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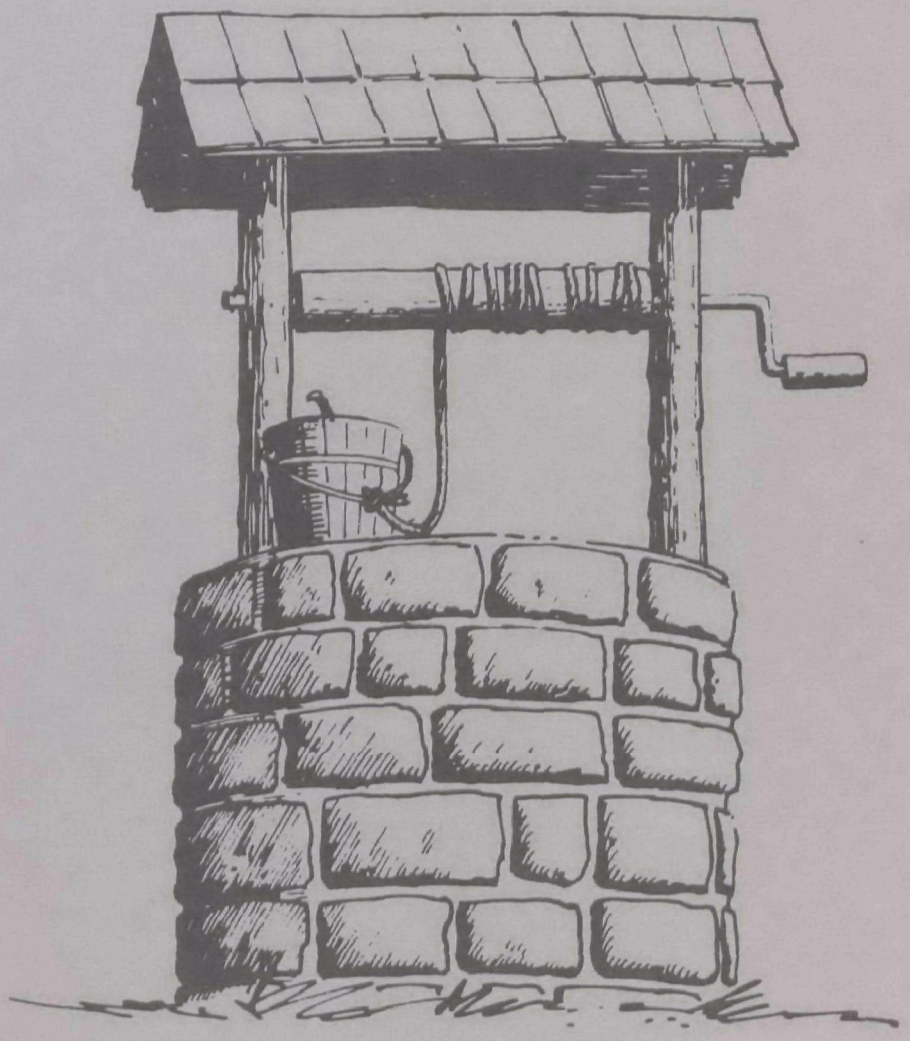
INTERACTION OF HERBICIDES AND SOIL MICROORGANISMS



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INTERACTION OF HERBICIDES AND SOIL MICROORGANISMS

by

Boyce Thompson Institute for Plant Research, Inc.
1086 North Broadway
Yonkers, New York 10701

for the

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ABSTRACT

In pure culture and in soils the addition of 2,3,5,6-tetrachloroterephthalate (DCPA) had little or no effect upon bacterial growth, and several microorganisms appeared to utilize the herbicide as a carbon source. Methyl-2,3,5,6-tetrachloroterephthalate and 2,3,5,6-tetrachloroterephthalic acid were identified as degradation products.

A fungus, Penicillium paraherquei Abe, was isolated from soil that had been treated previously with 5-bromo-3-sec-butyl-6-methyluracil (bromacil) and found to degrade bromacil in culture. When added to sterile bromacil treated soil, the fungus resulted in enhanced bromacil degradation. When added to non-sterile soil, probably due to competition from other organisms, the fungus was ineffective in hastening the degradation of bromacil.

The encouragement of the soil microflora by the addition of nutrient broths resulted in a reduction of toxicity to plants of a number of herbicides. These results indicate that the decontamination of soil by the degradative activities of the natural microflora may be accelerated by the addition of suitable nutrient sources.

A mixture of organisms cultured on isopropyl N-phenylcarbamate (IPC) as the sole carbon source was used to study the influence of ring chlorine on the degradation of a series of anilide herbicides. The retarding effect of ring chlorine on the rate of ring degradation, and for the most part, on microbial respiration, increased according to the configuration sequence: 0 > 2,4 > 2,4,5 > 3 > 4 > 3,4. The implications of this and the effects of other structural features on degradation are discussed.

The IPC-degrading organisms were used in a series of experiments which demonstrated that when added to soil they accelerated the degradation of IPC and related compounds. A membrane "biological filter" device for reducing waterborne biodegradable pollutants was also demonstrated using these organisms.

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and type of ultrafilter material in the Plexiglas model. Comparison is also made to the theoretical maximum calculated for these conditions from Equation 5.

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SECTION I

CONCLUSIONS

The soil contains a rich and varied population of microorganisms capable of degrading a vast number of natural and synthetic chemicals. But many man-made compounds such as the herbicides studied are new to the environment, and the natural soil microorganisms may not have the capabilities required to degrade them. However, by continually exposing a population of soil microorganisms to a particular molecule, organisms can frequently be derived by selection or adaptation that have such capabilities. These organisms can then be cultured and used to rid soil or water of the chemical and in some cases structurally related chemicals as well. The methods used here in demonstrating the potential of such organisms for decontaminating soil and water are probably not at this time ready for practical application, but we believe may point the way to the future development of more practical methodology.

The studies reported here, as well as those done by others on the relationship of chemical structure to persistence in the soil in a series of isomers and homologs, should be useful to chemists seeking to develop molecules that combine herbicidal potency with minimum persistence potential. Only with such information available can synthesis chemists intelligently design the types of pesticides needed in the years ahead if agriculture is to continue to produce the food and fiber required by an expanding population but without adverse environmental effects.

SECTION II

RECOMMENDATIONS

The studies reported here and elsewhere have demonstrated that microorganisms that are capable of degrading pesticides and other synthetic organic molecules can be obtained from the soil. We recommend that, since such organisms can be found or developed, studies on their possible use in decontaminating soils and water should be encouraged. Likewise, the continued development of information on the relationship of chemical structure to environmental persistence should be encouraged since chemists will then have the information necessary for the synthesis of biodegradable nonpersistent pesticides.

SECTION III

INTRODUCTION

The use of herbicidal chemicals to control plant growth is increasing at a rapid rate. They have become an integral part of cultural practices on farms, home gardens, parks, and roadways. With the ever-increasing use of herbicides and other pesticidal chemicals, it has become obvious that there are many problems associated with the introduction of such large volumes of toxic chemicals into the environment. Herbicide residues in the soils may be picked up by plants which when used as food or fodder represent a hazard to man and animals. Rain may wash them into streams where they may contaminate our surface water supplies or they may leach downward into underground water sources. Although they may be present only in sub-toxic quantities, the long-term effects of such materials are not known and their presence can be considered only as undesirable. Microbiological degradation of herbicidal chemicals in the soil appears to be one of the prime routes for their dissipation from the environment. The rate and ease of their degradation is known to vary, but the influence of environmental factors and the chemistry of these processes is poorly understood. A study of some of these processes was therefore undertaken with the aim of giving us a better understanding of the problem. A better understanding of the effects of chemical structure on decomposition kinetics should be useful to those involved in the designing of new, active, but more biodegradable, herbicides. The study of some of the organisms involved indicated that it may be possible to use cultural practices that will favor their activity and thus decrease the amount of herbicide residues in the soil. The introduction of microorganisms with specific degradative capacities into the soil was shown to be a possible means of ridding the soil of contaminating chemicals. Using these same organisms a "biological filter" system effective in removing a herbicidal chemical from water was developed. The procedures involved in these studies, the results obtained, and their possible significance are discussed in Sections IV-XI.

An investigation of the interactions of soil microorganisms and several groups of herbicidal compounds, primarily chlorinated derivatives, was made. Specific objectives were: 1) to isolate and characterize microbial species responsible for complete or partial herbicide degradation; 2) to characterize the rates of degradation of structurally related herbicides; 3) to study the influence of environmental factors such as supplemental substrates, previous adaptation of microorganisms, etc., upon herbicide degradation; 4) to identify the routes of degradation and fate of degradation products; and 5) to determine if microorganisms found capable of rapidly degrading a herbicide can be used to decontaminate soil and water.

INTERACTIONS OF SOIL-BORNE MICROORGANISMS AND DIMETHYL TETRACHLOROTEREPHTHALATE

Since the discovery at Boyce Thompson Institute (Limpel, et al., 1959) of the pre-emergence herbicidal activity of dimethyl-2,3,5,6-tetrachloroterephthalate (DCPA or Dacthal), its use for the control of crabgrass, other grass annuals and certain broadleaved weeds in a number of crops has increased rapidly. Although its efficacy as a herbicide has been the subject of many investigations, its interactions with soil microorganisms had not been thoroughly investigated. The latter area was, therefore, selected for study.

Methods

General. Soil samples were collected from the Boyce Thompson Institute Farm to which DCPA had been applied previously at a rate of 19 lb/A each year for five years and from similar plots that had not been treated with DCPA. An additional soil sample was obtained from a Colorado field where DCPA had been ineffective as a herbicide. Untreated Colorado soil was not available.

Populations of microorganisms in soil samples were determined by dilution plate techniques. Commercial nutrient agar was used for these platings as well as for maintaining stock cultures. A minimal medium used in many of the studies contained 0.05 M glucose, 5.0×10^{-3} M dibasic potassium phosphate, 1.0×10^{-4} M magnesium sulfate and 0.01 M sodium nitrate.

Effect of DCPA upon the populations of soil microflora. Determinations of soil microbial populations were made immediately after the soil samples had been collected. Subsequently, samples of these soils were mixed with DCPA at the rate of 20 lb/A and incubated at 37° C in nearly air-tight containers to maintain moisture content. Samples treated in a similar manner but without herbicide treatment served as controls. Sub-samples of each soil treatment were removed periodically and the populations of bacteria and actinomycetes determined. Each treatment was replicated five times.

The DCPA tolerance of microorganisms in pure culture was determined by incorporating DCPA into the minimal medium defined above at 1, 10, 100 and 1000 ppm. The agar used for these experiments had been extracted previously with 80% ethanol by refluxing for 16 hours to free the agar of extraneous carbon sources. Cultures of bacteria and actinomycetes were transferred to plates containing the media of varying DCPA concentrations using the replicate plate technique

(Lederberg and Lederberg, 1952).

The ability of selected microorganisms to utilize DCPA as a sole carbon source was investigated by substituting various concentrations of DCPA for glucose in the minimum medium. Studies on solid media (1.8% agar) were supplemented by using a liquid medium of a similar constitution. In the case of the liquid medium, aliquots were subsequently removed and plated to determine any change in numbers of viable cells.

Degradation studies. Selections were made from the bacterial isolates based upon their ability to utilize DCPA as a sole carbon source. Cultures were incubated for 96 hours and extracted as follows. The microbial cells were removed and the ambient solution adjusted to pH 12 with 5 N sodium hydroxide in order to form the sodium salt of the acid forms of DCPA. The cells and the supernatant were each extracted with dichloromethane. The aqueous solution was then adjusted to approximately pH 3 with concentrated hydrochloric acid and extracted with ethyl acetate. The quantities of DCPA and methyl-2,3,5,6-tetrachloroterephthalate were analyzed by gas liquid chromatography (GLC).

For GLC determinations, the dichloromethane and ethyl acetate solutions were evaporated to dryness and the residues dissolved in acetone. A Wilkins Gas Chromatograph Model 204 equipped with an electron capture detector was used for the GLC determinations. The following column conditions were used: 5% Dow 11 on 60 to 80-mesh Chromosorb W (HMDS), oven temperature 180° C, injector temperature for dichloromethane fractions 250° C, for ethyl acetate fractions 325° C, and the nitrogen flow rate was 40 ml/min.

To study the removal of chlorine from the ring ^{36}Cl-labeled DCPA (0.1 μc/100 ml of solution) was used. Two bacterial selections which tolerated high concentrations of DCPA and appeared to degrade it were used for these studies. The concentration of cells when the labeled herbicide was added was approximately 1 X 10^7 cells/ml. After incubation the cells and the ambient solution were extracted separately with dichloromethane and ethyl acetate. After extraction with ethyl acetate, the aqueous solutions were adjusted to pH 12 with sodium hydroxide to prevent the loss during drying of any chlorine present. Aliquots of each of the extracts were evaporated to dryness at room temperature in a hood. Radioactivity was determined with a low background (2 cmp) gas flow counter.

Results

Interaction studies. Plate counts showed that the ratio of actinomycetes to bacteria was higher in soils previously treated with DCPA

than in untreated soils (Table 1). In New York soils, the percentage of actinomycetes was three times greater than in untreated soils. In Colorado soil, the percentage and population of actinomycetes were much higher than in the New York soil. Gram-negative rod bacteria were absent in the New York soil treated with DCPA, but comprised a high percentage of the microbial population in untreated soils. However, Gram-negative rods were prevalent in the previously treated Colorado soils.

Table 1. Distribution of microorganisms in selected soils from New York and Colorado

Microorganism	New York		Colorado
	Treated %	Untreated %	%
Bacteria, Gram + coccoid	29	27	6
Bacteria, Gram + rods	41	45	19
Bacteria, Gram - rods	0	27	19
Actinomycetes	30	9	56

The initial population of microorganisms in previously treated New York soil was slightly lower than in untreated soil (Table 2). The addition of DCPA to previously treated soil caused a depression in the rate of microbial increase, whereas, treatment of previously untreated soil resulted in a slower increase in microbial population than for the control. These results indicate that, although DCPA has little effect upon the total microbial population, it may select the organisms which predominate in the soil.

Table 2. The effect of DCPA on the populations of microorganisms in the soil as measured by dilution plate technique and expressed as colonies per sq cm

Soil sample	Dilution		Time in days					
			0	1	4	7	10	14
Colorado	10^5	Treated	72	172	∞	∞	∞	∞
Colorado	10^5	Control	70	64	70	90	150	200
New York[a]	10^4	Treated	69	98	198	340	520	650
New York	10^4	Control	69	150	348	522	853	∞
New York[b]	10^4	Treated	59	120	406	750	805	∞
New York	10^4	Control	59	134	475	890	∞	∞

[a] No previous DCPA treatment. [b] Previous DCPA treatment.

When the herbicide was incorporated into a minimal medium containing a carbon source, it appeared to have little detrimental effect upon the growth of the Gram-positive and Gram-negative rod bacteria at concentrations of 100 ppm (Table 3). DCPA had no effect upon any of the organisms at 1 and 10 ppm, thus those values are not reported. Fewer of the coccoid bacteria grew on media containing DCPA at 100 and 1000 ppm than at 1 and 10 ppm. Slightly more selections of the actinomycetes grew on the media containing 100 and 1000 ppm than at concentrations below 100 ppm.

Table 3. The effect of DCPA on growth of microorganisms as measured by the percentage of 20 different isolates which grew on media containing various quantities of DCPA

Microorganism	MM[a]	MM + 100 ppm	MM + 1000 ppm	Water agar	Water agar extracted[b]
Actinomycetes	90	100	100	85	5
Bacteria, Gram + rods	90	85	30	35	15
Bacteria, Gram - rods	75	65	45	25	6
Bacteria, Gram + coccoid	65	25	25	25	5

[a] Minimal medium.
[b] Extracted with 80% ethanol by refluxing for 18 hours before preparation of agar medium.

When the isolates were grown on an agar medium containing DCPA as the sole carbon source, few bacteria grew (Table 4). More bacterial isolates grew at 1000 ppm than at 100 ppm or less. However, several of the actinomycetes grew at 100 ppm and nearly all actinomycetes selections grew at 1000 ppm. These data were confirmed by growing the organisms in liquid media and subsequently plating an aliquot to determine the increase in cell population.

Table 4. The ability of microorganisms to utilize DCPA as a sole carbon source as measured by the percentage of different isolates which grew on the respective media from the 20 tested

Microorganism	MM[a]	MM minus carbon source	MM without glucose		
			100 ppm	1000 ppm	10,000 ppm
Actinomycetes	95	15	25	90	90
Bacteria	80	5	5	15	15

[a] Minimal medium.

Degradation studies. Less than one-third of the DCPA was recovered as the parent compound or the monomethyl ester (Table 5). Most of the monomethyl ester was recovered from the cells, and only a trace was recovered from the ambient solution, thus it appears that degradation occurs intracellularly. All selections of microorganisms tested appeared to be equally capable of degrading DCPA.

Table 5. The degradation of DCPA by microorganisms as measured by the recovery of the parent compound and the monomethyl ester by gas liquid chromatography

Microorganism	DCPA Recovery			Monomethyl ester Recovery			
	Liquid	Bacterial cells	% Recovery	Liquid	Bacterial cells	% Recovery	Total % Recovery
Bacteria, Gram + coccoid	21	13	17	0	10	5	22
Bacteria, Gram + rod	14	8	11	1	15	8	26
Bacteria, Gram variable rod	20	5	13	1	1	1	14
Actinomycete	14	2	8	0	3	2	10
Bacteria, Gram + rod	29	trace	15	trace	5	3	18
Bacteria, Gram + rod	15	18	17	trace	1	1	18
Actinomycete	12	3	15	trace	5	-	10
Control	188	trace	94	trace	-	0	94

When ^{36}Cl-labeled DCPA was incubated with two bacterial selections, very little radioactivity was found in the aqueous solution (Table 6) which indicated that little if any chlorine was freed from the ring. It is probable that the activity remaining in aqueous solution was a result of incomplete extraction with dichloromethane or ethyl acetate rather than a dechlorination of the ring moiety.

More than 80% of the radioactivity was found in the ethyl acetate fraction for both selections. This fraction would contain both the monomethyl ester and acid fragments of DCPA. Nearly all of the radioactivity was associated with the microbial cells, thus confirming the previous supposition that DCPA is degraded intracellularly.

Table 6. The degradation of DCPA using ^{36}Cl-labeled herbicide as measured by the radioactivity of the various extracts

	Ambient solution			Bacterial cells		
	Dichloro-methane	Ethyl acetate	Water	Dichloro-methane	Ethyl acetate	Water
Bacterium, Gram + coccoid	1200[a]	1400	58	670	10200	413
Bacterium, Gram variable rod	1800	1700	48	1580	14110	256

[a] Counts per minute.

Discussion

The data show that DCPA has little or no adverse effects upon the population of soil-borne microorganisms. The data also indicate that growth of some of the actinomycetes is enhanced by the presence of high concentrations of DCPA, which possibly serves as a carbon source. When the herbicide was added to a liquid medium and its disappearance studied little or no difference could be found between the ability of the microorganisms to degrade DCPA. All cultures appeared to degrade DCPA to about the same extent; however, it is doubtful that this herbicide is an important carbon source for microorganisms. At the low concentrations which usually would be encountered in the soils, there was almost no stimulatory effect and the amount of carbon being supplied would be negligible.

The stimulatory effect on growth when high concentrations of DCPA were used was unexpected in view of the fact that its solubility is approximately 0.5 ppm. Thus, at concentrations above 0.5 ppm, aqueous solutions would be saturated. It is possible that the bacteria set up a microenvironment around the herbicide particles, and concentrations of 100 ppm and above are required to supply sufficient carbon to result in measurable growth. Another possibility is that, at these high concentrations, the quantity of DCPA dissolves more quickly as it is being removed from the microenvironment by the microorganisms.

The data (Tables 5 and 6) indicate that DCPA is being degraded by microorganisms and two degradation products are monomethyl-2,3,5,6-tetrachloroterephthalate and 2,3,5,6-tetrachloroterephthalic acid. These two degradation products have been found to occur under field conditions by Skinner *et al.*, (1964), who proposed that the hydrolysis of the methyl esters occurs mainly in the soil and very little hydrolysis occurs in plants and animals.

Fields et al. (1967) found that several groups of organisms including paramecia, fungi, bacteria, and algae would tolerate DCPA and a stimulatory effect often was observed. They also found no buildup of DCPA when applied over a 7-year period.

Unfortunately, a quantitative method could not be employed succesfully to determine the quantity of 2,3,5,6-tetrachloroterephthalic acid in these studies, and the method for determining monomethyl-2,3,5,6-tetrachloroterephthalate was only semi-quantitative. When the injector oven was raised to a temperature sufficiently high to decarboxylate this compound to methyl tetrachlorobenzoate, a constant temperature for the column oven could not be maintained. When known amounts of the monomethyl compound were analyzed, decarboxylation varied greatly with temperature.

SECTION V

STUDY OF THE DEGRADATION OF DIMETHYL TETRACHLOROTEREPHTHALATE

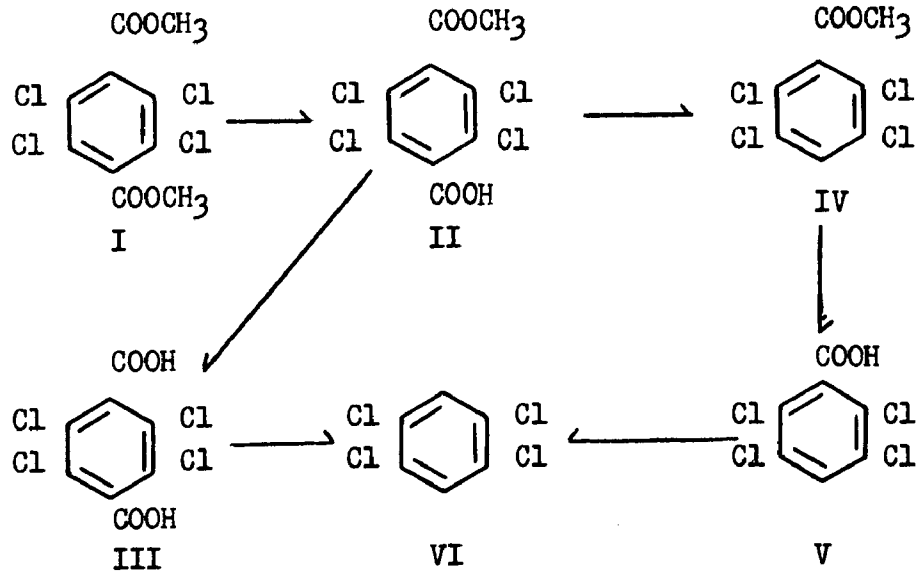
It is frequently possible, upon examination of the structure of a herbicide molecule, to hypothesize a plausible series of degradative steps. Thus a rational approach for the study of the decomposition of such compounds as herbicides in a biological environment can be developed. Identification of specific intermediates may allow for the reconstruction of a possible degradative pathway.

The proposed degradation path of dimethyl tetrachloroterephthalate (I) to 1,2,4,5-tetrachlorobenzene (VI) is shown in Scheme I below. Likely points of attack on the dimethyl tetrachloroterephthalate (I) (DCPA) molecule are the ester linkages which may be cleaved singly or simultaneously, resulting in monomethyl tetrachloroterephthalate (II) and tetrachloroterephthalic acid (III), respectively. II may be decarboxylated to yield methyl 2,3,5,6-tetrachlorobenzoic acid (IV), and III may also be mono or didecarboxylated to yield 2,3,5,6-tetrachlorobenzoic acid (V) and 1,2,4,5-tetrachlorobenzene (VI), respectively. The disappearance of VI from soil may be partly due to its high volatility, according to Josephs et al. (1957). Hydrolysis of the chlorine atoms followed by ring opening with subsequent degradation to innocuous small molecules should also be considered.

Previous work on the presence of degradation products of I in plants grown in I-treated soil was carried out by Skinner et al. (1964). II and III were found in plant tissues; but compounds I, IV, V and VI were not detected. This may be due, in part, to the extraction procedure employed in which the plant extract was partitioned between ether and water at pH 6. The ether phase, which was discarded, could have contained I, IV, and VI.

The work reported here was concerned with the development of an analytical method for the detection and identification of compounds I to VI, and some preliminary data on findings in I-treated soil.

Since, among the proposed degradation products, II, III, and V would be susceptible to thermal decarboxylation in the gas chromatograph, the products formed could be confused with proposed soil degradation products. Consequently, all free carboxylic acids were converted to the acid chlorides, by means of thionyl chloride, which in turn were treated with propanol to form the propyl esters. These derivatives would be stable, sufficiently volatile, and unlikely to be found in soil as such.



Scheme I. Proposed degradation pathways of dimethyl tetrachloroterephthalate (I) to 1,2,4,5-tetrachlorobenzene (VI).

Methods and Materials

Compounds. I, II (as sodium salt), III, and tetrachloroterephthaloyl chloride were obtained from Diamond Shamrock Company, Painesville, Ohio; V and 2,3,5,6-tetrachlorobenzoyl chloride were obtained from Hooker Chemical Corp., Niagara Falls, N. Y.; and VI was purchased from Aldrich Chemical Company, Inc., Milwaukee, Wis. The remaining esters, IV, propyl 2,3,5,6-tetrachlorobenzoate (VII), methyl propyl tetrachloroterephthalate (VIII), and dipropyl tetrachloroterephthalate (IX) were prepared.

Methyl 2,3,5,6-tetrachlorobenzoate (IV). A mixture of 2.0 g. (0.0076 mole) of 2,3,5,6-tetrachlorobenzoic acid, 20 ml of thionyl chloride, and 1 drop of pyridine was heated under reflux overnight. Excess thionyl chloride was removed by vacuum evaporation, and to the residue was added 40 ml of methanol. The solution was again heated overnight, after which the solvent was flash evaporated. The yield of crude product was 1.85 g (88%), m.p. 66° to 68° C. An analytical sample was crystallized from aqueous ethanol, m.p. 68° to 69° C. A similar yield of product was obtained from the preformed acid chloride.

Anal. calcd. for $C_8H_4Cl_4O_2$: C, 35.08; H, 1.47; Cl, 51.77. Found: C, 35.36; H, 1.45; Cl, 51.33.

Propyl 2,3,5,6-tetrachlorobenzoate (VII) was prepared from 2,3,5,6-tetrachlorobenzoic acid, as well as from the preformed acid chloride in 86 to 88% yield. A sample was crystallized from aqueous ethanol for analysis, m.p. 74° to 75° C.

Anal. calcd. for $C_{10}H_8Cl_4O_2$: C, 39.77; H, 2.67; Cl, 46.96. Found: C, 39.94; H, 2.45; Cl, 46.72.

Methyl propyl tetrachloroterephthalate (VIII) was prepared from sodium methyl tetrachloroterephthalate in 95% yield in the same manner as IV. The sample for analysis was crystallized from aqueous ethanol, m.p. 65.5° to 66.5° C.

Anal. calcd. for $C_{10}H_{10}Cl_4O_4$: C, 40.03; H, 2.80; Cl, 39.39. Found: C, 40.26; H, 2.60; Cl, 39.09.

Dipropyl tetrachloroterephthalate (IX) was prepared from tetrachloroterephthalic acid, as well as from the preformed acid chloride in 84 to 86% yield. An analytical sample was obtained by crystallization from aqueous ethanol, m.p. 88.5° to 89° C.

Anal. calcd. for $C_{14}H_{14}Cl_4O_2$: C, 43.33; H, 3.64; Cl, 36.55. Found: C, 43.38; H, 3.39; Cl, 36.65.

Analytical Procedure. All separations were carried out in an Aerograph Model 204 gas chromatograph fitted with an electron capture detector. The column employed was 5 ft X 1/8 in. o.d. stainless steel packed with 5% hexamethyl disilazane on Chromosorb W (80/100 mesh) (QF-I). Retention data were obtained under isothermal conditions. For the standard compounds the column temperature was kept at 193° C., and the detector and injector temperatures were 199° C. and 247° C., respectively, with a flow rate of nitrogen at 25 ml/min. The soil extract was chromatographed at column, detector, and injector temperatures of 180° C., 190° C., and 205° C., respectively, and the flow rate of nitrogen was 31.5 ml/min.

Preparation of Soil Extract. A 100-kg soil sample collected to a depth of 24 in. was obtained from a plot of land which had been treated with a cumulative total of 94 lb/acre of I in five annual treatments, and then remained untreated for three additional years. The sample was twice extracted with acetone at pH < 2 overnight. The acetone extract was evaporated to near dryness under vacuum, and residual water was removed by azeotroping with benzene. After flash evaporation of the benzene, the residue was dried over sulfuric acid for one week, and 83.5 g. of dry product resulted. For analysis, 5.0 g. of soil extract was heated under reflux with 50 ml of thionyl chloride overnight. The unreacted thionyl chloride was removed by vacuum distillation, and the residue was refluxed with 50 ml of propanol for 48 hours until very

little hydrogen chloride continued to evolve. The propanol was flash evaporated and the residue dissolved in carbon tetrachloride. The solution of esters was washed several times with water and dried by azeotropic distillation. The carbon tetrachloride was flash distilled, and the dry residue was dissolved in a known volume of acetone and was chromatographed.

Results and Discussion

A mixture of I, IV, and VI to IX was resolved in the gas chromatograph. The feasibility of separating and identifying a mixture of compounds related to the proposed degradation products of I was thus demonstrated. When the extract of I-treated soil was chromatographed peaks 1, 2, and 3, on the basis of retention times were suspected as being due to the presence of compounds, I, VIII, and IX, respectively. Since these peaks were reasonably well separated from one another, small portions of authentic samples of I, VIII, and IX were added to another portion of extract, and the mixture chromatographed. Upon co-chromatographing the extracted compounds in the presence of true samples, no separation was apparent. Thus peaks 1, 2, and 3, were tentatively identified as compounds I, VIII, and IX, which indicates the presence of dimethyl tetrachloroterephthalate (I) as a residue in soil and monomethyl tetrachloroterephthalate (II) and tetrachloroterephthalic acid (III) as degradation products. Further work would be necessary to establish whether or not the remaining proposed degradation products of dimethyl tetrachloroterephthalate can be identified in soil extracts.

SECTION VI

MICROBIAL DEGRADATION OF BROMACIL

Since bromacil, 5-bromo-3-sec-butyl-6-methyluracil, is recommended for use in general weed control in noncropland areas at rates up the 24 lb/A, its use, unless degraded readily by soil microorganisms, may present an environmental hazard. Studies were, therefore, initiated on the microbiological degradation of bromacil and its effects on soil microorganisms.

Soils having no history of exposure to bromacil were treated in the laboratory with 40 ppm of bromacil in a soil perfusion system (Audus, 1946 and 1960) or by mixing at rates up to 200 lb/A and allowed to incubate. Using dilution plate techniques (potato-dextrose agar for fungi and nutrient agar for bacteria), fungi and bacteria were isolated from the various treated soils. Fifty-five fungus and 73 bacterial cultures were selected and their ability to degrade bromacil studied by growing them in a Czapek-Dox broth containing 20 ppm of bromacil and periodically determining the amount of bromacil present using a bioassay system in which bromacil at concentrations down to less than 1 ppm can be detected using buckwheat as the test organism. None of the bacterial cultures appeared to degrade bromacil, but four of the fungus cultures exhibited this capability. One of these cultures which has been identified by the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, as Penicillium paraherquei Abe. was particularly active and was selected for further study. No significant amounts of bromacil could be detected 15-20 days after Czapek-Dox broth containing 20 ppm of the herbicide had been inoculated with this organism. Sterile soil treated with bromacil at a rate of 3.1 lb/A was still toxic to buckwheat after 56 days but no herbicidal effects could be detected 21 days after treatment with 6.2 lb/A or 28 days after treatment with 12.5 lb/A in sterile soil inoculated with P. paraherquei. Herbicidal effectiveness of the 25 and 50 lb/A applications was reduced to 50 and 80%, respectively, after 56 days. This organism was also able to degrade Terbacil (3-tert-butyl-5-chloro-6-methyl uracil), a closely related compound. However, when non-sterile soil was inoculated with P. paraherquei, no degradation of either Bromacil or Terbacil was observed. Apparently this organism is not sufficiently aggressive to compete with the natural soil microorganism population.

When liquid and solid Czapek-Dox media containing Bromacil were inoculated with P. paraherquei, the fungus grew normally at concentrations up to 200 ppm, but at higher levels growth was slower and more compact in appearance. Transfers from liquid media containing 800-1000 ppm of Bromacil yielded variant cultures characterized by the lack of the yellow pigment associated with the original culture. When

inoculated into a liquid mineral salts medium containing Bromacil as the sole carbon and nitrogen source, the variant grew slowly, whereas, the original culture failed to grow. When included in the medium at 2,000 ppm, Bromacil crystals precipitated out of solution, and mycelia of the variant fungus were observed growing on their surface. Additional studies on the degradative properties of the variant and the ability of Bromacil to produce variants are underway.

Following continued maintenance in culture, our isolates of P. paraherquei lost their ability to degrade Bromacil. By continual growth on culture media containing Bromacil, this ability could be regenerated but was soon lost again when grown on non-Bromacil containing media. Due to this instability and lack of aggressiveness in unsterile soil and because more interesting and promising organisms were being isolated in other aspects of the program, work was discontinued with P. paraherquei.

SECTION VII

PROMOTION OF HERBICIDE DEGRADATION IN SOIL BY THE APPLICATION OF MICROBIAL NUTRIENT BROTHS

Pesticides applied to cropland often remain in the soil longer than is desirable, and may find their way into water resources or other ecosystems via groundwaters and harvested foliage. Herbicides may remain in the soil until the next growing season and limit land use and crop rotation options. To control the amounts and lifetimes of these substances in soil, a preventive approach, such as superior design and practice, would be far more favorable to a corrective approach. However, several corrective approaches have been suggested including the application to soil of absorbants, chemicals, and even UV light as well as rotation, tillage and irrigation practices (Foy and Bingham, 1969; Kearney, et al., 1969). Application of microbes having the capacity to degrade herbicides has also been suggested (Audus, 1951) and attempted (Kearney, et al., 1969; MacRae and Alexander, 1965). Stimulation of the indigenous microflora to attack pollutants is yet another possibility. For example, it has long been known that the decomposition of resistant soil organic matter is increased by the addition of easily degraded materials (Bartholemew, 1957). It has also been noted that microbial degradation of certain herbicides and related compounds is accelerated by the addition of supplementary nutrients such as those found in ordinary microbial culture broths (Bartha, et al., 1967). In this context, the application of nutrient materials to lands that have been polluted may in some instances be a practical possibility. The purpose of the research reported below was to seek data in support of this suggestion.

Materials and Methods

The principal objective of our experimental approach was to contrast residue levels on broth treated soils with those on no-broth treated soils. Two major experiments were undertaken which were similar in design and differed only in the herbicides studied, the kinds of nutrient broths added, and the lengths of incubation time between broth addition and measurement of residual herbicide. It was decided that a bioassay rather than a chemical assay would be used to measure residue level after treatment. This choice, although sacrificing accuracy and precision, has the advantages of convenience and biological significance.

The herbicides arbitrarily chosen for study were diphenamid (N,N-dimethyl-2,2-diphenylacetamide), monuron [3-(p-chlorophenyl)-1,1-dimethylurea], atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine), dicamba (2-methoxy-3,6-dichlorobenzoic acid), CIPC [iso-

propyl N-(3-chlorophenyl)carbamate], and amiben (3-amino-2,5-dichlorobenzoic acid). These herbicides were applied to the surface of a composted Norfolk dry loam which was contained in small rectangular flats (7.5 in. wide, 9 in. long, 4 in. deep). Rates of application were selected to give an adequate response but not an excessive one under these conditions. The rates chosen were calculated on a surface basis and were at the lower limit of the dosage range recommended for field soils in the WSA handbook (Weed Society of America, 1967) and were as follows in lb/A: diphenamid 3, monuron 1.5, atrazine 1.5, dicamba 0.9, CIPC 1.8, and amiben 1.8. Test plant species selected on the basis of their sensitivity to all the herbicides used in the study were foxtail grass (Alopecurus pratensis L.), Johnson grass [Sorghum halepense (L.) Pers.], crabgrass [Digitaria sanguinalis (L.) Scop.], oats (Avena sativa L.), velvet leaf (Abutilon theophrasti Medic.), and mustard (Brassica sp.). Uncertainty over which species was the most sensitive indicator for any particular herbicide and the desirability of having comparable estimates of all residue levels for all six herbicides led to the decision to use all six species simultaneously in the bioassay. Therefore, the estimate of residual activity was made by collectively harvesting any living plant material remaining in the soil flat. The assumption was made that under these conditions and for the rates used the yield was acceptably proportional to the effective soil concentration and that any abnormalities in growth were also manifested as a yield effect.

In Experiment I, each herbicide was applied to 12 soil flats by spraying onto the moist soil surface 40 ml of a formulated suspension. Immediately following treatment, 4 of the 12 flats were sprayed with 200 ml of water, 4 were sprayed with 200 ml of double strength Czapek Dox broth, and 4 were sprayed with 200 ml of double strength Difco nutrient broth. The Czapek Dox broth contained the following substances in grams per liter: sucrose 30, $NaNO_3$ 3, K_2HPO_4 1, $MgSO_4$ 0.5, KCl 0.5, $FeSO_4$ 0.01. The Difco nutrient broth contained 3 g/l of beef extract and 5 g/l of peptone. The flats were then incubated at 30° C. Two of the flats (replicates) in each group of 4 were planted 7 days after treatment (Group I); the remaining 2 (Group II) were planted 21 days after treatment. Group II flats also received two additional broth treatments 7 and 14 days after the original treatment. Thus Group II flats represented a more intensive treatment and a longer time interval through which the treatment might act. Three weeks after planting, all green foliage was collectively harvested without regard to species, dried and weighed.

Experiment II was designed identically to Experiment I with the following exceptions: diuron [3-(3,4-dichlorophenyl)-1,1-dimethyl urea] was tested at the rate of 1.5 lb/A in addition to diphenamid, monuron, and atrazine at the same rates as in Experiment I. Instead of 2 groups

there were four groups differing both in incubation time and in the amount of nutrient broth subsequently added after the initial broth treatment. Group I flats were incubated 2 weeks after the initial broth treatment. Group II flats were incubated 4 weeks and received one additional broth treatment after 2 weeks. Group III flats were incubated 7 weeks and received additional broth treatments at 2, 4, and 6 weeks. Group IV flats were incubated 15 weeks and received additional broth treatments at 2, 4, 6, 8, and 10 weeks. Thus, as in Experiment I, Groups II, III, and IV represented successively more intensive broth treatment as well as longer incubation times. Finally, instead of applying two different kinds of broth, as in Experiment I, only one kind of broth was added. This was a combination of double strength Czapek Dox broth and Difco nutrient broth in the proportion of three parts to one, respectively. After the stated incubation time, all flats were planted and harvested 4 weeks later.

Results and Discussion

The dry weight yields of plants in each flat for Experiments I and II are recorded in Tables 7 and 8, respectively. The data at each time period are representative of a bioassay at that time for the amount of herbicide remaining in the soil after treatment. Yields of replicate broth and no-broth treated flats are grouped in adjoining columns along with their means for easy comparison. Although the quantitative relation between residue levels and yield is not known, it may be assumed nevertheless that the two are in some straightforward inverse relationship. Conversely, the toxicity and the residue level are directly related. Therefore, any reduction in herbicide toxicity with time and/or broth treatment may be interpreted as a reduction in residue level as a result of a particular rate of microbial degradation.

It is evident that there was an apparent beneficial effect of broth treatment alone and also an effect due to variable greenhouse conditions during the different bioassay periods. For this reason all the data of Table 7 and 8 have been re-expressed in Table 9 as the percentage difference between the means of broth treated and no-broth treated flats. Any percentage figure which does not substantially exceed its appropriate no-herbicide control value is probably insignificant as a measure of accelerated herbicide degradation by the broth. Percentage differences preceded by a negative sign signify a lower yield on the broth treated flats than on the no-broth treated flats. Positive figures are a measure of the effectiveness of the broth treatment relative to the level of toxicity in the soil after any incubation time.

Table 7. Experiment I. Effect of broth addition and incubation time on the yield of test plants grown in soil flats treated with different herbicides.

Herbicide applied	Rate lb/A		Yield of foliage after 3-week growing period (g. dry wt/flat)					
			Incubation time and number of broth treatments					
			Group I 1 week - 1 treatment			Group II 3 weeks - 3 treatments		
			No-broth control	Czapek Dox broth	Difco nutrient broth	No-broth control	Czapek Dox broth	Difco nutrient broth
Diphenamid	3	R_1	0.78	0.63	1.30	0.66	1.65	0.96
		R_2	0.66	1.00	0.94	0.34	1.85	0.94
		$\bar{x}$	0.72	0.82	1.12	0.50	1.75	0.95
Monuron	1.5	R_1	0.01	0.02	0.01	0.18	0.24	0.05
		R_2	0.06	0.01	0.01	0.05	0.12	0.02
		$\bar{x}$	0.04	0.02	0.01	0.12	0.18	0.04
Atrazine	1.5	R_1	0.17	0.15	0.15	0.09	0.20	0.15
		R_2	0.12	0.12	0.17	0.12	0.09	0.23
		$\bar{x}$	0.14	0.14	0.16	0.10	0.14	0.19
Dicamba	0.9	R_1	0.51	1.78	1.06	1.77	3.14	1.99
		R_2	0.40	1.72	0.50	2.31	3.10	1.48
		$\bar{x}$	0.46	1.75	0.73	2.04	3.12	1.74
CIPC	1.8	R_1	1.33	2.40	2.37	2.58	3.56	2.78
		R_2	1.09	1.78	2.23	2.34	3.89	2.80
		$\bar{x}$	1.21	2.09	2.30	2.46	3.72	2.79
Amiben	1.8	R_1	0.46	0.84	0.50	0.57	1.50	0.55
		R_2	0.50	0.92	0.63	1.19	1.86	0.78
		$\bar{x}$	0.48	0.88	0.56	0.88	1.68	0.66
No-herbicide - control		R_1	1.74	2.06	2.29	2.24	2.35	2.92
		R_2	1.82	2.24	2.28	2.08	3.12	1.64
		$\bar{x}$	1.78	2.15	2.28	2.16	2.74	2.28

Table 8. Experiment II. Effect of broth addition and incubation time on yield of test plants grown on soil flats treated with different herbicides

Herbicide applied	Rate lb/A		Yield of living foliage after 4-wk growing period (g. dry wt/flat)							
			Incubation time and number of broth treatments							
			Group I 2 wk - 1 treatment		Group II 4 wk - 2 treatment		Group III 7 wk - 4 treatment		Group IV 15 wk - 6 treatment	
			No-broth control	Combined broth	No-broth control	Combined broth	No-broth control	Combined broth	No-broth control	Combined broth
Diphenamid	3	R_1	0.00	0.00	0.01	0.02	0.04	0.25	0.35	0.16
		R_2	0.00	0.00	0.01	0.08	0.16	0.89	0.31	0.49
		$\bar{x}$	0.00	0.00	0.01	0.05	0.10	0.57	0.33	0.32
Monuron	1.5	R_1	0.08	0.20	0.53	1.95	1.35	-	-	2.88
		R_2	0.05	0.18	0.82	2.25	1.67	2.68	2.11	2.55
		$\bar{x}$	0.06	0.19	0.68	2.10	1.51			2.72
Atrazine	1.5	R_1	0.03	0.03	0.14	0.13	0.60	1.30	1.80	1.49
		R_2	0.03	0.03	0.10	0.23	0.37	2.41	1.94	1.24
		$\bar{x}$	0.03	0.03	0.12	0.18	0.48	1.86	1.87	1.36
Diuron	1.5	R_1	0.22	-	0.38	2.65	1.65	4.25	2.16	2.40
		R_2	0.23	0.26	0.14	3.38	2.05	4.56	2.60	1.98
		$\bar{x}$	0.22		0.26	3.01	1.85	4.40	2.38	2.19
No-herbicide control	-	R_1	2.34	3.80	1.60	3.96	3.23	5.69	3.48	4.84
		R_2	2.50	3.41	2.10	3.64	3.64	5.47	3.50	3.43
		$\bar{x}$	2.42	3.60	1.85	3.80	3.44	5.58	3.49	4.14

Table 9. Percentage difference between the mean foliage yield of broth treated and no-broth control flats in Experiment I and Experiment II

Herbicide applied	Experiment I			
	Percentage difference			
	Group I		Group II	
	Czapek Dox broth	Difco nutrient broth	Czapek Dox broth	Difco nutrient broth
Diphenamid	14	56	250	90
Monuron	-50	-75	50	-67
Atrazine	0	14	40	90
Dicamba	280	59	53	15
CIPC	73	90	51	13
Amiben	83	17	91	-25
No-herbicide control	21	28	27	6

	Experiment II			
	Percentage difference			
	Group I	Group II	Group III	Group IV
Diphenamid	0	400	470	-30
Monuron	122	210	78	29
Atrazine	0	50	288	-27
Diuron	78	1060	138	-8
No-herbicide control	48	105	62	19

Broth addition has clearly accelerated the rate of degradation of most of the compounds. A study of the three tables reveals the following highlights: Czapek Dox broth addition reduced dicamba toxicity by a factor of about 4 after one week of incubation. Difco nutrient broth was not as effective. Toxicity of CIPC was almost halved by both broths after one week. Only with these two herbicides did degradation appear to proceed to the point where the soil concentrations were so low as to be stimulatory. Amiben toxicity was nearly halved after one and three weeks by Czapek Dox broth but Difco nutrient broth was ineffective. Only after more intensive time and broth treatment was diphenamid toxicity reduced 3.5 fold by Czapek Dox broth. Again, Difco nutrient broth was less effective. In Experiment II the toxicity of diphenamid was apparently greater or else the combination of the two broths was less effective than Czapek Dox broth alone. Nevertheless, there is a decided effect of the combined broth treatment on the

degradation of diphenamid after 7 weeks' incubation and 4 broth treatments. Monuron toxicity was significantly reduced by the combined broth treatment after 4 weeks of incubation and 2 broth treatments, but little thereafter. Atrazine toxicity was not affected by the combined broth treatment until after 7 weeks of incubation and 4 broth treatments. Finally, the addition of the combined broths so reduced the toxicity of diuron after 4 weeks' incubation that the yield on these flats approached 80% of the no-herbicide controls compared to 14% for no-broth treated flats. This was the most outstanding broth effect of the experiment.

The data show Difco nutrient broth alone to be an apparently inferior degradation accelerator compared with Czapek Dox broth alone. This may be only an artifact of the method of measurement of herbicide degradation as the yield of foliage. In the case of nutrient broth the yield of foliage may have been reduced by bacterial fixation of available mineral nutrients. Unlike Czapek Dox, Difco nutrient broth contains no mineral nutrients to offset the loss to plants created by bacterial fixation. This phenomenon is probably operating also in Experiment II after 15 weeks of incubation and 6 broth additions as evidenced by the predominance of negative percentage differences. The addition of any large amount of carbonaceous or nitrogenous material to soil probably requires a supplementary mineral nutrient addition for fertility balance.

An incidental benefit that may accrue to the practice of adding microbial nutrient broths to soil is the greatly improved soil structure that was seen to result. Broth treated soils were more granular and porous and showed less tendency to puddle than soil in no-broth treated flats. This may be the principal reason why the yield on the broth controls was, on the average, about 40% greater than that on the no-broth control. Presumably, the change in structure followed an increase in the colloidal organic matter on the soil particles. Such a change would be expected to increase the adsorptive capacity of the soil and thus aid in its detoxification (Upchurch, 1966). The extent to which this increased adsorptive capacity, if any, plays a role has not been estimated but cannot be overlooked.

However, assuming that microbes are probably the principal agents of herbicide degradation in soil (Audus, 1964; Foy and Bingham, 1969), it follows that any factor that stimulates microbial activity will also influence the duration of herbicide activity in soil. The total system is surely very complex and one can speculate in a general way about what the mechanism may be for accelerated herbicide degradation by added broths. Most simply the additions of readily available carbon sources may act to increase the populations of those organisms active on the herbicide, thus accelerating its disappearance from soil. The apparent effects of length of incubation and type of broth added would be expected to operate through the control of these factors on both the succession and the kinds of populations which proliferate in response

to the added carbon sources. Also, it is conceivable that the metabolism of any less preferred substrate, such as the herbicide, is dependent on the concurrent cometabolism of the preferred substrate such as the sugars and the amino acids in microbial broths.

These data indicate both the need and the promise of more work in this area. The contamination of land by organic chemicals of various kinds threatens to become more, not less, serious. The rapid destruction of such contaminants may, in many instances, be both necessary and feasible by methods suggested by this research. Studies employing a wider combination of pesticides and liquid manures, or other economically feasible materials, may yield information that would significantly strengthen the case for this novel approach to the problem of pesticide pollutants in soil.

SECTION VIII

ACCELERATED DEGRADATION OF PHENYLCARBAMATES IN SOIL BY THE APPLICATION OF A MIXED SUSPENSION OF IPC-CULTURED MICROORGANISMS

In the previous section the possibilities of stimulating the indigenous soil microflora to attack herbicide residues by the application of nutrient broths to soil were discussed. This section also deals with the elimination of herbicide residues but differs in that microorganisms known to degrade certain phenylcarbamates have been successfully applied in macroquantities directly to soil containing these compounds.

The difficulties in establishing a functioning population of introduced microorganisms in natural ecosystems are well known. MacRae and Alexander (1965) attempted to protect alfalfa seedlings from 2,4-dichlorophenoxy butyric acid by inoculating the seed with a Flavobacterium known to degrade the latter. The bacterium afforded protection to the plants only in sterile soil indicating the Flavobacterium was not able to function in the presence of the native soil population. Kaufman and Kearney (1965) isolated pure cultures of soil bacteria which degraded and utilized IPC and CIPC as the sole carbon source. Clark and Wright (1970) applied pure cultures of IPC and CIPC degrading organisms to herbicide treated soil in Petri dishes and, using a barley root assay technique, observed rates of degradation exceeding controls.

The work reported here supports and adds to these findings. We have isolated a number of species of microorganisms which degrade phenylcarbamates and acylanilides in pure culture on agar or in liquid media and also in mixed cultures using unsterile agar, liquid, and soil media. This section is concerned with the action of unsterile mixed cultures in soil from the standpoint of their possible utility in detoxifying polluted soils.

Materials and Methods

IPC (isopropyl N-phenylcarbamate) was added at concentrations of 1 mg/ml to a flask containing a dilute suspension of a composted Gloucester sandy loam soil in a mineral salts solution (MSS) buffered with phosphate at pH 7.1. The MSS contained the following salts in grams per liter: K_2HPO_4 1.6, KH_2PO_4 .4, $MgSO_4$ .2, $CaSO_4$ .1, $NaNO_3$ .1, NH_4NO_3 .5, $FeCl_3$ .002. After about two weeks on a rotary shaker, a white turbidity was observed in the flask. Successive transfer of a few drops of inoculum from this and succeeding flasks to fresh liquid media resulted each time in a dense, complex microflora able to subsist on IPC as the sole carbon source. The concentration of organisms produced by this method was commonly in the neighborhood of .5 mg/ml dry weight of bacteria as measured after cheesecloth filtration. The

studies to be described were carried out using this population in unsterile media.

A trial experiment (Expt. I) was conducted to determine whether the bacterial population would accelerate the degradation of IPC in soil as determined by a plant bioassay. Rectangular wooden flats (7.5" X 9" X 4" deep) were filled with composted Gloucester sandy loam and planted to 6 IPC-sensitive plant species. These were corn (Zea mays L.), rye grass (Lolium perenne L.), oats (Avena sativa L.), foxtail grass (Alopecurus pratensis L.), rice (Oryza sativa L.), and crabgrass [Digitaria sanguinalis (L.) Scop.]. Rates of IPC equivalent to 4 and 12 lb/A were homogenized in MSS and sprayed onto the soil surface in a 40 ml volume. The mixed, unsterile bacterial suspension as described above was sprayed onto the soil surface in a 60 ml volume. All treatments were replicated twice. The flats were placed in the greenhouse and the foliage, if any, was harvested, dried and weighed after two weeks.

The promising results of this study led to a second greenhouse experiment (Expt. II) having the same objective, but broadening the range of herbicides employed. Five, 10 and 15 lb/A of the herbicides IPC, CIPC (isopropyl N-[3-chlorophenyl]carbamate), Swep (methyl N-[3,4-dichlorophenyl]carbamate) and Fenuron (3-phenyl-1,1-dimethyl urea) were formulated in 40 ml of 100 ppm Triton X155 (alkyl aryl polyether alcohol, Rohm and Haas, Inc.) and sprayed onto soil flats described previously. Sixty ml of bacterial suspension having a concentration of 0.3 mg dry wt/ml was sprayed onto the flats. Half of the flats were sprayed with a steam sterilized suspension of the same organisms to serve as a control. The flats were incubated for 1 week at 30° C. They were then planted to the same bioassay species used in Expt. I. After two weeks' growing time all living foliage was harvested, dried and weighed.

A third experiment was undertaken to determine how long the applied microorganisms remain functional in the soil. Twenty-four soil flats were sprayed with 100 ml of a suspension of IPC-grown microorganisms at a concentration of 0.4 mg dry wt/ml. The flats were divided into four groups. The first group was sprayed with IPC at a rate of 15 lb/A and planted to corn. The second, third, and fourth groups were treated the same way 3, 7 and 38 days, respectively, after the bacterial application. In the meantime, they remained together in the greenhouse, and the soil surface was prevented from drying by periodic wetting. In each group there were suitable no-bacteria and no-IPC controls. Each treatment was replicated twice. Plants in each group were harvested 15 to 18 days after planting. In this way the effectiveness of the applied population after four time intervals could be estimated by measuring corn yields.

Attempts were made to isolate and identify the major microbial components of the mixed, unsterile population used in these studies. IPC agar was prepared by homogenizing solid IPC on a rotary pestle in about 50 ml of MSS. The homogenate was then added to the final volume of MSS and agar such that the final IPC concentration was .7 to 1 mg IPC/ml agar. The mixture was then autoclaved and transferred to Petri plates. At the higher levels of IPC, solid crystals formed in the Petri plates. Isolations and transfers were made by streaking or pipetting an inoculum onto fresh agar and incubating three weeks at 30° C.

Results and Discussion

The data from Expt. I are presented and summarized in Table 10 as the dry wt yields of foliage on each soil flat. The small grasses, oats, rice, foxtail, crabgrass, and rye grass, were collectively harvested as their individual response to the treatments appeared comparable. Corn data are presented separately in the table because of their larger biomass and different response patterns. It is obvious that the application of IPC degrading bacteria to the soil resulted in the near complete dissipation of IPC toxicity to the test plants. It is interesting that the bacteria degraded the low rate to a concentration apparently stimulatory to corn whose yield is double that of the control value.

The results of Expt. II are presented in Table 11 and show a similar striking effect of the applied microorganisms on the effectiveness of both IPC and CIPC. Compared with a control of killed cells, application of microorganisms reduced even high rates of herbicides to levels at or approaching the vanishing point as indicated especially by the data for corn. Swep was not toxic to corn except at the highest rate. This toxicity was neutralized by the microorganisms which presumably did not eliminate Swep, but reduced it to a level tolerable to the corn. That Swep was still present in the soil flats was indicated by the more sensitive grasses, especially at the higher rates. This suggests that Swep is more resistant to degradation by the microorganisms.

The flats treated with Fenuron were not harvested, as a careful visual inspection revealed no differences between bacteria treated flats and no-bacteria treated flats. Thus the applied microorganism appeared unable to attack Fenuron despite similar structural aspects shared in common with IPC. This result is in agreement with that of Kearney (1965) who found that an enzyme of bacterial origin capable of hydrolyzing several phenylcarbamates was ineffective on the corresponding dimethylphenylurea.

Table 10. The effect of an IPC-grown mixture of microorganisms on IPC toxicity to test plants

Experiment I

Rate of IPC (lb/A)	Yield g dry wt/flat						% Increase
	No organism applied			Treated with organism			
	R_1	R_2	$\bar{x}$	R_1	R_2	$\bar{x}$	
	Yield of grasses [a]						
0	1.0	1.4	1.2	1.7	1.4	1.6	33
4	0.8	0.8	0.8	1.9	1.4	1.6	100
12	0.6	0.4	0.5	1.1	1.1	1.1	120
	Yield of corn						
0	1.5	1.9	1.7	1.8	1.5	1.7	0
4	0.2	0.9	0.6	4.0	3.7	3.8	530
12	0.2	0.1	0.2	1.8	1.1	1.5	650

[a] Cumulative weight of oats, rice, foxtail, crabgrass, rye grass,

Table 11. The effect of an IPC-grown mixture of microorganisms on IPC, CIPC, and Swep toxicity to test plants

Rate (lb/A)	Killed cells applied R_1	R_2	$\bar{x}$	Viable cells applied R_1	R_2	$\bar{x}$	% Increase
			Experiment II				
			Yield g dry wt/flat				
			IPC treated flats				
			Yield of grasses [a]				
0	0.7	0.8	0.8	0.7	0.6	0.7	0
5	0.2	0.3	0.2	0.5	0.5	0.5	150
10	0.2	0.2	0.2	0.6	0.3	0.5	150
15	0.2	0.1	0.2	0.4	0.3	0.4	100
			Yield of corn				
0	1.8	1.5	1.6	1.9	1.9	1.9	20
5	0.2	0.8	0.5	1.9	1.5	1.7	240
10	0.3	0.2	0.2	2.0	2.3	2.2	950
15	0.1	0.0	0.1	2.0	2.1	2.1	2000
			CIPC treated flats				
			Yield of grasses [a]				
0	0.8	0.8	0.8	0.7	0.6	0.6	0
5	0.2	0.1	0.2	0.5	0.6	0.6	200
10	0.1	0.1	0.1	0.8	0.7	0.7	600
15	0.1	0.0	0.1	0.9	0.7	0.8	700
			Yield of corn				
0	1.4	1.5	1.5	1.8	1.8	1.8	20
5	1.6	0.7	1.1	1.7	1.4	1.5	36
10	0.6	1.0	0.8	1.7	1.8	1.8	125
15	0.5	0.8	0.7	1.4	2.0	1.7	143
			Swep treated flats				
			Yield of grasses [a]				
0	0.8	0.8	0.8	0.7	0.6	0.6	0
5	0.3	0.4	0.3	0.7	0.4	0.6	100
10	0.2	0.2	0.2	0.4	0.4	0.4	100
15	0.1	0.1	0.1	0.4	0.2	0.3	200
			Yield of corn				
0	1.4	1.5	1.5	1.8	1.8	1.8	20
5	1.5	1.6	1.5	1.5	1.3	1.4	0
10	1.6	1.6	1.6	1.7	1.2	1.4	0
15	1.1	1.2	1.1	2.2	1.8	2.0	82

[a] Cumulative weight of oats, rice, foxtail, crabgrass, rye grass.

The results of Expt. III are shown graphically in Figure 1 by expressing the mean yields of corn on the IPC-treated microorganism-treated flats as a percentage of the mean yields of corn on the control flats (no IPC applied, microorganisms applied) at each of the four sampling periods. The effectiveness of the applied microorganisms clearly decreases in a somewhat exponential fashion as their residence time in the soil increases. By extrapolation of the curve the applied population was estimated to have lost all of its effectiveness in about 2.5 months. Presumably under these conditions, the less hardy, more exacting species in the mixed population perish first when deprived for long of the carbon source to which they have been adapted, followed by the remainder of the population. This is completely fortuitous for agricultural practice since a return to the normal biological balance in a short time is much to be preferred. However, it is apparent that this may be only one of a number of studies necessary to determine to what extent and for how long an applied population of microorganisms affects normal soil processes.

Isolation and Identification of Microorganisms. Streaks of the unsterile IPC-degrading organisms were made on sterile agar media containing IPC as the sole carbon source. After incubating for three weeks at 30° C, the dissolution of the IPC crystals in the vicinity of microbial was apparent. Dilution plates on sterile IPC containing agar were made and subcultures of representative types made. Eight isolates which appeared to be distinctly different on IPC containing agar were sent to three authorities for identification (Dr. Ruth E. Gordon, Institute of Microbiology, Rutgers University, New Brunswick, N. J.; Dr. Norvel M. McClung, Dept. of Botany and Bacteriology, University of Southern Florida, Tampa, Florida; and Mr. Gerard M. Thomas, Dept. of Entomology, University of California, Berkeley, California). The results of the identifications were not consistent and must be considered as tentative. Some of the isolates appeared to be mixed cultures. In the areas of greatest divergence of opinion (i.e., Corynebacterium, Arthrobacter and Mycobacterium), the taxonomy of these and similar forms has not been established (Gordon, 1966) which makes identifications rather meaningless. A summary of the identifications of the eight cultures is given in Table 12 with divergence of opinion being indicated by the word "or" and a mixed culture by the word "and".

The contamination of land by organic chemicals of various kinds threatens to become more, not less, serious. Although the phenylcarbamates themselves are not notable for their residual longevity in soil, the principle and implications are nonetheless clear: if microorganisms can be found which attack and degrade particular classes of compounds without threats to health or major disruptions in soil ecology, then it may be possible to "mass inoculate" contaminated soil with the results that residue levels are reduced. This report

demonstrates that there is, at least, no theoretical reason for the impossibility of such a practice. The suspensions of microorganisms used in this study were easily cultured under nonsterile conditions, were able to function in a soil environment, and became nonfunctional in a relatively short time. The feasibility of this approach points the way to yet more extended and complex uses of these microorganisms. For example, their local application to the root zone or to seed (as Rhizobium is now applied to legumes) may serve to protect crops from a herbicide to which the crop is sensitive. Such a practice would, therefore, reduce the selectivity requirements of a given herbicide and extend the conditions under which it might be employed.

Table 12. Summary of identification of eight isolates taken from an unsterile suspension of IPC-cultured microorganisms

Isolate number	Identity
12	Fusarium solani
21	Nocardia sp. and an Arthrobacter sp.
22	Aspergillus sp. or Penicillium stoloniferum and Corynebacterium or Arthrobacter or Mycobacterium
23	Corynebacterium or Arthrobacter or Mycobacterium
24	Penicillium stoloniferum and Corynebacterium or Arthrobacter or Mycobacterium
32	Corynebacterium or Arthrobacter or Mycobacterium
33	Corynebacterium or Arthrobacter and/or Salmonella
34	Streptomyces sp.

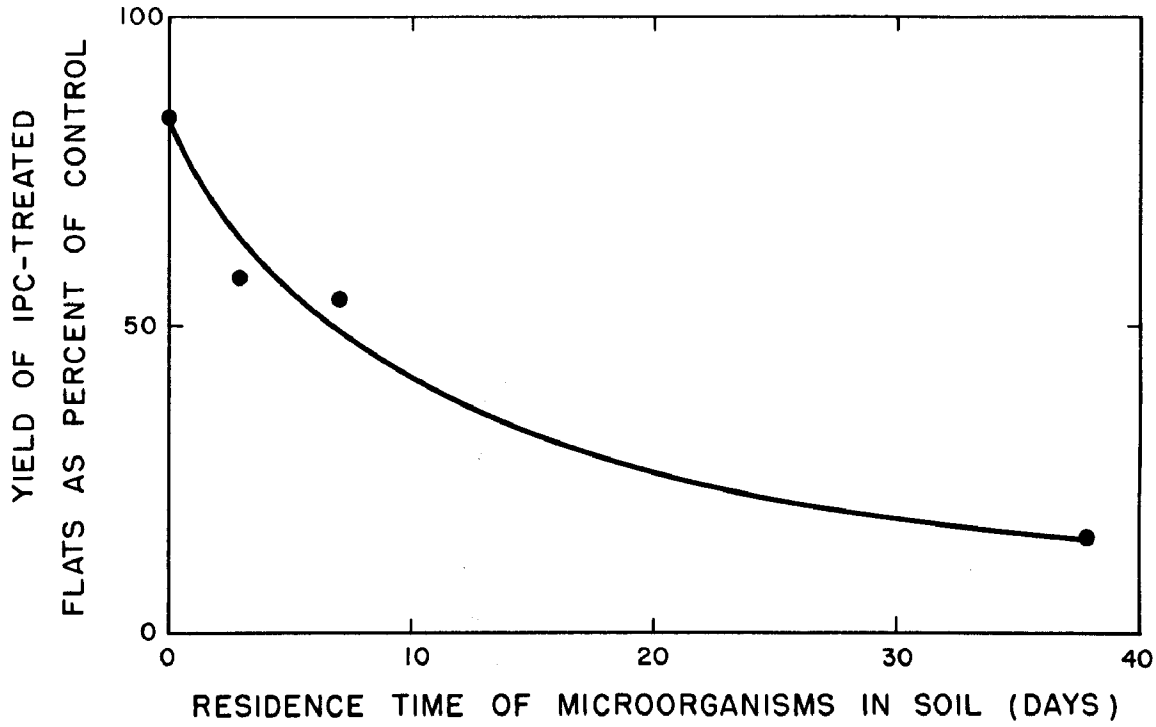


Figure 1. The Effect of Residence Time in the Soil on the Ability of Applied Microorganisms to Degrade IPC as Measured by Corn Yield.

SECTION IX

INFLUENCE OF RING CHLORINE ON THE DEGRADATION OF SOME ANILIDE HERBICIDES AND RELATED COMPOUNDS BY IPC-CULTURED MICROORGANISMS

In the previous section, the capacity of a group of microorganisms cultivated exclusively on a mineral salts medium containing IPC (isopropyl N-phenylcarbamate) to degrade phenylcarbamates when added to soil was discussed. This mixture of microorganisms was found to contain the gram positive coccoids of a Mycobacterium sp., an Arthrobacter sp., and possibly a Corynebacterium sp. in greatest abundance followed by Fusarium sp., Nocardia sp., Streptomyces sp., Aspergillus sp., and Penicillium sp. In the work reported below, the activities of these organisms are described, especially as regards the influence of molecular structure and ring chlorine on their ability to degrade compounds structurally related to IPC.

Considerable interest has centered around the degradation of three classes of anilide herbicides, namely, the dimethylphenylureas, phenylcarbamates, and acylanilides, because chloroaniline is produced as an intermediate in their degradation (Bartha, et al., 1967; Dalton, et al., 1965; Kaufman, 1967; Kearney and Kaufman, 1965). Not only are some chloroanilines quite persistent in soil (Alexander and Lustigman, 1966), but it appears that a substantial number of the chloroanilines may condense to form chloroazobenzene (Bartha, 1968; Bartha and Pramer, 1967). The formation of chloroazobenzenes is of concern in view of the carcinogenic activity of some of them. Bartha, et al. (1968) studied this reaction further and found the formation of chloroazobenzenes to be markedly dependent on the chlorine configuration of the aniline. For these reasons the influence of position of ring chlorine on the degradation of anilines or of compounds degrading to anilines is pertinent.

Methods and Materials

The microorganisms used in these studies were originally derived from a mineral salts medium containing IPC which had been inoculated with a small quantity of soil. The composition of mineral salts media in grams per liter used throughout these studies was: K_2HPO_4 1.6, KH_2PO_4 .4, $MgSO_4$ .2, $CaSO_4$ .1, $NaNO_3$ .1, NH_4NO_3 .5, and $FeCl_3$ .002. IPC was added to obtain concentrations usually ranging from 1 to 10 mg/ml. Fresh cultures were easily generated by inoculating the mineral salts-IPC containing medium in Erlenmeyer flasks with a few ml of suspension from an older culture and incubating on a rotary shaker. After an incubation period of approximately two weeks, a conspicuous turbidity developed. The final dry weight concentration of microorganisms normally ranged from 0.3 to 1.0 mg/ml of suspension.

The chemicals used in these studies were obtained from commercial suppliers and donors. Except when of analytical grade they were recrystallized from ethyl alcohol until a satisfactory melting point was obtained. Because they were difficult to obtain, isopropyl N-4-chlorophenylcarbamate (4-IPC) and isopropyl N-2,4-dichlorophenylcarbamate (2,4-IPC) were synthesized in this laboratory by refluxing the appropriate chloroaniline with isopropylchloroformate in pyridine and ether. The products were recrystallized from ethyl alcohol and gave melting points in agreement with those in the literature. For testing, compounds were formulated in 100 ppm of Triton X-155 surfactant (Rohm and Haas) as solutions or suspensions containing 50 μmol/ml. One ml or less of this stock solution or suspension was then added to 30 ml of basic mineral salts medium in duplicate 125 ml Erlenmeyer flasks. Concentration of chemicals used ranged from 0.5 to 0.7 μmol/ml (90 to 125 ppm). However, in one experiment a concentration as high as 1.7 μmol/ml was used. After being inoculated the flasks were incubated at 30° C on a rotary shaker. Periodically, 1 ml samples were removed for assay and placed in an 8 ml screw-top vial. Prior to sampling, each flask was weighed and sufficient water added to replace that lost by evaporation. Samples were either extracted immediately or stored in a freezer until extracted. The extraction procedure consisted of adding directly to the sample vial 2 ml of a spectrograde hexane:chloroform (2:1) mixture. The vials were then agitated vigorously for 10 minutes on a reciprocating shaker. The solvent layer was then poured from the vial into a silica cuvette and scanned in the UV (ultraviolet) from 320 to 220 microns using a Beckman DBG spectrophotometer. Extraction into solvent was found to have advantages over direct scanning of the centrifuged aqueous sample (Whiteside and Alexander, 1960) because of the elimination of water soluble background components and better peak definition.

The information sought in the scans was whether or not the compound degraded, how fast it did so, and whether intermediates accumulated in quantity. Intermediates were detected by changes in the absorption maximum and ring degradation was detected by decreases in absorption at the maximum. Rate of degradation was determined by estimating the amount of phenyl ring (a direct function of the UV absorption) remaining after a period of time, usually one or two weeks. To estimate rate, the final absorption at the maximum was expressed as a percentage of the initial absorption at the maximum. This method was applied even if the initial and final maxima were different, as in transitions, since it was found the molar absorptions of parent and intermediate in these studies were not appreciably different. In this way ring degradation could be quantitated. Rate of transition to the intermediate was equivalent to rate of peak shift. But this could be estimated in only a roughly qualitative way since the relative contribution of parent and intermediate to the total absorption during the transition could not be determined by these methods.

It was of some interest to know whether the composite effect of the isolates together in the unsterile suspension would be similar to the individual effect of any single species under sterile conditions. Accordingly, four isolates which had been continuously cultured on IPC agar (1 mg IPC per ml of basic mineral salts agar) and selected for their vigor, were transferred to 30 ml of a sterile medium containing 1 mg IPC per ml of mineral salts solution. The four isolates were a Fusarium sp., Mycobacterium sp., Arthrobacter sp. and Penicillium sp. that was later found to contain an Arthrobacter sp. also. When the IPC was exhausted 3 ml of the sterile suspensions were transferred to 50 ml flasks containing 12 ml of MSS. Compounds dissolved in methyl alcohol were added giving a final concentration of 0.7 µmol/ml. Samples were taken at intervals and analyzed as described above.

Warburg respirometer studies were conducted to determine the relative effect of the chemicals on the respiration of the microorganisms and as a further measure of their susceptibility to degradation. A 3.5 ml sample of microbial suspension was added to a Warburg flask and .5 ml of formulated compound suspension was added to the sidearm. The final concentration of compound in the flask after tipping ranged from 4 µmol/ml in some experiments to .3 µmol/ml in others. Each compound was assayed in triplicate at 30° C by measuring any O_2 uptake occurring during the course of metabolism.

Results and Discussion

Throughout these studies the microorganisms could be cultured in liquid or agar mineral salts media using unchlorinated anilides such as IPC, aniline, formanilide or propionanilide as the sole carbon source. All attempts to substitute chlorinated analogs of these molecules resulted in failure to produce visible cell masses of any kind even though degradation of the substrate molecule did occur in most cases. One interpretation is that the metabolism of the chlorinated ring is fundamentally different from that of the unchlorinated ring resulting in bypass or blockage of some critical growth process. Another is that the rate of metabolism of chlorinated substrates is simply too slow to support sustained observable growth. A third possibility is that chloroanilines or their derivatives are simply toxic at the concentrations employed.

In several respirometry studies, the use of chlorinated anilide substrates at a concentration of 4 µmol/ml consistently resulted in O_2 uptake that were at or below that of endogenous controls. Some chlorinated substrates were less toxic at lower substrate levels. Unchlorinated anilide substrates resulted in O_2 uptake considerably above these controls. The results of one experiment using a substrate concentration of .3 µmol/ml are shown in Figure 2. It is seen that the three unchlorinated substrates IPC, propionanilide and aniline all exhibit

high rates of uptake relative to the chlorinated substrates. The curve for aniline falls off rapidly after a steep initial rise, presumably because its molar carbon content is lower relative to IPC or propionanilide. Giving unexpectedly high rates of respiration was isopropyl N-2,4-dichlorophenylcarbamate. The lowest respiration rates were observed for the monochloroanilines and the monochloroisopropyl N-phenylcarbamates.

Measurement of Degradation Rates. Shown in Figures 3 and 4 are groups of UV scans of the hexane-chloroform extract of a particular culture flask sampled at different times. Many of the scans show a leftward shift of the UV absorption maximum from that of the parent compound time to that of a degradative intermediate at later times. In Figure 2, the dotted line indicates an identity between the absorption maximum of the intermediate and that of authentic aniline or chloroaniline. After initial independent verification by the method of Pease (1962), this identity was always taken as sufficient evidence that degradation intermediate was aniline or chloroaniline. The initial attack on molecules of this type is assumed to be a hydrolysis at the carbonyl carbon followed by spontaneous decarboxylation resulting in aniline or chloroaniline (Kearney and Kaufman, 1965).

The degradation of dimethylphenylurea and its chlorinated analogs was little or none in this system. Typical is the unchlorinated herbicide, dimethylphenylurea (fenuron), in which very little degradation was observed within 60 days (Figure 4). The failure of these organisms to degrade fenuron in soil had been observed previously (see previous section). Assumed intermediates of this degradation, formanilide and phenylurea, however, degraded with relative ease, especially the former (Figure 3). Thus it appears the two methyl groups are responsible for the degradative recalcitrance of these compounds. This is in agreement with Geisbuhler et al. (1963) who demonstrated that dealkylation of the two methyl groups precedes hydrolysis of the urea linkage.

All phenylcarbamates and acylanilides tested were degraded, usually with an accompanying production of aniline or chloroaniline. Whether or not aniline or chloroaniline actually appeared is viewed as a kinetic phenomenon involving the relative rates of two processes. The first is hydrolysis of the side chain at the carbonyl carbon by exogenous hydrolytic enzymes. The second is metabolic degradation of the aniline or chloroaniline formed by hydrolysis. If the hydrolysis is rapid or if the ring does not degrade, aniline or chloroaniline appear in the medium. On the other hand, if the hydrolysis is slow, the ring will degrade as soon as formed, and no intermediate appears. The relative rates of both processes appeared to be influenced primarily by the structure of the compound undergoing degradation, particularly its chlorine configuration.

Effect of Chlorine Configuration on Side Chain Hydrolysis and Ring Degradation. A simple structural effect of the side chain was noted in the difference between the rate of hydrolysis of IPC and propionanilide. Aniline never appeared during IPC degradation (Figure 3) but did appear during the degradation of propionanilide (Figure 4). Aniline, itself, unlike chloroaniline, was rapidly metabolized by the organisms (Figure 3). According to our kinetic hypothesis aniline was metabolized as fast as it was formed from IPC hydrolysis, but propionanilide hydrolysis was so much more rapid that aniline momentarily accumulated in the medium. Thus side chain hydrolysis is rate limiting in IPC degradation but not in propionanilide degradation. Also rapid was the hydrolysis of the herbicide 3,4-dichloropropionanilide (3,4-Prop) and N-(3,4,-dichlorophenyl)-2-methylpentanamide (Karsil) as seen in Figure 4. These repeated observations suggest that acyl bonds are more rapidly broken than ester bonds in these systems. This idea finds parallel expression in the Warburg data (Figure 2).

The effect of ring chlorine on the rate of hydrolysis is less clear in these studies. Slow transitions were noted for isopropyl N-3,4-dichlorophenyl carbamate and especially for isopropyl N-4-chloroisopropylcarbamate as shown in Figure 3. More rapid shifts were noted for the other isopropyl phenylcarbamates. Kaufman (1967) also noted an effect of ring chlorine on hydrolysis of phenylcarbamates. However, our data indicate that the principal role of ring chlorine for most compounds tested was its effect on the rate of degradation of the ring. Without exception this effect was to greatly retard that rate.

The retarding effect of ring chlorine on ring degradation was not equal for all configurations. Figure 3 shows scans of various chloro-IPC's and chloroanilines which were subjected to microbial attack over a period of 9 days (Expt. A). The percentage of ring remaining after 9 days is given in the figure at the lower right of each group of scans. It is seen that chlorine configuration has the same effect on rate of chloro-IPC degradation as it does on the rate of chloroaniline degradation. This is reasonable since chloroaniline is the principal degradation intermediate of chloro-IPC and the effect of ring chlorine on the hydrolytic production of chloroanilines is relatively small (with the possible exception of the 4-chloroanalog). Thus the chloroanilines and chloro-IPC's may be ranked by chlorine configuration according to their rapidity of degradation: $0 > 2,4 > 2,4,5 > 3 > 4 > 3,4$.

This order has been arrived at by consideration of five additional experiments as well as Experiment A shown in Figure 3. These are summarized in Table 13. The percentage of compound remaining at the indicated time increases according to the above order. Three kinds of exceptions are noted: 1) In 8 out of 11 instances, 2,4 degraded faster than 2,4,5 according to the above order. However, in one instance (Expt. R_1) the reverse was true, and in two instances little

difference was noted. 2) In 8 out of 11 instances, 3 ranked fourth in order of degradation according to the above sequence. However, in three instances it ranked second after the 0 configuration (Expt. R_1 and AP_3). 3) In 4 out of 11 instances, 4 degraded faster than 3,4 according to the above order. However, in one instance the reverse was true (Expt. R_2), and in 3 instances little difference was noted. In two instances neither compound was degraded owing to a particularly inactive suspension of microorganisms (Expt. R). In a final instance 3,4-dichloro IPC hydrolyzed faster than the 4-chloroanalog but little ring degradation was noted. One possible source of these differences may be that there were differences in the behavior of the organisms themselves toward a given compound because of unspecified environmental changes and/or adaptive phenomena.

On balance, however, the evidence is strong that the indicated sequence is the true one with some uncertainty attached to the relative position of the 4-chloro and 3,4-dichloro configurations. It is noteworthy that this order finds some parallel expression in the Warburg data, particularly the primacy of 2,4-dichloro IPC among the chlorinated substrates.

The results of the degradation experiment using four selected isolates under sterile conditions are shown in Table 14. The high percentage of ring remaining in this experiment is due to the 5-fold dilution of organisms involved in the experimental procedure. However, it is clear that the isolates differ little from each other in their ability to hydrolyze the substrate or degrade the resultant chloroaniline. Precise comparisons are not possible as accurate estimates of the relative cell concentrations in the suspensions were not made. Notable is the degree to which the order of degradation of the substrates according to chlorine configuration correspond to that already described for the composite population. One exception was that no hydrolysis of Swep was observed for any of the four isolates.

The Toxicity Hypothesis. The observed sequence of decreasing degradation rates as a function of ring chlorine configuration is difficult to explain in terms of any uniform variation in physiochemical properties of the molecules themselves such as solubility, partition coefficient, etc. Kearney (1965) was able to correlate enzymatic rates of hydrolysis of several phenylcarbamates with steric properties and electron density at the reactive site. However, no single approach is likely to succeed with intact cells because of the greater complexity of a system in which absorption, transport and concurrent effects on related processes are likely to play a role. Nor is explanation made easier by revising the sequence according to one or more of the three discrepancies noted above. Kaufman (1967) observed the order of degradation of chlorophenylcarbamates in perfused muck soil to be

Table 13. Degradation of IPC, Aniline and five of their chloroanalogs[a]

Compound	Percent "ring" remaining after indicated no. of days					
	Days	22	14	8	9	8	14
	Expt.	R	R_1	AP_3	A	B[f]	R_2
IPC		42[b]	0(3)[c]	0(1)	0(2)	0(1)	0(1)
2,4-dichloro		62[b]	27	33	12	56	24
2,4,5-trichloro		85[b]	63[b]	39	14	73	29
3-chloro		100[b]	11[b]	0(1)	33	80	44
4-chloro		100[b]	86[b]	65	60	100[d]	64
3,4-dichloro		100[b]	100[b]	79	58	100[d]	58
Aniline		11	0(3)	0(1)	0(2)	--[e]	--
2,4-dichloro		14	8	0(4)	7	--	12
2,4,5-trichloro		20	3	27	9	--	19
3-chloro		80	0(14)	28	24	--	31
4-chloro		100	35	46	40	--	44
3,4-dichloro		100	38	100	40	--	54

[a] All experiments replicated twice except A, AP_3 and B.
[b] Little or no hydrolysis noted.
[c] Figures in parenthesis are no. of days within which depletion occurred.
[d] Some hydrolysis but no ring degradation noted.
[e] -- not measured.
[f] Mean values of 4 isolates. See Table 14.

Table 14. Degradation of eight compounds by four isolates

Compound	Isolate No.[a]	Percent remaining after 8 days
IPC	12	completely degraded after 1 day
	23	
	24	
	33	
2,4-dichloro	12	60
	23	60
	24	49 $\bar{x} = 56$
	33	63
2,4,5-trichloro	12	74
	23	69
	24	73 $\bar{x} = 73$
	33	76
CIPC	12	73
	23	slow hydrolysis
	24	86
	33	80 $\bar{x} = 80$
4-chloro	12	slow and incomplete hydrolysis
	23	
	24	
	33	
3,4-dichloro	12	hydrolysis slow but complete
	23	
	24	
	33	
3,4-Prop	12	89
	23	90
	24	95 $\bar{x} = 92$
	33	95
Swep	12	
	23	
	24	little hydrolysis
	33	

[a] Identity of isolates as follows: 12, Fusarium sp.; 23, Mycobacterium sp.; 24, Arthrobacter sp.; 33 Penicillium sp. later found to be contaminated with Arthrobacter sp.

0 > 3 > 4 > 2. Dichlorophenylcarbamates were not degraded. If the soils were enriched with CIPC prior to treatment the order was 3,5> 4 > 2 > 2,4 > 3,4 > 2,5. This sequence like the one found in our studies for what is in essence an IPC adapted system, is also puzzling and no explanation was offered.

A clue to the rationalization of such sequences was the discovery by Bartha et al. (1967) that a substantial fraction of the chloroaniline derived from the degradation of 3,4-dichloropropionanilide in soil condensed to form a toxic residue that depressed soil respiration. This was subsequently identified as 3,3',4,4'-tetrachloroazobenzene (Bartha and Pramer, 1967). Moreover, evidence was offered that the condensation reaction was catalyzed by peroxidase but that the enzyme was not active on all chloroanilines (Bartha et al., 1968). Among the anilines not converted to chloroazobenzenes by peroxidase were those having a 2,4-dichloro and a 2,4,5-trichloro configuration. However, 3-chloro and 4-chloro and 3,4-dichloroanilines, among others, were converted to the corresponding chloroazobenzenes.

Unfortunately, we are not able to substantiate the presence of these compounds in our systems using the extraction and UV assay techniques described. However, conspicuous colorations ranging from yellow-orange to red were observed in certain Experiment R suspensions. This was a unique experiment because the substrate concentrations were high (1.7 μmols/ml as opposed to about 0.6 in other experiments) and the suspension was particularly inactive indicating a greater degree of toxicity. The 3-chloro and 4-chloroaniline suspensions were red colored and the 3,4-dichloroaniline suspensions were orange colored, but the 2,4-dichloro and 2,4,5-trichloroaniline suspensions were colorless. Since the 3,3',4,4'-tetrachloroazobenzene crystals isolated by Bartha and Pramer (1967) were "orange colored" this would seem to provide some presumptive evidence for the presence of such compounds in our systems.

These compounds are, apparently, especially evident and toxic at the higher substrate concentrations. The possibility that a desirable degradative sequence is being blocked by an intermediate whose toxicity depends on the chlorine configuration of the parent molecule bears further investigation.

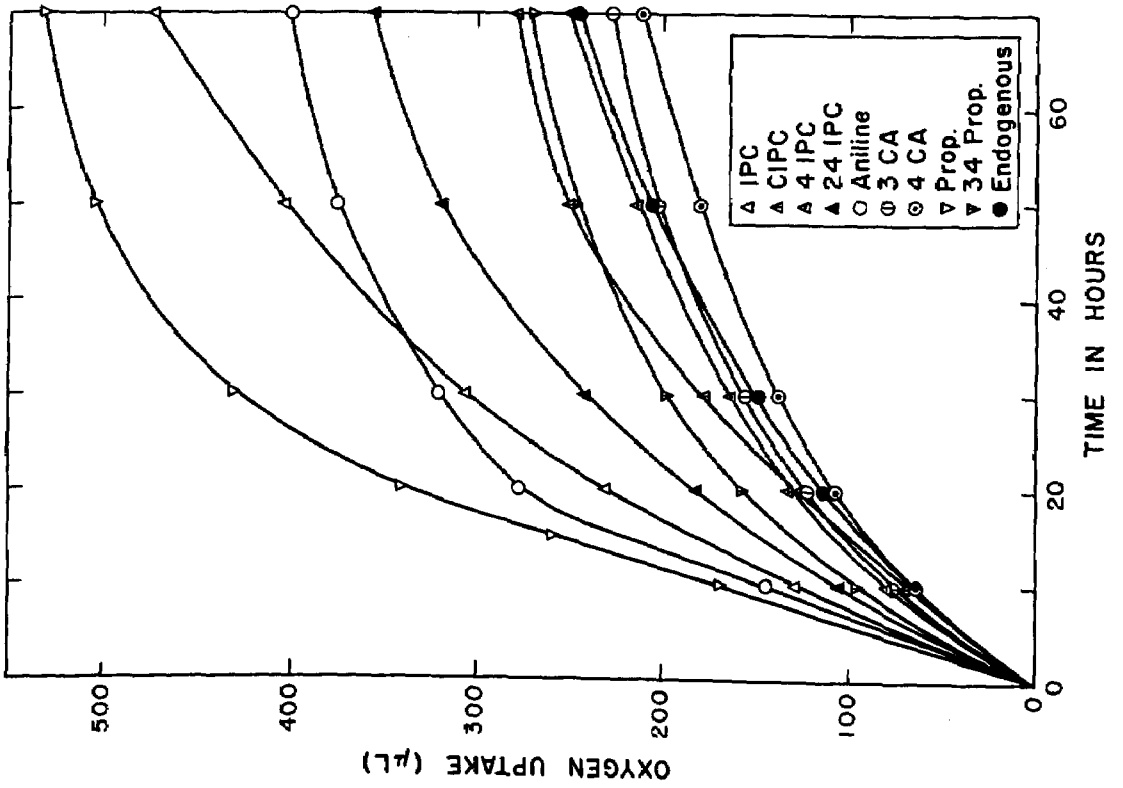


Figure 2. Influence of various anilide substrates on the respiration of IPC-cultured suspensions of microorganisms.

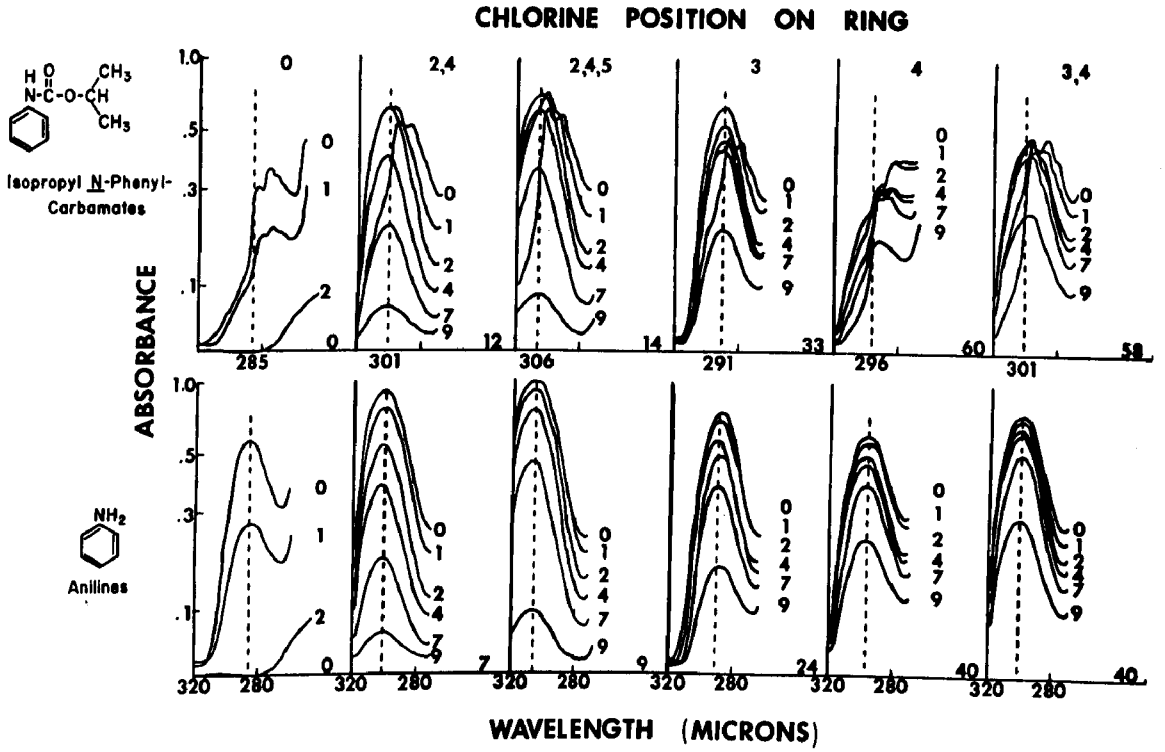


Figure 3. Influence of ring chlorine on the degradation of IPC's and Anilines. Shown are UV scans of samples taken from culture flasks at the indicated number of days after compound addition. Figures at lower right of each group are the percentage ring remaining after 9 days.

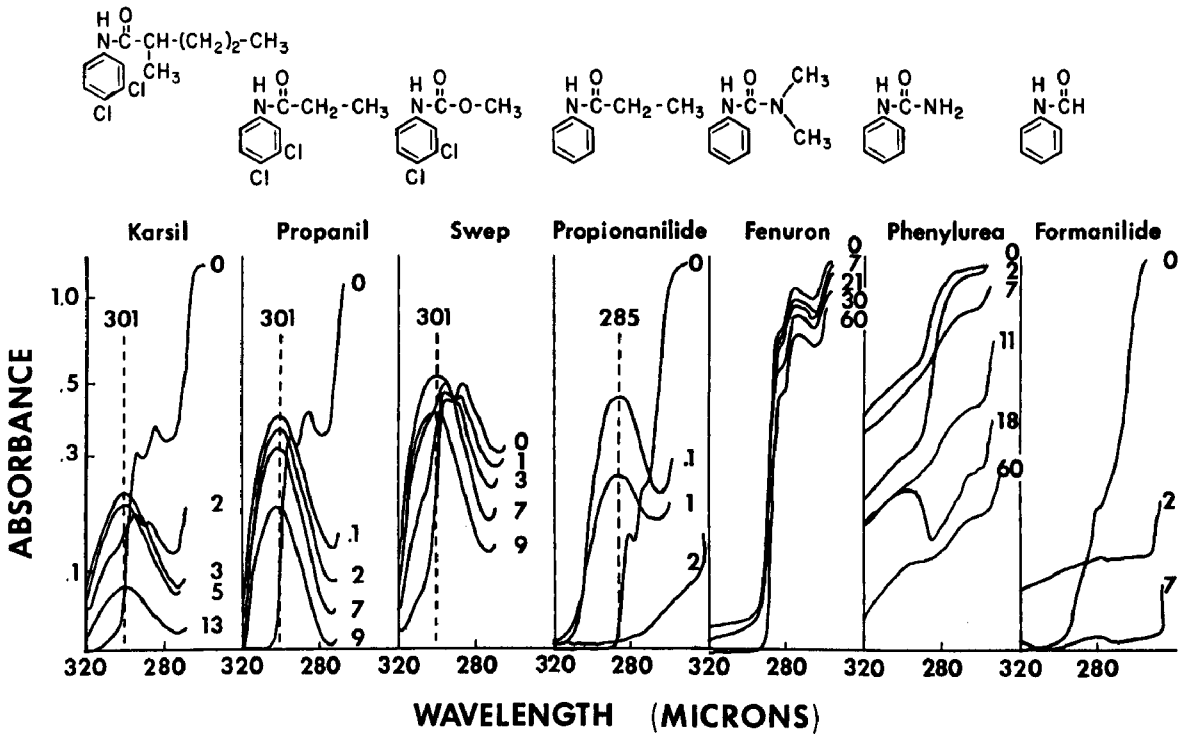


Figure 4. Influence of differing structures on rates of hydrolysis and ring degradation. Shown are UV scans of samples taken from culture flasks at the indicated number of days after compound addition.

SECTION X

A MEMBRANE BIOLOGICAL FILTER DEVICE FOR REDUCING WATERBORNE BIODEGRADABLE POLLUTANTS

The increasing use of pesticides and other synthetic organics together with the known resistance of many of them to microbiological attack (Alexander, 1965; Kearney, et al., 1969) indicates that the quantity of these substances in the environment will increase. The ultimate fate of most pollutants not fixed or degraded by soil is a water resource. The problem of pollutants in water is aggravated because many of the molecules which are more or less readily degraded by microbes in soil, such as the phenylcarbamates and phenoxyacetic acids, may be much more resistant to degradation in water (Schwartz, 1967).

As the result of work originally initiated as a study of the kinetics of herbicide degradation, an interest was developed in studying the possibility of using a "biological filter" system for the removal of pesticides from water. The term "biological filter" is customarily employed to designate the trickling film reactors common in sewage treatment plants. A different type of biological filter was studied in this laboratory in which the herbicide IPC (isopropyl N-phenylcarbamate) was removed from a flowing water source by a concentrated suspension of microorganisms retained behind an ultrafilter barrier.

The fundamental concept of the membrane biological filter device is that a pollutant substance in a feed stream is transferred by diffusion or mass flow across a barrier behind which is a bath containing an active suspension of microorganisms able to attack the pollutant. The microorganisms may be a very general population or a highly specific and selected one depending upon the resistance of the pollutant to biodegradation. In any case the destruction of the pollutant by the microorganisms results in an effluent containing a reduced pollutant concentration. The fundamental requirements for a practical device are as follows: 1) a population of microorganisms able to rapidly attack the molecule at hand; 2) a population relatively unfastidious in its nutritive requirements; 3) an ultrafilter material which is rugged, inexpensive and permits efficient exchange of pollutant from feed stream to bath while protecting and preventing loss of the population; and 4) a high flow rate output containing a significantly reduced concentration of the pollutant. What follows is a discussion of how a phenylcarbamate metabolizing population of microorganisms contained in a laboratory ultrafilter device successfully modeled these requirements.

Methods and Results

The derivation and constitution of the mixture of IPC-degrading microorganisms used in these studies were described in Sections VIII and IX.

It was necessary to determine the rate at which a unit weight of the microorganisms degraded a unit weight of IPC in order to have some basis for predicting the relation between flow rate of the feed stream, population size, and effluent concentrations. A population of microorganisms was grown as usual by inoculating 1 liter of MSS containing a gram of IPC (well over the solubility) with liquid inoculum from a previous culture. The MSS had a pH of 7.1 and contained the following in mg/l: K_2HPO_4, 400; KH_2PO_4, 100; $MgSO_4$, 50; $CaSO_4$, 25; $NaNO_3$, 25; NH_4NO_3, 125; and $FeCl_3$, 2. When the medium became turbid after incubation on a rotary shaker at 30° C, the suspension was filtered through cheesecloth, centrifuged and resuspended in fresh MSS. Amounts of suspension were added to one-liter beakers containing various quantities of soluble IPC, such that the final microbial concentrations determined gravimetrically were 30, 20, and 10 µg dry wt/ml and the final IPC concentrations were 110, 75, and 40 µg/ml in a total volume of 600 ml of MSS. The beakers were aerated and the decrease in IPC concentration was followed over a period of several hours at room temperature. This was done by withdrawing 1 ml samples and extracting them in 8 ml vials with 2 ml of hexane: chloroform (2:1). The UV absorbance of the solvent was then read at 276 mµ and related to a standard curve derived by carrying through identical procedures on known IPC concentrations.

After a short lag period of 3 to 4 hours the rate of destruction of IPC was found to be linear over all concentrations of IPC and microorganisms employed. The rates of IPC-degradation are shown in Table 15. The rates of IPC metabolism per unit of bacteria vary somewhat depending on both the concentration of microorganisms and IPC. The mean rate over all concentrations was .22 mg IPC detroyed/mg of microorganisms. The range was from .13 at the lowest concentration of IPC and the highest concentration of IPC and the highest concentration of microorganisms to .39 at the highest concentration of IPC and the lowest concentration of microorganisms. The values may be expected to increase at higher IPC concentrations. On the other hand, the values may be expected to decrease at high microbial concentrations, but the rate of decrease outside the range tested here is not known.

The mineral nutrition requirements of the microorganisms were not rigorously determined but the ability of the organisms to function in a nutrient environment of various strengths was estimated over a period of 2 days. This point is relevant because the mineral nutrient content of the bath must soon come to some sort of equilibrium with that of the feed stream. Therefore, the nutrient content of the feed stream must be sufficient for the level of functioning expected in the bath. In the event the bath is deficient in either mineral or organic nutrients, the slow metered addition of supplementary materials to the bath to insure adequate levels of cometabolism or maintenance cannot as yet be ruled out.

Five hundred ml of various dilutions and multiples of the MSS described above were placed in aerated liter beakers. A microbial suspension prepared as described above was added to give a final concentration of

200 µg/ml. Five hundred mg of solid IPC was added to each beaker. After 2 days the solid IPC remaining was harvested, washed and weighed and the percentage metabolized determined. The results are shown in Table 16. The data described a skewed convex curve with a maximum in the vicinty of the standard MSS in which the organisms were originally cultured. The significance of the data is that a depleted or exhausted mineral nutrient environment is not without some effect but, on the other hand, it is not by any means disastrous for the destruction of IPC over a limited period of time.

Table 15. Rate of IPC degradation (µg IPC/µg microorganism/hr) at three levels of IPC and microbial concentrations.

Microbial concentration µg/ml	IPC concentration µg/ml			
	40	75	110	$\bar{x}$
10	.20	.24	.39	.27
20	.17	.24	.29	.23
30	.13	.20	.24	.19
$\bar{x}$	.15	.22	.28	.22

Table 16. Influence of Mineral Salts concentration on a quantity of IPC degraded over a two-day period.

Concentration Factor	Percent IPC degraded
4	48
1	88
½	80
¼	74
0 (dist. H_2O)	50

The first and simplest laboratory "biological filter" system used was what may be called the dialysis tubing model. Mineral salts solution containing IPC was pumped through 65 feed of ¼ inch dialysis tubing. The tubing was loosely wound on a lattice spool and immersed in an 8-liter bath containing a stirred suspension of microorganisms at a concentration of .3 mg/ml. Feed stream IPC concentrations were .12 and .20 mg/ml and flow rates, achieved by a Technicon proportioning pump were .25 and 1.50 l/hr. Effluent was sampled two or three times

after each change in feed stream flow rate or IPC concentration to be certain the device had reached an equilibrium. IPC concentration of the samples was measured as described above. The device was operated 2 days at room temperature.

The dependence of IPC concentration of the effluent on the flow rate of the feed stream and its IPC content is shown in Table 17. The relation was so predictable over the ranges employed that a constant was evaluated for the equation:

$$[IPC]_{E.} = K'[IPC]_{F.S.}(FR) \qquad \text{(Eq. 1)}$$

where: $[IPC]_{E.}$ = IPC concentration of effluent in mg/ml
$[IPC]_{F.S.}$ = IPC concentration of feed stream mg/ml
FR = flow rate in l/hr
K' = a constant

Constant variation did not exceed 6% and was attributable to random errors. IPC in the effluent was reduced 94% at the lowest flow rate and about 66% at the highest. Presumably higher flow rates would result in even less efficient reductions unless the tubing lengths were increased. Effluent concentration was naturally increased at higher flow rates because the time and, therefore, the chances for any given molecule to contact the membrane surface were reduced. Therefore, it is clear that the physical rates of transfer of IPC across the membrane surface were the limiting factors in this model and not the rate of the microbial destruction of IPC.

Table 17. Dialysis Tubing Model--Influence of flow rate and IPC concentration of the feed stream on IPC concentration of the effluent.

Feed Stream		Effluent Stream	K'
FR l/hr	$[IPC]_{F.S.}$ mg/ml [a]	$[IPC]_{E.}$ mg/ml [a]	See Eq. 1
.25	.12	.007	.234
.50	.12	.014	.234
.75	.12	.021	.234
1.00	.12	.029	.242
1.25	.12	.032	.214
1.50	.12	.042	.234
.25	.20	.011	.220
.50	.20	.023	.230
.75	.20	.034	.226
1.00	.20	.044	.220
1.25	.20	.057	.228
1.50	.20	.066	.220

[a] FR = flow rate in l/hr
$[IPC]_{F.S.}$ mg/ml = mg/ml of IPC in feed stream
$[IPC]_{E.}$ mg/ml = mg/ml of IPC in effluent stream

A second model was developed with the aim of achieving greater durability and versatility as well as higher IPC transfer rates at increased feed stream velocities. The efficiency of such a system should hopefully be limited only by the capacity of the microbes to destroy IPC. Such a system would require 100% transfer of pollutant across the ultrafilter barrier. An equation describing such a system is derived below.

To meet the condition of 100% transfer, assume a feed stream containing IPC is emptied directly into the bath. Further assume that effluent leaving the bath at a rate equal to the input, is filtered in some way. Thus the volume and microbial concentration remain constant during the operation. It is clear that, as the feed stream rate increases, a point will be reached where the destructive action of the microbes can no longer keep pace with the input so that the IPC concentration of the bath begins to increase. At this point IPC appears in the effluent and is identical from then on to the bath concentration. We may write:

IPC input = IPC metabolized + IPC not metabolized (Eq. 2)

furthermore: $FR[IPC]_{F.S.} = K(V)(C) + FR[IPC]_{E.}$ (Eq. 3)

where FR = flow rate of feed stream = flow rate of effluent in ml/hr
$[IPC]_{F.S.}$ = IPC concentration of the feed stream
$[IPC]_{E.}$ = IPC concentration of the effluent
C = concentration of microorganisms in mg/ml
V = volume of microorganism bath
K = a constant for given conditions in mg IPC/mg microbes/hr

collecting and rearranging: $[IPC]_{E.} = [IPC]_{F.S.} - \frac{K(V)(C)}{FR}$ (Eq. 4)

Dividing by $[IPC]_{F.S.}$ and multiplying by 100 gives another useful form:

$$\% \text{ IPC remaining} = 100 - \frac{K(V)(C)100}{FR[IPC]_{F.S.}} \quad \text{(Eq. 5)}$$

This equation then defines the relation between the major variables of any filter device in which complete transfer of pollutant from the feed stream to the bath occurs. The term C would tend to slowly increase over a period of days if the pollutant is of a type that can be used for growth of new microbial cells. The term K is seen to be identical in meaning to that evaluated earlier at a mean of .22 mg IPC/mg microbes/hr.

The second membrane biological filter device which may be called the Plexiglas model is shown in Figure 5. The heart of the model consisted of 3 one-foot square, $\frac{1}{4}$ inch thick Plexiglas plates. Parallel $\frac{1}{4}$ inch wide channels were cut zigzag fashion into the plates (see inset) so that, when the plates were stacked, the channels all coincided. The total channel length of each plate was approximately 14 feet and the

total channel area 40%. An entrance and exit hole was cut into the wide dimension of each plate so that liquids could enter and exit from the channels through tygon tubing. Membrane filter sheets were placed between the plates, thus separating the channels of the center plate from those of the two outer plates. Solid $\frac{1}{2}$ inch thick plates were stacked above and below the three channeled plates and the entire device was squeezed together with throat clamps. The feed stream containing IPC at a concentration of .2 mg/ml was pumped through the center plate channels and the bath stream containing .28 mg/ml of microbes was pumped in countercurrent flow from a bath through the two outer plate channels and back into the bath. Bath stream rates were held between 5 and 6 l/hr by regulating the flow from a centrifugal circulating pump located in the bath. Total bath volume was 2700 ml. Feed stream rates through the device were achieved by a Harvard proportioning pump and varied from .25 to 3.5 l/hr. A peculiar feature of the device was the rerouting of the effluent tubing back through the proportioning pump next to the feed stream tubing. This was necessary in view of the unavoidably different pressures between the feed stream channels and the microbial bath channels. In this way, effluent and feed stream rates were kept equal as were channel pressures.

Results for the Plexiglas unit are shown in Figure 6. Each point on the graph is the mean of two determinations. Data are effluent concentration expressed as a percentage of the feed stream concentration. As predicted by Equation 5, all data show a decreasing percentage of IPC remaining as the flow rate decreases. Where membrane parchment was used as the barrier between feed stream and bath stream the effluent concentration dropped below 50% feed stream concentration at about .2 liter/hr. Where Millipore Ultrafilter material of pore size .45 micron was employed, effluent concentration dropped below 50% feed stream concentration at approximately .8 liter/hr. Thus a higher transfer rate was achieved using the higher porosity materials. Two feed stream center plate widths were tested, one .25 inch and the other .10 inch. It is seen that virtually no difference in efficiency resulted from using the narrower channels under these conditions.

The performance of this model may be evaluated to some extent by calculating the theoretical maximum efficiency using Equation 5. For the conditions of run V = 2700 ml, C = .28 mg/ml and $[\text{IPC}]_{\text{F.S.}}$ = .20 mg/ml. The value of K is taken at .22 mg IPC/mg microbes/hr which is the mean value found earlier for a limited range of microbial and IPC concentrations. Since the microbial and IPC concentrations employed in the filter device were different than those for which the constant was evaluated, the resulting curve can only be an approximation. This curve is plotted in Figure 6 as the theoretical maximum for these conditions and shows that the effluent concentration drops below 50% of the feed stream concentration at a flow rate of approximately 1.7 liters/hr. At low flow rates the rate of IPC

transfer through the material appears to be limiting. But at higher flow rates the efficiency approaches the maximum. One explanation for this is that the amount of turbulence in the feed stream channels increases at higher velocity causing a higher transfer of IPC between the feed and bath streams.

Discussion

Improving the Plexiglas model. Although the transfer rates at higher velocities approach 100%, the percent IPC remaining in the effluent at these velocities is unacceptably high. Clearly then, the unit must operate at lower feed stream velocities. At low velocities, however, the transfer efficiency of IPC to the bath stream is poor and indicates that we have not yet succeeded in building the efficient model desired. Several possibilities for improvement exist. 1) The data show an increase in transfer as the porosity of the ultrafilter increases. Since the size of Mycobacterium and Arthrobacter cocci is not under 1 micron, the pore size diameter could be safely increased from its current .45 micron. 2) The feed stream channels could be made more tortuous to increase channel mixing. They could be lengthened to increase the time in which a unit of volume resides in the device. They could also be greatly narrowed below .1 inch to increase the S/V ratio and thus the likelihood of transfer. 3) A pressure differential might be imposed to facilitate transfer. This could be done by applying a positive pressure to the feed stream or by manipulating the relative velocities of feed and bath streams. However, any device causing the mass flow of liquid volumes between the feed stream and bath stream would require a net transfer of zero within the device. Otherwise the bath volume would fluctuate.

The volume output of the filter can be increased by increasing the number of feed stream plates alternating with bath stream plates. For example a single unit of three plates operating at a feed stream velocity of 6.3 l/hr has reduced the IPC concentration by 70% according to Figure 6. The same reduction can be achieved at a flow rate of 3 l/hr by operating 10 feed stream plates and 11 bath stream plates simultaneously from common manifolds. Under such conditions the quantity of microbes must be increased to handle the enlarged input. Equation 5 permits prediction of the bath volume necessary for specified percentage reductions, flow rates, and concentrations. In the example above, the new volume required is calculated to be 6.8 liters. Assuming the ability to construct less unwieldy units having high transfer efficiencies, we may ask what bath volume would be required to achieve 70% reduction of IPC at total flow rates approaching pilot capacity such as 2000 l/hr. The equation predicts a bath volume of 4500 liters, roughly the dimensions of a tank 2 meters tall and 2 meters in diameter.

The "Separated" and "Mixed" Options. The models just described are examples of what may be termed the "separated" option. In this approach feed stream and bath are kept separated at all times by an ultrafilter barrier. An alternative is the "mixed" option in which the feed stream is emptied directly into the bath followed by ultrafiltration of the entire mix at a rate equal to the input. This approach was foreshadowed during the derivation of Equation 5. Both approaches would require considerable prefiltration by sand or diatomaceous beds. Whereas, separated systems would pass the bulk of the remaining solids directly through the device, mixed systems would face formidable filtration problems in that all solids down to bacterial size must be retained if functional organisms are not to be lost from the bath. The problem is aggravated if the solids content of the feed stream is already high. In addition, the accumulation of solids in the bath may appreciably interfere with the degradation of pollutant particularly if the population is in any way a selected one. At some point the bath will approach the characteristics of a sludge which would have to be removed following diversion of the feed stream to a fresh microbial bath.

The separated approach would be indicated in situations where a high solids content of the feed stream overtaxes existing filtration capabilities or would interfere with bath functioning owing to a selected or fastidious microbial population. It would also be an advantage where it is clearly undesirable to remove all forms of life from the effluent. The mixed approach would be called for if the resource requirement associated with the achievement of high transfer efficiencies of pollutant across the ultrafilter are prohibitive. It would also be of advantage if a general microbial population were not inhibited by accumulating solids and if the removal of such solids from the effluent were also an objective of the treatment.

In connection with the concept of multiple usage, an interesting variant is the potential adaptability of devices of this kind to the removal of inorganic nutrients such as nitrogen and phosphorus from water. The eutrophication of fresh water resources by algal and bacterial growth has its origin in the increased levels of mineral nutrients finding their way into water from fertilized field runoff as well as wastewater effluents. The problem is so severe in some sections that N and P, once innocuous, must be considered nothing less than pollutants under these conditions. A question for which data are needed is whether mineral nutrients could be removed from feed streams upon transfer to a microbial bath by either the mixed or separated approach. The nutrients would be immobilized in fast growing bacterial tissue by the addition to the bath, if necessary, of readily assimilable carbon sources. The rate of addition could be adjusted to keep cell proliferation populations expanding at a

sufficient rate to maintain low levels of nutrients in the bath. Not the least of the problems inherent in this approach is the periodic disposal of the large biomass so produced. Various approaches to this problem have been compared and reviewed by Eliassen and Tchobanoglous (1969).

The Developing Maturity of Ultrafiltration Technology. Regardless of approach, the purely physical aspects of ultrafiltration of large volumes at high rates would at first appear to be more formidable than is actually the case. The Battelle Memorial Institute (1969) on membranes for industrial, biological and waste treatment processes made clear that ultrafiltration process technology is evolving rapidly. Sophisticated ultrafiltration systems now available from companies such as Amicon Corporation (Lexington, Mass.) and Dorr Oliver Corporation (Stamford, Conn.) employ ingenious methods of handling high flux rates. In one process the feed stream under low pressure is pumped through thin (.25 to 2.5 mm), spiral channels parallel to the ultrafilter surface. The channel configuration induces laminar flow that maximizes fluid shear stresses at the membrane surface. Also minimizing the buildup of solids is an ultrafilter microstructure that promotes separations at the surface, avoiding the pore plugging that causes conventional filters to lose permeability after long use. The adoption of these advanced systems to model the biological filter process and to evaluate various system configurations and operating parameters on a laboratory or pilot scale would be within current capabilities.

The concepts developed here are in need of more exploration. As water pollution problems intensify the evaluation of the claims of competing technologies can be made only on the basis of a more adequate understanding of the more innovative approaches as well as the existing ones.

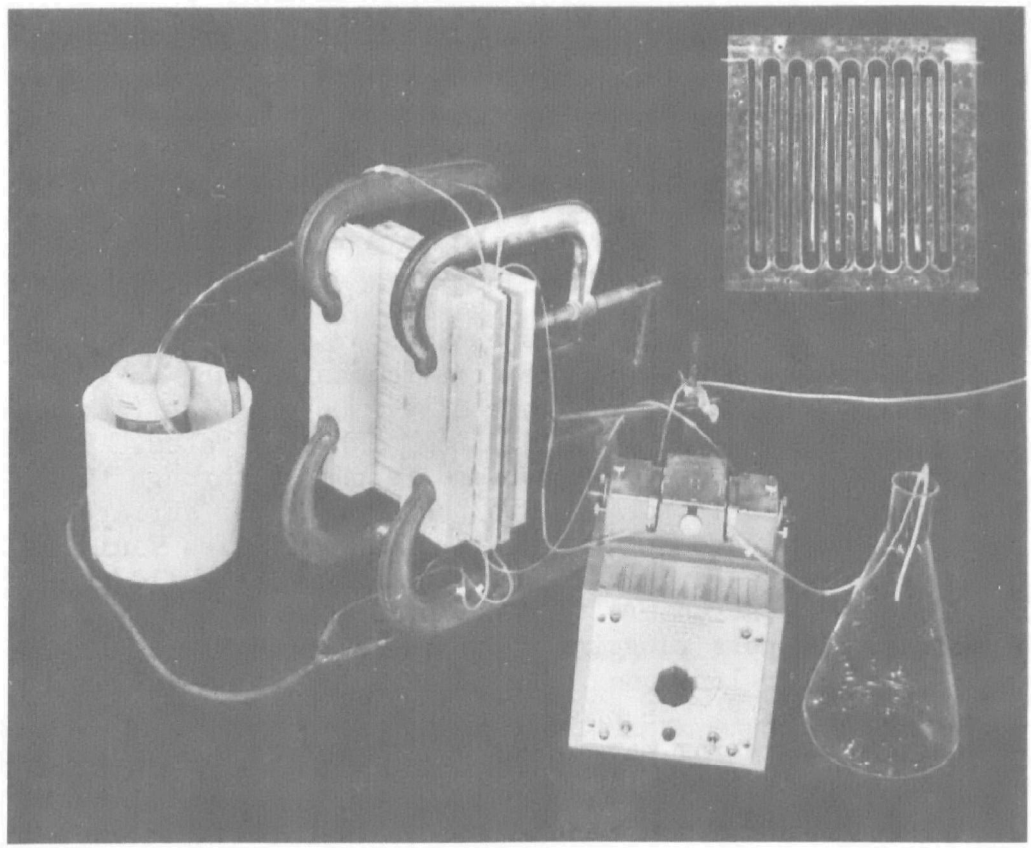


Figure 5. View of Membrane Biological Filter Device. Insert at upper right shows detail of channels in Plexiglas plates. At far left is bacterial bath and pump. At right coming from pump is effluent line and reservoir. At far right the feed stream line leads in from reservoir which is not shown.

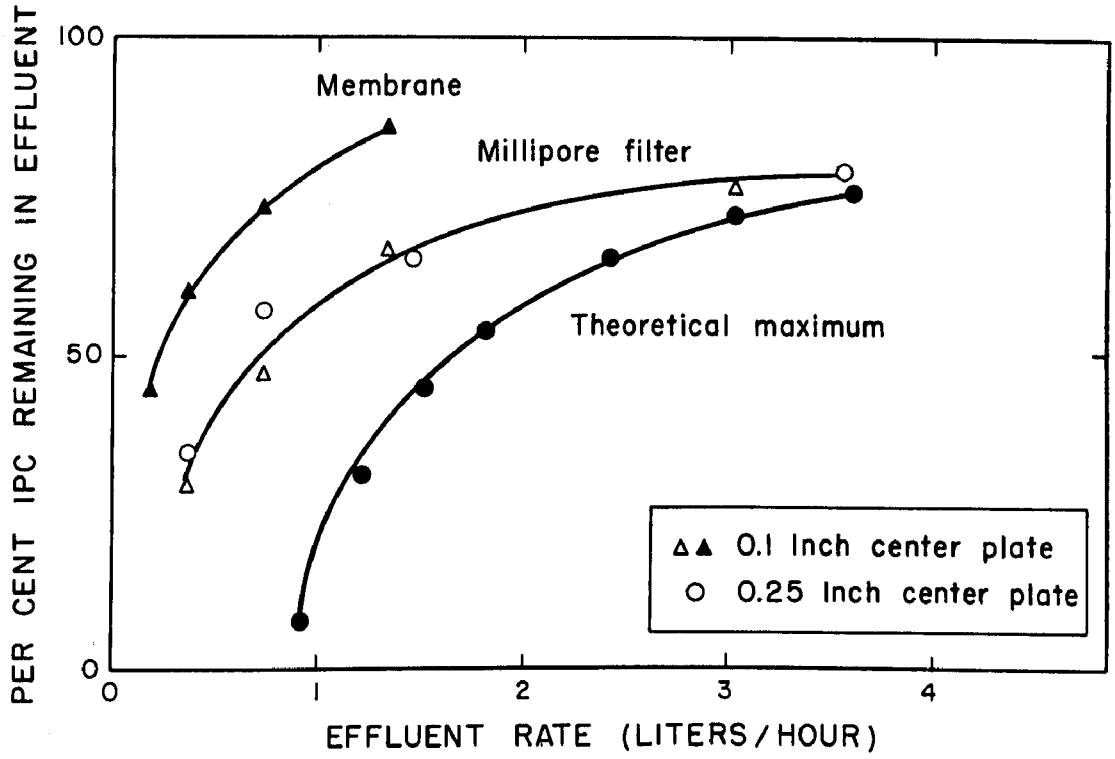


Figure 6. Dependence of Effluent IPC Concentration on Flow Rate and Type of Ultrafilter Material in the Plexiglas Model. Comparison is also made to the theoretical maximum calculated for these conditions from Equation 5.

SECTION XI

RELATIONSHIP OF CHEMICAL STRUCTURE OF HERBICIDES TO DEGRADATION BY MICROORGANISMS

As part of the overall objectives of this research program, an attempt was made to develop a body of knowledge on the relationship of chemical structure to persistence in the soil of a series of isomers and homologs which should be helpful to chemists seeking to synthesize molecules that combine herbicidal potency with minimum persistence potential. For this study, sixty compounds were chosen. They were selected because they: 1) were used commercially as herbicides (about half of those selected), 2) were presumed degradation products of these pesticides, or 3) contributed to a structurally homologous series between two or more of the herbicides selected. All of the compounds selected had a single aromatic nucleus with a side chain of varying complexity and nearly all had varying numbers of chlorine atoms in different positions on the aromatic ring. Among the classes of compounds represented were the chlorinated phenols, benzoic acids, anilines, phenoxyacetic acids, phenylureas and phenylcarbamates. The compounds were either purchased from chemical supply houses or, in the case of proprietary herbicides, were procured from the manufacturer. Compounds of questionable purity were recrystallized prior to use. The compounds used in this study are listed below in groups having similar structural features:

Phenols

- Phenol
- 3-Chlorophenol
- 4-Chlorophenol
- 2,4-Dichlorophenol
- 3,4-Dichlorophenol
- 2,4,5-Trichlorophenol
- 2,3,4,5,6-Pentachlorophenol

Benzoic Acids

- Benzoic acid
- 3-Chlorobenzoic acid
- 4-Chlorobenzoic acid
- 2,4-Dichlorobenzoic acid
- 3,4-Dichlorobenzoic acid
- 2,4,5-trichlorobenzoic acid
- 2,3,4-Trichlorobenzoic acid
- 2,3,6-Trichlorobenzoic acid
- 2,3,5,6-Tetrachlorobenzoic acid

Anilines

Aniline
3-Chloroaniline
4-Chloroaniline
2,4-Dichloroaniline
3,4-Dichloroaniline
2,4,5-Trichloroaniline

Phenoxyacetic acids

Phenoxyacetic acid
3-Chlorophenoxyacetic acid
4-Chlorophenoxyacetic acid
2,4-Dichlorophenoxyacetic acid (2,4-D)
3,4-Dichlorophenoxyacetic acid
2,4,5-Trichlorophenoxyacetic acid (2,4,5-T)
2,3,4,5,6-Pentachlorophenoxyacetic acid

Phenyl ureas

3-Phenyl-1,1-dimethylurea (fenuron)
3-(3-Chlorophenyl)-1,1-dimethylurea
3-(4-Chlorophenyl)-1,1-dimethylurea (monuron)
3-(2,4-Dichlorophenyl)-1,1-dimethylurea
3-(3,4-Dichlorophenyl)-1,1-dimethylurea (diuron)
3-(2,4,5-Trichlorophenyl)-1,1-dimethylurea
3-(3,4-Dichlorophenyl)-1-butyl-1-methylurea (neburon)
3-(3,4-Dichlorophenyl)-1-methoxy-1-methylurea (linuron)

Phenyl carbamates

Isopropyl-N-phenylcarbamate (IPC)
Isopropyl-N-(4-chlorophenyl)carbamate
Isopropyl-N-(3-chlorophenyl)carbamate (CIPC)
Isopropyl-N-(2,4-dichlorophenyl)carbamate
Isopropyl-N-(3,4-dichlorophenyl)carbamate
Isopropyl-N-(2,4,5-trichlorophenyl)carbamate
Methyl-N-(3,4-dichlorophenyl)carbamate

Phenoxybutyric acids

4-(2,4-Dichlorophenoxy)butyric acid (2,4-DB)
4-(2,4,5-Trichlorophenoxy)butyric acid

Phenoxyethyl sulfates

Sodium(2,4-dichlorophenoxy)ethylsulfate (sesone)
Sodium(2,4,5-trichlorophenoxy)ethylsulfate (2,4,5-TES)

Anilides

Propionanilide
3,4-Dichloropropionanilide (propanil)
3,4-Dichloro-2-methylacrylanilide (dicryl)
N-(3,4-Dichlorophenyl)-2-methylpentamide (karsil)

Miscellaneous Compounds

2,2-Dichloro-2-(2,4,5-trichlorophenoxy)ethylester of propionic acid (erbon)
O-(2,4-Dichlorophenyl)-O-methyl isopropylphosphoroamidothioate (zytron)
Dimethyl-2,3,5,6-tetrachloroterephthalate (dacthal)
4-Amino-3,5,6-trichloro-picolinic acid (tordon)
2,3,6-Trichlorophenylacetic acid (fenac)
2,3,6-Trichlorobenzyloxypropanol (tritac)
3,6-Dichloro-2-methoxybenzoic acid (dicamba)
3,5,6-Trichloro-2-methoxybenzoic acid (tricamba)

Since all of the compounds of interest absorb in the ultraviolet region, their rate of degradation by microorganisms was followed using UV spectrophotometry. Basically the compounds were exposed in a medium containing a biological phase, samples were withdrawn from the medium at various intervals, extracted with hexane-chloroform (2:1), and the quantity of compound present determined. This rather simple assay technique has provided a means of rapidly assaying a large number of the compounds.

The sixty compounds were exposed to soil suspensions in flasks on rotary shakers. These experiments generally lasted three to four months and were repeated with minor variations in measuring conditions, soil inoculum, mineral salts composition, test compound concentration, supplementary nutrients, type of controls, etc. In order to have a diversity of microorganisms present, the flasks were usually inoculated with a composite sample of soils obtained from various locations. The aim of basic experiments such as these was not only to measure relative rates of microbiological degradation under varying conditions but also to create conditions of long-term exposure under which microorganisms of unusual degradative capacities could develop or be selected over a period of time and could be isolated for further study. Efforts in this direction led to the consideration and trial of many methods and combinations of methods which seemed to have some promise of inducing microorganisms to attack these compounds. In one experiment the inoculum consisted of soil samples taken from areas under long-term exposure to chlorinated aromatic compounds in a chemical plant. In other experiments, various supplementary nutrients such as broths, sugars and yeast extract were added in order to stimulate degradation of the compound of interest. A continuous culture technique in which a mixed active population of microorganisms was slowly starved of substrate in an effort to initiate metabolism of a herbicidal compound of interest was tried. It appears, at least in theory, that the latter technique properly pursued might yield results of great interest.

Because the termination date of this program was made earlier than had originally been anticipated, it was not possible to obtain as complete

data as necessary but several trends did emerge. Using the data at hand to determine the effect of chemical structure on the rate of degradation of these compounds, a regression line was computer-calculated from the spectrophotometer measurements of the herbicide cultures over a period of time. The slope of the linear component of the line was taken as a measure of the rapidity of degradation. By ranking the slope values from greatest to least (most rapid to least rapid degradations), several conclusions were made. One was that the nature of the side chain or R-groups exerts a greater influence on degradation than the number and position of chlorine on the benzene ring nucleus. Slope values were distributed over a range of 126 units for R-group configurations as compared with a range of 68 units for chlorine configurations. The mean order for R-group configuration was: phenols > anilines > phenylcarbamates > benzoic acids > phenoxyacetic acids > phenyl ureas. The addition of supplementary nutrients to the degrading media did not significantly change this order. The mean order of degradation for chlorine configurations was: 4-chloro > 2,4-dichloro > 2,4,5-trichloro > 3-chloro > 3,4-dichloro. In this case, however, supplementary nutrients caused marked changes in the order of degradation with there being no current explanation of the effect.

An attempt at constructing and testing a hypothesis that would explain these observed differences in rates of degradation was initiated but, although these studies were not completed for the reason mentioned above, they are mentioned here as a matter of record. Conceivably the rate at which a molecule is degraded is dependent on a number of factors such as: 1) the ease with which the molecule can penetrate the cell and reach the appropriate enzyme site, 2) the extent to which steric effects interfere with enzyme bonding, and 3) the extent to which electronic effects of molecular substituents either interfere with enzyme bonding or alter the energy required to break the critical bonds in the molecule. The first factor may be estimated by the lipid solubility of the compounds. Lipid solubility in the form of the partition coefficient can be determined or learned from the literature. Electronic effects of a given functional group can be estimated by Hammet functions, many of which are available from the literature. Steric effects may be approximated by the Taft Steric Parameter. This approach has been used quite successfully by toxicologists attempting to correlate structure and toxicity in certain systems (Hansch, et al., 1963; Hansch and Deutsch, 1966). By using an equation of the type:

$$\log BR - k_1(\log P_x)^2 + k_2(\log P_x) + P\sigma + k_3E_s + k_4$$

where BR = biological response
P_x = partition coefficient of compound x
P = the Hammet constant
σ = the appropriate Hammet substituent value
E_s = the appropriate Taft steric parameter
k_1, k_2, k_3, k_4 = computed regression coefficients

it is possible to estimate the relative importance of the various factors that contribute to the biological response as defined by the equation. The our knowledge, no workers have attempted to use this approach to explain the microbiological degradation of a series of homologous compounds, but it appears to be an approach worthy of investigation. When sufficient data are collected, it may be possible to use this approach to estimate the susceptibility to degradation of other compounds.

SECTION XII

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SECTION XIII

REFERENCES

Alexander, M. 1965. Persistence and biological reactions of pesticides in soils. Soil Sci. Soc. Amer. Proc. 29: 1-7.

Alexander, M., and B. K. Lustigman. 1966. The effect of chemical structures on microbial degradation of substituted benzenes. J. Agr. Fd. Chem. 14: 410-413.

Audus, L. J. 1946. A new soil perfusion apparatus. Nature 158: 419.

Audus, L. J. 1951. The biological detoxication of hormone residues in soil. Plant Soil 3: 170-192.

Audus, L. J. 1960. Microbiological breakdown of herbicides in soils. In Woodford, E. K. and G. R. Sager, ed. Herbicides and the soil. Blackwell Scientific Publications, Oxford. pp. 1-19.

Audus, L. J. 1964. Herbicide behaviour in the soil. II. Interactions with soil microorganisms. pp. 163-206. In Audus, L. J., ed. The physiology and biochemistry of herbicides. 555 pp. Academic Press, New York.

Bartha, R. 1968. Biochemical transformations of anilide herbicides in soil. J. Agr. Fd. Chem. 16: 602-604.

Bartha, R., and D. Pramer. 1967. Pesticides transformation to aniline and azo compounds in soil. Science 156: 1617-1618.

Bartha, R., R. P. Lanzilotta, and D. Pramer. 1967. Stability and effects of some pesticides in soil. Appl. Microbiol. 15: 67-75.

Bartha, R., H. A. B. Linke, and D. Pramer. 1968. Pesticide transformations. Production of chloroazobenzenes from chloroanilines. Science 161: 582-583.

Bartholemew, W. V. 1957. Maintaining organic matter. U. S. Dept. Agr. Yearb. Agr. 1957: 245-252.

Batelle Memorial Institute. 1969. Conference on membranes for industrial, biological, and waste treatment processes. Oct. 20, 21, 1969. Columbus, Ohio.

Clark, C. G., and S. J. L. Wright. 1970. Detoxication of IPC and CIPC in soil, and isolation of IPC-metabolizing bacteria. Soil Biol. Biochem. 2: 19-26.

Hansch, C., and E. W. Deutsch. 1966. The use of substitution constants in the study of structure-activity relationships in cholinesterase inhibitors. Biochem. Biophys. Acta. 126: 117-128.

Hansch, C., R. M. Muir, T. Fujita, P. P. Maloney, F. Geiger, and M. Streich. 1963. The correlation of biological activity of plant growth regulators and chloromycetin derivatives with Hammet constants and partition coefficients. J. Amer. Chem. Soc. 85: 2817-2824.

Dalton, R. L., A. W. Evans, and R. C. Rhodes. 1965. Disappearance of diuron in cotton field soils. Proc. Southern Weed Conf. 18: 72.

Eliassin, R., and G. Tchobanoglous. 1969. Removal of N and P from waste water. Environ. Sci. Tech. 6: 536-541.

Fields, M. L., R. Der, and D. D. Hemphill. 1967. Influence of DCPA on selected soil microorganisms. Weeds 15: 195-197.

Foy, C. L., and S. W. Bingham. 1969. Some research approaches toward minimizing herbicidal residues in the environment. Residue Rev. 29: 105-135.

Geissbuhler, H., C. Haselback, H. Aebi, and L. Ebner. 1963. The fate of N-(4-chlorophenoxy)-phenyl-N,N-dimethylurea in soils and plants. Weed Res. 3: 277.

Gordon, R. E. 1966. Some strains in search of a genus - Corynebacterium, Mycobacterium, Nocardia or what? J. Gen. Microbiol. 43: 329-343.

Josephs, M. J., D. R. Mussell, J. K. Leasure, and R. L. Warden. 1957. 1,2,4,5-Tetrachlorobenzene for the control of wild oats. Proc. North Central Weed Control Conf. 14th, pp. 37-38.

Kaufman, D. 1967. Degradation of carbamate herbicides in soil. J. Agr. Fd. Chem. 15: 582-591.

Kaufman, D. D., and P. C. Kearney. 1965. Microbial degradation of isopropyl-N-3-chlorophenylcarbamate and 2-chloroethyl-N-3-chlorophenylcarbamate. Appl. Microbiol. 13: 443-446.

Kearney, P. C. 1965. Purification and properties of an enzyme responsible for hydrolyzing phenylcarbamates. J. Agr. Fd. Chem. 13: 561-564.

Kearney, P. C., and D. D. Kaufman. 1965. Enzyme from soil bacterium hydrolyzes phenylcarbamate herbicides. Science 147: 740-741.

Kearney, P. C., E. A. Woolson, J. R. Plimmer, and A. R. Isensee. 1969. Decontamination of pesticides in soils. Residue Rev. 29: 137-149.

Lederberg, J., and Ester M. Lederberg. 1952. Replica plating and indirect selections of bacterial mutants. J. Bact. 63: 399-406.

Limpel. L. E., P. H. Schuldt, and R. F. Lindemann. 1959. DAC-893, a promising new pre-emergence herbicide for use on turf. Agron. Abstr. p. 90.

MacRae, I. C., and M. Alexander. 1965. Microbial degradation of selected herbicides in soil. J. Agr. Fd. Chem. 13: 72-76.

Pease, H. L. 1962. Separation and colorimetric determination of monuron and diuron residues. J. Agr. Fd. Chem. 10: 279-281.

Schwartz, H. G. 1967. Microbial degradation of pesticides in aqueous solutions. J. Water Poll. Control Fed. 39: 1701.

Skinner, W. A., D. E. Stallard, and W. E. Priddle. 1964. The isolation and identification of the metabolites of Dacthal herbicide. Abstr. of papers, 147th National Meeting, Amer. Chem. Soc., Philadelphia, Pa., pp. 4A-5A.

Upchurch, R. P. 1966. Behavior of herbicides in soil. Residue Rev. 16: 47-85.

Weed Society of America. 1967. Herbicide Handbook, 1st ed. 293 pp. Geneva, New York.

Whiteside, J. S., and M. Alexander. 1960. Measurement of microbiological effects of herbicides. Weeds 8: 204-213.

SECTION XIV

PUBLICATIONS

Gershon, H., and G. W. McClure, Jr. 1967. Approach to the study of the degradation of dimethyl tetrachloroterephthalate. Contrib. Boyce Thompson Inst. 23: 291-294.

McClure, G. W. 1969. The dissipation of phenyl carbamate toxicity by a bacterium. Proc. N. E. Weed Cont. Conf. 23: 419.

McClure, G. W. 1970. Accelerated degradation of herbicides in soil by the application of microbial nutrient broths. Contrib. Boyce Thompson Inst. 24: 235-240.

McClure, G. W. 1971. A membrane biological filter device for reducing waterborne biodegradable pollutants. J. Water Pollution Control Federation. In manuscript.

McClure, G. W. 1971. Influence of ring chlorine on the degradation of some anilide herbicides and related compounds by IPC-cultured microorganisms. J. Agr. Fd. Chem. In manuscript.

McClure, G. W. 1972. Degradation of phenylcarbamates in soil by a mixed suspension of IPC-cultured microorganisms. J. Environ. Quality. In press.

Torgeson, D. C., and H. Mee. 1967. Microbial degradation of bromacil. Proc. N. E. Weed Cont. Conf. 20: 584.

Torgeson, D. C. 1969. Microbial degradation of pesticides in soil. p. 58-59. In: Gunkel, James E., ed. Current Topics in Plant Science. 461 pp. Academic Press, New York.

Tweedy, B. G., and Nikki Turner. 1965. Effect of Dacthal on microorganisms. Phytopathology 55: 1080.

Tweedy, B. G., Nikki Turner, and Miriam Achituv. 1968. The interactions of soil-borne microorganisms and DCPA. Weed Science 16: 470-473.

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27 Abstract
The herbicide 2,3,5,6-tetrachloroterephthalate (DCPA) had little effect on soil microflora but was degraded by a number of bacteria and fungi. Methyl-2,3,5,6-tetrachloroterephthalate and 2,3,5,6-tetrachloroterephthalic acid were identified as degradation products. Penicillium paraherquei Abe isolated from soil previously treated with bromacil (5-bromo-3-sec-butyl-6-methyluracil) was found to degrade bromacil in culture. When added to sterile bromacil treated soil accelerated degradation was also obtained.

A mixture of microorganisms capable of degrading and utilizing IPC (isopropyl N-phenylcarbamate) was used to study the influence of ring chlorine on the degradation of a series of aniline herbicides. The retarding effect of ring chlorine on the rate of degradation increased according to the configuration sequence: 0 > 2,4 > 2,4,5 > 3 > 4 > 3,4. When added to the soil these organisms accelerated the degradation of IPC and related compounds. A membrane "biological filter" device for reducing water-borne biodegradable pollutants was also demonstrated using these organisms.

The addition of microbial nutrient broths to herbicide treated soils resulted in increased degradation of some of the herbicides, presumably as the result of increased microbial activity.

Abstractor D. C. Torgeson
Institution Boyce Thompson Institute for Plant Research, Inc., Yonkers, N.Y.

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