Microbial Ecology

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INTRODUCTION

No individual exists as an isolated entity. All life is dependent on its surroundings, both animate and inanimate, and the biological specialty that is concerned with the interactions of organisms and environments is called ecology. Interest in ecology has intensified in recent years, and society increasingly demands that ecologists contribute to decisions regarding resource management and environmental quality.

Because of their small size, microorganisms are more intimately related to their environment than most other forms of life. Microbial ecosystems, therefore, have many attractive features and provide exceptional possibilities for extending our understanding of structure and function in nature. Microbial ecology is exceedingly complex, however, since it includes not only the individual, but also the community and the environment as they vary and interact in place and time. Ideally, microbial ecology is the integration of all knowledge concerning the microbe and its surroundings.

Ecology is among the oldest of the microbiological arts and the youngest of the sciences. In the past, food microbiologists, soil microbiologists, marine microbiologists and their colleagues in other fields were concerned with the characterization of microorganisms and their activities in natural habitats. Today, however, it is fashionable to deemphasize the diversity of habitats, to generalize rather than specify, and to focus on basic phenomena that are common to most if not all ecological systems.

Only special topics in microbial ecology are considered here. The book is not intended to be definitive, but the contributions of the four authors are somewhat related, and they mirror various levels of sophistication encountered in contemporary microbial ecology. Dr. Meers' paper defines and describes associative and antagonistic interrelationships. It is initially theoretical but ultimately concerned with microbial interactions in various natural environments, including sludge, wine, beer, soil, water, and the rumen. Dr. Stotzky focuses on soil. He discusses the influence of physicochemical and biological factors on the growth and activity of microorganisms, and describes selected methods for studying microorganisms in soil. All this is then used as background for an analysis of clay minerals as ecological determinants, an area of investigation which Dr. Stotzky pioneered. The two additional authors deal with the subject of pesticides. Dr. Pfister summarizes present knowledge of the effects of halogenated compounds on the growth and survival of microorganisms in the laboratory as well as in aquatic and terrestrial environments. He also considers the ability of microorganisms to absorb, accumulate, and metabolize DDT and related substances. Dr. Bollag categorizes pesticide detoxification reactions mediated by soil fungi. His contribution is biochemical in emphasis. It warns that concern for environmental pollution and public health must extend beyond the effects of the pesticides themselves and include studies of biodegradation products and their toxicology.

The essays that comprise this anthology of the best in ecology from *Critical Reviews in Microbiology* describe principles and practices in considerable detail. There is no attempt, however, at a balanced presentation of the different groups of microorganisms or their activities. Likewise, some areas of current concern are considered cursorily and others not at all. Nevertheless, the book is an interesting and informative introduction to a growing endeavor. The combined experience and insight of the contributing authors will surely aid the reader to develop an ecological attitude, and to better appreciate microorganisms as determinants of environmental quality.

David Pramer
Rutgers University
New Brunswick, N.J.
October 1973

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THE EFFECTS OF HALOGENATED COMPOUNDS ON MICROORGANISMS

The effects of DDT on the soluble salts in the soil, the accumulation of nitrate, and the production of ammonia from organic materials on a population of organisms in soil were tested by Wilson and Choudhri (1946). In addition, the presence of nodulation on leguminous plants and the effect of DDT on the growth of a variety of pure cultures of legume bacteria were also examined. A number of molds including *Aspergillus, Penicillium*, and the green alga *Chlorella* were examined. High concentrations of DDT were used (up to ½%) and the results indicated that with these quantities there was no evidence that the pesticide was injurious to any of the microorganisms tested, to ammonification, or to the accumulation of nitrate or the normal concentration of salts in the soil. The authors concluded that, from their work, any further examination of the DDT seemed useless.

Samples of two types of soil were air dried and sieved (Jones, 1952). Each was seeded with moistened DDT in concentrations of 0.001 up to 0.1%. Sulfur oxidation, nitrate production, ammonification, plate counts, and nitrogen fixation determinations were made. There was no injury to nitrifiers, ammonifiers, and sulfur oxidizing microorganisms from concentrations of DDT ordinarily added to soil. In all cases, toxicity began to be noticed in concentrations of about 0.1%. Nitrogen fixing bacteria in soil containing DDT as high as 1% appeared to be unaffected and in fact may have been stimulated, as determined by plate counts. DDT added to the soil was remarkably stable during the first year of storage, but after two and three years, a decrease was noted.

Samples for plate counts were taken from a series of experimental plots laid out on several different soil types to examine the effects of insecticides on soil microorganisms in the field (Bollen et al., 1954).

Different insecticides were applied at different rates of application. The results indicated that different isomers of BHC when added to clay soil at 1000 ppm in the laboratory caused different numbers of bacteria and molds to develop after incubation. The isomers of BHC also had a different influence on ammonification and nitri-

fication. The gamma isomer increased the bacterial population except for *Streptomyces*. Where mold development was favored after the addition of dextrose, α and β forms of the compound caused a depression in numbers. In the absence of dextrose, where molds were fewer in number, all isomers except alpha were inhibiting. The delta and gamma isomers of BHC increased ammonification in peptone, while other isomers had no effect. Nitrification appeared to be stimulated by beta and gamma isomers of BHC except in the field where heavier concentrations were used. The evidence indicated that these compounds were insufficiently intensive to materially influence fertility of the soil.

Soil fertility and microorganisms were studied further by Fletcher and Bollen (1954). Ten Oregon soils and six soil classes were examined in the laboratory with aldrin applied at 200 and 1000 ppm. Aldrin appeared to stimulate the bacterial populations with the exception of *Streptomyces* and other molds. Aldrin did not affect the development of *Azotobacter*. The effects of aldrin on ammonification and nitrification of peptone were minor and irregular. Any losses from the laboratory treated soils were largely a result of factors other than microbial. These authors concluded that for insect control aldrin would not significantly affect the soil microorganisms and adversely affect soil fertility.

Gray and Rogers (1954) reported on a study to determine if the addition of BHC to the agar medium used to estimate bacterial numbers in the soil would interfere with the formation of colonies or might enhance the development of certain groups of bacteria. BHC greatly reduced the number of microorganisms in soil, but there appeared to be no outstanding differences in numbers of BHC resistant bacteria in different soils. This suggested to the authors that the indigenous microfloras of these soils were similar with regard to their degree of resistance. In addition, BHC prevented the growth of colonies of most groups of bacteria except for Gram negative short rods. There did not appear to be any outstanding differences in the physiological characters that were examined with respect to BHC resistance in comparison to the controls.

The toxicity of DDT, chlordane, BHC, dieldrin, aldrin, endrin, and methoxychlor to soil microorganisms was examined (Jones, 1956). None of these compounds was excessively inhibitory to the ammonifying organisms or to those responsible for the decomposition of organic matter with the liberation of ammonia. Toxicity appeared to be manifested at a concentration of about 0.1% which was equivalent to a ton of insecticide per acre thoroughly distributed throughout the first 6 in. of soil. Jones concluded that there was little likelihood of these compounds reaching concentrations toxic to ammonifying organisms. A soil fumigant, ethylene dibromide, stimulated the ammonifying organisms over a wide range of concentrations. The author concluded that the herbicide 2,4-D would not be toxic to ammonifying organisms in the concentrations used. Dieldrin, aldrin, and chlordane were the most toxic and inhibited nitrifying microorganisms at concentrations of 0.1%. Soil organisms capable of oxidizing sulfur compounds were less resistant than microbes decomposing organic matter and liberating ammonia. The author concluded that if these compounds were used in the recommended amounts, they can be used safely without serious injury to sulfur oxidizing microorganisms. There was no correlation between the numbers of organisms in the treated soil and the nitrifying, ammonifying, and sulfur-oxidizing activities of soil microbes. The addition of DDT, chlordane, and BHC in sufficient concentration to inhibit these transformations caused significant increase in the number of microorganisms. Ordinarily no harm would result to soil nitrifiers from the use of 2,4-D and the presence of organic matter in the soil tended to protect the organisms there against the toxic action of pesticides used.

In a study by Eno and Everett (1958), two gallon glazed pots were filled with sand at pH 6.65. Ten insecticides, heptachlor, chlordane, methoxychlor, lindane, aldrin, toxophene, dieldrin, TDE, DDT, and BHC were added at concentrations of 12.5, 50, and 100 ppm. Each was seeded with black valentine beans two weeks after the soil treatment and examination of the germination of the seeds was made at that time. Aliquots were taken to determine the numbers of fungi, bacteria, amount of carbon dioxide produced, nitrification, and the pH. A second crop of beans was planted 11 months after applying the insecticides and the experiment was repeated. In the first sample the numbers of fungi were not significantly changed from those of the control for any insecticide except dieldrin which appeared stimulatory. In addition, the release of carbon as

CO_2 was increased by toxophene, dieldrin, TDE, and DDT. No significant difference in bacterial numbers was detected. The nitrate data indicated a decrease in nitrification rate for heptachlor, lindane, and BHC and an increase for soils treated with toxophene, TDE, and DDT. Sixteen months after the application of the insecticides, another evaluation of microbial response was made, and there appeared to be no difference in the numbers of fungi, bacteria, or amount of carbon dioxide produced at that time. Nitrate production, however, was significantly decreased by DDT and BHC. The authors concluded that applications of as much as 200 lb per acre of most of these insecticides caused little or no damage to the microbial population as indicated by measurements of CO_2 evolution and total numbers of fungi and bacteria. They suggest that microorganisms may be stimulated by the addition of these compounds.

In an experiment where five annual applications of insecticides to two southern California soils and the cumulative effect of these applications on the soil microbe population were examined (Martin et al., 1959), dosages were chosen to approximate the maximum use in normal field practice. Insecticides used and their approximate annual application rates in pounds per acre were aldrin, 5; chlordane, 10; DDT, 20; dieldrin, 5; endrin, 5; heptachlor, 5; lindane, 1; and toxaphene, 20. These chemicals were applied to the surface of the soil in the fall of the year as a spray and then the ground was turned over to a depth of 6 in. The soil plot was sampled to a depth of 6 in. with a 1 in. coring tube and plating to estimate the number and kinds of microorganisms was done immediately. The soil was stored for further use. The authors concluded from their experiments that the microbial populations of the plots were remarkably uniform. There was no indication that pesticide applications influenced numbers or kinds of soil fungi or numbers of bacteria. In addition, the decomposition of organic materials as measured by the amount of CO_2 evolved from enriched soil, suggested that the decomposition rate was the same in all treated soils as in the control samples. The same results applied to the oxidation of ammonium to nitrate. In the opinion of the authors, the chemicals aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, lindane, and toxaphene exerted no measurable effect on numbers of soil bacteria and fungi, on kinds of soil fungi developing, or on the ability of the soil population to perform the normal functions of organic matter decomposition and ammonia oxidation.

Some conditions which accelerate the conversion of DDT to DDD in soil and some inhibitory effects of DDT and DDD on soil microorganisms were reported on by Ko and Lockwood (1968). The effect of DDT and DDD on soil bacteria, actinomycetes, and fungi was studied in three ways. Diluted cell or spore suspensions were transferred onto nutrient agar containing 1, 10, or 100 ppm of DDT or DDD. Visual estimations of growth after three to five days incubation were made by comparison of a medium containing the same amounts of the solvent acetone. Secondly, soil suspensions were plated on selected media containing different concentrations of the compounds and after three to ten days incubation, numbers were counted, and thirdly, DDT and DDD were added to small quantities of soil which was incubated and examined for total numbers after 8 days incubation. Soils were prepared by either moistening 5 g of dried soil with 0.5 ml water or submersion in 3 ml distilled water which resulted in a waterlogged condition. The authors concluded that anaerobic conditions appeared to enhance the decomposition of some chlorinated hydrocarbon pesticides and that this rate was enhanced by the addition of organic materials such as alfalfa. The supplement of alfalfa did not appear to affect the rate of conversion, under aerobic conditions. The authors concluded that the conversion of DDT to DDD in soils under anaerobic conditions may explain the detection of DDD in soils where only DDT was originally applied. This is dangerous in that DDD in the soil is more stable than DDT and this may contribute to the persistance of residues of this type in soil and water. These workers also showed that at concentrations as low as 1 to 10 ppm DDT was highly toxic to soil bacteria and *Actinomycetes* in culture. DDD, which is also used as an insecticide, had a broader antimicrobial spectrum and was more toxic to microorganisms in vitro than was DDT. Perhaps, due to the stability and high potency of these compounds against microorganisms, such habitats as the rumen in cattle and the rhizosphere where microbial activity is concentrated may be important.

A study has been made of the effects of DDT,

dieldrin, and heptachlor on selected bacteria (Collins and Langlois, 1968). *E. coli, Pseudomonas fluorescens*, and *Staphylococcus aureus* in either autoclaved trypticase soy broth (TSB) or dehydrated skim milk with pesticides were plated immediately after inoculation and 1, 2, 4, and 7 days of incubation. Results of their work indicate that in concentrations of 50 and 100 ppm neither DDT, dieldrin, or heptachlor affected the normal growth of *E. coli* in either TSB or skim milk. *Pseudomonas fluorescens* was inhibited by both 50 and 100 ppm DDT in TSB after 2 days. Growth in the skim milk was not affected by any of the three pesticides. The growth of *S. aureus* was not affected by dieldrin in either TSB or in skim milk containing any of the pesticides, but was sharply inhibited by heptachlor.

The factors affecting the inhibition of growth of *Staphylococuss aureus* by heptachlor were examined further (Langlois and Collins, 1970). The pesticides used were 4 different concentrations of heptachlor, from 72 to 99% and heptachlor epoxide at a concentration of 99%. Fifteen to eighteen-hour TSB cultures of *Staphylococcus aureus* were incubated in the presence of the chemicals. Growth of the organism in the medium containing the pesticide and the appropriate control was determined by plating each sample on plate count agar. Direct microscopic counts were also made to check results of plating and in all cases the results obtained by both methods were in agreement. The final concentrations of pesticides used were between 1 and 100 ppm. The researchers summarized their results by stating that growth of the organisms was inhibited for 1 day by less than 3 ppm technical heptachlor, and almost completely stopped for 7 days by 10 ppm or more. Heptachlor epoxide appeared to have no effect, while inhibition of growth was more pronounced for technical heptachlor than similar levels of the pure compound. Growth in skim milk was unaffected by up to 10 ppm of heptachlor epoxide or up to 100 ppm heptachlor or technical heptachlor. The addition of 3% casein to the broth medium eliminated heptachlor inhibition, but did not eliminate it completely when trypticase was omitted.

Using the standard TSB growth medium and static cultures incubated at 25 or 37°C, Langlois and Collins (1970) concluded that the growth of *Pseudomonas fluorescens* and *Staphylococcus aureus* in the medium was affected by DDT exceeding 50 μg/ml. The organisms growing in the skim milk appeared to be protected in concentrations up to 100 μg/ml of DDT. The concentration of 3 μg/ml of heptachlor increased the lag phase of growth of *S. aureus* up to 18 hr, while 15 to 20 μg/ml prevented growth up to 7 days. Inhibition of *S. aureus* was not observed in skim milk or in basic medium plus 3% whole casein or a mixture of α and β casein fractions containing up to 100 μg/ml of heptachlor. Heptachlor epoxide appeared to be noneffective, while 72% heptachlor was more inhibitory than the 99%. The authors concluded from their experiments that the levels of organo-chlorine pesticides, which may be in milk or milk products, will not affect the growth of the resident microflora.

In the work of Hurlburt et al. (1970), the compound Dursban was added 4 times at 2 week intervals to small ponds in California at a concentration of 0, 0.01, 0.05, 0.1, and 1 lb per acre. The authors concluded from their experiments, which were done mainly to study duck mortality, that the Dursban treatment caused a decline in the total amount of zooplankton and that the population required at least two weeks and probably a month to recover completely even at the lowest dosages.

Whether microorganisms which had been isolated from different marine environments would vary in response to three chlorinated insecticides was determined by Menzel et al. (1970). Four species in culture were examined for their response to DDT, endrin, and dieldrin. According to Weaver et al. (1965), these compounds are the most widely distributed chlorinated hydrocarbons in major U.S. river basins. The species of organisms examined included *Skeletonema costatum*, a costal centric diatom isolated from Long Island Sound, *Dunaliella tertiolecta*, a green flagellate typical of tidal pools and estuaries, a Cocolithaforid, *Coccolithus huxleyi*, and the diatom *Cyclotella nana*, both from the Sargasso Sea. In these experiments, carbon 14 was added to cultures which were illuminated by fluorescent lights. The cell carbon concentration of the medium was adjusted to between 100, 250, and 500 μg of carbon/l. and was considered to be within the range of naturally occurring carbon concentrations in surface oceanic water. After 24 hr of exposure to the light, the cells were filtered and counted in

a Geiger Muller end window counter. Longer-term effects of the DDT and endrin on cell division were studied by counting cells for seven days. Cultures were grown in 125 ml flasks, containing 50 ml portions of medium and 100 ppb of the insecticide was added daily to each flask. The authors showed that none of the insecticides that were tested up to 1000 ppb affected the cultures of *Dunaliella* and no change in the rate of cell division could be detected over the 7 day period. The rate of C^{14} uptake in *Skeletonema* and *Coccolithus* was significantly altered at concentrations above 10 ppb in the case of all three of the insecticides. At 100 ppb DDT had some effect on cell division in *Skeletonema*, but no effect on *Coccolithus*. Endrin appeared to have little effect on the final concentration of *Skeletonema* cells, but the rate of growth over the first five days was slower than the controls. Reduced growth rates were also noticed with the *Coccolithus*. In contrast to these other species, *Cyclotella* was inhibited by all three insecticides and the slopes of the dose response curves for dieldrin and endrin suggest that quantities as low as 0.01 ppb may have been effective. Cell division was completely inhibited by dieldrin and endrin, while cells exposed to DDT divided more slowly than the controls. Because of the solubility difficulties with these compounds, the authors could not conclude much about the incorporation of these substances into the cell material, but felt because some species responded to concentrations above solubility limits that this indicated they were capable of incorporating these compounds as small particulates or that the saturation was quickly maintained while they concentrated the pesticide from solution. Although chlorinated hydrocarbons may not be universally toxic to all species, they may exert an influence on the dominance or succession of the individual forms.

Cowley and Lichtenstein (1970) used 17 species of fungi isolated from Wisconsin prairie soils. These organisms were grown on Czapek nutrient agar that had been treated with the chlorinated insecticides aldrin and lindane. Aldrin at a concentration of 20 ppm inhibited growth of *Fusarium oxisporum* by 37 to 44%. The addition of yeast extract, asparagine, ammonium sulphate, ammonium nitrate, or ammonium sulphamate to the medium resulted in a suppression of the growth inhibitory effect. Substitution of a vitamin mixture in place of the yeast extract had no effect.

The interaction of DDT and river fungi and the effects of the insecticide on the growth of four aquatic hyphomycetes have been studied (Dalton et al., 1970). *Heliscus submersus, Tetracladium setigerum, Varicosporium elodeae,* and *Clavariopsis aquatica* were obtained from river water as single spore isolates. DDT was added to a basal medium in concentrations that ranged from 0.1 to 60 μg of DDT/ml. The chlorinated hydrocarbon content of the medium before inoculation was determined by gas liquid chromatography. The extent of culture growth was determined at three day intervals by filtering through previously dried and weighed filter paper. The results indicate that the growth of *T. setigerum, V. elodeae*, and *C. aquatica* were unaffected by DDT concentrations below 2 μg/ml. At higher concentrations the growth rates were enhanced. *H. submersus* exhibited an enhanced rate of growth at each DDT concentration tested. Speculation about the results obtained (Dalton et al., 1970) suggests that the increase in growth may be the result of the use of DDT as a carbon source; that DDT may affect the permeability of the fungal cells to other nutrients; that DDT may increase the metabolic rate of the fungi in the capacity of a cofactor; and that this compound could have ecological significance since these fungi decompose organic debris and would be involved in the recycling of nutrients in the fresh water environment.

Dougherty et al. (1971) questioned what the sensitivity of *Bacillus thurengiensis* was to a number of pesticides as compared to other substances already known, such as antibiotics and sulfonomides. The method used for testing was to inoculate the surface of a petri plate containing nutrient agar with bacillus spores at a concentration of 7000 per plate. Sterile paper discs containing the appropriate insecticide or herbicide were placed aseptically on the surface of the medium. The following compounds did not inhibit the bacillus-DDT; methoxychlor; lindane; dieldrin; 2, 4-D and 2, 4, 5-T Significant inhibition was obtained with chloropropam.

METABOLISM OF HALOGENATED PESTICIDES

In experiments by Okey and Bogen (1965), metabolism was followed with a Warburg respirometer and by direct observation of sub-

strate removal. Culture material or cellular material was derived from soil and sewage inoculum and maintained on a single carbon source which was the unsubstituted homolog of the chlorinated test substrate. Their purpose was to develop a biological system capable of assimilating a specific carbon frame so that steric and substituent effects of a molecule could be properly evaluated. As the result of examination of a variety of chlorinated parafins, chlorinated napthalene, benzene, chlorinated benzoic acids, catechol, chlorinated catechol, chlorinated benzaldehyde, benzoic acid, and chlorinated phenoxyacetic acids, it was concluded that the presence of chlorine on a microbial substrate does not necessarily impede microbial metabolism. The effect of the chlorine is apparently regulated by such features as molecular size, the nature, and the number and position of other substituents. It appeared that there was no necessary development of any special enzyme system for the removal of chlorine and that the length of the chlorinated molecule had a significant bearing on its assimilability. No metabolism of the four carbon chlorinated parafins was detected while longer substances were completely utilized. The controlling feature was the number of carbon atoms between the site of chlorine substitution and the terminal carbon. In aromatic compounds, the chlorine substitution is altered by the oxidative state of the molecule. One chlorine atom affected the fate of chlorobenzene, chlorobenzaldehyde, and napthalene, but if the molecule was presented in a more oxidized form, monochlorination appeared to become less significant, until as in the case of chlorocatachol, little or no effect was observed at all. The location of the chlorine also appeared to affect the rate of metabolism. Metachlorobenzoate was metabolized completely, while ortha and para analogs were attacked partially. Because chlorine is such a strongly electronegative substituent, it is known to have a profound effect on chemical reactivity. Chlorine is known to alter the resonant properties of aromatic substances and in turn alter the electron densities of specific sites. This alteration of electron densities may increase or reduce the activation energy barrier through which a molecule passes during a reaction. For further clarification of these theories, the reader is referred to the work of Nakamura.[1] After searching the literature on detoxification, Okey and Bogen agree with the concept that hydrolysis is not the basic mechanism for the effect. Available reports indicate that chlorine is removed from alkyl substances in later catabolic steps (chloroacetic acid) and from aromatic substances during one of the electron rearrangements between muconic and adipic acid. The authors concluded that, the metabolism of monochlorinated parafins was impeded when six carbons or less are between the site of substitution and the terminal carbon, or when the presence of three or more chlorine atoms are an aromatic ring. They believe that the inhibition noted during microbial metabolism of these substrates can be explained by the electronic effects of the chlorine on the substrate. The initial attack on an intact aromatic ring appears to be electrophilic in nature. In general, an increase in oxidative state of chlorinated molecule appears to render it more available for attack. The completeness of metabolism of refractory substances may be regulated in part by the quantity of cellular material present and by the availability of the easily assimilable substrates present. The resistance to attack previously reported for lindane and dieldrin type compounds and 2, 4, 5,-T might be explained by the electron poor nature of the unsubstituted carbon atoms.

Stenersen (1965) performed experiments to investigate whether absorption, metabolism, or excretion of DDT was the cause of resistance in the stable fly *Stomoxys calcitrans*. These particular flies develop very high resistance to DDT, methoxychlor, and DDD when kept under selection pressure in the laboratory. *Serratia marcesens, Alcaligenes faecalis*, and one unidentified bacterial strain were isolated from excreta of these flies kept in sterile cages. These isolated strains and other laboratory strains of *E. coli, Bacillus brevis,* and *Aerobacter aerogenes* were cultured under anaerobic conditions in meat extract under nitrogen atmosphere, in unshaken cultures (oxygen deficiency), and in fully aerated cultures. The medium contained C^{14} labeled DDT and after incubation the mixture was extracted with hexane and analyzed for radioactivity. When growing anaerobically or in oxygen deficiency, the facultative anerobes *S. marcesens* and *E. coli* and their unidentified strain converted DDT almost completely to DDD (about 90%) and DDE (5%). In the aerated cultures, neither the facultative anaerobes nor the obligate aerobes had any effect.

An attempt was made (Matsumura and Boush,

1967) to discover microorganisms that were capable of degrading dieldrin, known to be extremely stable in the environment. Microorganisms isolated from various soils were exposed to 0.01 μM of C^{14} labeled dieldrin. These mixtures were incubated for 30 days without shaking and the reactions stopped by adding trichloroacetic acid to each tube. The tubes were extracted with chloroform and distribution of radioactivity between the aqueous and solvent phases was reported. Eight cultures which appeared to be promising in their ability to degrade dieldrin were extracted with chloroform and analyzed with thin layer chromatography. The results indicate the definite existence of a variety of breakdown products of dieldrin and suggest that in soil there are microbes capable of degrading dieldrin.

In a search for metabolites which might be produced by microorganisms detoxifying dieldrin, Matsumura et al., (1968) isolated and chemically identified the chief products of degradation of dieldrin involved with one microorganism. A *Pseudomonas* species, originally isolated from a soil sample near a dieldrin factory belonging to the Shell Chemical Co. near Denver, Colorado, was shaken in liquid culture with dieldrin in acetone for seven days. The experimental culture and the soil known to contain 100 ppm of dieldrin were extracted and the metabolites purified using a column of Florisil and thin layer chromatographic separation with silica gel HF. The metabolites that were separated from others were purified on a gas chromatographic system. In all cases the identity of the metabolites that were produced in the *Pseudomonas* culture were compared to those from soil by matching the R_f values and the retention times on various gas chromatographic or thin layer systems. Infrared analysis of the metabolites and mass spectroscopic analysis were carried out with the result that the following (Figure 1) proposed degradation for dieldrin was established. With the exception of aldrin, the metabolites that were discovered do not exist in nature and the authors felt that it was extremely important to study their toxological effects on various ecosystems in our environment.

In 1968 the question of whether the conversion of DDT to DDD involved a step in reductive dechlorination or was a two step reaction with DDE as an intermediate product was examined (Plimmer et al., 1968). *Aerobacter aerogenes* was the organism of choice and was grown at 37°C in shake flasks containing 3% trypticase soy broth medium. Logarithmically growing cells were exposed to C^{14} and deuterium labeled DDT in separate experiments. Incubation of these cultures was continued in still culture for 86 hr. Extracts from the medium were examined gas chromatographically and with silica gel G chromatographic plates. In addition, for mass spectroscopic studies, thin layer chromatographic plates were developed and bands scraped from the plate, extracted with ether and portions of these extracts injected into the gas chromatograph. At the same time, a portion of the sample was placed in a glass capillary tube in the probe of the mass spectrometer. The use of the deuterium labeled DDT enabled the investigators to demonstrate that with *Aerobacter aerogenes*, conversion of DDD occurred with the replacement of chlorine by hydrogen, indicating that there were no unsaturated intermediates involved. If an alternate

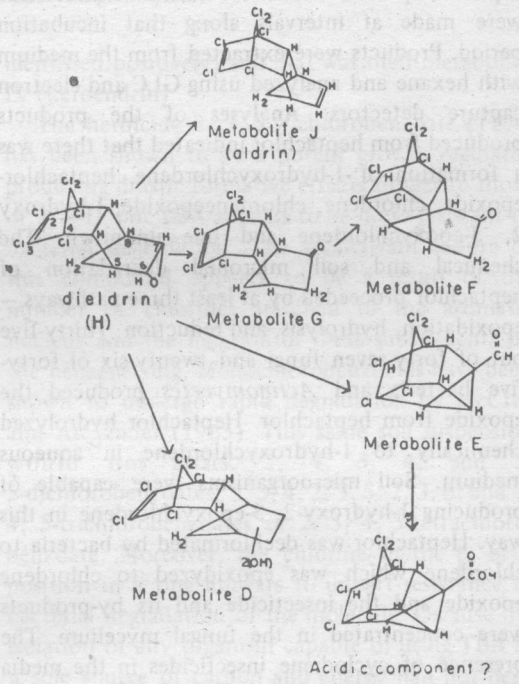

FIGURE 1. Proposed dieldrin degradation pathways by the microorganism in soil. The structures of metabolites G and F were established, F being one of the stable "terminal residues." The structures shown for E and the resulting acid metabolite are the most likely ones. The structural arrangement of those two hydroxyl groups in metabolite D is still unknown except that one of them is at the 6 carbon position. (From French, A. L. and Hoopingarner, R. A., *J. Econ. Entomol.*, 63, 756, 1970. With permission.)

route for the breakdown had occurred (the path through DDE to DDD), then the deuterium which was attached to the carbon atom would have been released and detected in their experiments. It was apparent that *Aerobacter aerogenes* affected the conversion of DDT to DDD by a process of reductive dechlorination. The thin layer chromatography indicated that the ether layer extracted in the experiment contained DDT and DDD as the only radioactive materials. Continuous ether extraction of the aqueous layer and the cellular debris revealed also only DDT and DDD.

Miles et al. (1969) attempted to isolate and identify the microorganisms which convert heptachlor to its epoxide in soil, and also to determine if there were other pathways of chemical and microbial degradation of heptachlor. The study was performed with 92 species of microorganisms originally isolated from soil. One ppm of the insecticide and/or its metabolites were incubated at 28°C in an aqueous basal medium for a period up to 6 weeks. Additions of insecticide were made at intervals along that incubation period. Products were extracted from the medium with hexane and analyzed using GLC and electron capture detectors. Analyses of the products produced from heptachlor indicated that there was a formation of 1-hydroxychlordene, heptachlor-epoxide, chlordene, chlordeneepoxide, 1-hydroxy 2, 3-epoxychlordene and one unknown. The chemical and soil microbial degradation of heptachlor proceeded by at least three pathways – epoxidation, hydrolysis, and reduction. Thirty-five out of forty-seven fungi and twenty-six of forty-five bacteria and *Actinomycetes* produced the epoxide from heptachlor. Heptachlor hydrolyzed chemically to 1-hydroxychlordene in aqueous medium. Soil microorganisms were capable of producing 1-hydroxy-2, 3-epoxychlordene in this way. Heptachlor was dechlorinated by bacteria to chlordene which was epoxidyzed to chlordene epoxide and the insecticide and its by-products were concentrated in the fungal mycelium. The presence of cyclodiene insecticides in the media appeared to influence some microbial metabolic processes. Previous to this work, it had been assumed heptachlor could be degraded in two ways, either by volitilization or by conversion to heptachlor epoxide. This work suggests that there are two additional pathways of degradation: chemical hydrolysis to 1-hydroxychlordene followed by microbial epoxidation to 1-hydroxy-2, 3-epoxychlordene and conversion to an unknown product and bacterial dechlorination to chlordene and then oxidation to chlordene epoxide. The authors felt that the pathway leading to the unknown product through hydrolysis and microbial decomposition was the major degradation route (Figure 2).

Twenty microbial isolates previously found capable of degrading dieldrin were tested on their ability to degrade endrin, aldrin, DDT, Baygon, and gamma BHC (Patil et al., 1970). The isolation of the microorganisms had been described by Matsumura and Boush (1967). Each isolate was cultured in a 10 ml solution of yeast extract and mannitol and maintained at 30°C for 57 hr. These suspensions were inoculated with labeled insecticide and incubated for 30 days without shaking. In the final analysis, the distribution of radioactivity between aqueous and solvent phases was recorded and suggested that all the isolates were capable of degrading DDT and endrin, while 13 degraded aldrin. None were able to degrade Baygon or Gamma BHC. Since a number of Gamma BHC degrading microorganisms did exist, the authors concluded that it was likely that the enzyme systems metabolizing dieldrin, endrin, and DDT were different from those for Gamma BHC. It is not entirely surprising that dieldrin degrading microorganisms could degrade endrin, because endrin is an endo isomer of dieldrin.

Metabolism of the insecticide lindane by *Chlorella vulgaris* and *Chlamydamonas reinhardtii* in axenic culture was ascertained by Sweeny (1968). The organisms were grown for 14 days in bristol solution containing lindane. After incubation the medium was extracted with hexane and the extract examined using gas and thin layer chromatographic analysis. A substance was identified as 1, 3, 4, 5, 6-pentachlorocyclohex-1-ene a known nontoxic lindane metabolite which suggested that these organisms under certain conditions could utilize lindane.

The comparative metabolism of methoxychlor, methiochlor, and DDT was studied in mice, insects, and in a model ecosystem (Kapoor et al., 1970). A model ecosystem constructed for evaluating pesticide biodegradability consisted of a 10 x 12 x 18 in. glass aquarium containing a shelf of 15 kg of washed white quartz sand which was molded into a sloping soil air water interface. The lower portion was covered with 12 l. of standard reference water which had satisfactory nutrient

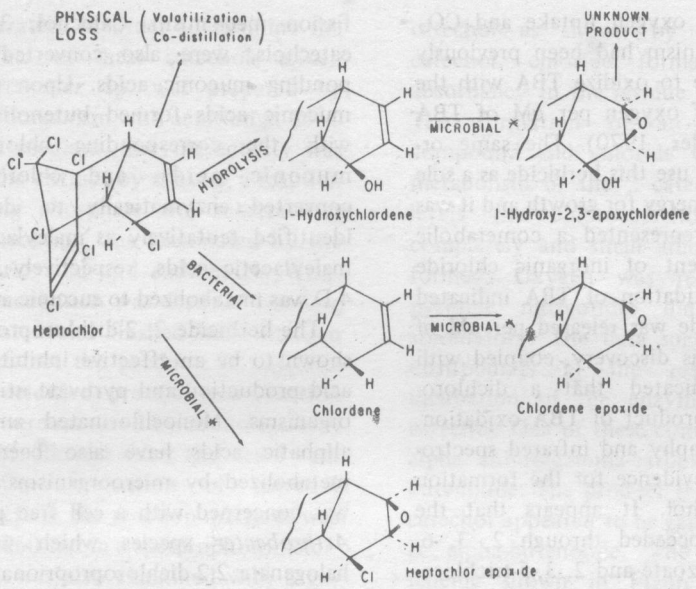

FIGURE 2. Scheme for chemical and microbial degradation of heptachlor. (From Matsumura, F., Boush, G. M., and Tai, A., *Nature*, 219, 965, 1968. With permission.)

naterial for the growth of *Sorghum halpense*, on he aerial portion and the alga *Oedogonium cardiacum* in the aquatic portion. The latter was seeded with a complement of plankton and also contained *Daphnia magna* and snails. The aquarium was provided with aeration and kept in an environmental growth chamber at 80°F with 12-hour exposure to 5000 foot candles of light. This model system was not fully evaluated but the workers were able to provide evidence of the relative biodegradability of the various pesticides examined.

About 150 isolates from various soil samples were screened to investigate the role of soil microorganisms in degrading endrin (Matsumura et al., 1971). Using techniques that they had described prior to this (Matsumura et al., 1968) they found that of the total cultures tested 25 were active in degrading endrin. At least seven metabolites of endrin were further isolated from the mass culture of a *Pseudomonas* species. In addition, all dieldrin degrading microbes which had earlier been reported to be highly active in degrading dieldrin were found to be also active in degrading endrin (Patil et al., 1970). On the basis of spectroscopic analyses, the conclusion was that the majority of the microbial metabolites of endrin were ketones and aldehydes with five to six chlorine atoms. Only one of the metabolites was identified positively, and that was their metabolite IV (ketoendrin).

The herbicide 2, 3, 6-trichlorobenzoate (TBA) has been shown to be a potent growth regulator producing drastic formative effects similar to those of 2, 4-D, but TBA appears to be more resistant to bacterial degradation. The recalcitrant nature of this compound appears to be a result of the number of chlorines attached to the aromatic nucleus and the location of these substituents on the benzene ring. Monochlorobenzoates have been shown to undergo rapid degradation by McCrea and Alexander (1965). This same soil suspension would not attack 2, 4; 3, 4; and 2, 5-dichlorobenzoates; 2, 3, 4; 2, 3, 5; 2, 3, 6; and 2, 4, 5-trichlorobenzoates or 2, 3, 4, 5-tetrachlorobenzoate. Moreover, the chlorine in the 3- or 5 position in a ring appears to impart resistance to bacterial degradation of the molecule. Because the isolation of any organism capable of using TBA as a sole source of carbon and energy had not been reported, Horvath (1971) thought that the process of cometabolism may be an important phenomenon in this case. His investigation attempted to demonstrate cometabolic degradation of TBA, its mechanism, and the pathway of its degradation with an organism identified as *Brevebacterium* sp. cultured on benzoate salts agar. Standard manometric techniques were

employed to measure oxygen uptake and CO_2 release. The microorganism had been previously described as being able to oxidize TBA with the uptake of one μM of oxygen per μM of TBA (Horvath and Alexander, 1970). The same organism was not able to use this herbicide as a sole source of carbon and energy for growth and it was considered that this represented a cometabolic mechanism. Measurement of inorganic chloride released during the oxidation of TBA indicated that one μM of chloride was released per μM of substrate oxidized. This discovery, coupled with manometric data indicated that a dichlorocatachole could be a product of TBA oxidation. Thin layer chromatography and infrared spectrophotometry provided evidence for the formation of 3, 5-dichlorocatachol. It appears that the oxidative pathway proceeded through 2, 3, 6-trichloro-4-hydroxybenzoate and 2, 3, 5-trichlorophenol to the end product 3, 5-dichlorocatachole which accumulated in the medium. 3, 5-dichlorocatachol was toxic to whole cells, but did not inhibit 2, 3, 6-trichlorobenzoate oxidizing enzymes or the pyro catechase enzyme. Horvath concluded that the specificity of pyrocatechase enzyme for an unsubstituted catechol was the cause of the phenomenon of cometabolism which this isolate exhibited.

The early experiments of Audus (1955) using *Bacterium globiforme* isolated from garden soil indicated that there were at least two intermediates produced in 2, 4-D breakdown which demonstrate phytotoxic properties.

While studying the metabolism of 2, 4-D by *Aspergillus niger*, the main product of the 2, 4-D metabolism was 2, 4-dichloro-5-hydroxyphenoxyacetic acid (Faulkner and Woodcock, 1964). Through the use of infrared spectrophotometry and mixed melting points, a second metabolite was identified as 2, 5-dichloro-4-hydroxyphenoxyacetic acid.

The products formed while the biological degradation of chlorinated catechols occurred and the establishment of the pathway by which the chlorophenoxyacetic acids and intermediates are metabolized was of interest to Tiedje et al., (1969). An enzyme preparation was prepared from 40 l. of actively growing *Arthrobacter* sp. grown in the presence of 2, 4-D. The enzymatic preparation catalyzed the conversion of 4-chloro and 3, 5-dichlorocatechols to *cis*-3-chloro and *cis, cis*-2, 4-dichloromuconic acids, respectively, by an ortho fission mechanism. Catechol, 3- and 4-methylcatechols, were also converted to the corresponding muconic acids. Upon acidification the muconic acids formed butenolides which along with the corresponding chlorinated *cis, cis* muconic acids, and chlorocatechols were converted enzymatically to identical products identified tentatively as maleylacetic and chloromaleylacetic acids, respectively. Ring labeled 2, 4-D was metabolized to succinic acid.

The herbicide 2, 2-dichloropropionate has been shown to be an effective inhibitor of panothenic acid production and pyruvate utilization in microorganisms. Monochlorinated and dichloronated aliphatic acids have also been shown to be metabolized by microorganisms. Kearney (1964) was concerned with a cell free preparation of an *Arthrobacter* species which was able to dehalogenate 2,2-dichloroproprionate; and a number of other pure cultures which were capable of decomposing this compound. These are listed as representatives of the following genera: *Pseudomonas, Agrobacterium, Bacillus, Alcaligenes, Arthrobacter*, and *Nocardia*. The enzyme was partially purified by ammonium sulfate precipitation and showed a substrate specificity with nine chlorinated aliphatic acids. The enzyme (the substrate specificity was tested with nine) had its greatest activity on 2, 2-dichloroproprionate with less activity on 2-chloroproprionate, dichloroacetate, and 2, 2-dichlorobutyrate. There was no activity on any beta-chloro substituted aliphatic acid. The basis of activity was made upon the number of μg of chloride ion liberated per mg of protein per 10 min. The biotransformation of 2, 4-dichlorophenoxy alkanoic acids and related compounds by soil microflora is perhaps the most extensively studied. A fine reference source for the metabolism of these compounds and pesticides in general has been compiled by Menzil (1969).

The soil bacterium *Arthrobacter* sp. can cleave the 2, 4-D molecule enzymatically to the corresponding phenol. In this case, the 2, 4-D and related compounds such as 4-chlorophenoxyacetate and 4-chloro-2-methyl phenoxyacetate (MCPA) are converted to 2, 4-dichlorophenol, 4-chlorophenol and 4-chloro-2-methylphenol. In other work (Bollag et al., 1968a), it was shown that these particular phenols can be converted to catechols by a mixed function oxidase. In the case of 4-chlorophenol, 4-chlorocatechol resulted and in the case of 2, 4-dichlorophenol, 3, 5-dichloro-