relation to water solubility and vapor pressure. Can. J. Botany 38:163-175.

Roberts, J. E., and W. B. Bollen. 1955. A microplanting method for soil molds and its use to detect some effects on certain insecticides and herbicides. Appl. Microbiol. 3:190-194.

Smith, N. R., and M. E. Wenzel. 1947. Soil microorganisms are affected by some of the new insecticides. Soil Sci. Soc. Am. Proc. 12:227-233.

Tu, C. M., J. R. W. Miles, and C. R. Harris. 1968. Soil microbial degradation of aldrin. Life Sci. [Oxford] 7:311.

Wilson, J. K., and R. S. Choudri. 1946. Effects of DDT on certain microbiological processes in the soil. J. Econ. Entomol. 39:537-538.

Wilson, J. K., and R. S. Choudri. 1948. The effect of benzene hexachloride on soil organisms. J. Agri. Research 77:25-32.

VITA

Nabil B. Shamiyeh was born on September 2, 1940. He graduated from Fulton High School, Knoxville, Tennessee, in 1957. He received the B. S. degree in Liberal Arts in 1962 from The University of Tennessee, Knoxville. He entered the United States Air Force in 1962 and was medically retired in 1964. He held a position as a social worker until he accepted a position with the Department of Agricultural Biology, The University of Tennessee, in May 1965 where he is currently employed. In March 1977, he received the Master of Science degree with a major in Agricultural Biology.

Mr. Shamiyeh is married to the former Anna Jean Selvy and has one son, Paul, and one daughter, Kimberly.

GRADUATE SCHOOL CREST