Insecticide Microbiology

Edited by Rup Lal

Foreword by V. Krishnamoorthy

With 42 Figures

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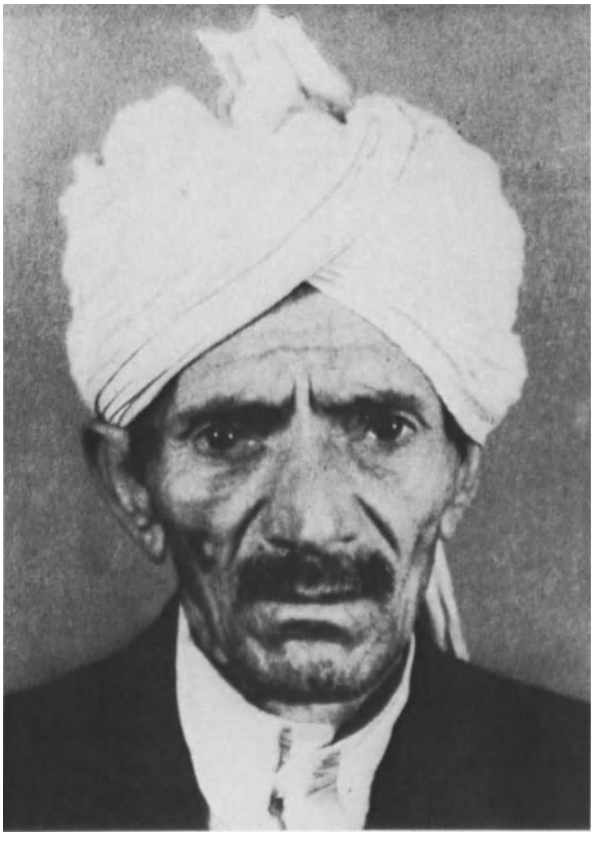

Dedicated to
RAM CHANDER (1898 – 1971)

Foreword

Of all the food produced in the world one third is lost to insect pests, weeds and diseases, and the total world population is estimated as growing from 4000 million in 1975 to about 6000 million by the year 2000. To satisfy these needs, the world's farmers must meet the extra requirement every year. The easiest way in which farmers can increase the amount of food they produce is to prevent the loss due to pests. The biological control measures which were once thought to be the safest methods of pest control have, as we now know, not proved successful on a commercial scale. In such a dismal situation the only solution is to use pesticides to save the losses from pests and to increase the crop yield. Apart from agriculture, pesticides have also contributed much to human comfort by controlling the vectors of typhoid, malaria, sleeping sickness, filariasis, dengue hemorrhage fever, plague etc.

On the other hand, the indiscriminate use of insecticides and their harmful effects on nontarget organisms has attracted much attention from people in all walks of life, for example, scientists, administrators, the press and the public. The harmful effects of insecticides on higher organisms such as birds, fish and mammals are easy to observe and have received much attention. However, the interactions of insecticides with microorganisms such as bacteria, fungi, algae and protozoa have gone unnoticed until recently. In both aquatic and terrestrial ecosystems these microbes are responsible for many basic ecological processes such as biogeochemical cycling, decomposition processes, energy production, energy transformations through trophic levels and numerous microbe-microbe, microbe-animal and microbe-plant interactions. The effects of insecticides on these microorganisms may have a marked effect on the productivity of the biosphere. Thus the challenge is to select and apply insecticides in such a way that target organisms are controlled by causing minimum damage to microorganisms. This is possible only by gathering sufficient information on this particular subject. The compilation of data on interactions of microbes with insecticides in a book is a timely step in this direction.

New Delhi — Dr. V. KRISHNAMOORTHY

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Preface

Insecticides are important to increase, protect, and improve the quality and the quantity of our food commodities, building materials, clothing, and animal health and to combat certain diseases transmitted to man. Thus the insecticides are deliberately designed to alter the ecological balance, i.e., to eliminate or restrict undesirable species for man's continued existence. The ubiquitous nature of so many biological and biochemical processes makes it unlikely that even highly specific insecticides will not affect some nontarget organisms. Earlier optimism regarding the potential of insecticides has been dampened to some extent by the increasing recognition of the potentially deleterious effects of insecticides on nontarget organisms. Among these, microorganisms are of major concern for their role in sustaining the productivity of biosphere.

A comprehensive understanding of microbe/insecticide interactions is necessary for the accurate indentification and assessment of environmental problems. Further the field of microbe/insecticide interactions has expanded rapidly during the last decade and has assumed new dimensions in the disciplines that it now encompasses. Hence there is an urgent need for a book that compiles data on this aspect and ascertains the directions in which current and future research should be carried out.

With the help of several authors I have attempted to produce *Insecticide Microbiology*, whose aim is to present microbe-insecticide interactions in a wider environmental context. The book brings together in an integrated fashion the findings on bioaccumulation, metabolism, detoxification, and effects of insecticides on microorganisms. The coverage of genetic engineering in detoxification of insecticides will convey to the readers some exciting and stimulating discoveries that have come to light in recent years.

New Delhi

RUP LAL

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CHAPTER 1

Introduction

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Insecticides are used in agriculture, health, household, and industry. When first developed around 1940, insecticides such as DDT, dieldrin, and mirex were received with open arms and they quickly became popular as effective economic agents against pests. The benefits of insecticide usage reflected as enhanced farm productivity and control of insect vector-borne diseases were so overwhelming that the real awakening to the problem of their persistent nature and toxic residues came into focus only around 1960. The indiscriminate use of these chemicals has caused serious concern about the toxic effects of the residues of these insecticides on most of the nontarget organisms. This subject today constitutes one of the most serious challenges to public health and environmental pollution.

Insecticides, by virtue of their nature, are toxic not only to the insects or pests against which they are used, but also to other organisms. Undesirable side effects, such as damage to nontarget organisms, perturbation of structure and function of ecosystem, and general environmental contamination by persistent insecticides have become increasingly apparent in recent years.

In the context of agriculture, the major concern is the harmful effects of insecticides and their residues on soil microflora and hence soil fertility. Microorganisms are intimately involved in several soil processes such as decomposition of organic matter and recycling of essential plant nutrients, humus formation, soil structural stability, pathogen survival and pesticide detoxification. In an aquatic environment, microorganisms play a significant role not only in the production of organic matter through photosynthesis, but also participate in several biogeochemical processes in water and sediments. The efficient functioning of these processes involves a delicate equilibrium between microorganisms and their environment and any change in their physical, chemical, and biological processes due to the introduction of insecticides may disturb the equilibrium, which may have far-reaching consequences.

The present extensive use of insecticides is unlikely to decrease. It is, therefore, a matter of priority to obtain adequate information on the interaction of insecticides with microbes. The period from 1960 to 1975 has witnessed most of the research on the toxic effects and metabolism of the persistent insecticides, particularly DDT, on higher organisms such as fish, birds, and mammals. During the same period extensive surveys of residual levels of insecticides in different components of the environment were also conducted. However, microbe-insecticide interactions have received very little attention in the past. The toxicity of insecticides to microorganisms and the potential of microorganisms to bioconcentrate, biomagnify, and metabolize insecticides has received more attention recently. Further, the studies on microbe-

insecticide interactions are not as simple as they appear to be. While there is a considerable impetus to perform such studies they may be complicated by several commonly encountered problems.

Whenever an insecticide comes into contact with microorganisms it accumulates. Since microorganisms are present at the base of the food chain, the accumulated insecticide may be transferred to higher trophic levels through food web and chain organisms. Another major aspect of microbe/insecticide interactions which has been extensively studied is insecticide metabolism. Once thought to be recalcitrant, molecules are now believed to be completely broken by microorganisms. Recently, attempts have made to characterize and isolate microbial enzymes responsible for the metabolism of these insecticides. The work in the field of insecticide detoxification and gene technology can further help to solve the problem posed by the persistent insecticides. Although little has been done in this direction, this is the area which will, increasingly claim the attention of insecticide microbiologists in the near future.

Although laboratory studies on toxicity have some scientific value, they cannot claim much success in terms of the actual consequences of insecticide application, as the microbial environments are very complex and it is difficult to simulate them in the laboratory. In order to fully understand the nature of microbe-insecticide interactions we must appreciate the habitat in which the microorganisms live. This area has recently received considerable attention, with the result that scientists have now realized that the subject has to be viewed in the light of both abiotic and biotic factors that interact with each other and finally decide the outcome of interactions. As mentioned earlier, the importance of environmental factors is not easy to assess from the study of the effect of any single factor or microbes in laboratory experiments. In a natural environmental an alteration in any single factor may trigger off consequential changes which have a more profound effect on microbe insecticide-interactions. This is particularly important in soil, which receives the major part of the insecticides and also the fertility of which is dependent on microbial activity. In aquatic environments, algae are more sensitive to insecticides. However, this field also requires much more work, particularly with insecticides other than DDT. Finally, the cytological and biochemical effects of insecticides on microorganisms has also not been studied extensively although the microorganisms can be used as a model to predict the mode of action of these compounds.

CHAPTER 2

Insecticides and Microbial Environments

S. K. GUPTA

1 Introduction

By and large, insects have been the most successful of living forms, the class Insecta having more species than all other classes of animals combined. Even now, every year, insects account for a major loss to our food and property and are a serious health hazard, as vectors of many diseases. Hence, it is little wonder that insecticides (pesticides used to control insects) are the largest class of pesticides manufactured and used throughout the world, in an array of forms, to control the insects and thereby the destruction caused by them. The U.S. Federal Environmental pesticide Control Act defines a pesticide as "Any substance or mixture of substances intended for preventing, destroying, repelling or mitigating any insect, rodent, nematode, fungus, weed or any other form of terrestrial or aquatic plant or animal life or virus, bacteria or other micro-organism which the Administrator declares to be a pest, except viruses, bacteria or other micro-organism on or in living man or other animals ..." Thus, the term pesticide-insecticide includes all chemicals intended for use in agriculture or horticulture except fertilizers and chemicals used to control pests of any kind except veterinary products and drugs for internal parasites or pests of man and animals. The use of insecticides has made an enormous contribution to agriculture and public health. They have brought tremendous benefits through increased food and fiber production, control of vectors of human and livestock diseases, and protection of structures from insect damage.

Microbial processes play an important role in bringing about the biological transformations of insecticides (Matsumura 1974). A large percentage of insecticides applied find their way into the aquatic and terrestrial environments where microorganisms are prevalent, whether they are directly aimed at them or not. Aquatic as well as terrestrial habitats are dominated by microorganisms, and due to their ability to adapt and proliferate in diverse situations, their total biomass, and surface area, they are considered to represent the principal force of environmental alteration of insecticidal residue. Further, insecticides can be termed micropollutants, since residues left after their application are generally too low to contaminate the environment physically but are high enough to bring about a change in the physiological process of microorganisms which come in contact with them. Further, insecticide residues may act as a carbon source to the microorganisms in natural environments.

2 Classification of Insecticides

Insecticides are naturally occurring or synthetic inorganic or organic chemicals and can be subclassified depending on their mode of action, mode of entry into insects, or their chemical nature. For convenience of presentation, in the following pages, insecticides are discussed in brief on the basis of their origin (synthetic or natural) and chemical nature. However, detailed information on various groups of insecticides can be had from O'Brien 1967; Casida 1973; Brooks 1974; Corbett 1974; Eto 1974; Moriarty 1975; Crosby 1976.

2.1 Inorganic Insecticides

Almost all the insecticides used before World War II were inorganic compounds. The major categories of these insecticides are:

2.1.1 Arsenical Insecticides

A crystal compound of acetate and arsenite of copper having an approximate composition of Cu_4 $(CH_3CO_2)_2(ASO_2)_2$ popularly known as Paris Green, was one of the earliest insecticides, used for the control of potato beetle. Later, several compounds containing the element arsenic were synthesized and used for insect control. The insecticidal property and effectiveness of these compounds is related to the percentage content of arsenic, and their phytotoxicity is correlated with their solubility in water. Other important arsenical compounds include white arsenic (AS_2O_3), lead arsenate, calcium arsenate, etc. In general, arsenicals are stomach poisons and they react with the thiol group (–SH) of various respiratory enzymes (Webb 1966). Arsenicals are persistent chemicals which build up on plant or fruit surface exposed to repeated application and slowly accumulate in the soil and lead to very high residues.

2.1.2 Fluoride Insecticides

Fluorides came into use with the discovery of sodium fluoride as a potent stomach poison in cockroaches. It has limited value for outdoor application as it is very soluble in water and is a persistent compound. Other fluoride compounds used for insect control include silicofluoride (Na_3SiF_6) and cryolite (Na_3AlF_6). Fluorides are believed to be enzyme inhibitors, and a relatively high concentration is required to cause death in insects (O'Brien 1967). Corbett (1974) suggested that their toxicity is due to simultaneous inhibition of several enzymatic processes having iron, calcium or magnesium as a prosthetic group.

2.1.3 Thiocyanates

All alkyl thiocyanates (R–S–CHN) prepared by reaction of an alkyl halide with sodium thiocyanate have insecticidal properties. In general, they are extremely rapid in action; they have, however, an irritant effect on human skin. Thanite is the only compound of this series which has some commercial importance and is used mainly as a constituent of fly sprays to protect livestock.

2.1.4 Other Inorganic Compounds

Several other inorganic substances have been used on a low or moderate scale for killing insects. Some of the important compounds used for pest control include lead chromate and boric acid.

2.2 Insecticides of Natural Origin

2.2.1 Nicotine and Related Alkaloids

The use of tobacco as an insecticide is of very long standing. The active ingredient, nicotine, and related alkaloids, nornicotine and anabasine, can be obtained from several species of *Nicotiana (N. tabaccum, N. rustica, N. glauca* etc.). They are readily soluble in water and mainly act as a contact insecticide. However, their activity is restricted to aphids and some species of ticks and mites. Nicotine has a high mammalian toxicity.

2.2.2 Rotenone

Rotenoid insecticides obtained from the roots of *Derris* sp. *(D. elliptica, D. malaccensis, D. urucu)* and *Lonchocarpus utilis* derive their main virtues from their low mammalian toxicity and short persistence. However, they are very toxic to fish and pigs. They act as stomach and contact poisons.

2.2.3 Ryanodine

Powdered roots and stems of *Ryania speciosa,* a shrub indigenous to tropical South America, are highly toxic to lepidopterous larvae, caterpillars and several insect species. The effective constituent is ryanodine, a natural substance having a chemical formula $C_{25}H_{35}NO_9$. Ryanodine acts as a contact and stomach poison. It is nonphytotoxic and has low toxicity to mammals.

2.2.4 Natural Pyrethrins

Extracts from the flowers of *Chrysanthemum* species *(C. rosum, C. coccineum, C. carneum* and *C. cinerariaefolium)* have insecticidal properties. Flowers are dried and powdered or they are subjected to extractive processes with organic solvents. The commercial product thus obtained, referred to as pyrethrum, is a mixture of six esters. The active ingredients which form 25% of the pyrethrum extract contains pyrethrin I-35%, pyrethrin II-32%, cinerin I-10%, cinerin II-14%, jasmolin I-5% and jasmolin II-4%.

Pyrethrins are used mainly as space sprays against household flying insects like mosquitoes and flies, for control of flies on cattle and as dusts for plant pests. Pyrethrum is a very powerful contact insecticide having rapid knock-down properties. Pyrethrum acts on the peripheral and central nervous system and, interestingly, it has a negative temperature coefficient, having maximum efficiency at low temperature (Corbett 1974). In general, natural pyrethrins have very low mammalian toxicity and are readily detoxified in mammalian systems. The major weakness of natural

pyrethrins is that they are unstable and rapidly decompose when exposed to light and oxygen. However, in spite of this, their use has increased in recent years mainly due to their outstanding rapid knock-down action and low mammalian toxicity (Green et al. 1977).

2.2.5 Azadirachtin

Azadirachtin is a naturally occurring insect repellent compound obtained from the "Neem" tree *(Azadirachta indica)* and Persian lilac or China berry, common in India and South America. It is generally applied by sprinkling the dried and powdered seeds and leaves of these plants over the crops. Azadirachtin acts principally as repellent of common insect pests including caterpillars and locusts. However, if consumed internally, it upsets the molting cycle due to its fairly close chemical structure to the insect molting hormones, the ecdysones, slows down the growth rates and eventually kills the insects. Azadirachtin is a cheap and promising insecticide for developing countries.

2.3 Organochlorine Insecticides

2.3.1 DDT and Related Compounds

DDT [2,2bis(p-chlorophenyl)-1,1,1-trichloroethane] is the cheapest and most effective of the synthetic insecticides. Apart from being cheap and easy to make, it is a chemically stable compound having a low vapor pressure of 1.5×10^{-7} mm of mercury at 20 °C (Woodwell et al. 1971). Due to its efficiency as an insecticide, DDT was considered a boon to the field of agriculture and medical entomology. DDT has a persistent and potent nature and it offers the possibility of prolonged control from a single application. However, due to the development of resistance in insects to DDT and extreme persistence of the insecticides in the environment it has led to widespread environmental contamination. It is because of these reasons that its use has been minimized or terminated in technologically advanced countries.

The efficiency and effectiveness of DDT has led to the synthesis of numerous analogs, related compounds such as lindane, perthane, prolan, bulan etc., which have useful properties which make them desirable for specific uses. In general, these compounds are more toxic to insects and less toxic to nontarget organisms.

2.3.2 Cyclodienes

Cyclodienes form an important group of cyclic hydrocarbons containing an endomethylene bridge. They are produced by the Diels-Alder reaction on hexachlorocyclopentadiene. The first commercial insecticide of this type, chlordane, was produced by the reaction of hexachlorocyclopentadiene with cyclopentadiene and further chlorination in carbon tetrachloride. Like DDT, it is also a very persistent insecticide and acts as a contact and stomach poison and it is toxic to a wide range of insects. Other important insecticides of this group include heptachlor, aldrin, dieldrin, endrin, mirex, chlorodecone, endosulfan etc. Aldrin, dieldrin and endrin are very powerful general insecticides and are particularly effective where contact ac-

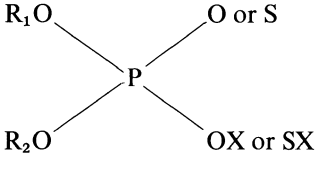
tion and long persistence are required. However, in general, all these compounds are significantly more toxic to mammals than DDT, and several restrictions have been placed on their use in Canada and United States.

2.3.3 Chlorinated Terpenes

Bicyclic terpenes on chlorination produce a mixture of compounds which have insecticidal properties. The most important of these compounds is toxaphene, which is a mixture of camphenes chlorinated to 67–69%. It has biological properties similar to DDT and lindane but is more soluble in petroleum hydrocarbons. Moreover, toxaphene has a reasonably low mammalian toxicity, does not accumulate persistently in body fat, and is eliminated in a short period after the intake is stopped.

2.4 Organophosphorus Insecticides

Organophosphorus insecticides form the largest and most diverse group of insecticides. These compounds have phosphorus as the active nucleus and are esters of alcohols with phosphoric acid or anhydrides of phosphoric acid with another acid (O'Brien 1967). The structure of most active compounds of this class can be represented by the general formula:



Early compounds like schradan, dimefox, and mipafox had the $(CH_3)_2$ N group in place of R_1O and R_2O and F in place of OX or SX. However, these compounds have a very high mammalian toxicity and are not used nowadays. In some of the compounds the alkoxy groups R_1O and R_2O are replaced by alkyl or aryl groups as in the case of mercarphon, trichloronate, leptophos, cyanolate etc. In several new organophosphorus compounds, e.g., methamidophos, acephate, phosfolan, mephosfolan, etc. The SX group is replaced by NXY. Other commercially important organophosphorus insecticides are phosphoric anhydrides (TEPP, sulfotep), phosphates (paraoxon, fospirate, dichlorvos, phosphamidon, chlorfenvinphos etc.), o-phosphorothioates (parathion, diazinon, parathion-methyl, demeton etc.), S-phosphorothioates (demephion-S, omethoate, endothion, amiton, acetofos, thiochrophos, cyanthoate etc.), and phosphorodithioates (disulfoton, malathion, phorate, azinphos-methyl, ethion, dimethoate etc.). In general, all these organophosphorus insecticides act on the nervous system by inhibiting the action of several ester-splitting enzymes particularly acetylcholinesterase at the synapse (for details see O'Brien 1967 and Corbett 1974).

2.5 Carbamates

Carbamates are closely related to the organophosphorus insecticides in their biological action and act as inhibitors of acetylcholinesterase enzyme in insects. This property of carbamates is due to their structural similarity with acetylcholine and the attraction of the C=O group to the OH site of the enzyme acetylcholinesterase. The major classes of carbamates which are generally used as insecticides are as follows:

A) N-methylcarbamates of phenols: Important insecticides of this class include carbaryl, MTMC, isoprocarb, metalkamate, promecarb.

B) N-methylcarbamates of oximes (e.g., aldicarb, methomyl, thioxamyl, thiocarboxime etc.).

C) N-methylcarbamates and N,N-dimethylcarbamates of hydroxyheterocyclic compounds (important insecticides are carbofuran, decarbofuran, bendiocarb, primicarb, dimetilan etc.).

In general, carbamate is a diverse group having a wide range of mammalian toxicity and broad spectrum. They are easily metabolized by plants and animals and do not bioaccumulate or persist in the environment.

2.6 Synthetic Pyrethroids

The effectiveness of the natural pyrethrins in controlling a wide variety of insects led to the synthesis of several new related compounds. Allethrin, tetramethrin, K-othrin, kadethrin, proparthrin and prothrin were among the early synthetic pyrethroids. All these compounds, although effective against several pest species, failed to extend their range of application over natural pyrethrum, and found only a limited use either due to high mammalian toxicity or due to their unstable nature in light and air. However, several recent compounds, e.g., permethrin, S-5439, fenvalerate, cypermethrin, decamethrin, developed by U.S. and Japanese workers, are not only active against a number of insect species, but are quite stable in light and oxygen compared to natural and earlier synthetic pyrethroids, and exert a prolonged residual action, opening new possibilities of large-scale use in crop protection. These compounds, in general, have very low toxicity to mammals and birds, but they are highly toxic to fishes and their nonspecific toxicity to insects may prove hazardous to bees and other beneficial insects.

3 Microbial Environments

Microorganisms exists in almost all natural environments including terrestrial, aquatic, atmosphere, animal, and extreme environments, e.g., hot springs, acid and salt lakes, and antarctica. These habitats are characterized by the presence of a diverse microbial flora engaged in various activities of functional significance. Terrestrial, aquatic, and atmosphere are the major microbial habitats which are generally considered in relation to microbe-insecticide interactions and hence in the following pages only these three principle habitats are discussed.

3.1 Terrestrial Habitats

Soil is a dynamic natural medium for terrestrial plant growth, composed of varying proportions of inorganic and organic components originating as a result of numerous interactions between many complex processes including weathering of rocks, decomposition of plant materials, and redistribution of materials by water movement. These physical and chemical processes modify the microenvironments as they greatly influence the nature and amount of food substrates for the microbes. The nature and type of microbial population are largely governed by the availability and type of food. In soils, food substrate is available in the form of a variety of organic materials originating from the death of plants and animals, roots and root exudates of living plants, leaching from plants, corpses, and dropping of the soil and aboveground fauna and the microbes themselves. Further, the availability of the food sources is determined by the type of vegetation.

Changes in the physicochemical characteristics as well as environmental factors may influence the soil metabolism and may have a profound effect on the microbial populations therein (Stotzky 1975). Any shift in the amount of water in a soil will affect aeration by flooding air spaces which may lead to a change in pH and in the oxidation-reduction states of several inorganic minerals.

3.1.1 Soil Microflora

A wide variety of microorganisms are associated with the soil. Bacteria are the most numerous (10^6–10^9cells cm^{-3}) on the basis of cell number. However, owing to their very small size, generally they are not the major component of soil biomass. Bacteria constitute the most important part of the soil microorganisms as they are regarded as largely responsible for the cycling and transformation of all the important elements including carbon, nitrogen, phosphorus, iron and sulfur. Fungi are another group probably as important as bacteria. The ability of several fungi to tolerate low pH makes them particularly significant in acid soils. Fungi play a major role in plant residue decomposition and plant pathogenesis in terrestrial environments. Other components of soil microflora are actinomycetes, protozoa, and algae. Algae are of special interest in certain habitats, e.g., rice fields, where they fix atmospheric nitrogen and contribute toward soil fertility.

The habitats of microalgae are varied and diverse, including aquatic environments, soil surface, other exposed surfaces, and antarctica. Some microalgae live in association with other organisms such as invertebrates, fungi (forming lichens) and plants such as several ferns, cycads, azolla and *Anthoceros*. Further, blue-green algae can colonize exposed or barren surfaces and help to maintain soil fertility through carbon and nitrogen fixation and synthesis of substances supporting plant growth and binding of soil particles (Shields and Durrell 1964). Protozoa occur in soil as flagellates, amoebae, ciliates and testacea to the order of 10^3 g^{-1} wet soil for each group. They have ecological significance as predators of soil bacteria and their ecology has been discussed by Darbyshire (1975).

3.1.2 Microflora of Aerial Plant Parts

In terrestrial environments, microorganisms are not confined to the soil alone and may occur on living or dead leaves, branches, cut grasses, hay etc. The biology and

ecology of microorganisms inhabiting the aerial plant surfaces has been reviewed by Ruinen (1961), Preece and Dickinson (1971), Dickinson and Preece (1976) and these special habitats are briefly discussed below.

3.1.2.1 The Phylloplane

Phylloplane can be defined as the external surface of plant leaves on which microbial growth occurs (Last 1955; Ruinen 1956). Almost all the major groups of microorganisms including fungi, algae, lichens, and bacteria have been recorded from the leaf surfaces (Last and Warren 1972). In general, the inoculum for the exposed plant surfaces is provided by the deposition of airborne and splash-dispersed microorganisms. Bacteria are the primary colonizers on newly emerged leaves and inflorescences and they are generally distinct from those of the soil. Major bacterial species recorded from the leaves include species of *Erwinia herbicola, Zanthomonas compestris* and *Pseudomonas fluorescens.* Several yeast species, e.g., *Sporobolomyces, Tilletiopsis, Cryptococcus* etc. replace the bacteria. Filamentous fungi are last to colonize the phylloplane. These fungi may include both saprophytic fungi and plant pathogenic fungi. *Cladosporium* spp. are the earliest and most frequently encountered colonizer (Diem 1974). Other fungi recorded from the phylloplane include species of *Alternaria, Verticillium, Acremonium, Epicoccum, Puccinia,* and *Fusarium.*

3.1.2.2 The Microflora of Cut Grass

The grasses cut for fodder, hay and silage, if left in the field for drying, are colonized by microorganisms. Due to mechanical damage to the cut plant tissues, cell sap is released which forms the substrate for the growth of several microbial species. Bacteria, especially lactic acid bacteria belonging to the genera *Streptococcus, Leuconostoc, Lactobacillus, Pediococcus,* etc., rapidly colonize and multiply in number. However, if the cut grass is left for drying for a prolonged period these microorganisms decrease in number. High relative humidity may enhance the populations of *Cladosporium, Epicoccum,* and *Aureobasidium* especially after the death of plant cells.

3.1.2.3 Silage Microflora

During partial fermentation of cut grass to preserve for later feeding, the aerobic microorganisms of cut grass are replaced by facultative anaerobic species after the utilization of available oxygen. Typical silage microflora includes species of *Streptococcus, Leuconostoc, Bacillus, Clostridium* and *Padiococcus.*

3.1.3 Sources and Substrates for Microbial Growth

Soil microorganisms show a great diversity in their nutritional behavior. Several microorganisms (especially algae and a few bacteria) are autotrophs and obtain their energy from light or oxidation of inorganic compounds and assimilation of carbon dioxide. However, a majority of the soil microorganisms are heterotrophs, requiring organic matter originating from a variety of sources. Soil microorganisms can be classified into the following groups on the basis of their nutrition, considering their source of energy, carbon and reducing equivalents:

a) Phototrophs–obtaining their energy directly from the sun by photophosphorylation.
b) Chemotrophs–capture energy from oxidation of organic and/or inorganic materials by substrate and oxidative phosphorylation.
c) Autotrophs–organisms capable of synthesizing their cell carbon from atmospheric CO_2 or inorganic carbon compounds.
d) Heterotrophs–organisms requiring fixed organic sources of carbon.
e) Lithotrophs–organisms capable of producing the reducing equivalents required for cell synthesis from inorganic materials such as hydrogen sulfite and ferrous iron.
f) Organotrophs–organisms obtaining their reducing powers from the oxidation of organic nutrients.

The major sources and substrates for microbial growth are presented below.

3.1.3.1 Inorganic Sources

Soil microorganisms are dependent on the mineral nutrients present in the soil for all the major and minor essential elements. Further, depending upon their mode of nutrition, they may require special inorganic compounds, e.g., lithotrophic populations require these as a source of reducing equivalents often in conjugation with light energy. Phytotrophs, particularly blue-green algae (which are present in the surface horizons and are capable of fixing atmospheric nitrogen) in addition to mineral nutrients require surface water and light energy for their growth and metabolisms.

3.1.3.2 Organic Material

a) Crop Residue and Leaf Litter. The dry matter produced by the plants reaches the soil through direct litter fall and death of the plant or it is removed by the herbivores including mammals, molluscs and insects, and some of it is eventually returned to the soil on the death of the herbivore. Further, the supply of organic matter to soils is largely governed by the litter fall patterns. In equatorial regions and warm temperature forests, litter fall is continuous although seasonal changes in quantity can occur, whereas in cool temperature forests the litter fall is correlated with cooling and decreasing light intensities during the autumn (Bray and Gorham 1964). Further, leaf and branch material arrive on soil surface at different times of the year (Bray and Gorham 1964). The rate of decomposition of litter varies not only with the type of vegetation but with the type of litter. Thus, even in similar environmental conditions coniferous leaves and woods decompose more slowly than those of deciduous trees. Also, the herbaceous material decomposes faster than the woody material (Ovington 1962; Bocock et al. 1960). Further, soil type as well as type of the leaf (e.g., sun and shade leaves of same tree) influences the rate of decomposition (Bocock 1964; Heath and Arnold 1966).

Water-soluble organic materials and inorganic ions like calcium, magnesium, potassium, sodium and phosphates are leached from the litter during the early stages of decomposition (Latter and Cragg 1967; Burges 1958; Gilbert and Bocock 1960). However, the loss of material from the litter varies with the species. Thus, whereas hot water-soluble material from *Quercus petraea* decreases slowly, in *Frazi-*

nus exulsion more than 90% material disappeared after only 1 month (Gilbert and Bocock 1960).

Hudson (1968) observed that during fungal succession on crop residue, species capable of utilizing simple soluble materials including monosaccharides and disaccharides (e.g., *Mucor* spp. and *Rhizopus* spp.) are among the primary colonizers. These species have relatively rapid growth as compared to the secondary colonizers, and Lynch and Harper (1974) observed that this is characterized by a high specific rate of sugar utilization. Secondary colonizers are capable of assimilating more complex materials, e.g., polysaccharides, whereas the tertiary colonizers use complex polymers such as lignin and keratin. However, as these complex polymers are not readily assimilated as energy sources, the growth of tertiary colonizers is very slow (Garrett 1951).

b) Root Exudates. Root surface and adjoining soils show intense microbial activities and the density as well as diversity of microorganisms is generally higher in this zone than in the adjacent soil (Starkey 1958; Rovira 1965, 1972). This selective stimulation of microorganisms is due to release of several nutrients through diffusion from the plant roots which are generally referred to as root exudates. These nutrients include carbohydrates, amino acids, vitamins, organic acids and several other organic compounds.

3.1.4 Physicochemical Conditions and Microorganisms

Soils are the most complex of microbial habitats. Several physicochemical characteristics show temporal and spatial variations which in turn affect the microbial growth in terrestrial habitats. The major physicochemical conditions which govern the successful growth and survival of microorganisms in terrestrial habitats are discussed briefly.

3.1.4.1 Soil Texture

Soil texture plays a significant role in determining microbial activity. Soil texture depends on the size and distribution of particles (sand, silt, clay) and it forms an important classification feature of soils. The principal clay minerals include kaolinite, montmorillonite, and illite. Clay particles are colloidal in nature, and they can interact with microorganisms as they are coated with metal hydroxides and sesquioxides. The surface of these particles is polarized. Microorganisms also carry polarized charge due to the presence of charged macromolecules lying on their surfaces, and this polarization of charges causes the microorganisms to be absorbed on clay particles. Moreover, water is also present around clay particles as it interacts with clay. Clay particles have exchangeable ions on or near their surface which are replaced by the H^+ ion of water leaving the clay surface and entering the water phase. In contrast to clay, sand and silt fractions of soils are composed of relatively large particles, their surfaces are generally inert and they carry very little moisture or organic matter and thus restrict the microbial activity on them.

3.1.4.2 Water

Microorganisms are generally considered to be aquatic, requiring high moisture content. However, the availability of water is often restricted in terrestrial habitats. Further, evaporation of water from the soil surface not only reduces the availability of water to microorganisms but also changes the diffusion pathways of gases contained in the voids between soil particles. As soil dries out, the resulting desiccation kills many microorganisms and several others modify themselves and survive as resting spores, sclerotia, resting cells or hyphae and rhizomorphs etc. (Warcup 1957). Rose (1966) and Griffin (1972) have discussed the importance of physical factors governing the soil water contents and the effect of water potential on microbial populations in soils. The availability of water to microorganisms depends upon the water potential of the soils, which in turn depends upon the osmotic potentials and matric potential and is usually expressed in bar.

The water potential of any soil determines to a large extent the survival, germination, and growth of various microorganisms. However, this effect of water potential varies from species to species (Griffin 1972). In general, the growth of bacteria is limited between water potentials of 0 and −100 bar, however some bacteria can grow at potentials up to −200 bar. Further, nitrification and sulfur oxidation is maximum at water potentials between −0.1 and −0.2 bar and −0.03 and −0.06 bar, whereas in drier conditions fungi like *Aspergillus* and *Penicillium* predominate where water potential is less than −145 bar (Griffin 1972). *Azobacter* sp., an aerobic microorganism capable of fixing molecular nitrogen, has lower intercellular osmotic pressure than fungi and actinomycetes and its activity is markedly reduced at matric potential of less than −5 bar (Dommergues 1962). Further, Kouyeas (1964) observed that *Pythium* spp. and *Mortierella* spp. also require very high water contents, whereas *Fusarium* spp., *Trichoderma* sp. and *Gliocladium* sp. were indifferent to the moisture contents of the soils.

Soil water content not only affects the growth and survival of microorganisms, but also exerts a profound effect on the movement of microorganisms. With a fall in the matric potential of soil, the movement of bacteria is restricted more than that of fungal mycelia which can anchor themselves to the older mycelia, which makes them more successful in dry condition than bacteria. Soil moisture affects the movement and redistribution of pesticides also in the soils which is generally directly related to the water content of soils (Griffin 1972). The movement of pesticides also depends upon their volatility and the water content of a soil may change the diffusion coefficients of pesticides in the vapor phase (Ehlers et al. 1969).

3.1.4.3 Soil Atmosphere

In normal, well-drained soils, the voids are filled with air. Microorganisms show considerable diversity in their response to the changes in the concentration of oxygen in their microenvironment. Several bacteria and fungi are strictly aerobic. However, the quantitative relationship between growth and oxygen concentration may vary between different genera or even species of the same genus. Some bacteria are strictly anaerobic and not capable of growth in aerobic environments. The amount of air in soil is largely determined by the particle size distribution. Further, the soil particles are generally aggregated together to form clods or crumbs and their spatial

arrangement gives the soil its structure. Thus, the aeration of soil may be considered in two different phases, the outer surface and spaces between adjoining crumbs and air between the crumbs. Greenwood (1961) and Greenwood and Berry (1962) observed that in water-saturated soil crumbs of 3 mm or more in radius, the oxygen present in the center of the crumbs is utilized by the respiratory activities of the microbial population present and they soon become anaerobic even though they may be surrounded by air. However, Allison (1968) opined that microbes in the center of a crumb utilize all the substrate available in the early stages of their colonization and thus most of the microbial activity in the later stages is confined to the outer aerobic surfaces. However, it is clear that anaerobic microenvironments do occur in otherwise well-aerated soils, allowing the growth of anaerobic microorganisms. Moreover, the concentration of oxygen affects the oxidation-reduction potential of the soil and may play a significant role in determining the microbial distribution patterns (Alexander 1964).

As a result of microbial activities the concentration of carbon dioxide changes in the soil atmosphere, which may have a profound effect on the microorganisms. Alexander (1964) opined that the concentration of carbon dioxide may change the pH in microhabitats, can act as a carbon source for the autotrophic organisms or may have an inhibitory effect on heterotrophic microorganisms. The concentration of oxygen and carbon dioxide in soils plays a significant role in determining the activity, density, and diversity of microorganisms in various soils (Gray and Williams 1971; Burges 1958; Stotzky et al. 1962; Stotzky and Goos 1965).

3.1.4.4 Acidity and Alkalinity

The density and diversity of soil microflora is highly dependent on the soil pH. Calcium content is one of the major determinants of soil pH as Ca^{2+} ions occupy the exchange sites on the soil minerals. Metabolic activities involving redox reaction, e.g., reduction of nitrates to elemental nitrogen, sulfates to sulfides etc. causes the soils to become more alkaline. However, carbon dioxide produced during respiration forms carbonic acid, making the soil more acidic. In general, high acidity decreases the growth of bacteria, and at pH below 5 bacterial activity is restricted mainly to acidophilic forms. One of the most remarkable acidophilic bacteria belongs to the genus *Thiobacillus,* which is capable of growing at pH as low as 0.6. However, in general, fungi are more tolerant to acid conditions than bacteria and actinomycetes, and several fungi can grow at pH 3. Fungal flora of soils having low pH includes species such as *Mucor ramannianus, Mortierella parvispora,* and *Trichoderma viride* (Williams and Parkinson 1964). The pH in soils is intimately involved with the availability of many nutrients. At low pH several substances such as phosphorus and calcium become more soluble and may be leached away. Similarly, nitrification is inhibited, and it was found that *Azobacter* is absent in soils with pH values below 5.6–5.8 (Rangaswami and Sadasivam 1964). Further, the concentration of several metal ions (Al, Mn, Ni, Fe) in soils may become toxic due to their increased solubility at low pH.

Variations of pH in the microenvironment may occur due to a variety of reasons and the pH may not be the same as that of the gross pH of the macroenvironment. William and Mayfield (1971) observed that the release of ammonia from the de-

composition of amino acids, urea or chitin added to an acid sandy soil accumulate around organic matter present in the soil, raising the pH from 4.0 to 7.0, sufficient to allow the growth of acid-sensitive streptomycetes. Similarly McLaren (1960) opined that negatively charged colloidal clay particles attracted H^+ ions, thus making the pH low around such particles. The enzymes released by microorganisms may also become adsorbed on the soil particles, decreasing the pH in their microenvironment in relation to the ambient solution (McLaren and Skujin 1963). Griffin (1972) observed that many fungal hyphae are coated with clay particles, and this can influence the pH at the surface as well as their growth patterns, due to their capacity to absorb not only hydrogen ions and other cations but several other organic molecules including humic compounds, enzymes, microbes, several other metabolic by-products and pesticides.

3.1.4.5 Temperature

The composition of the soil microflora and its activity is significantly affected by temperature. The temperature of any soil habitat is largely governed by its geographical location, direction and degree of surface slopes, exposure, soil color and density of vegetation cover. In general, the soil temperature at the surface is directly related to the ambient temperature (Travleev 1960), however, a steep temperature gradient occurs within the first few centimeters of the soil surface. Further, the temperature not only varies seasonally but large diurnal fluctuations also occur which have a profound effect on the surface microorganisms. These diurnal variations are more marked at the surface, whereas deeper layers are comparatively buffered. Wilkins and Harris (1947) reported that the average monthly temperature varied from 2° to 19 °C on the surface of a forest soil, whereas the range of variations decreased to 4–14 °C only at a depth of 7.5 cm. The moisture content of the soil also influences its temperature regime, and a close association between the water content and the temperature was demonstrated by Raney (1965). Thus the temperature variations in moist soils are considerably less than in dry soils.

Microorganisms can be classified in the following categories depending upon their survival potentials and optimum growth temperature:

a) Psychrophiles–microorganisms capable of growth at extremely low temperature (below 5 °C). However, they may have higher optimum growth temperatures (see Eddy 1960).

b) Mesophiles–microorganisms capable of growth within normal soil temperature. They cannot survive in low (5 °C) or high (40 °C) temperature, usually have optimum growth temperature between 25° and 37 °C.

c) Thermophiles–microorganisms having a high optimum growth temperature (55 °–65 °C). They can grow at temperatures between 45° and 75 °C. However, due to the rigidity of their macromolecular cell components, there is very little growth below 40 °C (Brock 1969).

The soil temperature not only affects the general metabolic activity of the microorganisms present but it may be responsible for the shifts in the microbial composition of the soils as it exerts a profound effect on the nature and type of microorganisms present in any soil habitat (Okafor 1966).

3.1.4.6 Agricultural Practices

Common agricultural practices such as plowing, rotovating and application of pesticides not only decrease the population of soil organisms but also bring about a redistribution of undecomposed and partially decomposed organic matter in agricultural soils. These practices may also change the aeration of soil and its water-holding capacity and consequently may alter the soil microbial populations (Russell 1968; Powlson 1975). Moreover, application of insecticides may have a diverse effect on different microorganisms. It may increase or decrease the microbial populations due to its selective inhibitory or stimulating effect on various metabolic processes. Removal of vegetation is another factor which changes the microbial environment by decreasing the amount of organic matter in the soil and also making it more acidic.

Burford (1976) studied the effects of application of cow slurry on the composition of soil atmosphere and demonstrated that it not only provided a substrate for microbial growth, but also decreased the concentration of oxygen and increased the concentration of carbon dioxide, methane, and nitrous oxides at a depth of 10 cm. In general, application of manure slurries may drastically change the microbical populations as it may lead to blockage of soil pores with organic matter carried into the soil in water, accumulation of toxic metals and the creation of anaerobic conditions (Skinner 1975; Burford 1976).

3.2 Aquatic Environments

Aquatic environments can be defined as those habitats where water is the principal external as well as internal medium (E. P. Odum 1971). Aquatic microbiology refers to the study of those microorganisms which live in the earth's natural water bodies ranging from small and transient rain water pools to great oceans. Oceans contain most of the earth's water (97.1–97.6%), whereas smaller quantities are present in polar ice and glaciers (2.1%) and groundwater (0.3–0.8%). Still much smaller quantities are present in inland freshwaters including lakes (0.009%) and rivers (0.00009%) (Vallentyne 1974). However, in spite of such a vast range of habitats and general conditions prevailing in them, in practically all of them microorganisms can be encountered. Considerable work has been done in the area of aquatic microbiology, and it is impossible to do justice in the space available, only a very brief account of the principal aquatic habitats, namely marine, estuarine and freshwater is presented. For detailed discussions and ecological information the readers are referred to Hutchinson (1957, 1967), Hynes (1970), Golterman (1975), E. P. Odum (1971), Reid and Wood (1976), Willoughby (1976) and Wood (1965).

3.2.1 Physicochemical Characteristics of Aquatic Habitats

The characteristics of an aquatic system are largely governed by its geographical location, geology of the area, morphometry and land management practices in the catchment area of the water body. Further, the light and temperature regimens, which are usually closely related in freshwater habitats (Hutchinson 1957; Talling 1962), are dependent mostly upon their geographical location (Hutchinson 1957).

Thus, tropical and subtropical lakes are quite distinct in their thermal behavior from temperate lakes (Golterman 1975; Hutchinson 1957). The formation of stratification in any water body depends upon its surface area, depth of the lake, and the degree of exposure to wind. Thus, in small shallow lakes, when they are exposed to wind action, no persistent stratification has been found to occur (Craven and Brown 1970; Davis 1972; Hutchinson 1957; Macan and Maudsley 1966); but in the absence of such a wind-induced mixing, protected nature, and local topography, shallow waters have been found to show thermal stratification (Moss 1969). Physicochemical characteristics determine the nature, amount and diversity of biotic components in an aquatic system. The temperature of a water body affects the distribution of various species (Soeder and Stengel 1974); it regulates their growth rates and several physiological and biochemical processes as well. Blue-green algae have been reported to be very successful in tropical conditions usually at high (35 °–40 °C) temperature optima (Fogg 1965; Singh 1955). The transparency of a lake determines the overall production potential of any aquatic system.

The existence of different biotic composition in freshwaters with varying chemical characteristics has been shown by Hutchinson (1967). Thus, waters of hard rock regions have many species of desmids in contrast to waters of soft rock areas where phytoplankton communities dominated by diatoms and blue-green algae have been reported (Moss and Moss 1969; Moss 1972, 1973; Talling and Talling 1965). Several workers have shown a correlation between physicochemical and biological components. A number of inorganic and organic substances are also required by phytoplankton to grow in natural waters (Gerloff 1963; Healey 1973; Hunter and Provasoli 1964; Lewin 1962; O'Kelly 1968; Nicholas 1963).

The pH of a water body can have a profound effect in a mixed community (Golterman 1975). Golterman (1969, 1975) further opined that pH is related to the CO_2–HCO_3–CO_3 system and that in aquatic systems its relation with diverse reactions is multifaceted. The pH dependence of dissociation rates and the ionic state of polar and nonpolar inorganic and organic compounds affects the availability of CO_2 and iron (Stengel 1970).

In general, freshwaters are dilute solutions of alkali and alkaline-earth bicarbonates and carbonates, sulfate and chloride (Hutchinson 1957). A number of minor constituents and a variety of coloidal materials, both organic and inorganic, are also present in freshwater. On the basis of the relative concentrations, Golterman (1975) classified the elements and compounds normally encountered in natural waters in dissolved state under several categories, such as major, minor, trace elements, gases, and organic compounds. Thus, Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, HCO_3^{-}, SO_4^{2-}, Cl^{-} have been categorized as major ions, N, P, Si as minor ions and Fe, Cu, Co, Mo, Mn, Zn, B, V, etc. as trace elements. Golterman further suggested that whereas the concentration of major elements and compounds in lake water determines the nature and types of organism present in that system, the minor elements or compounds control the relative and absolute number of these organisms. Based on the relative proportions of various major and minor ions, the freshwaters of the world have been classified into different categories, such as "hard" or "soft", "bicarbonates of chloride-sulfate", "oligotrophic" or "eutrophic" (Hutchinson 1957; Golterman 1975). Additionally, the productivity and trophic status of a water body is primarily governed by the amount of these vital elements available to the biotic community. Phosphorus and

nitrogen as raw material for protein synthesis are often considered limiting to organic production (Edmondson 1972; Golterman 1975; Vollenweider 1961, 1965, 1969).

Dissolved oxygen, water transparency, conductivity, summer alkalinity differences between surface and bottom waters, C.O.D., algal pigment, primary productivity, P, N, Fe, and Mn have been reported to be related to the trophic status in lakes (Lueschow et al. 1970; Sakamoto 1966a; Shannon and Brezonik 1972; Vollenweider 1968). McColl (1972) observed little or no relationship of total dissolved solids, pH, Ca, Mg, Na, K, SO_4, Cl, Cu, Zn and Si and trophic status in seven New Zealand lakes. However, Zafar (1959), based on the observations of Storm (1930), developed a system of trophic classification and suggested that Pearsall's basic ratio (Na + K/Ca + Mg) can be used to separate lakes into three (oligotrophic, mesotrophic and eutrophic) basic trophic groups. It is for these reasons that the quality and quantity of the dissolved salts have been of fundamental interest to ecologists and limnologists. Moreover, differences in physicochemical characteristics, either vertical or horizontal, may depend on the morphometry, the pattern of detritus and silt sedimentation, and decomposition and mineralization of organic matter. The significance of diurnal variations of temperature and their influence on the formation of stratification, vertical distribution of oxygen, CO_2 and other physicochemical characteristics was noticed earlier (Ganf and Viner 1973; O'Connell and Andrews 1976; Reynolds 1976). Similarly, the responses of plankton to light conditions could be a factor in regulating the diurnal vertical migration of these organisms (Berman and Rodhe 1971; Talling 1971).

Hynes (1960) opined that "the rivers are strict individualists, each of which varies in its own way" and therefore, no two rivers have exactly the same water quality. These variations are due to the differences in geographical regions, geological formations and differences in the degree and kind of human disturbances along the river course. Introduction of industrial wastes and sewage in the rivers and streams acts in a variety of ways. These changes mainly include increase in dissolved nutrients, increase or decrease in oxygen concentration, increase in temperature, change in the nature of river bottom, increase in the activity of heterotrophic organisms and many others (Klein 1962; Hynes 1960; Hawkes 1976; Mason 1981). Zonations in river waters are due to addition of effluents and have been characterized and classified on the basis of both physicochemical and biological characters of waters (Fjerdingstad 1964, 1971).

Golterman (1975) and Viner (1976) suggested that the sediments control the chemical composition as well as productivity of the shallow water bodies. The role of sediments in regulating the nutrient cycling in water bodies is also significant, as several chemical and biological processes, due to microbial activity, produce raw material from complex organic matter and mineral constituents of clay fraction of the sediments (Golterman 1975; Hutchinson 1957).

3.2.2 The Marine Environment

The sea covers approximately 70% of the earth's surface. Various oceans are interconnected and are in a state of continuous circulation. The depth of the sea is variable and ranges from approximately 200 m at the continental shelves to more than 2000 m in the open sea, and can reach up to 10,000 m or more at the Pacific

Trenches. Sea waters are constantly in movement in well-defined patterns. Surface water movements are dominated by great ocean currents which are generated by wind patterns in the lower atmosphere and the transfer of heat energy from tropical and subtropical regions toward the poles. Other factors influencing the movement include the Coriolis effects, salinity gradients and lunar periodicity. Further, due to their great depth, and penetration of light only in the upper euphotic zone, much of the sea remains permanently in near total darkness and maintains temperature only a few degrees above freezing. One other notable feature of the sea, important for the successful survival of marine biota, is its salt content. The salinity of sea water varies from 32 to 38% and averages approximately 35%. Sodium chloride contributes approximately 27% toward salinity, whereas the rest is due to calcium, magnesium, and potassium salts (Reid and Wood 1976). However, the concentration of dissolved nutrients, particularly nitrates and phosphates, is very low except in coastal waters and constitutes a major limiting factor for production. The comparatively high productivity in the near-shore regions is partly due to the process of upwelling caused by the movement of surface waters away from the coastal slopes due to wind action, thereby bringing the bottom cold water, rich in nutrients, to the surface.

The marine environment can be divided into a number of zones, both horizontally and vertically, based largely on physical factors. The zone between high and low tide is termed intertidal or littoral zone, followed by the neritic zone which is the shallow water zone on the continental shelf before the bottom drops off steeply as the continental slope (E. P. Odum 1971). The open region beyond the continental shelf is termed the Oceanic region. Vertically, oceans can be divided as the upper euphotic and lower aphotic zone. The lower boundary of the euphotic zone is determined by the effective light penetration where photosynthesis balances respirations. The euphotic zone forms a very thin layer of not more than 200 m over a very thick aphotic zone.

The marine microorganisms (phytoplankton, protozoa, fungi, and bacteria) make up a large proportion of the total biomass and play a primary role in influencing the physicochemical, biological, and geological processes in the oceans and ocean floors. Several processes in the marine ecosystem involve a close interaction between plankton and bacteria. Parsons and Takahashi (1973) and Rheinheimer (1975) observed that bacteria play a significant role in the regeneration of inorganic nutrients from the organic matter, which is vital for sustaining the growth of autotrophic phytoplankton. Similarly, bacteria utilize photo-assimilated organic compounds released by phytoplankton (Williams and Yentsch 1976; Iturriaga and Hoppe 1977), and also act as a food source for several planktic filter-feeders (Jorgensen 1966). The density and diversity of microorganisms in the intertidal zone primarily depend upon the nature of the substratum, organic matter content and nutrient inputs (Dale 1974). Phytoplankton are the major primary producers in marine ecosystems. Diatoms and dinoflagellates are the most obvious and numerous elements of the phytoplankton, though in some situations photosynthetic microflagellates and blue-green algae may be equally important. Nannoplankton are the main contributors to the primary productivity in the oceanic euphotic zone. Further, Yentsch and Ryther (1959) opined that nannoplankton appear to play a significant role in the coastal waters also.

The density of marine bacteria may vary between less than one l^{-1} in the open oceanic zone to more than 10^8 ml^{-1} in the inshore waters and between 10^1 and 10^8 g^{-1} in marine sediments (Zoßell 1963). The great difference in the density of bacteria in the inshore and oceanic zone seems to be due to the fact that most marine bacteria are heterotrophic and their numbers are more where organic nutrients are abundant. Further, marine bacteria tend to differ characteristically from terrestrial and freshwater bacteria. Marine bacteria are strictly halophilic and generally have a specific requirement for sodium ion which appears to be necessary for the transport of substances in the cell (McLeod 1965). Some of the most common genera are *Micrococcus, Bacillus, Pseudomonas, Sarcina, Spirillum, Nocardia, Corynebacteria* and *Streptomyces* (Wood 1965).

Until recently, the active role of fungi in marine environments was seriously doubted by most scientists. However, investigations during the last few decades have established that they may play an active part in some habitats (for details see E.B.G. Jones 1976 and Hughes 1975). Most of the marine fungi, like the terrestrial forms, are heterotrophic and occur predominantly in those parts where organic matter is present in sufficient quantity (E.P. Odum 1971). However, some forms have been isolated from sand grains and from waters with low organic matter. Further, several fungi have been reported living parasitically in marine algae and animals. Thus, species of *Eurychasmidium, Ectrogella, Lagenidium, Lulworthia, Sphaceloma* etc. have been reported living on red and brown algae and diatoms (Johnson and Sparrow 1961; Drebes 1966; Kohlmeyer 1972, 1974). Several marine animals including fish, molluscs and crustacians are often infected by fungi and fungus-like organisms (Bruce and Morris 1973; Alderman 1976). However, very little is known about the interactions and role of fungi in marine enviroments.

3.2.3 The Estuarine Environment

An estuary is a semi-enclosed coastal body of water which has a free connection with the sea where sea water is diluted in a measurable degree by the influx of freshwater (E.P. Odum 1971; Cameron and Pritchard 1963; Pritchard 1967). Examples of an estuary include river valleys and river delta, tidal marshes, coastal bays and shallow basins enclosed by barrier islands having connection with the sea through small channels or inlets. Although an estuary can be considered as a transitional zone between freshwaters and sea, it has several unique characteristics not shared by either of the other two habitats. Salinity and its distributional pattern within an estuary are governed by local conditions and range from a gradual salinity gradient to distinct stratification with freshwater floating over the more saline, high density sea water. Additionally, due to the large movement of water, and the constant stirring of bottom sediments and nutrients carried by the rivers, estuarine environments are very productive. However, despite the fact that nutrients are generally not limiting, the species diversity is low and only a specialized biota is present due to varying physical stress created by wide ranges and fluctuations of salinity, temperature, and other environmental factors within a short period.

Estuaries can be classified in a number of ways, depending upon the characteristic selected for classification. More common classifications are based on the following characteristics:

a) Geomorphology: Examples of classification based on morphological characteristics include fjord type, bar-built, river delta, drowned river valley and estuaries produced by tectonic processes (see Pritchard 1967 for details).

b) Stratification and water circulation patterns: Estuaries can be classified into various categories depending upon the degree of mixing of fresh and saline waters. They can range from highly stratified salt wedges to partially mixed or completely homogenous estuaries depending upon the river water currents, tidal action, surface winds and other morphometric factors.

c) Ecosystem energetics: H.T. Odum et al. (1969) classified estuarine ecosystems and other coastal waters on the basis of system energetics. They recognized several categories such as physically stressed systems, natural arctic, temperate coastal and tropical coastal ecosystems.

3.2.3.1 The Microbial Flora

Estuaries of brackish waters form an environment of a transient nature colonized from both adjacent habitats, the freshwater and marine, through the infiltration of euryhaline species (Lenz 1977). Though the diversity of microorganisms is comparatively low, estuaries tend in general to be more productive than either freshwaters or the sea (E.P. Odum 1971). The high productivity is due to the fact that estuaries act as a nutrient trap and sustain a year-round photosynthetic activity due to the presence of macrophytes (including sea weeds, sea grass and marsh plants), benthic microphytes and phytoplankton.

In general, the microbial community in estuarine environments is composed of a mixture of endemic species, halophilic marine organisms and, to a lesser extent, freshwater species having osmoregulatory capabilities. However, the number of genuine brackish water species is very small although the overall density may be very high (Remane and Schlieper 1971). Autecological studies on several saprophytic brackish water bacteria have demonstrated that, in general, they have salinity optima between 10 and 20% and they are unable to grow in either fresh or sea waters (Ahrens and Rheinheimer 1967; Rheinheimer 1975). Thus, a large proportion of allochthonous microbes carried into the estuarine environments via waste water in the coastal waters dies within a short time. However, a few osmophilic bacteria which develop optimally at osmotic potentials equal to brackish or sea water may survive for a longer duration, though in contrast to halophilic bacteria, they do not require sodium or chloride ions for their development.

The estuarine environments generally have favorable conditions for the growth of sulfur-reducing and oxidizing bacteria. The sulfate-reducing bacteria are strictly anaerobic and they are abundant in bottom waters and sediments. Bansemir and Rheinheimer (1970), during their investigations on a fjord in the Baltic Sea, observed that the sulfate-reducing microorganisms (*Desulfovibrio* sp.) were considerably higher in sediments than in the water. The oxidation of reduced sulfur compounds in marine and estuarine environments is primarily carried out by *Thiobacillus, Beggiatoa,* and *Thiothrix* species. Further, several different autotrophic nitrifying bacteria (e.g., *Nitrosococcus mobilis, Nitrospina gracilis, Nitrosocystis oceanus,* etc.) have been recorded from estuarine and marine habitats (Watson 1965; Watson and Waterbury 1971; Koops et al. 1976).

Phytoplankton in estuarine habitats are also dominated by dinoflagellates and diatoms, as in marine environments. However, the diversity of euryhaline species decreases with decrease in salinity, and those species which are able to grow show a reduction in their size (Remane and Schlieper 1971). Important phytoplankton include species of *Cerataulina, Chaetoceros, Coscinodiscus, Nitzschia, Skeletonema, Rhizosolenia, Thalassiosira Ceratium, Dinophysis* and *Peridinium.* Lenz (1977) recorded the presence of certain freshwater green algae, whereas Stewart (1965) has demonstrated the presence of nitrogen-fixing blue-green algae. Jones (1974) opined that the blue-green algae are the most important nitrogen-fixers, although bacterial nitrogen fixation may take place to a certain extent.

The presence of fungi and yeasts has been frequently recorded from the estuarine habitats (Johnson and Sparrow 1961; E.B.G. Jones 1976; Norkrans 1966; Van Uden 1967). Colwell (1972) opined that yeasts are widely distributed in estuarine environments.

3.2.4 Freshwater Environments

Freshwater habitats contain only approximately 0.009% of the total earth's water (Vallentyne 1974) and cover relatively small areas as compared to marine and terrestrial habitats. However, they are of paramount importance to man as a source of potable water, industrial and domestic use, and waste disposal. Freshwater habitats can be divided into two main types: the standing waters or lentic habitats including lakes, ponds, swamps etc. and running waters or lotic habitats, e.g., springs, rivers, streams etc.

3.2.4.1 The Microbial Flora

Microorganisms exist in a variety of conditions in aquatic habitats. Bacteria, algae and fungi make up a large proportion of the total biomass in freshwaters (Brock 1966). In aquatic habitats, bacteria are both autochthonous and allochthonous (derived from the terrestrial soils, plants, and animals), although most of the invading terrestrial bacteria die off quickly. In lotic habitats, the input of allochthonous materials is very important, due to their close interaction with surrounding terrestrial habitats (Rheinheimer 1974). Kaushik and Hynes (1968) observed that leaves from terrestrial vegetation provide an important source of energy for several bacteria and fungi. Similarly, bacteria and fungi are primary colonizers on rock surfaces, leaves of submerged plants, sediments, and also in the mucous sheaths of colonial algae followed by diatoms and protozoa.

In general, most of the aquatic bacteria are heterotrophic; however, phototrophic and chemolithotrophic bacteria are also widely distributed. The density and diversity of heterotrophic bacteria is primarily governed by the amount of organic material present. Thus, in nutrient-poor springs and groundwaters, the numbers of bacteria are quite low, dominated by Gram-negative rods and prosthecate bacteria. Important species present in these habitats include species of *Hyphomicrobium, Caulobactor, Gallionella, Pseudomonas* etc. Input of organic materials in rivers has a profound effect on the microbial flora, increasing the number of bacteria and yeasts severalfold (Cooke 1961; Woollett and Hendrick 1970) and creating conditions favorable for the growth of communities of sewage fungus (Curtis 1969). In lentic

habitats, Gram-negative rods form the major component of the heterotrophic bacteria (Collins 1960). The frequently encountered genera include *Achromobacter, Flavobacterium, Micrococcus, Sarcina, Bacillus, Pseudomonas, Nocardia, Streptomyces* etc. (Rheinheimer 1974). I.G. Jones (1971, 1972, 1973) is of the opinion that a number of physicochemical and biological characteristics determine the type and number of bacteria in freshwater habitats. Hypolimnetic deoxigenation, concentration of nutrients, and underwater light climate, all seem to be important in controlling the microbial flora (Talling 1971; Goulder 1974). Phototrophic bacteria are generally encountered in the oxygen-depleted hypolimnion of eutrophic waters in the presence of sufficient light and hydrogen sulfide (J.G. Jones 1972). In stratified lakes, sulfate-reducing bacteria are found most abundantly in the bottom deposits (Kusnezow 1959). Purple or green sulfur bacteria, due to their autotrophic nature, prefer to grow in upper water layers, provided electron donors are available and usually restricted to habitats where hydrogen sulfide is available (Genovese et al. 1962; Overbeck 1974).

In aquatic habitats, microalgae play an extremely important role in the microbial community as primary producers of organic compounds. In general, microalgae are autotrophic; however, organic substances such as sugars and organic acids can be utilized by several algae to maintain growth in the absence of light or as a sole or supplementary source of carbon in the presence of light (Fogg 1966). Physiological and biochemical studies have demonstrated that apparently obligate phototrophs (e.g., *Chlorella vulgaris, Chlamydomonas eugametos)* may function as heterotrophs or photoheterotrophs in nature (Willoughby 1976). Further, Smith et al. (1967) opined that blue-green algae can also utilize organic sources of carbon although the light-dependent biochemical pathways may be different. The most important phytoplankton in freshwaters belong to blue-green algae (Cyanophyta), green algae (chlorophyta) particularly chlorococcales and yellow-brown algae (Crysophyta) including the diatoms. As discussed in the earlier section, the distribution of microalgae is largely determined by the environmental factors and the occurrence of a species in any particular habitat depends upon its suitability to the sum total of the ranges for various factors in the habitat (Golterman 1975; Hutchinson 1967). In general, light, temperature, and nutrient supply are the most important factors. In fast-flowing lotic habitats, epilithic algae growing on stones or benthic algae (living in the bottom sediments) are more frequent, whereas planktonic algae are more successful in lentic habitats. Further, if conditions are favorable, the growth of planktonic algae in lentic habitats is very rapid and several blue-green and green algae (e.g., *Microcystis, Anabaena, Aphanizomenon, Scenedesmus, Chlamydomonas* etc.) may form very conspicuous accumulation of cells (popularly termed algal blooms) in the surface waters.

Fungi, due to their heterotrophic nature, play an important role as decomposers in aquatic habitats. The distribution and number of fungi depend upon the availability of substrate in aquatic habitats. In general, lower fungi and yeasts are common near sewage discharge points (Woollett and Hendrick 1970). Phycomycetes, particularly belonging to orders Chytridiales and Saprolegniales, are common in lentic habitats.

3.3 The Atmosphere

The atmosphere is one of the major media for the dispersal of microorganisms within and between various components of the total environment. It is a gaseous mixture of nitrogen, oxygen, carbon dioxide, traces of other inert gases, and water vapor. The aerial habitat, in general, is not particularly suited to have a permanent aerial microbial flora of its own due to low availability of nutrients and water. However, Gregory (1973) opined that the aerial habitat is not beyond the range of microbial exploitation. Further, a wide range of microorganisms including microalgae, bacteria, protozoa and spores of fungi may be encountered in the air (Gregory 1973). The degree of adaptation to airborne dispersal varies greatly between different groups of microorganisms. Thus, whereas bacteria, viruses and algae are poorly adapted, fungi have developed several mechanisms to enable their spores to become readily airborne and remain in the air for longer periods. The different methods of liberation and take-off have been reviewed by Ingold (1965, 1966, 1971), and Gregory (1973).

The atmosphere is probably the most important medium for dispersion of pesticides also. Several processes, e.g., drift and evaporation during aerial application, volatilization from soils, wind erosion of contaminated soils etc., constantly add insecticides and other pesticides to the atmosphere. A great opportunity exists for microorganisms in the air to encounter and react with these pesticides, thus making the atmosphere a potential medium for pesticide-microbe interaction.

3.3.1 Characteristics of the Atmosphere

The atmosphere surrounding the earth is made up of a series of concentric shells separated by narrow transitional zones. The first shell or layer, the troposphere has a maximum altitude of 17 km at the equator and 6–8 km at the poles. It comprises 80% of the total mass of the atmosphere. The gaseous composition is 78% nitrogen, 21% oxygen, and 0.03% carbon dioxide, with traces of other inert and pollutant gases and water vapors; other layers in ascending order are the stratosphere, mesosphere, thermosphere, and exosphere. However, there is little exchange of air or particles from the trophosphere except as a result of volcanic activity or nuclear explosions. Dust is present in the outer layers of the atmosphere but this is largely extraterrestrial and meteoric in origin (Gregory 1973). Also only the troposphere contains particles of terrestrial origin, including microorganisms, dust and organic matter, and is the only layer which is biologically significant.

The troposphere can be further divided into several zones and the successful liberation, dispersal, and deposition of airborne microorganism depends, to a great degree, on the changes occurring between various zones. The zones or layers of the troposphere in an ascending order have been discussed by Gregory (1973).

3.3.2 Origin and Dispersal of Microorganisms

Diverse microorganisms including bacteria, viruses, protozoa, myxomycetes spores, microalgae, and spores of fungi can exist in any air sample. However, in general, the density, diversity and survival of these organisms encountered at any time depends upon several physical and environmental factors such as location, weather con-

ditions, time of the day, relative humidity, wind speed, temperature, and desiccation. Spores of fungi predominate in the air due to their several adaptation mechanisms for the liberation of spores.

The important sources of bacteria in the air are soil, water bodies, plants, and animals. Wind and wind-induced turbulence causes the bacteria present in soils or on plant surfaces to become airborne. Cut grasses for silage, hay grains etc. are other sources providing substratum for the growth of bacteria which can later become airborne. Bacteria are very common on the skin and hair of animals. They become airborne when skin scales are released as a result of friction. Man is an important vector of microbes, and helps in their dispersal through skin scales released following bathing and friction with towels and clothes, and during talking, coughing, and sneezing. Fungal spores in the air are mainly derived from fungi growing on vegetation surfaces, litter, paint works, wall paper, decaying woods and other substances above ground level. Active and passive liberation of spores by several fungi from their spore-bearing hyphae is the major source of fungi in the atmosphere. Algal flora of air is also mainly derived from soils and algae growing on vegetation. Aquatic habitats also contribute to a smaller extent. Water droplets containing microalgae may enter air through rain-splash, wave-action, boating activities etc. and later on may be carried away to long distances.

The dispersal of airborne particles in the aerial environment can be considered in the following three stages:

3.3.2.1 Liberation, Entry and Take-Off

For a successful liberation and entry of microbes, energy is required to overcome adhesive forces and then to cross the laminal boundary layer. Further, the fate of any airborne microorganism once it enters the atmosphere depends upon several metereological factors and morphological characteristics of the microbe. Spores of fungi, the most successful group of airborne particles, can be liberated by a number of indigenous mechanisms (Ingold 1971; Gregory 1973). The important mechanisms of liberation and take-off are gravity-shedding, mechanical disturbances, rain-splash etc. (For details, see Gregory 1973; Ingold 1953, 1965, 1966, 1971).

3.3.2.2 Dispersion

Once airborne, the fate of spores or other microorganisms depends upon their physical characteristics such as size, shape, degree of surface roughness, density, and electrostatic charges carried by them. Environmental factors including turbulence, air currents, wind speed, air viscosity, relative humidity, and wind gradients also affect the dispersion pattern of air microorganisms.

3.3.2.3 Deposition

The ultimate stage of any airborne microbe is its deposition on the earth's surface. They are returned to the boundary layer of soil, plant surfaces or other structures so that they can no longer be blown off by normal winds. Sedimentation under gravity, turbulent deposition, impaction, boundary layer exchange and wash-out through rains are some of the important mechanisms through which the microbes present in the air are deposited back on the ground.

3.3.3 Seasonal and Diurnal Periodicity of Microorganisms

Seasonal and diurnal periodicity patterns in the abundance of microorganisms have been established depending upon the method of liberation and their growth behavior (Hirst 1953; Last 1955; Shenoi and Ramalingam 1975). Seasonal variations in airborne bacteria are related to agricultural activities. In general, density of spores and bacteria is higher during summer and lower during winter and spring. Several plant pathogens occur at distinct seasons, e.g., *Erysiphe* spores are more abundant during early summer, *Ustilago* and *Puccinia graminis* have maximum number during the flowering period of their grass host. Many ascospores and basidiospores have maximum densities during the autumn. Similarly, species having spore-liberation mechanisms depending upon changing relative humidity shed their spores in the morning, e.g., *Phytophthora* and *Deightoniella* sp. *Cladosporium, Alternatia, Ustillago* etc., release their spores at mid-day when temperature and wind turbulence are comparatively high. Species which require water for liberation of their spores, e.g., *Sporobolomyces* spp., release their spores during the night.

Further, the spore types of any region depend upon its geographical location. *Cladosporium* spp. predominate through temperate and several tropical regions. Several species such as *Curvularia, Pyricularia, Nigrospora* are restricted to tropical regions in their distribution, whereas *Alternaria* spp. is most abundant in warm and dry regions.

3.3.4 Survival of Aerial Microflora

The successful survival of aerial microbes depends upon several stress factors acting upon them. A major factor of their survival in the air is the problem of remaining airborne. Only microbes having a diameter of less than 1 mm can remain permanently suspended in air (Madelin and Linton 1971). Low availability of water and nutrients, temperature variations, ultraviolet and other shortwave radiations etc. cause stress on the aerial flora. A wide range of relative humidity ranging from 10–20% in warm deserts to near saturation or even supersaturation may be encountered by the microbes. The lower limit of fungal growth is approximately 65%, whereas bacteria require even more moisture (Kouyeas 1964). Interestingly, prokaryotic microalgae are better adapted to survive low relative humidities than eukaryotic algae; this is probably due to their closer resemblance to bacteria in their mode of arid survival (Ehresmann and Hatch 1975). Further, ultraviolet radiations are potentially hazardous to organisms and can cause death or mutation in bacteria in aerosols (Gregory 1973; Anderson and Cox 1967). Desiccation and temperature also interact in affecting the survival of airborne microbes. However, the interaction is little understood, but in general, freezing at high altitudes and desiccation may protect and preserve the microbes, particularly against radiation damage.

From the foregoing account it is thus clear that although our knowledge of a true microbial population inhabiting the atmosphere is limited, the air can, however, be considered as a microbial habitat probably tolerated by specialized microorganisms which somehow remain in suspension. Even in the absence of a true aeroplankton flora, the air constitutes a very important dispersal medium for a vast array of microorganisms, and during their stay in the atmosphere they may encounter and react with several pesticide particles co-existing with them.

4 Entry and Dispersal of Insecticides in Microbial Environments

An insecticide may be applied to the soil or soil-grown crops, water bodies, atmosphere, or animals, depending on the purpose for which it is used. All these form components of the total environment (in the case of animals it includes both the outer exposed surface and the inner parts) which are all inhabited by microorganisms. Thus, in the process of reaching the target organisms, at which they are generally aimed on application, the insecticides also encounter microorganisms in the specific component of the environment.

In addition, depending upon the mode of application, temperature, humidity, and wind velocity, and the subsequent physical, chemical or biological transformation of these chemicals, a major quantity of the insecticides applied to specific components of the environment never reach their intended target and are redistributed either in their original form or as transformed products into the other components of the environment. It thus becomes inevitable that insecticides, either in their original or modified forms, in the process of being cycled within and between components of the environment, come into contact with microorganisms present in the natural environment and interact with them.

In this context it is necessary to examine the various routes of entry of insecticides to specific components of the microbial environment and the route of their redistribution (dispersal) either within the environment or from one environment to the other, as components or the total environment. While the entry of the insecticides into a specific component of the environment may be through direct, accidental or indirect routes, their redistribution or dispersal within and between components of environment is influenced by various factors.

4.1 Soil Environments

4.1.1 Entry of Insecticides in the Soil Environment

The major route by which insecticides find entry into the soil environment is through their direct or intentional application to the soil or to the foliage of crops and weeds, and sometimes even through seeds treated with insecticides during storage. In such instances insecticides reach the soil by missing the targets (only a fraction of the enormous quantities applied actually reach their intended targets), by run-off from treated plant surfaces or by spillage during application. The various indirect routes which facilitate entry of insecticides into soil are: (a) root exudation, (b) death and deposition of the treated or contaminated plant, (c) drifts of airborne remnants subsequent to application or through volatilization from soil and foliar surface, (d) use of contaminated manure (from treated plants or feces of treated animals), (e) death of animals grazing on treated plants, and (f) spillage while washing and disposing of containers etc.

4.1.2 Dispersal of Insecticides in the Soil Environment

Various factors, including the properties of the soil, the insecticide and the ambient climatic conditions, determine the redistribution of the insecticides and their prod-

ucts both within the soil environment and from the soil environment to the other components of environment. These factors can be broadly classified as follows.

4.1.2.1 Erosion by Wind and Water

Insecticides can be moved in a lateral fashion over soil surfaces by wind and water. Insecticides both adsorbed to soil particles (wash-off) and in solution (run-off) may be transported along with soil during erosion of soil by water (Epstein and Grant 1968). Also, insecticides adsorbed to soil particles may be blown over great distances by wind erosion. Such lateral movement of insecticides over soil surfaces are in turn influenced by topography, soil permeability, rainfall, degree of adsorption of insecticides to the soil particles, formulation and application rate of the insecticide, method of soil cultivation and the prevailing vegetational cover.

4.1.2.2 Adsorption

Insecticides, on entering the soil, became redistributed on the solid soil surfaces, in the soil solution and in the soil atmosphere. Depending on the properties of the soil and the insecticide, insecticides become adsorbed to mineral and organic soil particles in the soil to varying degrees. This intensity of adsorption determines whether the insecticide undergoes any distortion and diffusion in soil solution or remains adsorbed to the soil particles. The amount of organic matter is the most important soil characteristic that influences adsorption of insecticides to soil particles, although ion exchange capacity and pH are also known to be important factors. Adsorption of insecticides to soil particles not only reduces their transport through water and wind erosion, but also volatilization from soil surfaces. Further, adsorption may also hinder the biological activity of the insecticide.

4.1.2.3 Leaching

Leaching is the downward or vertical movement of insecticide in solution through the soil profile in the zone above the water table and is generally a consequence of rainfall or flooding of land. The movement of the insecticide may be either by diffusion or by bulk transfer, involving a mass flow of water containing the pesticide. Further, the extent of leaching is determined by the properties of the insecticide such as solubility, adsorptive properties and rate of degradation of the insecticide and by the physical and chemical characteristics of the soil. In general, most of the insecticides are resistant to leaching.

4.1.2.4 Volatilization

Volatilization or the loss of insecticides to the atmosphere is governed by the vapor pressure of the insecticide, time, formulation and rate of application, water content of soil, ambient temperature, wind velocity, relative humidity, plant cover etc. In certain cases, as in DDT, the transformation products (DDE) may be more volatile than the parent insecticide (W.F. Spencer and Cliath 1972).

4.1.2.5 Plant Uptake

Another route of dispersal of insecticides from the soil environment is by their uptake by plants. The quality and rate of uptake are influenced by the ability of the plant to absorb the insecticide and the availability of the same to the roots. However, few insecticides are lost from the soil environment in this manner (Edwards 1974). Moreover, the insecticides taken up by the plants are invariably returned to the soil either directly following the death of the plant or indirectly via food chains as excretions or by the death of the recipient animals.

4.1.2.6 Animal Uptake and Transport

Animals may receive insecticides either by direct, intentional application or by accidental contact or indirectly by feeding on treated plants and animals. Soil invertebrates may ingest insecticides adsorbed to soil particles and may even accumulate them in their tissues (Edwards 1974). Such animals, however, are able to transport those insecticides over short distances only which are eventually returned to the soil through excreta and death of the animals. The quantity of insecticides that these invertebrates accumulate is dependent on the degree of exposure to the chemicals, the ability of the animals to absorb and retain the insecticides in their tissues, and other soil and insecticidal properties.

The contaminated invertebrates may also be eaten by other animals (especially birds), in which case they may be transported over long distances (Evans 1974) and eventually may return to the soil through death and excretion.

4.2 Aquatic Environments

4.2.1 Entry of Insecticides in Aquatic Environments

The entry of insecticides into aquatic environments may again occur directly or indirectly. They may be intentionally applied to the water bodies for sanitizing purposes or for controlling the spread of insects. Other routes of direct entry of insecticides into the aquatic environment are through domestic and industrial insecticidal wastes, disposal of unused insecticides subsequent to application, washing and disposal of containers and commodities having residues of insecticides etc.

Insecticides may also unintentionally enter aquatic environments by: (a) drift from aerial and ground applications, (b) volatilization from treated surfaces and their entry by precipitation, (c) erosion of contaminated soil by wind and water into aquatic systems (run-off, wash-off, leaching etc), (d) irrigation water from contaminated areas, (e) spillage during application, (f) accidents in transport of insecticides as well as during application, and (g) domestic and industrial effluents.

Aerial drift of insecticides as a result of spraying application may travel long distances before entering water bodies through precipitation. This and run-off from agricultural lands are the two major routes of entry of insecticides into aquatic environments (Nicholson 1967).

4.2.2 Dispersal of Insecticides in Aquatic Environment

4.2.2.1 Volatilization

Volatilization is one of the major routes by which insecticides are rapidly lost from the aquatic environment. By this process, even "nonvolatile" insecticides may be released to the atmosphere (W.F. Spencer and Cliath 1972), and may then be either deposited by rainfall to soil or aquatic environments or may remain in the atmosphere. Volatilization is influenced by various factors such as the vapor pressure of the insecticide, ambient temperature, water solubility, and adsorption characteristics (C.W. Spencer et al. 1973; Kenaga 1972). Lichtenstein and Schulz (1970) have shown for the insecticide dyfonate that volatilization increased with concentration although no direct relationship occurred between insecticide solubility and volatilization. Further, they have demonstrated that in general the addition of soil, phytoplankton or detergent to water reduced the loss by volatilization.

4.2.2.2 Adsorption

Several insecticides have a tendency to adsorb to organic material and other nonliving and animate particles in nature. Several organochlorine insecticides, though comparatively insoluble in water, can be present in much higher concentrations in the sediments of aquatic habitats than their concentration in the overlying waters (Edwards 1974) and it is possible that anaerobic microbes present in the bottom mud and sediments play a significant role in their degradation in nature (Gerakis and Sficas 1974). Further, insecticides may be directly adsorbed into the sediments or may also reach the bottom sediments through adsorption and subsequent death of algae, invertebrates, fishes, and other vegetation. The lipophilic nature of several insecticides and a very high surface to volume ratio of unicellular plankton and other microorganisms may be responsible for a rapid sorption of insecticides and their removal from the aquatic media (Valentine and Bingham 1974).

4.2.2.3 Absorption

Phytoplankton and many other aquatic vegetations have the capacity to absorb and accumulate pesticides from the water bodies. The pesticides can enter either directly through absorption in most aquatic microbes, plankton, and higher flora or may enter higher organisms indirectly via the food chain. Edwards (1974) demonstrated that organochlorine insecticides can be absorbed by invertebrates in fresh and saline waters and accumulate to varying degrees, reaching very high concentrations in oysters and sea squirts, and it has been suggested that such a direct intake of insecticides may be more important than biomagnification through the food chain (Moriarty 1975).

4.3 The Atmosphere

4.3.1 Entry of Insecticides in the Atmosphere

The atmosphere is one of the most important mediums for long-distance dispersion of insecticides. There are several ways by which insecticides reach the atmosphere.

Drift and volatilization during application, volatilization from treated crops and soils, wind erosion of contaminated soils, and escape from manufacturing and formulating plants are some of the major sources of atmospheric input of insecticides. The major routes of the entry of insecticides into the atmosphere are briefly discussed below.

4.3.1.1 Spray Drift During Application

Enormous quantities of insecticides and other pesticides enter the atmosphere during spray application to agricultural crops. The application usually involves the dissemination of insecticides dissolved in some dispersion medium or carrier in the form of finely divided droplets or particles to provide a uniform distribution of small quantities of insecticides over a large surface area. The extent of drift is influenced by many factors, such as the formulation of the insecticide, the atmospheric conditions during application, the type of equipment used for application, and the direction of application with respect to the ground and the height from which applied. High wind speeds and turbulence during spray applications lead to extensive particle drift and determine the distance a spray droplet of a given size and weight is carried. Small particles and droplets have low settling velocities and remain in the atmosphere for long periods, during which they are highly susceptible to drift and can be transported by air currents into even the higher layers of the troposphere.

Depending on the equipment used, a range of droplet/particle size may be produced during application. While large droplets are less effective in terms of the insecticide reaching its target, small droplets remain for a longer time in the atmosphere. In general, the time is inversely proportional to the size and weight of the droplet/particle. Moreover aqueous spray droplets can evaporate during application to a smaller size, thereby becoming more susceptible to drift. High temperature and low relative humidity together hasten the process of evaporation.

The direction and height of discharge determine the time taken for the spray droplets/particles to reach the target, thereby influencing evaporation and drift. Although most spray procedures use water as a carrier, evaporation and particle drift can be considerably reduced by using insecticide formulation with low volatility carriers and by ultra-low volume spraying (using high concentration of insecticides in minimum volume of the solvent).

4.3.1.2 Volatilization During Application

Depending on the volatility of the insecticide, losses of up to half the applied quantity may occur in the vapor phase (Menzer et al. 1970, Kiigemagi and Terriere 1971). Although because of practical difficulties in experimentation absolute estimations of losses during application have not been possible, Decker et al. (1950) have shown that excepting for the least volatile insecticides, a portion of the insecticide applied enters the atmosphere in the vapor phase.

4.3.1.3 Volatilization from Treated Surfaces

Volatilization from treated agricultural soils and plants is another major route of entry of insecticides into the atmosphere. The major factors that affect volatilization

are the nature of the treated surface, air movement and temperature, and the volatility of the insecticide itself (W.F. Spencer 1975; Wheatley 1973). Woodwell et al. (1971) have reported that volatilization is the principal mechanism by which DDT is lost to the atmosphere.

4.3.1.4 Wind Erosion of Contaminated Soil

Wind-blown dust contributes substantially to the occurrence and transport of insecticides in the atmosphere. Insecticides adsorbed to the soil particles may be distributed in the air by wind erosion (Cohen and Pinkerton 1966). However, such entrained soil particles (also microbial propagules, pollen and pieces of foliage) are transported only relatively short distances (Seiber et al. 1975; Freed et al. 1972).

4.3.1.5 Escape from Manufacturing and Formulating Plants

Young et al. (1976) have reported that escape of DDT from manufacturing plants and the associated dumping sites are one of the major sources of the atmospheric contamination with insecticides. Possibilities of atmospheric pollution are thus associated with the manufacture, formulation, and packaging of insecticides.

4.3.2 Dispersal of Insecticides in the Atmosphere

From the detection of low concentrations of insecticides from the air and rainfall samples of many urban and rural areas (Cohen and Pinkerton 1966; Stanly et al. 1971; Sodergren 1972) and detection of DDT in the snows of antartica (Peterle 1969), it appears that air acts as a global dispersal agent of insecticides. Gaseous and particulate insecticides may travel long distances from the areas of application, and such airborne insecticides are generally deposited through precipitation, sedimentation and dry deposition. However, insecticides transported over long distances are rapidly diluted to extremely low concentration and have negligible environmental significance.

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CHAPTER 3

Experimental, Methodological and Analytical Approach to the Study of Microbe-Insecticide Interactions

RUP LAL and N. P. AGNIHOTRI

1 Introduction

The heterogenity of biological, chemical and physical properties of microbial environments has presented many problems to understand the microbe-insecticide interactions. In fact, many studies have examined such interactions only under laboratory conditions and thus the scientific literature on this subject is dominated by in vitro studies with pure and mixed cultures of microorganisms (Ware and Roan 1970; Cox 1972; Tu and Miles 1976; Wainright 1978; Lal and Saxena 1980, 1982; Lal 1982; Lal and Dhanaraj 1984). Such studies have considerable scientific value but there has been an unfortunate and undesirable tendency to assume that they also reflect the interaction of insecticides with microorganisms in natural environments.

It has already been discussed in Chapter 2 that there are many factors, both biotic and abiotic, which either interact with microorganisms or with insecticides prior to the microbe-insecticide interactions. It is also understandable that we can neither consider all the factors together in our studies nor study the interaction of all insecticides on all microorganisms. However, realizing these difficulties, our experimental and methodological approach should be more realistic. In this chapter an attempt is made to summarize and point out the lacunae in our experimental and methodological approach.

2 Accumulation

The accumulation of insecticides has been mainly studied in aquatic microorganisms in pure cultures particularly with algae. A generalized scheme which involves different steps during the assessment of bioconcentration of the insecticide is shown in Fig. 1.

Normally the accumulation of the insecticides has been studied by the addition of unlabeled (Lal et al. 1981; Saxena et al. 1982; Agarwal et al. 1982) and labeled insecticides to the medium (Barry 1968; Dalton 1971; Hansen 1979; Goulding and Ellis 1981). The organisms are either centrifuged or filtered, and pellet is obtained which is subjected to further analysis. The main drawback of this method is that most of the insecticide sticks to the surface of the glassware and/or on the filter paper. The solvents used to extract the insecticide from the microbial pellet also remove the insecticide from the glassware or the filter papers, thus making it extreme-

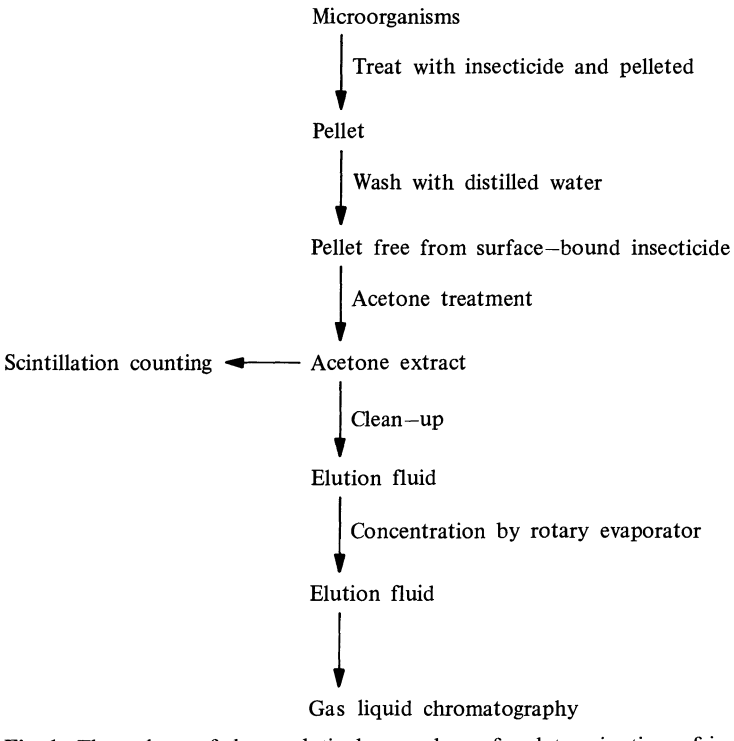

Fig. 1. Flow chart of the analytical procedures for determination of insecticides in the microorganisms

ly difficult to establish the exact amount accumulated at a particular time. After centrifugation the protozoans come up again and become suspended in the medium, making it difficult to collect all the organisms in a pellet (Lal et al. 1981; Agarwal et al. 1982).

Despite the fact that there are certain difficulties which cannot be avoided, the method described above, if performed properly, can give good results. The major difficulty with filtration is that most membrane filters are highly hydrophobic and often have a large mass as compared to biomass. Consequently, they can sorb large quantities of the insecticides very efficiently. However, centrifugation is a more straightforward method if adequate samples are available. Some degree of error will always be associated with the biomass that is not pelleted or that becomes resuspended on decanting the supernatant solution. The use of labeled compounds can also sometimes present special problems in measuring insecticide accumulation. The difficulties arise from the fact that measurement of radiation is not specific since labeled compounds are not noted for high purity.

3 Metabolism

A considerable amount of work has been directed to studying the metabolism of insecticide in microbial cultures. Laboratory studies of microbial metabolism of insecticides have utilized organisms either obtained from culture collections or isolated from soil, aquatic system, sewage etc. When radiotracer methodology was still in its infancy, microbial studies with unlabeled insecticides were useful to obtain information on the biodegradability of insecticides. However, the question may be raised as to the relevance of these studies for the persistence and fate of insecticides under natural conditions. Pure culture studies carried out with unrealistically high concentrations very often do not reveal the true fate of insecticides in natural environments. Thus observations in culture can rarely be extrapolated to field circumstances and that organisms able to metabolize insecticide in situ are not necessarily active in vitro. One possible exception was reported by Gunner and Zuckerman (1968). They found that diazinon in soil resulted in a selective enrichment of an *Arthrobacter* sp. and a *Streptomyces* sp. In cultural conditions neither organism could cleave the pyrimidine ring, whereas in a mixed culture of both organisms, 15–20% of the radiolabeled ring carbon was evolved as $^{14}CO_2$. There was then good, although circumstantial, evidence that these two microorganisms were stimulated in soil because of their synergistic ability to degrade the insecticide. If such synergistic interactions are common, and there is every possibility that they are, they may be responsible for the inability of many investigators to demonstrate in pure cultures the metabolism of insecticides that are degraded under field conditions. Thus a mixed microbial population offers many advantages in insecticide metabolism due to the participation of multiple degrading enzymes and the nature of the metabolism of insecticides can be studied under laboratory-simulated conditions corresponding to natural environments.

Isolation of microbial cultures, mixed or pure, from the natural ecosystem is instrumental in deciding the ability of the organism to metabolize insecticide. Recently enrichment of specific group of microorganisms has proved to be the best practical approach. Several investigators have isolated insecticide-degrading organisms from soil sediments and water which had received repeated applications of insecticides (Munnecke et al. 1982; Sethunathan et al. 1977).

Following isolation from natural environments, microorganisms are selected that are capable of metabolizing the insecticide in solid or liquid culture media. The methods of assessment of metabolism differ widely. Metabolic studies, however, have mainly followed traditional methodology, the organisms being incubated in a variety of media containing the insecticide in the presence or absence of other sources of carbon, nitrogen, and energy. The fastidious requirements of some microbes can be accommodated by the inclusion of, for example, amino acids and growth factors (Matsumura et al. 1976; Ohisa and Yamaguchi 1978; Ohisa et al. 1980).

Many workers have used the disappearance of insecticide from the soil or medium as the criterion to study the metabolism of insecticides (Tu and Miles 1976; Sethunathan et al. 1977). The disappearance of an insecticide from soil or culture supernatant cannot itself be taken as a satisfactory indication of metabolism. Losses

of unchanged insecticide from the medium can occur by volatilization, intracellular accumulation, and by absorption to cell surfaces (Gregory et al. 1969). The detection of insecticide products is thus an essential criterion for confirming the metabolism of insecticides. The use of a radiolabeled substrate not only facilitates analysis but, when used in an enclosed incubation system, allows the collection of volatilized products evolved from the culture medium into the air and enables the preparation of a "balance sheet" for recovery of insecticides and their transformation products.

Further the observations of the presence of insecticide products in microbial cultures and their absence in appropriate controls is not necessarily an indication of microbial metabolism. Changes in the chemical structure of the insecticide can be caused by the physicochemical properties of the medium following microbial growth of intracellular constituents of the organism. Proof of metabolism is only conclusively obtained following the isolation and, wherever possible, the purification of active enzyme systems.

4 Analytical Implications

Since a large number of organic compounds are used as pesticides, a variety of procedures are available for this analysis. However, no single method is adequate for the analysis of insecticides and their metabolites. Shortcomings can be pointed out in each method, leaving scope for future development. The choice of the method depends on the chemical nature of the insecticide. Basically, analysis comprises three main steps (1) extraction (2) clean-up and (3) identification and estimation. Each step is crucial for satisfactory analysis. An incomplete extraction results in low recoveries, an inadequate clean-up leaves co-extractives which may interfere in the final analysis or lead to mistaken identification, and finally if the choice of method is not appropriate, no confidence can be had in the analytical data. These analytic steps are discussed below.

4.1 Extraction

The extraction is the first step in residue analysis of pesticides. The choice of the suitable solvent depends on the polarity of the insecticide, the nature of the sample and sometimes on the method employed for the final estimation. When a very low quantity of the insecticide is used, the bulk of the microbial sample has to be extracted for obtaining sufficient quantities for final estimation.

During extraction, many unwanted materials such as lipids, proteins, carbohydrates etc. are also co-extracted along with insecticide metabolites, and interfere in the final analysis. The problem of analysis is particularly aggravating in microbial samples which contain high amounts of co-extractions. These interfering materials have to be removed from the extracts by some suitable clean-up procedure before the final estimation is carried out. A proper selection of solvent/solvent mixture can greatly reduce these co-extractives, but these cannot be completely eliminated. For

example, impurities such as lipids can be reduced by extracting the sample with a polar solvent such as acetonitrile.

The efficiency of extraction depends on how efficiently solvent can penetrate microbial cells. Initially after application, insecticide is present largely in unbound state (adsorption) and can be dislodged from the microbial sample with any suitable solvent. However, as time progresses, insecticide molecules tend to be absorbed into the microorganisms, thus moving to the cell or cell organelles. Hence, simple agitation, mixing, and tumbling of the sample with solvent may not be enough to yield quantitative extraction, as only loose and unbound residues are extracted. Efforts to increase percentage recovery by increasing agitation time, increasing the ratio of weight of the sample to the volume of the solvent, using a mixture of a polar and a nonpolar solvent to secure more intimate contact between the surfaces and the solvent has not ensured complete extraction. It is now generally accepted that simple tumbling, agitation, or soaking leaves appreciable quantities of residues unextracted, and that these techniques of extraction are inefficient and unreliable.

The invariable efficiency of an extraction is ensured by fortifying the sample with pesticide just prior to extraction. This gives misleading information because there is not enough time for residues to reach equilibria with the substrate. The recoveries are then generally higher than those obtained under actual working conditions. It is recommended therefore that recoveries be checked by fortifying the sample with radiolabeled material and that extraction be carried out after sufficient time to allow the sample to equilibrate with the insecticide molecules. Later, a balance sheet can be prepared on the amount of pesticide extracted and the portion retained in the sample.

For quantitative extraction, blending and Soxhlet extraction are considered to be the most efficient and satisfactory techniques. Blending is generally used for "wet" or high moisture-containing samples and Soxhlet extraction for dehydrated or partially dehydrated samples. Soxhlet extraction is the most thorough procedure for quantitative recoveries. The recoveries from other methods are compared with the Soxhlet method only. In Soxhlet, the sample is finally ground to expose the maximum surface area and extraction is carried out with a suitable nonpolar solvent. A sample containing light moisture is extracted after mixing with anhydrous sodium sulfate prior to grinding. Residues from microbial samples containing high moisture contents can also be extracted efficiently by a mixture of a nonpolar and a polar solvent such as methanol and chloroform (1 : 1) (Porter and Burke 1969; Burke et al. 1971). Limitations in this procedure are that it is unsuitable for thermally unstable compounds and is time-consuming.

Blending or maceration is a simple, rapid and efficient technique for extraction of residues from "wet" biological materials. The solvent comes in intimate contact with both internal and external residues. However, the solution of solvent(s) is very critical. The choice depends on the polarity of the chemical, nature and moisture content of the sample and the location of the residues in the sample. With the blending technique, generally a mixture of a nonpolar and a polar solvent, such as hexane-acetone, hexane-isopropyl alcohol and benzene-acetone has been found to be superior to a single solvent (Hardin and Starter 1962; Beynon and Eglar 1966). The polar solvent wets on a bridge between a nonpolar solvent and the water of the sample. However, invariably emulsions are formed which are hard to break, causing

problems in subsequent filtration (Goodwin et al. 1961). Some workers therefore prefer to extract the samples first with a polar, then with a nonpolar solvent. In the latter analysis, acetonitrile has been used extensively for the extraction of organochlorine, organophosphorus (Mills et al. 1963; Storherr et al. 1971), carbamate (Porter et al. 1969; Holden 1973) and synthetic pyrethroids (Braun and Stanek 1982). The extraction with acetonitrile is preferred because recoveries are high and extracts are clearer than obtained by other solvents. The amount of lipids extracted is minimal. However, acetonitrile has been reported to be an unsatisfactory solvent for extracting dehydrated samples (Bertuzzi et al. 1967; Porter and Burke 1969; Burke et al. 1971). The recoveries, however, could be improved by the addition of water. Bertuzzi et al. (1967) have reported that recoveries with 35% water acetonitrile are equivalent to thorough extraction by Soxhlet with methanol-chloroform mixture (1 : 1).

The level of moisture has also been reported to be crucial in the quantitative extraction of pesticide residues from the soil. Apparently, in dry soil pesticide molecules are too lightly sorbed to be extracted by routine extraction procedure. A residue of a small quantity of water helps release these sorbed molecules, water molecules being preferentially sorbed over nonionic pesticides. Lichtenstein (1965) has reported best recoveries of pesticides from soil at 12–16% moisture.

The blending technique is not satisfactory for extraction of residues which are translocated inside the biomass (Wheeler et al. 1967; Burke 1971; Burke et al. 1971). However, these residues could be extracted quantitatively by Soxhlet extraction technique.

In the microbial environment, nonionic pesticides are frequently degraded into products which are anionic in character. For example, DDT is converted into DDA (2,2-bis [p-chlorophenyl] acetic acid). Microbial hydrolysis and oxidation products of organophosphorus and carbamate insecticides lead to the formation of products which are anionic in nature (Lal 1982). These anions have more adsorption capacity than their parent compounds. Their metabolite and degradation products are also conjugated with other substances. Consequently the method must also take these aspects into consideration. The extraction of anionic pesticides is relatively difficult from samples. Usually substrate is acidified prior to extraction with a polar solvent so that the partition coefficient is shifted in the direction of the aqueous phase.

Finally, the analyst must be clear in his mind about the nature of the compound, different isomeric forms in which it may be present and the polarities of the toxic metabolites formed. If necessary, samples must be extracted sequentially with series of solvents with increasing or decreasing polarity. Invariably the polar compounds form conjugates and they must be hydrolyzed prior to extraction. The validity of the extraction procedure must be confirmed by conducting recovery experiments, preferably by using radiolabeled material, not only with the parent compound, but also with all the metabolites of interest and possible conjugates.

4.2 Clean-Up

Insecticides and their metabolites extracted from microbial samples are analyzed by gas liquid chromatography (GLC). Luke et al. (1981) have reported analysis of pesticides, without any clean-up. Organophosphorus compounds are analyzed by

flame photometric detector and organochlorine and organonitrogen compounds by Hall electrolytic detector. However, usually it is necessary to clean up the sample to remove the major interfering co-extractives to avoid deterioration of column performance, poor resolution, undue contamination of the detector and confusing peaks, and to keep the instrument running properly. The clean-up procedures are laborious, lengthy, and recoveries are seldom 100%. Therefore, a minimum clean-up for reasonable reduction of interference should be carried out to give recoveries of more than 85%.

A variety of clean-up procedures have been utilized. These have been reviewed by Gunther (1962) and Lantos et al. (1981). The initial purification of pesticide residues involves the partitioning of the pesticide in a nonpolar solvent to remove the co-extractives. The subsequent clean-up depends on the nature of the substrate and properties of the insecticides such as polarity, stability, volatility, acidity, basicity etc. The techniques commonly used are liquid-liquid partition, adsorption chromatography, and gel permeation. The scope of chemical treatment is limited, as most of the insecticides are destroyed or altered on reaction with strong acid or alkali. However, for most insecticides, lipids from the extracts are removed by liquid-liquid partitioning. The residues after extraction are partitioned between acetonitrite (polar phase) and hexane or petroleum ether (nonpolar phase). The pesticide is passed into the polar phase and the fatty materials pass into the nonpolar phase. The insecticides and its metabolites are recovered from the polar phase by re-extraction with a nonpolar solvent or by evaporation. However, the technique is not efficient for removal of polar lipids, pigments, and other interfering compounds. Consequently, adsorption chromatography is used to remove these materials. For samples of nonfatty material adsorption chromatography is the most effective technique. A variety of adsorbents are available to select. The most frequently used are florisil, alumina, charcoal and silica gel. Amongst these florisil has been used most extensively as single or in combination with other adsorbents such as charcoal and celite 545 (Aoki et al. 1975; Brown 1975). The mixed columns are occasionally used in an effort to combine the best features of the adsorbents. However, such columns pose problems of reproducibility and, therefore, as far as possible it is better to use a single adsorbent. For reproducible results, it is essential that adsorbent be standardized as to particle size activity. The activity of florisil changes from batch to batch; no two batches of florisil give identical clean-up. Therefore, the commercial product must be standardized before use. Methods for standardization of florisil have been discussed in great detail by the U.S. Environmental Protection Agency (1974) in their manual for pesticide residue analysis.

A successful clean-up does not always depend on the selection of the right adsorbent or its activity, but also on the use of an efficient eluting system. For example, normal elution of florisil with 6% and 15% ethyl ether in petroleum ether recommended for organochlorine and organophosphorus insecticide (Mills et al. 1963; Burke et al. 1971) was not found sufficient to elute relatively polar insecticides and metabolites (Mills et al. 1972). These compounds could be eluted quantitatively if the polarity of the eluting solvent is increased by using 25% or 30% ethyl ether in petroleum ether. Mills et al. (1972) have suggested the use of these eluants consisting of a mixture of methylen chloride, hexane and acetonitrile to recover insecticide and their metabolites with a great range of polarity.

The florisil columns can also be used for purification of anionic compounds with some modification. A sample extract is passed through an adsorbent column. The insecticides, being polar, are more strongly bound than the impurities. Hence it is possible to elute the impurities from the column while leaving the polar insecticides on the column. The pesticide is then recovered by eluting on a strong polar solvent.

Thin layer chromatography (TLC) is also used for clean-up of sample extracts. The method is especially suited for clean-up of soil and nonfatty material samples. Except for the limitation that this method is not suitable for samples containing lipids, it is more rapid and less tedious than column chromatography. In the TLC technique, extracts are normally applied as a streak along with a spot of standard compound on the side of the plate to locate the region where pesticide will be found. After development, the band is scraped and eluted. The efficiency of the method is reportedly further improved if plates are developed twice with the same or a different solvent system (Faucheux 1968). Usually a polar solvent such as acetonitrite is used. The plant coextratives remain in place or move only slightly, while the insecticide and its metabolites move to the front.

Recently, gel permeation technique (Gel Filtration Chromatography) has been reported as an alternative to partition and adsorption chromatography to remove lipid pigments and other interfering materials. Recoveries are claimed to be better than by acetonitrile-petroleum ether partitioning and florisil clean-up (Griffritt and Graun 1974). The utility and scope of this method has been greatly increased by the introduction of automation and the improved gel solvent system (Ault et al. 1979; Buttler and Hormann 1981; Hopper 1981). The method is based on the molecular size of the pesticide and the co-extractives. There is no problem of adsorption of polar compounds on the column. Hence, the method is particularly suitable for clean- up of insecticides and metabolites which are polar and are liable to be adsorbed on the adsorbent. Another advantage of this method is that gel column can be used repeatedly without losing it effectiveness.

4.3 Identification and Estimation

Analysis of pesticide residue is commonly carried out quantitatively by thin layer chromatography (TLC), by gas-liquid chromatography (GLC) and more recently by high pressure liquid chromatography (HPLC). It is essential that the identity of the pesticide residue is unequivocally confirmed by suitable tests. Recently, mass spectrometry, coupled with GLC or HPLC, is being used for the identification of insecticides and their metabolites. The spectroscopic methods have very limited use in insecticide residue analysis, as they are nonspecific and their sensitivity is relatively low. Co-extractives and even solvent at times give absorbance in the spectrum region that is characteristic of the insecticide.

The use and scope of chromatographic methods employed in insecticide residue analysis is given below.

4.3.1 Thin Layer Chromatography

Thin layer chromatography has been the most widely used technique both for the analysis of multi-residues of various class of pesticides and individual residues of a

specific compound. Basically, it is a qualitative technique used for the separation and confirmation of residues tentatively analyzed by GLC or HPLC. The confirmation is based on the comparison of Rf values of the pesticide in question with an authentic pesticide standard obtained with different solvent systems and a given adsorbent. The method is indispensable for separation, identification, and quantitative estimation of radiolabeled compounds used in metabolic studies. The method requires an adsorbent layer fixed on a solid support usually a glass plate. The compound adsorbent used are alumina, silica gel and florisil. Alumina is known to adsorb the insecticides and has been used for identification purposes only. For experiments where insecticide has to be recovered from the chromatographic plate, silica gel is preferred. For validification, Rf value should be taken at least on two or three solvent systems.

4.3.2 Gas Liquid Chromatography

Gas liquid chromatography is undoubtedly the most versatile technique in the analysis of pesticide residues. It offers both qualitative and quantitative analysis with speed, accuracy and sensitivity in subnanogram levels. The technique can be used for analysis of multi-residues. The GLC is utilized for separation of insecticides and their metabolites which are again analyzed by infrared or mass spectrometry for further identification and confirmation. The identification of insecticides and their metabolites is the most important aspect of GLC analysis. The identity of the compound is based on the retention time or Rf value of its peak and the quantity on the peak area. For identification, retention time of the unknown compound is compared with the known standard. It is desirable to examine the retention time on two or more different columns to secure additional proof of identity. One of these columns should preferably contain a nonpolar liquid phase (e.g., DC 200, OV-1, SE-30) and the other column a polar liquid phase (e.g., QF-1). The identity of the compound is almost certain if the retention time of the unknown and the standard is matched on both the columns. Comparison of retention times provides valuable confirmatory evidence. This multiple column technique can also be used to resolve confusing overlapping peaks. A complete resolution of all the pesticides even within a group is virtually impossible on a single column. It is recommended, therefore, to use one column for primary analysis and an alternate column to provide verification of identity. For multiple residue analysis, mixed phase columns (e.g., 10% DC-200 + 15% QF-1 for organophosphorus compounds and 0.5% OV-1 + 2% OV-25 for organochlorine compounds) gave better separation than single phase columns (Aoki et al. 1975; Yamato et al. 1976). It may be necessary to use more than one mixed phase column.

Further valuable confirmatory evidence can be obtained through the use of selective detectors which respond to a group or class of insecticides. For example, identity of organophosphorus compounds can be confirmed by use of flame photometric detector (P mode) and organochlorine, organosulfur and organonitrogen compounds by the use of Hall electrolytic detectors (HECD), specific for halogen, nitrogen and sulphur compounds (Luke et al. 1981). It is also useful to compare results obtained on two detectors preferably one specific and another nonspecific. For example, organochlorine insecticides can be analyzed by electron capture detector,

which has a very high sensitivity, and HECD detector, which is selective. Another advantage of a specific detector is that clean-up of the sample can be eliminated or reduced to a minimum. Luke et al. (1981) have shown that organophosphorus and organonitrogen pesticides, after extraction with acetone and partitioning with methylene chloride petroleum ether (to remove water and polar impurities), could be analyzed directly by GLC without the usual florisil clean-up.

Despite the case of multiple columns and selective detectors, gas-liquid chromatography can give only tentative identification. Exact identification of the compound, however, can be accomplished by mass spectrometer-gas-liquid chromatography. However, this technique has not been used for routine analysis to identify insecticides and their metabolites because the cost of the instrument is prohibitive. Although gas-liquid chromatography is a versatile technique applicable to a wide range of insecticides and their metabolites, it has limitations when samples are highly polar or nonvolatile, or thermally unstable. Some of these problems can be overcome by the use of derivatization of some functional groups in the insecticides.

4.3.3 High Performance Liquid Chromatography

Lastly, high performance liquid chromatography is becoming a standard technique in pesticide residue analysis. It is gradually replacing gas-liquid chromatography, the main reason being that this method is nondestructive. In GLC, pesticide or its derivative is heated to volatilize and in the process many thermally unstable compounds are degraded. Also the method is not suited for the separation of polar compounds. On the other hand, HPLC separation is brought about on a solubility basis. The choice of the solvent(s) (mobile phase) is of prime importance in separation. An appropriate solvent is selected that does not react with the pesticide to be separated. Thus compounds like carbamates, ureas and chlorophenoxy acids, which are being used increasingly in modern agriculture and are not easily analyzed by GLC, can now be analyzed by HPLC. The resolution is more on HPLC column because of the higher number of theoretical plates (5000–6000 plates/foot) than GLC-column (500–1000 plates/foot). The separation of DDT analogs from PCB's (Arochlors 1254 and 1260) has been achieved by HPLC. The sensitivity of detectors, a limiting factor in the past, has been greatly improved recently. The sensitivity of ultraviolet and fluorescent detectors is up to subnanogram range.

HPLC has also proved to be an excellent technique for removing the interfering co-extractives from the sample extracts. Gel columns using Bio-beads SX-3 have been used for separation of lipids from samples prior to GLC analysis (Johnson et al. 1976). More recently, SEP-PAK cartridges have been used for the routine clean-up of sample extracts. The cleaned extract is passed through the chromatographic column for further separation and identification. When the cleaned extracts have been collected, they can be concentrated and taken in suitable solvent for further analysis by TLC, GLC, infrared spectrophotometer and mass spectrometry.

Application of HPLC in separation of insecticides has been reviewed by Lawrence and Turton (1978) and Hank and Colvin (1981). The method has been reported to be suitable for separation of most pesticides and their metabolites. However, detection, particularly by UV mode, is not satisfactory for residue estimation of certain pesticides. The sensitivity of some organophosphorus and carbamate insecti-

cides is only in the μg range, although higher detection limits can be obtained if specific detectors like the chlorine esterase inhibitor Auto Analyzers are used (Ranteiner and Hormann 1975). The compounds that yield phenols on alkaline hydrolysis can be derived by dansyl chloride and estimated by fluorescence detector in nanogram range (Lawrence et al. 1976). Thus, HPLC can be used as an alternative method for pesticide samples that cannot be analyzed on GLC and as a secondary confirmation technique.

5 Effects

5.1 Soil Microorganisms

The problem of assessing the impact of insecticides on soil microflora is complicated not only by the highly complex system of multiple interactions which occur between insecticide and soil, but also by the diversity both of types of microorganisms in soil and of their activities. Further microorganisms co-exist in soil with other species, thus it is illogical to presume that the study of a single species in pure culture will demonstrate the probable effect of insecticide on those organisms in the soil environment. The simple chemical and physical factors that influence solid microbial activity have been listed in Chapter 2.

The example of organochlorine insecticides, particularly DDT and γ-HCH with their strong adsorption characteristics, has underlined both the futility of extrapolation of the results from pure cultures to soil and the need for studies to be conducted in the presence of soil or in soil. It is interesting to mention here that most of the studies conducted under field conditions report no noticeable effect of the insecticides, whereas similar doses of the insecticides have been reported to affect the growth of microorganisms drastically under laboratory conditions in pure cultures (Lal and Saxena 1982). This reflects that the insecticide applied to the soil becomes adsorbed first and is either not available at all or available in very small quantities, whereas in pure culture studies the actual amount of the insecticide is available to the microorganisms.

It is also important to emphasize here that currently the availability of precise and reproducible methods for analysis of a large number of interrelated biological activities leaves much to be desired. This is by no means the fault of the researchers, on the contrary, it is due to the difficult medium in which they must work. Thus, in attempting to evaluate the effects of insecticides on soil microorganisms an anomaly is presented to the researcher, namely to have the confidence of an analysis offered by pure cultures or the uncertainty of results offered by in situ soil experimentation. Compromising with semi-artificial media may also compromise the degree of doubt attached to each system. The only satisfactory solution to this anomaly appears to be in the use of both pure cultures and in situ soil tests.

Johnen and Drew (1977) pointed out that any system for evaluating insecticidal effects must reflect the situation that occurs in natural environments. In soil many factors affect the interactions that occur between insecticide and microorganisms and their environment. For instance clay contents, cation exchange capacity, pH,

and organic matter all influence the adsorption, leaching, and volatility of insecticides and hence may increase or decrease any potential harmful effects. Pure culture systems have technical advantages and often provide valuable biochemical information, but their results cannot be extrapolated to the natural habitat – a difficulty already mentioned.

All tests apply insecticides to freshly sampled soil in the laboratory in preference to treating soil in the field, sampling at intervals and transporting the samples to the laboratory for testing. In the field, fluctuations in temperature and moisture and local differences in physical and chemical soil properties may modify microbial responses to insecticides. Indirect consequences such as the removal of other plants and animals by insecticides and subsequent changes in soil moisture, temperature etc, may have a far greater effect on the microorganisms than the insecticide application itself. In contrast, laboratory conditions allow greater control of variables. Insecticide or insecticide-treated material should be thoroughly mixed with the soil. Re-infestation by microorganisms from areas that have missed treatment is minimized in the more homogenous laboratory soil with suitable processing replicate laboratory soil samples.

Though there are many difficulties in the laboratory test, which ultimately make it more difficult to interpret the results, laboratory tests are still considered to be more rigorous than field tests and more likely to reveal any insecticide effects. They also require less time, since microbial processes proceed much faster at the higher incubation temperature normally used and time saved in routine tests can be re-allocated to further studies if required. Further, if the adverse effects are confirmed in the laboratory, the severity and persistence of the effects will then be checked by field studies before judgement is made.

The following discussion of methods is not a complete coverage of those used to estimate the interaction of insecticide with soil microflora. It aims to highlight some of the principal methods used and to present some of their advantages and disadvantages.

5.1.1 Estimation of Soil Populations

The study of microbial populations and the influence of insecticides in them has both quantitative and qualitative aspects. In recent years measurements of changes in total microbial number by dilution plate procedures has received less attention than previously. This is due primarily to the well-known disadvantage of the technique which depends on the growth of organisms on agar medium (Nitikin and Makarieva 1970). Another difficulty is the unsuitability of this method for counting filamentous organisms or those which produce numerous spores. It is also known that some fungi are sensitive to the presence of volatile metabolites produced by bacteria (Moore-Landecker and Stotzky 1972). Despite these criticisms, however, use is still made of plate-counting. Indeed, when it is necessary or desirable to isolate organisms showing any response to insecticide, this is frequently the only method available. In addition, plate method can be used to detect species shift within a total population or by the use of selective media to follow effects on particular physiological groups. A frequently more convenient and accurate method for this sort of estimation, particularly for unicellular microorganisms, is the Most Probable Number (MPN).

The methods referred to so far are means of counting viable microbial propagules. An alternative approach is to count the total numbers of organisms with the light microscope. The original techniques relied on stains such as aniline blue, and failed to differentiate dead from viable cells and often cells from soil organic matter. Improvements have been made by using fluorescent stains such as acridine orange, which is assumed to make at least some differentiation between dead and viable cells, as it conjugates specifically with nucleic acids. It is assumed that nucleic acids disappear rapidly after cell death. Differentiation between living or recently dead cells is possible, with a europium-based stain. Such direct counting methods allow the microbial biomass in the soil to be calculated approximately, but give no estimate of metabolic active cells, nor do they give any information about potentially important species shifts which may occur. The development of fluorescent antisera that react with specific microbial species or even stains now allows identification of these species in field soils. As yet, however, the technique has not been widely applied in soil microbiology and not at all in insecticide studies. This is due in part to the difficulty of preparing fluorescent antisera that react only with specific microorganisms.

5.1.2 Measurement of the Biomass

Biomass estimates have received much attention from soil microbiologists in recent years because they indicate the response of total microflora to changes in level of available energy in the environments. Biomass changes also provide a measure of changes in the microflora induced by such factors as the introduction of insecticide. As direct microscopic counting of population smears is a tedious operation, attention has been focused on methods of estimating biomass based on determination of commonly occurring cell constituents such as chlorophyll or adenosine triphosphate (ATP). Chlorophyll naturally gives information only about the algal flora in the soil. In contrast, ATP has been suggested as a general measure of biomass since it is present in all living cells. It may be, however, that except in certain highly specialized situations ATP gives no valid measure of biomass. Its main value seems to be as an indicator of the effects of factors such as insecticides on total microbial life flux of the environment. Moreover, ATP concentration is a useful measure of total microbial biomass (algal, bacterial and microzooplankton), but gives little information on the individual biomass.

Doxtader (1969) described a method in which microbial cells are lysed. ATP was partitioned in buffered alcohol and then introduced into a luciferin-luciferase solution contained in scintillation vials. The emission of a quantum of light from the reaction of enzyme substrate with ATP was measured in the scintillation counter. Further improvement of the methods for estimation of ATP have been reported by Ausmus (1973) and Anderson and Davies (1973).

5.1.3 Measurement of Microbial Activity

While studies of microbial populations and pure cultures can provide a great deal of essential information, the results are most valuable when considered in conjunction with those studies of biological activity in soil. In any assessment of the effects of insecticide on soil microorganism, the prime directive is to establish whether these

effects are likely to influence crop production. It is by measuring biological activity in treated soil that we are most likely to obtain useful information.

Two broad approaches have been made to measure activity: the study of specific enzyme activities and the use of overall estimates such as respiration. In both, some attempt has been made to measure the actual activity on undisturbed soil in field sites, but more often laboratory samples have been studied.

5.1.3.1 Enzymes

The search for rapid methods of measurement of soil biological activity has increasingly turned to the study of soil enzymes. Such studies must be approached with a considerable degree of caution because the interpretation and extrapolation of the results, especially with regard to soil fertility, are very difficult. Active enzymes exist in the soil not only within the confines of living cells but free in the matrix of the soil. These enzymes are mainly microbial products in soil. Skujins (1967) reviewed the technique available for assay of many enzymes and indicated that soil enzymes are contributed by microorganism plant roots, soil animals, and living roots.

The measurement of the effects of insecticides on enzyme activities is markedly affected by the technique used. Thus many methods introduce an artificial substrate into the soil and this has to be considered while analyzing the data. Methods that examine the effects of insecticides on such activities under the most natural conditions are to be favored, but as yet are not widely used. Current methods often do not include any environmental factors. How close the results obtained are to natural soil values is open to discussion.

The practical significance of the effects of insecticides on soil enzymes can only be considered when the relationship between these enzymes and fertility is fully understood. Enzymes in soil, in general forms, bring about the cleavage of macromolecules to promote humus formation and provide readily available nutrients for plants and other organisms. As yet, however, there is only limited knowledge of how these enzymes are distributed and localized amongst the soil constituents and of their availability for catalysis. Attempts have been made by various workers to correlate the activity of enzymes with other measures of biological activity. Further the enzyme essays in soil use either buffered systems at the known optimum pH for the specific enzyme under study or, more frequently, unbuffered mixtures of soil and substrate. The latter ignores any change in pH caused by the substrate and assumes, usually wrongly, that the average pH of reaction is that of the bulk soil. Obviously both systems, particularly the latter, have disadvantages when considering the results in terms of what might occur in the field.

5.1.3.2 Metabolic Processes

By far the most commonly used techniques for assessing the effect of insecticide on the soil microflora are those measuring parameters which are indicative of processes occurring in soil. In this context soil respiration and nitrogen transformation are most widely studied. In common with all other methods used to study insecticide-microorganisms interactions these usually involve soil samples removed from the field and treated under defined conditions. Consequently such investigations are actually measuring potential activity of the soil biotic rather than the actual activity due to microbial population.

The metabolic processes measured in determinations of biological activity in soil are common to many living things in soil. Thus the results obtained arise from the sum of contributions from microflora, microfauna, plant roots, animals etc. Attempts have been made to distinguish the relative contributions of individual groups of organisms to the particular biological activity. Such attempts are usually based on the use of relative inhibitors and subsequent measurements of the residual activity. It is, however, difficult to ensure that any inhibitor added to a soil will come into contact with all susceptible organisms. Furthermore it is at present not possible to select an inhibitor that will affect all organisms in a particular group, even if efficient contact is certain. Nor is it possible to select an inhibitor that will not affect organisms from other groups. For these reasons it is still usual to concentrate on measurements of total activity in a particular process. Respiration is usually measured from carbondioxide evolution (Runkles et al. 1958) and oxygen uptake (Stotzky 1965; Stotzky 1972).

5.1.3.3 Nitrogen Transformation

The conversion of organic nitrogen to inorganic forms in the soil is the result of a series of processes which are extremely sensitive to the action of pollutants and environmental factors. It also bears an obvious relationship to soil fertility and is consequently the most frequently studied criterion to study the effects of pesticides on microorganisms. In most instances the transformations are measured as the production of nitrate during incubation of soil. This, it should be realized, is merely an overall measure of all the processes involved and thus gives no idea of which single process or group of processes is affected by a particular treatment. This lack of specificity can be overcome to some extent by making simultaneous measurements of ammonium nitrite and nitrate.

Any survey of the literature quickly reveals that there is often confusion between the terms mineralization, ammonification, and nitrification. Mineralization is a general term which is applied to the process of conversion of organic nitrogen to any form of inorganic nitrogen. As such it embraces ammonification, which specifically relates to the conversion of organic compounds to ammonium compounds. Usually the subsequent conversion of the ammonium to nitrite and finally nitrate is very rapid and hence ammonium may be a purely transitory stage in the transformation of the original nitrogen. This process of oxidation of ammonium to nitrite and nitrate is termed nitrification. Nitrification originally signifies both mineralization of organic nitrogen to ammonium and subsequent conversion to nitrate. The present confusion of terms appears to be due to the continued use of nitrification. It should be remembered that the measurement of the products of nitrogen mineralization and nitrification is not a true measure of the activity of these processes, but is the difference between production and utilization. Some mention must also be made of denitrification, i.e., the conversion of nitrate to gaseous nitrous oxide and nitrogen during respiration. This process, achieved by nitrate reduction and cytochromes is inhibited by oxygen. Generally the highest rates of denitrification are to be found in waterlogged soils, especially when nitrate fertilizer has been supplied or when nitrification has occurred.

Only comparatively few investigators have measured the effects of insecticides on nitrogen changes in soil in field plots. The principal method of following trans-

formation in soil has been, and still is, the measurement of nitrate production during incubation of the soil and a number of examination perfusion techniques have been used. One major drawback is that conditions in a column of soil perfused with a solution bear little relationship to those that occur in field soil. It is, therefore, even more difficult to extrapolate the results obtained to field conditions than when using standard soil incubation techniques. The perfusion technique has been applied using unamended soils and soils amended with nitrogen compounds, particularly ammonium salts. The chief advantage of amending soils with ammonium salts is that it allows results to be obtained faster and can reveal any possible stimulation of nitrification. Such stimulation may not be detected in unamended soil if the rate of ammonification is so low. It might be argued, however, that this advantage is outweighed by the fact that addition of a substrate can cause major shifts in the microbial equilibrium in the soil and thus give misleading results.

5.1.4. Organic Matter Decomposition

One of the most important processes in soil is the decomposition of organic matter and plant residues. The effects of insecticides on these processes have not been studied both in soil and in pure microbial cultures. The most promising technique available at present for the routine assessment of the effects of chemicals on the decomposition of organic residues in soil is the measurement of $^{14}CO_2$ from ^{14}C-labeled substrates. This technique allows a continuous measurement to be made of decomposition of the substrate without periodically removing it from the soil.

5.2 Algae

Like many other organisms, algae also exist in soil and water in association with other species; it is illogical to presume that study of single species in pure culture will demonstrate the probable effects of an insecticide on that organism in the natural environment. Whereas simple chemical and physical factors that influence algae are many, it is difficult to consider all of them together.

Tchan (1952) described a method of enumerating algae using a hemocytometer and a standard light microscope modified to provide the required wave length for chlorophyll excitation. In pure culture or unialgal cultures, algae are maintained under laboratory conditions under fluorescent lamps and treated with insecticide at different concentrations and the samples are examined periodically to determine the survival potential of algae in the presence of insecticide compared to that of untreated controls.

Algae either enumerated under microscope for cell number measurements or dry or wet weights are estimated by simple techniques. Mostly algal growth is estimated by extraction and quantification of photosynthetic pigment. Tchan (1959) used organic solvents to extract chlorophyll and measured spectrophotometrically.

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CHAPTER 4

Microbial Accumulation of Insecticides

SUKANYA LAL

1 Introduction

The extensively used insecticides are common contaminants of microbial environments, and their residues are rapidly picked up by the microorganisms. This is particularly important in aquatic environments where these residues are transferred through the food chain to higher organisms, resulting in an ecological hazard. However, it is surprising to find that very little attention has been given to the study of bioaccumulation in microorganisms. Possibly the lack of interest stems from the fact that much attention has been paid to the role of microbial metabolism of insecticides.

The literature on bioaccumulation of insecticides has been reviewed recently (Boughman and Paris 1981; Lal and Saxena 1982; Lal 1982, 1983). In this chapter the subject of microbial bioaccumulation is critically analyzed, with major emphasis on quantitative aspects of accumulation, factors influencing accumulation and mechanisms of accumulation.

2 Quantitative Aspects

The process of accumulation has been quantitatively expressed by the use of many terms such as bioaccumulation, bioconcentration and ecological magnification (Bevenue 1976). The definitions of these terms are summarized as follows:

Bioconcentration – the amount of insecticide residue accumulated by an organism by adsorption and/or absorption and via oral or other routes of entry. This has been quantitatively expressed in terms of percentage accumulation and bioconcentration factors. Percentage accumulation is the amount of substance accumulated in percent of the amount initially added to the medium.

Bioconcentration factor – the ratio of the concentration of substance in biological materials expressed in ppm(dry/wet weight) to its concentration in water in ppm.

Ecological magnification or biomagnification – the occurrence of a substance at increasingly higher concentration with increasing trophic levels in the food chain.

Although the direct approach which involves the use of percentage accumulation and bioconcentration factor is more accurate, an indirect approach which involves the use of partition coefficient has also been suggested. The partition coeffi-

cient (K_B) is the ratio of equilibrium concentration of solute in the cell $(P)_{cell}$ and medium $(P)_{H_2O}$

$$K_B = \frac{(P)_{cell}}{(P)_{H_2O}} \quad (1)$$

According to Boughman and Paris (1981) K_B is also equal to the ratio of rate constants that describe the transfer between solution and sorbed phase if $(P)_{cell}$ is directly in equilibrium with pollutant in solution.

$$(P)_{H_2O} \underset{K_1}{\overset{K_1}{\rightleftharpoons}} (P)_{cell} \quad (2)$$

Thus Eqs. (1) and (2) provide a mathematical statement of the definition of bioaccumulation if the metabolism is negligible.

In 1909 Freundlich proposed the following equation to describe adsorption phenomenon.

$$(P)_{cell} = K_f (P)^{1/n}_{H_2O}$$

Since a major part of the insecticide accumulates by adsorption in microorganisms, Freundlich's equation has also been used to express the bioaccumulation of insecticides in microorganisms. The use of this equation probably stems from the lack of distinction between adsorption and absorption. The value of the constant. 1/n, approaches unity for many systems at low concentration. In that case the Freundlich constant K_f is equivalent to K_B and one obtains the same results as with Eq. (1). Further, the use of partition coefficients to express the bioaccumulation of insecticides is becoming popular as the partitioning of insecticide between lipids of the cell and water is nearly equal to the partitioning of insecticides between organic solvents and water. Thus one can get an idea about the accumulation of the insecticides in biological systems simply from the values of partition coefficients between organic solvent and water. This is further evident from the following data on toxaphene bioaccumulation (Paris et al. 1977).

Organism	Bioconcentration factor
Bacillus subtilis	0.34×10^4
Flavobacterium harrisonii	0.52×10^4
Aspergillus sp.	1.7×10^4
Chlorella pyrenoidosa	1.7×10^4
Average of axenic cultures	1.1×10^4
Field samples (bacteria and algae)	0.66×10^4
n-Octanol/water partition coefficient	0.33×10^4

Hamelink et al. (1971) have suggested that the water insolubility of a highly lipid-soluble insecticide provides the driving force in producing lipid storage through a series of simple partitioning from water to lipids. Metcalf et al. (1975) have correlated the ecological magnification values for DDE and PCB's from fish in model ecosystems with both water solubilities and with the octanol/water partition values of the insecticides. They found that bioconcentration values for PCB's and DDE fall

closely in relation to their partitioning in octanol and water. Johnson and Kennedy (1973) reported that in bacteria DDT was accumulated two to three times more than methoxychlor. The partition coefficient values for DDT and methoxychlor in a hexane acetonitrile system were 0.38 and 0.069 respectively, indicating that methoxychlor is more polar or less lipid-soluble than DDT, which thus provides an explanation why two to three times as much DDT was accumulated than methoxychlor.

3 Bioconcentration

Although the uptake of insecticides from solutions by microorganisms has been studied extensively, there have been relatively few attempts to establish a quantitative relationship to describe bioconcentration. In many cases microbial bioconcentration has been inferred from the decrease in solution concentration after incubation with microorganisms. The limitation of this type of approach is that insecticide metabolism and volatilization occur in the medium and the investigator is not certain whether the insecticide is being accumulated directly, metabolized, or excreted out.

In general, microorganisms show a marked capacity to accumulate insecticides from the medium and the degree of accumulation varies with insecticide concentration in the medium and the type of microorganism (Table 1). There also exists a wide difference in the accumulation capacities between different groups of microorganisms. For instance, three species each of fungi, streptomycetes, and bacteria differed markedly in their ability to accumulate DDT and dieldrin from distilled water (Chacko and Lockwood 1967). After 4 h of incubation the fungi accumulated 60% to 83% of the DDT and dieldrin. The streptomycetes accumulated 63% to 83% of the DDT and 73% to 83% of dieldrin. However, during the same period bacterium *Agrobacterium tumefaciens* accumulated 100% of the DDT and 90% of the dieldrin, whereas *Serratia marcescens* and *Bacillus subtilis* each accumulated only 60% of the dieldrin and 48% and 38% of the DDT respectively. The incomplete recovery of DDT and dieldrin from cultures of these bacteria was probably due to elution of a portion of the insecticides during the washing of their cells.

Ciliate protozoans have been reported to accumulate DDT to a varying degree. *Stylonychia notophora* (Lal et al. 1981) and *Tetrahymena pyriformis* (Lal et al. 1980; Agarwal et al. 1982) accumulated less than 0.5% of DDT, whereas *Blepharisma intermedium* (Saxena et al. 1982) accumulated 90% of DDT under similar experimental conditions. Further studies on the accumulation of DDT metabolites in *Tetrahymena pyriformis* revealed that under similar experimental conditions metabolites of DDT were accumulated more readily than DDT. The order of accumulation was p,p′-DDD > p,p′-DDE > p,p′-DDT > DDMU > p,p′-DDT.

The ability to accumulate DDT is common to a variety of fungi under different experimental conditions (Boush and Batterton 1972). The results obtained by Ko and Lockwood (1968) indicate that species of different fungi differ in their capacity to accumulate DDT. However, it is not possible to compare the abilities of many fungi to accumulate DDT, since either different amounts of mycelia were used, or

Table 1. Accumulation of organochlorine insecticides by microorganisms

Insecticide	Microorganism	Insecticide in medium	% Removed from medium or bio-concn. factor	References
Aldrin	Lake bacteria	1 ppm	80 – 100	Leshniowsky (1970)
	Zalerion maritimum	10 – 100 ppm	2,000	Sguros and Quevedo (1978)
	Anabaena cylindrica	1 ppb	1.29×10^2	Schauberger and Wildman (1977)
	Anacystis nidulans	1 ppb	9.9×10^2	Schauberger and Wildman (1977)
Chlordane	*Scenedesmus quadricauda*	0.1 – 100 ppb	6,000 – 15,000	Glooschenko et al. (1979)
	Caulobacter vibrioides	0.1 ppb	55,900	Grimes and Morrison (1975)
DDT	*Mucor remannianus*	0.1 – 1 ppm	87	Chacko and Lockwood (1967)
	Glomeralla cingulata	0.1 – 1 ppm	70	Chacko and Lockwood (1967)
	Trichoderma viride	0.1 – 1 ppm	60	Chacko and Lockwood (1967)
	Streptomyces lavendulae	0.1 – 1 ppm	83	Chacko and Lockwood (1967)
	Streptomyces venezuelae	0.1 – 1 ppm	70	Chacko and Lockwood (1967)
	Bacillus subtilis	0.1 – 1 ppm	38	Chacko and Lockwood (1967)
	Serratia marcescens	0.1 – 1 ppm	48	Chacko and Lockwood (1967)
	Agrobacterium tumefaciens	0.1 – 1 ppm	100	Chacko and Lockwood (1967)
	Rhodotorula gracilis	2 ppm	97	Boush and Batterton (1972)
	Torulopsis utilis	2 ppm	94	Boush and Batterton (1972)
	Dunaliella tertiolecta	80 ppb	97.8	Bowes (1972)
	Cyclotella nana	80 ppb	96.6	Bowes (1972)
	Thalassiosira fluviatilis	80 ppb	94.4	Bowes (1972)
	Amphidinium carteri	80 ppb	93.2	Bowes (1972)
	Rumen protozoans	40 ppm	95	Kuches and Church (1971)
	Bacillus subtilis	1.7 ppm	75	Hicks and Corner (1973)
	Stylonychia notophora	1 ppm	0.5	Lal et al. (1981)
	Tetrahymena pyriformis	1 ppm	0.5	Lal et al. (1980)
	Blepharisma intermedium	1 ppm	90	Saxena et al. (1982)
	Aerobacter aerogenes	1 ppb	1140 – 3400	Johnson and Kennedy (1973)
	Bacillus subtilis	1 ppb	1140 – 3400	Johnson and Kennedy (1973)
	Microcystis sp.	1,000 ppb	230	Vance and Drummond (1969)
	Anabaena sp.	1,000 ppb	268	Vance and Drummond (1969)

	Scenedesmus sp.	1000 ppb	134	Vance and Drummond (1969)
	Oedogonium sp.	1000 ppb	207	Vance and Drummond (1969)
	Amphidinium carteri	Ambient	80,000	Cox (1972)
	Syracosphaera sp.	Ambient	25,000	Cox (1972)
	Anacystis nidulans	1 ppm	849	Gregory et al. (1969)
	Scenedesmus obliquus	1 ppm	626	Gregory et al. (1969)
	Euglena gracilis	1 ppm	99	Gregory et al. (1969)
	Paramecium multimicronucleatum	1 ppm	964	Gregory et al. (1969)
	Paramecium bursaria	1 ppm	264	Gregory et al. (1969)
	Pythium ultimum	42 ppm	0.22	Ko and Lockwood (1968)
	Rhizocotonia solani	42 ppm	0.2	Ko and Lockwood (1968)
	Aphanomyces eutraiches	42 ppm	0.15	Ko and Lockwood (1968)
	Fusarium solani	42 ppm	0.1	Ko and Lockwood (1968)
	Cladosporium cladosporoides	20 ppm	86	Dalton (1971)
	Isoachlya sp.	20 ppm	85	Dalton (1971)
	Heliscus submersus	20 ppm	79	Dalton (1971)
	Cyclotella nana	0.7 ppb	63	Rice and Sikka (1973a)
	Isochrysis galbana	0.7 ppb	50	Rice and Sikka (1973a)
	Olisthodiscus luteus	0.7 ppb	50	Rice and Sikka (1973a)
	Amphidinium carteri	0.7 ppb	28	Rice and Sikka (1973a)
	Tetraselmis chuii	0.7 ppb	55	Rice and Sikka (1973a)
	Skeletonema costatum	0.7 ppb	93	Rice and Sikka (1973a)
	Cyclotella nana	0.7 ppb	37,600	Rice and Sikka (1973a)
	Isochrysis galbana	0.7 ppb	11,300	Rice and Sikka (1973a)
	Olisthodiscus luteus	0.7 ppb	4600	Rice and Sikka (1973a)
	Amphidinium carteri	0.7 ppb	4300	Rice and Sikka (1973a)
	Tetraselmis chuii	0.7 ppb	5200	Rice and Sikka (1973a)
	Skeletonema costatum	0.7 ppb	31,900	Rice and Sikka (1973a)
Diazinon	*Chlorella* sp.	1 ppm	96	G. L. Butler et al. (1975)
	Golinkiniopsis sp.	1 ppm	82	G. L. Butler et al. (1975)
	Monoraphidium sp.	1 ppm	82	G. L. Butler et al. (1975)
	Actinastrum sp.	1 ppm	78	G. L. Butler et al. (1975)
	Scenedesmus sp.	1 ppm	83	G. L. Butler et al. (1975)
	Nitzschia sp.	1 ppm	77	G. L. Butler et al. (1975)

Table 1 (continued)

Insecticide	Microorganism	Insecticide in medium	% Removed from medium or bio-concn. factor	References
Dieldrin	*Mucor ramannianus*	0.1 – 1 ppm	13	Chacko and Loockwood (1967)
	Glomeralla cingulata	0.1 – 1 ppm	25	Chacko and Loockwood (1967)
	Trichoderma viride	0.1 – 1 ppm	7	Chacko and Loockwood (1967)
	Streptomyces lavendulae	0.1 – 1 ppm	83	Chacko and Loockwood (1967)
	Streptomyces grisens	0.1 – 1 ppm	70	Chacko and Loockwood (1967)
	Streptomyces venezuelae	0.1 – 1 ppm	81	Chacko and Loockwood (1967)
	Bacillus subtilis	0.1 – 1 ppm	16	Chacko and Loockwood (1967)
	Serratia marcescens	0.1 – 1 ppm	16	Chacko and Loockwood (1967)
	Agrobacterium tumefaciens	0.1 – 1 ppm	90	Chacko and Loockwood (1967)
	Anabaena cylindrica	1 ppb	200	Schauberger and Wildman (1977)
	Anacystis nidulans	1 ppb	500	Schauberger and Wildman (1977)
	Nostoc muscorum	1 ppb	1800	Schauberger and Wildman (1977)
	Cyclotella sp.	0.1 ppb	80 – 90	Werner and Morschel (1978)
	Microcystis sp.	1 ppm	130 – 270	Vance and Drummond (1969)
	Anabaena sp.	1 ppm	130 – 270	Vance and Drummond (1969)
	Scenedesmus sp.	1 ppm	130 – 270	Vance and Drummond (1969)
	Oedogonium sp.	1 ppm	130 – 270	Vance and Drummond (1969)
	Ankistrodesmus amalloides	0.72 ppb	320	Neudorf and Khan (1975)
	Zalerion maritimum	10 – 100 ppm	2000	Sguros and Quevedo (1978)
	Skeletonema costatum	1.7 ppb	42	Rice and Sikka (1973b)
	Tetraselmis chuii	1.7 ppb	16	Rice and Sikka (1973b)
	Isochrysis galbana	1.7 ppb	15.5	Rice and Sikka (1973b)
	Olisthodiscus luteus	1.7 ppb	13	Rice and Sikka (1973b)
	Cyclotella nana	1.7 ppb	13	Rice and Sikka (1973b)
	Amphidinium carteri	1.7 ppb	23	Rice and Sikka (1973b)
	Skeletonema costatum	1.7 ppb	15,882	Rice and Sikka (1973b)
	Tetraselmis chuii	1.7 ppb	8588	Rice and Sikka (1973b)
	Isochrysis galbana	1.7 ppb	8235	Rice and Sikka (1973b)
	Olisthodiscus luteus	1.7 ppb	4900	Rice and Sikka (1973b)
	Cyclotella nana	1.7 ppb	4810	Rice and Sikka (1973b)
	Amphidinium carteri	1.7 ppb	982	Rice and Sikka (1973b)

Fensulphothion sulfide	*Klebsiella* sp.	40 ppm	9.8	Timms and MacRae (1983)
	Escherichia coli	40 ppm	14.1	Timms and MacRae (1983)
	Pseudomonas fluorescens	40 ppm	12.4	Timms and MacRae (1983)
	Nocardia opaca	40 ppm	28.8	Timms and MacRae (1983)
	Leuconostoc mesenteroides	40 ppm	11.0	Timms and MacRae (1983)
	Leuconostoc plantarum	40 ppm	7.2	Timms and MacRae (1983)
γ-HcH (Lindane)	*Chlorella pyrenoidosa*	0.01 – 1 ppm	153 – 267	Canton et al. (1977)
	Chlamydomonas sp.	0.01 – 1 ppm	310	Cantom et al. (1977)
	Dunaliella sp.	0.01 – 1 ppm	1500	Canton et al. (1977)
	Achromobacter delicatus	0.1 ppm	200	Grimes and Morrison (1975)
	Bacillus megaterium	0.1 ppm	70	Grimes and Morrison (1975)
	Bacillus subtilis	0.1 ppm	100	Grimes and Morrison (1975)
	Caulobacter vibrioides	0.1 ppm	300	Grimes and Morrison (1975)
	Chromobacterium violaceum	0.1 ppm	70	Grimes and Morrison (1975)
	Enterobacter aerogenes	0.1 ppm	10	Grimes and Morrison (1975)
	Escherichia coli	0.1 ppm	20	Borghi et al. (1973)
	Chlorella sp.	10.6 ppb	2301 – 6561	Hansen (1979)
Methoxychlor	*Aerobacter aerogenes*	1 ppb	1140 – 3400	Johnson and Kennedy (1973)
	Bacillus subtilis	1 ppb	1140 – 3400	Johnson and Kennedy (1973)
	Bacillus subtilis	0.008 – 0.05 ppm	48,000	Paris et al. (1975)
	Flavobacterium hurrisonii	0.008 – 0.05 ppm	1200	Paris and Lewis (1976)
	Aspergillus sp.	0.008 – 0.05 ppm	5200	Paris and Lewis (1976)
	Chlorella pyrenoidosa	0.008 – 0.05 ppm	8400	Paris and Lewis (1976)
	Chlorella sp.	0.01 ppm	34	G. L. Butler et al. (1975)
	Golinkiniopsis sp.	0.01 ppm	63	G. L. Butler et al. (1975)
	Monoraphidrum sp.	0.01 ppm	40	G. L. Butler et al. (1975)
	Actinastrum sp.	0.01 ppm	40	G. L. Butler et al. (1975)
	Scenedesmus sp.	0.01 ppm	37	G. L. Butler et al. (1975)
	Nitzschia sp.	0.01 ppm	57	G. L. Butler et al. (1975)
Mirex	*Chlorococcum* sp.	5 ppb	88	Hollister et al. (1975)
	Dunaliella tertiolecta	5 ppb	79	Hollister et al. (1975)
	Thallasiosira pseudonana	5 ppb	79	Hollister et al. (1975)
	Chlamydomonas sp.	5 ppb	55	Hollister et al. (1975)
	Chlorococcum sp.	5 ppb	7300	Hollister et al. (1975)

Table 1 (continued)

Insecticide	Microorganism	Insecticide in medium	% Removed from medium or bio-concn. factor	References
Mirex	*Dunaliella tertiolecta*	5 ppb	4100	Hollister et al. (1975)
	Thallasiosira pseudonana	5 ppb	5000	Hollister et al. (1975)
	Chlamydomonas sp.	5 ppb	3200	Hollister et al. (1975)
	Tetrahymena pyriformis	0.9 ppb	82–193	Cooley et al. (1972)
Parathion	*Anacystis nidulans*	1 ppm	50	Gregory et al. (1969)
	Scenedesmus obliquus	1 ppm	72	Gregory et al. (1969)
	Euglena gracilis	1 ppm	62	Gregory et al. (1969)
	Paramecium bursaria	1 ppm	94	Gregory et al. (1969)
	Paramecium multimicronucleatum	1 ppm	116	Gregory et al. (1969)
Toxaphene	*Bacillus subtilis*	0.1 – 5 ppm	3.4×10^3	Paris et al. (1977)
	Flavobacterium harrisonii	0.1 – 5 ppm	5.2×10^3	Paris et al. (1977)
	Aspergillus sp.	0.1 – 5 ppm	1.7×10^4	Paris et al. (1977)
	Chlorella pyrenoidosa	0.1 – 5 ppm	1.1×10^4	Paris et al. (1977)

insufficient data were reported. Barry (1968) found that the rate at which DDT is taken up by *Tetracladium setigerum* increases with increasing amounts of mycelium, indicating that the biomass of the organisms must be taken into consideration in order to make any comparison of insecticide accumulation.

The bioconcentration of DDT is rapidly achieved by both dead and living fungi. Dalton (1971) investigated the persistence of ^{14}C-DDT in a medium to which living and ethylene oxide-killed mycelia of *Heliscus submersus, Isoachlya* sp and *Cladosporium cladosporioides* were added. Between 62% and 83% of the insecticide was lost from the medium within 15 min. The loss increased over a period of 24 h to between 71% and 95%. Both the living and dead fungi accumulated the insecticide, but after 24 h the live culture contained up to 5% more DDT mg^{-1} mycelium. Kallman and Andrews (1963) also found that living cultures of *Saccharomyces cerevisiae* accumulated much more insecticide than control-containing boiled cells.

Algae also vary considerably in their response to accumulate insecticides. Rice and Sikka (1973a) tested different species of algae for their ability to accumulate DDT from the medium initially containing 1 ppb. All the algae tested accumulated DDT rapidly and in the diatom *Skeletonema costatum* and *Cyclotella nana* the accumulation of DDT was linearly related to its concentration in the medium. However, an increase in cell density decreased the capacity of individual cells to accumulate DDT. Rice and Sikka (1973b) also studied the accumulation of dieldrin in algae. The uptake of dieldrin showed a linear relationship between accumulation and cell density in *Amphidinium,* but it was not linear in *Skeletonema, Tetraselmis, Cyclotella, Isochrysis* and *Olisthodiscus.*

Hill and McCarty (1967) reported that DDT accumulation by a mixed algal culture was approximately twice that absorbed from water by bentonite clay. Since algae are numerous in lakes and streams it was considered that these algae containing the insecticide would contribute to the eventual anaerobic decay of the insecticides in bottom sediments by settling down to the bottom after death. Further indications that algae could collect DDT or its metabolites from water was provided by Ware et al. (1968), who examined DDT levels in algae from an irrigation canal. The algae, especially the filamentous *Cladophora,* accumulated higher residues of the DDT and its metabolite DDE than did plants, suggesting that *Cladophora* could serve as an indicator of DDT contamination of water.

Synechococcus elongatus, a blue-green algae (Cyanobacterium) isolated from a stream, was found to contain DDT (Worthen 1973). The culture of this species was raised under laboratory conditions. The algae rapidly removed a high percentage of DDT from the medium and the accumulated levels were subsequently retained. Earlier, Ware et al. (1968) also reported that the alga *Cladophora* accumulated DDT and retained it without any apparent excretion. They suggested that this organism could serve better than *Oscillatoria* as an indicator of DDT contamination in surface irrigation water.

Very little information is available on the microbial bioaccumulation of organophosphorus insecticides. This may be partly due to the less persistent and biodegradable nature of these insecticides. Ahmed and Casida (1958) found that certain bacteria, yeasts and algae rapidly accumulated Thimet and then released it slowly from living and dead cells. The bacteria *Pseudomonas cepacia, Xanthomonas* sp., *Commomonas terrigera* and *Flavobacterium meningosepticum* accumulated mala-

thion more rapidly than did fungus *Aspergillus oryzae* (Paris et al. 1975). The rate of malathion accumulation in bacteria was approximately 5000 times faster than in *Aspergillus oryzae* under similar experimental conditions. However, malathion concentration in the organisms declined subsequently, since most of the malathion was metabolized by the microorganisms.

4 Bioconcentration Factors and Biomagnification

To survey the bioconcentration factors that have been measured for microorganisms, values are selected from the data mainly on laboratory experiments (Table 1). It is very difficult to present a generalized account of the research work on this aspect, due to the entirely different conditions under which the experiments have been carried out. Ideally of course, the same species should be used for the whole range of insecticides under similar conditions but at present such complete information is not available, or if available is not expressed in suitable form.

Cox (1970) determined the bioconcentration factor of DDT in pure cultures of marine phytoplankton at concentrations equivalent to natural low ambient levels. The bioconcentration factor obtained was 25,000-fold and 80,000-fold for *Syracosphaera* sp. and *Amphidinium carteri* respectively, for exceeding that reported for the diatom *Cylindrotheca closterium* by Keil and Priester (1969). The accumulation of dieldrin by benthic algal communities was studied in laboratory structures by Rose and McIntire (1970). Dieldrin concentration ranging from 0.5 to 7.0 ppb were maintained in the water of laboratory streams for a period of 2–4 months. Analysis of algal samples by gas liquid chromatography revealed dieldrin concentration ranging from 0.1 to 200 ppm giving a bioconcentration factor up to 30,000 times. Gregory et al. (1969) exposed the cultures of blue-green alga *(Anacystis nidulans),* a green alga *(Scenedesmus obliquus),* a flagellate *(Euglena gracilis)* and two ciliates *(Paramecium bursaria* and *Paramecium multimicronucleatum)* to DDT and parathion at 1 ppm. These algae and protozoa concentrated DDT to 99 to 964 times and parathion to 50 to 116 times during 7-day exposure period. *Paramecium multimicronucleatum* accumulated the highest levels of both the insecticides. Only small amounts of DDT or parathion remained in the supernatant liquid after 7 days. In *Ankistrodesmus amalloides,* the bioconcentration factor (1000 cells ml^{-1}) was time-dependent and was highest for DDT (Wilkinson et al. 1964), followed by dieldrin (Harris 1969) and photodieldrin (Blanke et al. 1978).

High residues of certain insecticides have been found frequently in fish and birds. Some investigators have hypothesized that the food chain magnification of these chemicals may act as an important causative factor in producing such residue levels in higher vertebrates. The food chain magnification hypothesis was predicted on the assumption that chemical pollutants enter the food chain and are accumulated and magnified as they pass through succeeding trophic levels, resulting in the high chemical residues in top level consumers. Conceivably this could mean that a chemical just within the detection limits in water, essentially innocuous, may rapidly reach lethal or detrimental concentration as a result of biomagnification. The direct evidence for such a biomagnification of insecticides was provided by P.A. Butler

(1967). He observed that DDT and its metabolites applied to estuarine waters were absorbed by plankton almost immediately. The biomagnification for residues in food levels progressed from an estimated 1.0 ppb DDT and related metabolites in water to 70 ppb in plankton to 15 ppm in fish and up to 800 ppm in porpoise blubber. The clam *Rangia cuneata,* which was allowed to feed on dieldrin-contaminated *Dunaliella peircei* for 48 h, exhibited a biomagnification of dieldrin residues in tissues up to 54 times greater than the concentration resulting from the resuspension of contaminated algal cells in clean sea water (Petrocelli et al. 1975a). Further, the food chain transfer of dieldrin residues from contaminated *Rangia cuneata* tissues to the blue crab *Callinectes sapidus* has also been demonstrated (Petrocelli et al. 1975b). Metcalf et al. (1975) studied the biomagnification of DDE in alga, snail, mosquito, and fish. The biomagnification increased from 11,251 in alga to 36,342 in snail to 59,390 in mosquito to 120,370 in fish. Therefore, organisms feeding on contaminated materials will have an increased body burden of these insecticides, which may cause mortality or significantly affect their normal physiological function.

Hansen (1980) examined the uptake, transport and accumulation of lindane directly and through a freshwater food chain consisting of *Chlorella* sp. *Daphnia magna* and *Gasterosteus aculeatus.* He, however, reported that the uptake directly from the water was rapid, while the uptake via the food chain was relatively slow and dependent on the duration of the experiment and the feeding rate.

5 Patterns of Accumulation

A few attempts have been made to investigate the patterns of uptake of insecticides in microorganisms. In general the rate of uptake of insecticide is very rapid and in certain cases it takes only a few seconds to accumulate the insecticide to the highest level. However, the time required to reach equilibrium differs from organism to organism and among microorganisms bacteria show an extraordinary ability to rapidly accumulate the insecticides. For instance, *Agrobacterium tumefaciens* took only 15 min to accumulate 90–100% DDT and dieldrin. However, within the same period *Serratia marscens* and *Bacillus subtilis* each accumulated only 16% of dieldrin and 40% and 38% of DDT respectively (Chacko and Lockwood 1967). The rate of accumulation of DDT and dieldrin by mycelia of streptomycetes and *Trichoderma* was also rapid. Streptomycetes and *Trichoderma* accumulated 80% and 76% respectively of the dieldrin in 15 min. These organisms took up DDT at a somewhat slower rate, accumulating 70% and 60% respectively in 4 h. The algae *Ankistrodesmus amalloides,* however, required 1 to 3 h to accumulate maximum levels of DDT (Neudorf and Khan 1975). DDT was also rapidly taken up in *Tetraselmis chuii* (Rice and Sikka 1973a). About 90% of the maximum uptake occurred within 2 h after exposure. Similar rapid uptake of DDT was observed in *Cyclotella nana, Isochrysis galbana, Olisthodiscus luteus, Amphidinium carteri,* and *Skeletonema costatum.* In alga *Chlorella pyrenoidosa* (Södergren 1968) and the yeast, *Torulopsis utilis* (Boush and Batterton 1972) the accumulation of DDT was very rapid: these species required only 15 s and 3 min respectively to accumulate DDT to the highest levels. Yeasts *Saccharomyces cerevisiae* (Voerman and Tammes 1969) and *Rhodotorula gracilis* (Boush

and Batterton 1972) also required 3 min to obtain equilibrium. In ciliate protozoans, unlike many microorganisms, the rate of uptake of DDT is not so rapid. In *Stylonychia* and *Tetrahymena* maximum accumulation of DDT occurred within 1 and 12 h respectively (Lal et al. 1980, 1981; Agarwal et al. 1982). However, in *Blepharisma intermedium,* although the rate of uptake of DDT was rapid in the beginning, equilibrium was not reached until the end of the experiment, which lasted for 10 days (Saxena et al. 1982).

Microorganisms have also been reported to eliminate the insecticide rapidly when transferred to a toxicant-free medium. The desorption of DDT and its metabolites when *Tetrahymena pyriformis* was transferred to a toxicant-free medium occurred in two phases: initial rapid phase followed by the slow phase (Lal et al. 1980). During the initial rapid phase more than 50% of the DDT or its metabolites was eliminated from the organism and during the slow phase about 30% DDT residue was lost. In *Aerobacter aerogenes* and *Bacillus subtilis* exposed to DDT and washed with DDT-free medium, the DDT residue decreased by 55% and 70% respectively (Johnson and Kennedy 1973). However, DDT residues were retained and not released into the fresh medium in *Chlorella* (Södergren 1968) and *Euglena* (Dekoning and Mortimer 1971).

Pattern of uptake of dieldrin in *Dunaliella peircei* is shown in Fig. 1 (Petrocelli et al. 1975a). The rate of uptake increased rapidly till 24 h, followed by a rapid decline afterwards till 48 h and slow but regular decrease till the end of the experiment which lasted for 96 h. During the first 24 h, 12.1 μg g^{-1} dieldrin was accumulated, which corresponded to 45% of the initial concentration of dieldrin added to the culture. This also represents a general picture of the pattern of accumulation in most of the microorganisms. However, the pattern of uptake of dieldrin in *Cyclotella* from a solution containing 1 ppb dieldrin was slightly different (Rice and Sikka 1973b). Maximum uptake of dieldrin occurred within the first 24 h of its addition to the culture and no significant change in the accumulation of dieldrin was observed afterwards. These data corresponded to those of Reinert (1972) on uptake of dieldrin by a freshwater alga *Scenedesmus obliquus.* Reinert reported an equilibrium which was established at some time between 24 and 36 h of exposure.

A common observation which emerges from the data is that in general the rate of uptake is rapid in the beginning and the equilibrium is attained within a short

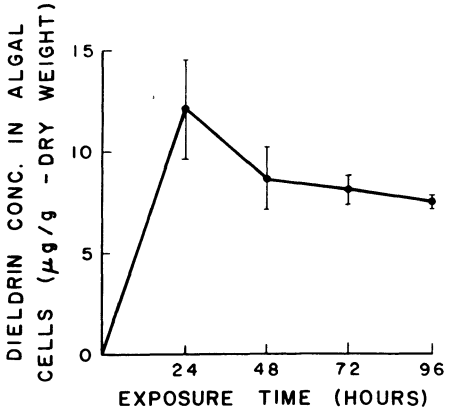

Fig. 1. Pattern of dieldrin uptake in *Dunaliella peircei.* (Petrocelli et al. 1975a)

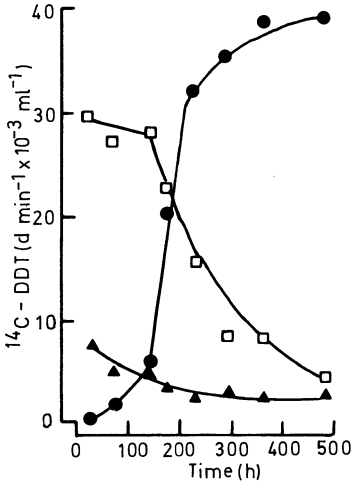

Fig. 2. The pattern of accumulation of ^{14}C-DDT in cultures of *Chlorella fusca.* Cultures of *Chlorella fusca* (initial inoculum 10^4 cells ml^{-1}) received ^{14}C-DDT to give an initial specific activity of 1 μg and 0.02 μCi ml^{-1}. At 2- or 3-day intervals samples were withdrawn and ^{14}C present in the culture medium (▲) the hexane wash (□), and the algal cells (●) was determined. (Goulding and Ellis 1981)

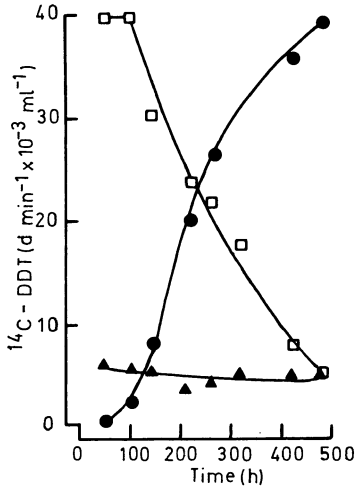

Fig. 3. The pattern of accumulation of ^{14}C-DDT in cultures of *Anabaena variabilis.* Cultures of *Anabaena variabilis,* (initial inoculum 10^6cells ml^{-1}) received ^{14}C-DDT to give an initial specific activity of 1 μg and 0.02 μCi ml^{-1}. At 2- or 3-day intervals samples were withdrawn and ^{14}C present in the culture medium (▲), the hexane wash (□), and the algal cells (○) was determined. (Goulding and Ellis 1981)

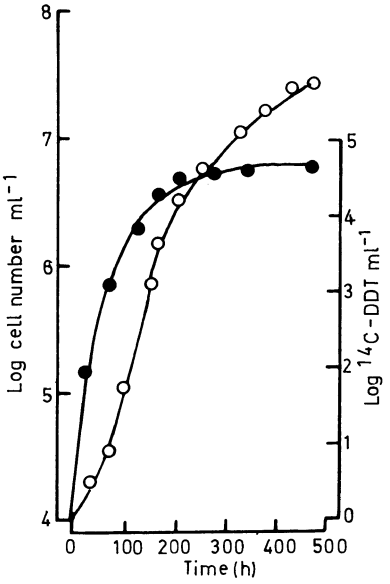

Fig. 4. Relationship between ^{14}C-DDT accumulation (●) and growth (○) or *Chlorella fusca.* (Goulding and Ellis 1981)

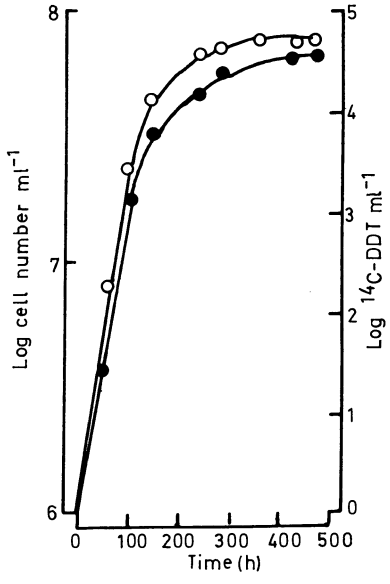

Fig. 5. Relationship between ^{14}C-DDT accumulation (●) and growth (○) in *Anabaena variabilis.* (Goulding and Ellis 1981)

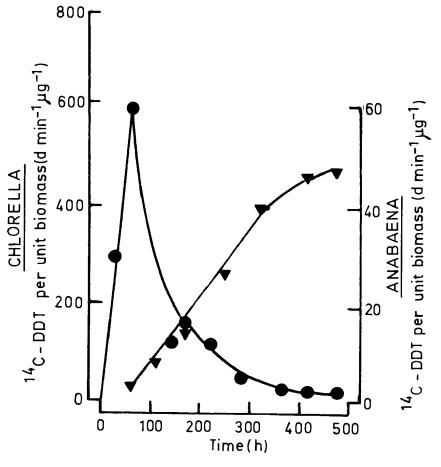

Fig. 6. The amount of ^{14}C-DDT accumulated by *Chlorella fusca* and *Anabaena variabilis* expressed on a unit biomass basis. The amount of ^{14}C-DDT µg^{-1} cells is shown for *Chlorella fusca* (●) and *Anabaena variabilis* (▲). (Goulding and Ellis 1981)

period of time. This is then followed by a decline. This decline, however, does not indicate the decrease in the absolute amount of insecticide taken up by the microorganisms, but a decrease in the amount per unit weight as the biomass increases but the rate of uptake does not increase proportionately. Hansen (1979) carried out a detailed study in *Chlorella* sp. to reveal the pattern of uptake of lindane. The levels of lindane concentration per cell were very high on the first day, fell off on the third day and increased somewhat again until the sixth day. The pattern in terms of bioconcentration factor was also in the same order, i.e., bioconcentration factor of 1236 after 1 day decreased to 429 on the third day and then increased to 710 on the sixth day. In contrast, Goulding and Ellis (1981) reported that although uptake of C^{14}-DDT was rapid in *Chlorella,* which continued to increase till the the equilibrium was obtained after 200 h (Fig. 2), in *Anabaena variabilis,* however, there was a continuous increase in the amount of DDT accumulated till the end of the experiment which lasted for about 500 h (Fig. 3). There was a linear relationship between cell number and the amount of DDT accumulated only till 200 h in *Chlorella* (Fig. 4). In contrast, a linear relationship between DDT accumulation and cell number was observed throughout the experimental period in *Anabaena* (Fig. 5). In this case also the pattern of uptake of C^{14}-DDT was reversed when expressed in terms of bioconcentration factor in *Chlorella* (Fig. 6). However, there was a continuous increase in the bioconcentration factor in *Anabaena variabilis* (Fig. 6).

6 Factors Affecting Microbial Accumulation of Insecticides

The accumulation of insecticides by microorganisms can be influenced by the physical and chemical characteristics of the insecticide, physical factors, and the type of microorganism. It is, however, surprising that very little attention has been given to studying the effect of these factors on microbe/insecticide accumulation. Only a few reports are available with a wide range of results and interpretations. In the follow-

ing account the role of abiotic and biotic factors that influence microbial accumulation of insecticides is discussed.

6.1 Insecticide Solubility

It is well known that an inverse relationship exists between water solubility and the extent of bioaccumulation in higher organisms. A similar relationship between water solubility of insecticide and bioaccumulation exists also in microorganisms. Metcalf et al. (1975) in their studies with microorganisms and carbaryl, diazinon, malathion, parathion, methoxychlor, and toxaphene showed that the latter two insecticides were accumulated more than others. The distribution coefficients of these two insecticides were greater than 10 and these two insecticides are 50 times less soluble than others. Paris et al. (1975) also reported that the bacterial bioconcentration of DDT, methoxychlor, occurred in the order DDT > methoxychlor > toxaphene, which is inversely related to their solubilities in water (DDT 1.2 $\mu g\ l^{-1}$, methoxychlor 50 $\mu g\ l^{-1}$and toxaphene 500 $\mu g\ l^{-1}$ from Johnson and Kennedy (1973).

The relationship between water solubilities and accumulation of the insecticides in bacteria was also studied by Grimes and Morrison (1975). Their studies also revealed that the degree of accumulation of insecticides was inversely related to water solubilities. The cellular contents of chlorinated hydrocarbon insecticides in 8 of the 13 bacteria were found to have the following order of magnitude: α- or β-chlordane > dieldrin > heptachlor expoxide > γ-HCH. The same relationship has been observed or is apparent from the data of others (Hill and McCarty 1967; King et al. 1969; Voerman and Tammes 1969; Södergren 1968, 1971; Gregory et al. 1969; Chacko and Lockwood 1967; Rice and Sikka 1973a, b).

Not only the total accumulation but the rate of accumulation was reported to be faster in microorganisms (bacteria, *Chlorella* and *Aspergillus)* with methoxychlor

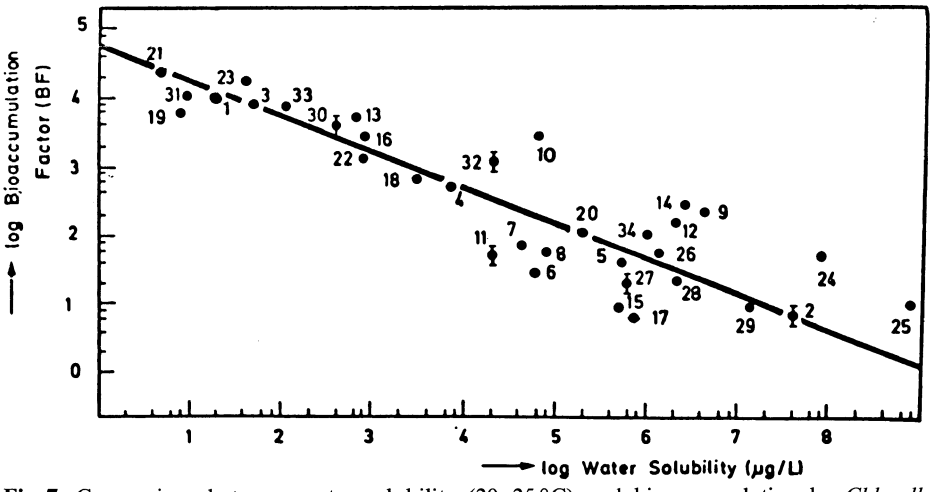

Fig. 7. Comparison between water solubility (20–25 °C) and bioaccumulation by *Chlorella* after exposure to 50 $\mu g\ l^{-1}$ for 24 h. (Geyer et al. 1981)

than with toxaphene, which is less soluble (Paris et al. 1977). A similar relationship between solubility and time exists in the data of Neudorf and Khan (1975) for freshwater alga *Ankistrodesmus amalloides* exposed to DDT, dieldrin, and photodieldrin. Recently Geyer et al. (1981) examined the accumulation of many organic chemicals, including aldrin and carbaryl, in *Chlorella.* They reported a clear inverse relationship between the water solubility of 34 chemicals, the solubility of which ranges between 5 $\mu g\ l^{-1}$ to 800 $g\ l^{-1}$(Table 2 and Fig. 7). That calculated bioconcentration factors were found to be within the order of magnitude of the experimentally elucidiated bioconcentration factor and water solubility, however, held true only for the insecticides which are minimally or not at all metabolized. Thus the water solubility data seem to be a suitable parameter for estimating the bioaccumulation of

Table 2. Comparison between water solubility (20 – 25 °C) and bioaccumulation by *Chlorella* after exposure to 50 $\mu g\ l^{-1}$ for 24 h. (Geyer et al. 1981)

No.	Chemical	Water solubility ($\mu g\ l^{-1}$)	Bioaccumulation factor	
			Experimental	Calculated
1	Aldrin	17	12,600	9620
2	Aniline	3.66×10^7	4	12
3	Anthracene	53	7800	5730
4	Biphenyl	7.48×10^3	540	587
5	p-tert-Butylphenol	6.5×10^5	34	75
6	p-Bromobenzoic acid	5.6×10^4	25	231
7	Carbaryl	4.0×10^4	73	271
8	p-Chlorobenzoic acid	9.0×10^4	63	187
9	p-Chloroaniline	3.9×10^6	260	33
10	Chlorhexidin (Hibitane)	4.2×10^4	2560	265
11	Cortisone acetate	2.0×10^4	40	372
12	Coumarin	2.0×10^6	42	45
13	Di-(2-ethylhexyl)-phthalate	6.0×10^2	5400	1863
14	2,6-Dichlorobenzamide	2.7×10^6	320	39
15	2,4-Dichlorobenzoic acid	4.7×10^5	9	87
16	2,2-Dichlorobiphenyl	7.9×10^2	2700	1640
17	2,4-Dichlorophenoxyacetic acid	9.0×10^5	7	65
18	2,6-Di-tert-butylphenol	2.5×10^3	800	966
19	Dodecane	8.4	6300	13,360
20	Na n-Dodecylbenzene sulfonate	3.0×10^5	64	107
21	Hexachlorobenzene (HCB)	5.0	24,800	16,866
22	Hexachlorocyclopentadiene	8.05×10^2	1090	1623
23	Hexadecanol	41.2	17,000	6433
24	Hydroquinone	7.0×10^7	65	9
25	Maleic acid	7.88×10^8	14	3
26	Metribuzin(Sencor)	1.22×10^6	59	56
27	Monolinuron	5.8×10^5	33	79
28	Nitrobenzene	1.9×10^6	24	46
29	p-Nitrophenol	1.5×10^7	11	18
30	Pentachloronitrobenzene	4.4×10^2	3100	2160
31	2,2′,4,4′,6-Pentachlorobiphenyl	31	11,500	7305
32	Pentachlorophenol	1.4×10^4	1250	437
33	2,5,4′-Trichlorobiphenyl	1.1×10^2	8960	4079
34	2,4,6-Trichlorophenol	9.0×10^5	51	65

chemicals by microorganisms. Further it would be of greater advantage to predict the bioaccumulation factors of the insecticides from such physicochemical properties, since it is difficult to test the bioconcentration factors of all the existing or new insecticides on different organisms.

6.2 Insecticide Concentration

Concentration of the insecticide in the medium has a profound effect on accumulation. For instance, the effect of DDT concentration on bioaccumulation in *Cyclotella* showed that the amount of DDT accumulated increased linearly with an increase in the concentration of DDT upto 100 ppb (Rice and Sikka 1973 a). The increase in uptake was not proportional to the increase in the amount of DDT in the medium beyond 100 ppb. This was attributed to the fact that all the DDT above 100 ppb was not in soluble form. However, in *Amphidinium carteri* the pattern of uptake was linear for DDT, only in the range from 0.17 to 1.08 ppb (Rice and Sikka 1973 b). In *Tetraselmis chuii* and *Amphidinium carteri* the uptake of dieldrin also increased linearly with an increase in the dieldrin concentration upto 1000 ppb (Rice and Sikka 1973 b).

Johnson and Kennedy (1973) examined the insecticide biomagnification in *Aerobacter aerogenes* and *Bacillus subtilis* in relation to the concentrations of insecticides ranging from 0.5 to 5 ppb. When total insecticide residues taken up by the organism were plotted against concentration (0.5 to 5 ppb) a linear relationship was obtained. In addition, the bioconcentration factor was dependent on biomass of microorganisms. When the biomass of *Aerobacter aerogenes* was decreased by 50% (from 200 to 100 $\mu g\ ml^{-1}$), the bioconcentration factor of methoxychlor increased by 68%. A similar inverse relationship was noted with DDT. The bioconcentration factor of the insecticide was almost doubled from 2666 to 5235 times when the biomass of *Aerobacter aerogenes* was reduced by one half. Thus the bioconcentration factor is not absolute, but is correlated to both DDT or methoxychlor concentration in water and the biomass of bacteria.

6.3 Chemical Structure of the Insecticides

The extent of accumulation of HCH isomers in *Saccharomyces cerevisiae* appeared in the order: $\beta < \gamma < \alpha < \delta$, which was different from their order of solubilities in organic solvents and also from their order of toxicity to the microorganisms (Lyr and Ritter 1969). The order of toxicity being $\delta < \gamma < \beta < \alpha$ when the cells were exposed to 3×10^5 M of individual isomers in the medium, the intracellular concentration of α, β, γ and δ-isomers reached constant levels corresponding to the uptake of 60,18,40, and 24% respectively of the compound in the medium. δ-isomer was taken up marginally more readily than the slightly active β-isomer. For these reasons the stereochemical difference between isomers were considered to have an overriding influence on accumulation. On the contrary two isomers of chlordane (α and β) were essentially taken up to the same extent and at the same rate by *Zoogloea ramigera* and *Enterobacter aerogenes* (Grimes and Morrison 1975).

6.4 Temperature and pH

Variation in temperature does not have much influence on the accumulation process. This is evident from the studies of Smith et al. (1978) where they found that the bioconcentration factor for methoxychlor in bacteria decreased from about 480 to 220 during the change in temperature from 25 ° to 2 °C.

The pH of the medium has a marked influence on the uptake of insecticides. For instance, Chacko and Lockwood (1967) reported that *Streptomyces venezuela* took up 90% to 100% of DDT and dieldrin at pH 5.5, 8.0 and 11.0. At pH 3 the accumulation dropped to 75% and 40% for dieldrin and DDT respectively. On the other hand, *Trichoderma viride* accumulated 90% to 100% of each compound at pH 3.0, 5.5 and 8.0. At pH 11.0 accumulation dropped to 80% for dieldrin and 52% for DDT. Grimes and Morrison (1975) also reported that variation in pH influenced the uptake of α and β-chlordane significantly in bacteria and reported pH 7 to be optimum for maximum accumulation of the insecticides.

6.5 Type and Nature of Microorganisms

In addition to solubility of insecticide, its concentration, pH of the medium and temperature, the amount of insecticide accumulated is also dependent on the nature and type of organism. Though no detailed studies are available on this aspect, that the nature of organisms plays an important role is evident from many investigations (Chacko and Lockwood 1967; Södergren 1968; Leshniowsky 1970; Canton et al. 1977; O'Kelley and Deason 1976; Smith et al. 1978; Lal 1980; Saxena et al. 1982; Agarwal et al. 1982).

In general the equilibrium between insecticide concentration in the medium and in the organism is obtained very quickly. The time required to obtain equilibrium is maximum in protozoa (French and Roberts 1976; Lal et al. 1980; Saxena et al. 1982; Agarwal et al. 1982) and less for fungi and algae (Chacko and Lockwood 1967, Paris et al. 1975; Paris and Lewis 1976, Paris et al. 1977) and least for bacteria (Chacko and Lockwood 1967; Leshniowsky 1970; Johnson and Kennedy 1973; Paris and Lewis 1976). Rice and Sikka (1973a) showed that various species of algae differed significantly in their capacity to accumulate DDT. The order of accumulation was: *Skeletonema* > *Cyclotella* > *Isochrysis* > *Olithodiscus* > *Amphidinium* > *Tetraselmis*. The difference was attributed to the taxonomic characteristics of the cell and cell constituents. Werner and Morschel (1978) compared four species of diatoms (*Cyclotella* and *Nitzschia* sp.) with ten species of algae *Chlamydomonas* and *Scenedesmus* for their dieldrin accumulation capacities. The diatoms accumulated 85% to 90% of C^{14}-dieldrin within 1 h whereas the algae accumulated only 35% dieldrin in 1 h, suggesting that diatoms are better absorbers of insecticides than are algae.

6.6 Biomass, Cell Number and Cell Size

In most of the studies insecticide accumulation in microorganisms has not been considered in relation to biomass. The effect of cell number along with uptake has also

not been considered. Rice and Sikka (1973a) found that in algae the accumulation of DDT was linearly related to its concentration in the medium and the number of cells present in the medium. They further emphasized that the surface area exposed to insecticides was higher in dilute cell cultures than in concentrated cultures, also that smaller cells yielding more cell/unit weight should adsorb/absorb more insecticide than a similar culture of species with larger cells. However, their data do not support the latter hypothesis because in their experiments, *Isochrysis galvana* (4×4 μ cell size) at 1.4×10^6 cells ml^{-1} absorbed/adsorbed 12.5 ng DDT mg^{-1} dry weight giving magnification ratio of 17,900, while *Skeletonema costatum* (7×14 μ cell size) at 1.19×10^6 cells ml^{-1}adsorbed/absorbed 18.4 ng DDT mg^{-1} dry weight giving a magnification ratio of 26,800. Also *Amphidinium carteri* (cell size 15×15 μ) at 0.178×10^6 cells ml^{-1} adsorbed/absorbed 6.6 ng DDT mg^{-1} dry weight giving a magnification ratio of 9400 while *Tetraselmis chuii* (9×14 μ cell size) at 0.134×10^6 cells ml^{-1} accumulated only 4.0 ng DDT mg^{-1} dry weight and gave a magnification of only 5700. The findings of Rice and Sikka (1973a) also showed that the uptake of DDT in algae was greatly influenced by the cell density. Increasing the cell density resulted in an increase in the amount of DDT accumulated, although the increase in uptake was not directly proportional to the increase in cell density. Similarly the uptake of dieldrin by algae increased with an increase in the amount of cells (Rice and Sikka 1973b). However, the relationship was found to be linear in the culture of *Amphidinium* but nonlinear in *Skeletonema, Tetraselmis, Cyclotella,* and *Isochrysis.* Cox (1970) exposed a culture of *Dunaliella solina* to 0.015 ppb C^{14}-DDT using a special apparatus which allowed the density of the cells to be gradually increased. After cells were added up to a density about 750 μg l^{-1} the mean concentration of ^{14}C-DDT per cell declined, indicating that no more DDT was available for uptake. At density lower than this figure the uptake was proportional to cell density, indicating that the cells had taken up a maximum amount. Neudorf and Khan (1975) also found that the total amount of ^{14}C-DDT accumulated from the medium containing 0.72 ppb was higher, with denser cell suspensions in *Ankistrodesmus amalloides.*

7 Mechanisms of Accumulation

It is evident from the literature that most of the studies have been designed to show the amount of insecticide accumulated in microorganisms and have indicated little about the mechanisms of accumulation. Södergren (1968) studied the mechanism of uptake of DDT by a *Chlorella* sp. and found that ^{14}C-DDT at a concentration of 0.6 ppb was rapidly taken up by *Chlorella.* The primary uptake was attributed to a passive physical process of adsorption rather than active assimilation. This observation was also supported by Rice and Sikka (1973a), Södergren concluded that the rate of penetration of DDT into algal cells was probably equal to its rate of diffusion in water. Cox (1970) interpreted Södergren's (1968) results of rapid passive ^{14}C-DDT uptake by *Chlorella* in terms of a partition mechanism and considered that the much higher concentration of DDT used by some workers might affect the partition coefficients of organisms for DDT residues in water. In general, the rate of uptake of in-

secticide in microorganisms is very rapid and in certain cases it takes only a few seconds to attain the equilibrium.

The rapid accumulation has been attributed either to adsorption and/or absorption and little has been done to understand whether insecticide adsorbed and/or was absorbed by the microorganisms. Most investigators have avoided this question and some have assumed adsorption (Hill and McCarty 1967, Shin et al. 1970; Voerman and Tammes 1969), whereas others have referred to absorption (Walsh et al. 1977; Wheeler 1970; Cox 1971, 1972), Sguros and Quevedo (1978) have suggested that lindane accumulation by bacteria occurs via adsorption followed by internal diffusion. Adsorption of colloidal aldrin by flocculant bacteria as a removal mechanism was proposed by Leshniowsky (1970).

However, insecticide accumulation in microorganisms seems to be due to both adsorption and absorption. This was demonstrated by Johnson and Kennedy (1973) in bacteria. When the insecticide– exposed cells were washed with insecticide-free water, DDT residues decreased by 45% in *Aerobacter aerogenes* and by 30% in *Bacillus subtilis* and methoxychlor decreased by nearly 75% in the organisms. Subsequent washing did not reduce the insecticide residues, indicating that a part of the insecticide was accumulated by absorption. Wheeler (1970) also demonstrated that dieldrin is accumulated both by adsorption and absorption in *Chlorella.* The demonstration of dieldrin absorption by *Chlorella pyrenoidosa* was based on the following reasoning. Firstly that the quantity of radiolabeled dieldrin increased for varying periods of time after the insecticide had been introduced to the culture. Secondly, the insecticide became more difficult to extract with time, indicating movement, perhaps into subcellular organelles. These two facts strongly support the idea that the insecticide, in addition to adsorption, is also being absorbed by *Chlorella.*

Johnson and Kennedy (1973) proposed that some chemical and physical characteristics of insecticide and the nature of bacteria also play an important role in accumulation. These factors proposed by them are: p-value, gas chromatography retention time of the chemical, surface area, and lipid contents of microorganisms. Ether-extractable lipid contents of *Aerobacter aerogenes* and *Bacillus subtilis* were similar approximately 5% to 6% per unit dry weight. With this similarity one would expect little difference in accumulation of insecticide between these two organisms. However, Johnson and Kennedy (1973) observed no significant differences in DDT accumulation, but *Bacillus subtilis* magnified methoxychlor twice as compared to *Aerobacter aerogenes.* This rules out the possibility that lipid contents of the organism alone decide the amount of insecticide accumulated. Shin et al. (1970) treated the soil with ether and ethanol for removal of lipoidal materials. The treatment, however, increased the adsorption of DDT to the soil, suggesting that components other than the lipoidal material played a larger role in the sorption. *Aerobacter aerogenes* has a surface area ten times greater than *Bacillus subtilis,* which actually accumulated no more residue than *Bacillus subtilis* (Johnson and Kennedy 1973), indicating that surface area alone does not play a significant role. However, the accumulation of these insecticides followed the lipid partition hypothesis for magnification. The partition coefficient values (p-value) for DDT and methoxychlor in a hexane acetonitrile solvent are 0.38 and 0.069 respectively, and indicate that methoxychlor is more polar or less lipid-soluble than DDT. This may explain why two or three times as much DDT as methoxychlor was accumulated by both the organisms.

The possibility that insecticides are incorporated into microorganisms by active or passive mechanisms also provokes special concern. However, many reports indicate that the accumulation of insecticides is almost the same in living and dead cells. Johnson and Kennedy (1973) found that the accumulation rate of DDT and methoxychlor by autoclaved cell was greater than that for living bacteria. After autoclaving the cells of *Aerobacter aerogenes,* the uptake of methoxychlor was double the amount accumulated by living cells. Experiments with yeasts *Saccharomyces cerevisiae* also showed that the accumulation of lindane and dieldrin increased after boiling the organisms and that the two insecticides could be removed by washing (Voerman and Tammes 1969). Rice and Sikka (1973a) found that several species of heat or mercuric chloride-killed algae accumulated almost the same amounts as viable cells except in the case of *Amphidinium carteri,* for which the accumulation of DDT increased from 1.9 to 2.5 times. This has led many investigators to conclude that it is mainly the partitioning of the insecticide between the medium and lipids of the cells that influences accumulation and that no metabolic factor is involved. On the contrary, Chacko and Lockwood (1967) observed that organochlorine insecticides are accumulated against concentration gradient in soil system, and therefore suggested an active transport in microorganisms. This assumption needs to be explained on the basis of the findings of Hansen (1979) on lindane accumulation in *Chlorella pyrenoidosa.* He reported that the rate of accumulation of lindane was very rapid in *Chlorella* sp. during the first 24 h, decreasing until the third day, and increasing somewhat again until the sixth day. The higher accumulation rate of the whole culture was assumed to be due to a higher proportion of young cells with a high initial accumulation. The increase in the accumulation from the third to the sixth day was attributed to the metabolic activities of the algae. The algae in the lethal range had been so seriously damaged that they simply maintained the accumulation level which had been reached by the third day, whereas algae in the sublethal range which could additionally take up the insecticides were in a different metabolic physiological state.

8 Conclusions and Future Prospects

It is evident from the data reviewed that little research has been done on microbial bioconcentration of insecticides. Most investigations are incomplete, with many inconsistencies. For instance, the same organism has been reported to accumulate the same insecticides at different levels. This may in part be due to the different experimental conditions under which these studies have been conducted. Another drawback which has been frequently noticed is the use of a very high concentration of insecticides, making it difficult to draw any conclusion of ecological significance.

The kinetics of insecticide uptake have also not been studied by many investigators. In general, uptake of insecticides occurs in two stages. The first stage, which seems to be passive (physical adsorption), is very rapid and occurs within a short time after the microorganisms come in contact with the insecticide. The second stage is possibly active, related to the metabolic activity of the organisms.

The rapid uptake could be the result of adsorption. But microorganisms can adsorb the insecticide into their surfaces by many processes. The insecticide can be adsorbed by complexing with the lipids of the membranes, as most of the insecticides are lipophilic and membranes are rich in lipids.

By comparison, microorganisms have also received little attention regarding their ability to biomagnify the insecticides through the food chain. Indeed, since some of the microorganisms are known to accumulate these insecticides at a level many times higher than that of the insecticides in the surrounding medium, there is reason to believe that such activities may be far more common than has apparently been considered by the investigators. The potential of the microorganisms to biomagnify insecticides should therefore be given more attention.

Many factors, such as insecticide solubility, its concentration, pH and temperature of the medium, and the nature of the microorganisms influence the bioaccumulation. Such factors must be considered in order to make our studies more reliable. There is also a need to study the mechanisms of accumulation, which can be better done by using labeled insecticides.

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CHAPTER 5

Metabolism of Insecticides by Microorganisms

SUDHAKAR BARIK

1 Introduction

During the past 40 years the use of agrochemicals including pesticides[1] has contributed to a significant increase in major crop production. According to an EPA report prepared by Lawless et al. (1975), there are at least 550 different pesticide chemicals commercially available in the United States alone. Moreover, about 8000 different pesticide "formulations" are sold on the market and over 500 of these products contain two or more "active ingredients". Pesticide usage over recent years has also increased dramatically, from 1.1×10^9 lb yr^{-1} in 1971 to 1.5×10^9 lbs at the end of the decade (Storck 1980). A recent prediction shows that the worldwide expenditure on pesticides will steadily increase from U.S. $ 11.5 billion in 1980 to $ 14.3 billion in 1985. The United States alone will be spending nearly $ 4.5 billion on pesticides by the year 1985 (Farm Chemicals 1981). Thus our environment will remain under continuous pressure from the intensive applications of massive quantities of these toxic chemicals.

Pesticides reaching the soil, sediment or water ecosystems can be degraded by chemical or biological agents (Fig. 1). Biological and chemical decomposition of xenobiotic compounds in such environments are always influenced by the changes of many physicochemical forces such as pH, temperature, ion concentration and redox potential. Consequently, these toxic compounds are decomposed through photometabolism, oxidation, reduction, and hydrolysis: the sum total mechanism of both chemical and biological phenomena. However, it is difficult to differentiate these processes under natural environments unless supported by model laboratory studies.

For many years the fate and metabolism of pesticides in the environment were considered as a natural phenomenon, and only when their long-term presence led to many chronic toxicity problems and affected the food chain system was more extensive research directed to study the pathways of degradation under natural and laboratory conditions. Microorganisms are now believed to be the principal agents which can cleave and modify the complex lipophylic pesticide molecules, once considered recalcitrant to simple water-soluble products. This process has been shown to reduce the toxicity of the xenobiotics from 2 to 800 times (Munnecke et al. 1982). These water-soluble intermediates are usually attacked by primary or secondary group(s) of organisms to form inorganic end-products resulting in complete biode-

1 Chemical names of pesticides mentioned in the text are indicated in the Appendix.

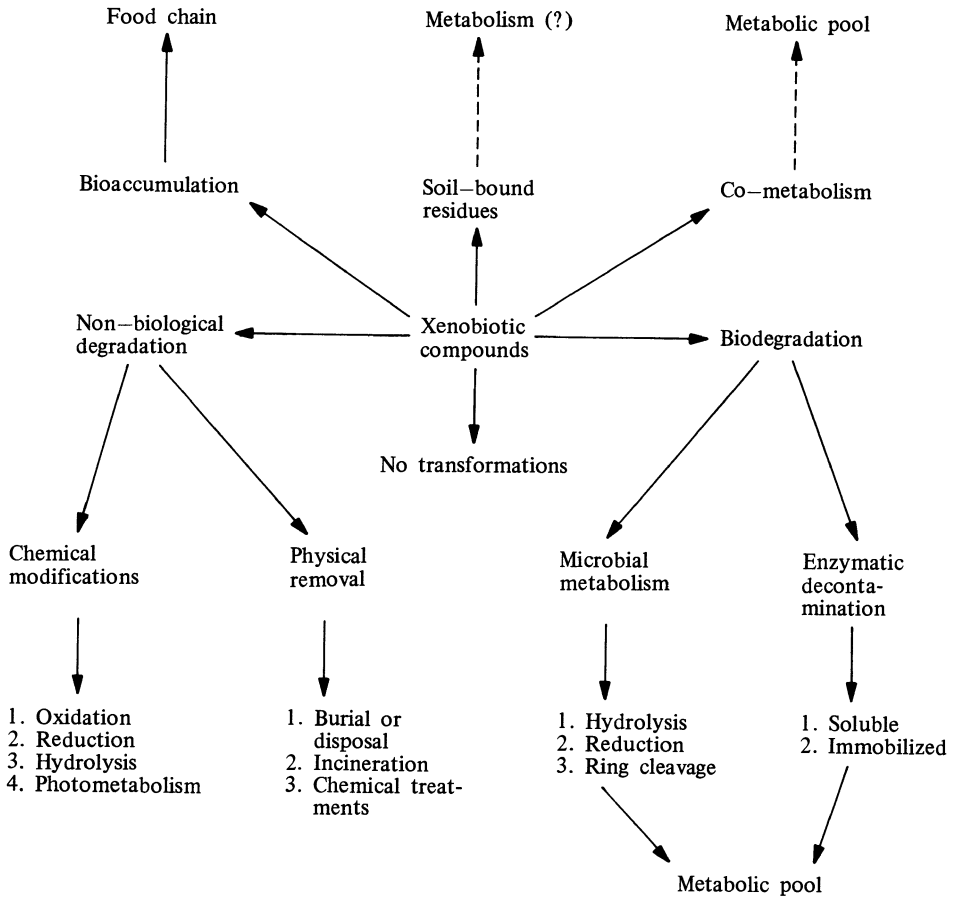


Fig. 1. Fate of xenobiotic compounds in soil, aquatic and microbial systems

gradation. Several mechanisms by which microorganisms adapt and degrade pesticides have been suggested and discussed by many authors (Alexander 1969, 1973, 1974, 1980, 1981; Bollag 1974, 1982; Kaufman 1974; Kaufman et al. 1970; Paris and Lewis 1973; Sethunathan and Siddaramappa 1978).

Microorganisms first attack these organic chemicals by the enzymatic apparatus acquired during the course of enrichment when they are exposed to these specific or structurally related compounds. Presence of these chemicals in the environment either induces or derepresses the enzymatic function of a microorganism (Dagley 1978). This capability largely depends upon the selective microbial community as well as on the structural and functional groups of the xenobiotic compounds. In many instances, when these parameters were carefully considered, novel microbial cultures were isolated for future industrial application.

The intent of this paper is to discuss the major microorganisms isolated and their involvement in insecticide metabolism in natural habitats and/or under laboratory studies. More emphasis will be given to the most commonly used organophosphates,

dithioates, and carbamate insecticides; whereas the organochlorines will be briefly discussed. The central theme of this review will be oriented on the well-established fact that the majority of the pesticides are susceptible to degradation by microorganisms and microbial cultures could be easily isolated employing standard techniques.

2 Adaptation and Enrichment Techniques

In natural habitats, complete biodegradation of many pesticide molecules to inorganic end products is channeled with the participation of indigenous microbial biomass. This process is achieved through the occurrence of stepwise pesticide transformations. A mixed microbial population offers many advantages in pesticide transformation, due to the participation of multiple degrading enzymes. The nature of pesticide-microbial interaction can be studied in the laboratory under simulated conditions corresponding to natural environments. Isolation of microbial cultures, mixed or pure, from natural ecosystems is, therefore, instrumental to establish the pathways of pesticide degradation. Among the several methods used "enrichment" for specific microflora in the presence of test pesticide compounds has proved to be the best practical approach. Microbial cultures have been isolated using pesticides as main source of carbon and energy, nitrogen or phosphorus. Several investigators have isolated pesticide-degrading microorganisms after repeated applications of pesticides to flooded paddy fields, soils, sediments and water samples. More recently, both batch culture or continuous culture techniques have been employed for bacterial enrichment and isolation (Munnecke et al. 1982; Sethunathan et al. 1977).

Model ecosystems are now being extensively used for the evaluation of pesticide disappearance and to predict their physical and microbiological transformations under natural conditions (DiGeronimo et al. 1979; Metcalf 1974; Metcalf et al. 1971; Virtanen et al. 1980). Microcosm or ecocores were subsequently designed and natural microflora were acclimated to many xenobiotics (Pritchard et al. 1979; Spain et al. 1980; Spain and Van Veld 1983). Adaptation of microflora under these test conditions has resulted in the isolation of many microorganisms able to degrade some micropollutants (Spain et al. 1980; Spain and Van Veld 1983), indicating the feasibility of these processes in isolating pesticide-degrading microorganisms.

Many microorganisms, however, do not use these toxic chemicals as the main source of carbon and energy, but possess enzyme systems to make structural modifications. This phenomenon is called co-metabolism (Alexander 1973; Horvath 1972) and can be defined as "a microbial process where an organism transforms organic compounds without using them as energy for their metabolism, resulting in product accumulation with little or no biomass production". The resultant compounds are more vulnerable to microbial attack than the parent molecules. For the cultivation of these co-metabolic organisms in the laboratory, additional energy sources other than pesticide compounds must be provided. Many reactions in nature are now thought to be co-metabolic. Though co-metabolism does not necessarily lead to complete detoxification of a compound, the initial resistance of pesticides to microbial attack is often overcome during this process. Although the feasibility of providing extra co-energy substrates in studying pesticide metabolism is debatable (Hulbert

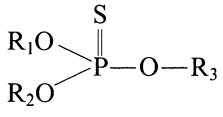
and Kraweic 1977), this process can offer a possible alternative for biodegradation of many toxic xenobiotic compounds (Alexander 1980, 1981).

Other groups of microorganism possess enzymes which favor conjugate reactions between the xenobiotic compounds and naturally occurring substances such as humus and other organic matter. Such reactions do not occur very often, but can significantly prevent other microbial transformations of these toxic compounds.

A fourth important group of organisms is known to incorporate xenobiotics into their cells by the action of active or passive accumulations, leading to biomagnification. This bio-accumulation of toxic chemicals, particularly in freshwater and marine environments, can drastically affect higher food chain systems. For more details on this process, the reader is referred to the review articles published elsewhere (Boush and Batterton 1972; Kenega 1974; Lal and Saxena 1982) and Chapter 4, this Volume.

3 Organophosphates

Pesticides belonging to organophosphates have more broad-spectrum insecticidal activities than many other pesticide chemicals. The general chemical formula of the pesticides of this class is of the type:



where R_1 and R_2 are alkyl (either ethyl or methyl) moieties; and R_3 is a substituted aryl or alkyl group. Organophosphates, such as diazinon, fenitrothion, methyl parathion and parathion are perhaps the most extensively used insecticides under many agricultural practices. Pesticides belonging to organophosphates are known to be rapidly metabolized in plant and animal tissues (Alexander 1969). Organophosphates are also found to be very short-lived in soil and aquatic ecosystems, but our knowledge of metabolism of these compounds prior to 1970 was fragmentary (Alexander 1969). More recently considerable attention has been directed toward a better understanding of their fate and metabolism in these ecosystems with special emphasis on the microbial participation in their degradation under such environments. So far, microorganisms have been shown to be the major contributors to pesticide metabolism and chemical degradation has been considered of minor importance in natural habitats. Microbial degradation through hydrolysis of P-O-alkyl and P-O-aryl bonds are now considered as the most significant steps in the detoxification of organophosphorus compounds.

3.1 Diazinon

Diazinon was introduced for chemical evaluation in the early 1950's and since then has been widely used to control insects in rice, corn, vegetables, fruits and many

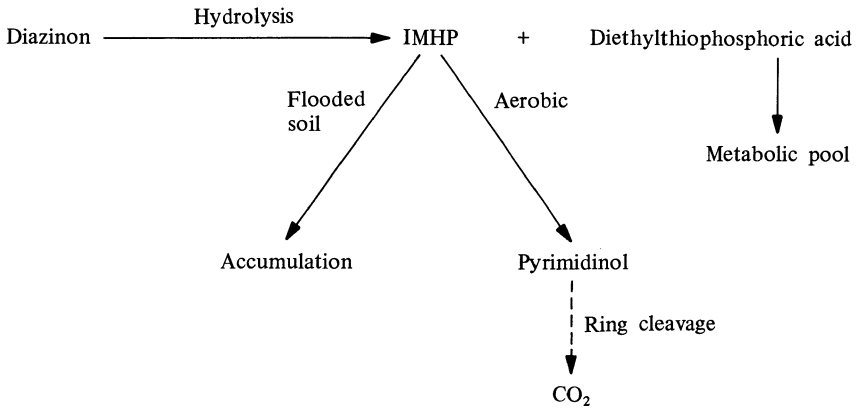


Fig. 2. Proposed pathway for diazinon metabolism in soil, aquatic and microbial systems

other cash crops. Hydrolysis, the major means of detoxification of diazinon, can be chemical and biological (Sethunathan 1972; Fig. 2). The significance of ester bond hydrolysis and participation of native microflora in the detoxification of these compounds was realized in the early 1970's. Hydrolysis of diazinon in soils was thought to be a chemical process (Getzin 1967, 1968; Getzin and Rosefield 1968; Kearney and Helling 1969) until Sethunathan and associates (Sethunathan and MacRae 1969; Sethunathan and Pathak, 1972; Sethunathan and Yoshida 1973a) reported direct involvement of bacterial isolates in such a mechanism. Microbial degradation of diazinon occurred in a flooded acid soil (Sethunathan and MacRae 1969). Diazinon was hydrolyzed to IMHP and diethylthiophosphoric acid in upland soils. IMHP accumulated in flooded anaerobic soils, but in nonflooded soils the pyrimidine ring was mineralized to CO_2 (Getzin 1967). Rapid degradation of diazinon was observed in nonsterile muck soil when treated with marl (a mixture of finely powdered calcium carbonate and clay) but the rate of diazinon disappearance was much slower in autoclaved soils (Kageyama et al. 1972).

An *Arthrobacter* sp. and a *Streptomyces* sp. isolated through selective enrichment technique, synergistically utilized diazinon and released $^{14}CO_2$ from the pyrimidinyl ring (Gunner and Zuckerman 1968). Neither of the species could utilize diazinon or affect the pyrimidinyl ring cleavage individually. However, almost 84% of diazinon was lost from the medium within 7 days when both the species were inoculated together. Sethunathan and Yoshida (1973a) isolated a *Flavobacterium* sp. from a diazinon-treated paddy field, which hydrolyzed diazinon to IMHP in mineral solution. This bacterium also hydrolyzed parathion and Dursban to more unstable products but could not metabolize malathion. *p*-Nitrophenol, the major hydrolytic product of parathion degradation, did not serve as energy source for this *Flavobacterium* sp. whereas IMHP was completely mineralized to CO_2 (Sethunathan and Yoshida 1973a). Adhya et al. (1981a) reported that *Flavobacterium* sp. also hydrolyzed methyl parathion and fenitrothion in addition to diazinon and parathion. A cell-free crude enzyme from this bacterium could hydrolyze diazinon, parathion, chlorpyrifos, and paraoxon but not aminoparathion, malathion or 4-aminophenol. This enzyme has been purified and identified as phosphotriesterase (Brown 1980).

Rosenberg and Alexander (1979) obtained two pseudomonads which could use diazinon or malathion as a sole source of phosphorus. These bacteria had hydrolytic activity toward many organophosphates including aspon, monocrotophos, fensulfothion, diazinon, malathion, acephate, parathion, and trithion, but they failed to hydrolyze dimethoate, trichlorfon, methyl parathion, and dichlorvos.

3.2 Fenitrothion

Since 1967, fenitrothion has been in use as a replacement chemical for DDT in controlling forest defoliators in addition to its use on agricultural crops. Contamination of soil and aquatic ecosystems can occur during frequent aerial spraying and contaminated-surface run-off following rainfall. Fenitrothion, like other organophosphates, can undergo chemical and microbiological degradation (Symons 1977). Reports of its microbial metabolism in natural environments are, however, limited. Microbially, fenitrothion can undergo hydrolytic reactions forming 3-methyl-4-nitrophenol or can be reduced to form aminofenitrothion (Fig. 3). Chemical hydrolysis of fenitrothion is pH and temperature-dependent (Sundaram 1973 a).

The residence period of fenitrothion in forest soils varied from 30–64 days (Spillner et al. 1979; Yule and Duffy 1972). Residues of fenitrothion in forest soil samples reached minimum level (0.01 ppm) after 64 days of its application (Yule and Duffy 1972). The disappearance of fenitrothion from the forest soil environment was probably due to the action of soil microflora. Similar observation of microbial degradation of fenitrothion in soil was also made by Salonius (1972). In an agricultural soil fenitrothion was rapidly degraded under both aerobic and anerobic conditions (Takimoto et al. 1976). Aerobically, ^{14}C-fenitrothion was hydrolyzed to 3-methyl-4-nitrophenol which was further metabolized to $^{14}CO_2$. Similar results on fenitrothion degradation were obtained in two moist soils (Spillner et al. 1979). Almost 50% of fenitrothion disappeared within 3 days in an organic and sandy loam soil giving rise to 3-methyl-4-nitrophenol, 3-methyl-4-nitroanisole, CO_2 and soil-bound fractions. The hydrolytic product, 3-methyl-4-nitrophenol was nonpersistent in both

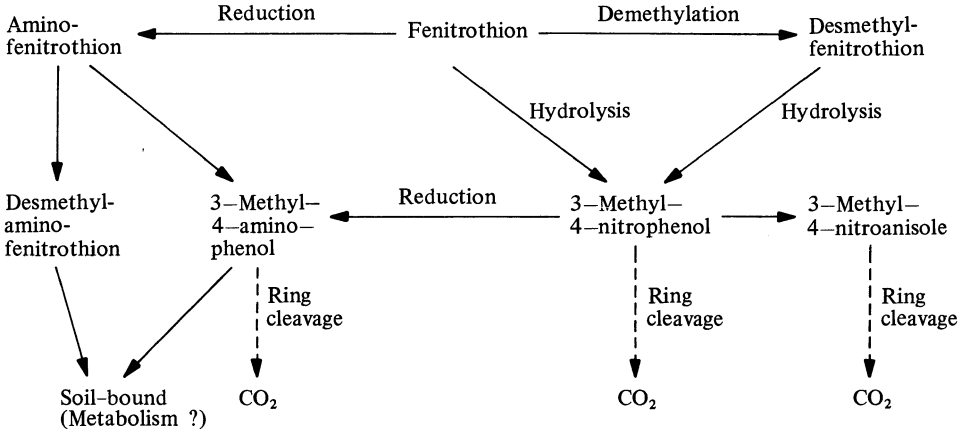


Fig. 3. Proposed pathway of fenitrothion metabolism in soil, aquatic and microbial systems

the soils and degraded to CO_2 and some soil-bound residues. 3-Methyl-4-amino phenol and desmethylfenitrothion were not detected in moist forest soils, but were the degradation products of fenitrothion in agricultural soils (Takimoto et al. 1976).

Fenitrothion was also short-lived in pond and stream water samples, having a half-life of 0.3 to 3.5 days (Sundaram 1973 b). In a buffered distilled water, natural water and buffered lake water, the hydrolysis of fenitrothion was pH- and temperature-dependent (Greenhalgh et al. 1980). Biological degradation of fenitrothion via nitro group reduction has been reported. Yasuno et al. (1965) isolated bacteria from polluted water which degraded fenitrothion to aminofenitrothion. A soil bacterium, *Bacillus subtilis,* also decomposed fenitrothion via nitro group reduction, forming aminofenitrothion as the major metabolite (Sumitomo Chemical Co. 1973). Zitko and Cunningham (1974) detected aminofenitrothion and desmethylaminofenitrothion as the major products of fenitrothion metabolism in a river water incubated under laboratory conditions. In an aquatic ecosystem, fenitrothion was converted to aminofenitrothion (Moody et al. 1978). When sprayed to a pond water, fenitrothion was hydrolyzed to *p*-nitro-*m*-cresol and was found only in water; whereas aminofenitrothion was detected in sediment fractions and not in water (Magurie and Hale 1980).

3.3 Methyl Parathion

Methyl parathion is a nonsystemic insecticide, having lower mammalian toxicity than its ethyl analog, parathion. The degradative pathway of methyl parathion is analogous to parathion (Fig. 4). Methyl parathion was rapidly decomposed in natural soils but was persistent in sterile soils and 99.9% degradation was observed at 0.5% to 1% concentration level (Naumann 1970 a). Chemical factors which influence the longevity of methyl parathion in soil ecosystems include various soil types, rates of application and moisture content (Burkhardt and Fairchild 1967; Harris et al. 1977).

Biological hydrolysis of methyl parathion was the principal means of degradation observed in soil (Lichtenstein and Schulz 1964). Sterilization of soil samples by autoclaving destroyed this capacity (Getzin and Rosefield 1968; Lichtenstein and Schulz 1964). However, γ-radiation (4 mrad) had very little effect on biological degradation (Getzin and Rosefield 1968). Kishk et al. (1976) showed that soil enzymes, probably of microbial origin, hydrolyzed methyl parathion to *p*-nitrophenol, but this activity in soil was either lost or partially reduced upon heat treatment.

In an artificial salt marsh ecosystem, Bourquin et al. (1977) showed degradation of ^{14}C-methyl parathion with an evolution of $^{14}CO_2$. Evolution of $^{14}CO_2$ from methyl parathion was influenced by the microbial community as no such phenomenon was observed in sterile systems. Pritchard et al. (1979) studied methyl parathion degradation in sediment-water microcosm experiments; and they demonstrated rapid microbial adaptation to methyl parathion with the formation of amino-methyl parathion and CO_2. Methyl parathion was more stable under sterile conditions, indicating little chemical and/or abiotic decomposition. As expected, the methyl parathion-adapted microorganisms also degraded *p*-nitrophenol without any lag. In this sediment-water ecosystem, the rate of methyl parathion degradation was

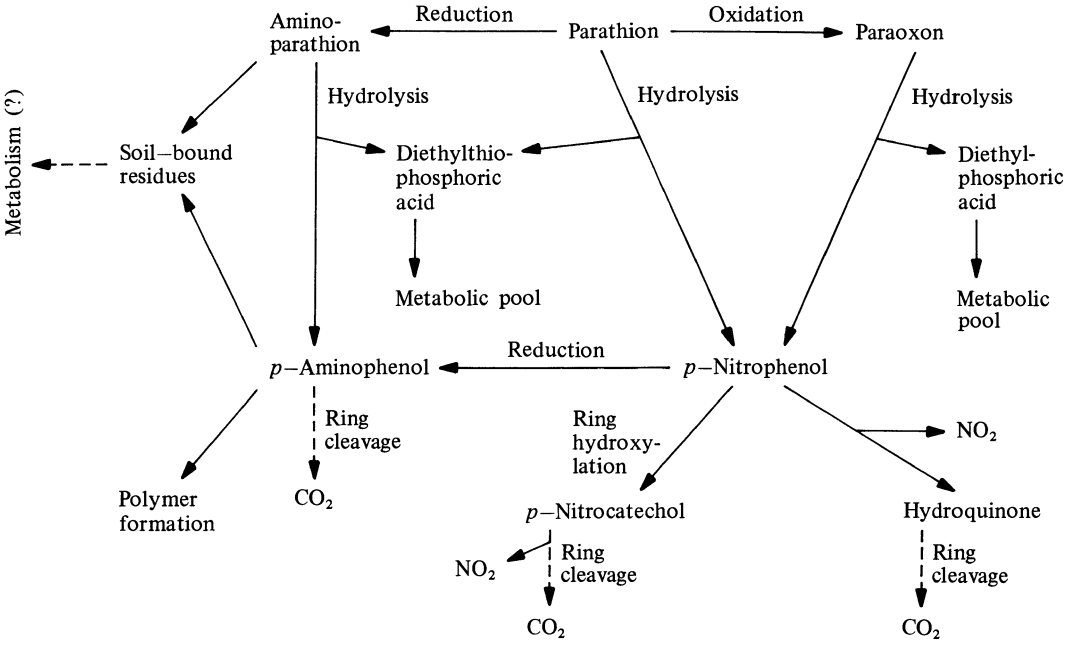


Fig. 4. Proposed pathway for parathion metabolism in soil, aquatic and microbial systems

slower when sediment to water ratios were increased. Methyl parathion was co-metabolically degraded by a mixed bacterial culture obtained from oligotrophic or eutrophic waters (Chou and Bohonos 1979). They noticed rapid degradation of methyl parathion in the presence of 0.1% nutrient broth, but surprisingly the activity of the cultures was very much retarded upon subsequent transfers. In another study, Spain et al. (1980) used water-sediment systems (ecocores) from a salt marsh and a river, to study the possible adaptation of natural microflora and their role in the metabolism of methyl parathion and *p*-nitrophenol. This study showed that pre-adapted ecocore microorganisms from river sites mineralized both methyl parathion and *p*-nitrophenol to carbon dioxide. Microbial adaptation was poor in a salt marsh ecocore system for methyl parathion. *Pseudomonas* and *Acinetobacter* spp. were isolated from the Escambia River cores which degraded *p*-nitrophenol but not methyl parathion.

In a nonsterile flooded soil, methyl parathion and fenitrothion were hydrolyzed to *p*-nitrophenol and 3-methyl-4-nitrophenol respectively. When the soil was further reduced upon rice-straw amendment, the insecticides were decomposed via the reductive pathway. In contrast, aminoparathion was the principal metabolite of parathion degradation under both conditions (Adhya et al. 1981 b). Although sufficient evidence was not presented, substantial recovery of the hydrolytic products of the insecticides after a 12 h lag, and the formation of amino analogs, suggested the significant role played by the microorganisms under these conditions.

A pesticide-degrading *Bacillus* sp. was isolated from sewage sludge. This culture had biochemical and morphological characteristics similar to that of *Bacillus cereus* and co-metabolized methyl parathion. Many *Pseudomonas* sp. isolated from this

sewage sludge could not utilize methyl parathion as sole source of carbon and energy. Addition of peptone or other organic compounds also did not favor the disintegration of this insecticide (Maleszewska 1974).

Aufwuchs microorganisms grown in a batch culture were adapted to methyl parathion and diethyl phthalate (a plasticizer) (Lewis and Holm 1981). Loss of methyl parathion from the culture vessel was due to microbial action, as no degradation occurred with autoclaved aufwuchs biomass. In addition, the rate of methyl parathion degradation was almost equivalent when samples were incubated in the dark, indicating little photooxidation or degradation by algae. The degradation of methyl parathion was about three times faster in the presence of nutrient broth. The influence of soil-water tension on the mineralization of methyl parathion was studied by Ou et al. (1983) using a sandy loam (Cecil) and silty clay loam (Webster) soils. Methyl parathion was degraded very rapidly when soil-water tension was at 10 and 33 kPa. Methyl parathion was first hydrolyzed to *p*-nitrophenol and then *p*-nitrophenol was subsequently reduced to *p*-amino phenol. Acceleration of methyl parathion degradation in soil when soil-water ratio was increased probably favored increased microbial activity. A similar phenomenon has been observed for parathion degradation (Sethunathan et al. 1977).

3.4 Parathion

Parathion, a nonsystemic insecticide and acaricide was introduced in 1947 and is used for controlling major insect pests on many agricultural crops including rice and vegetables. As with diazinon, hydrolysis is the major process involved in detoxification of parathion (Fig. 4). Both chemical and microbial hydrolysis of parathion has been found to occur in natural environments. Parathion can also be reduced to form aminoparathion, a process exclusively mediated by microorganisms (Sethunathan et al. 1977).

3.4.1 Residues in Soil

There are conflicting reports about long-term persistence of parathion in soil (Chisholm and MacPhee 1972; Iwata et al. 1973; Kearney et al. 1969; MacPhee et al. 1960; Sethunathan and Siddaramappa 1978; Stewart et al. 1971; H.R. Wolfe et al. 1973). Despite these reports on parathion persistence in soil, our knowledge of its biological degradation in soil, sediment, and water ecosystems is vast and somewhat more complete than most of the organophosphorus insecticides. Recently, significant contributions have been made toward better understanding the various aspects of parathion-microbial interactions in flooded soils, sediments and water environments.

Soil persistence of parathion was shown to be a factor of the types of soils used, water regime, and chemical additives apart from microbial participation in the degradation process. Sethunathan and Yoshida (1973a) reported more rapid degradation of parathion on four different flooded soils than in nonflooded soils. In contrast, parathion was lost rapidly in only one of the four soils under flooded conditions (Iwata et al. 1973). But an accelerated rate of parathion degradation occur-

red both in flooded and nonflooded soils when inoculated with parathion-acclimated bacterial cultures (Daughton and Hsieh 1977). However, the faster disappearance of parathion from flooded soils than from nonflooded soils can be attributed to the interaction of bacteria, yeast, and fungi (Sethunathan et al. 1977).

Hsu and Bartha (1979) demonstrated an accelerated evolution of $^{14}CO_2$ from ^{14}C-labeled parathion and pyrimidinyl ring labeled ^{14}C-diazinon in a nonflooded bean rhizosphere soil. A similar phenomenon was also observed in a rice rhizosphere soil (Reddy and Sethunathan 1983). Ring-labeled ^{14}C-parathion was metabolized to $^{14}CO_2$, via hydrolysis and ring cleavage, in rhizosphere soils under both flooded and nonflooded conditions. However, flooded rhizosphere soil showed greater activity than that of nonflooded rhizosphere soil. Parathion disappearance in nonrhizosphere soils was relatively slower. Flooded rhizosphere soils apparently favor microbial proliferation and secretion of increased root exudates to facilitate higher rate of pesticide degradation probably through co-metabolic and/or synergistic actions.

The soil-residence period of parathion can be increased upon autoclaving and/or chemical treatments, in spite of flooding (Sethunathan and Yoshida 1973b). Many workers (Barik and Sethunathan 1978a; Getzin and Rosefield 1968; Lichtenstein et al. 1968), studying the stability of parathion or methyl parathion in autoclaved and nonautoclaved soils, found evidence for the indirect participation of microorganisms. More convincing evidence for the role of microorganisms in parathion metabolism in mineral and organic soils was obtained from the study of Miles et al. (1979). The authors compared the persistence of eight organophosphates in sterile and nonsterile soils and observed very rapid disappearance of chloropyrifos, parathion, fensulfothion, chlorfenvinphos, trichloronat, fonofos and ethion in nonsterile soils.

Chemical and/or sterilizing agents such as sodium azide (Lichtenstein et al. 1968), methyl bromide (Sacher et al. 1972), ABS, LAS (Lichtenstein et al. 1968), or irradiation of soils also resulted in a slower rate of parathion degradation (Getzin and Rosefield 1968; Lichtenstein et al. 1968; Nauman 1970a, b; Sacher et al. 1972; Sethunathan and Yoshida 1973a), presumably due to reduced microbial activities. Use of these parameters unquestionably reduces microbial population but can also cause drastic changes in the physicochemical properties of the soil that catalyze pesticide degradation.

3.4.2 Microbial Hydrolysis of Parathion

In vitro studies on parathion metabolism using microbial cultures isolated from soil, sediment, and water ecosystems have now been demonstrated, suggesting widespread occurrence of biological agents capable of degrading parathion and other organophosphorus compounds. Sethunathan (1973) obtained bacterial enrichment from a flooded alluvial soil which rapidly degraded parathion but lost the activity upon autoclaving. A *Pseudomonas* sp. isolated from this enrichment completely mineralized parathion to nitrite (Siddaramappa et al. 1973) and CO_2 (Barik et al. 1976) via hydrolysis; but was unable to metabolize methyl parathion or fenitrothion (Adhya et al. 1981a). Interestingly, the crude cell-free preparations of this bacterium hydrolyzed parathion, but the resulting hydrolytic product, *p*-nitrophenol, was not metabolized further (Barik and Sethunathan, unpublished).

Bacterial enrichments have been obtained when several types of soil, sediments or water samples were successively treated with parathion (Barik and Sethunathan 1978a). Parathion disappeared very rapidly when repeated additions of parathion were made to the samples. This led to a tremendous increase (from 1.4×10^4 g^{-1} soil after first addition to 14.6×10^6 and 4300×10^6 g^{-1} soil after second and third applications respectively) in the numbers of parathion-hydrolyzing bacteria (Barik et al. 1979). Heat treatment of this enrichment inactivated parathion hydrolyzing capacity and parathion was very stable in all the samples (Barik and Sethunathan 1978a). In a slightly modified method, parathion-hydrolyzing enrichments were obtained when flooded soil was treated with *p*-nitrophenol, the hydrolysis product of parathion. When parathion was added to these soil samples, the insecticide disappeared more rapidly in soil pre-treated with *p*-nitrophenol (Barik et al. 1979). In a similar study, using ^{14}C-parathion in a Cranberry soil, Ferris and Lichtenstein (1980) showed complete mineralization of parathion in soil samples with a history of *p*-nitrophenol treatment. This is completely surprising, but convincingly demonstrates that parathion-hydrolyzing bacterial enrichment in soils can occur if treated with a metabolic product of the insecticide.

In an attempt to isolate a parathion-hydrolyzing bacterium, Hsieh and Munnecke (1972) used a continuous culture method for bacterial enrichment and obtained a consortium of bacteria which hydrolyzed parathion to *p*-nitrophenol. The bacterial isolates from these adapted mixed cultures utilized *p*-nitrophenol but not parathion as sole source of carbon. Daughton and Hsieh (1977) isolated two species of Pseudomonads, *P. stutzeri* and *P. aeruginosa* from this bacterial consortium. *P. stutzeri* co-metabolized parathion to *p*-nitrophenol and could not metabolize *p*-nitrophenol, whereas *P. aeruginosa* mineralized only *p*-nitrophenol to nitrite and CO_2. The crude cell-free extract (Munnecke 1976) of this mixed bacterial culture exhibited exceptionally broad substrate specificity and hydrolyzed diazinon, EPN, chlorpyrifos, triazophos, parathion, paraoxon, aminoparathion, methyl parathion, Dursban, fenitrothion and cyanophos; but could not hydrolyze fenthion, ethion, azinophosmethyl, or chlormephos. This enzyme was later shown to be an important pesticide-hydrolyzing enzyme which could be used for biological decontamination of pesticide containers, highly contaminated soils or artificial polymers and industrial waste effluents (Barik and Munnecke 1982; Munnecke 1979a, b; 1980).

Nelson (1982) isolated 50 different bacterial isolates mainly belonging to *Bacillus* and *Arthrobacter* sp. from parathion-treated Israeli (Gilat) soil which hydrolyzed parathion to *p*-nitrophenol. When the *Bacillus* sp. was reinoculated to the sterile soil treated with parathion, little parathion decomposition occurred; whereas *Arthrobacter* sp. rapidly hydrolyzed parathion under similar situation. Moreover, *Arthrobacter* sp. utilized parathion and *p*-nitrophenol as sole carbon source. In a subsequent study, Nelson et al. (1982) showed rapid increase in bacterial populations when parathion (10–160 μg g^{-1} dry soil) was applied to the nonsterile soil. The bacterial population reached the highest level within 4–5 days and then declined. The authors also showed that the rate of parathion hydrolysis was independent of the substrate concentration (10 to 160 μg parathion g^{-1} dry soil) and proposed a model to predict the relationship between soil-parathion concentration, microbial population and hydrolysis kinetics.

3.4.3 Microbial Reduction of Parathion

Parathion degradation can also proceed biologically via nitro-group reduction, forming aminoparathion. This occurs primarily in reduced conditions or under conditions of low oxygen tension. Many microorganisms, including bacteria, fungi and yeasts, have been isolated, which carry out parathion reduction in pure culture. Aminoparathion is a short-lived, less toxic compound than parathion and can be easily hydrolyzed to *p*-aminophenol for subsequent metabolism (Fig. 4).

Parathion was metabolized to aminoparathion in a soil (Lichtenstein and Schulz 1964). The nitro group reduction of parathion was associated with yeast cell proliferation, indicating the possible role of microorganisms in such conversions. Several groups of soil microorganisms (Nauman 1970a, b), including algae (Ahmed and Casida 1958; Iwata et al. 1973; Mackiewicz et al. 1969), have been shown to form amino derivatives from parathion and methyl parathion.

The soil fungi *Trichoderma viride* (Matsumura and Boush 1968) and *Penicillium waksmanii* (Rao and Sethunathan 1974) can convert parathion to aminoparathion in pure cultures. Adhya et al. (1981a) determined that glucose-grown cultures of a *Pseudomonas* sp. co-metabolized parathion to aminoparathion. When glucose was depleted, this bacterium used parathion as sole carbon and energy sources (Barik et al. 1976; Siddaramappa et al. 1973).

3.4.4 Parathion in Sediment and Aquatic Ecosystems

Sediment and aquatic ecosystems are the final repository of pesticide run-off, and native microflora play a major role in parathion metabolism under such environments. A strain of *Bacillus subtilis* isolated from a polluted sewage water system can reduce parathion, fenitrothion, methyl parathion and sumithion to their amino analogs (Miyamoto et al. 1966; Yasuno et al. 1965). Microorganisms from lake sediments degraded parathion to aminoparathion when incubated under both aerobic and anaerobic conditions. Under aerobic condition, aminoparathion was converted to an unidentified metabolite, but was stable under anaerobic conditions (Graetz et al. 1970).

McIntyre et al. (1981) observed rapid disappearance of parathion, diazinon and malathion in raw sewage, and they attributed this disappearance partly to the action of microorganisms.

Parathion is also very short-lived in raw river water (Barik and Sethunathan 1978a; Eichelberger and Lichtenberg 1971) and pond water (Barik and Sethunathan 1978a). The rapid metabolism of parathion in these aquatic ecosystems is presumably due to microbial activity. Weber (1976) reported that parathion was metabolized in a raw estuarine water due to the development and action of native microflora. He also suggested that biological detoxification was more predominant than chemical hydrolysis under this situation. A freshwater alga, *Chlorella pyrenoidosa proteose,* was reported to reduce parathion to its amino analog (Ahmed and Casida 1958). Sharom and associates (1980a, b) showed that parathion disappeared more quickly in natural water than in distilled water, sterilized distilled or sterilized natural waters, indicating the importance of microbial action. Aerobic bacteria present in natural water were adapted to hydrolyze parathion, but addition of co-energy substrates such as glucose or ethanol favored the nitro group reduction of parathion (Sharom and Miles 1981).

3.5 Other Organophosphates

Many other organophosphorus compounds are now available in the market as a replacement chemical for more persistant, unsafe, and toxic insecticides belonging to other groups. Preliminary studies on the fate and stability of these compounds under natural environments, though not conclusive, indicate that they, too, can be microbially unstable. But much more data has to be generated under various situations to determine the metabolic pathways involved and their safe use in natural environments.

Chloropyrifos has been extensively used against a coniferous leaf defoliator. Very little information is available on the microbial degradation of this compound in soil, water, and sediments. From the studies on the persistence in soil (Chapman and Harris 1980; Szeto and Sundaram 1982), aquatic models (Hughes et al. 1980; Szeto and Sundaram 1982); microbial breakdown of chloropyrifos under natural (Szeto and Sundaram 1982) and laboratory conditions (Miles et al. 1979) have been suggested.

In a study of phosdrin and phorate degradation in five Sacramento Delta soils, phosdrin disappeared more rapidly in most nonautoclaved soils than in autoclaved soils, indicating possible microbial participation in the degradation process (Burns 1971). While examining the microbial activity in these organic matter-depleted soils, Burns observed the necessity for a minimum level of organic matter to achieve active participation of pesticide-degrading microorganisms.

A *Pseudomonas putida* culture isolated from soil and grown with disodium formate degraded more than 98% of demeton S-methyl sulfoxide (Metasystox-R) within 14 days (Ziegler et al. 1980). A *Nocardia* sp. isolated from the same soil showed similar results. Another organophosphate, dichlorvos, was metabolized to dichloroethanol, dichloroacetic acid, and ethyl dichloracetate by a mixed bacterial population enriched from sewage sludge (Lieberman and Alexander 1983). Dichlorethanol, dichloroacetate and inorganic phosphate were produced from the insecticide exclusively in the presence of microbial cells and were not the products of nonenzymic or chemical degradation.

Despite reports of long-term persistence of a few organophosphates, it is generally believed that they seldom pose any residual problems in the environment. Microbial rather than chemical processes are the principal means of detoxification of organophosphates observed in natural ecosystems and in laboratory studies. A rapid build-up of organophosphate-degrading microorganisms can occur in flooded soil, sediment, and aquatic ecosystems during successive applications of these compounds (parathion, methyl parathion) or their metabolic products (*p*-nitrophenol), leading to rapid pesticide breakdown. Biological hydrolysis of the ester bond of organophosphates is the most common and widespread detoxification mechanism found in natural ecosystems and in pure cultures. However, under low oxygen tension some organophosphorus compounds can undergo reductive transformations forming their amino analogs. These metabolic products are rapidly degraded by microorganisms completing the process of detoxification.

3.6 Metabolites of Organophosphates

Microbial and chemical decomposition of organophosphates in the environment result in the formation of many organic and inorganic products. These compounds, if persistent in the environment, can influence the microbial equilibria of the ecosystem. Thus, there is a need for their further metabolism in order to achieve complete detoxification of the xenobiotics. Biological degradation of these intermediary products occurs widely and the fate and metabolism of these compounds has been studied in detail.

3.6.1 Aminoparathion

Pesticides with a nitro group can be reduced to their amino analogs by chemical or biological means. Aminoparathion, for example, is the major metabolic product of parathion reduction in soil ecosystems (Sethunathan et al. 1977). Aminoparathion can be biologically hydrolyzed (Sethunathan et al. 1977) unless it is soil-bound (Katan et al. 1976; Katan and Lichtenstein 1977) and therefore unavailable for microbial attack. Aminoparathion was rapidly degraded in soil (Lichtenstein and Schulz 1964), and in microbial cultures (Graetz et al. 1970). *p*-Aminophenol, the hydrolytic product of aminoparathion was, however, not detected. In contrast, a parathion-adapted mixed culture hydrolyzed aminoparathion to *p*-aminophenol and diethylthiophosphoric acid (Munnecke and Hsieh 1976).

3.6.2 Paraoxon

An oxygen analog of parathion, paraoxon, is formed during chemical (Gomma and Faust 1972) and photolytic (Joiner et al. 1971) reactions. A parathion-adapted mixed bacterial culture oxidatively converted parathion to paraoxon (Munnecke and Hsieh 1976). Paraoxon is very short-lived in the environment and can be easily hydrolyzed to *p*-nitrophenol and diethyl phosphoric acid, and this process is mediated by chemical and/or biological agents. Gunther et al. (1977) have reviewed the residual effect of paraoxon under field conditions. In a bioassay study Lichtenstein and Schulz (1964) observed hydrolysis of paraoxon in a soil. *p*-Nitrophenol was the only major metabolite formed during this hydrolysis. Paraoxon was hydrolyzed to *p*-nitrophenol and diethylphosphoric acid by parathion-adapted mixed culture (Munnecke and Hsieh 1976).

3.6.3 Phosphonates and Other Phosphorus Compounds

For many years, several synthetic alkyl- or aryl-phosphonate compounds have been used as industrial, agricultural, and commercial products, but the ultimate fate and metabolism of these chemicals in natural environments has only recently become an academic concern (Cook et al. 1978 a, 1979; Daughton et al. 1979 a, b). Some trialkyl phosphorothioate, dialkyl-alkyl- and aryl-phosphonothioate esters are highly toxic and their presence in trace amounts could potentiate or antagonize mammalian toxicity (Fukuto 1983).

Cook et al. (1978 a) obtained enrichments from soil and sewage samples that were able to utilize several alkylphosphonates, *o*-alkyl alkylphosphonates and *o*-,*o*-dialkyl alkylphosphonates as sole sources of phosphorus. A bacterial isolate, *Pseudomonas putida* also utilized AEP as sole carbon, nitrogen and phosphorus source. An inducible phosphonatase obtained from this culture was able to release orthophosphates from AEP, but the reaction was dependent on the presence of co-factors, pyruvate and pridoxal phosphate. In another study, Cook et al. (1978 b) obtained several bacterial species which utilized the products of many organophosphorus pesticides containing ionic phosphorus groups. The compounds tested included dialkyl phosphate, dialkyl phosphorothioate, dialkyl phosphorodithioate, alkyl arylphosphonate, alkyl arylphosphonothioate, and alkyl alkylphosphonate as phosphorus sources (Cook et al. 1978 b) or as a sole source of sulfur (Cook et al. 1980).

Another isolate, *Pseudomonas testoteroni,* used *o*-alkyl-alkylphosphonates as sole source of phosphorus and produced more natural products such as alcohols, alkanes and inorganic phosphates (Daughton et al. 1979 a). This bacterium cleaved the C-P bond of various phosphonates and formed methane or ethane from phosphorus-bound methyl or ethyl groups under aerobic conditions (Daughton et al. 1979 b). The C-P bond cleavage mechanism acquired by a bacterium during the course of enrichment clearly suggests that the ionic-phosphorus-containing metabolic products can be easily mineralized under natural environments. Moreover, these bacterial isolates or enzymes derived from them could be applied in future detoxification mechanisms of contaminated pesticide spills or industrial effluents, as described for parathion and other organophosphates (Munnecke 1977; Munnecke et al. 1982; Talbot et al. 1982).

3.6.4 *p*-Nitrophenol and Other Nitrophenolics

p-Nitrophenol, the major hydrolytic product of parathion and methyl parathion, like many other nitro-phenolic compounds poses odor problems and is toxic to aquatic inhabitants. Nitrophenols are very susceptible to biodegradation, since the presence of additional polar groups such as NO_2^-, OH, NH_2, N–C–(O)–, and COO^- in the benzene nucleus provides a new focal point for microbial attack (Helling et al. 1971; Woodcock 1971). However, the type, position and the number of derivatives in the benzene ring also contribute in determining the rates of microbial degradation of several organic compounds (Alexander and Lustigman 1966).

Several reports on microbial metabolism of nitroaromatic compounds including picric acid (trinitrophenol) and trinitroresorcinol (Erikson 1941); DNOC (Gundersen and Jensen 1956; Jensen and Gundersen 1955; Teuteberg 1964); 2,4-dinitrophenol (Madhosingh 1961; Teuteberg 1964) are available. But in recent years, the bacterial metabolism of *p*-nitrophenol has drawn much more attention. Simpson and Evans (1953) isolated two species of pseudomonads from the filter beds of an industrial effluent which decomposed *o*- and *p*-nitrophenol to nitrite; but no other nitrophenols were used by these bacteria. Another bacterial isolate could also release nitrite from 2,4-dinitrophenol, through oxidative elimination of nitro group prior to the ring cleavage.

A culture of *Corynebacterium simplex* was obtained from DNOC-treated soil and this organism utilized DNOC as sole carbon and energy source, liberating more than 70% of the nitrogen in DNOC as nitrite (Gundersen and Jensen 1956; Jensen and Gundersen 1955). This bacterium also utilized *p*-nitrophenol, 2, 4-dinitrophenol and 2, 4, 6-trinitrophenol as the sole carbon source with the formation of nitrite. Various strains of *Pseudomonas* sp., *Flavobacterium* sp. and *Nocardia* sp. utilized *o*- and *p*-nitrobenzoic acid and *o*- and *p*-nitrophenol as nutrient sources. Arylamines, ammonia, and nitrite were detected as the metabolic products during this process (Cain 1958, 1966; Cartwright and Cain 1959; Durham 1958; Germanier and Wuhrman 1963; Gracia-Acha and Villanueva 1962). Several pseudomonads isolated from parathion-treated soils degraded *p*-nitrophenol but did not use parathion as sole source of carbon (Griffiths and Walker 1970). Raymond and Alexander (1971) reported that a soil bacterium utilized *p*-nitrophenol as the sole source of carbon and energy with a stoichiometric formation of nitrite. In this case, the reaction did not proceed past the 4-nitrocatechol stage, when the resting cells were treated with chloroform and incubated with *p*-nitrophenol. This bacterium also converted *m*-nitrophenol to nitrohydroquinone in a co-metabolic reaction.

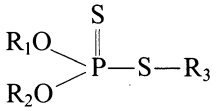
Barik and Sethunathan (1978 b) reported the complete disappearance of *p*-,*o*- and *m*-nitrophenols in a basal salts medium when inoculated with a parathion-hydrolyzing enrichment from a flooded alluvial soil (Barik and Sethunathan 1978 a). A similar enrichment obtained from another soil type was very specific in degrading only *p*-nitrophenol. However, the substrate specificities of this enrichment culture were least pronounced when tested under flooded soil conditions. Moreover, the authors also demonstrated the enhanced evolution of $^{14}CO_2$ from uniformly labeled ^{14}C-*p*-nitrophenol from the inoculated flooded soils under aerated (stirred) conditions (Barik and Sethunathan 1978 b).

Two bacterial cultures, a *Pseudomonas* sp. and a *Corynebacterium* sp. isolated from parathion-amended flooded soil (Siddaramappa et al. 1973), utilized *p*-nitrophenol as the sole carbon and energy source and metabolized to nitrite (Siddaramappa et al. 1973) and CO_2 (Barik et al. 1976). These two bacterial isolates differed markedly with regard to their specificities in metabolizing various other nitrophenols. The *Pseudomonas* sp. metabolized only 2, 4-dinitrophenol, while *Corynebacterium* sp. metabolized only *m*-nitrophenol (Barik et al. 1976). Similarly, Munnecke and Hsieh (1976) obtained a *Pseudomonas* sp. from a parathion-hydrolyzing enrichment which utilized *p*-nitrophenol as a carbon source and formed nitrite via hydroquinone metabolism. The details of aromatic ring cleavage and *p*-nitrophenol metabolism through ring hydroxylation pathways have been discussed elsewhere (Dagley 1977, 1978; National Academy of Sciences Report 1972).

4 Dithioates

Another important class of organophosphorus compounds are the dithioates. These compounds have recently attracted much attention for their action against a wide range of insect pests in many major crops such as corn, cotton, or vegetables. Dithioates, as the name implies, contain two S groups attached to P; and their general

structural formula is of the type:



where R_1 and R_2 = alkyl moiety and R_3 = substituted alkyl or aryl moiety.

Degradation of dithioate insecticides, like other organophosphates, can be by chemical and biological processes. Many reports indicate that the chemical degradation of dithioates is primarily pH- and temperature-dependent. As with chemical decomposition, microbial metabolism of dithioate compounds occurs most significantly in natural environments.

4.1 Azinophosmethyl (Gusathion)

Gusathion, commonly known as azinophosmethyl, was first introduced in Mexico in 1954 and was used against cotton insects in the United States in 1956. This is a broad-spectrum, nonsystemic insecticide and is now widely used on fruit, potatoes, sugarcane, vegetables and on many forage crops including alfalfa. Reports of its persistence in soil are very few but they indicate that Gusathion metabolism can be influenced by chemical or biological processes (Yaron et al. 1974). In a study conducted by the Chemagro Division Staff of Baychem Corporation (1974), the average half-life of Gusathion in soils was approximately three months. In addition, there was no evidence of residue build-up when the soil persistence studies were conducted using different soil types (Chemagro Division Staff of Baychem Corporation 1974). Staiff et al. (1975) studied the persistence of different formulations and concentrations of Gusathion in a sandy soil. Their study showed that undiluted insecticide can remain in soils at least up to 4 years. The effect of light, temperature and pH on the degradation of Gusathion was studied by Liang and Lichtenstein (1972). The authors reported that exposure to UV light enhanced Gusathion degradation; but the effect was negligible when exposed to sun light. Rapid degradation of Gusathion was observed in temperatures higher than 37 °C which was further enhanced by the presence of water. The insecticide was very stable in water below pH 10.0; but above pH 11.0, was rapidly converted to anthranilic acid, benzamide and a few other unidentified metabolites (Liang and Lichtenstein 1972). In a study with irrigated and nonirrigated field conditions, Yaron et al. (1974) reported no remarkable effect of flooding on the persistence of azinophosmethyl and tetradifon, probably due to the combinations of effects of physicochemical and microbial processes.

Engelhardt et al. (1981) screened many soil bacteria representing the genera *Pseudomonas, Nocardia, Arthrobacter, Corynebacterium, Brevibacterium,* and *Bacillus* for ability to degrade Gusathion and, surprisingly, all the strains transformed the insecticide to several metabolites. Among them *Pseudomonas fluorescens* DSM 1976 hydrolyzed Gusathion and cleaved the heterocyclic ring forming anthranilic acid. The cell extracts of this bacterium also formed anthranilic acid from Gusathion, which was not further degraded by whole cells or the cell extracts. In a recent study, Barik et al. (1983) obtained mixed bacterial cultures which degraded Gusathion

alone or in the presence of yeast extract. The crude-cell-free extract of this bacterial consortium not only had hydrolytic activity for Gusathion but also toward other dithioate compounds (e.g., malathion and dimethoate).

4.2 Malathion

The metabolic fate of malathion has been most extensively studied in recent years because of its heavy applications to many crops, particularly fruits and vegetables. The hydrolysis of malathion can be chemical or microbiological (Fig. 5). Konrad et al. (1969) reported that malathion in soil was hydrolyzed principally due to chemical mechanism before the adaptation of native microflora can occur. The authors also observed malathion hydrolysis in a soil-free system and this reaction was completely pH-dependent. Malathion was very stable at acid or natural pH but was susceptible to hydrolysis with an increase in pH (Konrad et al. 1969; W.W. Walker and Stojanovic 1973; N.L. Wolfe et al. 1977) and temperature (N.L. Wolfe et al. 1977). Malathion degradation in Tope, Snyder and Shell soil samples (Matsumura and Boush 1966) and in three nonautoclaved Mississippi soils (W.W. Walker and Stojanovic 1973) was faster than in autoclaved soils. This is probably due to the action of soil microflora in malathion dissipation as compared to chemical breakdown.

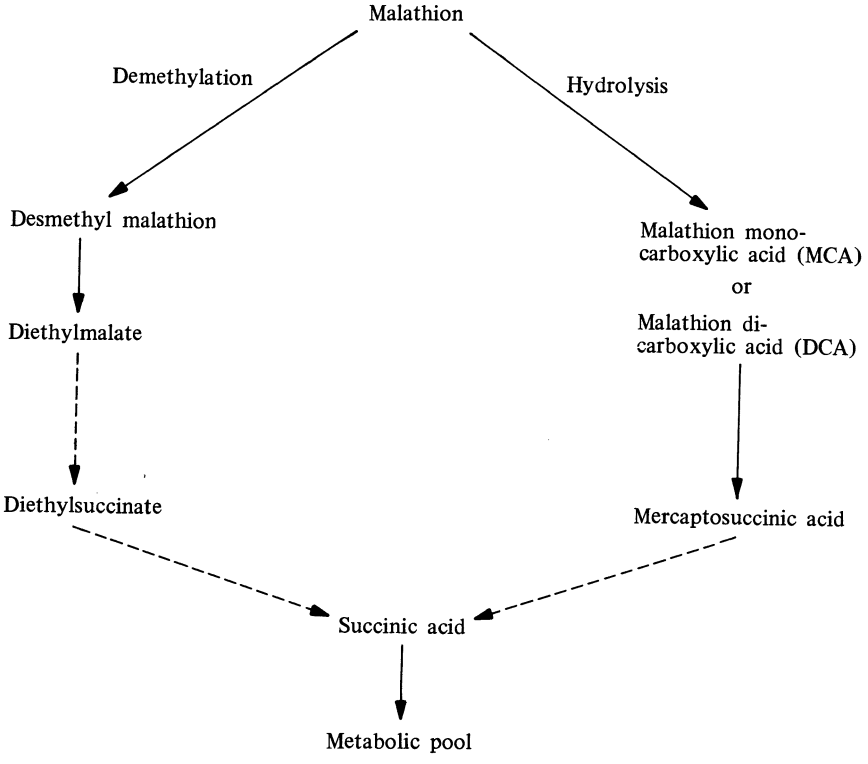


Fig. 5. Proposed pathway of malathion metabolism in soil, aquatic and microbial systems

Malathion degraded rapidly in nonsterile soil samples with a half-life of only 0.4 days compared to 8.3 days in sterile soils (Paschal and Neville 1976). Malaoxon, an oxygen analog of malathion, decomposed in both sterile and nonsterile soils at equal rates. The microbial population in soil amended with malaoxon indicated malaoxon as a potent anti-microbial agent which decreased bacterial and fungal populations in soil to 50% and 90% respectively. Co-metabolism of malathion by soil microflora was observed by Merkel and Perry (1977). Increased rate of malathion biodegradation occurred in soils when provided with other co-substrates such as n-heptadecane. These and more recent studies on malathion metabolism clearly indicate the microbial involvement in malathion hydrolysis in soil, sediment and estuarine ecosystems.

A strain of *Trichoderma viride* and a *Pseudomonas* sp. were isolated from soil hydrolyzed malathion (Matsumura and Boush 1966); both organisms possess soluble carboxyesterase activity, the most likely mechanism involved in malathion hydrolysis. Another soil fungus, *Penicillum notatum,* metabolized nearly 76% of the applied insecticide to a hydrolytic metabolite (Mostafa et al. 1972a). Two species of *Rhizobium, R. leguminosarum* and *R. trifolii,* isolated from an Egyptian soil, hydrolyzed malathion using a carboxyesterase and a demethylation process (Mostafa et al. 1972b). *Aspergillus oryzae* was isolated from a freshwater pond and degraded malathion to beta-malathion monoacid and malathion dicarboxylic acid (Lewis et al. 1975); products similar to bacterial decompositions (Chen et al. 1969). The degradation of malathion by *A. oryzae* was slow, but the rate of malathion transformation was proportional to the increase in dry weight of fungal biomass, suggesting that the fungus utilized malathion as a nutrient source.

W.W. Walker and Stojanovic (1974) isolated five bacterial isolates from agricultural soils having high capacity for malathion hydrolysis. Among them, an *Arthrobacter* sp. was found to be the most efficient and could degrade almost 95% of added malathion within a period of 7 days. A similar *Arthrobacter* sp. isolated from acclimated soil and aerated sludge samples rapidly degraded malathion when grown on malathion alone or with yeast extract (Barik et al. 1984). This bacterium was found to possess a hydrolytic enzyme which converted malathion and two other dithioates, azinophosmethyl and dimethoate (Barik et al. 1982).

There are a few reports of malathion metabolism in estuarine environments (Bourquin 1977; W.W. Walker 1976). Malathion completely disappeared both in sterile and nonsterile estuarine water samples within 18 days of incubation. In an estuarine sediment, complete degradation of malathion occurred in nonsterile samples but was persistent in sterile sediments, strongly indicating the participation of natural microflora in the degradation of malathion (W.W. Walker 1976). Two bacterial isolates obtained from these acclimated sediment samples also utilized malathion as sole carbon source (W.W. Walker 1976). Bourquin (1977) documented the biological degradation of malathion in salt marsh environments and isolated several bacterial species having the capacity to degrade malathion as sole or as coenergy substrates. These bacterial isolates possessed carboxyesterase and phosphatase enzyme activities which hydrolyzed malathion to different unstable metabolites.

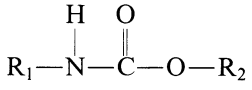
Phenthoate, a broad spectrum insecticide-acaricide having many common structural features with malathion, was very unstable in flooded non-sterile soils, but the degradation was retarded in autoclaved soil samples. ^{14}C-phenthoate degraded very

rapidly in soils via hydrolysis under both aerobic and anaerobic conditions. The major metabolic product, phenthoic acid, was further metabolized to CO_2 and other polar metabolites under both conditions; but rather slowly under anaerobic conditions (Iwata et al. 1977).

Like other organophosphorus pesticides, dithioate chemicals are short-lived in natural ecosystems. Chemical transformation of these compounds seems to be of minor importance as compared to biological degradation. Several microorganisms exist which can degrade malathion and few other dithioates under laboratory conditions. Further study on microbial detoxification of dithioate pesticides in highly contaminated areas and in industrial effluents is needed.

5 Carbamates

Pesticides belonging to this group are the ester derivatives of N-substituted carbamic acid, with a general formula



where $R_1 = CH_3$ or aryl group and R_2 = aryl or alkyl groups. Carbamate insecticides, nematicides, fungicides, and herbicides are extensively used as a substitute or "alternative" pesticide in place of more persistent organochlorine pesticides.

5.1 Carbaryl

Carbaryl, a methyl carbamate insecticide, is the active ingredient of Sevin, and is known for its wide applications and low mammalian toxicity. Biological decomposition of carbaryl in soil (S.Y. Liu and Bollag 1971; Bollag and S.Y. Liu 1972a; Caro et al. 1974; Rodriguez and Dorough 1977), by marine microorganisms (Sikka et al. 1975) and by *Pseudomonas melophthora,* a bacterial symbiont of the apple maggot (Boush and Matsumura 1967) have been reported. In a field study, carbaryl persisted in soil for 25–116 days after its application but then was rapidly degraded. This degradation was probably due to microbial adaptation (Caro et al. 1974). Rodriguez and Dorough (1977) reported rapid disappearance of carbaryl in soils with a history of pre-applications. Biological degradation of ^{14}C-carbaryl occurred in these soils and liberation of $^{14}CO_2$ due to ring cleavage was a result of microbial attack. Several bacterial and fungal isolates were obtained from carbaryl-treated soils, and these organisms degraded the insecticide in a mineral medium, but at a slower rate. The metabolism of carbaryl in flooded rice soils was faster but was dependent on soil types, pH, and redox potential (Venkateswarlu et al. 1980). A bacterium, *Pseudomonas cepacia,* was isolated from a carbofuran-amended flooded soil and this bacterium co-metabolized carbaryl in presence of yeast extract or soil extract. This bacterium was unable to utilize carbaryl as sole carbon and energy sources.

S.Y. Liu and Bollag (1971) isolated a fungus, *Gliocladium roseum*, which hydroxylated carbaryl to different metabolic products. In a following report, Bollag and S.Y. Liu (1972a) obtained several fungal isolates belonging to the genera *Aspergillus, Mucor, Penicillium* and *Fusarium* which degraded carbaryl either by side-chain or ring hydroxylations, producing water-soluble metabolites. An isolate of *Pseudomonas melophthora* degraded carbaryl to several metabolites (Boush and Matsumura 1967) via oxidative pathway. Another soil fungus, *Trichoderma viride*, degraded carbaryl through ring hydroxylations to several polar metabolites (Matsumura and Boush 1968). Several microorganisms isolated from marine environments were capable of using carbaryl as source of energy and produced hydroxylated water-soluble products. In contrast, species of *Brevibacterium* and *Spirillum* degraded carbaryl and produced only ether-soluble metabolites (Sikka et al. 1975). *Aspergillus terreus* degraded carbaryl at low concentrations, but above 200 ppm level no degradation of carbaryl occurred, possibly due to toxicity (Bollag and S.Y. Liu 1972b). An *Achromobacter* sp. used carbaryl as sole carbon source. This bacterium converted carbaryl to 1-naphthol, hydroquinone and catechol (Sud et al. 1972).

Microbial degradation of acephate and carbaryl was reported in natural pond and creek water and sediments (Szeto et al. 1979). Carbaryl, like acephate, disappeared rapidly from nonsterilized water samples, indicating microbial degradations. Addition of pond and creek sediments to pond and creek water samples respectively further accelerated the dissipation of the insecticides. Apparently, additions of sediments allowed more indigenous microflora to take part in the process of carbaryl degradation.

Osman and Belal (1980) studied the persistence of carbaryl in irrigation and drainage canal water. Carbaryl and 1-naphthol disappeared completely in raw river water within 6 days of their application, possibly due to microbial action. Abiotic degradation of carbaryl was observed in a mountain stream with depleted organic matter but both biotic and abiotic transformations occurred in polluted water. The metabolism of carbaryl was enhanced in the presence of added nutrients (e.g., glucose and peptone) under both aerobic and anaerobic conditions (D. Liu et al. 1981).

5.2 Carbofuran

Carbofuran, a broad spectrum systemic insecticide belonging to the N-methyl carbamate family, is used against numerous pests of rice, corn and on many common vegetable plants. Breakdown of carbofuran occurs primarily either via hydrolysis of the carbamate ester, resulting in the formation of a phenol or by the oxidation of the methylene group of the furanyl ring forming alcohols and ketones (Fig. 6). Due to the water-soluble nature of carbofuran and resulting metabolites, no serious bio-accumulation or other toxic problems of carbofuran appears in natural environments. The dissipation of carbofuran in soil (Getzin 1973; Miles et al. 1981; Ou et al. 1982) and water (Seiber et al. 1978) ecosystems can be influenced by pH, temperature, and the extent to which the microorganisms can be adapted for its metabolism (Felsot et al. 1981).

Hydrolysis is one of the most important mechanisms of carbofuran degradation in soil and water ecosystems. Hydrolysis of carbofuran can be mediated by chemical

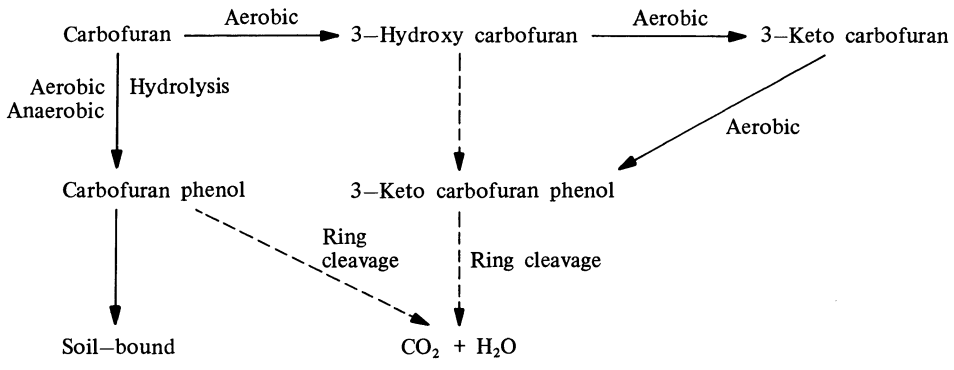


Fig. 6. Proposed pathway of carbofuran metabolism in soil, sediment and microbial systems

and/or microbiological processes (Getzin 1973; Miles et al. 1981; Siddaramappa et al. 1978; Siddaramappa and Seiber 1979). In a model ecosystem, carbofuran was degraded via chemical and biological processes to carbofuranphenol, 3-ketocarbofuran, 3-hydroxycarbofuranphenol, N-hydroxy-methylcarbofuran, and 3-hydroxycarbofuran. This study also reported the formation of N-methylcarbamic acid, which was further degraded to CO_2 (Yu et al. 1974).

Getzin (1973), studying the degradation of carbofuran in different soil types, observed that in alkaline soils chemical hydrolysis was the predominant mechanism of carbofuran degradation. Both chemical and microbiological processes were involved in its metabolism in acid and neutral soil types. Although there was no remarkable difference in the disappearance of the insecticide from irradiated or nonirradiated soil types, indirect evidence for biological degradation of carbofuran via phenol formation was documented. Carbofuran and one of its metabolites, 3-hydroxycarbofuran, disappeared faster in natural muck and loam soils than in sterilized soil samples (Miles et al. 1981), indicating the participation of soil microflora. Surprisingly, the authors failed to notice any stimulation of bacterial or actinomycetal populations. The degradation of another metabolite, 3-keto carbofuran, was found to be due largely to chemical rather than to microbiological processes.

Venkateswarlu et al. (1977) reported rapid breakdown of carbofuran in flooded rice soils, but the process was retarded upon autoclaving. The authors isolated a bacterium, *Pseudomonas cepacia,* from carbofuran-amended flooded soil that decomposed the insecticide in basal salts medium. In another study, Venkateswarlu and Sethunathan (1978) reported that in spite of an increase in bacterial populations in carbofuran-treated rhizosphere soil, the rate of the insecticide disappearance was almost similar to that in nonrhizosphere soil samples. Furthermore, the initial lag of carbofuran degradation could not be eliminated, although the loss of the insecticide was faster when carbofuran was applied repeatedly to a flooded soil. Although carbofuran was degraded more rapidly under anaerobic than under aerobic conditions, much information is needed on the specific mechanisms of the insecticide dissipation under such anoxic conditions. Although carbofuran breakdown occurred rapidly in pre-treated soils after an initial lag, Siddaramappa et al. (1978) observed no acceleration in carbofuran degradation in paddy water pretreated with the insecticide. Formation of carbofuran phenol in both sterile and nonsterile paddy wa-

ter was essentially a chemical reaction. But the metabolism of carbofuran phenol was perhaps associated with a microbiological process. Siddaramappa and Seiber (1979) studied carbofuran degradation in paddy soil and water systems and they failed to notice any acceleration in carbofuran degradation after its repeated applications to a paddy field. In a laboratory study, however, carbofuran disappeared more rapidly in nonautoclaved soils, indicating the occurrence of microbial metabolism, but isolation of microorganisms was not attempted.

Additions of rice-straw to flooded rice soils created anaerobic conditions and helped to promote rapid decomposition of carbofuran under such conditions. Complete anaerobic or alternate aerobic-anaerobic conditions affected carbofuran degradation in a soil. In a study with ^{14}C-carbaryl and ^{14}C-ring-labeled carbofuran, Venkateswarlu and Sethunathan (1979) showed that a significant amount of carbofuran disappeared from flooded soils; but the ring decomposition did not occur, as evidenced by low $^{14}CO_2$ evolution and high soil-bound residues. The degradation of ^{14}C-carbofuran to 3-hydroxy-carbofuran, 3-ketocarbofuran, carbofuran phenol and 3-ketocarbofuran phenol occurred in both Cecil and Webster soils when incubated aerobically. Although the metabolism of carbofuran was influenced by soil moisture and temperature, no statistical correlation was found between carbofuran degradation and the number of aerobic bacteria, number of aerobic fungi, soil pH, organic matter content, clay content or CEC (Ou et al. 1982).

Williams et al. (1976a, b) showed rapid degradation of ^{14}C-labeled carbonyl carbofuran in nonsterile soils with the evolution of $^{14}CO_2$. Of the eight pure cultures of microorganisms isolated, actinomycetes were most active in metabolizing carbofuran in the medium. Microorganisms were also shown to be responsible for the breakdown of 3-hydroxycarbofuran, a metabolic product of carbofuran. Three fungal isolates, *Helminthosporium* sp., *Aspergillus niger* and *Trichoderma viride,* were shown to degrade carbofuran to form 3-hydroxycarbofuran (Kandaswamy et al. 1977). Felsot et al. (1981) also reported the rapid loss of carbofuran in soils previously treated with the insecticide (Furadan 10 G) and phorate; but this capacity was completely lost when the soils were sterilized by autoclaving. Two bacteria, an *Achromobacter* sp. and a *Pseudomonas* sp. were isolated from carbofuran-treated corn soils and these bacteria degraded carbofuran in mineral medium and also when inoculated to sterile amended soils.

5.3 1-Naphthol

1-Naphthol is formed during the biological and chemical breakdown of carbaryl and can be decomposed biologically (Bollag and S.Y. Liu 1971; N. Walker et al. 1975; Sjoblad et al. 1976). A soil fungus, *Fusarium solani,* used 1-naphthol as a nutrient source and degraded the compound in a mineral medium. When spore suspensions of this organism were used as inocula nearly 50% of the carbaryl was lost from the medium within 12 h of incubation. The autoclaved spore suspensions did not decompose 1-naphthol. The cell extracts from spores and the mycelium of *F. solani* degraded 1-naphthol and the enzyme was shown to be constitutive (Bollag and S. Y. Liu 1972b). A *Pseudomonas* sp. isolated from soil used 1-naphthol as a sole carbon source and produced 3, 4-dihydrodihydroxy- 1(2 H)-naphthalene as the

major metabolite (N. Walker et al. 1975). Another soil fungus, *Rhizoctonia praticola* (Sjoblad et al. 1976; Sjoblad and Bollag 1977) grown in modified Czapak Dox medium transformed 1-naphthol to other polymerized products. The culture filtrate of this fungus and other species of *Rizoctonia* also produced a compound from 1-naphthol which was totally unextractable with benzene or diethyl ether. This high molecular weight compound was not produced when the culture filtrate was subjected to boiling or incubated under anaerobic conditions (Bollag et al. 1976). The extra-cellular enzyme fraction derived from this fungus also acted similarly and polymerized 1-naphthol to high molecular weight polymers through oxidative coupling. The details of this and other phenolic polymerization by this enzyme has been described elsewhere (Bollag et al. 1976; Sjoblad and Bollag 1977; D. Liu et al. 1981; Sjoblad and Bollag 1981).

In summary, carbamate insecticides do not pose environmental residue problems and persistence studies on carbamates suggest that they are easily biodegradable. Microbial degradation of carbofuran, carbaryl and 1-naphthol is most common and widespread in flooded soil and aquatic ecosystems. Under field conditions, however, carbofuran does not necessarily lead to microbial proliferation.

6 Organochlorines

Prior to the 1970's, organochlorine compounds were used as biocides more than any other pesticide chemical. Their long-term persistence created environmental problems and before long these compounds or their halogenated metabolic products were detected in groundwater and wastewater systems. Apart from causing health hazards, they are now known to be mutagens and carcinogens. Therefore, the use of organochlorine insecticides has now been restricted or completely banned in many countries. Despite limited use, research is continuing on their mode of degradation and/or interactions with natural biota. Lal and Saxena (1982) have discussed some mechanisms of organochlorine degradation and have described the details of microflora-organochlorine pesticide interactions. They have shown how the presence of these micropollutants in the ecosystem affect various chemical and/or biological processes.

Many organochlorine insecticides, including DDT, lindane (γ-BHC), endrin, endosulfan, aldrin, dieldrin, chlordane, heptachlor and methoxychlor were frequently used in different agricultural practices. Although the mode of degradation of these compounds in natural environments can be mediated through chemical or biological methods, the later processes perhaps play the most significant role in their dissipation. More often, microbial degradation of these chemicals is hindered by their low water solubility and thus is not readily accessible to microbial or enzymatic attack. Biological conversion of organochlorines via dechlorination, dehydrochlorination, oxidation and/or isomerization, are the common mechanisms of their degradation in natural habitats and these reactions are described by several authors (Johnsen 1976; Kaufman 1974; Kearney and Kaufman 1972; Lal and Saxena 1982; Matsumura 1973; Pfister 1972; Ware and Roan 1970) in detail.

6.1 DDT

Chlorinated pesticides are well known for their resistance to chemical and biological degradation. These compounds remain unaltered for many years after their application to soil and aquatic ecosystems (Edwards 1973; Menzie 1978). Residues of DDT and other chlorinated hydrocarbons have been found recently in farm soils (del Rosario and Yoshida 1976; Harris et al. 1977; Miles and Harris 1978), rice seeds and plants (del Rosario and Yoshida 1976), oligotrophic lake ecosystems and sediments (Andersen et al. 1982; Kveseth 1981; Veith et al. 1977), aquatic environments (Bjerk and Brevik 1980), marine ecosystems (Young et al. 1976) and in lagoons (Rosales and Escalona 1983), even though usage of these chemicals ceased in the early 1970's.

The persistence of DDT or its major metabolic products in soil vary from 6 months to many years (Edwards 1973; Kuhr et al. 1972; Nash and Harris 1973; Nash and Woolson 1967; Voerman and Besemer 1975). Addition of organic matter to soil accelerated DDT metabolism (Guenzi and Beard 1967; Farmer et al. 1974; Parr et al. 1970) and pH had very little effect on its conversion (Fleming and Maines 1953) and dissipation under natural conditions. About 62% of DDT applied to a soil was converted to DDD when incubated anaerobically (Guenzi and Beard 1967). In a subsequent study, they showed that alfalfa enhanced this conversion of DDT in anaerobic soil, but had little effect under aerobic conditions (Guenzi and Beard 1968). Similar results were obtained by Ko and Lockwood (1968) when submerged soil was amended with alfalfa or a mixture of glucose and peptone. DDD was the main product of DDT conversion in the fortified soil and was not detected in sterile alfalfa-amended soil, implicating biological conversion of DDT.

DDT disappeared rapidly in a moist nonautoclaved soil under anaerobic conditions (Burge 1971). Addition of glucose or distillates of alfalfa to the anaerobic soil enhanced DDT conversion to DDD. This conversion was biological, as DDT was not metabolized in sterile soils. In contrast, addition of co-energy substrates accelerated soil anaerobiosis, but had little effect on DDT metabolism (Parr et al. 1970). These studies demonstrate that certain obligate or facultative anaerobic microflora harboring in the soil can play an important role in destroying DDT from these habitats.

In an Everglades muck DDT was converted to DDD and DDE, but slowly. Addition of lime enhanced this process, probably by stimulating growth of DDT-degraders or accelerating DDT desorption from the muck (Parr and Smith 1974). Both flooding and addition of organic matter to DDT-amended soil shifted the formation of DDE to DDD (Farmer et al. 1974). Aquatic ecosystems, sewage and sediments were unfavorable environments for DDT decomposition. For example, DDT conversion to DDD or DDE was quite slow in an aquatic ecosystem. No degradation occurred in a bottled river water even after 8 weeks of incubation (Eichelberger and Lichtenberg 1971).

Zoro et al. (1974) observed rapid metabolism of DDT to DDD under anaerobic conditions. The authors suggested that under reduced conditions, DDT forms a complex with iron-porphyrins which is more susceptible to degradation. Under aerobic conditions, no such complex is formed, making DDT recalcitrant and inaccessible to microbial attack. This finding confirms a similar observation made by

Glass (1972), who demonstrated the role of iron-redox system in DDT degradation under anaerobic conditions. Albone and co-workers (1972 a) showed the conversion of DDT to DDD in an anaerobic sludge incubated at 35 °C under H_2-atmosphere. However, when incubated at 20 °C under N_2 atmosphere, DDT was converted to DDCN and DDD in the sewage sludge (Jensen et al. 1972). In situ degradation of DDT in Severn estuary sediment was found to be slow (Albone et al. 1972 b). DDT degradation was also not observed in a sewage lagoon (Halvorson et al. 1971).

6.1.1 Microbial Degradation of DDT

Microorganisms usually convert DDT via two major pathways, (a) reductive dechlorination, a process favored under anaerobic environments; and (b) dehydrochlorination, occurring in the presence of oxygen (Fig. 7). In vitro catabolism of DDT occurred with intact cells or with cell-free extracts of several bacterial species. Three facultative anaerobes, *Aerobacter aerogenes, Escherichia coli,* and *Klebsiella pneumoniae* dechlorinated DDT to DDD (Langlois 1967; Plimmer et al. 1968;

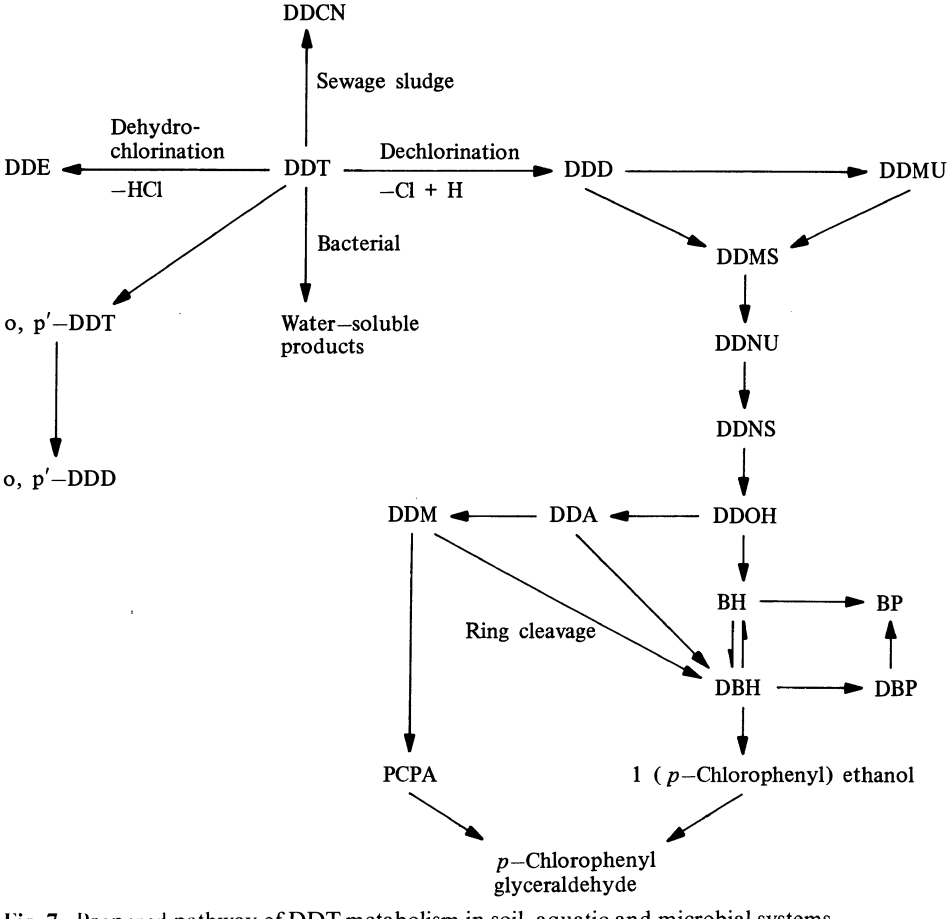


Fig. 7. Proposed pathway of DDT metabolism in soil, aquatic and microbial systems

Wedemeyer 1966). Complete anaerobiosis greatly enhanced this conversion (Wedemeyer 1966). Another bacterium, *Proteus vulgaris,* isolated from the gut of a DDT-resistant female mouse, dechlorinated DDT to DDD (Barker et al. 1965). A *Clostridium* sp. isolated from a γ-BHC-amended flooded soil converted DDT to DDD anaerobically (Sethunathan and Yoshida 1973c). The cell-free extracts of *A. aerogenes, Pseudomonas fluorescens, E. coli,* and *K. pneumoniae* dechlorinated *p,p'*-DDT to DDE, DDD, DDMU and DDNU exclusively under anaerobic conditions. Dechlorination of DDT was slightly accelerated in the presence of light-activated flavin mononucleotide (Wedemeyer 1967). DDT conversion by *E. coli* was completely inhibited when skim milk was present (Langlois 1967).

DDT was dechlorinated to DDD by six actinomycetes belonging to *Nocardia* and *Streptomyces.* But none of the tested fungi could metabolize DDT under laboratory conditions (Chacko et al. 1966). In contrast, *Mucor alternans* in shake cultures converted DDT to five unidentified metabolites. The spores of *M. alternans* failed to convert DDT in soil (Anderson et al. 1970). A baker's yeast converted DDT to DDD through reductive dechlorination, without forming DDE as an intermediate (Kallman and Andrews 1963).

Many soil microorganisms, mainly bacteria, were able to degrade DDT, endrin, aldrin, and dieldrin (Patil et al. 1970). DDT was primarily converted to TDE or a dicofol-like compound. DDE was not produced by any of these isolates. Juengst and Alexander (1976) obtained several bacterial species from surface water and sediment of marine environments which degraded DDT to water-soluble products. However, efforts to reconstitute organisms into a model marine ecosystem failed to result in DDT degradation. Marine microorganisms isolated from water and bottom sediments actively degraded DDT to TDE as the major metabolite. DDNS and DDOH were also formed in minor quantities (Patil et al. 1972).

6.2 DDT Analogs

DDM, an analog of DDT, was co-metabolized by a sewage sludge bacterium, *Hydrogenomonas,* under aerobic conditions. Ring cleavage of DDT did not occur with this bacterium and DDM, or *p,p'*-dichloro benzophenone were not utilized as carbon sources (Focht and Alexander 1971). In a later study, Pfaender and Alexander (1972) showed extensive dechlorination of DDT to DDD, DBP, DDMS, DDMU and DDE under anaerobic conditions. When oxygen was provided, PCPA was formed from one of the metabolites, implicating bacterial ring cleavage. Interestingly, ^{14}C-DDT was converted to similar metabolites detected with *Hydrogenomonas* sp. when DDT was added to sewage and freshwater sediment model systems. *Hydrogenomonas* sp. could cleave the aromatic ring of DDM and produced *p*-chlorophenyl acetate under aerobic conditions (Focht and Alexander 1970). The resting cells of another bacterium, *Arthrobacter* sp., isolated from lake water, converted *p*-chlorophenyl acetate to 4-chloro-3-hydroxyphenyl acetate and an unidentified metabolite (Deo and Alexander 1976).

A *Pseudomonas* sp. which utilized diphenyl ethane as sole carbon source, also metabolized several monochlorinated and nonchlorinated analogues of DDT affecting ring cleavage (Francis et al. 1976). Aryl-chlorine and a-carbon substitution de-

termined the biodegradability of DDT by *Pseudomonas* sp. as postulated by Focht and Alexander (1971). This bacterium did not utilize *p,p'*-dichlorophenyl analogs as sole carbon source, but bis(*p*-chlorophenyl) methane and 1,1-bis(*p*-chlorophenyl) ethane were co-metabolized in the presence of DPE (Francis et al. 1978).

Pseudomonas putida growing on diphenyl methane and benzhydrol, co-metabolized bis(*p*-chlorophenyl) methane (Subba-Rao and Alexander 1977a) producing several dehalogenated products. This bacterium also affected ring cleavage on certain products, but failed to co-metabolize these in the presence of DPM. In another study, Subba-Rao and Alexander (1977b) reported the formation of BHE by *P. putida* grown in DPM salts broth. BHE was again co-metabolized by the resting cells of this bacterium to BH and BP.

DDT and its analog, methoxychlor, were degraded much faster in an anaerobic flooded soil than in upland (nonflooded) soils. DDD was formed in anaerobic soils and resisted further degradation (Castro and Yoshida 1971).

Two nonhalogenated insecticides, having structural analogy with DDT, were first sorbed to soils but the insecticides disappeared with prolonged incubation. The rate of degradation of these compounds was dependent on the soil types, moisture content and redox potential. The breakdown intermediates of these analogs were degraded faster under aerobic conditions but were persistent in water-logged (anaerobic) and sterilized soils (Kimber 1980). These studies clearly indicate that soil microflora actively participate in the metabolism of these compounds.

Mixed bacterial cultures containing *Pseudomonas, Acetomonas* and *Acinetobacter* sp. were isolated from sewage sludge which degraded 1,1-diphenyl-ethylene, another analog of DDT. Bacterial metabolism of the DDT-analog was achieved through hydration and oxidation forming 2,2-diphenyl ethanol and atropic acid (Focht and Joseph 1974).

In general, biological conversion of DDT in soil and sediments accelerates upon organic matter amendments and anaerobiosis. Dechlorination is perhaps the major pathway of DDT metabolism by microbial cultures or with cell-free extracts. Bacteria degrading several analogs of DDT can metabolize DDT via co-metabolic reactions affecting ring cleavage.

7 Pesticide Combination

Most earlier studies relating to persistence of pesticides in soil and aquatic ecosystems involved the application of a single pesticide. But in agricultural practice, simultaneous or successive application of more than one pesticide is made for effective control of a wide variety of pests. This can lead to the build-up of combined residue problems in the environment. The interaction of this combination can have either a deleterious or beneficial effect on their persistence in nature.

The toxicity of parathion to certain insects increased significantly in the presence of atrazine (Lichtenstein et al. 1973; Liang and Lichtenstein 1974). When applied together, methyl carbamate insecticides increased the soil persistence of a herbicide, chlorpropham by nearly two to four fold, perhaps inhibiting the carbanilate-hydrolyzing enzymes (Kaufman et al. 1970). Likewise, azides, diazinon, phorate, and

carbaryl inhibited the degradation of porpham and chlorpropham in soil and in bacterial cultures (Kaufman 1977).

Hubbell et al. (1973) observed an additive effect on the fungal populations when DDT, parathion, and Zineb were applied to soil. Bacterial, actinomycetes, and algal populations remained the same under these treatments. The application of combined pesticides had little effect on the persistence of the individual pesticides in soil. Barik and Sethunathan (1979) studied different combinations of pesticides on the biological degradation of parathion in a flooded soil. In this case, benomyl increased the soil persistence of parathion by inhibiting both ester bond hydrolysis and nitro group reduction of parathion. Addition of benomyl to flooded soil also inhibited soil reduction even after prolonged incubation. In contrast, BHC, atrazine, and 2,4-D did not influence the biological degradation of parathion in flooded soil.

The effect of fertilizers and other nitrogenous compounds can also influence the persistence of pesticides in soil. Rajaram et al. (1978) observed that biological hydrolysis of parathion in soil amended with rice-straw was inhibited by the addition of potassium nitrate and ammonium sulfate. Additions of rice straw in combination with only potassium nitrate or only ammonium sulfate exhibited no such inhibition, and parathion was degraded via hydrolysis as in control soils. The degradation of fonofos and parathion was affected in the presence of fertilizers, captafol and atrazine. Addition of cow manure, sewage sludge and atrazine to soil treated with fonofos increased the unextractable soil-bound residues. Ammonium sulfate, atrazine or captafol, when combined with parathion, retarded the formation of aminoparathion. Evolution of $^{14}CO_2$ from ^{14}C-labelled fonofos and parathion was inhibited significantly when these compounds were applied with insecticides (Lichtenstein et al. 1982).

Thus, soil persistence of pesticides can be altered when applied in combination with other pesticides, fertilizers, and nitrogen-containing compounds. Long-term pesticide residues in the natural environment are undesirable. But, as many commercial formulations of pesticide combinations are available for use in agricultural practices, adverse effects from such combinations may be inevitable. Therefore, careful formulations of pesticide combinations are necessary for integrated pest-control programs. Microbiological aspects of pesticide degradation should also be kept in mind while adapting this method to agricultural practice.

8 Microorganisms and Pesticide Waste Treatment

The use of synthetic biocides in controlling agricultural pests continues to rise. Because of continuous pest problems, their usage possibly cannot be discontinued in the near future. Thus soil and aquatic environments will be the continuous sink for the millions of pounds of the world's pesticides. Large amounts of additional hazardous wastes can also be generated both at the producer and the consumer levels. Disposal of excess pesticides, empty pesticide containers, and accidental spillage during their transportation can create further environmental and ecological hazards. Present technology for detoxifying these unwanted pesticide wastes from ecological systems is very limited or does not exist.

Biological processes were recognized as one of the potent methods for detoxification of industrial wastes in the late 1960's (Howe 1969). Chemical treatment of low concentrations of pesticides is cost-prohibitive, but these wastes can be effectively treated biologically. Microbial degradation of the xenobiotics from sewage sludge (McIntyre et al. 1981), pesticide disposal pit (Johnson and Hartman 1980), and polluted water systems (Daorai and Menzer 1977) has been reported. McIntyre et al. (1981) reported the degradation of several organophosphates (diazinon, ethyl parathion, and malathion) in sewage sludge, attributed partly to biological phenomenon. A *Pseudomonas* sp. isolated from a sewage lagoon metabolized Abate into water-soluble compounds (Daorai and Menzer 1977). Johnson and Hartman (1980) studied the microbiology of several pesticide disposal pits and found increased numbers of bacterial populations in them. Furthermore, the authors suggested a possible pesticide utilization by these microorganisms.

Enzymatic decontamination of pesticides from waste water (Munnecke 1978, 1980) and highly contaminated soils (Barik and Munnecke 1982; H.F. Fisher et al. 1980) has been discussed. Munnecke (1979a, b) suggested detoxification of contaminated areas and pesticide-industrial effluents using soluble or immobilized enzymes. Enzymatic detoxification has been attempted using parathion hydrolase. The presence of high concentrations of salts, solvents, and detergents (also present in the commercial formulations of pesticides) had little effect on pesticide hydrolysis (Munnecke 1980). This enzyme was further used to decontaminate pesticide containers, suggesting its higher catalytic properties even at elevated concentrations. Detoxification of very high concentrations (up to 1%) of parathion (H.F. Fischer et al. 1980) and diazinon (Barik and Munnecke 1982) in soils by enzymatic treatment was also successful. The insecticides were hydrolyzed to less toxic and unstable compounds within a short period of incubation and were not detected in the soil after 24 h. Without treatment, high concentrations of this type of residue are expected to persist in the soil for many years (Davis et al. 1977; Staiff et al. 1975; H.R. Wolfe and Durham 1966; H.R. Wolfe et al. 1973).

The presence of high salt, solvent and other micropollutants, particularly in the sewage sludge and industrial effluents, may not favor fast microbial growth and effective pesticide decomposition. Therefore, the use of microbial cells in such areas may not be feasible and therefore is not recommended without a pre-treatment. Microbial enzymes could be the only alternative to physical and chemical decontamination for detoxifying pesticides in these ecological areas. More efforts are needed to determine the potential application for enzymatic detoxifications to clean up used pesticide containers and contaminated areas, and to reduce pesticide concentrations in industrial waste disposals to acceptable levels. Enzymatic detoxification of pesticide residue treatment can be a viable alternative to the physical and chemical methods recommended for the decontamination of toxic chemicals (Lawless et al. 1974).

9 Conclusions

The use of pesticides has become an integral part of modern agricultural systems. However, government regulations for the safe usage and disposal of pesticides have

become more stringent in many countries. Pesticides reaching soil, sediment, and aquatic ecosystems can be influenced by many physical, chemical, and biological factors. But their behavior and the nature of their residence period in these ecosystems determines their potential usefulness against target pests and hazardous effects on nontarget organisms. However, their fate and metabolism under such conditions cannot be predicted unless supported by laboratory studies.

Basic research regarding pesticide transformation in natural environments and under laboratory conditions has established the importance of biological degradation as a major mechanism of pesticide detoxification. Microorganisms are now believed to be the major catalytic forces that degrade pesticides in soil, sediments, and aquatic ecosystems. Once acclimated to these synthetic chemicals, their versatility in degradation of the xenobiotic compound is intensive. Biological hydrolysis is one of the major reactions to occur in the decontamination process of many pesticides. This is particularly true in the metabolism of organophosphates, dithioates, and carbamates. This process leads to complete destruction of parent pesticide molecules. Reductive transformations of some pesticides (organophosphates, organochlorines) are also biologically mediated and occur widely in soil, aquatic ecosystems, and in isolated microbial cultures. These reactions include, transformations of nitro to amino group, reduction of sulfoxides to sulfide, reductive dehalogenation and reduction of certain metals. Formation of these reduced products often leads to soil-bound residues (Lichtenstein 1981). Our knowledge of the fate and metabolism of bound residues is limited. More research is needed in this area, involving the usefulness of microbial cells or their enzymes for the detoxification of these residues.

Although co-metabolic reactions are not a direct process of pesticide detoxification, co-metabolism can lead to ultimate degradation of a xenobiotic compound. An initial microbial attack on the pesticide molecule determines its stability under environmental conditions and in some cases co-metabolism can trigger this biodegradability. Anaerobic or alternate aerobic-anaerobic conditions often accelerate pesticide decomposition, both under soil environments and in microbial cultures. However, our knowledge of the fate and metabolic pathways of pesticides under complete anaerobiosis is fragmentary. Present knowledge of reductive cleavage of the benzene ring under complete anaerobic conditions (Evans 1977; Mountfort and Bryant 1982) should be extended to pesticide research for the complete discovery of their metabolic pathways under such anoxic situations. Interesting phenomena occur when the biodegradation of pesticide compounds is tested in combination with other pesticides, fertilizers or other organic substances. More careful research emphasizing the microbial role on such situations is needed before predicting the safety of a pesticide in natural environments. Also, microbial participation (either intact cells or enzymatic) on the degradation of xenobiotic contaminated sewage sludge and waste water systems needs much more attention.

The ultimate fate of the xenobiotics in the environment cannot be totally determined by studying their behavior under controlled laboratory conditions or vice versa. Model ecosystems are helpful to extrapolate biodegradation studies and predict metabolic pathways of organic pesticides in natural environments. In recent years, yet another technique, using bacterial plasmids, has drawn much research attention for its application in pesticide degradation (see Chap. 7). Plasmids are found to be distributed throughout the bacterial world, and they can be coded for pesti-

cide-degrading enzymes. In fact, the usage of bacterial plasmids has resulted in the degradation of more toxic phenoxyacetate herbicides such as 2,4-D, 2,4,5-T, MCPA, and many naturally occurring aromatic and aliphatic compounds (Chakrabarty 1982; Chatterjee and Chakrabarty 1983; P.R. Fisher et al. 1978; Pemberton 1979). Kellog et al. (1981) also reported that molecular genetic breeding for new plasmids in bacterial species can be performed under laboratory conditions. This process could be implicated for plasmid creation, evolution and for obtaining multitude pesticide-degrading capabilities in bacterial species. Integration of molecular biology for better understanding the degradative pathways of pesticide metabolism will probably enhance the potential for the use of microorganisms in the detoxification of xenobiotic compounds.

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Appendix

Chemical names of pesticides mentioned in the text.

Common name	Chemical name
Abate	0,0,0′,0′-Tetramethyl 0,0′-thiodi-*p*-phenylene phosphorothioate
ABS	Alkyl benzenesulfonate
Acephate	O-S-Dimethyl acetylphosphoramidothioate
Aldrin	1,2,3,4,10,10-Hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-endo, exo-5,8-dimethanonaphthalene
Aspon	1,2,4,5,6,7,8,8,-Octachlor-2,3,3,3a,4,7,7a-hexahydro-4,7-methanoindane
Atrazine	2-Chloro-4-(ethylamino)-6-(isopropyl amino)-*s*-triazine
Azinophosmethyl	O,O-Dimethyl-*s*-(4-oxo-3-H-1,2,3-benzotriazine-3yl)-methyl-dithiophosphate
Benomyl	Methyl 1-(butylcarbamoyl)-2-benzimida-zolecarbamate
BH	Benzhydrol
BHC	A mixture of isomers of 1,2,3,4,5,6-hexa-chlorocyclohexane
γ-BHC (Lindane)	γ-1,2,3,4,5,6-Hexachlorocyclohexane
BHE	1,1,1′,1′-Tetraphenyldimethyl ether
BP	Benzophenone
Captafol	*N*-(1,1,2,2-Tetrachloroethylthio) cyclohex-4-Ene-1,2-dicarboximide
Carbaryl	1-Naphthyl methylcarbamate
Carbofuran	2,3-Dihydro-2,2-dimethyl-7-benzofuranylmethyl-carbamate
Chlordane	1,2,3,4,5,6,7,8-Octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene
Chlorfenvinphos	2-Chloro-1-(2,4-dichlorophenyl) vinyl diethyl phosphate
Chlormephos	S-Chlormethyl-O,O-diethyl phosphoro-thiolothionate
Chlorpropham	Isopropyl-N-3-chlorophenyl-carbamate
Chlorpyrifos	O,O-Diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate
2,4-D	(2,4-Dichlorophenoxy) acetic acid
DBP	4,4′-Dichlorobenzophenone
DDCN	bis(*p*-Chlorophenyl) acetonitrile
DDD	2,2′-bis(*p*-Chlorophenyl)-1,1-dichloroethane
DDE	2,2′-bis(*p*-Chlorophenyl)-1,1-dichloroethylene
DDM	bis(*p*-Chlorophenyl) methane
DDMS	2,2-bis(*p*-Chlorophenyl)-1-chloroethane
DDMU	1-Chloro-2,2-bis(*p*-chlorophenyl) ethylene
DDNS	2,2-bis(*p*-Chlorophenyl) ethane
DDNU	2,2′-bis(p-Chlorophenyl) ethylene
DDOH	2,2′-bis(*p*-Chlorophenyl) ethanol
DDT	1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl) ethane
Diazinon	O,O-Diethyl O-(2-isopropyl-4-methyl-6-pyrinidinyl) phosphorthioate

Dichlorvos	2,2-Dichlorovinyl O,O-dimethyl phosphate
Dicofol	2,2-bis(*p*-Chlorophenyl)-1,1,1-trichloroethanol
Dieldrin	1,2,3,4,10,10-Hexachloro-6,6-epoxy-1,4,4a,5,6,7,8,8a-Octahydro-1,4-endo,exo-5,8-dimethanonaphthalene
Dimethoate	O,O-Dimethyl S-(N-methylcarbamoylmethyl) phosphorodithioate
DPE	Diphenylethane
DPM	Diphenylmethane
DNOC	Dinitro-ortho-cresol
Dursban	O,O-Diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate
Endosulfan	6,7,8,9,10,10-Hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin 3-oxide
Endrin	1,2,3,4,10,10-Hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-*endo, endo*-5,8-dimethanonaphthalene
EPN	O-Ethyl O-(4-nitrophenyl) phenyl phosphonothioate
Ethion	O,O,O,O-Tetraethyl-S,S-methylene bisphosphorodithioate
Fenitrothion	O,O-Dimethyl O-(3-methyl-4-nitrophenyl) phosphorothioate
Fensulfothion	O,O-Diethyl-O- 4-(methylsulfinyl)phenyl phosphorothioate
Fenthion	O,O-Dimethyl-O- 3-methyl-4-(methylthio)phenyl phosphorothioate
Heptachlor	1,4,5,6,7,8,8-Heptachloro-3a,5,7,7a-tetrahydro-4,7-methanoindene
IMHP	2-Isopropyl-4- methyl-6-hydroxy pyrimidine
LAS	Linear alkyl benzenesulfonate
Lindane	see γ-BHC
Malathion	O,O-Dimethyl-S(1,2-dicarbethoxyethyl)-phosphorodithioate
MCPA	4-Chloro-2-methyl phenoxyacetic acid
Metasystox-R	O,O-Dimethyl S-2-(ethylsulfinyl) ethyl phosphorothioate
Methoxychlor	1,1,1-Trichloro-2,2-bis(*p*-methoxyphenyl) ethane
Methyl parathion	O,O-Dimethyl-O-*p*-nitrophenyl phosphorothioate
Monocrotophos	(E)-Dimethyl 1-methyl-3-(methylamine)-3-oxo-1-propenyl phosphate
Paraoxon	O,O-Diethyl-O-*p*-nitrophenylphosphate
Parathion	O,O-Diethyl-O-*p*-nitrophenyl phosphorothioate
PCPA	*p*-Chlorophenyl acetic acid
Phorate	O,O-Diethyl S-(ethylthio) methyl phosphorodithioate
Phenthoate	O,O-Dimethyl S-α-(carboethoxy)-benzyl phosphorodithioate
Phosdrin	2-Methoxycarbonyl-1-methylvinyl dimethyl phosphate
Porpham	Isopropyl-N-phenylcarbamate
Sevin	see Carbaryl
Sumithion	see Fenitrothion
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
TDE	1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl) ethane
Tetradifon	S-*p*-Chlorophenyl 2,4,5-trichlorophenyl sulfone
Triazophos	1-Phenyl-1,2,4-triazolyl-3-(O,O-diethylthionophosphate)
Trichlorfon	Dimethyl (2,2,2-trichloro-1-hydroxyethyl) phosphonate
Trichloronat	2,4,5-Trichlorophenyl ethyl phosphonothioate
Trithion	S-(*p*-Chlorophenyl)thio methyl O,O-diethyl phosphorodithioate
Zineb	Zinc ethylenebis (dithiocarbamate)

CHAPTER 6

Enzymes Associated with the Microbial Metabolism of Insecticides

L. M. JOHNSON

1 Introduction

Until the second World War, chemicals that were used as pesticides[1] were typically inorganic compounds (Bollag 1982). After this period, the chemical revolution occurred and as a result, the production and use of organic chemicals as pesticides increased dramatically. Although some non-carbon containing, inorganic pesticides and metallo-organic pesticides are in use, this review will focus on the biodegradation of organic insecticides.

The magnitude of the problem that arises from pesticide use can be appreciated by noting the quantity of chemicals used annually. In 1964; approximately 6.8×10^8 pounds of active pesticidal chemicals were manufactured (Alexander 1969). In 1971, about 8.0×10^8 pounds of pesticides were applied to the environment (Munnecke et al. 1976). Synthetic pesticides will continue to be used in tremendous quantities (Fox and Delvo 1972). Approximately 1.2 billion pounds of pesticides (about 6 pounds per person) were being used annually in the United States (United States Department of Agriculture 1977). It has been projected that a 50% increase in the amount of pesticides used in 1976 would be seen by 1984 (Muhm 1976).

Each increase in the use of crop-protection chemicals accentuates an already severe problem of how to dispose of unwanted pesticides and pesticide containers. In 1968, about 2.4×10^8 empty pesticide containers were produced from agricultural practices (Munnecke et al. 1976). In California, approximately 350,000 small (1–5 gallon) metal containers, 98,000 30-gallon and 8300 55-gallon metal containers accumulated as a result of the use of pesticides in agriculture during 1969 (Archer and Hsieh 1973). After being used, these containers contain 0.02 to 0.37% pesticide residue (Hsieh et al. 1972) and must be properly disposed of to avoid environmental and public health hazards (Roper 1972). Reviews of pesticide disposal methods were presented by Day (1976) and Munnecke et al. (1976).

Presently, it is difficult to determine the amount and types of chemicals that are manufactured and used. Chemical technology has made a wide variety of crop-protection chemicals available to agriculture, and to a large extent, the agricultural industry is the major consumer of pesticides. During the 1970's, it was recognized that agricultural chemicals could damage the environment and public health. By the end of that decade, nearly 1.5×10^9 pounds of pesticides were used annually (Storck 1980). Because such large amounts of pesticides are used, adverse responses to the

1 Chemical names of pesticides mentioned in the text are given in the Appendix

chemicals were evoked and problems of improper waste disposal (Munnecke et al. 1976), environmental damage (Munnecke 1979a) and chronic exposure (Murry 1976) began to arise.

Several disposal methods have been recognized for treating pesticide wastes, and these include: (1) use as a diluent in spray tanks, (2) incineration at high temperatures (1000 °C), (3) soil injection by ordinary farm equipment (plows, harrows, etc.), (4) photodecomposition, by placement of the chemical on the soil surface or in a lagoon, (5) chemical degradation by chemical oxidation or reduction and alkaline or acid hydrolysis, (6) biodegradation under controlled microbial culture conditions, (7) disposal pits, (8) special landfills, (9) storage (which merely delays disposal), and (10) enzymatic treatment. The least expensive and most convenient methods are usually sought and the particular method should be chosen to minimize environmental damage. Biological systems are advantageous because they are capable of metabolizing a wide variety of pesticides under mild conditions and the resulting detoxification is generally more complete than physical or chemical procedures. Also, facilities that utilize biological methods for pesticide disposal are more easily constructed than pesticide incinerators or chemical treatment plants.

Howe (1969) described several elaborate biological systems capable of specifically degrading toxic wastes present in industrial effluents. Data were also reported on degradation processes of mixed culture microorganisms which were acclimated. Munnecke (1976) presented a possible pesticide disposal method capable of enzymatically hydrolyzing organophosphate insecticides. A crude cell extract from a mixed bacterial culture grown on parathion hydrolyzed this chemical 2450 times faster than chemical hydrolysis by 0.1 N sodium hydroxide. Still further refined enzyme preparations were reported by Munnecke (1977); an immobilized bacterial enzyme system was capable of hydrolyzing nine organophosphate insecticides. Although these laboratory systems were not practical for immediate routine use, their importance has been determined with time. It is the intent of this article to review the advances that have been made in the area of enzymes associated with the metabolism of insecticides. This review will concentrate on those reactions that are mediated by cell-free extracts and isolated enzyme systems in order to demonstrate the potential of microbial enzyme systems. Microbial enzymes have been isolated

Table 1. Toxicity of selected insecticides and major products of enzymatic hydrolysis

Pesticide	Major metabolite	Toxicity ($mg\ kg^{-1}$)[a]	Detoxification factor
Organophosphates			
Parathion	–	6	
	p-Nitrophenol	350	122
Azinphos-methyl	–	13	
	Anthranilic acid	4630	824
Carbamates			
Carbaryl	–	500	
	1-Naphthol	2590	7

[a] Toxicity reported: LD_{50} oral, rat, $mg\ kg^{-1}$ (Data from Christensen 1976)

that are effective in converting toxic or persistent insecticides to less toxic or persistent compounds. Hydrolases are instrumental for organophosphate and carbamate insecticide degradation and are excellent candidates for industrial or large-scale use because they are stable over wide ranges of pH and temperature and require no co-factors. The toxicity of some insecticides is listed in Table 1, as well as the toxicity of their major product of enzymatic hydrolysis (Christensen 1976). For example, when *p*-nitrophenol is produced via the hydrolysis of parathion, a 122-fold decrease in toxicity occurs. Hydrolysis of organophosphate and carbamate insecticides will generally result in a significant decrease in toxicity. In addition, the bioactivity of the parent insecticidal compound is destroyed and the metabolites of the hydrolytic reactions are most typically less stable in the environment than the original insecticidal molecule.

2 Fundamental Reactions of Insecticide Metabolism

The enzyme-mediated metabolism of pesticides by microorganisms is best discussed in the light of information that has been gathered which describes the microbial activities that cause pesticide transformations. An early summary of microorganisms responsible for degrading pesticides was provided by Alexander (1969) and an excellent review of organophosphorus and carbamate insecticide degradation has been prepared by Laveglia and Dahm (1977). The general modes of transformation of pesticides have been presented by Bollag (1974, 1982) and Matsumara (1974). Recently, Lal and Saxena (1982) summarized the interactions of organochlorine insecticides and microorganisms.

Because microorganisms possess the unique biochemical asset of being able to catalzye the oxidation of numerous chemical structures with molecular oxygen (Dagley 1975), enzymatic, oxidative reactions in pesticide metabolism are frequently observed. Man-made molecules will be biodegradable only if they are susceptible to attack by enzymatic capabilities acquired during the evolution of the organisms involved. The natural development of enzyme systems has been observed (Vandenbergh et al. 1981) and sheds light on the infallibility of microorganisms to adapt and destroy man-made compounds.

Frequently, a primary transformation of a molecule involves a hydroxylation reaction (Bollag 1974, 1982, Dagley 1975, Matsumara 1974) and such hydroxylase or oxygenase reactions are of great importance during the early steps of aromatic biodegradation, But, since these enzymes require co-factors, they are not under consideration for large-scale use at the present time. Matsumara (1974) believed that microorganisms exhibit hydrolytic activities more frequently than hydroxylation for opening ring structures. Similar hydrolytic activities have been investigated in detail and have the greatest potential for use as enzyme systems for detoxifying pesticides.

Dealkylation reactions are important in the transformation of phenylureas, carbamates and triazines (Bollag 1974, 1982; Bartha et al. 1967). Hill et al. (1955) were among the first to describe dealkylation reactions, and subsequently Ross and Tweedy (1973) and Weinberger and Bollag (1972) also reported demethylation reactions. Because many pesticidal compounds possess alkyl moieties which produce

desired toxic influences, dealkylation reactions are the first steps in the detoxification of these types of molecules.

Epoxidation reactions are commonly observed in the metabolism of insecticides. Such reactions occur when an oxygen atom is added to a double bond. Frequently, this reaction is observed in the metabolism of aromatic substances, and during the epoxidation of cyclodiene insecticides (Lal and Saxena 1982). Epoxidation is one of the more thoroughly studied microbial oxidation systems because of the relative refractory nature of cyclodiene insecticides.

One metabolic reaction that is frequently observed during the biodegradation of certain organophosphate insecticides is that of sulfoxidation (Bollag 1974, 1982; Laveglia and Dahm 1977). This reaction consists of the oxidation of divalent sulfur to the sulfoxide and sometimes to the sulfone.

Several groups of pesticides are subject to reduction, but these types of reactions have been less studied than oxidative types. In many instances, a conversion of a nitro group to an amine occurred with many bacteria and fungi, and this type of reaction was observed during the transformation of parathion (Mick and Dahm 1970; Munnecke and Hsieh 1974).

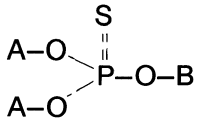
Several mechanisms are also involved in the anaerobic dissimilation of pesticides and some mechanisms have been presented by Bollag (1974). A review by Williams (1977) presented anaerobic transformations by several microorganisms on over 130 different pesticidal molecules. Anaerobic mechanisms responsible for the degradation of pesticides include reduction, dealkylation, dehalogenation and hydrolysis reactions. Although Ko and Lockwood (1968) reported that anaerobic conditions enhanced the degradation of many chlorinated hydrocarbons, it has only been until recently that more information has been acquired to describe the actual mechanisms of dehalogenation. For example, Suflita et al. (1982) recently described the strictly anaerobic dehalogenation of halobenzoates by a stable methanogenic consortium. The results of these studies indicate that such mechanisms may be important for removing some chlorinated compunds from the environment. The enzymes involved in these metabolic processes have not been isolated or characterized, but they may serve to detoxify chloroaromatics without requiring cofactors.

The laccase enzymes system have been described for removing xenobiotic compounds from the environment. These enzymes incite the polymerization of phenols and other aromatic substances into water-insoluble polyaromatics. This enzyme is produced by the fungus *Rhizoctonia praticola* and has been described by Bollag et al. (1979).

3 Organophosphates

Organophosphate pesticides have been used as replacement chemicals for the more persistent organochlorine and inorganic mercurial chemicals. Reviews have been published discussing the environmental fate of organophosphate pesticides and the role microorganisms play in their degradation (Adamson and Inch 1973; Kaufman 1974; Laveglia and Dahm 1977; Paris and Lewis 1973; Sethunathan et al. 1977; Tu and Miles 1976). The organophosphates are characterized as having the general

structural formula of the type shown in Fig. 1. Parathion (O,O-diethyl-O-*p*-nitrophenyl phosphorothioate) is the most extensively studied organophosphate insecticide because it is used extensively in agriculture. Indirect evidence of microbial participation in the degradation of parathion was demonstrated when the stability of parathion in autoclaved and nonautoclaved soil and water samples was examined (Barik and Sethunathan 1978; Lichtenstein et al. 1968).



A = alkyl moiety; B = substituted aryl or alkyl moieties

Fig. 1. Structure of organophosphate insecticides

In some cases, heat-labile agents of microbial origin were isolated from the soil, which helped in rapid degradation of parathion. Methyl parathion, like ethyl parathion, also disappeared rapidly in nonsterilized but not in sterilized soils (Getzin and Rosefield 1968). Conclusive evidence for the involvement of microorganisms in parathion degradation was obtained when microbes were isolated from soil which could degrade parathion in vitro.

The initial point of organophosphate metabolism is the hydrolysis of the aryl phosphate bond. The hydrolase responsible for catalyzing this reaction (EC 3.1.3), also referred to as an esterase, aryl esterase, or phosphotriesterase, is the most important enzyme in the bacterial metabolism of organophosphates. An analogous enzyme has been extensively studied in both insects and mammals (Eto 1974), since this reaction is a major mechanism of resistance and detoxification. In one of the earliest studies of organophosphate hydrolases, Mounter et al. (1955) reported that a group of microorganisms contained enzymes capable of hydrolyzing dialkylfluorophosphates. The DFPase activity (EC 3.7.2.1) was found in 10 of 14 microbes tested and was activated by Mn^{2+} and inhibited by Ca^{2+}.

Zech and Wigand (1975) isolated two phosphohydrolases from *E. coli* and one of the enzymes, DFPase (EC 3.8.2.1) could detoxify di-isopropylfluorophosphate while the other, paraoxonase (EC 3.1.1.2) hydrolyzed paraoxon. Both enzymes had a very low substrate specificity ($K_m = 1.7 \times 10^{-2}$ and 5×10^{-3} mM l^{-1} for DFP and paraoxon, respectively), had activity at pH 8 to 8.5 and were unstable at room temperature.

A cell-free preparation isolated from *Flavobacterium* sp. ATCC 27551 was described by Sethunathan and Yoshida (1973) and this preparation hydrolyzed diazinon, parathion, and chlorpyrifos. The aromatic or heterocyclic moieties produced by the hydrolysis of these insecticides could not be further metabolized by the *Flavobacterium*. This phosphoesterase (EC 3.1.3) did not hydrolyze amino parathion or malathion, a dithioate insecticide. In another investigation with this bacterium, Brown (1980) reported that the constitutive phosphotriesterase was composed of two protein units, one with a molecular weight of 50,000, and the other with a molecular weight of greater than 100,000.

The larger protein unit was associated with an orange gum which was produced by the bacterium and most likely contained flavoprotein. The enzyme expressed ac-

tivity with those organophosphate substrates having an electron withdrawing aromatic or heterocyclic leaving group (such as parathion, paraoxon, and diazinon). The enzyme could not hydrolyze compounds with weakly electrophilic groups like 4-aminophenol. This phosphoesterase showed activity at pH values of 8 to 10 and was unaffected by the presence of EDTA, NaF, or NaN_3 or metal ions such as Zn^{2+}, Cu^{2+}, Co^{2+}, Mg^{2+} or Mn^{2+}. Irreversible enzyme inhibition was seen with non-ionic detergents such as Triton X-100 and Tween 80.

A crude enzyme extract obtained from a mixed bacterial culture that was grown on parathion showed exceptionally high activity for hydrolysis of at least ten organophosphates (Munnecke 1976). The rate of parathion hydrolysis was 3000 nmol min^{-1} mg^{-1} protein and was 3000 times faster than chemical hydrolysis by 0.1 N NaOH. This phosphoesterase (EC 3.1.3) was unaffected by high substrate concentration or metabolite accumulation, did not require co-factors, and was active between pH 7 and 10.5. The temperature optimum of the enzyme was 35 °C, and the crude enzyme preparation was stable at room temperature but was denatured by freezing.

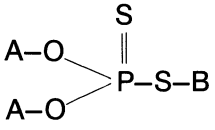
The presence of various solvents at 15 to 450 ppm accelerated hydrolysis, but enzyme activity was inhibited at solvent concentrations of 1000 ppm (Munnecke et al. 1976). Cleavage of the arylphosphoester bond was influenced more by alkyl substituents on the phosphorus atom than by functional groups on the aromatic ring. In the case of diazinon, EPN, chlorpyrifos, and triazophos, the effect of aromatic functional groups did not prevent enzymatic hydrolysis, but a $-SCH_3$ group in the para position of fenthion inhibited hydrolysis. Both dimethyl- and diethyl-substituted dithiophosphates (ethion, azinphosmethyl, chlormephos) were not hydrolyzed by this crude enzyme preparation.

Munnecke (1980) and Talbot et al. (1982) covalently bound this crude enzyme extract to porous glass, silica beads or alumina and then examined the properties of the enzyme. Simulated waste waters containing concentrated pesticide solutions were studied. The immobilized enzyme exhibited approximately 50% of its soluble enzyme activity and displayed minor changes in pH and temperature from the characteristics of free enzyme. Each immobilized enzyme reactor had a volume of 200 ml and could hydrolyze 90% of a 10 ppm parathion solution at flow rates up to 96 l h^{-1}. Although reactors containing enzyme bound to glass exhibited good characteristics for the parameters examined, the reactor containing enzyme immobilized to alumina was superior at high flow rates, such as those rates that are encountered in industrial situations (Talbot et al. 1982).

Rosenberg and Alexander (1979) obtained an inducible phosphoesterase from two *Pseudomonas* species grown in the presence of either diazinon or malathion as sole phosphorous sources. A crude enzyme preparation could hydrolyze several other compounds, including aspon, monocrotophos, fensulfothion, diazinon, malathion, acephate, parathion, and trithion. The enzyme preparation could not hydrolyze dimethoate, trichlorfon, methyl parathion or dichlorvos. The cleavage of the common phosphorous-oxygen bond of these pesticides was not influenced by the type of alkyl substituents. This enzyme also expressed activity against the dithioate insecticides malathion and carbophenothion in contrast to the parathion hydrolase reported by Munnecke (1976).

All of the above organophosphate hydrolysis studies deal with the primary hydrolysis of the parent molecule. Clearly this is an important step in the mineraliza-

tion of pesticides because such degradation primes the compounds for further metabolism. For example, *p*-nitrophenol, the major metabolite formed by hydrolysis of parathion, paraoxon, or methylparathion is readily metabolized by numerous microorganisms, as are other phenol metabolites produced from the initial hydrolysis of other organophosphates (Barik and Sethunathan 1978; Barik et al. 1976; Gundersen and Jensen 1956; Jensen and Lautrup-Larsen 1967; Munnecke and Hsieh 1974).



A = alkyl moiety; B = substituted aryl or alkyl moieties

Fig. 2. Structure of dithioate insecticides

Dithioates constitute a major group of organophosphates. They are characterized by the general chemical formula shown in Fig. 2. These pesticides are in great demand due to their broad spectrum and high insecticidal activities. However, not much work has been reported regarding their microbial metabolism except for one compound, malathion [O,O-dimethyl-S-(1,2-dicarbethoxyethyl)phosphorodithioate]. Two species of the genus *Rhizobium, R. leguminosarum* and *R. trifolii,* isolated from an Egyptian soil, demonstrated powerful carboxyesterase activity (EC 3.1.1.1) toward malathion (Mostafa et al. 1972 b). Five hydrolytic products, inorganic phosphate and thiophosphate, were identified as metabolites. In a study by Mostafa et al. (1972 a), three fungal isolates, including *Aspergillus niger, Penicillium notatum* and *Rhizoctonia solani,* expressed carboxyesterase activity as indicated when malathion was degraded to different carboxylic products which constituted nearly 40% of the total metabolites. Monomethyl phosphate and thiophosphate were formed in the culture medium due to the action of two other enzymes, an esterase (EC 3.1.1.2) and a phosphatase (EC 3.1.3). The esterase cleaved the sulfur carbon bond of the mono- and di-acids to dimethyl phosphorothioates and dimethyl phosphorodithioates, which were ultimately demethylated through phosphatase activity.

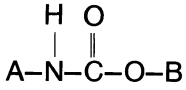
Matsumura and Boush (1966) examined a strain of *Trichoderma viride* and a *Pseudomonas* sp. and found that malathion was rapidly metabolized by the action of a soluble carboxyesterase (EC 3.1.1.1) as evidenced by the presence of carboxylic acid derivatives in the culture medium in addition to other demethylated products and products of hydrolysis. Cell-free enzymatic hydrolysis of malathion at the phospho-sulfur bond was reported by Rosenberg and Alexander (1979). This enzyme was obtained from a *Pseudomonas* species grown on either diazinon or malathion which hydrolyzed carbophenothion and malathion, but failed to metabolize dimethoate. An alkali-extractable, heat-labile organic entity from soil, which transformed malathion to malathion monoacid, was isolated by Getzin and Rosenfield (1971) from both irradiated and nonirradiated soil. This soil-free, extracellular esterase was quite stable, exhibited a pH optimum of 6.8 and displayed normal Michaelis-Menten kinetics. The enzyme was not denatured with increased acidity (pH < 2.0) and was active over a wide temperature range (20 °– 70 °C).

Recently, Barik et al. (1982) reported the enzymatic hydrolysis of malathion and other dithioate pesticides. In these studies, a constitutive, cell-free enzyme extract was obtained from two strains of *Arthrobacter.* The two enzyme extracts showed a broad pH optimum (6–9), temperature optima of 25 °C and specific activities of 1.3 and 2.0 μmol mg^{-1} protein min^{-1} for the substrate malathion.

4 Carbamates

The carbamates are a structurally and physiologically heterogeneous group of compounds. Their classification is based upon the fact that they are all derivatives of carbamic acid (Fig. 3). Carbaryl and carbofuran act as insecticides whose mode of action is that of inhibiting acetylcholinesterase, whereas chlorpropham and diallate act as herbicides and control plant growth by interfering with cell division. More is known about the microbial enzymology of phenylcarbamate herbicides than any other group of the carbamate pesticides. The best-characterized carbamate-hydrolyzing enzyme was obtained by Kearney and Kaufman (1965) when they isolated a strain of *Pseudomonas striata* Chester from soil which could metabolize the phenylcarbamate chlorpropham [isopropyl-N-(3-chlorophenyl) carbamate, CPC] as a sole source of carbon and energy. Enzymatic cleavage of the ester and/or amide linkage of chlorpropham was quantified by the appearance of 3-chloroaniline. The esterase activity (EC 1.1.1) contained in a cell-free extract from the pseudomonad also cleaved propham, isoproyl-N-phenylcarbamate (IPC), as well as nine other carbamates, including alpha-carbo (2,4-dichlorophenoxyethoxy) ethyl N-(3-chlorophenyl) carbamate, isopropyl-N-(3,4-dichlorophenyl) carbamate, sec-butyl-N-(3,4-dichlorophenyl) carbamate, and alpha-carboisopropoxyethyl-N-(3-chlorophenyl) carbamate.

Either 3-choroaniline, aniline, or 3,4-dichloroaniline was detected from all of the carbamates after esterase activity, Kearney (1965) later reported the purification and characterization of this enzyme. With chlorpropham as the carbon source, the pseudomonad isolate was mass-cultured and the cells were sonicated. Ammonium sulfate precipitation, gel filtration and ion exchange were used to purify the enzyme. After purification the pH optimum was 8.5 and the enzyme had a broad range of activity between pH 6.0 and 9.5. With chlorpropham and propham as substrates, the esterase had specific activities of 6 and 5 nmol min^{-1} mg^{-1} protein, respectively. The metal ions Mg^{2+}, Mn^{2+}, Ca^{2+} and Co^{2+} were not necessary for enzyme activity. The esterase was inhibited 42% with diisopropylfluorophosphate at a 10 to 6 M concentration, which implied that the hydroxyl group of serine possibly was part of the active site of enzyme–substrate interaction. The broad substrate specificity of the enzyme was demonstrated by its esterase activity against the carbamates, plus the fact



A = methyl or aryl moiety; B = typically alkyl or aryl moiety

Fig. 3. Structure of carbamate insecticides

that it also showed amidase activity. As an amidase (EC 3.5.1.4), the enzyme hydrolyzed 3,4-dichloroacetanilide and 3,4-dichloropropionanilide. It was proposed that large, bulky substituents in the area of the carbonyl carbon could cause steric hindrance and inhibit the hydrolysis of compounds with this property.

5 Miscellaneous Enzyme Reactions

Several other enzymatic reactions have been observed and are responsible for detoxifying pesticides. Besides those enzymes that catalyze reactions to detoxify insecticides, there are catalytic proteins that exhibit the capacity to degrade herbicides and other pesticidal molecules and these have been presented by Munnecke et al. (1982).

As mentioned previously, the enzyme laccase has shown a remarkable propensity for catalyzing the oxidative coupling of phenols and substituted phenols (Bollag et al. 1979). Such enzyme systems may prove to be very useful pesticide-detoxifying enzymes, and it is anticipated that commercial interest in this enzyme will develop in the future. The laccase enzyme forms dimerized or polymerized products that exhibit reduced toxicity and biospecificity.

6 Discussion

Many insecticide-degrading enzymes have been described in this chapter, but only one of these enzymes is practical for immediate routine industrial use: parathion hydrolase. The potential importance of the development of several enzymes for pesticide disposal has been indicated. Enzyme-mediated pesticide disposal can be applied to many situations. In agricultural practices, methods for detoxifying pesticides are nonexistent, yet unwanted pesticides are often generated by miscalculations in mixing or spraying, or by residues remaining in containers. At times, crop damage dictates that soil be detoxified before future planting is allowed. One way of detoxifying soil could be by the use of enzymes. Soil spills are not uncommon on farms either, and enzymatic methods for detoxifying spilled pesticides could easily be developed. In any of these situations a suitable pesticide-detoxifying enzyme preparation could be used by the consumer to allow safe and efficient disposal of unwanted chemicals. One major problem is the commercialization and marketing efforts that would be required to allow for such disposal. It seems that in many cases the technology for enzymatic treatment of certain pesticides, especially the organophosphates, has been developed. It appears that enzymatic treatment is also economically feasible. The enzymatic removal of parathion from pesticide containers, for example, has already been demonstrated (Munnecke 1980) when it was observed that 85 g of residual parathion in a 200-l drum were hydrolyzed after parathion hydrolase was added. Within 16 h, 90% of the parathion present in the drums as a 48% emulsifiable concentrate was hydrolyzed. Methyl parathion, diazinon, and dursban at concentrations up to 1% technical compound could also be enzymatically hydrolyzed.

The feasibility of using enzyme preparations to clean up soil spills has also been demonstrated. Studies by Domsch et al. (1979) and by Barik and Munnecke (1982) indicated that when parathion hydrolase was added to soils, it efficiently hydrolyzed 1% parathion or diazinon within 24 h to less toxic and unstable products. Unfortunately, real world situations indicate that use of such enzymes does not occur because they are not made available to farmers or commercial applicators.

Enzymes capable of degrading pesticides could also be used in agriculture to prevent carry-over of crop-protection chemicals. Spray tanks and other equipment containing phenoxyacetate, phenylurea, acylanilide, or other herbicides could be treated with an appropriate enzyme to remove residual pesticide. This would prevent carry-over and accidental phytotoxicity during subsequent spraying procedures.

Detoxification by enzymes rather than by whole microbial cells is beneficial since enzymes can sometimes better tolerate environmental extremes. Extremes of pH and temperature, as well as high salt and solvent concentrations, are often encountered in pesticide production wastewaters. The enzyme parathion hydrolase, for example, could tolerate salt concentrations up to 10%, solvent concentrations of 1% and temperatures up to 50 °C. These parameters could prevent the growth of the *Pseudomonas* sp. that produced the hydrolase (Munnecke 1979b, 1980). The use of enzymes is also attractive because the transport of pesticides into whole microbial cells can be problematic. Such membrane transport problems could be avoided when soluble enzymes are employed in disposal processes. It seems quite feasible that the combination of whole cells and enzymes may be particularly beneficial for detoxifying spills such as those that occur in soils as the result of transportation or handling accidents.

The use of immobilized enzymes in a continuous flow bioreactor for detoxification of pesticide production wastewaters has also been described (Munnecke 1979b; Talbot et al. 1982). These systems were examined to determine their ability to hydrolyze residual parathion in process wastewaters before discharge into waste treatment systems. The enzyme columns were effective in reducing parathion levels from 10 ppm to 500 ppb in approximately 1 min and the enzyme remained active under continuous use conditions for over 70 days.

Enzymes that show the greatest potential for pesticide detoxification are those that can function without co-factors or co-enzymes and can detoxify a pesticide molecule by a hydrolytic or other simple enzymatic reaction. Presently, there are some enzymes that would be useful for detoxification reactions but are not likely candidates for immediate development because they require co-factors or co-enzymes. These chemicals make such use economically prohibitive. For example, oxygenase enzymes (EC 1.14.12 and EC 1.14.13) require molecular oxygen and either NADPH or NADH to initiate aromatic ring fission. The development of oxygenase enzymes for large-scale applications will be prohibited until economical methods for co-factor regeneration are discovered. Carboxy-lyase enzymes (EC 4.1.1) could also be important degradative enzymes for industrial pesticide detoxification use, but these require thiamin-diphosphate or pyridoxal-phosphate, and until an inexpensive source of these co-factors is available, carboxy-lyase enzymes cannot be developed for treatment systems.

In most cases where insecticide detoxification is mediated by enzymes, metabolites are generated that are less toxic than the parent molecule. In addition, the metabolites may be more easily degraded by other microorganisms. One other advantage of enzyme treatment is that the biospecificity of the parent molecule is destroyed.

7 Directions and Goals

It has been estimated that the use of industrial enzymes would average a growth rate of 8% over the 6-year period between 1979 and 1985 (O'Sullivan 1981). The use of enzymes for detoxifying pesticides has been limited on the industrial scale, however. Large-scale production and use of detoxification enzymes is quite feasible, and interest has been expressed by chemical companies to develop and expand this enzyme technology further. Most of this interest has not reached the stage of commercialization, although several enzymes exhibit a variety of different functions and it is most likely that other enzymes that have yet to be characterized in depth may also serve to detoxify pesticides and other forms of hazardous wastes. One such group of enzymes are the halidohydrolases. It would be desirable if enzyme systems could be developed to dehalogenate aromatic compounds since the basic nucleus of many pesticides is a halogenated aromatic ring. Perhaps an enzyme system exists in the anaerobic populations described by Suflita et al. (1982). Two halidohydrolases have been described that were induced by chloroacetate and dichloroacetate, and these enzymes used a hydroxyl group from water to replace the halogen in the substrate (Goldman et al. 1968). The aerobic dechlorination of aromatic compounds has been described by Janke and Fritsche (1979).

Chemical industries have engaged in active programs to utilize detoxification enzymes. In addition, many of these industries are also examining the possibility of using whole cells to detoxify their process waste streams.

Investigators are also searching for new detoxification enzymes in several academic laboratories. These enzymes are being sought in hopes of detoxifying several forms of toxic compounds, not only those chemicals commonly classified as pesticides. The enzymes that catalyze simple hydrolytic reactions will best be used for immediate development for large-scale use. There is particular interest in developing enzymes for detoxifying a wide variety of wastes because such treatment is gentle and safe. Public sentiment is sympathetic to natural, biological treatment and once existing detoxification enzymes and whole cells have been developed further and used on a large scale, then the search for and development of other new enzymes, including those that dehalogenate or require co-factors, may occur. The current revolution in genetic engineering is sure to have an impact on such detoxification process and in time the true potential for biodecontamination will be realized (see Chap. 7).

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Appendix

Common name	*Chemical name*
Acephate	O-S-Dimethyl acetylphosphoramidothioate
Aminoparathion	O,O-Diethyl-O-p-amino phenyl phosphorothioate
Aspon	1,2,4,5,6,7,8,8-Octachlor-2,3,3,3a,4,7,7a-hexahydro-4,7-methanoindane
Azinphos-methyl	O,O-Diethyl-s-(4-oxo-3-H-1,2,3-benzotriazine-3yl)-methyl-dithiophosphate
Carbaryl	1-Naphthyl methylcarbamate
Carbofuran	2,3-Dihydro-2,2-dimethyl-7-benzofuranyl-N-methylcarbamate
Carbophenothion	S-[(p-Chlorophenylthio) methyl] O-diethyl phosphorodithioate
Chlormephos	S-Chlormethyl-O,O-diethyl phosphorothiolothionate
Chlorpropham	Isopropyl-N-3-chlorophenyl-carbamate
Chlorpyrifos	O,O-Diethyl O-(3,5,6-trichloro-2-pyridyl)-phosphorothioate
Diallate	S-(2,3-Dichloroallyl)-diisopropylthiocarbamate
Diazinon	O,O-Diethyl O-(2-isopropyl-4-methyl-6-pyrimidinyl)-phosphorothioate
Dichlorvos	2,2-Dichlorovinyl O,O-dimethyl phosphate
Dimethoate	O,O-Dimethyl S-(N-Methylcarbamoylmethyl) phosphorodithioate
Dursban	O,O-Diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate
EPN	O-Ethyl-O-(4-nitrophenyl)-phenyl phosphonothioate
Ethion	O,O,O,O-Tetraethyl-S,S-methylene bisphosphorodithioate
Fensulfothion	O,O-Diethyl-O-[(4-methylsulfinyl) phenyl] phosphorothioate
Malathion	O,O-Dimethyl-S(1,2-dicarbethoxyethyl)-phosphorodithioate
Methylparathion	O,O-Dimethyl-O-p-nitrophenyl phosphorothioate
Monocrotophos	(E)-Dimethyl1-methyl-3-(methylamino)-3-oxo-1-propenyl-phosphate
Paraoxon	O,O-Diethyl-O-p-nitrophenylphosphate
Parathion	O,O-Diethyl O-p-nitrophenyl phosphorothioate
Propham	Isopropyl-N-phenylcarbamate
Triazophos	1-Phenyl-1,2,4-triazolyl-3-(O,O-diethylthionophosphate)
Trichlorfon	Dimethyl (2,2,2-trichloro-1-hydroxyethyl) phosphonate
Trithion	S-[(p-Chlorophenyl thio) methyl] O,O-diethyl phosphorodithioate

CHAPTER 7

Genetic Engineering and Biological Detoxification/ Degradation of Insecticides

J. M. PEMBERTON and E. C. WYNNE

1 Introduction

Gene cloning technology, or genetic engineering as it is more commonly known, has contributed much to modern biology in the 10 years since the first experiments were devised and performed (Jackson et al. 1972). For the bacterial geneticist, genetic engineering has provided an unparalleled opportunity to clone a wide range of genes from almost any strain of bacterium. Gene cloning has enabled scientists to circumvent the normal evolutionary barriers which prevent genetic exchange between the various species and genera of Gram-positive and Gram-negative bacteria. These techniques have permitted the cloning of bacterial genes into the genetically well-characterized, Gram-negative bacterium *Escherichia coli.* Bacterial genes for antibiotic resistance, enterotoxin production, cellulose degradation, nitrogen fixation and plant pathogenicity, to name but a few, have been cloned into *Escherichia coli.* The ability to dissect out the genes conferring a particular bacterial phenotype, e.g. nitrogen fixation, has led to a greater understanding of the number of genes involved, their regulation and synthesis at the genetic level. Information gained by these techniques has been used to study evolutionary relationships between genes conferring the same phenotype in different bacteria, and for a variety of other purposes, including vaccine production, strain improvement in the fermentation industries, construction of novel strains of bacteria useful in waste water treatment and the recycling of industrial, agricultural and urban wastes such as cellulose and certain pesticides, pesticide residues and by-products of pesticides.

A number of problems have arisen from the manufacture, distribution and use of synthetically produced insecticides; these range from the more obvious cases of accidental poisoning in man and his animals, to the more subtle, long-term and less easily defined increases in particular types of cancer due to accidental or occupational exposure to insecticides. There are many ways of limiting both the short- and long-term effects of the manufacture and use of insecticides, such as terminating or limiting the use of the more persistent molecules, e.g. DDT, by synthesizing insecticides which are rapidly degraded/detoxified in the environment, or by employing less elegant approaches for disposal of unwanted toxic molecules, such as burying them (Munnecke 1979).

Environmental pollution is an everyday occurrence, a fact that highlights a pressing need to devise methods for rapid degradation/detoxification of certain insecticides, as well as a multitude of other real and potentially harmful synthetic molecules generated as products or by-products of expanding chemical industries. For

the very recalcitrant molecules, the method of last resort is to incinerate them at sea; a very expensive method. Few novel approaches have been developed to tackle the problem of widespread pollution on land and in water. The approach canvassed in this article is to utilize and further develop genetic engineering techniques to harness the extensive array of degradative capabilities possessed by bacteria; to use cloned degradative genes to construct strains of bacteria which will completely and rapidly degrade/detoxify those toxic insecticide molecules which are real or potential threats to both man and his environment.

2 Insecticides, Microbes and Enzymes

One of the major characteristics of bacteria as a group is their extensive biochemical versatility. Bacteria break down and recycle a wide variety of complex organic molecules, exhibiting a vast array of degradative functions. Bacteria have been shown to play a major and indispensible role in the degradation of the products and by- products of the activities of animals and plants as well as other microorganisms; well-known examples are the degradation of sewage, breakdown of oil wastes from industry, as well as the degradation and recycling of pesticides, including all three groups of insecticides – the organophosphorus, carbamate, and organochlorine insecticides (Table 1). With certain insecticides, such as the organochlorine, DDT, the rate of recycling is very slow and there is accumulation and biomagnification in the environment, leading to the problem of environmental pollution.

In contrast to the minority of insecticides, which exhibit molecular recalcitrance, most insecticide molecules which find their way into soil and water are rapidly degraded by a variety of microorganisms. An examination of the literature will show that many of the strains of bacteria which degrade/detoxify insecticides have been identified as species of *Pseudomonas* and related genera (Table 1).

It is these genera which have provided the initial genetic material for the cloning of degradative genes. In this context, it is useful to examine the phylogenetic relationships between bacteria of known degradative functions and those which have not been screened intensively for such characteristics.

2.1 Degradative Bacteria – Taxonomy and Phylogeny

The pioneering work of Stanier et al. (1966) in the 1960's highlighted the degradative functions of one particular genus of soil microorganisms – the pseudomonads. Although this genus contains many species which possess considerable degradative versatility, nevertheless insecticide-degrading bacteria are spread over a range of genera of soil microorganisms. Indeed, as recent studies on the degradation of the organochlorine herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), amply demonstrate, other genera, in this instance the genus *Alcaligenes,* do possess degradative abilities of considerable interest to microbiologists (Pemberton 1983). Recent history has seen a rapid change in the theory of cellular evolution of microorganisms, and this has particular significance in expanding the number of genera of soil bacteria

Table 1. Organoinsecticide metabolism by microorganisms

Insecticide group	Insecticide	Microorganism	References
Organo-phosphates	Bromophos	Soil microorganisms	Stenersen 1969
	Diazinon	*Arthrobacter* spp.	Gunner and Zuckerman 1968; Sethunathan 1969; Sethunathan and Pathak 1971
		Flavobacterium sp.	Sethunathan and Yoshida 1973; Adhya et al. 1981
		Mixed microbial culture	Munnecke 1976
		Pseudomonas spp.	Rosenberg and Alexander 1979
		Pseudomonas melophthora	Boush and Matsumura 1967
		Soil Microorganisms	Getzin 1967; Bro-Rasmussen et al. 1968; Gunner and Zuckerman 1968; Hsu and Bartha 1979
		Streptomyces griseus	Gunner and Zuckerman 1968
	Dichlorvos	*Pseudomonas melophthora*	Boush and Matsumura 1967
	Dimethoate	Soil microorganisms	Congregado et al. 1979
	Fenitrothion	*Bacillus subtilis*	Miyamoto et al. 1966
		Mixed microbial culture	Munnecke 1976
		Soil microorganisms	Spillner et al. 1979
	Malathion	Estuarine microorganisms	Walker 1976
		Flavobacterium sp.	Sethunathan and Yoshida 1973
		Pseudomonas spp.	Matsumura and Boush 1966; Rosenberg and Alexander 1979
		Rhizobium spp.	Mostafa et al. 1972
		Salt marsh microorganisms	Bourquin 1977
		Soil microorganisms	Spillner et al. 1979
	Methyl parathion	*Bacillus subtilis*	Miyamoto et al. 1966
		Flavobacterium sp.	Adhya et al. 1981
	Parathion	*Flavobacterium* spp.	Sethunathan and Yoshida 1973; Adhya et al. 1981
		Mixed microbial culture	Munnecke 1976; Munnecke and Hsieh 1976
		Pseudomonas spp.	Siddarmappa et al. 1973; Munnecke and Fischer 1979; Adhya et al. 1981; Talbot et al. 1982
		Pseudomonas aeruginosa	Gibson and Brown 1974
		Pseudomonas diminuta	Serdar et al. 1982
		Pseudomonas melophthora	Boush and Mutsumura 1967
		Pseudomonas stutzeri with *Pseudomonas aeruginosa*	Daughton and Hsieh 1977
		Rhizobium japonicum	Mick and Dahm 1970
		Rhizobium meliloti	Mick and Dahm 1970
		Soil microorganisms	Sudhakar-Barik and Sethunathan 1978; Hsu and Bartha 1979; Sudhakar-Barik et al. 1979; Ferris and Lichtenstein 1980
		Water microorganisms	Walker 1976; Sudhakar-Barik and Sethunathan 1978
	Phorate	*Bacillus megaterium*	Le Partourel and Wright 1976
		Soil microorganisms	Getzin and Shanks 1970

Table 1 (continued)

Insecticide group	Insecticide	Microorganism	References
Carbamate	Carbaryl	*Pseudomonas cepacia*	Venkateswarlu et al. 1980
		Pseudomonas melophthora	Boush and Mutsumura 1967
		Soil microorganisms	Liu and Bollag 1971; Rodriguez and Dorough 1977
	Carbofuran	*Pseudomonas cepacia*	Venkateswarlu et al. 1980
		Soil microorganisms	Caro et al. 1973; Getzin 1973; Venkateswarlu et al. 1977; Venkateswarlu and Sethunathan 1978
Organo-chlorine	Aldicarb	Soil microorganisms	Kandasamy et al. 1977
	DDT	*Actinomyces*	Chacko et al. 1966
		Aerobacter aerogenes	Meksongsee and Guthrie 1965; Wedemeyer 1966; Johnson et al. 1967; Plummer et al. 1968
		Agrobacterium tumefaciens	Johnson et al. 1967
		Arthrobacter sp.	Patil et al. 1970
		Bacillus sp.	Patil et al. 1970
		Bacillus cereus	Johnson et al. 1967; Plummer et al. 1968; Langlois et al. 1970
		Bacillus coagulans	Langlois et al. 1970
		Bacillus megaterium	Plummer et al. 1968
		Bacillus subtilis	Johnson et al. 1967; Langlois et al. 1970
		Clostridium pasteurianum	Johnson et al. 1967
		Clostridium michiganense	Johnson et al. 1967
		Enterobacter aerogenes	Langlois et al. 1970
		Erwinia sp.	Johnson et al. 1967
		Erwinia amylovora	Johnson et al. 1967
		Erwinia ananas	Johnson et al. 1967
		Erwinia carotovora	Johnson et al. 1967
		Erwinia chrysanthemi	Johnson et al. 1967
		Escherichia coli	French and Hoopingarner 1970; Langlois et al. 1970
		Hydrogenomonas sp.	Focht and Alexander 1970, 1971
		Klebsiella pneumoniae	Wedemeyer 1966
		Kurthia zopfii	Johnson et al. 1967
		Micrococcus sp.	Plummer et al. 1968; Patil et al. 1970
		Nocardia sp.	Chacko et al. 1966
		Proteus vulgaris	Barker and Morrison 1965
		Pseudomonas sp.	Patil et al. 1970
		Pseudomonas fluorescens	Meksongsee and Guthrie 1965; Johnson et al. 1967
		Pseudomonas glycinea	Johnson et al. 1967
		Pseudomonas marginalis	Johnson et al. 1967
		Pseudomonas morsprunorum	Johnson et al. 1967
		Pseudomonas syringae	Johnson et al. 1967
		Pseudomonas tabaci	Johnson et al. 1967

Table 1 (continued)

Insecticide group	Insecticide	Microorganism	References
		Sewage sludge	Albone et al. 1972;
		microorganisms	Jensen et al. 1972
		Streptococcus sp.	Ledford and Chen 1969
		Streptomyces annamoneus	Chacko et al. 1966
		Streptomyces aureofaciens	Chacko et al. 1966
		Streptomyces viridochromogenes	Chacko et al. 1966
		Xanthomonas pruni	Johnson et al. 1967
		Xanthomonas stewartii	Johnson et al. 1967
		Xanthomonas uredovorus	Johnson et al. 1967
		Xanthomonas vesicatoria	Johnson et al. 1967
	γ-BHC (lindane)	*Aerobacter aerogenes*	Meksongsee and Guthrie 1965
		Bacillus cereus	Meksongsee and Guthrie 1965
		Bacillus megaterium	Meksongsee and Guthrie 1965
		Citrobacter freundii	Jagow et al. 1977
		Clostridium spp.	Raghu and MacRae 1966; MacRae et al. 1969; Sethunathan et al. 1969
		Clostridium rectum	Jagnow et al. 1977; Ohisa and Yamaguchi 1978; Ohisa et al. 1980
		Escherichia coli	Francis et al. 1975; Vonk and Quirjins 1979
		Mixed microbial culture	Engst et al. 1979
		Pseudomonas fluorescens	Meksongsee and Guthrie 1965
		Pseudomonas putida	Benezet and Matsumura 1973
		Soil microorganisms	Tu 1975
	Aldrin	*Bacillus* sp.	Tu et al. 1968; Patil et al. 1970
		Corynebacterium sp.	Tu et al. 1968
		Micrococcus sp.	Patil et al. 1970
		Micromonospora sp.	Tu et al. 1968
		Nocardia sp.	Tu et al. 1968
		Pseudomonas sp.	Patil et al. 1970
		Streptomyces sp.	Tu et al. 1968
		Thermoactinomyces sp.	Tu et al. 1968
	Dieldrin	*Aerobacter aerogenes*	Wedemeyer 1968
		Arthrobacter sp.	Jagnow and Haider 1972
		Bacillus sp.	Matsumura and Boush 1967; Jagnow and Haider 1972
		Corynebacterium sp.	Jagnow and Haider 1972
		Micrococcus sp.	Jagnow and Haider 1972
		Mycobacterium sp.	Jagnow and Haider 1972
		Nocardia sp.	Jagnow and Haider 1972
		Pseudomonas sp.	Matsumura and Boush 1967; Matsumura et al. 1968; Jagnow and Haider 1972
		Pseudomonas melophthora	Boush and Matsumura 1967
		Soil microorganisms	Patil et al. 1970
	Endrin	*Aerobacter aerogenes*	Meksongsee and Guthrie 1965
		Arthrobacter sp.	Patil et al. 1970
		Bacillus sp.	Patil et al. 1970
		Bacillus cereus	Meksongsee and Guthrie 1965

Table 1 (continued)

Insecticide group	Insecticide	Microorganism	References
		Bacillus megaterium	Meksongsee and Guthrie 1965
		Micrococcus sp.	Patil et al. 1970
		Pseudomonas sp.	Patil et al. 1970; Matsumura et al. 1971
		Pseudomonas fluorescens	Meksongsee and Guthrie 1965
	Toxaphene	*Aerobacter aerogenes*	Meksongsee and Guthrie 1965
		Bacillus cereus	Meksongsee and Guthrie 1965
		Bacillus megaterium	Meksongsee and Guthrie 1965
		Pseudomonas fluorescence	Meksongsee and Guthrie 1965
	Heptachlor	*Arthrobacter* sp.	Miles et al. 1969
		Bacillus sp.	Miles et al. 1969
		Corynebacterium sp.	Miles et al. 1969
		Micromonospora sp.	Miles et al. 1969
		Nocardia sp.	Miles et al. 1969
		Soil microorganisms	Carter and Sringer 1970
		Streptomyces sp.	Miles et al. 1969
		Thermoactinomyces sp.	Miles et al. 1969

which might be useful in future studies on bacterially encoded degradative functions. Work by Woese and his co-workers (Stackebrandt and Woese 1981) on the 16S ribosomal RNA's of a variety of microorganisms suggests that there should be a re-alignment between various groups of Gram-positive and Gram-negative microorganisms. It appears that the genus *Pseudomonas,* as described by Stanier et al. (1966), is not a phylogenetically cohesive group, in that the fluorescent pseudomonads are not related to the remaining organisms allocated to this genus. Moreover the so-called fluorescent pseudomonads are more closely related to members of other genera such as *Azotobacter vinelandii* which, apart from its importance as a free-living nitrogen-fixing bacterium, does possess interesting degradative capabilities (Thompson and Skerman 1979).

As microbiologists are well aware, many photosynthetic bacteria such as species of the nonsulfur, photosynthetic genus *Rhodopseudomonas,* do play a major role in the recycling of certain complex organic molecules in nature. From Woese's work it appears that the taxonomic separation of photosynthetic and nonphotosynthetic bacteria is arbitrary. Photosynthetic bacteria such as *Rhodopseudomonas sphaeroides* and *Rhodopseudomonas capsulata* appear to be related to the nonphotosynthetic bacterium *Paracoccus denitrificans.* While the plant pathogen *Agrobacterium tumefaciens,* the symbiotic nitrogen fixer *Rhizobium leguminosarum* and *Pseudomonas diminutia* (all nonphotosynthetic), are related to the photosynthetic *Rhodopseudomonas viridis, Rhodopseudomonas palustris* and *Rhodomicrobium vaneilli.* Other important members of the metabolically versatile genus *Pseudomonas* such as *Pseudomonas acidovorans* and *Pseudomonas testosteroni* are related to the photosynthetic *Rhodopseudomonas gelatinosa* and *Rhodospirillum tenue,* as well as the nonphotosynthetic *Sphaerotilus natans, Comamonas terrigena* and *Aquaspirillum gracile.* Such projected relationships indicate the pool of soil and water organisms which could provide degradative pathway genes in strain construction.

Of particular interest is the relationship between bacterial species known to degrade synthetic organochlorine pesticides, since these are among some of the most recalcitrant of pesticides. Recent studies have shown that strains of *Alcaligenes eutrophus* rapidly and completely degrade the organochlorine pesticides 2,4-D and 2-methyl-4-chlorophenoxyacetic acid (MCPA) as well as a range of chlorinated aliphatic and aromatic compounds (Don and Pemberton 1981; Pemberton 1983). Stackebrandt and Woese (1981) have shown that *Alcaligenes eutrophus* is related to *Pseudomonas cepacia* and *Chromobacterium lividum.* Although little is known of the degradative capabilities of *Chromobacterium lividum, Pseudomonas cepacia* is considered to be the epitome of a degradative organism having utilized nearly two-thirds of the 160 carbon sources fed to it as a sole source of carbon and energy. The recent isolation of a strain of *Pseudomonas cepacia* which degrades the extraordinarily recalcitrant, organochlorine herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Kellogg et al. 1981), indicates that both *Alcaligenes eutrophus* and *Pseudomonas cepacia,* and perhaps *Chromobacterium lividum* will be useful in the rapid degradation/detoxification of the more recalcitrant organochlorine insecticides.

2.2 Insecticides

2.2.1 Organophosphates and Dithionates

It is interesting to note that the synthesis of highly toxic nerve gases for chemical warfare led to the development of the first organophorus insecticides. Of some 100,000 different organophosphorus compounds synthesized over the last 30–40 years only 100 have been marketed. Many were rejected because of their toxicity and lack of species selectivity (Hassall 1982). Since the primary site of action of organophosphorus and dithionate compounds is the nervous system enzyme acetylcholinesterase, present in both insects and vertebrates, there is some disquiet over accidental and occupational exposure to these chemicals. Indeed, because of the severe neurotoxic side-effects of many organophosphorus molecules, molecular recalcitrance and subsequent build-up in the environment is of major concern to biologists (see Chap. 4). Consequently, it is important to ensure that any organophosphorus insecticides released into the environment are detoxified/degraded rapidly (for an extensive review of microbial metabolism of organophosphorus insecticides see Chap. 5).

As Munnecke (1981) has pointed out, the first enzymatic reaction in the metabolism of a particular pesticide can result in considerable detoxification. In the case of the organophosphorus insecticides parathion and paraoxon, the hydrolytic cleavage of the phosphodiester bond leads to between a 60- and 200-fold decrease in toxicity. In consequence, hydrolases active against organophosphates are of major importance in any research directed toward rapid detoxification processes. In a number of instances the initial hydrolysis of the phosphodiester bond of parathion has led to complete degradation of this molecule.

Although the synthesis of this type of hydrolase, commonly referred to as an esterase, forms the basis of insecticide resistance and detoxification in insects and

mammals, many bacteria produce similar enzymes. A species of *Flavobacterium* was able to hydrolyze diazinon, parathion, and chlorpyrifos, although this initial detoxification was not followed by complete degradation (Sethunathan and Yoshida 1973). Yet another strain of *Flavobacterium* was able to hydrolyze methyl parathion, fenitrothion, diazinin and parathion (Adhya et al. 1981), indicating that the hydrolases produced by these two strains differed significantly in their substrate specificities; this is an important point to remember if attempts are to be made to clone the structural genes for such enzymes. Species of *Pseudomonas* can also be a source of organophosphorus hydrolases.

A report by Munnecke and Fischer (1979) suggests that a strain of *Pseudomonas* carries the structural gene(s) for parathion hydrolase on a plasmid; such a strain would prove invaluable in initial cloning experiments involving these genes. Another source of such genetic material could be the strain of *Pseudomonas* isolated by Rosenberg and Alexander (1979) which synthesizes an inducible phosphoesterase capable of hydrolyzing diazinon and malathion; in addition this strain can completely degrade these two organophosphorus insecticides, indicating that it may be a suitable host for cloning and expression of a variety of hydrolases from other species leading from detoxification to complete degradation of a range of organophosphorus molecules.

2.2.2 Carbamates

For those carbamates, such as carbaryl and carbofuran, which act as insecticides, their mode of action, like that of the organophosphates, is to inhibit acetylcholine esterase. Although many fungi have been shown to metabolize various carbamate insecticides, few reports exist on the role of bacteria in the detoxification/degradation of this group of insecticides. In fungi, at least the major route for metabolism is via a hydrolase or an acylamidase. In the case of naphthyl N-methyl carbamate (carbaryl), Boush and Matsumura (1967) isolated a strain of *Pseudomonas melophora,* an obligate symbiont of the apple maggot, that hydrolyzed the carbaryl to naphthol. Indeed such an observation indicates that insecticide resistance may not be due solely to changes in the insects genetic make-up, but could result from the evolution and spread of resistance (detoxification genes) among bacteria associated with the insect itself. If this is the case then such symbionts could be screened for detoxification genes suitable for use in cloning experiments.

2.2.3 Organochlorines

Organochlorine insecticides are known for their toxicity and persistence in the environment, and in recent years many have been either banned or had their use as insecticides severely limited. Among the many organochlorine insecticides synthesized and used over the last 40 years are some well-known environmental pollutants – DDT, γ-BHC, aldrin, dieldrin, endrin, chlordane and heptachlor (for an extensive review of microbial metabolism of organochlorine insecticides see Lal and Saxena 1982).

For each of the major organochlorine insecticides – DDT, γ-BHC, aldrin, dieldrin, endrin and heptachlor – there is at least one report of a strain of bacterium which will metabolize it. An interesting feature of many of the studies on the metab-

olism of the various organochlorine insecticides is that many of the strains were hydrogen autotrophs and allocated to the now defunct genus *Hydrogenomonas* (invalid). It would be reasonable to expect that many of these strains could be reallocated to the genus *Alcaligenes,* in particular to the species *Alcaligenes eutrophus;* or to the nine species of hydrogen autotrophic pseudomonads (Aragno and Schlegel 1981). Recent studies, first by Pemberton and co-workers (Pemberton and Fisher 1977; Fisher et al. 1978; Pemberton et al. 1979, Pemberton 1980, Don and Pemberton 1981, Pemberton 1983), have emphasized the role of *Alcaligenes eutrophus* and *Alcaligenes paradoxus* in the metabolism of chlorinated aromatic and aliphatic compounds, and then by Bull and his co-workers, which have emphasized the role of *Pseudomonas putida* in the metabolism of certain aliphatic, chlorinated molecules (Slater et al. 1976, 1979). Since oxygenases and dehalogenases perform the initial detoxification steps in organochlorine insecticide metabolism, it is to the genera *Alcaligenes* and *Pseudomonas* that microbiologists should look for suitable degradative genes for strain construction.

Having identified some of the enzymes and some of the bacteria involved in the initial detoxification of organophosphorus, carbamate, and organochlorine insecticides, the question now arises as to the best cloning strategy for construction of bacterial strains which will degrade and recycle insecticide and other pesticide molecules. Cloning of degradative genes would be greatly facilitated if prior investigation ascertained their location within the bacterial cell.

3 Cellular Location of Detoxification/Degradation Genes

3.1 Conjugation

Although many species of fungi are known to degrade insecticides, the apparent genetic simplicity of bacterial genomes is particularly appealing for initial studies aimed at cloning and genetic analyses not only of genes involved in the initial detoxification reactions, but also those degradative pathway genes which enable the host bacterial cell to completely degrade and recycle the insecticide.

Ostensibly, the genetic simplicity of bacteria is such that the gene or genes encoding a particular degradative function can only be located in one of two places in the bacterial cell; either in the single, circular main chromosome or on those small, circular, additional chromosomes, the plasmids. The possibility also exists that the degradative pathway genes are distributed between these two components of the bacterial genome.

In the emerging studies on the evolution and spread of genetic material through bacterial populations, the gene transfer process known as bacterial conjugation plays a major role. During the conjugal process, the cell possessing the self-transmissible (conjugative) plasmid (the donor) produces a conjugal tube which attaches to a second cell which lacks the plasmid (the recipient). The donor cell produces a replicate copy of its plasmid DNA, transferring one copy to the recipient and retaining the other copy. When transfer via the conjugal tube is complete, the conjugating cells separate. In this way the plasmid and the information it carries can be transferred rapidly from one population of bacteria to another.

Conjugation, as a means of transfer of degradative genes, has a number of major advantages. First, there is virtually no limit to the amount of DNA that can be carried on a plasmid. This is important since many degradative pathways can be encoded in excess of 20 genes. Second, since plasmids are replicated and maintained in the bacterial cell separate from the main chromosome, transfer and survival of the plasmid usually does not require host cell recombination and replication functions. Third, compared with the majority of plasmids isolated from species of the Enterobacteriaceae which have a limited host range, the number of hosts which will accept, maintain, and express degradative plasmid genes appears to be extensive.

In emerging studies on the cloning and genetic analysis of degradative genes, it was the finding that many naturally occurring strains of *Pseudomonas* and *Alcaligenes* carry degradative pathway genes on plasmids, which has proven of central importance in research directed toward an understanding of the ecology, evolution, and spread of degradative genes through soil populations (Pemberton 1983). For the microbial geneticist, the real value of plasmid-located genes lies in the relatively small size of plasmids compared with the main chromosome, making them easily accessible to both genetic and biophysical analyses (Broda 1979; Hardy 1981).

3.2 Degradative Plasmids

3.2.1 Incompatibility and Host Range

A characteristic of closely related plasmids is that they are unable to stably co-exist in the same cell – they are incompatible with one another (Datta 1979). Use has been made of this characteristic to group most known plasmids of the Enterobacteriaceae into 20 or so incompatibility (Inc) groups; the various groups have been denoted by letters of the alphabet, IncA, IncB, IncC, etc. Because of the narrow host range of many of the antibiotic resistance plasmids isolated from species of *Pseudomonas,* a separate incompatibility grouping was devised, using the notation IncP-1, IncP-2, etc. (Jacoby and Shapiro 1977); so far this classification extends to IncP-10.

Although the majority of plasmids isolated from members of the Enterobacteriaceae have a narrow host range, being only transferred between closely related strains of the same species or between closely related species, plasmids of the IncP-1, IncQ, IncW and IncN groups can be transferred among and between a large number of Gram-negative bacteria. These few groups of plasmids are termed broad host range plasmids. Such broad host range plasmids have proven all too efficient in the spread of single and multiple antibiotic resistance genes, providing a major route for the generation and dissemination of new combinations of antibiotic resistances, particularly among primary and opportunistic pathogenic species of bacteria in response to selection pressures generated by injudicious use of most antibiotics.

By way of contrast, a relatively large proportion of the degradative plasmids isolated from species of the genera *Pseudomonas* and *Alcaligenes* (Pemberton 1983) possess a broad host range. Such a broad host range provides an important route for the evolution and spread of degradative functions through soil microorganisms. The circulation of plasmid-encoded degradative genes allows novel combinations of plasmid-borne and chromosomally located degradative pathway genes to be

generated, which in turn ensures a rapid breakdown and recycling of most organic molecules (both naturally occurring or synthetically produced) entering the soil ecosystem.

3.2.2 Plasmids and Insecticides

Insect repellants and insecticides, such as camphor and naphthalene, were to figure prominently in the earliest studies of plasmid-borne degradative genes. Camphor, an active ingredient in mothballs and embalming fluid, was shown to be degraded by a strain of *Pseudomonas putida;* part of the degradative pathway genes was shown to be plasmid-borne – this was the first report of plasmid-borne degradative genes among soil bacteria (Chakrabarty and Gunsalus 1971). Soon after this discovery came the isolation of a naphthalene (NAH)-degradative plasmid by Dunn and Gunsalus (1973); naphthalene is both a moth-repellant and insecticide, and the most abundant single constituent of coal tar, from which it is obtained. The isolation of the NAH plasmid raises the possibility that it could be used in strain construction for degradative organisms capable of the degradation of carbaryl (naphthyl N-methyl carbamate) using conventional genetic methods, such as plasmid transfer.

Studies have resulted in the isolation and continuing characterization of plasmids which encode the degradation of a large number of naturally occurring aromatic and aliphatic compounds. Degradative plasmids have been isolated which degrade such diverse compounds as xylene and toluene (Wong and Dunn 1974; Worsey and Williams 1975), salicylate, in the acetate form better known as the analgesic, aspirin (Farrell et al. 1978), and nicotine/nicotinate, used in veterinary medicine as an ectoparasiticide.

More recently came the first report of plasmids which encoded the degradation of synthetic chlorinated hydrocarbons – the controversial herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 2-methyl-4-chlorophenoxyacetic acid (MCPA) (Pemberton and Fisher 1977; Fisher et al. 1978; Pemberton et al. 1979; Don and Pemberton 1981; Chatterjee et al. 1981; Pemberton 1983). An interesting feature of the seven 2,4-D plasmids so far isolated from strains of *Alcaligenes eutrophus* and *Alcaligenes paradoxus* is that they have a broad host range. One particular plasmid, pJP4, can be transferred to such diverse Gram-negative bacteria as the photosynthetic bacterium *Rhodopseudomonas sphaeroides,* the symbiotic nitrogen fixeer *Rhizobium,* the plant pathogenic *Agrobacterium tumefaciens,* the gut inhabitant *Escherichia coli,* and the soil saprophyte *Pseudomonas putida* (Don and Pemberton 1981; Pemberton 1983; Don 1983; Don and Pemberton unpublished data).

A genetic and biophysical map of pJP4 has been generated (Don and Pemberton unpublished data; Don 1983). The 2,4-D degradative genes map in a single region of the plasmid, and these genes have been cloned into pBR322. Subcloning and further analyses of these genes will hopefully provide useful genetic material for the development of bacterial strains capable of the complete degradation of a wide range of organochlorine pesticides. Of major interest is to determine if pJP4 encodes dehalogenase enzymes, an important activity required for the detoxification of organochlorine insecticides.

With an expansion of the number of naturally occurring plasmids which encode degradative functions, has come the opportunity to genetically analyze and clone

the genes responsible for these functions. Although *Escherichia coli* has proven particularly suitable for cloning and expression of a number of genes, initial experience suggests that degradative pathway genes from *Pseudomonas* and *Acaligenes* are poorly expressed in this organism. Clearly there is a need for alternative strategies for cloning in Gram-negative organisms other than *Escherichia coli.*

4 Genetic Engineering

4.1 Microorganisms and Cloning Strategies

A large number of species of soil organisms are involved in the degradation and recycling of naturally occurring and synthetically produced aromatic and aliphatic molecules (Table 1). The bacteria belong to such widely diverse genera as *Alcaligenes, Flavobacterium, Pseudomonas, Rhizobium, Paracoccus, Agrobacterium, Azotobacter, Chromobacterium* and *Rhodopseudomonas,* to name but a few. One thing that each of these genera has in common is a collective lack of well-characterized systems of genetic exchange and genetic analysis. This deficiency, coupled with the general lack of expression of heterologous degradative genes in *Escherichia coli,* has led to the development of two strategies for the cloning and expression of degradative genes from diverse groups of Gram-negative (and perhaps Gram-positive) bacteria.

The first strategy involves setting up a system for cloning in the organism which possesses an interesting degradative function, e.g., *Pseudomonas putida.* Such self-cloning experiments require at least some rudimentary knowledge of the genetics of the organism, as well as a variety of suitable mutants. Among the Gram-negative soil microorganisms, *Pseudomonas putida, Pseudomonas aeruginosa, Rhizobium meliloti, Agrobacterium tumefaciens,* and *Rhodopseudomonas sphaeroides* appear to fulfill the basic genetic requirements for self-cloning. These are: the ability to be transformed by plasmid or bacteriophage DNA's, and the availability of mutants which lack host cell restriction systems. To a lesser extent there is a need for mutants lacking host cell recombination enzymes (Rec-) to prevent any cloned segment of DNA being lost from its vector plasmid by homologous recombination with the identical segment on the main chromosome. In addition, such host strains should lack endogenous plasmids which would confuse any molecular analyses of cloned material. Although a good adjunct to the second alternative, this method has limitations in terms of the species of bacteria which can be analyzed.

A second alternative involves cloning into a broad host range vector in *Escherichia coli,* and then transferring the cloned material back into its original host for expression. Such an approach has a number of advantages. First, once cloned, degradative genes can be transferred to a wide range of Gram-negative bacteria using such vectors as RSF1010. Second, the newly cloned material can be tested for expression in a variety of genetic backgrounds, enabling the construction of strains of bacteria with combinations of plasmid and chromosomally encoded degradative pathways. Third, the levels of expression can be altered by employing a variety of RNA polymerase binding sites (promotors), ribosome-binding sites and regulatory genes.

4.2 Gene Cloning – the Process

To date, there have been few reports of the transfer of "foreign" DNA into the cells of plants and animals. The so-called "recombinant DNA" experiments have mainly involved the insertion of DNA into bacteria, particularly the genetically well-characterized, gut inhabitant *Escherichia coli.* Many procedures are currently employed, but their main purpose is to produce large quantities (clones) of individual genes; the availability of bacterial cells carrying many copies of the cloned material allows the biologist to examine not only the structure of the cloned gene but also the protein which it encodes (Old and Primrose 1980).

The first step is to extract DNA from cells harboring the gene or genes encoding the property of interest; extraction of whole-cell DNA from Gram-negative, organisms is a fairly straightforward process. This DNA is then cut, using biological catalysts known as restriction enzymes. The next step is to insert this DNA into a sequence of DNA known as a cloning vector, which facilitates the maintenance and replication of the "foreign" DNA in the bacterial cell. Usually, the small circular plasmid DNA's are isolated for this purpose, and are cut at a single point with a restriction endonuclease, thus opening the circle. The linear plasmid vector DNA and the "foreign" DNA fragments are then mixed in a test tube. Treatment with the joining enzyme, DNA ligase, completes the linkage of the plasmid, with "foreign" DNA inserted to form a circle. Finally, this hybrid of plasmid and "foreign" DNA's is introduced into the bacterial cell by the process of transformation.

4.2.1 Ligases and Restriction Endonucleases

Perhaps the most significant contribution to cloning technology was the isolation and exploitation of enzymes that catalyze reactions involving DNA. Bacteria produce a multitude of enzymes for the replication, repair and general functioning of cellular DNA. Some of these enzymes are used in various steps of the genetic engineering technique. First, there is the joining enzyme, DNA ligase. Second, there are the restriction enzymes, which cut DNA and are produced by bacterial cells to restrict infection by lethal viruses.

The most useful types of restriction enzymes for genetic engineering purposes are those which recognize a specific sequence of bases in the DNA molecule, and cut at a precise point within that sequence. For example, the action of the restriction enzyme EcoR1, isolated from *Escherichia coli.* This enzyme cuts the double stranded DNA molecule asymmetrically at the sequence GAATTC, leaving a single-stranded region of four bases at both ends of the molecule. These ends are termed "sticky" as they can adhere to any other end formed by EcoR1 cutting, by linkage between their complementary four base sequences. A wide range of restriction enzymes have been isolated which recognize a variety of sequences in DNA (Roberts 1976).

The enzyme EcoR1 aids the insertion of foreign DNA into the plasmid vector DNA as follows. First, both ends of the "foreign" DNA fragment are made "sticky", by cutting either side of the required "foreign" gene(s) with EcoR1. Second, the plasmid vector is cut by EcoR1 at a single point, thus opening the plasmid circle. Finally, the "foreign" and plasmid DNA's are mixed, allowing the plasmid to recircularize with the "foreign" DNA now inserted as part of the plasmid. The hybrid

plasmid is then made permanent by treatment with the joining enzyme, DNA ligase, which essentially repairs the initial cuts made by the restriction enzyme. The hybrid plasmid created in a test tube and containing the desired "foreign" gene, can now be transferred back into a living bacterial cell, by the process known as transformation.

4.2.2 Plasmids as Cloning Vectors

Small antibiotic resistance plasmids have proven ideal vectors for adding "foreign" DNA to bacterial cells, by fulfilling three major requirements. Firstly, large quantities of plasmid DNA can be readily isolated from bacterial cells. Secondly, plasmids can be efficiently re-introduced into the bacterial cell, and their acceptance detected by testing for the antibiotic resistances they confer. Thirdly, these plasmids can be stably maintained in the bacterial cell, being replicated independently of the main chromosome.

4.2.3 Transformation of the Hybrid Plasmid

The importance of transformation as a means of gene transfer between bacteria in their natural environments is uncertain. However, it serves an indispensable role in many laboratory manipulations. This process is readily achieved with *Escherichia coli,* by treatment with calcium chloride (rendering the cell wall more permeable to DNA), prior to the addition of the hybrid plasmid DNA solution to the bacterial culture. The uptake of the hybrid plasmid by the cell is detected by testing for the acquisition of plasmid-conferred properties such as antibiotic resistance. If a cell accepts the hybrid plasmid, then it can be tested for the production of the desired "foreign" protein.

4.2.4 Cloning Vectors of Escherichia coli

4.2.4.1 Small Amplificable Vectors of Escherichia coli

Plasmid pBR322 is a small, 4000 base pair plasmid whose natural host is *Escherichia coli* (Bolivar et al. 1977). This plasmid has a number of advantages common to the more useful cloning vectors used in microbial genetics. First, the number of copies per cell in an *Escherichia coli* culture varies from 50–100 under normal growth conditions, hence any gene cloned into this vector will also be present in the cell at 50–100 copies per cell. Given that bacterial cultures can reach 10^{10} cells ml^{-1}, then the number of potential DNA clones ml^{-1} can exceed 10^{12}, providing an abundant source of the gene for molecular analysis. In addition, using amplification techniques, the number of copies of pBR322 per cell can be increased in excess of 1000. If the cloned gene is expressed, that is a protein is produced from the cloned DNA, then multicopy, amplifiable cloning vectors such as pBR322, can allow substantial production of particular proteins.

Second, pBR322 contains unique cleavage sites for the more commonly used restriction endonucleases, such as EcoR1, BamHI, HindIII, and PstI. Foreign DNA cloned into any of these three unique sites results in insertional inactivation of the antibiotic resistance genes carried by pBR322; "foreign" DNA cloned into the PstI site inactivates ampicillin resistance, and into the HindIII or BamHI sites inactivates

tetracycline resistance. Finally, entry of the plasmid plus its cloned DNA can be assayed by using the drug resistance gene not subject to insertional inactivation during the cloning process; cloning into the tetracycline gene, select for ampicillin resistance to detect plasmid, and vice versa. The major drawback to using pBR322 as a general-purpose cloning vector for a range of Gram-negative bacteria, is its narrow host range. pBR322 will only replicate and be maintained in *Escherichia coli.*

4.2.4.2 Intergeneric Transfer of pBR322

Transposable elements are small segments of DNA, usually encoding such characteristics as antibiotic resistance, which can move freely from one replicon (plasmid/virus/main chromosome) to another. The process of transposition (transposon movement) occurs when a donor replicon (carrying the transposon) and a recipient replicon (lacking the transposon) interact physically at the site of the transposable element. When the donor and recipient replicons separate they both now possess a copy of the transposon; in this way the transposon (or a copy of it) moves from one replicon to another; when inserted in transferable replicons such as conjugative plasmids and bacterial viruses, the transposons can move from one bacterial cell to another, their presence being detected by the property they confer on their host cell, e.g. resistance to an antibiotic, resistance to heavy metals, ability to use certain compounds as sole sources of carbon and energy, enterotoxin production etc. (Kleckner 1981).

For transposable elements such as *Tn*3 (ampicillin resistance), *Tn*501 (mercury resistance) and *Tn*21 (Kleckner 1981) there is an intermediate stage in the transposition process where the donor and recipient replicons form one large replicon. This replicon contains two copies of the transposon, one at each point of attachment between the two replicons; such a structure is known as a co-integrate. A transposon such as *Tn*3 encodes two functions essential for transposition; a transposase, which enables interaction of the two replicons at the site of the transposon and duplication of the transposon; a resolvase, which leads to resolution of the co-integrate into two replicons, each now contains a copy of the transposon. In the case of *Tn*501 the resolvase activity is weak, and once co-integrates form, they are slowly resolved (De La Cruz and Grinsted 1982). In 1981, Pemberton and Bowen demonstrated that *Tn*501 enabled the broad host range, drug resistance plasmid RPI, to promote chromosome transfer in *Rhodopseudomonas sphaeroides.* It was assumed that this transfer occurred by co-integrate formation between RPI and the main chromosome promoted by *Tn*501. That this assumption was correct comes from the observation that *Tn*501 could promote similar chromosome transfers in the causative organisms of whooping cough, *Bordetella pertussis* (Weis and Falkow 1983) and in the soil saprophyte *Pseudomonas putida* (Dean and Morgan 1983). In *Bordetella pertussis* the chromosomally inserted, conjugative plasmid carried flanking copies of *Tn*501, the characteristic of co-integrate formation.

Stable co-integrate formation is one way of promoting not only chromosome transfer but also plasmid transfer; such an observation was made by Morrison et al. (1983) for the interaction of *Tn*501 and nodulation plasmids in *Rhizobium.* An additional interest would be the transfer of the widely used, but narrow host range, cloning vector pBR322. Although co-integrates between R751::*Tn*501 and pBR322

allowed transfer of pBR322 within and between strains of *Escherichia coli,* interspecific or intergeneric transfer was not detected (Pemberton unpublished data). However, using a mutant of the related transposon *Tn*21, deleted for its resolvase function (Grinsted personal communication) very stable co-integrates could be generated between R751::*Tn*21 del and pBR322. In this combination pBR322 was transferred to such diverse genera as the facultative phototroph, *Rhodopseudomonas sphaeroides,* cellulolytic saprophyte, *Cellvibrio mixtus,* amylolytic *Aeromonas punctata* and the pesticide-degrading *Alcaligenes eutrophus* (Bowen and Pemberton unpublished data). Transfer and maintenance of pBR322::*Tn*21 del::R751 co-integrates in a range of Gram-negative bacteria have a number of possible advantages, from R-prime promoted chromosome transfer using in vitro cloning in pBR322 to complementation and expression of DNA's already cloned into this popular cloning vector.

4.2.4.3 Broad Host Range Cloning Vectors

Of more immediate use are those plasmids which already have a broad host range, small size, and high copy number. Using as their starting material the broad host range, nonconjugative plasmid RSF1010, which encodes streptomycin and sulphonamide resistances, Timmis and his co-workers have generated a number of particularly useful cloning vectors for use among Gram-negative bacteria (for review, see Bagdasarian and Timmis 1982).

Plasmid RSF1010 and the cloning vectors derived from it have a number of advantages for cloning genes from Gram-negative bacteria, particularly degradative genes involved in insecticide detoxification/degradation. First, these small nonconjugative plasmids can be mobilized with high efficiency by the IncP-1 group of conjugative plasmids, e.g., RP4, into a wide range of Gram-negative organisms, including *Pseudomonas aeruginosa, Pseudomonas putida, Rhodopseudomonas sphaeroides, Rhizobium meliloti, Agrobacterium tumefaciens, Alcaligenes eutrophus, Cellvibrio mixtus, Aeromonas punctata,* and *Flavobacterium* sp. (Bagdasarian and Timmis 1982; Pemberton unpublished data).

The concomitant transfer of RP4 has been overcome by Puhler and co-workers (Puhler personal communication) by inserting RP4 in the main chromosome of the RSF1010 donor strain. Second, these plasmids have high copy numbers, allowing for ready isolation of plasmid DNA's and elevated levels of expression of cloned DNA's. Third, they can be transformed into *Escherichia coli, Pseudomonas putida* and *Pseudomonas aeruginosa* allowing self-cloning of DNA's in these three species. In addition, they provide an ideal tool for detection of transformation in bacterial species which otherwise have little genetic characterization.

Finally, being broad host range plasmids, they have evolved strategies for maintenance, replication, and expression of their DNA's independently of host cell functions, and these properties can be exploited to allow for the maintenance, replication, and expression of any genes added to them by genetic engineering techniques. A good example is the cloning of the gene encoding 2,3-oxygenase, a major ring-splitting enzyme.

Among the broad host range cloning vectors constructed by Timmis and coworkers (Bagdasarian and Timmis 1982) is the vector plasmid pKT230. This plasmid

is 11.9 kb in size, was derived by in vitro cloning techniques, and can be used for cloning *Hind*III, *Xma*I and *Xho*I fragments by insertional inactivation of the kanamycin resistance gene, and for cloning *Eco*R1 and *Sst*I generated DNA fragments by insertional inactivation of the streptomycin resistance gene. Cloning into the kanamycin resistance gene has a major advantage in that this gene carries four strong promotors, three of which lie upstream from the *Hind*III, *Xma*I and *Xho*I cleavage sites; cloned DNA inserted at any of these three cleavage sites benefits from increased levels of transcription, which can lead to substantial increases in the levels of proteins encoded in the cloned DNA's.

The value of cloning degradative genes into such broad host range cloning vectors can be seen from cloning experiments using pKT230 and the gene for 2,3-dioxygenase derived from the TOL plasmid. When this gene was cloned away from its regulatory genes and into the kanamycin resistance gene of pKT230 at the *Xho*I cleavage site, high levels of uninduced 2,3-oxygenase were produced in *Escherichia coli* and *Pseudomonas putida* (Bagdasarian and Timmis 1982).

4.2.4.4 Cloning of Insecticide Detoxification/Degradation Genes

Availability of genetically engineered microorganisms which produce large quantities of major detoxification enzymes for insecticides and other pesticides is of primary importance in procedures using either immobilized enzymes or immobilized bacteria for biological conversion processes. The distinction must be made between detoxification and degradation. Detoxification usually involves the first enzymatic reaction in the metabolism of a particular pesticide and can result in significant reductions in the toxicity of the pesticide, e.g. hydrolytic cleavage of the phosphodiester bond of both parathion and paraozon, results in significant reductions in the toxicity of these pesticides. In this instance perhaps one or two structural genes encode the enzymes performing this primary detoxification process, and these could be cloned into pKT230, to provide a result similar to that obtained for the catechol 2,3-dioxygenase gene of TOL. Such an approach would be recommended for the hydrolases which detoxify organophosphorus and dihionates, hydrolases, and acylamidases which detoxify carbamates and oxygenases and dehalogenases which detoxify organochlorine insecticides; the availability of such strains may simplify detoxification of these insecticides.

If, however, it is desirable to completely degrade any of these insecticides, then the cloning and genetic manipulation becomes more complex. In these circumstances the rate-limiting steps in the degradative pathway will have to be identified and the genes cloned. Using the broad host range of the cloning vectors, combinations of chromosomally encoded dissimilatory and plasmid encoded degradative pathways can be constructed to give optimal levels or in industrial waste water treatment plants.

5 Conclusion

In almost every aspect of modern living insect pests are an almost universal preoccupation. From the lice which inhabit school children's hair and clothes, to the

remorseless invasion of household pests such as cockroaches, ants, blowflies, spiders etc., to a range of agriculturally and therefore economically important insects that infect domesticated plants and animals. In addition there are a large number of medically important insects, such as the mosquito vector of the malarial parasite, which each year transmit a range of debilitating diseases to hundreds of millions of people around the world.

To combat encroaching insect plagues, man has developed potent chemical weapons – collectively known as insecticides. The three major groups of insecticides – organophosphorus (and related dithionate), carbamate, and organochlorine – contain many molecules which are highly toxic to nontarget organisms such as man. Any accumulation in the environment poses a real threat – both immediate through poisoning and long-term through possible carcinogenic effects.

There is a need to balance the use of insecticides against any derived benefits, e.g., crop protection versus higher yields, but at the same time ensure that any molecules derived in either the manufacture or use of insecticides will decimate their target organisms quickly and equally, quickly be degraded either completely or at least to a much less toxic form.

Disposal of unwanted or accumulating insecticide molecules in industrial complexes or agricultural areas is therefore of high priority if pollution is not to overtake and destroy many nontarget plants and animals. By the judicious use of in vivo and in vitro genetic engineering techniques, we suggest that it is possible to achieve the goal of rapid recycling of many of the more persistent (recalcitrant) insecticide molecules – and indeed other pesticide molecules.

Such genetically engineered strains will be useful in confined, wastewater treatment plants, and even in agricultural and industrial areas showing high levels of insecticide and other pesticide molecules. If only a small proportion of the funds used in pollution control were directed toward development of biological recycling of potential pollutants, much of the expense involved in the storage or incineration of recalcitrant pesticides would become unnecessary.

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CHAPTER 8

Effects of Insecticides on Soil Microorganisms

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1 Introduction

Insecticides are being used extensively to control plant pests and vectors of human and live-stock diseases. Whatever may be the mode of application, and whether they are used in agriculture or in public health, insecticides never remain at the site of application and ultimately sink into the soil (See Chap. 2). In the soil, they come across the soil flora and fauna and the stage is set for their interaction with the soil ecosystem.

The soil biota is a complex entity comprising several genera of bacteria, fungi, actinomycetes and algae, whose populations, always in dynamic equilibrium, continuously influence each other. These microorganisms are identified with a variety of basic ecological processes such as recycling of essential plant nutrients, trash decomposition and humus formation, soil structural stability, pathogen survival, pesticide degradation, formation of organic matter by chemo- and photosynthesis, nitrogen fixation, mineralization of carbon, nitrogen, phosphorus, and other elements. The efficient functioning of these processes is the result of a delicate equilibrium between microorganisms, soil, and plants. The balance between all these processes results in a state generally referred to as soil fertility. Consequently any interference in such microbe-mediated processes by insecticides may generally affect the soil fertility. Such chemical controls become self-defeating if the insecticides are applied without taking the complex microbiological systems into account.

The interaction of insecticides with soil microorganisms has been the subject of several reviews (Bollag 1961; Cox 1972; Pfister 1972; Johnsen 1976; Tu and Miles 1976; Butler 1977; Williams 1977; Wainwright 1978; Sylvestre and Fournier 1979; Lal and Saxena 1980, 1982). In the present chapter an attempt is made to survey the recent literature with more emphasis on the effects of insecticides on microbial populations and their activities.

2 Soil Microbial Populations

The literature on insecticides and soil microbial interactions shows results which span from inhibition to stimulation. In this range come the qualitative effects: stimulation or inhibition limited to a group of organisms; reversible inhibition where the organisms are first inhibited but later recover and sometimes reach levels higher than controls and finally show no apparent effects.

At field doses, insecticides are not harmful to soil microflora. Visalakshi et al. (1980) found carbofuran to be harmless to rice rhizospheric microflora at a field dose of 0.5 kg a.i. ha^{-1}. Diazinon similarly did not affect the growth and root colonization of soybean by mycorhizal fungus *Glomus mosseae* in an Andover clay loam soil (Burpee and Cole 1978). Eight organophosphorous insecticides (fonofos, trichloronate, chlorfenvinfos, fenitrothion, parathion, chlorpyrifos, diazinon) were also harmless to bacteria and fungi in an organic and a sandy loam soil (Miles et al. 1979). Tu (1978a) found no harm to soil fungal populations by leptophos, terbufos, WL 24073 and carbofuran at 5 μg a.i. g^{-1} in a sandy loam soil. Even at high concentrations some insecticides are harmless. Salonius (1972) observed no apparent damage to aerobic and anaerobic populations in a forest soil treated with a massive dose of 112 kg ha^{-1} of DDT formulation and fenitrothion. There were no qualitative changes in bacteria, fungi, and actinomycetes. This could possibly be due to the presence of organic matter in the soil which adsorbs the chemicals. Calcium carbonate present in the formulation was also suspected to have stimulated the microflora. Similarly, Martin (1966) found no depressive effects on a sandy loam soil populations by aldrin, chlordane, DDT, and toxaphene at 5 and 2 lb $acre^{-1}$. Even a much higher dose of 200 kg ha^{-1} or 2000 ppm of dieldrin were not harmful to soil populations (Harris 1969; Tu and Miles 1976). Similar results were observed with aldrin at 120 ppm (Jonsson and Fahreus 1960). Tenfold rates of acephate and methamidophos did not influence the microflora (Pathak et al. 1960–61). Fungi were tolerant to 200 ppm DDT (Juneja and Dogra 1978) and 200 ppm endrin in a black cotton soil (Varade and Nakat 1977). Bollen and Tu (1971) also found similar results with endrin at three times the normal dose.

Several other reports, however, show that insecticides were depressive to soil microflora. Rennie (1977) found that lindane, dieldrin, and heptachlor reduced the bacterial populations at 20 ppm. Similarly Stojanovic et al. (1972) also found that technical DDT, carbaryl, dieldrin, malathion and paraquat decreased bacteria in a calcarious loam. However, formulation of carbaryl and DDT increased the bacterial growth.

Fungi were inhibited by 5 μg a.i. g^{-1} of parathion in a sandy loam after the 6th week of treatment (Tu 1978a) and by technical DDT, carbaryl, dieldrin, and paraquat (Stojanovic et al. 1972). At higher doses also, the insecticides were harmful. Bacteria isolated from an estuary sediment and grown on agar were reduced substantially by 10 ppm DDT along with its mixed isomers (Albone et al. 1972). Fungal population of a red loamy soil was also depressed by Dasanit at 10 ppm (Sreenivasulu and Rangaswamy 1973).

At much higher doses total populations are inhibited. Misra and Gaur (1977) found that lindane at 1, 10, and 100 ppm depressed bacteria and actinomycetes. However at 1 and 10 ppm the addition of farm yard manure neutralized the effect. Similarly carbofuran at five times the field dose (1.25 kg a.i. ha^{-1}) was toxic to bacteria, fungi and actinomycetes. The chemical was applied in two split doses. While bacterial and fungal populations were inhibited by the first dose itself, actinomycetes were resistant which were depressed by the second dose (Kandasamy et al. 1975). Lethbridge and Burns (1976) showed that fensulfothion and malathion at 100 and 200 ppm respectively inhibited total microflora of a sandy loam and a silt loam.

But they developed resistance after 21 days. At 4000 ppm pyrolan, α and β-endosulfon and dieldrin depressed microflora of an irrigation soil (El Beit et al. 1981).

Some authors, using pure cultures of organisms isolated from the soil, studied the effects of insecticides. These studies often conclude that insecticides are harmful to soil organisms. For example, DDT at 1 ppm was inhibitory for bacteria isolated from a garden and nursery soil, and grown in the laboratory (Kokke 1970). At 100 ppm it was toxic to aerobic and anaerobic bacteria isolated from a mud (Albone et al. 1972) and also to *Pseudomonas fluorescence* and *Staphylococcus aureus* (Collins and Langlois 1968). Heptachlor also inhibited these organisms (Collins and Langlois 1968) and bacteria, fungi, and actinomycetes isolated from a silt loam at 10 and 100 ppm (Shamiyeh and Johnson 1973). Aldrin, lindane, parathion, carbaryl, and phorate at 40 ppm slightly depressed the fungi isolated from a prairie soil (Cowley and Lichtenstein 1970). The laboratory studies with pure or mixed cultures are unlikely to reproduce accurately the complex environmental conditions prevailing in the field conditions and hence cannot be extrapolated on to the field conditions. However, they yield good biochemical information on the mode of action of the insecticide.

Some reports, however, show important ecological disturbances which these insecticides can produce in natural environment. Pudelko (1978) found that enolphos and tritox, a mixture of DDT, BHC, and methoxychlor, were harmful to fungal populations of a potato rhizosphere but not to other groups of microorganisms. Raghupathy (1977) observed a selective effect of aldicarb on different groups of microorganisms. Aldicarb decreased the fungal population while bacteria and actinomycetes were stimulated. Phorate and terbufos and their metabolites also showed selective effect on different groups of microorganisms (Tu 1980a), while phorate, terbufos and terbufos sulfoxide, DOWCO 275 stimulated bacteria, terbufos sulfone and DOWCO 275 were stimulatory to fungal populations. Permethrin inhibited both bacteria and fungi. Similarly diazinon at 3000 ppm inhibited bacteria but its degradation product was stimulatory (Robson and Gunner 1970). Naumann (1971) found that diazinon inhibited bacterial population without any effect on fungal populations.

Within the same group of organisms insecticides may also have selective effect. Tanton et al. (1981) found changes in the fungal genera of prairie soil by carbofuran. Sreenivasulu and Rangaswami (1973) found rather severe effects of three insecticides thimet, dasanit and disyston on fungi, bacteria, and actinomycetes. They observed that all the three insecticides stimulated only Gram-positive bacteria without affecting Gram-negative bacteria. At later stages, however, Gram-negative bacteria were increased, especially *Pseudomonas* which could degrade all three insecticides. Similarly Audus (1970a) found that aldrin, chlordane, dieldrin, endrin, and heptachlor inhibited Gram-positive bacteria while Gram-negative bacteria were not affected. This was attributed to the differences in chemical composition of cell walls of these bacteria. On the contrary, Naumann (1971) found stimulation in the population of Gram-positive bacteria and reduction in Gram-negative bacteria when treated with methyl parathion. Mohanty and Evans (1980) also reported a differential toxicity to bacterial isolates treated with DDT at 0.25 ppm. Gunner et al. (1966) found that diazinon at 0.3 and 3 lb acre^{-1} favored a particular coccoid bacterium for 124 days. However, those fungal populations were not affected that could

utilize this chemical as sulfur, phosphorous, carbon and nitrogen sources. Among the actinomycetes, *Streptomyces* significantly increased after 180 days of treatment due to the stimulatory effects of the degradation products of diazinon.

Actinomycetes populations also responded qualitatively to Thimet, disyston and Dasanit (Sreenivasulu and Rangaswami 1973). *Streptomyces* increased in all treatments while *Nocardia* increased only in Thimet and disyston-treated soils. Among the fungal populations *Aspergillus* was abundant in all three treated soils while *Penicillium* was more in thimet treated soils. *Fusarium* was stimulated by Thimet and Dasanit, *Verticellum* by disyston and Dasanit and *Trichoderma* by disyston. It was suggested that these organisms utilize these insecticides as phosphorous source. Tu (1977) also reported that carbofuran at 1 mg g^{-1} of sandy loam soil slightly depressed fungal populations which later increased.

Insecticides have been reported to inhibit the growth initially with the populations recovering quickly afterwards. Dipterex and Nuvacron at 0.5 g m^{-2} depressed the rhizospheric microflora initially but the counts increased thereafter. The phosphate dissolvers, however, showed an increased growth, which indicates that these utilized the chemicals as energy source which lead to recovery (Mahmoud et al. 1981). Tu (1980b) also reported an initial depression of population in a sandy loam by five pyrethroid insecticides, permethrin, FMC 45498, WL 41706, WL 43467 and WL 43775 at 0.5 and 5 ppm followed by a recovery and stimulation in 2 to 4 weeks. Carbofuran and chlorpyrifos also had similar effects. In another experiment Tu (1978b) studied the effects of organophosphorus and chlorinated insecticides in laboratory conditions using sandy loam soil. A significant decrease in bacterial numbers 2 days after treatment with parathion and triazophos at 10 mg g^{-1} soil was reported. Soil fungi were inhibited in samples treated with chlordane and lindane at 5 and 10 ppm and with parathion, thionazin, metalkamate and permethrin only at 10 μg g^{-1} after 2 days. The populations of these microorganisms subsequently recovered to the levels of the controls. In the case of bacteria the recovery was fast with ethoprop, malathion, trichloronate at 5 μg g^{-1} and chlorpyrifos and fensulfothion at 10 μg g^{-1}.

In Delhi loamy sand soil with carbofuran and Dasanit and in an organic soil with fonofos and fensulfothion, the growth of microorganisms was stimulated (Tu 1972, 1979). Sreenivasulu and Rangaswami (1973) found that Thimet, disyston and Dasanit applied to red sandy loamy soil at 10 and 20 ppm a.i. in granular formulations, increased the bacterial populations. They suggested that bacterial population might have started utilizing the insecticide as a carbon source after their acclimatization to the insecticides. After 10 days, Thimet at 10 ppm significantly increased the fungal populations, while disyston had no effect and Dasanit decreased the fungi after 10 days. The observed recovery after an initial depression may be due to the degradation and disappearance of the insecticide. The recovery period, though fast, may last for as many as 30 days (Domsch et al. 1977). Sometimes the added insecticide is utilized as an energy source and only the organisms capable of utilizing them show stimulation (Bourquin 1977; Sreenivasulu and Rangaswami 1973).

The actual stimulation of microbial growth by insecticides is not very common. Bourquin (1977), in a simulated laboratory study of salt marsh environment, found that the bacterial populations which utilized malathion as a sole carbon source in-

creased significantly when malathion was added. The increase was dose-dependent. Congregado et al. (1978) also reported stimulation of microbial populations with technical malathion and dimethoate at 10 and 100 μg g^{-1}. Similarly, the population of bacteria were stimulated with endrin, lindane, and toxaphene (Naumann 1971; Misra and Gaur 1977; Varade and Nakat 1977). Abdel-Kader et al. (1978) found that Dipterex promoted soil and rhizospheric fungi of cotton plants at 0.5 g m^{-2}. Disyston, Thimet, and Dasanit also stimulated the actinomycetes of a red loamy soil at 10 and 20 ppm and the fungal populations were stimulated only with Thimet (Sreenivasulu and Rangaswami 1973). Diazinon (Sethunathan and MacRae 1969) and leptophos (Tu 1978 b) have also been reported to stimulate microbial populations.

The acquisition of resistance to a gradual exposure to insecticides has also been reported in laboratory studies though it may also occur in field conditions. Kokke (1970) found that bacteria isolated from nursery soil were susceptible to DDT at 1 ppm but became tolerant after gradual exposure to 0.6 and 0.8 ppm. Such an adaptation to insecticides has also been reported by Lethbridge and Burns (1976).

The effects of insecticides on soil algae have not been extensively studied (see Chap. 9). The algicidal action of endosulfan, malathion, hexidrin, and BHC on 20 algae isolated from cotton fields was studied by Tarar and Salpekar (1980). Among these *Chlorococcum humicola* was resistant and *Cylindrospermum musicola* was highly susceptible. Several other algae including *Anabaena cylindrica, Anacystis nidulans, Nostoc muscorum* (Schauberger and Wildman 1977), *Chlorella* (Ellis and Goulding 1973) and *Chlorococcum* (Torres and O'Flaherty 1976) have been reported to be sensitive to insecticides. However, Raghu and MacRae (1967 a) and Sethunathan and MacRae (1969) found a stimulation of algae in the presence of insecticides. Diazinon at field rate (2 kg a.i. ha^{-1}) and a higher rate (20 kg a.i. ha^{-1}) substantially increased algal population in flooded Maahas clay loam (Sethunathan and MacRae 1969). Similar results were reported with BHC (Raghu and MacRae 1967 a). This was attributed to elimination of algal predators such as small crustaceans by these insecticides. Similarly, carbofuran enhanced the survival, growth, and nitrogen fixation in *Nostoc muscorum* at lower doses but gradually inhibited at higher doses of 50 to 1000 μg ml^{-1} (Kar and Singh 1978).

The interactions of insecticides and soil microflora are subject to the influences of several environmental and edaphic factors and agricultural practices. In assessing an insecticide for its desirability with reference to its adverse effects on nontarget soil microflora, it is essential to take such factors into consideration. Though such an awareness was not evident in the past, the recent literature reflects an interest by soil microbiologists on this aspect.

Tu (1981 a) observed that flooding altered the effects of DDT. Anaerobic bacteria were stimulated by the insecticide at 10 mg g^{-1} of soil while fungi were inhibited. In such observations the observed effect cannot be attributed to the insecticide alone, because anaerobic bacteria are stimulated by flooding in natural conditions also.

Agricultural practices, often neglected in assessments, may also have profound effects. Common agricultural practices, such as addition of synthetic fertilizers and organic farmyard manure may substantially increase the populations even in the presence of the insecticides. While the former may substantially increase the specific

microflora dependent on such substrates such as urea, the latter may increase a wide spectrum of microbes. The organic matter added as manure to the soil may also influence the interactions by adsorbing the chemicals and thus reducing the possibilities of their interactions with the soil microbes. Misra and Gaur (1977) showed that the toxicity of lindane to the bacteria at 1 and 10 ppm was neutralized by the added farmyard manure. This was attributed to the insecticide-adsorbing capacity of the manure. They also speculated that this neutralization may be associated with the degradation of the chemical by the increased number of the microorganisms stimulated by the manure. Similarly, the amendments of the soil in the laboratory experiments also often mask the real effects of the insecticide. Cowely and Lichtenstein (1970) reported that at 40 mg ml^{-1} aldrin, lindane, parathion, phorate and carbaryl which were toxic to *Fusarium oxysporium* in laboratory, were not harmful when the medium was supplemented with asparagine, ammonium salts and yeast extract. Richardson and Miller (1960) decidedly showed that the properties of the insecticide are important in determining its effects. They found that lindane, heptachlor, chlordane, and aldrin were toxic to *Rhizoctonia solani* at 25 ppm due to their high solubility and vapor pressure. Of these the toxicity of lindane was highest and DDT, methoxychlor, dieldrin and endrin were relatively less toxic. The vapor pressure of the insecticides and their toxicity toward the fungus were proportionately related. Further, in case of the formulations, the carrier substances may have more profound effects on the microflora than the insecticide. This was substantiated by Stanlake and Clark (1975), who showed that the petroleum distillates present in the malathion formulation were more toxic to the pure cultures of bacteria than the pure insecticide. However, in field conditions both the insecticide and the carrier were nontoxic. On the other hand, Salonius (1972), who found no toxic effect of formulated DDT and fenitrothion on microflora, attributed this to the presence of an additional substance ($CaCO_3$) in the formulation, which was supposed to have stimulated the microbes, thus masking the action of insecticide. Mathur et al. (1980) inferred from their experiments that disulfoton and prometryne did not affect the microflora in soil plots on which lettuce were grown, because of their disappearance. But permethrin was persistent till the time of harvesting and was toxic to microflora. Such a relation between persistence of a pesticide and type of soil was also shown by Raghupathy (1977). He found that aldicarb was more persistent in black clay soil than in alluvial clay or red silty loam. Further, this persistence was related not to the amount of rainfall, but to its distribution.

Mathur et al. (1980) found that the mode of application also decides the effect of insecticides. In a mesi soil, fungal populations were decreased by carbofuran and dyfonate when applied as bands under the seeds, while carbofuran, applied as a broadcast, instead stimulated the population up to 52% higher than the controls. Banded application, however, was favorable to bacteria.

The edaphic factors such as soil temperature, moisture, pH, soil type and plant cover also affect the experimental result. The microbes become more susceptible to the insecticide in the presence of temperature stress. Lindane was toxic to bacteria, fungi, actinomycetes, and *Azotobacter* at 1 ppm at 30 °C but not at 15 ° and 45 °C (Misra and Gaur 1977). Similarly, methyl parathion was toxic to actinomycetes at 12 °–15 °C for a longer time than at 20 °C. Fungi, on the other hand, were depressed at 20 °C but tolerant at 12 °–15 °C (Naumann 1972). Verstraeten and Vlassak (1973)

suggested that to eliminate such interferences from natural stress conditions, the laboratory experiments should be done at optimum temperatures where the activities of the test organisms are maximum. This also applies to the other controllable factors such as soil pH, moisture, aeration etc. The pH altered the toxicity of lindane. It was more toxic to *Azotobacter* populations and fungi in acidic and alkaline than in neutral soils. However, bacteria and actinomycetes were stimulated by the acidic conditions (Misra and Gaur 1977).

With reference to the changes in soil moisture, Misra and Gaur (1977) found that lindane at 10 ppm was stimulatory under high soil moisture conditions to *Azotobacter,* but inhibitory to soil fungi. Naumann (1971) also found that formulations of lindane and toxaphene had a stimulatory effect on bacteria in general but depressed anaerobic populations and spore-forming bacteria in dry conditions.

Afifi and Abdulla (1979) reported that endrin applied to okra and lupin shoots disturbed fungal populations by changing the plant root exudates. Nayak and Rao (1980) found that specific groups of nitrogen-fixing organisms responded to carbofuran and BHC, depending on the soil type. The microbes were stimulated by carbofuran in an alluvial soil (pH 6.2), laterite (pH 5.0) and acid saline soils (pH 4.2) and by BHC in alluvial soil and acid saline soils, while in other soils (Kara pH 3.2 and Karapadam pH 5.0) there was considerable inhibition. Similarly dyfonate at 8.3 kg ha^{-1} was completely inhibitory to populations in calcareous clay and clay soils. The differences could be attributed to the soil texture and the organic content.

The soil being a repository for several pesticides, there is a possibility of synergistic effect and sometimes antagonistic effects. To test such a possibility in the field, Hubbel (1973) tried the effects of different combinations of pesticides. He found that parathion with DDT and Zineb increased fungal populations, while Zineb alone was highly toxic. Fungitoxicity of Zineb was slightly negated by parathion and completely negated by DDT. On the other hand, they observed harmful synergistic effects. DDT and parathion alone had only a slightly depressing effect, whereas in combination they were substantially toxic. Stojanovic et al. (1972) also found a similar synergistic effect. A combination of DDT, dieldrin and carbaryl severely depressed bacteria, while a combination of malathion, with trifluralin and 2,4,5-T, was slightly depressive.

3 Ammonification

Ammonification is the first step in the mineralization process during which NH_4^+ ions are liberated from the organic nitrogen compounds. This is a key step in soil, supplying readily available nitrogen to plants and microorganisms. Since the process is associated with many physiologically dissimilar microorganisms, the application of insecticides seldom jeopardises their activities altogether. Hence, it is not surprising that ammonification is relatively tolerant to insecticide treatments. However, various reports show a diversity in the effects of insecticides, depending on the microorganisms, the type of xenobiotic, its concentration and the experimental conditions.

A few workers reported an accumulation of NH_4^+ compounds in soil treated with insecticides (Sivasithamparam 1969; Tu 1970, 1972, 1973a; Bollen and Tu 1971;

Endo et al. 1982). It is possible that due to their heterogenity, the ammonifying microorganisms continue their activities for several months even in the presence of insecticides, whereas the comparatively sensitive nitrifying microorganisms slow down the conversion of NH_4^+ compounds into NO_2^--N and NO_3^--N and hence the observed accumulation in NH_4^+-compounds. After the nitrifying organisms acclimatize to the added insecticides, the NH_4^+ level falls to normal. Several reports substantiate that the ammonification process is comparatively tolerant to insecticide applications. Raghu and MacRae (1967b) did not find any inhibitory effect of lindane at 5 and 50 kg ha^{-1} under submerged soil conditions. Endrin, at rates three times that which might be expected from seed application of the insecticides, did not affect ammonification appreciably in three different soils (Bollen and Tu 1971). Endrin and chlordecone, each at 22 kg ha^{-1} and fenophos or pirimiphosethyl at 11 kg ha^{-1} in a sandy clay loam or a clay soil caused ammonification to fluctuate for 4 weeks, but the activity returned to normal after 7 to 8 weeks (Gawaad et al. 1972b, 1973a). Similarly, terbufos, at rates of 0.1 to 10 kg ha^{-1} in three soil types, did not show any effect on ammonification (Laveglia and Dahm 1974). Application of DDT also caused no effect on ammonifying bacteria (Ross 1974).

Stimulation or a delayed stimulation of ammonifiers on insecticide treatments has also been reported (Naumann 1970a; Sundram et al. 1977). Part of this effect may be due to the killing of soil insects as well as of sensitive microorganisms which would provide a greater amount of substrate material for the resistant ones. Chlorpyrifos had a stimulatory effect on population of ammonifiers as well as ammonia production in clay soil of pH 5.3 (Sivasithamparam 1970). In a soil supplemented with 2% compost, lindane at 0.22 and 4.4 kg ha^{-1} or dieldrin at 2.5 and 50 kg ha^{-1} initially suppressed and subsequently increased ammonification (Mahmoud et al. 1970). A similar trend is shown by methylparathion at 15, 150 and 300 kg ha^{-1} in a loam soil (Naumann 1970b). Such a fluctuation in ammonifying organisms was shown to depend on soil moisture level (Naumann 1971, 1972). In a red soil carbofuran at 35 kg ha^{-1} or more, inhibited ammonification process in a red soil which was restored after 2 months and an amendment of nitrogen source was found to be beneficial for the recovery of ammonifiers (Sundram et al. 1977). Tu (1970, 1972, 1973a) studied the effect of six organophosphorus insecticides (chlorpyrifos, diazinon, ethoprop, fensulfothion, thionazin and trichloronate) on ammonification and most of the treatments increased ammonia production from added peptone, and mineralization of soil organic nitrogen was equal to or more than that of control soils.

Harmful influence of insecticides on ammonification process is often manifested at higher doses. Nuvacron at 200 and 600 g a.i. ha^{-1} and Supracids at 10 and 20 oz $acre^{-1}$ did not alter ammonification in a laboratory study (Idris 1973) whereas at higher doses (Nuvacron 1000 g ha^{-1} and Supracids 30 oz $acre^{-1}$) showed depressive effect on the process. Similarly endrin at 10 and 50 ppm stimulated the soil microflora and ammonification in a black cotton soil, and the insecticide at 100 and 200 ppm showed inhibitory effect for 25 days (Varade and Nakat 1975). However, Audus (1970b) reported that Demeton was the most toxic insecticide as it inhibited ammonification even below field rate. However, Tu (1972) reported that ammonification of soil organic nitrogen was depressed significantly at 1 and 5 ppm of carbofuran, whereas ammonification of added peptone was unaffected by the treatment.

4 Nitrification

Nitrification is an indicator of good soil biological activity and fertility and is mainly brought about by two important species of chaemosynthetic bacteria, the *Nitrosomonas* sp. oxidizing NH_4^+ into NO_2^- and *Nitrobacter* sp. oxidizing NO_2^- into NO_3^-. The resulting NO_3^- is the major nitrogen source assimilated by the plants. Unlike ammonification, nitrification is more sensitive to the changes in the environment, probably due to the more susceptible nature of micro-organisms involved in the process. Several studies have been carried out to evaluate the deleterious effects of the insecticides on the process and due to the diversity of the results reported, it is difficult to reach a general conclusion.

Many insecticides, when applied at field rates, failed to show any depressive action on nitrification or on the growth of the microorganisms involved. Pathak et al. (1960–1961) did not find any impairment of nitrification with DDT, chlordane or aldrin. A similar result was also reported by Shaw and Robinson (1960) with DDT, aldrin, dieldrin, and heptachlor.

Insecticide applications generally exhibited an initial inhibition of nitrification which often was followed by a gradual recovery of the process. The decreased effect of insecticides after a lapse of time may indicate that the insecticides might have been metabolized and detoxified by the nitrifiers or that they adapted themselves to the insecticides. Chandra (1967) reported that dieldrin and heptachlor inhibited nitrification for 8 weeks in a heavy clay, a sandy loam, and two loam soils. The toxic effect disappeared and nitrification increased in the heavy clay and a mountain loam by 16 weeks. It was also noted that the inhibitory effect of insecticides decreased with increasing time and temperature and that the toxic effect was least in soil with high values of organic matter content, clay content, and cation exchange capacity but neutral pH. Probably, the above-mentioned factors increased the physical, chemical, as well as microbial degradation of the insecticides as a result of which the microorganisms would have recovered. In a sandy loam soil Bhardiya and Gaur (1970) found that the insecticide concentration at 25 ppm or a higher dose exerted a temporary inhibition of nitrification of the added NH_4SO_4, which appeared in the order lindane > dieldrin > aldrin. Also the decrease in NO_3^- level was accompanied by NO_2^- accumulation, indicating that *Nitrobacter* sp. was more sensitive than *Nitrosomonas* sp.

In a series of experiments, Tu (1970, 1980a) and Tu and Miles (1976) observed a similar effect of insecticides on nitrifiers, wherein the time needed for the recovery of nitrification varied with the insecticides tested. In a sandy loam Bay 27289, diazinon, Dursban and Zinophos at 10 and 100 ppm depressed nitrification of soil organic nitrogen for 2 weeks and to a lesser extent after 3 weeks (Tu 1970). Similarly in loam soil, dieldrin and aldrin exerted an inhibitory influence on nitrification at concentrations as high as 2000 ppm for 2 weeks and by 3 months the nitrification gradually rose to normal (Tu an Miles 1976). Tu (1980a) arrived at an almost comparable result with a few organophosphorus insecticides as well as their metabolites. The nitrifiers in loamy soil needed 2 to 3 weeks for adaptation. However, after 3 weeks, most samples treated with phorate, phorate sulfoxide, phorate sulfone, terbufose, terbufose sulfoxide, terbufose sulfone, permethrin and DOWCO 275 at

5 ppm recovered from the insecticidal effects. After 6 weeks, the nitrifiers recovered completely.

The dilution of pesticide by degradation in soil to a point at which it could exert a stimulation is speculated as a cause for delayed stimulation of the NH_4^+ oxidiers. With chlorpyrifos, Sivasithamparam (1969) observed a suppression of NH_4^+ oxidation after 3 weeks of treatment and a stimulation of the process after a lapse of 3 months. Similar delayed stimulation in plots of sandy clay loam and clay soil was reported by Gawaad et al. (1972 b, 1973 b). With chlordecone or endrin at 22 kg ha^{-1} and fonofos or pirimiphosethyl at 11 kg ha^{-1}, nitrification decreased initially and then returned to normal afterwards.

The effect of insecticide on nitrification is also shown to depend on its concentration. Carbofuran at 1 ppm was innocuous on nitrification in a Delhi loamy soil, typical in Ontario, Canada, whereas at 5 ppm it exerted a stimulatory effect (Tu 1972). Similarly, Varade and Nakat (1977) reported that in a black cotton soil, endrin at 100 and 200 ppm temporarily inhibited nitrification, but at lower doses (10 and 50 ppm) stimulated the process. On the contrary, stimulation of nitrification is also associated with higher concentrations of lindane, heptachlor, parathion, and disulfoton (Audus 1970 b). Stimulation of nitrifiers by the highly toxic and short-lived insecticide, methyl parathion (Nauman 1970 a), and an increased NO_2^- in volcanic ash soil with disulfoton treatment at 60 kg ha^{-1} (Kobayashi and Katsura 1968) for 25 to 50 days has also been reported.

It has also been shown that formulated products in pure culture studies are more toxic to *Nitrosomonas europea* and *Nitrobacter agilis* than the pure unformulated pesticides (Caseley and Luckwill 1965). These observations were attributed to the ability of the wetting agent to enhance the effect of the pesticide. For instance, nitrification was severely inhibited by granular (formulated) propoxur and aldicarb at 500 ppm, but much less effective with the technical grade insecticde (Kuseske et al. 1974). The metabolism of corn cob grits which was used as the carrier in the formulated carbofuran and the resultant mobilization of nitrogen might be the cause of apparent inhibition of nitrification. The corn cob grits carrier alone applied in equivalent amounts to achieve 500 ppm of the toxicant also resulted in the total inhibition of the nitrification process. On the contrary, Sethunathan and Ramakrishna (1982) noted that the application of carbofuran (technical or formulated) at 10 and 100 ppm a.i. distinctly stimulated autotrophic oxidation of ammonium in rice rhizosphere soil suspension. *Nitrosomonas* sp. was also enriched by the insecticide treatment. Calcium carbonate present in the formulated carbofuran (Furadon 3G) was held responsible for a more pronounced stimulation on ammonium oxidation.

Application of insecticides in combination may be of significance as combination can synergistically influence the soil microbial activities. Ray et al. (1980) reported that in a simulated oxidized zone of flooded soil, HCH at 5 ppm effected only a transitory inhibition (26% control) and virtually innocuous effect with 500 ppm of carbofuran when applied singly. But a combination of HCH and carbofuran effected a synergistic decrease in the formation of NO_2^- from NH_4^+ (61% over control) after 12 days, which increased to 71% after 40 days. This inhibition of nitrification in the oxidized zone of flooded soil due to pesticides may not be agronomically deleterious, since it is reported that ammonium nitrogen applied as basal or top dressing to flooded soil is oxidized at the top layer to nitrate which is leached to the reduced

zone and lost by denitrification as volatile form (Ponnamperuma 1972). The formation of small amounts of NO_3^- in soil amended with 100 ppm HCH, despite the complete inhibition of NH_4^+-oxidizing autotrophs, as reported by Ray et al. (1980), could be due to the activity of more tolerant heterotrophic microorganisms. Such heterotrophic nitrification may be important in situations where more sensitive autotrophic nitrifiers are inhibited under adverse conditions of high pesticidal pressures.

Nitrification, as mentioned earlier, involves the conversion of organic nitrogen compounds to NO_2^--N by *Nitrosomonas* sp. and NO_2^--N to NO_3^--N by *Nitrobacter* sp. However, not much work has been done to understand the effects on these two organisms separately. In a pure culture study Garretson and SanClemente (1968) reported that aldrin, TDE and parathion at 10 ppm completely inhibited NO_3^- oxidation by *Nitrobacter agilis,* whereas lindane and malathion at 1000 ppm caused only a delay in the process. They also noticed that lindane, Baygon and malathion were more toxic to *Nitrosomonas europea* than *Nitrobacter agilis,* since NH_4^+ oxidation by *Nitrosomonas europea* was completely inhibited by these insecticides at 10 ppm. Similarly heptachlor and chlordane partially inhibited NO_2^- oxidation by *Nitrobacter agilis,* the insecticides being more inhibitory in cell-free extracts than in cell suspensions (Winely and San-Clemente 1968). It was also concluded in this experiment that the insecticides chlordane, DDD, heptachlor, lindane, and aldrin exerted their effects by inhibiting the cytochrome-c oxidase of the microbes. Pure culture studies on *Nitrobacter agilis* and *Nitrosomonas europea* indicated that propoxur and aldicarb at 500 ppm were toxic to both organisms, particularly, to the latter and the toxicity to *Nitrosomonas* sp. was more severe in pure culture than in soil studies (Kuseske et al. 1974). In liquid culture, carbofuran inhibited autotrophic NO_2^- oxidation for 12 days, whereas in unamended medium NO_2^- completely disappeared in 7 days (Ramakrishna et al. 1978). In contrast, NH_4^+ was readily oxidized to NO_2^- in cultures of NH_4^+-oxidizing autotrophs, despite the presence of 100 ppm of carbofuran and in fact more NO_2^- accumulated in carbofuran-amended medium than in unamended medium, suggesting a stimulation of the NH_4^+ oxidizers.

It is tempting to predict toxicity under field conditions on the basis of data obtained from pure culture studies. However, factors such as solubility, adsorption on soil colloids, and physical, chemical, and microbial degradation of the toxicant should also be considered. Also some degradation products may be more active than the parental compound. Jones and Hood (1980) reported that the parent compounds of thiophosphorus pesticides had little effect on ammonium oxidation in estuarine sediments, whereas the metabolites which accumulate from decomposition of these pesticides, especially in sediments under low O_2 levels, may significantly reduce the process. The activity of the indigenous ammonium oxidizers from estuarine sediments was inhibited after 14 days of treatment with methylparathion. The pesticide was aged under aerobic, anaerobic, and microaerobic conditions and the axenic cultures of ammonium oxidizers were exposed to the resulting metabolites which then resulted in a 10 to 20% reduction of the activity. It was also reported that though methylparathion caused little effect, its degradation product, *p*-aminophenol, inhibited ammonium oxidation at a concentration as low as 0.01 ppm.

Insecticide application at field rates does not appear to have any adverse effect on nitrification. However, when used at higher doses, it inhibits nitrification. Eno

and Everett (1958) reported a reduction in nitrification with BHC, lindane, and heptachlor at 100 ppm. Nitrification was unaffected by lower concentrations of Nuvacron and Supracids whereas at a higher dose (1000 g Nuvacron ha^{-1} and 30 oz. Supracids $acre^{-1}$) it was depressed (Idris 1973). Lin et al. (1972) reported that trichlorfen at 5 and 50 kg ha^{-1} inhibited nitrification for a short period whereas aldicarb and propoxur at 50 kg ha^{-1} showed a marked depression in nitrification. Elliot et al. (1972) showed that the population of NH_4 oxidizers were lower with ethoprop and of NO_2^- oxidizers were lower with carbofuran. Similarly, in a simulated oxidized zone of flooded alluvial soils, NH_4^+ was oxidized fairly rapidly in unamended soil and in soils amended with 10 and 100 ppm carbofuran during a 40-day incubation (Ramakrishna et al. 1978). But carbofuran partially inhibited NO_3^- formation at 1000 ppm and strongly at 5000 ppm (0.5%). Interestingly, in soils amended with 0.5% carbofuran, NO_2^- accumulated in large quantities from NH_4^- oxidation concomitant with the inhibition of NO_3^- formation. In a similar fashion, at 100 and 1000 ppm cartap HCl diminished NO_2^--N and NO_3^--N in soil, although the insecticide was ineffective at 10 ppm level on nitrification (Endo et al. 1982).

To sum up the section on nitrifying bacteria, we may refer to the laboratory studies of Bartha et al. (1967) on different pesticides and their interpretation of data on nitrification. They found a relationship between the chemical configuration and the endurance of these products and their effects in the soil. With the inhibitory products, several levels of reaction were noted. They proposed that (a) the toxic effects may decrease with time indicating that the products undergo a transformation or an increasing activity of resistant species, (b) the inhibition of nitrification remains constant throughout the test period. Such products are chemically and biologically stable, (c) the toxicity may increase with time suggesting that the metabolite is more harmful than the parent compound.

5 Denitrification

Microbial denitrification, the reduction of NO_3^- and NO_2^- into nitrous oxide or nitrogen gas which is lost from soil into atmosphere represents a net loss of nitrogen to microorganisms and plants. Although denitrification is a common and important activity in soil, it is least investigated.

The insecticides in general are nontoxic to denitrifying bacteria. In soils previously incubated anaerobically to increase denitrifiers, lindane had no effect on denitrification of added nitrate and parathion was innocuous on denitrifiers at normal to 100 times the field rate (Audus 1970b). Similarly methylparathion up to 300 kg ha^{-1} had little effect on denitrifier population in a loam soil (Naumann 1970a). Gawaad et al. (1972a; 1973a) reported stimulation of denitrification in sandy loam, clay loam, and clay soils with chlordecone or endrin at 22 kg ha^{-1} and fonofos or pirimiphosethyl at 11 kg ha^{-1}. On the contrary Mitsui et al. (1962; 1964) reported that BHC at 20 ppm caused comparatively minor inhibition of denitrification.

6 Biological Nitrogen Fixation

Biological nitrogen fixation plays an important role in returning gaseous nitrogen to soil. The symbiotic association between *Rhizobium* and leguminous crops, as well as the activities of asymbiotic N_2-fixing bacteria such as *Azotobacter, Beijerinckia, Clostridium* etc., and nitrogen-fixing blue-green algae, have received little attention in relation to insecticide treatments.

6.1 Symbiotic Nitrogen Fixation

Seed dressing, as well as soil-applied insecticides, are recommended to control various insect pests of leguminous crops. Inoculation of seeds of leguminous crops like groundnut with *Rhizobium* to increase nodulation and crop yield is also becoming popular (Kulkarni et al. 1974). There are varying reports on the effects of insecticides on rhizobia, nodulation of legume (nodule number as well as weight) and the efficiency of nitrogen fixation measured in terms of nitrogenase activity, incorporation of $^{15}N_2$ and the total nitrogen assimilated by the eukaryotic symbiont etc.

Since nitrogen fixation is a growth-linked process, the effect of insecticide on the growth of either member of the symbiotic association may indirectly indicate the influence of the compunds on nitrogen fixation. A few reports indicate the absence of any negative influence of the insecticides on the growth and yield of leguminous crops or on *Rhizobium* when applied at lower doses or at recommended field rates. For instance, Pareek and Gaur (1969, 1970) did not find any reduction in growth and yield of *Phaseolus aureus* with normal doses ($<$ 40 ppm) of DDT. Similarly in pot experiments with clay soil Selim et al. (1970) found that dieldrin (25 and 500 ppm) and lindane (2.22 ppm) had no effect on the morphology of shoot and root systems of the leguminous crop, whereas at a higher dose (44.4 ppm) lindane had a harmful effect. Soil application, foliar spray or a combination of both methods with certain systemic insecticides viz., phorate, carbofuran and methomyl at a rate of 1 lb $acre^{-1}$, did not affect germination, number of mature pods per plant or yield of soybean plants (Wheeler and Bass 1971; Shehane and Bass 1974). In a pot study with red soil, application of four insecticides at recommended rates (Carbofuran 16 kg ha^{-1}, phorate 8 kg ha^{-1}, Dasanit 20 kg ha^{-1} and heptachlor 16 kg/ha) did not affect yield, dry matter content or total nitrogen content of *Arachis hypogea* (Kulkarni et al. 1974). Tu (1977, 1981b) studied the effects of insecticide as well as insecticide and fungicide combinations on soybean-*Rhizobium* association. Soybean seeds were treated with the insecticides lindane at a rate of 14 g $bushel^{-1}$ (27 kg seed), and chlorpyrifos 8.5 g $bushel^{-1}$ alone or in combination with the fungicide thiram at 28 g $bushel^{-1}$ and incubated in yeast extract mannitol agar medium for 48–72 h (Tu 1977). The germinated seedlings were planted in moist sterile silica sand in plastic containers which were inoculated with broth cultures of *Rhizobium japonicum*. Lindane alone or in combination with chlorpyrifos did not affect the weights of stem and leaf 3 weeks after treatment. But in combination with thiram,

the insecticides effected a harmful influence on plant growth. However, no significant effect was observed after 8 weeks in all treatments. In an identical study with lindane, diazinon and thiram, Tu (1981b) reported no deleterious effect in the weights of leaves plus stem or roots of soybean plants 7 weeks after seed treatments. It was suggested that under good moisture conditions, in a free draining soil, the potentially toxic doses were mitigated by the bacterial migration from toxic zones.

When a significant amount of organic matter is added to soil, it may decrease the biological activity of the insecticides (Harris 1972a, b) and under these circumstances a reduction in toxic effect of the insecticides is expected. In a pot experiment with sandy loam soil, aldrin application at 1.5 and 10 ppm reduced the straw-grain yield as well as total nitrogen content of *Cicer arietinum* after 7 weeks (Kapoor et al. 1977). Addition of farm yard manure (FYM) at 0.5% on carbon basis along with all treatments improved nitrogen fixation in terms of yield and total nitrogen. At 5 ppm aldrin, presence of FYM eliminated the adverse effect and was comparable to control, whereas at 10 ppm level, even though better plant growth and yield were obtained, the toxic effect of the insecticide was not completely eliminated. However, in another study, the inhibitory effect of lindane at field rate or higher dose on the growth as well as yield of *Cicer arietinum* could not be alleviated by the addition of organic matter in the form of grain-straw (Misra and Gaur 1975). Seed application of deltamethrin resulted in stunted growth of soybean seedling and the insecticide at concentrations greater than 1000 ppm adversely affected the emergence and growth of seedlings (Tu 1982a). Iswaran (1975) reported that seed application of aldicarb together with *Rhizobium* inoculation increased the yield of *Phaseolus aureus* as well as in controlling infection.

Several strains of rhizobia have been found to be resistant to insecticides, some are stimulated while other are sensitive. The implication is that for legume inoculation, strains should be chosen which are resistant to the insecticide to be used (Audus 1970b). Although the results of the studies on the effect of insecticides on a rhizobia are often at variance, from the data we can infer a general inhibition of rhizobia especially in pure culture experiments. Five insecticides were tested against four strains of rhizobia by Daitloff (1970) using insecticide emulsions absorbed on beads which were applied wet or dry to agar plates seeded with rhizobia. The order of toxicity of wet beads was dimethoate > lindane > isobenzan > endrin > dieldrin, and wet beads produced large zones of inhibition than dry beads, especially in the case of dimethoate. Gillberg (1971) showed that dinoseb, a contact herbicide which may be also used as an insecticide, at 150 ppm inhibited most of the strains of *Rhizobium meliloti, Rhizobium leguminosarum,* and *Rhizobium trifolii.* Lindane and fonofos at 10 and 50 ppm respectively, inhibited glucose utilization of an effective strain of *Rhizobium trifolii* and increased it in an ineffective strain; conversely diazinon (50 ppm) increased glucose utilization in the effective strain (Salem 1971). A group of insecticides were tested for inhibition of rhizobia on agar medium and in all cases *Rhizobium trifolii* and *Rhizobium leguminosarum* were found to be the most sensitive (Lin et al. 1972). Kapusta and Rouwenhorst (1973) tested the interaction of ten insecticides and *Rhizobium japonicum* strains in pure culture and found that only disulfoton at 7.5 ppm inhibited a mixture of strains while others had no effect. Individual strains when screened separately, however, showed different sensitiveness to disulfolon and carbaryl. Disulfoton reduced growth of all strains successfully, at

the highest rate (24 ppm), while carbaryl inhibited two strains at the highest rate (24 ppm), and one strain was resistant.

With increasing concentration of disulfoton, carbofuran and endrin, Oblisami et al. (1973) reported an inhibition of *Rhizobium* sp. Two strains of *Rhizobium meliloti* attained normal growth with 1.89 ppm dimethoate, whereas 3.89 ppm completely inhibited the growth (Staphorst and Strijdom 1974). The inclusion of trichlorfon in media at rates up to 1285 ppm suppressed the growth of *Rhizobium leguminosarum* and *Rhizobium trifolii* and shifted their optimum pH and temperature for growth (Salama et al. 1973). In a liquid culture study Hamed and Salem (1977) observed that the application of Dipterex, endrin and carbaryl at 8 ppm generally inhibited the growth of *Rhizobium leguminosarum* and the effect varied with the strains. Endrin at 0.5 to 8 ppm inhibited the growth of *Rhizobium leguminosarum* (R_v), whereas the insecticide relatively stimulated *Rhizobium leguminosarum* (R_L). Also the effect varied with the three insecticides, Dipterex being more inhibitory, followed by endrin and carbaryl. They concluded that Dipterex was more easily absorbed by *Rhizobium* as it was a semi-systemic insecticide and hence caused a comparatively higher inhibitory effect. Goss and Shipton (1965) also found systemic organophosphorus compounds to be very harmful to rhizobia. Tu (1977, 1982a, b) studied the inhibitory effect of several insecticides and fungicides alone or in combination on the growth of *Rhizobium* by filter paper disc method. The pesticides were applied to filter paper discs before placing the discs on the the agar surface inoculated with the bacteria and zones of inhibited growth surrounding the discs were measured after the incubation period. It was observed that chlorpyrifos at 1.4 and 14 g bushel^{-1} level had a small inhibition zone and at 140 kg bushel^{-1} level showed greater inhibition, whereas lindane at higher levels produced moderately toxic effects on bacteria (Tu 1977). But in combination with thiram, the toxicity of chlorpyrifos and lindane was enhanced. Of the five pyrethroid insecticides, namely cypermethrin, deltamethrin, fenpropanate, fenvalerate, and permethrin, the latter three had low toxic effect on the growth of *Rhizobium meliloti* (Tu 1982a). The development of resistance to pesticide without losing the ability to fix nitrogen is of great agronomic importance. Gupta and Shirkot (1981) reported that by serial passage procedure *Rhizobium* strains D-467, D-232 and CP-20, which are sensitive to carbaryl at 15 ppm, were made resistant to the insecticide up to 50 ppm. No decrease in nodulation capacity of the resistant strains compared to their sensitive counterparts was noticed. Dehydrogenase activity, which has been shown to be related to nitrogen fixation, increased with the development of resistance except in the case of D-467, where it decreased over its sensitive counterparts. In an earlier study, Gillberg (1971) also reported the isolation of mutant strains of *Rhizobium* sp. with higher doses of insecticides.

The tap root nodulation of leguminous crops such as soybean is a qualitative characteristic which appears useful as an index of the level of the adequacy and activity of bacterial inoculum at planting. Despite several reports on the delay in the emergence of nodulation, pesticide treatment for legume protection is still of prime importance.

Pareek and Gaur (1970) reported the absence of inhibitory influence of DDT at 1.5 and 10 ppm on nodulation in *Phaseolus aureus* (Green gram) although at 100 and 1000 ppm there was a complete inhibition. Similarly Dipterex at 15 kg feddan^{-1}

did not seriously affect nodulation of broad bean plants (Salama et al. 1974). Of the several organophosphorous (Gardona, Imidan, Supracids, and trichlorfon) and carbamate (aldicarb, carbaryl, carbofuran, methomyl and propoxur) insecticides, only methomyl and propoxur at 5 and 50 ppm and carbofuran at 50 ppm caused some phytotoxic effects (decolorization and spotting) on soybean plants grown in sandy clay loam soil under greenhouse conditions (Rodell et al. 1977). However, this effect was not correlated with the loss of nitrogenase activity or plant growth. Tu (1977, 1981b) also did not find any permanent deleterious effect on nodulation or acetylene reduction capacity of soybean with insecticide and/or fungicide treatments. However, seed treatments of lindane (14 g bushel^{-1}), chlorpyrifos (8.5 g bushel^{-1}) and thiram (21 g bushel^{-1}) alone or in combination showed a slight inhibitory effect on acetylene reduction activity of soybean plants 3 weeks after treatment (Tu 1977). Plants showed steady increase in nitrogenase activity, and after 6 weeks' growth, the level of acetylene reduction, with the exception of thiram-treated plants, was equal to or higher than the control values. It was also shown that nodulation, as well as acetylene reduction, was unaffected by seed treatments with lindane and diazinon alone or in combination (Tu 1981b). However, stimulatory effects on acetylene reduction were noted with the treatment of diazinon throughout the experiment and with diazinon plus lindane after 7 weeks.

Nitrogen fixation was stimulated with aldicarb and chlorpyrifos in broad bean plants (Salem et al. 1976). Endrin and chlorfenvinphos did not exhibit any inhibition on nodulation in cowpea *(Vigna sinensis)* and phorate enhanced nodulation by 313% over the control plants after 30 and 50 days, although the weight of the nodule did not progressively increase with number (Swamiappan and Chandy 1975). The breakdown and degradation of the organophosphorus moiety of phorate was reported to be the possible cause for the stimulatory effect on nodulation. Earlier, Gawaad et al. (1972a) reported under laboratory conditions a similar stimulatory effect by phorate on nodule number in bean and clover, but the insecticidal effect was absent on clover roots under field conditions and at high application rates. Goss and Shipton (1965) reported that in their trials with leguminous inoculation with *Rhizobium,* dimethoate used even months before the inoculation caused damage and prevented nodulation. Straphorst and Strijdom (1974) also reported that seed treatment of *Medicago sativa* with dimethoate (37.8 to 226.8 ml a.i./50 kg seed) 5 days before inoculation with two strains of *Rhizobium meliloti* in quartz sand culture decreased plant yield, but did not affect nodulation of plants except at higher doses. They also noticed that the yield of the plants was better when the inoculation was done 2 to 9 days after seed treatment with dimethoate (113.4 ml a.i./50 kg seed) than those inoculated immediately after seed treatment. Dimethoate at 0.3 ppm a.i. as a foliar spray applied to inoculated lucerne had no significant effect on nodulation, but sprayed plants were smaller than those not sprayed (Staphorst and Strijdom 1974). Under laboratory conditions lindane, DDT, heptachlor and aldicarb significantly reduced number of nodules per plant on bean and clover, whereas under field conditions the effect did not appear (Gawaad et al. 1972b). Application of aldrin, dieldrin, chlordane, DDT, BHC, lindane and parathion also caused a depressive effect on nodulation (Sylvestre and Fournier 1979). Kulkarni et al. (1974) noticed a significant reduction in nodule number in *Arachis hypogea* with phorate, heptachlor, and Dasanit at recommended rates, but the nodules were bigger and

weighed significantly more than the control plants and there was no quantitative variation in the leghemoglobin content of the nodules. Aldrin at 1, 5, and 10 ppm was reported to reduce nodulation (nodule number and weight) in *Cicer arietinum* grown in a pot with sandy loam soil (Kapoor et al. 1977). Eisenhardt (1975) noticed a significant reduction in symbiotic nitrogen fixation by alfalfa grown in sand agar medium when treated with phoxim. With 10, 100, and 1000 ppm of the insecticide the nitrogen fixation was inhibited by 44, 52, and 93%. Similarly, soil application or seed treatment of *Cicer arietinum* under greenhouse conditions with lindane at field rate level or higher doses inhibited nodulation (Misra and Gaur 1975).

The difference in the effect of various insecticides in different studies seems to indicate that some chemicals are safer used in conjugation with legume inoculants. Often no permanent deleterious effect of insecticides on leguminous growth and nitrogen fixation could be observed, especially when the insecticides were applied at recommended field rates. Also the toxicity of the pesticide is often low if applied to soil, intermediate if incorporated in the soil and high if maintained in direct contact in solution, suspension, or slurry with seeds. However, the results based on laboratory experiments should be related to field conditions with caution.

6.2 Asymbiotic Nitrogen Fixation

The effect of various insecticides on asymbiotic nitrogen fixers show a great diversity, the effect varying with different insecticides and with specific groups of nitrogen fixers. However, the available information indicates that at normal field rates the insecticides had no effect on asymbiotic nitrogen-fixing bacteria. DDT, chlordane and aldrin at application rates did not alter asymbiotic nitrogen fixation (Pathak et al. 1960–1961). Lindane (10 ppm) and fonopos (50 ppm) initially inhibited glucose utilization and N_2 fixation in *Azotobacter chroococcum* and *Azotobacter agile,* but later these functions increased (Salem and Gulyas 1971). Mackenzie and MacRae (1972) found that DDT and lindane had no effect on the growth and N_2-fixing activity of *Azotobacter vinilandi* even when applied at 50 times the field rate. Soil application of disulfoton, phorate, and carbofuran had an adverse effect on a population of *Azotobacter,* but it was not significant (Nirmal et al. 1977).

Schradan at field rates increased *Azotobacter* numbers whilst lindane or heptachlor at rates higher than normal field rates stimulated aerobic nitrogen-fixing bacteria (Jaiswal 1967). In a clay soil treated with 0.22 and 4.4 kg ha^{-1} of lindane or at 2.5 and 50 kg ha^{-1} of dieldrin increased *Azotobacter* numbers; anaerobic nitrogen fixers were also increased by dieldrin but lindane decreased them (Mahmoud et al. 1970). Parathion methyl (0.15 kg a.i. ha^{-1}) briefly increased N_2-fixing *Azotobacter* in a loam soil (pH 7.2), while at higher rates (15, 150, and 300 kg a.i. ha^{-1}) it increased the population after a transient decrease (Naumann 1970a). At field rates disulfoton also stimulated the N_2-fixing aerobic population in the rhizosphere of cotton plants, but inhibited anaerobic populations (Mahmoud et al. 1972). An inhibition of the growth of *Azotobacter* population under the influence of phosphamidon, malathion, fenthion, methylphosphorodithioate and parathion was also noticed when the insecticides were supplied as sole carbon source, but the inhibition turned into stimulation when the insecticides were added as phosphorus source (Kandasamy et al. 1977).

Depending on the concentration of the insecticide, several cases of inhibition have been reported. In soils inoculated with *Azotobacter macrocystogenes,* phoxim significantly depressed acetylene reduction activity after 5 and 14 days (Eisenhardt 1975), the levels of nitrogen fixation with the insecticide at 10, 100 and 1000 ppm being 82, 55, and 8% of the control respectively. Vlassak et al. (1976) reported a similar inhibition of nitrogenase activity with dinoseb. The insecticide at 3 ppm and 6 ppm produced 60% and 90% of inhibition respectively whereas at 9 ppm a complete inhibition was reported. With a crude extract of *Azotobacter vinilandii,* they demonstrated that the reduction in nitrogen fixation was due to the specific inhibition of the nitrogenase enzyme by dinoseb and not due to a general inhibition of the population of nitrogen fixers. A similar dose-dependent inhibition on the population of asymbiotic microorganisms *(Spirilla* and *Azotobacter),* as well as acetylene reduction activity, has been reported with aldicarb, chlorpyrifos and dinoseb in a laboratory experiment with clay loam soil under maize cultivation with glucose or malate amendent (Hagazi et al. 1979). At field rate applications, aldicarb and chlorpyrifos inhibited more than 65% of nitrogenase activity, whereas dinoseb inhibited 48 to 91% of the activity. They reported that the N_2-fixing bacteria were more susceptible to the insecticides with malate amendment than with glucose. Further soils, irrespective of the amendment, regained their normal nitrogenase activity and populations of N_2-fixers after 30 days of incubation, suggesting that the deleterious effects of added toxic pesticides disappeared, possibly because of the degradation of the toxic substances. Thus the addition of available carbon resources, e.g. plant refuse, can cause an enhancement of microbial activity and detoxification of the pesticide and can help in the restoration of normal nitrogenase activity. Of the several organophosphorus and carbamate insecticides tested only tetrachlorvinphos at 50 and 250 ppm was reported to inhibit nitrogenase activity of *Azotobacter vinelandii* (Rodell et al. 1977). Under normal (5 ppm in soil) and higher (10 ppm) field application rates, aldicarb, disulfoton and fensulfothion inhibited in vitro growth of the *Azotobacter chroococcum* whereas at 2 ppm the insecticides were ineffective (Balasubramaniyan and Narayanan 1980). In addition the insecticides at 5 and 10 ppm inhibited respiration (glucose oxidation), ^{14}C-glucose assimilation and nitrogen fixation.

Tu (1978a and 1979) studied the influence of various insecticides on the populations of N_2-fixers and their activities. The effects of the tested insecticides varied with different insecticides, their concentrations and duration of the trail period. In a short incubation study on the effects of 32 pesticides on nonsymbiotic N_2-fixers of a sandy loam (pH 7.8), Tu (1978a) reported that chlorfenvinfos, chlorpyrifos, carbofuran, metalkamate, and permethrin at 5 and 10 ppm and ethoprop, leptophos, and chlordane at 10 ppm significantly reduced nitrogenase activity. However, parathion or triazophos (5 and 10 ppm), ethion, thionazin or trichloronate (5 ppm) and chlordane or dieldrin (10 ppm) stimulated the N_2-ase activity. It is interesting to mention here that none of the insecticides used inhibited the population of nonsymbiotic nitrogen fixers, indicating that these two processes are independent of each other. This creates a doubt regarding most studies where only populations of nonsymbiotic nitrogen fixers were studied in relation to the effect of insecticides. Thus, to make the studies more reliable, these two parameters i.e., population and N_2-fixation, should be studied together.

The effect of insecticides on soil microorganisms and microbial processes depend upon physical, chemical, and biological conditions in soil. However, in most of the studies these factors have not been considered. As mentioned earlier, the population of asymbiotic N_2-fixers has not been studied together with nitrogen fixation. Further, neither the acetylene reduction technique nor the $^{15}N_2$-tracer technique alone can provide reliable information. Hence it has been suggested that nitrogen fixation should be evaluated simultaneously by both these techniques. Thus Najak and Rao (1980, 1982) studied the effect of different pesticides and their combination on the population of asymbiotic N_2-fixers and on N_2-fixation in submerged paddy soils employing both $^{15}N_2$-tracer technique and acetylene reduction method. The pattern of N_2-fixation was almost identical with both methods. Carbofuran treatment at 5 ppm significantly enhanced N_2-fixation in cellulose-amended alluvial soil of pH 6.2, while stimulation was less in a laterite soil of pH 5.0 (Nayak and Rao 1980). The insecticide completely suppressed N_2-fixation in acid sulfate Karapadam soil of pH 5.0. The variation in the effects of carbofuran in different soil types has been attributed to the pH condition and the selective effect of the insecticide on different asymbiotic N_2 fixers. It has also been reported that the higher the acidity of soil, the greater the persistence of carbofuran (Venkateswarlu et al. 1977). Another insecticide BHC under similar experimental conditions also stimulated N_2-fixation in alluvial soil, acid sulfate Pokkli soil, whereas it inhibited the process in other soil types (Nayak and Rao 1980). Raghu and MacRae (1967 b) also reported a similar stimulation of N_2-fixation in two Phillippines soils with γ-BHC.

Mahapatra and Rao (1981) reported a stimulation of soil nitrogenase activity as well as nitrogen fixers (*Azospirillum* sp. and *Azotobacter* sp. to a greater extent and anaerobic organisms to a lesser extent) in a submerged rhizosphere soil with BHC treatments equivalent to field rates (1–2.5 kg a.i. ha^{-1}) or twice the level. They attributed the stimulation of nitrogen fixation due to more favorable redox level developed in the soil by the insecticide treatment.

Recently, Nayak and Rao (1982) recorded the influence of commonly used pesticides and their combination in a submerged paddy soil (sandy loam; pH 6.2). Diazinon inhibited the population of *Azotobacter* and *Azospirillum* and a combination of carbofuran and benomyl inhibited only *Azotobacter,* while *Azospirillum* and anaerobic N_2-fixing microorganisms were stimulated. They also noted that the application of carbofuran, parathion, and γ-BHC and a fungicide, benomyl, at 5 ppm stimulated the incorporation of $^{15}N_2$, while diazinon was not stimulatory. Carbofuran, in combination with benomyl, exerted the maximum stimulation. Though diazinon did not retard the stimulation of carbofuran, it lowered the stimulatory effect by benomyl. These findings were further confirmed by the acetylene reduction method.

Under submerged conditions in fields, blue-green algae form the most promising system, where in addition to contributing nitrogen, they benefit the crop in many other ways. A few studies have been carried out to find the nitrogen contribution of the algal growth in rice fields (MacRae and Castro 1967; Henriksson 1971; Yoshida and Ancajas 1973; Venkataraman 1981). Although not much work has been done on the effect of insecticides on nitrogen fixation by blue-green algae, the available reports with insecticides do not indicate a permanent deleterious effect of insecticides on blue-green algae, if applied at recommended field rates. Malathion or DDT at

1 ppm in culture medium did not adversely affect the growth of blue-green algae (Gregory et al. 1969; Goulding and Ellis 1981). Ahmed and Venkataraman (1973) found that *Aulosira fertilissima* has been found to tolerate insecticides like lindane, parathion, endrin, diazinon and Sevin at recommended doses (1–2 ppm), although BHC at 1 ppm reduced the growth of algae to the extent of 50%. Raghava Reddy (1976) found no significant effect of insecticides like rogor, dimecron, and endrin on growth and N_2-fixation by blue-green algae like *Tolypothrix tenuis* and *Anabaena* sp.

Sardeshpande and Goyal (1982) studied the effect of carbofuran, endosulfan, phorate and Gamma BHC at 1, 5, 20, and 50 ppm on four algae, *Anabaena iyengarii, Haplosiphon intricatus, Calothrix membranacea* and *Calothrix bhardwajae.* They reported that growth was significantly reduced by BHC followed by endosulfan, phorate, and carbofuran. But nitrogen fixation by all four algal forms was significantly reduced in presence of endosulfan, while carbofuran had the least effect on this metabolic activity. The insecticides at different concentrations were found to be highly toxic to growth as well as to nitrogen fixation by *Haplosiphon intricatus,* the toxic effect being lowest in the case of *Calothrix bhardwajae.* It is interesting to note that carbofuran and phorate at a concentration as low as 1 ppm stimulated the growth and N_2-fixation in *Anabaena iyengarii* and *Calothrix membranacea.* The difference in sensitivity of algae to insecticides has been attributed to the nature of the organisms and their ability to accumulate and metabolize insecticides (Kar and Singh 1979). Further, while BHC reduced growth of algae significantly, another insecticide, endosulfan, reduced nitrogen fixation significantly. This suggests that also in the case of blue-green algae the effect of insecticides on nitrogen fixation is independent of that on growth. The application of BHC (5 kg ha^{-1}) selectively stimulated the growth of indigenous soil algae in rice fields (Raghu and MacRae 1967 b). This stimulation of blue-green algae was attributed to elimination by BHC of small predatory crustaceans and was considered an added benefit to the use of BHC in rice cultivation. A somewhat different picture emerges from the studies of Singh (1973) on the effects of insecticides on pure cultures of blue-green algae, where a commercial formulation of BHC was generally the most inhibitory and endrin was least inhibitory. Das and Singh (1977) also reported severe toxicity of BHC to growth and N_2- fixation by several bloom-forming algae.

The nitrogen-fixing blue-green algae *Cylindrospermum* sp. and *Aulosira fertilissima* grew well in the presence of 300 and 400 ppm of diazinon respectively (Singh 1973). This compound, used for insect control in rice fields, has been shown by Sethunathan and MacRae (1969) to stimulate and increase algal population in standing water of flooded soil.

Chlorpyrifos, when applied at the normal rate (1.2 ppm) to a pond, exerted a persistent reduction in growth of most of the phytoplankton (Brown et al. 1976). At 2.4 ppb, however, the insecticide has been found to stimulate growth of blue-green algae, in artificial ponds (Hulbert et al. 1972). However, DaSilva et al. (1975) reported with malathion (100 ppm) a depression in N_2-fixation by blue-green algae.

Tarar and Salpekar (1980) studied the algicidal potential of insecticides at various concentrations. The toxic order was found to decrease in the order endosulphon < malathion < hexadrin < BHC < Daimor < DDT. Of the algal forms, *Chlorococcum humicola* was found to be more resistant, while *Cylidrospermum muscicola* was found to be highly toxic to these insecticides.

7 Respiration

Although microbial respiration gives an overall idea of the metabolic activity of soil microbes, microbial biomass is not the sole contributor of soil respiration. Soil respiration measured in terms of oxygen consumption or CO_2 evolution, however, represents a good index of the activity of the microflora involved in organic matter decomposition.

Bartha et al. (1967) did not find any significant effect of DDT, DDD, aldrin, dieldrin, endrin, methoxychlor, and telodrin on CO_2 production after 30 days of incubation. Similarly, mexacarbamate application at 0.17 kg ha^{-1} did not alter respiratory activity in a forest soil with or without litter (Bollen et al. 1970). Salonius (1972) also reported that treatment of forest soils with heavy doses (112 kg ha^{-1}) of DDT or fenitrothion did not affect respiration by soil flora. In this study microbial utilization of pesticide as carbon source was not suspected during the 12 months' incubation, as fresh addition of the insecticides failed to increase the respiratory rate. Terbufos at a rate of 0.1 to 10 kg ha^{-1} had no effect on respiration in three soil types (Laveglia and Dahm 1974). Under field conditions DDT, fensulfothion, fenitrothion, and carbofuran at 2.24 kg ha^{-1} had no significant effect on respiratory activity, while in laboratory studies all the insecticides except carbofuran significantly reduced respiratory activity and the toxicity was maximum, with fenitrothion at 100 ppm or more (Tate 1974).

Other reports indicate a stimulatory effect of insecticides on soil respiratory activities. Methylparathion at 10 ppm caused a significant increase in CO_2 production and it is unlikely that the apparent stimulation is due to the utilization of pesticide as a carbon source (Pahr and Smith 1969). Robson and Gunner (1970) noted a stimulation in oxygen uptake with a hydrolysis product of diazinon, diethylphosphorodithioate. Tu (1970, 1972, 1973a, 1980a) reported a stimulation in soil respiration with various insecticide treatments. Chlorpyrifos, diazinon, thionazin, and trichlornate at 10 and 100 ppm enhanced O_2 consumption in proportion to insecticide concentration, suggesting a microbial degradation of the insecticides (Tu 1970). With carbofuran Tu (1972, 1973a, b) reported a similar response from soil with or without glucose amendment. In a loamy soil, phorate as well as terbufos at 5 ppm enhanced the O_2 consumption, whilst the oxidative analogs of the parent insecticides viz., phorate sulfoxide, phorate sulfone, terbufose sulfoxide, and terbufose sulfone showed a more pronounced activity (Tu 1980a). The increase in O_2 consumption might result from the increased oxidation of insecticides by microorganisms. However, Kuseske et al. (1974) reported that the stimulation in O_2 uptake by soil on application of formulated carbofuran at 500 ppm was due to the presence of an organic carrier (corn cob grits) present in the formulation, as technical grade insecticide was without any effect.

Depression in respiratory activity with insecticide treatments has been reported by several workers (Bartha et al. 1967; MacRae et al. 1967; Bhardiya and Guar 1968; Robson and Gunner 1970; Derby and Ruber 1970; Menzel et al. 1970). MacRae et al. (1967) reported that in a clay and loam soil γ-BHC at five times the recommended field rate retarded the evolution of CO_2, which might be due to the inhibitory effect of the insecticides on mineralization of native organic matter. Ap-

plication of carbamate insecticides isolan and carbaryl inhibited CO_2 evolution whilst malathion, parathion, and phorate also depressed CO_2 production but only after an initial stimulation (Bartha et al. 1967). Similarly under laboratory conditions, lindane at 100 and 1000 ppm depressed CO_2 production after 3 days, while dieldrin at 1000 ppm was significantly inhibitory from the first day itself (Bardiya and Gaur 1968). They also observed a significant reduction in respiration with endrin at 25 ppm or higher rates. O_2 uptake by a diazinon-resistant strain was unaffected by 30 ppm of the insecticide, while in some sensitive strains the O_2 uptake was reduced by up to 40% (Robson and Gunner 1970). Menzel et al. (1970) reported a depression in ^{14}C-uptake by DDT treatment at 0.001 ppm to *Cyclotella nana* and at 0.005 ppm for *Skeletonema costatum* 24 h after treatment. Derby and Ruber (1970) observed a depressive effect on O_2 production by algae with insecticides and the order of inhibitory effect was fenthion > propoxur > DDT > Abate. Aldicarb, disulfoton, and fenitrothion at field rate levels (5 ppm) and at double the rates depressed respiratory activity by soil microorganisms as well as ^{14}C-glucose assimilation (Balasubramaniyan and Narayanan 1980). In laboratory-conditioned upland and flood soils, though O_2 uptake was unaffected by cartap HCl at normal application rates (100 ppm), at a higher rate (1000 ppm) there was an inhibitory effect (Endo et al. 1982).

The application of insecticides also shows fluctuations in the soil respiratory activities. The difference in the toxicity of the parental compound and its metabolite can be one of the factors responsible for such fluctuations as a function of time. Also several physical, chemical, and biological factors which can influence the metabolism of the insecticides also alter the respiratory activities. Gupta et al. (1975) observed only a transient depression in CO_2 evolution with propoxur application at 2.5 and 12.5 kg ha^{-1}, whilst a pronounced depression after an initial stimulation was observed with 125 kg ha^{-1} of the insecticide. Bayer et al. (1982) reported a similar depression or stress-dependent increase in CO_2 release. They observed that propoxur, lindane, endrin, parathion, methidation, and omethoate stimulated CO_2 evolution up to 5 weeks after treatment, then a distinct decrease in activity was followed by an increase in activity. In sandy loam, calcareous clay and clay soils lindane at 22 kg a.i. ha^{-1}, thionazin or trichlornate at 1 and 10 kg ha^{-1} and fonofos or pirimiphosethyl at 11 kg a.i. ha^{-1}exerted an initial depression in CO_2 evolution, followed by a stimulation and then a normal level of activity (Gawaad et al. 1973 b).

Bartha et al. (1967) studied the influence of organochlorine, organophosphate and carbamate insecticides on microbial respiration in soil. With the help of model systems having established activities, they suggested that mechanisms responsible for changes in soil microbial respiration due to insecticide treatment might be a combination of the following: an insecticide acting to uncouple oxidative phosphorylation; or an insecticide without anti-microbial action is transformed to a stable toxic product or an insecticide with selective toxicity inhibits CO_2 production of some microorganisms but is oxidized by other microbes that are resistant to its initial action. They concluded that pesticide concentrations greatly in excess of those recommended for agricultural and home use were required to produce an effect on soil microbial respiration and supplementary organic matter reduced pesticide toxicity and increased microbial degradation of the pesticide in soil.

8 Soil Enzymes

Soil enzymes are often related to microbial activity and soil fertility. However, the information on insecticide effects on soil enzymes is limited, making it difficult to establish general trends. Of the many soil enzymes, dehydrogenases play a vital role in the decomposition of organic matter as well as electron transfer reactions, and are extensively studied using TTC (triphenyl tetrazolium chloride) reduction method. Many of the dehydrogenases are anaerobic in nature (Orten and Neuhaus 1970) and their activity is pronounced under flooding. However, the addition of commercial HCH and carbaryl at the time of flooding to an alluvial soil at 1, 10, and 100 μg g^{-1} soil prevented the accumulation of dehydrogenase activity (Chendrayan and Sethunathan 1980). Even though inihibition by HCH was attributed to a high redox potential, it may not be necessarily so, since carbaryl inhibited the activity despite a depression in potential. However, no appreciable inhibition was noted with 100 μg g^{-1} HCH in the soil pre-reduced by flooding for 15 days, indicating that insecticides have no effect on the already accumulated enzymes. The low level of dehydrogenase activity in soil treated with formulations under flooding was probably due to the inhibitory action on the proliferation of microorganisms.

Naumann (1970b) reported a stimulation of dehydrogenase activity with methyl parathion at 15 kg a.i. ha^{-1}, whereas higher doses (150–300 kg a.i. ha^{-1}) exhibited a complete inhibition, more so at 12 °–15 °C than at 20 °C (Naumann 1972). Citrolane at 1.5 kg ha^{-1}, when applied to a rice field soil caused a temporary inhibition of the enzyme activity for 5 days (Purushothaman 1974). The initial lag has been attributed to a decline in the microbial population which recovered after 20 days with acquired resistance. By serial passage procedure, Gupta and Shirkot (1981) demonstrated the development of resistance in *Rhizobium* strains to Sevin up to 50 μg/ml with a concomitant enhancement of dehydrogenase activity. Tu (1980b) did not find any inhibition of dehydrogenase activity with five pyrethroid insecticides in a sandy loam incubated for 2 weeks at 28 °C. In fact, formazan production was significantly greater than the controls after 3 weeks with WL 43467, WL 41706 and FMC 45498 at 0.5 μg g^{-1} and with WL 43775 and WL 41706 at 5 μg g^{-1}. None of these pyrethroids inhibited soil urease activity. Moreover, in most instances they were even stimulative. However, urease activity was found to be inhibited by the organophosphorus insecticides, Accothion, malathion and Thimet at rates of 50, 200, and 1000 ppm (Lethbridge and Burns 1976).

Laboratory experiments with an organic soil incubated at 28 °C for 2 weeks revealed that a few carbamate, chlorinated, and organophosphorus insecticides failed to show any permanent suppression of urease and dehydrogenase activities (Tu 1981c). However, terbufos, triazophos, and trichlornate inhibited dehydrogenase activity at 5 and 10 μg g^{-1}, while all the treatments except triazophos inhibited urease activity at 100 μg g^{-1} for 1 week. However, the activities recovered after 2 weeks with a stimulatory trend especially with the organophosphorus compounds. Phosphatase activity, involved in the decomposition of organic phosphorus, was depressed after 2 h of incubation with many insecticides with the exception of parathion, triazophos, carbofuran, metalkamate, oxyamyl, and permethrin, whilst chlordane at 5 μg g^{-1} soil stimulated the activity. In a later study, most of these insecti-

cides failed to depress the invertase or amylase activities involved in the hydrolysis of sugars and starch respectively (Tu 1982c). Diazinon, ethoprop, fensulfothion, fonofos, leptophos, phorate, terbufos, thionazin, triazophos, trichlornat at 5 or 10 μg g^{-1} increased the invertase activity on the first day. Similarly chlorpyrifos, chlorfenvinphos, diazinon, ethion, ethoprop, fensulfothion, fonofos, malathion, parathion, phorate, triazophos, chlordane, dieldrin, permethrin at 5 or 10 μg g^{-1} increased the amylase activity on the third day.

Abdel Yussif et al. (1976) reported that diazinon and carbathion increased the invertase and catalase activities, but decreased urease and phosphatase activities. Tsirkov (1970) reported an inhibition of urease and catalase activities in meadow soils by heptachlor, whilst lindane and dieldrin increased the enzyme activities. NADH oxidase activity of membrane fractions of many microbes was markedly inhibited (90 to 93%) by aldrin, dieldrin, and chlorane, whereas lindane and DDT showed a less severe effect (Trudgill and Widdus 1970). Succinate dehydrogenase and NADH dehydrogenase assays on membrane preparations from *Bacillus subtilis* and *Escherichia coli* had shown that only the NADH dehydrogenase of *Escherichia coli* was inhibited by chlordane. Endo et al. (1982) demonstrated that cellulose saccharase activities were not influenced by cartap hydrochloride even at 1000 ppm in laboratory-conditioned upland and flooded soils. However, phosphatase activity decreased initially and then recovered with 1000 ppm of the insecticide. Under flooded conditions, the protease activity was also depressed initially, to be stimulated later with 1000 ppm, while in upland conditions the enzyme activity was depressed throughout the experiment with 100 and 1000 ppm of the insecticide. Even though the available information is limited and in many cases contradictory, it can be presumed that the insecticides at lower concentrations are unlikely to have any detrimental effect on soil enzyme activities and fertility.

9 Plant Pathogenic Microflora

Interactions between the pesticides and soil pathogenic microflora have attracted much attention (Sylvestre and Fournier 1979). However, there have been very few reports on the influence of insecticides on plant pathogenic microflora in soil considering that several tons of grain are lost every year due to increased incidence and virility of plant diseases. The interaction between the soil pathogenic microflora and the insecticide may lead either to a stimulation or depression in the populations (Sylvestre and Fournier 1979). The former may result when the chemical itself stimulates the growth of the pathogens, or when the antagonists of the pathogens which keep them in check are destroyed by the treatments. This may also be due to an alteration of host plant sensitivity by the chemicals by making it more susceptible (Sylvestre and Fournier 1979). On the other hand, there are reports to show that sometimes the pathogens are depressed by insecticides which were not intended for such an action. This could possibly be due to the direct action of the chemicals on the pathogens or due to a stimulation of their antagonists.

Leach and Frank (1982) observed in a field study that rhizoctonia disease of potato was significantly higher in plots treated with aldicarb at 33.6 kg ha^{-1}, but car-

bofuran and disulfoton were not effective. Dipterex and Nuvacron at 0.5 g m^{-2} also increased the spread of root rot in a broad bean plant (Mahmoud et al. 1981). Carbofuran, however, encouraged *Trichoderma* on the first day although it was inhibited at later stages. An opposite effect was observed with *Acremonium, Fusarium,* and *Humicola* whose populations increased with increasing concentrations of carbofuran, whilst *Cladosporium* was depressed (Oblisami et al. 1979). Similarly root rot of barley caused by *Helminthosporium sativum* was enhanced by maleic hydrazide and heptachlor (Richardson 1957).

Interestingly, some insecticides reduced the incidence of pathogens in field trials as well as their growth in laboratory cultures, though the results obtained in the latter study may not occur in field conditions. Aldrin effectively controlled several pathogens: seedling disease of wheat caused by *Helminthosporium sativum* (Richardson 1957), tomato wilt caused by *Fusarium oxysporium lycopersicii* (Richardson 1959), take-all disease caused by *Ophiobolus graminis* (Slope and Last 1963; Grossmann and Steckham 1960) and club root of cabbage caused by *Plasmodiophora brassica* (Channon and Keyworth 1960).

Heptachlor also decreased take-all disease (Slope and Last 1963) and *Rhizoctonia* in pure culture (Richardson and Miller 1960), chlordane decreased *Helminthosporium sativum* (Richardson 1957), take-all disease caused by *Ophiobolus graminis* (Grossmann and Steckham 1960) and *Rhizoctonia solani* (Richardson and Miller 1960). *Helminthosporium sativum* was decreased by endrin (Richardson 1957), wilt disease by Nuvacron (Mahmoud et al. 1981), *Trichoderma* by disyston, *Verticellum* by disyston and Dasanit *Fusarium* by Thimet (Sreenivasalu and Rangaswami 1973) and laboratory cultures of *Rhizoctonia solani* by fensulfothion (Rodriguez-Kabana et al. 1976). Ocampo and Hagman (1980) reported that insecticides may also influence the competition between two pathogens. Aldicarb increased the vascular arbuscular mycorrhizal (VAM) infection. This was found to be due to the elimination of nematode competitors for the same root. Tabet and Lichtenstein (1976) found that *Trichoderma viride* metabolized photodieldrin to noninsecticidal compounds. Addition to aldrin ard dieldrin, however, decreased its capacity to degrade photodieldrin.

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CHAPTER 9

Effects of Insecticides on Algae

SUKANYA LAL

1 Introduction

Algae are common inhabitants of surface waters and are encountered in every water system that is exposed to sunlight. They range from unicellular to filamentous types. Most of the algae, including diatoms, green algae, dinoflagellates and chrysophytes, are significant organisms in the aquatic environment, while the blue-green algae are important in soil and associated environments such as irrigation and drainage systems and lakes and ponds. Blue-green algae help to maintain soil fertility, through carbon fixation and synthesis of substances supporting plant growth. Probably the most noted activity is the ability of some species to fix atmospheric nitrogen which is particularly significant in the productivity of rice paddy. Algae are also unique in having photosynthetic ability which is absent in typical bacteria, actinomycetes, fungi, yeast and protozoa.

Insecticide pollution of the aquatic environments arises from (1) measures taken to control the waterborne stages of insect vectors of animal diseases (Simmons 1959); (2) accident spillage (Nicholson et al. 1962; Marth 1965), (3) airborne drift and drainage from insecticide-treated land (Cope 1961; Briedenbach and Lichtenberg 1963; Grzenda et al. 1964; Grzenda and Nicholson 1965; Terriere et al. 1966; Hindin et al. 1966; Lichtenstein et al. 1966; Briedenbach et al. 1967), (4) the sedimentation of wind-blown contaminated dust particles (Abbott et al. 1965) and the precipitation of the insecticide by rainwater (Wheatley and Hardman 1965; Cohn and Pinkerton 1966).

The literature on the effects of insecticides on algae has been discussed by many workers (Ware and Roan 1970; Cox 1972; Butler 1977; Lal and Saxena 1980, 1982; Lal 1982, 1983; Lal and Dhanaraj 1984). In this chapter the effects of insecticides on algae are critically examined and discussed. The literature on the effects of insecticides on algae has been grouped as (1) direct effects on growth (populations, dry weight, wet weight, or on specific metabolic processes such as photosynthesis) and (2) indirect effects which may be due to the interaction of insecticides with organisms other than algae.

2 Direct Effects

In reviewing the literature on this aspect one finds discrepancies in results obtained and conclusions drawn (Table 1). For instance, one researcher may report that a

Table 1. Effects of insecticides on algae

Chemical	Algae	Concentration	Effect	References
Aldrin	*Microcystis aeruginosa*	5 ppm	Cell division inhibited	Vance and Drummond (1969)
	Euglena gracilis	50–100 ppm	Cell division inhibited	Poorman (1973)
	Scenedesmus quadricauda	20 ppm	None	Vance and Drummond (1969)
	Oedogonium sp.	15 ppm	None	Vance and Drummond (1969)
	Anabaena cylindrica	20 ppm	None	Vance and Drummond (1969)
	Chlorella ellipsoidea	100 ppm	None	Clegg and Koevenig (1974)
	Chlamydomonas sp.	100 ppm	None	Clegg and Koevenig (1974)
	Euglena gracilis	100 ppm	None	Clegg and Koevenig (1974)
	Euglena gracilis	50–100 ppm	Cell division inhibited	Poorman (1973)
BHC	*Ankistrodesmus braunii*	0.1–100 ppm	Cell division inhibited	Kopecek et al. (1976)
	Anacystis nidulans	0.1–100 ppm	Cell division inhibited	Kopecek et al. (1976)
	Anabaenopsis raciborskii	100, 1000 ppm	Cell division inhibited	Das and Singh (1978)
	Anabaena aphanizomenoides	100, 1000 ppm	Cell division inhibited	Das and Singh (1978)
	Anabaena spiroides	100, 1000 ppm	Cell division inhibited	Das and Singh (1978)
	Microcystis flosaquae	100, 1000 ppm	Cell division inhibited	Das and Singh (1978)
	Cylindrospermum sp.	10, 80, 300, 600 ppm	None	Singh (1973)
	Aulosira fertilissima	50 ppm	None	Singh (1973)
	Plectonema boryanum	200, 400, 600 ppm	None	Singh (1973)
Carbofuran	*Nostoc muscorum*	10 ppm	None	Kar and Singh (1978)
	Nostoc muscorum	25 ppm	Cell division and nitrogen fixation stimulated	Kar and Singh (1978)
	Nostoc muscorum	150, 1000 ppm	Cell division and nitrogen fixation inhibited	Kar and Singh (1978)
	Anabaena iyengarii	5, 20, 50 ppm	Cell division inhibited	Sardeshpande and Goyal (1982)
	Nostoc muscorum	7.5, 15, 30 ppm	Cell division inhibited	Kar and Singh (1979)
	Wollea bharadwajae	7.5, 15, 30 ppm	Cell division inhibited	Kar and Singh (1979)
	Hapalosiphon intricatus	1, 5, 20, 50 ppm	Cell division inhibited	Sardeshpande and Goyal (1982)
	Calothrix membranacea	5, 20, 50 ppm	Cell division inhibited	Sardeshpande and Goyal (1982)
	Calothrix bharadwajae	1, 5, 20, 50 ppm	Cell division inhibited	Sardeshpande and Goyal (1982)
Carbaryl	*Chlorococcum* sp.	0.5, 1 ppm	Cell division inhibited	Walsh and Alexander (1980)
	Nitzschia angularium	0.5–1 ppm	Cell division inhibited	Walsh and Alexander (1980)

	Skeletonema costatum	0.5–1 ppm	Cell division inhibited	Walsh and Alexander (1980)
	Chlorella sp.	0.5–1 ppm	Cell division inhibited	Walsh and Alexander (1980)
	Nitzschia clostridium	10 ppm	Cell division inhibited	Maly (1980)
	Amphora coeffeaeformei	10 ppm	Cell division inhibited	Maly (1980)
	Gonyaulax sp.	10 ppm	None	Maly (1980)
	Amphiphora sp.	10 ppm	Cell division inhibited	Maly and Ruber (1983)
	Chlorococcum sp.	10 ppm	Cell division inhibited	Maly and Ruber (1983)
	Euglena gracilis	10–100 ppb	Photosynthesis inhibited	Gillot et al. (1975)
	Chlorella pyrenoidosa	1 ppm	Cell division inhibited	Cole and Plapp (1974)
	Protococcus sp.	10 ppm	Cell division inhibited	Ware and Roan (1970)
	Chlorella sp.	10 ppm	Cell division inhibited	Ware and Roan (1970)
	Dunaliella euchlora	10 ppm	Cell division inhibited	Ware and Roan (1970)
	Phacodactylum tricornutum	0.1 ppm	Cell division inhibited	Ware and Roan (1970)
	Monochrysis lutheri	1 ppm	Cell division inhibited	Ware and Roan (1970)
	Anabaena sp.	10, 100 ppm	Cell division inhibited	Subramanian (1982)
Chlorfenvinfos	*Scenedesmus obtusiuculus*	5 ppm	None	Ordog (1979)
	Scenedesmus obtusiuculus	16 ppm	Cell division inhibited	Ordog (1979)
Chlorpyrifos	*Ankistrodesmus falcatus*	1.2 ppb	Cell division inhibited	Brown et al. (1976)
	Ankistrodesmus spiralis	1.2 ppb	Cell division inhibited	Brown et al. (1976)
	Dinobryan sp.	1.2 ppb	Cell division inhibited	Brown et al. (1976)
	Glenodinium sp.	1.2 ppb	Cell division inhibited	Brown et al. (1976)
	Gonatozygon sp.	1.2 ppb	Cell division inhibited	Brown et al. (1976)
	Scenedesmus dimorphus	1.2 ppb	Cell division inhibited	Brown et al. (1976)
	Tetraedron sp.	1.2 ppb	Cell division inhibited	Brown et al. (1976)
	Trachelomonas sp.	1.2 ppb	Cell division inhibited	Brown et al. (1976)
	Ceratium sp.	1.2 ppb	None	Brown et al. (1976)
	Nitzschia closterium	10 ppm	Cell division inhibited	Maly (1980)
	Amphora coeffeaeformis	10 ppm	Cell division inhibited	Maly (1980)
	Chlorococcum sp.	10 ppm	Cell division inhibited	Maly (1980)
	Amphora sp.	10 ppm	Cell division inhibited	Maly (1980)
	Gonyaulax sp.	10 ppm	Cell division inhibited	Maly (1980)
Chlordane	*Scenedesmus quadricauda*	0.1–100 ppb	Cell division stimulated	Glooschenko and Lott (1977)
	Chlamydomonas sp.	0.1–50 ppb	Cell division stimulated	Glooschenko and Lott (1977)
	Chlamydomonas sp.	100 ppb	Cell division inhibited	Glooschenko and Lott (1977)
	Chlorella ellipsoidea	100 ppm	None	Clegg and Koevenig (1974)
	Chlamydomonas sp.	100 ppm	None	Clegg and Koevenig (1974)

Table 1 (continued)

Chemical	Algae	Concentration	Effect	References
Chlordane	*Euglena gracilis*	100 ppm	None	Clegg and Koevenig (1974)
	Exuviella baltica	50 ppb	Cell division and chlorophyll contents inhibited	Magnani et al. (1978)
	Mixed species of estuarine phytoplankton	10 ppb	Cell division inhibited	Biggs et al. (1978)
DDE	*Exuviella baltica*	25 ppb	Cell division inhibited	Powers et al. (1979)
	Exuviella baltica	0.1–10 ppb	None	Powers et al. (1979)
DDT	*Euglena gracilis*	100 ppb	None	Mosser et al. (1972a)
	Porphyridium sp.	80 ppb	None	Bowes (1971)
	Thalassiosira pseudonana	50 ppb	None	Fisher (1975)
	Euglena gracilis	100 ppm	None	Clegg and Koevenig (1974)
	Microcystis aeruginosa	10 ppm	Cell division inhibited	Von Witsch et al. (1975)
	Coelastrum proboscideum	10 ppm	None	Von Witsch et al. (1975)
	Chlorella sp.	1 ppm	Cell division inhibited	Ellis and Goulding (1973)
	Chlorella sp.	5 ppm	None	Wilson and Choudhri (1946)
	Chlorella pyrenoidosa	0.3 ppb	Clumping of cells	Smith and Wenzel (1947)
	Chlorella pyrenoidosa	10–100 ppm	None	Christie (1969)
	Chlorella ellipsoidea	100 ppm	None	Clegg and Koevenig (1974)
	Chlamydomonas sp.	100 ppm	None	Clegg and Koevenig (1974)
	Anacystis nidulans	1 ppm	None	Gregory et al. (1969)
	Scenedesmus obliquus	1 ppm	None	Gregory et al. (1969)
	Scenedesmus quadricauda	0.1–1000 ppb	None	Luard (1973)
	Scenedesmus quadricauda	0.1 ppm	Cell division inhibited	Bradbury (1963)
	Chlamydomonas reinhardii	20 ppm	None	Morgan (1972)
	Dunaliella sp.	1 ppm	None	Menzel et al. (1970)
	Cyclotella sp.	1 ppm	Cell division inhibited	Mosser et al. (1972a)
	Chlamydomonas nivalis	1–25 ppm	Cell division inhibited	Czeczuga and Gierasimow (1977)
	Ankistrodesmus falcatus	1–25 ppm	Cell division inhibited	Czeczuga and Gierasimow (1977)
	Synechococcus elongatus	20 ppm	None	Morgan (1972)
	Euglena gracilis	1 ppm	None	Gregory et al. (1969)
	Euglena gracilis	100 ppm	None	Poorman (1973)
	Skeletonema costatum	1–10 ppb	Cell division inhibited	Menzel et al. (1970)

	Cyclotella nana	80 ppb	None	Bowes (1971)
	Monochrysis lutheri	1000 ppb	None	Ukeles (1962)
	Thalassiosira pseudonana	100 ppb	Cell division inhibited	Mosser et al. (1972 a)
	Phaeodactylum tricornutum	1000 ppb	None	Ukeles (1962)
	Thalassiosira fluviatilis	80 ppb	Cell division inhibited	Bowes (1971)
	Dunaliella euchlora	1000 ppb	None	Ukeles (1962)
	Dunaliella tertiolecta	1000 ppb	None	Bowes (1971)
	Amphidinium carteri	80 ppb	None	Bowes (1971)
	Chlorella sp.	1000 ppb	None	Ukeles (1962)
	Chlorella pyrenoidosa	100,000 ppb	None	Christie (1969)
	Protococcus sp.	1000 ppb	None	Ukeles (1962)
	Scenedesmus quadricauda	20,000 ppb	None	Vance and Drummond (1969)
	Oedogonium sp.	20,000 ppb	None	Vance and Drummond (1969)
	Chlamydomonas reinhardii	100 ppb	None	Mosser et al. (1972 a)
	Microcystis aeruginosa	20,000 ppb	None	Vance and Drummond (1969)
	Anabaena cylindrica	20,000 ppb	None	Vance and Drummond (1969)
	Anabaena variabilis	100 ppb	Cell division stimulated, cell size reduced	Goulding and Ellis (1981)
	Anabaena sp.	10–100 ppm	Cell division stimulated	Lal et al. (1982)
	Skeletonema costatum	1 ppb	None	Subramanian et al. (1979)
	Skeletonema costatum	4, 15 ppb	Cell division inhibited	Subramanian et al. (1979)
	Synedra sp.	Repeated aerial application	Cell division stimulated	Shane (1948)
	Euglena gracilis	10–100 ppb	Photosynthesis inhibited	Gillott et al. (1975)
	Chlorella pyrenoidosa	1–1000 ppm	Cell division inhibited	Cole and Plapp (1974)
	Anacystis nidulans	800 ppb	Cell division inhibited	Batterton et al. (1972)
DDVP	*Scenedesmus obtusiuculus*	5 ppm	None	Ordog (1979)
	Scenedesmus obtusiuculus	100 ppm	None	Ordog (1979)
Diazinon	*Chlorella ellipsoidea*	100 ppm	None	Clegg and Koevenig (1974)
	Chlamydomonas sp.	100 ppm	None	Clegg and Koevenig (1974)
	Euglena gracilis	100 ppm	None	Clegg and Koevenig (1974)
	Cylindrospermum sp.	10, 80, 300, 600 ppm	None	Singh (1973)
	Aulosira fertilissima	10, 200, 400, 600 ppm	None	Singh (1973)
	Plectonema boryanum	200, 400, 600 ppm	Cell division inhibited	Singh (1973)

Table 1 (continued)

Chemical	Algae	Concentration	Effect	References
Dieldrin	*Dunaliella tertiolecta*	1000 ppb	None	Menzel et al. (1970)
	Scenedesmus quadricauda	20,000 ppb	None	Vance and Drummond (1969)
	Oedogonium sp.	20,000 ppb	None	Vance and Drummond (1969)
	Microcystis aeruginosa	5000 ppb	Cell division inhibited	Vance and Drummond (1969)
	Anabaena cylindrica	20,000 ppb	None	Vance and Drummond (1969)
	Cyclotella nana	100 ppb	Cell division inhibited	Menzel et al. (1970)
	Chlorella ellipsoidea	100 ppm	None	Clegg and Koevenig (1974)
	Chlamydomonas sp.	100 ppm	None	Clegg and Koevenig (1974)
	Exuviella baltica	10 ppb	Cell division inhibited	Powers et al. (1977)
	Euglena gracilis	100 ppm	None	Clegg and Koevenig (1974)
	Ankistrodesmus braunii	0.1–100 ppm	Chlorophyll content, dry weight photosynthesis inhibited	Kopecek et al. (1976)
	Anacystis nidulans	0.1–100 ppm	Chlorophyll content, dry weight photosynthesis inhibited	Kopecek et al. (1976)
	Chlorella pyrenoidosa	1 ppm	Cell division inhibited	Cole and Plapp (1974)
Endosulfan	*Anabaena iyengarii*	1, 5, 20, 50 ppm	Cell division inhibited	Sardeshpande and Goyal (1982)
	Hapalosiphon intricatus	1, 5, 20, 50 ppm	Cell division inhibited	Sardeshpande and Goyal (1982)
	Calothrix membranacea	1, 5, 20, 50 ppm	Cell division inhibited	Sardeshpande and Goyal (1982)
	Calothrix bhardwajae	1, 5, 20, 50 ppm	Cell division inhibited	Sardeshpande and Goyal (1982)
Endrin	*Dunaliella tertiolecta*	1000 ppb	None	Menzel et al. (1970)
	Scenedesmus quadricauda	20,000 ppb	None	Vance and Drummond (1969)
	Oedogonium sp.	20,000 ppb	None	Vance and Drummond (1969)
	Microcystis aeruginosa	5000 ppb	Cell division inhibited	Vance and Drummond (1969)
	Anabaena cylindrica	20,000 ppb	None	Vance and Drummond (1969)
	Cyclotella nana	100 ppm	Cell division inhibited	Menzel et al. (1970)
	Cylindrospermum sp.	10, 80, 300, 600	None	Singh (1973)
	Aulosira fertilissima	10, 80, 300, 600	None	Singh (1973)
	Plectonema boryanum	200, 400, 600	Cell division inhibited	Singh (1973)
Fenthion	*Skeletonema*	100 ppb	Cell division inhibited	Derby and Ruber (1970)
	Cyclotella	100 ppb	Cell division inhibited	Derby and Ruber (1970)
	Dunaliella	100 ppb	Cell division inhibited	Derby and Ruber (1970)
	Phaeodactylum	100 ppb	Cell division inhibited	Derby and Ruber (1970)

γ-HCH (Lindane)	*Dunaliella tertiolecta*	9000 ppb	None	Ukeles (1962)
	Phaeodactylum tricornutum	5, 7.5 ppm	None	Ukeles (1962)
	Chlorella sp.	9 ppm	None	Ukeles (1962)
	Anabaena sp.	1,5 ppm	Cell division inhibited	Lal et al. (1982)
	Anabaena sp.	1, 10, 100 ppm	Cell division stimulated	Subramanian (1982)
	Anabaenopsis recibroskii	10–80 ppm	Cell division inhibited	Das and Singh (1978)
	Anabaenopsis aphanizomenoides	10–80 ppm	Cell division inhibited	Das and Singh (1978)
	Anacystis nidulans	10 ppm	Chlorophyll contents, dry weight, cell division inhibited	Kopecek et al. (1976)
	Ankistrodesmus braunii	0.1–100 ppm	Chlorophyll contents, cell division, dry weight inhibited	Kopecek et al. (1976)
	Chlorella sp.	10–100 ppb	Cell division inhibited	Hansen (1979)
	Chlorella pyrenoidosa	200 ppb	Cell division inhibited	Hansen (1979)
	Pavlova lutheri	7.5 ppm	Cell division inhibited	Escoubet (1978)
	Dunaliella primolecta	7.5 ppm	Cell division inhibited	Escoubet (1978)
	Chlorella sp.	511 ppb	Lethal	Hansen (1979)
	Amphidinium carteri	2 ppm	Lethal	Jeane-Levain (1979)
	Anabaena lyengarri	1, 5, 20, 50 ppm	Cell division inhibited	Sardeshpande and Goyal (1982)
	Hapalosiphon intricatus	15, 20, 50 ppm	Cell division inhibited	Sardeshpande and Goyal (1982)
	Calothrix membranacea	1, 5, 20, 50 ppm	Cell division inhibited	Sardeshpande and Goyal (1982)
	Calothrix bharadwajae	1, 5, 20, 50 ppm	Cell division inhibited	Sardeshpande and Goyal (1982)
	Euglena gracilis	50–60 ppm	Cell division inhibited	Jeanne-Levain (1974)
	Dunaliella bioculata	5 ppm	Cell division inhibited	Jeanne-Levain (1974)
	Cylindrospermum sp.	10, 80, 300, 600 ppm	None	Singh (1973)
	Aulosira fertilissima	200, 400, 600 ppm	None	Singh (1973)
	Chlorella sp.	1 ppm	None	Loosanoff et al. (1957)
	Chlamydomonas	1 ppm	None	Loosanoff et al. (1957)
	Proloccus sp.	60 ppm	Cell division inhibited	Ware and Roan (1970)
	Chlorella sp.	60 ppm	Cell division inhibited	Ware and Roan (1970)
	Dunaliella euchlora	60 ppm	Cell division inhibited	Ware and Roan (1970)
	Phaeodactylum tricornutum	60 ppm	Cell division inhibited	Ware and Roan (1970)
	Monochrysis lutheri	60 ppm	Cell division inhibited	Ware and Roan (1970)
	Nostoc muscorum	2, 3, 4 ppm	Cell division inhibited	Kar and Singh (1979)
	Wollea bhardwajae	2, 3, 4 ppm	Cell division inhibited	Kar and Singh (1979)
Heptachlor	*Exuviella baltica*	50 ppb	Cell division inhibited	Magnani et al. (1978)

Table 1 (continued)

Chemical	Algae	Concentration	Effect	References
Malathion	*Anabaena cylindrica*	100 ppm	Initial period of depression succeeded by an increased activity in nitrogen fixation	DaSilva et al. (1975)
	Aulosira sp.	100 ppm	Initial period of depression succeeded by an increased activity in nitrogen fixation	DaSilva et al. (1975)
	Calothrix elenkenii	100 ppm	Initial period of depression succeeded by an increased activity in nitrogen fixation	DaSilva et al. (1975)
	Chlorogloea fritschii	100 ppm	Initial period of depression succeeded by an increased activity in nitrogen fixation	DaSilva et al. (1975)
	Cylindrospermum muscicola	100 ppm	Initial period of depression succeeded by an increased activity in nitrogen fixation	DaSilva et al. (1975)
	Nostoc sp.	100 ppm	Initial period of depression succeeded by an increased activity in nitrogen fixation	DaSilva et al. (1975)
	Chlorella vulgaris	1 ppm	Cell division inhibited	Torres and O'Flaherty (1976)
	Chlorococcum hypnosporum	1 ppm	Cell division inhibited	Torres and O'Flaherty (1976)
	Tribonema sp.	1 ppm	Cell division inhibited	Torres and O'Flaherty (1976)
	Vaucheria geminata	1 ppm	Cell division inhibited	Torres and O'Flaherty (1976)
	Oscillatoria lutea	1 ppm	Cell division inhibited	Torres and O'Flaherty (1976)
	Amphiprora	10 ppm	Cell division inhibited	Maly and Ruber (1983)
	Amphora	10 ppm	None	Maly and Ruber (1983)
	Nitzschia	10 ppm	None	Maly and Ruber (1983)
	Chlorococcum	10 ppm	None	Maly and Ruber (1983)
	Gonyaulax	10 ppm	None	Maly and Ruber (1983)
Methoxychlor	*Chlorella pyrenoidosa*	100 ppb	Cell division inhibited	Kricher et al. (1975)
Methylparathion	*Chlorella protothecoides*	10–30 ppm	Cell division, cell volume, chlorophyll and carotenoid contents reduced	Saroja and Bose (1982)

Mevinfos	*Scenedesmus obtusiuculus*	100 ppm	Cell division inhibited	Ordog (1979)
	Scenedesmus obtusiuculus	5 ppm	None	Ordog (1979)
Mirex	*Chlorococcum* sp.	0.2 ppm	None	Hollister et al. (1975)
	Dunaliella tertiolecta	0.2 ppm	None	Hollister et al. (1975)
	Chlamydomonas sp.	0.2 ppm	None	Hollister et al. (1975)
	Nitzschia sp.	0.2 ppm	None	Hollister et al. (1975)
	Thallasiosira pseudonana	0.2 ppm	None	Hollister et al. (1975)
	Porphyridium eruentum	0.2 ppm	None	Hollister et al. (1975)
	Chlorella pyrenoidosa	100 ppb	Cell division inhibited	Kricher et al. (1975)
Parathion	*Euglena gracilis*	50–100 ppm	Cell division inhibited	Poorman (1973)
	Chlorella pyrenoidosa	1 ppm	Cell division inhibited	Cole and Plapp (1974)
	Euglena gracilis	1 ppm	None	Gregory et al. (1969)
	Anacystis nidulans	1 ppm	None	Gregory et al. (1969)
	Scenedesmus obliquus	1 ppm	None	Gregory et al. (1969)
Permethrin	*Anabaena inaequalis*	1.6–5 ppm	Cell division inhibited	Stratton and Corke (1982)
	Anabaena cylindrica	0–10 ppm	None	Stratton and Corke (1982)
	Anabaena variabilis	0–10 ppm	None	Stratton and Corke (1982)
	Chlorella pyrenoidosa	0–10 ppm	None	Stratton and Corke (1982)
	Scenedesmus quadricauda	0–10 ppm	None	Stratton and Corke (1982)
Phorate	*Anabaena iyengarii*	1, 5, 20, 50 ppm	Cell division inhibited	Sardeshpande and Goyal (1982)
	Hapolosiphon intricatus	1, 5, 20, 50 ppm	Cell division inhibited	Sardeshpande and Goyal (1982)
	Calothrix membranacea	1, 5, 20, 50 ppm	Cell division inhibited	Sardeshpande and Goyal (1982)
	Calothrix bhardwajae	1, 5, 20, 50 ppm	Cell division inhibited	Sardeshpande and Goyal (1982)
Tepp	*Dunaliella* sp.	100 ppm	Cell division inhibited	Ukeles (1962)
	Phacodactylum sp.	100 ppm	Cell division inhibited	Ukeles (1962)
	Monochrysis sp.	100 ppm	Cell division inhibited	Ukeles (1962)
	Protococcus sp.	100 ppm	Cell division inhibited	Ukeles (1962)
	Chlorella sp.	100 ppm	Cell division inhibited	Ukeles (1962)
	Proloccus sp.	500 ppm	Cell division inhibited	Ware and Roan (1970)
	Chlorella sp.	500 ppm	Cell division inhibited	Ware and Roan (1970)
	Dunaliella euchlora	500 ppm	Cell division inhibited	Ware and Roan (1970)
	Phacodactylum tricornutum	500 ppm	Cell division inhibited	Ware and Roan (1970)
	Monochrysis lutheri	500 ppm	Cell division inhibited	Ware and Roan (1970)
Toxaphene	*Microcystis aeruginosa*	2 ppm	Cell division inhibited	Palmer and Maloney (1955)
	Cylindrospermum sp.	2 ppm	Cell division inhibited	Palmer and Maloney (1955)
	Scenedesmus obliquus	2 ppm	None	Palmer and Maloney (1955)

Table 1 (continued)

Chemical	Algae	Concentration	Effect	References
Toxaphene	*Chlorella variegata*	2 ppm	None	Palmer and Maloney (1955)
	Nitzschia palea	2 ppm	None	Palmer and Maloney (1955)
	Monochrysis lutheri	1 ppb	Cell division inhibited	Ukeles (1962)
	Phaeodactylum tricornutum	40 ppb	None	Ukeles (1962)
	Dunaliella euchlora	150 ppb	Cell division inhibited	Ukeles (1962)
	Chlorella sp.	70 ppb	Cell division inhibited	Ukeles (1962)
	Protococcus sp.	150 ppb	Cell division inhibited	Ukeles (1962)
	Protococcus sp.	0.15 ppm	Cell division inhibited	Ware and Roan (1970)
	Chlorella sp.	0.07 ppm	Cell division inhibited	Ware and Roan (1970)
	Dunaliella enchlora	0.15 ppm	Cell division inhibited	Ware and Roan (1970)
	Phacodactylum tricornutum	0.04 ppm	Cell division inhibited	Ware and Roan (1970)
	Monochrysis lutheri	0.1 ppm	Cell division inhibited	Ware and Roan (1970)
Trichlorfon	*Dunaliella* sp.	100 ppm	Cell division inhibited	Ukeles (1962)
	Phacodactylum sp.	1000 ppm	Cell division inhibited	Ukeles (1962)
	Manochrysis	100 ppm	Cell division inhibited	Ukeles (1962)
	Protococcus	100 ppm	Cell division inhibited	Ukeles (1962)
	Chlorella	100 ppm	Cell division inhibited	Ukeles (1962)
	Scenedesmus obtusiuculus	5–15 ppm	None	Ordog (1979)
	Scenedesmus obtusiuculus	50 ppm	Cell division inhibited	Ordog (1979)
	Proloccus sp.	1000 ppm	Cell division inhibited	Ware and Roan (1970)
	Chlorella	500 ppm	Cell division inhibited	Ware and Roan (1970)
	Dunaliella euchlora	500 ppm	Cell division inhibited	Ware and Roan (1970)
Trichlorfon	*Phacodactylum tricornutum*	500 ppm	Cell division inhibited	Ware and Roan (1970)
	Monochrysis lutheri	100 ppm	Cell division inhibited	Ware and Roan (1970)

particular insecticide inhibits the growth, while another may conclude that the compound at similar concentrations has no effect. A valid comparison of these data is often impossible because of insufficient information of conditions under which experiments are carried out. It can be noted from Table 1 that in many of the studies levels of insecticides far exceeding their water solubility have been used, thus making it further difficult to draw any conclusion of ecological significance.

2.1 1,1,1-Trichloro-2,2-bis(p-Chlorophenyl)Ethane (DDT)

A critical examination on the effects of DDT using laboratory culture of algae shows many apparently conflicting results. For instance, a concentration of 100 ppm DDT has been reported to have no effect on the growth of *Chlorella pyrenoidosa* over periods up to 7 days (Christie 1969; Hannan and Patouillet 1972). Likewise using *Chlorella variegata,* Palmer and Maloney (1955) reported no effect of 2 ppm DDT up to 21 days exposure and 0.6 ppm had no effect on *Chlorella* after 10 days (Ukeles 1962). On the contrary Södergren (1968) reported that the growth of a *Chlorella* was inhibited by over 90% after 7 days at the remarkably low concentration of 0.004 ppm. Several other algae, however, have been consistently shown to be resistant to DDT. These include *Chlamydomonas* sp. at concentrations from 0.1 to 20 ppm (Egloff and Partridge 1972; Morgan 1972; Mosser et al. 1972a), and the green flagellate *Dunaliella tertiolecta* at concentrations up to 1 ppm (Ukeles 1962; Menzel et al. 1970; Bowes 1972; Mosser et al. 1972b) and *Coccolithus huxleyi* also at concentrations up to 1 ppm (Menzel et al. 1970; Bowes 1971, 1972; Fisher 1975).

Results with *Euglena gracilis* are also less consistent. Whereas DeKoning and Mortimer (1971) and Mosser et al. (1972a) have reported no growth effects by 0.01 ppb, 1 ppb, and 1 ppm DDT after 4 days, Poorman (1973) has found growth to be stimulated by two- to threefold after 7 days exposure to 100 ppm. With *Skeletonema costatum* and *Thalassiosira pseudonana* also the results of experiments on growth seems to be especially variable. Total inhibition of growth of *Skeletonema costatum* for periods up to 9 days at 0.08 ppb DDT has been recorded (Bowes 1972), while in other experiments, 0.1 ppm was found to cause only a slight initial growth inhibition which had completely disappeared after 4 days (Mosser et al. 1972a). Using the same species, the results of Menzel et al. (1970), Fisher (1975) and Subramanian et al. (1979) are more in accordance with those of Bowes (1972), since in each case inhibition of growth in excess of 80% was found at concentrations of less than 0.1 ppm DDT. Bowes (1972) also found that growth of *Thalassiosira pseudonana* was unaffected over a 14-day period by 0.08 ppm DDT. Conversely, Mosser et al. (1972b) recorded 30% inhibition of growth after 2 days at 0.05 ppm and Menzel et al. (1970) found that 0.1 ppm caused over 70% inhibition of growth after 7 days. The growth of the blue-green alga *Anabaena* sp., measured as optical density, was not affected at 1 ppm DDT (Goulding and Ellis 1981; Lal et al. 1982) and 10, 50, and 100 ppm DDT stimulated the growth (Lal et al. 1982). The stimulation was maximum, with 100 ppm DDT at the end of 35 days.

The effect of DDT on photosynthesis, measured as optical density after relatively short periods of exposure to insecticide, has been examined by many investigators. Yet again a critical examination of the literature reveals several inconsistent results.

According to Derby and Ruber (1970), 0.01 ppm DDT inhibited photosynthesis in *Dunaliella tertiolecta* by 30% while Menzel et al. (1970) and Luard (1973) reported that DDT even at 1 ppm had no effect. It is also noticeable that the results of short-term photosynthesis studies do not always reflect the results of longer-term growth studies. For instance, whereas there are no reports which indicate the inhibition of growth of *Coccolithus huxleyi* by DDT, Wurster (1968) has recorded 60% inhibition of photosynthesis by 0.1 ppm and Menzel et al. (1970) found a 20% inhibition. On the other hand, Fisher (1975) found photosynthesis to be unaffected at 0.05 ppm. As mentioned earlier, *Skeletonema costatum* has been reported to be very sensitive to DDT and even at 0.1 ppm photosynthesis was reduced by over 75% (Wurster 1968; Fisher 1975). Derby and Ruber (1970), however, have shown photosynthesis to be inhibited only by 30% at the same concentration of DDT.

Some of the apparent inconsistencies in the reported sensitivity of algal growth and photosynthesis to DDT may be due to species or strain differences. However, in view of some alarming predictions which have been made based on studies so far carried out, it is essential that a thorough study of the effects of DDT on algae is made. Many of the reports referred to do not mention the initial cell density of algae used in the work and in view of the well-known difficulties associated with accurate assessment of algae, it is surprising that so few studies have assessed growth in more than one way. All too frequently only one sampling time has been used rather than monitoring growth effects regularly over a long period. Many factors, such as the effect of pH, aeration, temperature and light intensity on the influence of DDT on algae have also been largely ignored. Any of these factors could be important and variations in such experimental parameters may account for apparently inconsistent results. Realizing such inconsistencies in the literature concerning the effect of DDT

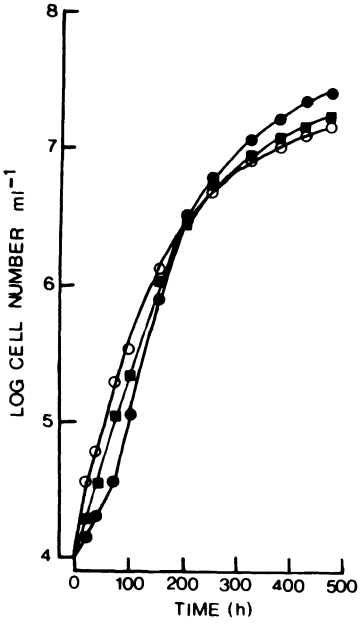

Fig. 1. The effect of DDT on the cell numbers of *Chlorella fusca*. Sufficient cells from a logarithmic phase culture of *Chlorella fusca* were added to 50 ml of culture medium in cotton wool-stoppered flasks to give a final concentration of 1.0 (●) or 0.1 (■) μg ml^{-1} DDT. Controls (○) received an equivalent amount of acetone. The cultures were continuously shaken (150 rpm) and illuminated (1500 lx) at 25 °C. (Goulding and Ellis 1981)

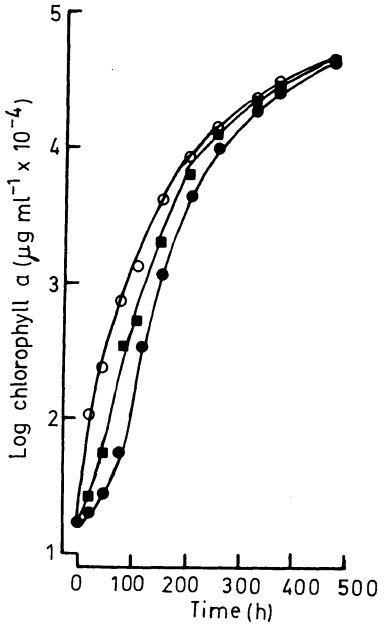

Fig. 2. The effect of DDT on the chlorophyll a contents of *Chlorella fusca*. Sufficient cells from a logarithmic phase culture of *Chlorella fusca* were added to 50 ml of culture medium in cotton wool stoppered flasks to give a final concentration of 1.0 (●) or 0.1 (■) µg ml^{-1} DDT. Controls (○) received an equivalent amount of acetone. The cultures were continuously shaken (150 rpm) and illuminated (1500 lx) at 25 °C. (Goulding and Ellis 1981)

on the growth of algae in laboratory cultures, Goulding and Ellis (1981) carried out a detailed study on the effects of DDT on the growth of laboratory cultures of two freshwater algae, *Chlorella fusca* and *Anabaena variabilis*. They used different parameters such as cell number, chlorophyll contents, cell volume and cell morphology. Concentrations of DDT up to 1 ppm had no effect on the growth of *Anabaena variabilis*. In contrast, 0.1 and 1 ppm DDT showed an initial inhibition of growth of *Chlorella fusca* (Fig. 1). The mean generation time as determined from the increase in cell numbers after 300 h was increased by two and three times in 0.1 and 1 ppm treated cultures respectively. However, the cell number was almost same both in the treated as well as control cultures after 200 h. The observations on the effect of DDT on chlorophyll contents followed a different pattern. There was an initial increase in the inhibition of chlorophyll contents and the chlorophyll contents only approached that of control after 336 h and 470 h with 0.1 and 1 ppm respectively (Fig. 2). At no time did the growth of the treated cultures based on chlorophyll data exceed that of control. However, the dry weight data showed neither inhibition nor stimulation of growth at the end of the experiment, in contrast to the cell number results, which showed approximately a 50% increase of growth at 1 ppm. Goulding and Ellis also observed that the cells in the treated cultures were smaller and more ovoid than those in the control cultures (Fig. 3). Further, the total culture biovolume (Fig. 4) followed the pattern of the chlorophyll data (Fig. 2) rather than cell number data (Fig. 1). The mean cell volume throughout the experimental period also showed that DDT indeed has a marked effect on cell size (Fig. 5). The results of this experiment clearly indicate that the growth should be monitored after regular intervals throughout the experimental periods, at least where batch culture studies are employed. It is also equally clear that more than one growth parameter should be used in order to draw any significant conclusion. The fact, reported by Goulding and Ellis, that the

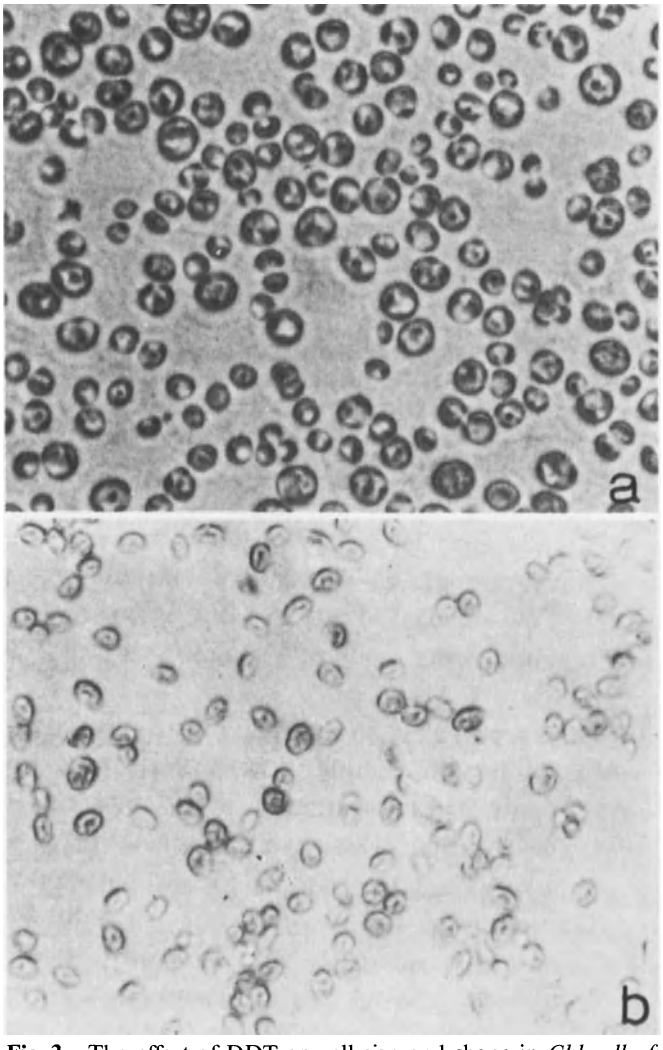

Fig. 3. The effect of DDT on cell size and shape in *Chlorella fusca* after 168 h. **a** Controls. **b** 1.0 μg ml^{-1} DDT treated cells. (Goulding and Ellis 1981)

Chlorella fusca cells treated with DDT were smaller than control cells might have gone unnoticed if only the effects on cell numbers had been considered.

Despite the fact that there are numerous reports on the effects of DDT on algae, very little is known about the possible adverse effects of DDT and its metabolites. The metabolites of DDT are generally considered as detoxified compounds, which actually they are not. For instance in *Anacystis nidulans* DDT, DDD, and DDE inhibited growth markedly (Boush and Matsumura 1975). In this case DDD was most toxic, followed by DDE and DDT. Mosser et al. (1974) reported the growth inhibition of diatom *Thalassiosira pseudonana* at 500 ppb of DDE. In *Exuviella baltica* DDE has been found to be highly toxic (Powers et al. 1979). The concentrations of

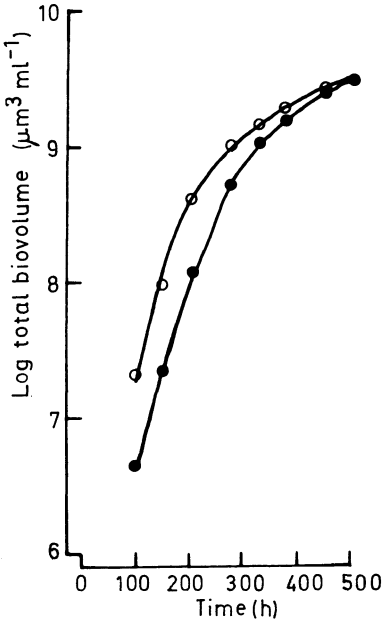

Fig. 4. The effect of DDT on the total biovolume of *Chlorella fusca.* Sufficient number of cells from logarithmic phase cultures of *Chlorella fusca* were added to 50 ml of culture medium in cotton wool-stoppered flasks to give a final concentration of 1.0 μg ml^{-1} DDT (●), control (○) received an equivalent amount of acetone. The cultures were continuously shaken (150 rpm) and illuminated (1500 lx) at 25 °C. (Goulding and Ellis 1981)

DDE that affected *Exuviella baltica* in this experiment are those which have been reported from natural marine phytoplankton communities (Cox 1972; Södergren 1971; Bowes 1972). The earlier inhibition and later recovery of the cells from DDE treatment as observed by Powers et al. (1979) was suggested to increase the cell number by reducing the availability of the toxicant to algal cells. Powers et al. (1979) suggested that in nature the brief period of inhibition as observed in their experiment may provide time for faster-growing, less susceptible organisms to gain a competitive advantage over more sensitive organisms such as *Exuviella baltica:* an

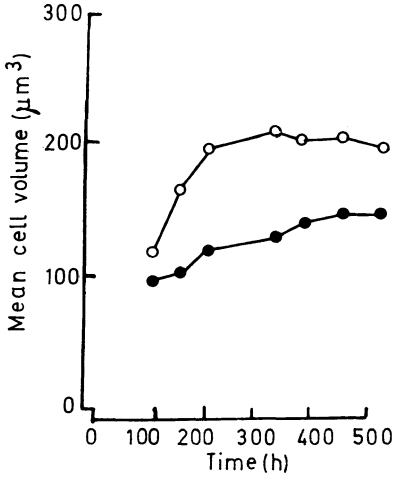

Fig. 5. The effect of DDT on the mean cell volume of *Chlorella fusca.* Sufficient number of cells from logarithmic phase cultures of *Chlorella fusca* were added to 50 ml of culture medium in cotton wool-stoppered flasks to give a final concentration of 1.0 μg ml^{-1} DDT (●) control (○) received an equivalent amount of acetone. The cultures were continuously shaken (150 rpm) and illuminated (1500 lx) at 25 °C. (Goulding and Ellis 1981)

effect demonstrated in laboratory studies in the phytoplankton community (Mosser et al. 1972b). However, Subba Rao and Alexander (1980) reported no statistically significant effects of DDT metabolites (DDM, DBM, DDE, DDT, BHE, PCPA and DDA) on *Chlorella vulgaris* at different concentrations ranging from 1 to 100 $\mu g\ g^{-1}$ soil except for PCPA and DDA which were toxic at 100 $\mu g\ g^{-1}$ of soil.

2.2 Lindane

Loosanoff et al. (1957) used 1 ppm lindane and found no effect on *Chlorella* and *Chlamydomonas* sp. In contrast, the growth of several algae isolated from surface waters of New York state was inhibited by lindane (Lazaroff and Moore 1966). Moore (1967), however, reported some isolates of algae to be sensitive to even 100 ppb of lindane, while others were resistant to concentrations up to 5 ppm.

Ukeles (1962) treated cultures of five unicellular marine algae with 1 to 9 ppm of lindane. *Protococcus* sp. was the most resistant, while *Monochrisis lutheri* was the most sensitive. Moore and Dorward (1968) reported the inhibition of growth of *Eudorine elegans* by 60% at 0.5 ppm of lindane and the algae were completely eliminated at 2.5 ppm, while the growth of *Gonium pectorale* was inhibited by 84% at 25 ppm. Singh (1973) reported complete elimination of blue-green algae at a concentration of 200 ppm of commercial lindane.

Ankistrodesmus braunii and *Anacystis nidulans* were exposed to lindane at 0.1 to 100 ppm (Kopecek et al. 1976). The chlorophyll content, dry weight, photosynthetic capacity and the formation of autosphere were reduced at all the concentrations used. Jeanne-Levain (1974) found that the dinoflagellate *Amphidinium carteri* was very sensitive to lindane, and 2 ppm has proved to be lethal to the organisms. In contrast, high concentrations of 50 to 60 ppm were required to inhibit the growth of *Euglena gracilis. Dunaliella bioculata* was also sensitive to lindane, as a 5-ppm dose was enough to inhibit cell division and at 10 ppm, organisms failed to divide. Subramanian (1982), however, reported stimulation of growth in *Anabaena* even at 1, 10, and 100 ppm of lindane.

Surface applications of lindane at low rates in water-logged soils under conditions simulating those of rice fields appeared to affect only the diatom populations while at higher concentration (10× normal) application rates, the nitrogen-fixing blue-green algae were also affected (Ishizawa and Mutsuguchi 1966). A distinct increase in the population of blue green algae was observed at 5, 6, and 50 kg h^{-1} to submerged Philippine rice soils (Raghu and MacRae 1967).

2.3 Aldrin, Dieldrin, and Endrin

Reports on the effect of aldrin, dieldrin, and endrin are also conflicting. For instance, Vance and Drummond (1969) found no significant effect on green and blue-green algae at 1 ppm of aldrin, dieldrin, and endrin. On the contrary, aldrin and dieldrin at 1 ppm inhibited the growth of *Anabaena cylindrica, Anacystis nidulans* and *Nostoc muscorum* without affecting the morphology of these organisms

(Schauberger and Wildman 1977). Only *Microcystis aeruginosa* was inhibited by less than 5 ppm aldrin, dieldrin and endrin, and many other algae resisted even much higher concentrations (100 ppm) without any apparent affect on the population densities (Clegg and Koevenig 1974). Four algal species tested by Menzel et al. (1970) differed markedly in their response to dieldrin. Whereas the estuarine green flagellate *Dunaliella tertiolecta* was insensitive to the insecticide at concentrations up to 100 ppb, the growth rates of *Skeletonema costatum* and *Coccolithus huxleyi* were reduced even at 100 ppb. *Cyclotella nana* was the most sensitive organism, its growth being completely inhibited by 100 ppb. Powers et al. (1977) reported that dieldrin treatment at 0.01 ppb inhibited the growth of dinoflagellate *Exuviella baltica* and caused disintegration of cells. The diameter of the surviving cells was, on the average, 11.2% smaller than controls.

Concentrations of endrin eliminating growth of four unicellular algae ranged from less than 5 ppm to more than 20 ppm (Vance and Drummond 1969). Dieldrin and its analogs were also found to inhibit the growth of *Anacystis nidulans* (Boush and Matsumura 1975). The organism was more susceptible to endrin, dieldrin, ketoendrin, and photodieldrin. Menzel et al. (1970) reported no effect on the growth of *Dunaliella tertiolecta* at concentrations up to 1000 ppb. However, the growth rate of *Skeletonema costatum* was reduced by 100 ppb endrin only during the early period of growth and 100 ppb reduced the growth rate of *Coccolithus huxleyi* throughout the experiment and completely inhibited the growth of *Cyclotella nana.* Endrin has also been reported to be more toxic than its metabolite ketoendrin to blue-green algae (Batterton et al. 1971). The growth of *Agmentellum quadruplicatum* was inhibited at all concentrations of endrin tested, whereas *Anacystis nidulans* was affected only at high concentrations. There was a slight stimulation of growth of *Agmentellum* by ketoendrin. However, *Cylindrospermum* sp. has been reported to tolerate even 600 ppm of endrin (Singh 1973).

2.4 Endosulfan

The toxicity of endosulfan toward algae differs from other insecticides mainly due to its different biological, physical, and chemical behavior. The effects of endosulfan and its metabolites, including endosulfan alcohol and endosulfan lacton, on *Chlorella vulgaris* revealed some interesting results (Goebel et al. 1982). The tests were conducted under long-term conditions in culture thermostat under daily renewal of culture solution. Under these conditions cell division was only reduced at concentrations of more than 2 mg kg^{-1}. This effect was reversible after the termination of the contamination of the culture solution. Test tube investigations with a very high endosulfan concentration were carried out only with *Selenastrum* and *Phormidium.* Injury to the *Selenastrum* only became apparent at a concentration of 80 mg kg^{-1}. No significant effect on growth was observed in *Phormidium* sp. exposed for a period of 14 days in a test tube to endosulfan and its metabolites (α-endosulfan, β-endosulfan, endosulfandiol, endosulfan ether, endosulfan sulfate, hydroxyendosulfan ether, endosulfan lactone) at 1.25 mg kg^{-1}. At 20 mg kg^{-1} a change of the shade of algae toward grey could be ascertained with metabolites possessing sulfur in the molecule (α-endosulfan, β-endosulfan and endosulfan sulfate).

Metabolites devoid of sulfur in the molecules (endosulfan alcohol, endosulfan ether and endosulfan lactone) caused no colour change in algal culture.

2.5 Toxaphene

Toxaphene at 2 ppm was reported to be highly toxic to *Microcystis aeruginosa,* and *Cylindrospermum* (Palmer and Maloney 1955). However, the same concentration did not affect the growth of *Scenedesmus, Chlorella variegata* and *Nitzschia palea.* Ukeles (1962) treated several marine phytoplankton with 0.1 to 0.15 ppm of toxaphene. Growth of *Monochrysis lutheri* was completely inhibited at 0.1 ppm, while the growth of many other phytoplanktons was suppressed only at concentrations higher than 0.1 ppm. Toxaphene at 0.1 and 1 ppm has also been reported to reduce the growth and biomass of *Scenedesmus quadricauda* (Stadnyk et al. 1971) and lower concentrations of toxaphene (1 and 10 ppb) were reported to have no effect on many freshwater algae (Butler 1977).

2.6 Diazinon

Very little information is available on the effects of diazinon on algae. Stadnyk et al. (1971) reported that diazinon had no significant effect on cell number, photosynthesis, and biomass of *Scenedesmus quadricauda.* Diazinon at concentrations ranging from 0.01 to 25 ppm, however, inhibited the growth of many phytoplanktons (Butler 1977).

2.7 Parathion and Methyl Parathion

Reports on the effects of parathion on algae are also conflicting. For instance, parathion at 0.1 to 1.2 ppm was noninhibitory to *Euglena gracilis* but 10 ppm parathion killed the cells within 1 to 2 h (Moore 1970). However, Gregory et al. (1969) reported no effect of parathion on *Euglena gracilis, Anacystis nidulans,* and *Scenedesmus obliquus* at 1 ppm. Parathion treatment even at 1 to 100 ppm did not affect the growth of *Euglena gracilis* till 24 h and at the end of 7 days of treatment the growth was even stimulated (Poorman 1973). While such discrepancies may be explained in experiments conducted under natural conditions, it is disturbing that they should also occur in pure culture conditions, illustrating the inherent variability of microbial response to the insecticides.

The unicellular green alga *Chlorella protothecoides,* grown autotrophically in the presence of methyl parathion showed inhibition in cell number, packed cell volume pigment and protein contents (Saroja and Bose 1982). Inhibition was slight at 10 ppm, moderate with 20 ppm and severe at 30 ppm. For any concentration of the insecticide, chlorophyll contents were inhibited more than any other parameter measured. Chlorophyll a appeared to be inhibited more than chlorophyll b, yielding a decrease in chlorophyll a/b ratio, and cell number was inhibited more than packed cell volume.

2.8 Malathion

Malathion at 100 ppm has been reported to inhibit the growth of *Chlorella pyrenoidosa* and the rate of inhibition decreased with time (Christie 1969). Moore (1970) treated *Euglena gracilis* with 0.15 to 7.25 ppm of malathion and only 7.25 ppm was inhibitory, whereas 0.15 ppm had no effect. The growth of several isolates of algae was inhibited at 25 and 50 ppm of malathion (Butler 1977). Torres and O'Flaherty (1976) reported the inhibition of growth at 1 ppm in *Chlorella vulgaris, Chlorococcum hypnosporum, Tribonema, Vaucheria geminata* and *Oscillatoria lutea.* However, at 0.1 and 0.5 ppm malathion stimulated chlorophyll production in *Tribonema* and *Oscillatoria.* In contrast, Poorman (1973) reported stimulation of growth in *Euglena gracilis* at 10 and 100 ppm malathion.

Malathion at 100 ppm markedly inhibited the ability of *Nostoc muscorum, Tolypothrix tenuis* and *Westiellopsis muscicola* to fix nitrogen (DaSilva et al. 1975). In general there was an initial period of depression succeeded by an increased activity. In another experiment, malathion has been reported to have no effect on growth of *Chlorella,* irrespective of inoculum size and sampling time (Ellis and Goulding 1973).

2.9 Permethrin

Toxic effects of the pyrethroid insecticide permethrin and ten of its degradation products on growth, photosynthesis and acetylene-reducing ability of two species of green algae and three species of cyanobacteria were reported by Stratton and Corke (1982). Permethrin was relatively nontoxic in all systems except for the growth of cyanobacterium *Anabaena inaequalis,* where it had an EC_{50} of 1.6 to 5.0 ppm. Metabolites of permethrin were more toxic. The most toxic metabolites were 3-phenoxybenzaldehyde and 3-phenylbenzyl alcohol, followed by benzoic acid and 3-phenoxybenzoic acid. The EC_{50} values for these compounds were as low as 1.4 ppm although more were in the range of 2 to 6 ppm.

2.10 Other Insecticides

Various organophosphorus insecticides have been reported to inhibit the growth of phytoplanktons to varying degrees (Sweeney 1968). Guthion and Meta-Systox did not have any effect, but methyl-trithion inhibited the natural population of phytoplankton significantly. The percent inhibition of growth by these insecticides is listed below.

Organophosphorus insecticides	Percent decrease in phytoplankton population at 1 ppm
Bayer 29493 (Baytex)	7.2
Diazinon	6.8
Dibrom	55.6

Organophosphorus insecticides	Percent decrease in phytoplankton population at 1 ppm
Di-syston	55.2
Ethion	69.0
Guthion	0.0
Meta-Systoxe	0.0
Methyl-trithion	85.9
Systox	7.1

Walsh and Alexander (1980) have described a simple algal bioassay for short- and long-term studies of organophosphorus insecticides (EPN, carbophenthion, ethoprop, DEF, methyl parathion and phorate). *Skeletonema costatum* was grown in optically matched culture tubes that fit directly into a spectrophotometer, allowing population density to be estimated by absorbance without removal of samples. The 96 h EC_{50} values reported were EPN 340 μg l^{-1}, carbophenthion 109 μg l^{-1}, DEF 366 μg l^{-1}, ethoprop 8.4 μg l^{-1}, methyl parathion 5.3 mg l^{-1} and phorate 1.3 mg l^{-1}. Venkataraman and Rajyalakshmi (1971, 1972), found that the majority of the 28 algal isolates tested against insecticides showed a high tolerance limit to all the compounds tested. Sardeshpande (1981) found that growth and nitrogen fixation by four blue-green algae was significantly reduced by lindane and endosulfan. He also reported that carbofuran and phorate at 1 ppm, appreciably increased the growth and nitrogen fixation by *Anabaena iyengarii* and *Calothrix membranacea.* Raghava Reddy (1976) observed that insecticides such as rogor, dimecron, and endrin at 25 ppm did not show any significant effect on *Tolypothrix tenuis* and *Anabaena* sp. Carbofuran and phorate at 1 ppm appreciably increased the growth and nitrogen fixation by *Anabaena iyengarii* and *Calothrix membranacea* (Sardeshpande and Goyal 1982), while phorate at this concentration increased nitrogen fixation in *Calothrix bharadwajae* without affecting the growth. However, BHC and endosulfan reduced the growth and nitrogen activity of blue-green algae at 1 ppm, which is well below the recommended dose of 1.5 of these insecticides in field. It was concluded from these observations that at the recommended doses of field application, these insecticides do not have any deleterious effect on algal contribution. It was also suggested that insecticides which effectively inhibit the growth and nitrogen fixation in the flask under laboratory conditions will not behave in the same way in the field because many physical, chemical, and biological forces may be acting on the insecticide and may inactivate it. Moreover, when the insecticide is dusted or sprayed on the crop, the actual quantity of it reaching the soil will be less than the field dose, suggesting that the insecticide active against algae tested at their field dose in the laboratory may not be active in the field.

3 Factors Influencing Microbe/Insecticide Interactions

The information on the effects of insecticides on algae is dominated by laboratory studies in axenic cultures. Such studies are of considerable scientific value, but may not reflect the actual fate of insecticides in natural environments because microbial

environments are heterogenous in their resources and conditions and so are able to sustain the growth of wide range of microorganisms with a broad spectrum of metabolic activity. The nature of microorganisms in a habitat is mainly dependent upon the nature of biotic and abiotic factors prevailing in that habitat. These factors can affect the microbes or the insecticides prior to microbe insecticide interactions. It is neither possible to assess all factors that affect microbe insecticide interactions nor to consider all these factors together in laboratory or field conditions. However, it is becoming increasingly clear that in order to extrapolate the laboratory results to what may happen in natural environments, due consideration must be given to the major biotic and abiotic factors which play a significant role in deciding the effects of insecticides on algae. The value of such studies also depends heavily on the methodological approach which has been discussed in detail in Chapter 3.

The interpretation of the significance of the laboratory results to natural environment is also extremely difficult. Direct comparison between insecticide concentrations in natural waters and those added to media may not be valid because various processes including absorption, and adsorption by planktonic and other organisms (Södergren 1968; Mecks 1968; Cox 1970; Hamelink et al. 1971; Södergren 1971), adsorption to particulates (Cox 1970) tend to remove the insecticides from water. In the following discussion factors which play an important role in relation to insecticide algae interactions are discussed.

3.1 Light

Much work has not been done on the influence of light on microbe–insecticide interactions, however, it seems to play an important role in deciding the sensitivity of algae to insecticides. For instance, the light intensity has been shown to influence the degree of inhibition of photosynthesis caused by DDT in the diatom *Nitzschia* (MacFarlane et al. 1971) and *Chlorella fusca* (Goulding and Ellis 1981). The high light intensity and DDT brought about a great growth depression in *Monocystis aeruginosa,* as compared to the effect of 10 ppm DDT under normal light intensities, whereas *Coelastrum proboscideum* was largely insensitive to light radiation as well as to the combined treatment with light and DDT (VonWitsch et al. 1975). Further studies by Kopecek et al. (1976) in *Ankistrodesmus braunii* and *Anacystis nidulans* exposed to lindane and dieldrin at concentration ranging from 0.1 to 100 ppm separately under light and dark revealed that these insecticides reduced the chlorophyll contents, dry weight, photosynthesis, and formation of autospheres. Lindane above 10 ppm decreased the chlorophyll pigment, dry weight, and cell number of *Anacystis* only under illumination. Low lindane concentration, however, stimulated photosynthesis.

3.2 Nutrients and Chemical Composition of the Media

Limited changes in the chemical and nutrients in the medium seem to have no significant influence of the effect of insecticide on algal growth. This is expected, as the algae are mainly dependent on light for growth. The variation in phosphate contents to one tenth of that in the normal medium did not alter the pattern of the effect of

1 ppm DDT on *Chlorella fusca* (Goulding and Ellis 1981). However, in the blue-green alga *Nostoc muscorum* the toxicity of BHC was reduced when K_2HPO_4, $Ca(NO_2)_2$ and $CaCl_2$ were added beyond normal quantities (Kar and Singh 1979).

3.3 pH and Temperature

Variation in pH does not influence the pattern of the effect of insecticides on algae. This was shown by Goulding and Ellis (1981) in *Chlorella fusca.* They reported that varying the pH between 5.5 to 7.5, although altering the growth rate of the culture considerably, did not alter the pattern of inhibition of growth reported for DDT at 1 ppm, this being 55% to 60% in the range of 5.5 to 7.5 pH. Carbaryl at 10 ppm and 20 °C (the temperature farther from the optimum) inhibited the growth of *Nitzschia closterium* and *Amphora coeffeaeformis* to a greater degree than controls at optimum temperature, whereas *Gonyaulax* sp. was inhibited at neither temperature (Maly 1980).

3.4 Aeration

Goulding and Ellis (1981) examined the effect of aeration on the degree of growth inhibition in *Chlorella fusca* treated with DDT. In their experiments all cultures were incubated in cotton wool-stoppered flasks with continued shaking to ensure adequate aeration. Cultures which were shaken but closed with rubber bungs showed very poor growth. In order to decrease aeration and still allow reasonable growth, cultures were left unshaken in the incubator. Table 2 shows that these cultures grew much less than the normal cultures, but then again DDT had an inhibi-

Table 2. Effect of aeration on the inhibition of growth of *Chlorella fusca* by DDT. Figures represent % inhibition of growth, as measured by cell number ml^{-1} at various sampling times and are the means of two replicates. (Goulding and Ellis 1981)

Time of growth (h)	Shaking	Standing
48	66.1±1.9	52.6±1.7
72	77.3±2.5	83.0±1.8
120	63.8±1.0	88.9±1.5
168	39.9±0.9	79.2±0.3
216	+ 8.2±0.2	50.0±1.4
240	+14.7±0	42.1±0.3
470	+48.8±2.6	8.9±1.6
Culture	Final cell number after 470 h growth ($\times 10^{-6}$)	
Control	14.1±0.19	1.26±0.03
+DDT	21.2±2.01	1.13±0.05

tory effect. The percentage inhibition was slightly higher than that in well-aerated cultures. In contrast to the normal well-aerated cultures, at no time did the growth in the poorly aerated DDT-treated cultures exceed that of poorly aerated control cultures.

3.5 Insecticide Concentration

In general, the toxicity of an insecticide is dose-dependent. However, beyond certain levels this relationship is not followed, as most of the insecticides are almost insoluble and if a greater amount of the insecticide is applied, the insecticide comes to the surface. In general, lower doses either stimulate the growth or have no effect, while higher doses of the insecticide inhibit the growth. For instance inhibition of cell volume, cell number, and protein contents in *Chlorella* was slight with 10 ppm methyl parathion, moderate with 20 ppm and severe with 30 ppm (Saroja and Bose 1982). In *Euglena gracilis,* malathion at 0.15 ppm had no effect, whereas 7.25 ppm inhibited the growth (Moore 1970). A similar correlation has been established or is evident from many other studies (Torres and O'Flaherty 1976; Goebel et al. 1982; Kopecek et al. 1976; Moore and Dorward 1968; Wurster 1968).

3.6 Insecticide Combination with Other Chemicals

Insecticides are present in combination with several chemicals in the microbial environments. These chemicals either interact with insecticides or with microorganisms, thereby altering the effect of insecticides. Mosser et al. (1974) added a mixture of chlorinated hydrocarbons (DDE and PCB's) to the axenic cultures of *Thalassiosira pseudonana* and found that PCB's and DDE in combination were more toxic to the organisms than they were individually, indicating that PCB's and DDE act synergistically, thereby increasing the toxicity. However, the addition of 50 ppb of PCB's restored the growth of organisms which had been affected by 500 ppb DDT. In the blue-green alga, *Nostoc muscorum,* the toxicity of BHC was reduced when K_2HPO_4, $Ca(NO_2)_2$, and $CaCl_2$ were added beyond normal quantities (Kar and Singh 1979). In this case it was suggested that BHC forms complexes with these compounds that might be less permeable. In *Exuviella baltica,* DDE was more toxic than PCB (Powers et al. 1975). However, when the two were combined, their effect was approximately additive.

3.7 Number, Type and Nature of Microorganisms

There is considerable diversity in algal sensitivity to insecticides. DDT was toxic to the diatom *Skeletonema costatum* even at very low concentration and the toxicity increases with the increase in insecticide concentration. It was concluded that low levels of DDT in natural waters might have deleterious effects on phytoplankton populations (Wurster 1968). A similar conclusion was drawn by Södergren (1968) from the studies on growth of *Chlorella* sp. which was affected by less than 0.3 ppb

of DDT. The results of other studies are somewhat at variance with these observations. DDT at 0.6 ppm was not toxic to marine phytoplankton (Ukeles 1962). Even higher concentrations (20 ppm) have been reported as not affecting the algal population (Vance and Drummond 1969). Christie (1969) found that DDT at 10 and 100 ppm did not affect the growth of *Chlorella pyrenoidosa.* The lack of effect of DDT in this case was attributed to the low solubility of DDT and the rapid degradation of this compound by the organisms. However, these cannot be taken as the only factors which make algae more sensitive or resistant to insecticide. Other intrinsic factors such as population size at the time of treatment, the lipid contents of the organisms, their inherent nature, physiological state and the experimental conditions are also equally important.

Whereas the blue-green alga *Anabaena variabilis* appeared to be insensitive to DDT even at 1 ppm, the growth of *Chlorella fusca* was inhibited by 0.1 ppm (Goulding and Ellis 1981). Although the initial inoculum of *Anabaena variabilis* was 10^6 cells ml^{-1} as opposed to 10 cells ml^{-1} of *Chlorella fusca,* and in term of biomass, the initial inoculum of *Anabaena variabilis* was only approximately six times that of *Chlorella fusca.* Further when *Chlorella fusca* was treated with 1 ppm DDT at an initial inoculum of 10^5 cells ml^{-1} inhibition was still noted. This prompted Goulding and Ellis (1981) to propose that the different sensitivities of the two were not due to the difference in inoculum size but were due to the different nature of these organisms.

Goulding and Ellis (1981) also examined the effects of initial cell inoculum on the degree of inhibition caused by DDT in *Chlorella fusca.* Table 3 shows that the size of initial inoculum markedly affected the degree of growth inhibition. The growth (as measured by cell numbers) was inhibited by 75% at an inoculum of 1×10^3 cells ml^{-1} whereas at 1×10^4, 1×10^5, and 5×10^5 cells ml^{-1} growth was inhibited by 60, 30, and 20% respectively.

Of the two algal species studied, *Pavlova lutheri* was found to be more resistant to lindane than *Dunaliella primolecta* (Escoubet 1978). Stratton and Corke (1982) reported that prokaryotic algae were more sensitive to pyrethroid insecticides and their metabolites than eukaryotic algae. This was also attributed to the differences in the basic cellular organization between these organisms. Menzel et al. (1970) also observed that different species of marine phytoplankton varied considerably in their response to the organochlorine insecticides DDT, dieldrin, and endrin. DDT at

Table 3. Effect of inoculum size on the initial inhibition of growth of *Chlorella fusca* by DDT. Figures represent the % inhibition of growth as measured by cell number ml^{-1} at the various sampling times and are the mean of four replicates. (Goulding and Ellis 1981)

Time of growth h	Inoculum size (cells ml^{-1})			
	10^3	10^4	10^5	5×10^5
72	96.2±3.1	76.0±3.1	61.1±0.1	34.2±1.4
96	75.1±3.3	59.3±1.7	30.3±1.2	19.6±6.5
160	59.8±0.2	41.0±0.4	9.6±0.8	4.2±0.3
200	29.7±1.5	5.9±0.6	0.5±0.2	–

100 ppb inhibited cell division in *Skeletonema costatum* (a coastal diatom), but had no effect at concentration of 0.1 to 1 ppb on *Coccolithus huxleyi* (an open-ocean alga). In contrast, endrin had little effect on cell division in *Skeletonema costatum. Dunaliella tertiolecta* (an estuarine species) was apparently insusceptible to all the compounds up to 1000 ppb. It was suggested that resistance of estuarine species compared with the more susceptible coastal and open-ocean forms may reflect their greater adaptability to insecticides. Bowes (1972) reported that with the exception of *Skeletonema costatum,* whose growth was inhibited at 80 ppb DDT, seven other species of marine phytoplanktons were not affected at all. In a related study Mosser et al. (1972 a) confirmed that *Dunaliella tertiolecta* was unaffected by 1000 ppb DDT as reported by Menzel et al. (1970) and that other organisms including *Chlamydomonas reinhardii* and *Euglena gracilis* were also relatively resistant (Mosser et al. 1972 a). They (Mosser et al. 1972 b) further investigated the effect of DDT on mixed cultures of marine algae containing the sensitive diatom *Thalassiosira pseudonana* (earlier known as *Cyclotella nana*) and a resistant alga *Dunaliella tertiolecta* in equal proportion. It was found that growth of *Dunaliella tertiolecta* was not inhibited at any DDT concentrations tested. However, *Thalassiosira pseudonana,* which grew faster and soon outnumbered *Dunaliella tertiolecta* in control cultures, was unaffected at 10 ppb to the extent that its competitive success was significantly diminished, even though *Thalassiosira pseudonana* was unaffected at 10 ppb DDT in pure culture.

Recently, Maly and Ruber (1983) reported that *Gonyaulax* was resistant to 10 ppm of temephos, propoxur, chloropyrifos, malathion, and carbaryl, whereas *Nitzschia* sp. was affected only by carbaryl. *Amphiprora* was affected by all the insecticides and *Amphora,* like *Nitzschia,* was sensitive to carbaryl but not to other insecticides. It is thus evident from the data that differential algal sensitivity may have no effect on certain species, but the elimination of sensitive species will decrease the competition, as a result of which the population of resistant species may show a tremendous rise.

3.8 Multispecies Interactions

A number of studies on the effects of insecticides in single species algal cultures have demonstrated that while some species show inhibition of growth when exposed to levels as low as a few ppb (Wurster 1968), other species are resistant to levels of several ppm (Ukeles 1962; Menzel et al. 1970; Derby and Ruber 1970; Poorman 1973). Because of the differential algal sensitivity one might predict that toxicants would have no long-term inhibitory effect on biomass of algae, instead a shift in community composition with more resistant species replacing the sensitive species might be expected. This has been demonstrated recently by Maly and Ruber (1983). In a three-species experiment using *Amphiprora, Nitzschia,* and *Chlorococcum* and carbaryl at 2 ppm, the densities of *Chlorococcum* and *Nitzschia* were not different from the controls but the number of *Amphiprora* was reduced. In another three-species experiment using *Amphiprora, Amphora,* and *Chlorococcum,* carbaryl treatment again reduced the number of *Amphiprora.* The cell number of *Chlorococcum* was

enhanced while *Amphora* was not affected. In a five-species experiment using *Amphiprora, Amphora, Nitzschia, Chlorococcum,* and *Gonyaulax,* the growth of *Amphiprora* was once again inhibited, *Gonyaulax* completely disappeared, *Amphora* was slightly inhibited, whereas the growth of *Nitzschia* and *Chlorococcum* was stimulated. Thus it is apparent from these studies that one cannot predict the effect of insecticides on components of multispecies solely from their effects in single species cultures. All four species were inhibited by carbaryl at 2 ppm in single-species but in multispecies cultures only two species were inhibited. There was also a difference in the rank of sensitivity in single and multispecies cultures. *Amphora* was more sensitive in multispecies communities and *Chlorococcum* was more sensitive in single-species cultures.

4 Secondary Effects

If the direct toxicological effects of insecticides on growth, survival, or reproduction of the organisms may be called primary, then ecosystem changes that follow as a result of primary effects are for convenience, termed secondary effects (Hurlbert 1975). Complete or partial removal of a species from an ecosystem is followed by changes in the prey-predator competition and as a result of interrelatedness of ecosystem components, a large number of secondary effects must be expected to occur whenever an ecosystem is exposed to insecticides.

Knowledge of secondary effects has lagged because little credence has been given to their potential ecological and economic importance and because of poor or no experimental design. In the majority of these studies, increased algal populations following insecticide treatments constitute some of the best evidence available on the importance of grazing herbivorous invertebrates as a regulating factor of algal populations. The phenomenon has been demonstrated by planktonic algae, benthic algae, and macroscopic filamentous algae suspended in water. Apparently the first insecticide-induced phytoplankton bloom to be reported was one that developed in a large drinking water reservoir after it had received repeated aerial applications of DDT during a city-wide treatment in Wilmington, Delaware, aimed at controlling a polio epidemic (Shane 1948). Soon after treatments were initiated, the diatom *Synedra* began increasing rapidly in the reservoir, finally attaining an average cell concentration 100 times higher for September than the average concentrations for the four previous Septembers. Ruber and Ferrigno (1964) observed increased photoplankton productivity, in salt marsh plots treated with DDT, endrin and fenthion at the rates used in mosquito control.

Large-scale phytoplankton cultures, used to feed marine molluscs and their larvae, are occasionally invaded and depleted by copepods. Loosanoff et al. (1957) demonstrated that insecticides which eliminate pests also produce phytoplankton blooms. These workers found that 1 ppm of TEPP, Dipterex, parathion, lindane, or DDT was sufficient to cause 100% mortality of the copepods within 2 days without any effect on the algal cultures.

Rotenone treatment of two lakes in Alberta, Canada, temporarily eliminated all planktonic crustaceans and reduced planktonic rotifer populations which resulted in

the formation of bloom of *Ceretium hirudinella* 1 month later in one lake and a smaller bloom of *Volvox* sp. in the second lake 9 months later (Anderson 1960). Hrbacek et al. (1961) demonstrated that insecticide treatment removed zooplanktivorous fish and zooplanktons, leading to the formation of phytoplankton blooms. Kiser et al. (1963) also observed that rotenone treatment of a shallow Washington lake eliminated all planktonic crustaceans and was followed by a slight increase in the phytoplankton.

In Minnesota ponds, DDT treatment resulted in a temporary and moderate decline in the number of cladocerans, copepods, and ostracods, while *Volvox* was more abundant in treated than in control ponds (Jones and Moyle 1963). Three successive applications of parathion to a clear lake in California reduced populations of *Daphnia, Cyclops,* and *Diaptomus* sp. and produced impressive surface blooms of the blue-green alga *Anabaena* (Cook and Conners 1963). Raghu and MacRae (1967) applied γ-HCH at 23 ppm to submerged tropical rice soils and noted a marked stimulation in the growth of indigenous algae. The enhanced algal growth was subsequently attributed to the insecticidal effect on small animals utilizing algae for food rather than a direct stimulatory effect on the algae.

Populations of phytoflagellate *Chlorogonium* persisted longer in experimental pools where larval mosquito (*Culex pipens*) populations were temporarily reduced by the application of dibutyl cresol at 1.8 ppb (a highly toxic substance being tested as potential insecticide) than in untreated control pools (Hurlbert 1975). On the

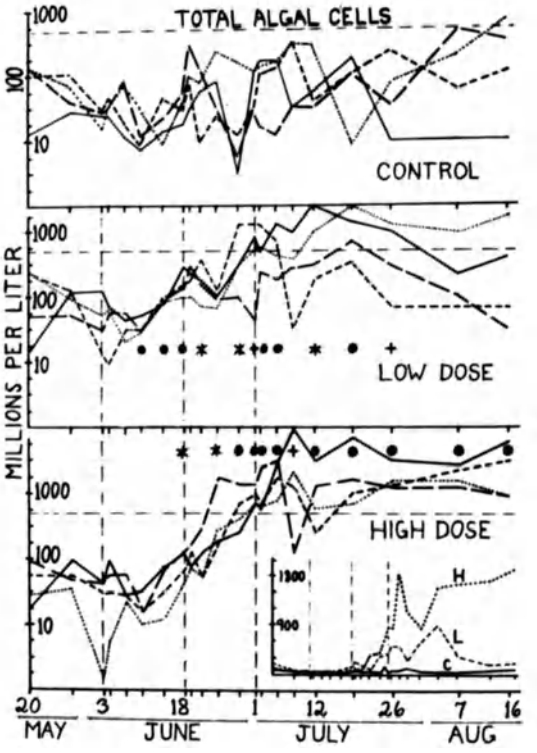

Fig. 6. The effect of Dursban treatment on the phytoplankton, expressed in total algal cells l^{-1}. Individual ponds are represented as follows: *dotted line* C-1, L-1, H-1; *short dashes* C-2, L-2, H-2; *long dashes* C-3, L-3, H-3; *solid line* C-4, L-4, H-4. *Dashed horizontal reference line* is drawn at 500 million cells l^{-1}. *Symbols across top of each chart* show a statistical significance. *Inset* shows geometric mean abundances plotted on an arithmetic scale. Low dose = 0.028 kg ha^{-1}, and high dose = 0.28 kg ha^{-1}. (Hurlbert et al. 1972). *C* control pond; *L* low dose pond; *H* high dose pond

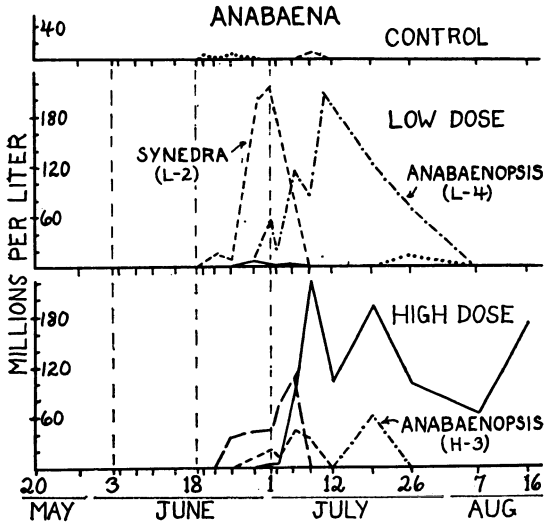

Fig. 7. Effect of Dursban treatment on diatom and filamentous blue-green algae populations. Unless otherwise indicated, all the lines represent *Anabaena* populations and individual ponds are represented as in Fig. 6. (Hurlbert et al. 1972)

other hand, the more numerous smaller phytoplankters became less abundant in treated than in control pools.

Clear evidence of the formation of algal bloom as an effect of insecticide on predators was provided by Hurlbert et al. (1972). Three successive treatments of Dursban spaced at 2 weeks' intervals reduced populations of crustaceans and were followed by large phytoplankton increase in ponds treated both at 0.028 and 0.28 kg ha^{-1} (Fig. 6). Although crustaceans were completely eliminated in high dose ponds, the phytoplankton increases in these ponds lagged behind those in low dose ponds at the beginning. During the later periods, the phytoplankton populations were always larger in high dose ponds. The different algal species responsible for the formation of blooms were *Anabaena, Anabaenopsis, Synedra,* and *Schroederia setigera.* The contribution of different algae to bloom formation was dependent on the time of treatment, concentration, and duration of treatment (Figs. 7 and 8).

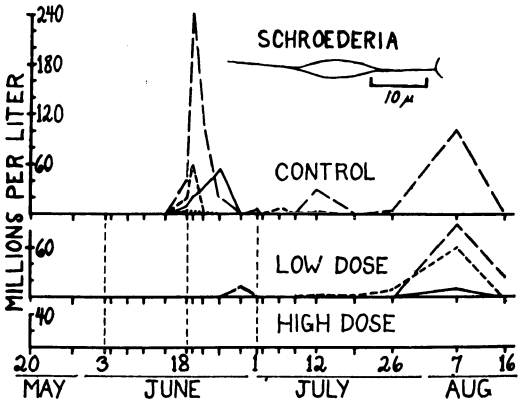

Fig. 8. Effect of Dursban treatment on the alga *Schroederia setigera.* Individual ponds represented as in Fig. 6. (Hurlbert et al. 1972)

5 Conclusions and Future Prospects

As insecticides are widely distributed in our environment and algae are an important component of the aquatic ecosystem, it is interesting to speculate on the possible ecological implications of their interactions. In doing so, it is acknowledged that laboratory investigations have obvious limitations in the study of field problems. However, it is generally accepted that pure culture studies have the advantage of separating the responses of one species in defined conditions from the interaction of many organisms in a complex environment. In natural waters the concentration of insecticide rarely exceeds their solubility limits and in most cases insecticides used below solubility levels stimulate the growth of algae. This will also affect the species balance in natural communities.

Sensitivity of algae to insecticides depends on the type and nature of the insecticide and the organism and the experimental conditions. With some insecticides sensitivity follows closely on dosage levels and at higher levels of insecticides algae are markedly injured or even killed. The insecticide toxicity of algae is also dependent on the ability of the organisms to react with the insecticide. If the organism is able to metabolize the insecticide, the toxicity may be due to the metabolites at a later state. On the contrary, the metabolites may not affect the organism and thus the effect may disappear. However, no work has been done to understand this particular aspect, although algae are known to metabolize the insecticides.

A striking feature encountered in the review of literature is the amount of apparent disagreement among various workers. Part of it may arise from the nonequivalent environment in which the studies are carried out. Additionally, most of the investigators are concerned with one or two environmental factors, whereas many factors have been ignored. Studies in which all the possible factors of the environment are under strict control have not been conducted.

In order to obtain a full picture of interaction of insecticides with algae, some emphasis can be given to certain aspects which have not received sufficient attention, in some cases for obvious reasons of practical difficulty. This includes the interaction of insecticides with algae with respect to biotic and abiotic factors. Also much information is not available on the effect of insecticides on nitrogen fixation in algae, though the growth of many blue-green algae which fix nitrogen has been reported to be affected by the insecticides.

Among the least-studied effects of insecticides in algae are the secondary effects. Experiments have only rarely assessed the influence of an insecticide on pre-predator interactions of aquatic populations where algae are in abundance.

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CHAPTER 10

Cytological and Biochemical Effects of Insecticides on Microorganisms

RUP LAL and S. SHIVAJI

1 Introduction

Insecticides are known to alter the growth and activities of microorganisms. These alterations are mediated through cytological and biochemical changes at cellular level. Although an enormous literature is available on the effects of insecticides on the growth and activities of microorganisms, little has been done to understand the effects of insecticides at cellular level. Some microorganisms, being unicellular and having many other attributes in experimental biology, are used as models to understand the mode of action of xenobiotic compounds. Thus information on the cytological and biochemical effects on such organisms may also present a picture of cytological and biochemical effects of insecticides in higher organisms.

Cytological and biochemical effects are either primary or secondary in nature. The primary change is the first metabolic reaction that is affected at the lowest concentration of the insecticide. Other reactions affected at higher insecticide concentrations or at a given low concentration after a lag are considered to be secondary in nature since they appear as a result of the effect of insecticide on a particular primary target component. For instance, insecticide whose primary site of action is at the level of synthesis of the energy-rich compound, ATP, would subsequently also inhibit many other reactions that require energy.

In higher organisms, such as insects and mammals, the sites of action of insecticides have been clearly identified. Organochlorine insecticides interfere by impairing the ionic permeability of nerve cell membranes, thus producing an unstable state in which spurious nerve impulses induce uncontrolled activity in the whole organism. The organophosphorus and carbamate insecticides owe their insecticidal properties to phosphorylation or carbamylation of the enzyme acetylcholinestrase. This poisons the enzymes so that it cannot catalyze the hydrolysis of acetylcholine at the synapse (which permits the continuous transmission of nerve impulses); effective nerve coordination breaks down, the insects or mammals suffer convulsions and finally death occurs. By contrast, microorganisms display a multitude of potential targets for insecticide action (Table 1).

Several reviews on the interaction of insecticides with microorganisms deal only with microbial growth and contain hardly any information on cytological and biochemical effects of insecticides (W. B. Bollag 1961; Ware and Roan 1970; Cox 1972; Pfister 1972; J. M. Bollag 1974; Butler 1977; Wainwright 1978; Lal and Saxena 1980a, 1982; Lal 1982, 1983; Lal and Dhanaraj 1984). This review therefore, focuses primarily on the cytological and biochemical effects of insecticides on microorganisms.

Table 1. Cytological and biochemical effects of insecticides on microorganisms

Compound	Organism	Concentration	Effect	References
Aldrin	*Chlorella ellipsoidea*	100 ppm	ATP contents reduced	Clegg and Koevenig (1974)
	Chlamydomonas sp.	100 ppm	ATP contents reduced	Clegg and Koevenig (1974)
	Euglena gracilis	100 ppm	ATP contents reduced	Clegg and Koevenig (1974)
	Escherichia coli	8.6 μM	Lipid composition of plasma membrane altered	Rosas et al. (1980)
Chlordane	*Scenedesmus quadricauda*	0.1–100 ppb	Oxygen production stimulated	Glooschenko and Lott (1977)
	Chlamydomonas	0.1–50 ppb	Oxygen production stimulated	Glooschenko and Lott (1977)
	Estuarine phytoplanktons	100 ppm	C^{14}-uptake inhibited	Biggs et al. (1978)
	Exuviella baltica	50 ppb	C^{14}-uptake inhibited	Magnani et al. (1978)
	Aeromonas proteolytica	10–100 ppm	ATPase activity inhibited	Nakas and Litchfield (1979)
	Chlorella ellipsoidea	100 ppm	ATP contents reduced	Clegg and Koevenig (1974)
	Chlamydomonas sp.	100 ppm	ATP contents reduced	Clegg and Koevenig (1974)
	Euglena gracilis	100 ppm	ATP contents reduced	Clegg and Koevenig (1974)
	Saccharomyces cerevisae	1×10^{-4} M	Mitochondrial oxidative phosphorylation affected	Nelson and Williams (1971)
Chlorpyrifos	*Azotobacter agilis*	20–200 ppm	Phosphatase and dehydrogenase activity inhibited	Pawlaczyk-Szpilowa and Lejczak (1978)
	Bacillus megaterium	20–200 ppm	Phosphatase and dehydrogenase activity inhibited	Pawlaczyk-Szpilowa and Lejczak (1978)
	Brevibacterium sp.	20–200 ppm	Phosphatase and dehydrogenase activity inhibited	Pawlaczyk Szpilowa and Lejczak (1978)
DDT	*Crithidia fasciculata*	425 ppm	Plasma membrane structure altered, DNA and RNA synthesis inhibited	French and Roberts (1976)
	Tetrahymena pyriformis	50, 100 ppm	DNA, RNA and protein synthesis inhibited	Saxena et al. (1981)
	Stylonychia notophora	50, 100 ppm	Production of nuclear incisions, DNA, RNA and protein synthesis inhibited	Lal and Saxena (1980b)
	Blepharisma intermedium	50, 100 ppm	DNA, RNA and protein synthesis inhibited	Lal et al. (1981)
	Skeletonema costatum	1100 ppb	C^{14}-uptake inhibited	Ware and Roan (1970)

	Coccolithus huxleyi	1100 ppb	C^{14}-uptake inhibited	Ware and Roan (1970)
	Pyramimonus sp.	1100 ppb	C^{14}-uptake inhibited	Ware and Roan (1970)
	Peridinium trochoideum	1100 ppb	C^{14}-uptake inhibited	Ware and Roan (1970)
	Escherichia coli	8.6 μM	Lipid and fatty acid composition altered	Rosas et al. (1980)
	Chlorella ellipsoidea	100 ppm	ATP contents reduced	Clegg and Koevenig (1974)
	Chlamydomonas sp.	100 ppm	ATP contents reduced	Clegg and Koevenig (1974)
	Euglena elastica	100 ppm	ATP contents reduced	Clegg and Koevenig (1974)
	Anacystis nidulans	800 ppb	Mg^{2+}-dependent adenosine triphosphatase activity inhibited	Batterton et al. (1971)
	Skeletonema costatum	1–100 ppm	C^{14}-uptake inhibited	Menzel et al. (1970)
	Coccolithus huxleyi	1–10 ppb	C^{14}-uptake inhibited	Menzel et al. (1970)
	Cyclotella nana	1 ppb	C^{14}-uptake inhibited	Menzel et al. (1970)
	Thalassiosira pseudonana	50 ppb	C^{14}-uptake inhibited	Fisher (1975)
	Skeletonema costatum	50 ppb	C^{14}-uptake inhibited	Fisher (1975)
	Coccolithus hyxleyi	50 ppb	C^{14}-uptake inhibited	Fisher (1975)
	Selanastrum capricornutum	3.6 and 36 ppm	C^{14}-uptake inhibited	Lee et al. (1976)
	Bacillus megaterium	10 ppm	Membrane lipids altered	Hicks (1976)
	Chlorella ellipsoidea	100 ppm	ATP contents reduced	Clegg and Koevenig (1974)
	Chlamydomonas sp.	100 ppm	ATP contents reduced	Clegg and Koevenig (1974)
	Euglena gracilis	100 ppm	ATP contents reduced	Clegg and Koevenig (1974)
	Chlamydomonas nivalis	1–5 ppm	Total amino acids reduced and tyrosine contents increased	Czeczuga and Gierasimow (1977)
	Chlamydomonas nivalis	10–25 ppm	Alanine, aspartic acid and threonine contents decreased	Czeczuga and Gierasimow (1977)
	Ankistrodesmus falcatus	1–5 ppm	Amino acid contents decreased	Czeczuga and Gierasimow (1977)
	Ankistrodesmus falcatus	10–25 ppm	Decrease in amino acids	Czeczuga and Gierasimow (1977)
	Chlamydomonas reinhardii	0.2–20 ppm	C^{14}-uptake not inhibited	Morgan (1972)
	Pyramimonas	200 ppb	C^{14}-uptake inhibited	Morel (1976)
	Dunaliella tertiolecta	0.1–1000 ppb	C^{14}-uptake not affected	Luard (1973)
	Scenedesmus quadricauda	0.1–1000 ppb	C^{14}-uptake not affected	Luard (1973)
DDVP	*Polyspondylium violaceum*	3×10^{-5} M	Number of aggregation centres and sorocarps increased	Clark (1977)

Table 1 (continued)

Compound	Organism	Concentration	Effect	References
DDVP	*Escherichia coli*	–	Mutagenic	Lofroth et al. (1969), Lofroth (1970), Bridges et al. (1973), Lawely et al. (1974), Wennerberg and Lofroth (1974), Green et al. (1974)
	Salmonella	–	Mutagenic	Carere et al. (1978)
Diazinon	*Chlorella ellipsoidea*	100 ppm	ATP content reduced	Clegg and Koevenig (1974)
	Chlamydomonas sp.	100 ppm	ATP content reduced	Clegg and Koevenig (1974)
	Euglena gracilis	100 ppm	ATP content reduced	Clegg and Koevenig (1974)
Dieldrin	*Skeletonema costatum*	10 ppb	C^{14}-uptake inhibited	Menzel et al. (1970)
	Coccolithus huxleyi	10 ppb	C^{14}-uptake inhibited	Mosser et al. (1972a)
	Cyclotella nana	0.1–1 ppb	C^{14}-uptake inhibited	Wurster (1968)
	Chlorella ellipsoidea	100 ppm	ATP content reduced	Clegg and Koevenig (1974)
	Chlamydomonas sp.	100 ppm	ATP content reduced	Clegg and Koevenig (1974)
	Euglena gracilis	100 ppm	ATP content reduced	Clegg and Koevenig (1974)
	Escherichia coli	8.6 μM	Plasma membrane lipids altered	Rosas et al. (1980)
Endosulfan	*Rhodotorula gracilis*	50 and 100 ppm	Efficiency of proton pump and transport of D-xylose reduced	
Endrin	*Skeletonema costatum*	10 ppm	C^{14}-uptake inhibited	Menzel et al. (1970)
	Coccolithus huxleyi	10 ppm	C^{14}-uptake inhibited	Menzel et al. (1970)
	Cyclotella nana	0.1 ppm	C^{14}-uptake inhibited	Menzel et al. (1970)
Fenthion	*Skeletonema* sp.	100 ppb	Oxygen production inhibited	Derby and Ruber (1970)
	Cyclotella sp.	100 ppb	Oxygen production inhibited	Derby and Ruber (1970)
	Dunaliella sp.	100 ppb	Oxygen production inhibited	Derby and Ruber (1970)
	Phaeodactylum sp.	100 ppb	Oxygen production inhibited	Derby and Ruber (1970)

γ-HCH (Lindane)	*Amphidinium carteri*	0.3 ppm	Abnormal nucleus, microtubule number increased, DNA, RNA and protein synthesis inhibited	Jeanne-Levain (1979)
	Dunaliella bioculata	10 and 20 ppm	Enlarged cells, endomultiplication of organelles, DNA, RNA and protein synthesis inhibited	Jeanne-Levain (1979)
	Saccharomyces cerevisiae	5, 10, 50 ppm	Cytochrome P-450 contents increased	Karenlampi et al. (1982)
	Kluyveromyces fragilis	5, 10, 50 ppm	No effect	Karenlampi et al. (1982)
	Dunaliella bioculata	10 ppm	DNA, RNA synthesis inhibited, enlarged cells, increased in Golgi apparatus, large vacuoles, abnormal partition of nucleus	Jeanne-Levain (1974)
Malathion	*Vaucheria geminata*	1 ppm	Production of brown crystal inside cytoplasm, formation of second cell wall, distortion of gametagia	Torres and O'Flaherty (1976)
	Bacillus subtilis	–	Mutagenic	Shiau et al. (1980)
	Salmonella typhimurium	–	Mutagenic	Shiau et al. (1980)
	Dictyostelium discoideum	5, 10 ppm	Macromolecular synthesis inhibited, DNA strand breaks	Green et al. (1974) Griffin and Walter (1978)
	Aspergillus niger	10, 50, 90 ppm	Citric acid production inhibited	Rahmatullah et al. (1979)
Temephos	*Skeletonema*	100 ppb	Oxygen production inhibited	Derby and Ruber (1970)
	Cyclotella	100 ppb	Oxygen production inhibited	Derby and Ruber (1970)
	Dunaliella	100 ppb	Oxygen production inhibited	Derby and Ruber (1970)
	Phaeodactylum	100 ppb	Oxygen production inhibited	Derby and Ruber (1970)
Zectran	*Chroococcus* sp.	10 ppm	C^{14}-uptake inhibited	Sheridan and Simms (1975)
	Spirogyra sp.	10 ppm	C^{14}-uptake inhibited	Sheridan and Simms (1975)
	Ulothrix	10 ppm	C^{14}-uptake inhibited	Sheridan and Simms (1975)
	Schizogonium sp.	10 ppm	C^{14}-uptake inhibited	Sheridan and Simms (1975)
	Mougeotia sp.	10 ppm	C^{14}-uptake inhibited	Sheridan and Simms (1975)
	Vaucheria sp.	10 ppm	C^{14}-uptake inhibited	Sheridan and Simms (1975)
	Zygonema sp.	10 ppm	C^{14}-uptake inhibited	Sheridan and Simms (1975)
	Oedogonium sp.	10 ppm	C^{14}-uptake inhibited	Sheridan and Simms (1975)

2 Cell Membranes

Lipophilic compounds are known to penetrate cell membranes easily. Hence it is obvious that cell membranes may be potential targets for the action of insecticides which are lipophilic in nature. Insecticides are also known either to partition into or adsorb on membranes, thus distorting the structural and chemical integrity of the membranes which is essential for selective permeability. Many of the acute toxic effects of insecticides can be related to disruption of one or more of the cell membrane systems.

The toxicity of DDT in *Bacillus subtilis* was dose-dependent and the toxicity was correlated positively in relation to the binding of DDT to membranes (Hicks and Corner 1973; Hicks 1976), suggesting that DDT treatment may alter the lipid backbone of the membranes. Rosas et al. (1980) established that DDT, aldrin, and dieldrin altered the ratio of polar phospholipid head groups and the composition of fatty acids in *Escherichia coli* (Table 2). Such changes in phospholipids may alter the architecture of plasma membrane as observed in *Crithidia fasciculata* (French and Roberts 1976) and consequently membrane-related functions.

In *Rhodotorula gracilis,* endosulfan at 50 ppm and above probably affected the pH by affecting some components of plasma membrane and as a consequence, the efficiency of the proton pump operating across the membrane was reduced (Srivastava and Misra 1981). The uptake of D-xylose, which is closely linked to the efficiency of the proton pump, was also significantly lowered (Fig. 1), followed by an increase in the rate of respiration (Fig. 2). Further, the optimum temperature for initial velocity of D-xylose transport did not change but the rate of transport was sharply lowered (Fig. 3). An Arrhenius plot of temperature dependence of xylose transport revealed that there was an insignificant change in the energy of activation between treated and control cells, suggesting that the carrier molecule (the component directly related with the transport of D-xylose) was hardly influenced by endosulfan. Thus in *Rhodotorula gracilis* endosulfan appears to react with membrane components not directly linked with D-xylose transport.

Table 2. Effects of pesticides on the phospholipid (P) composition of *Escherichia coli*[a] (Rosas et al. 1980)

Pesticide	μmol of Pg^{-1} of lyophilized cells					
	Total lipid phosphate	Phosphatidyl serine	Phosphatidyl ethanolamine	Phosphatidyl glycerol	Cardiolipin	Acidic/neutral
Control	42.50±4.19	0.65±0.06	26.20±1.60	1.70±0.10	4.38±0.18	4.56
Parathion	59.47±1.07	2.25±0.23	50.60±1.50	5.01±0.39	8.08±0.70	4.00
DDT	71.30±0.75	0.82±0.08	65.50±1.70	4.70±0.32	8.70±1.00	2.85
Aldrin	50.47±0.38	5.91±0.20	28.35±0.52	ND	7.89±0.25	4.26
Dieldrin	52.23±0.23	6.60±0.20	27.30±0.70	ND	5.50±0.10	6.14

[a] Pesticides were added to the medium to give a final concentration of 8.6 μM.

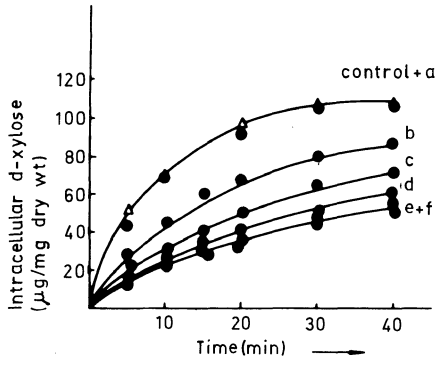


Fig. 1 D-Xylose uptake by *Rhodotorula gracilis* cells. Yeast suspensions in water (about 8 mg dry wt ml^{-1}) were incubated with different concentrations of endosulfan for 30 min. Equivalent amounts of alcohol (as present in endosulfan solution) were incorporated in normal cell suspensions and aerated for 30 min which served as control. The cell suspensions (about 4 mg dry wt ml^{-1}) were incubated with 75 mM phosphate buffer of pH 4.5 at 30 °C with 25.0 mM D-xylose. The sugar transport followed thus. Values are the mean of two parallel sets each in control and endosulfan treated samples; *a* 10 ppm; *b* 50 ppm; *c* 100 ppm; *d* 200 ppm; *e* 300 ppm; *f* 400 ppm. (Srivastava and Misra 1981)

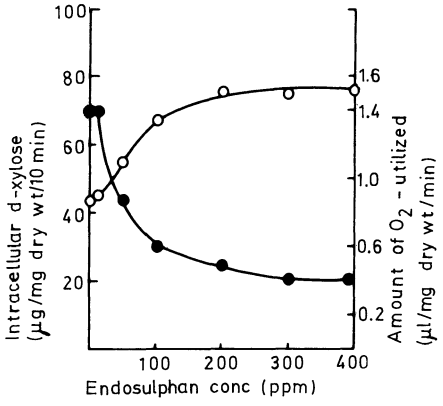


Fig. 2. Dose-response curves for D-xylose transport (closed circles) and O_2-uptake (open circles) in *Rhodotorula gracilis.* (Srivastava and Misra 1981)

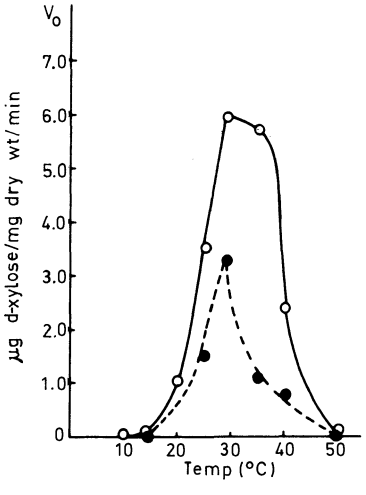


Fig. 3. Temperature dependence of the initial velocity of D-xylose transport (final conc. 25.0 mM) between 10 ° and 50 °C in control (open circles) and endosulfan-treated (50 ppm) cells (closed circles) in *Rhodotorula gracilis.* (Srivastava and Misra 1981)

3 Energy Production, Enzymes and Metabolic Pathways

Very few studies have been directed towards the effect of insecticides on membrane-bound or soluble enzymes. Based on indirect studies, Batterton et al. (1971) suggested that DDT inhibited the membrane-bound Mg^{+2} and Na^+, K^+ dependent ATPases in *Anacystis nidulans.* Chlordane was shown to inhibit the activities of ATPase, succinate dehydrogenase, NADH dehydrogenase (Trudgill and Widdus 1970; Widdus et al. 1971) endopeptidase (Nakas 1977; Nakas and Litchfield 1979) in *Bacillus subtilis, Escherichia coli,* and *Aeromonas proteolytica.*

Nelson and Williams (1971) observed that organochlorine insecticides (chlordane, aldrin, dieldrin, heptachlor, heptachlor epoxide, endrin, and mirex) inhibited cell division of *Saccharomyces cerevisiae* on all nonfermentable substrates (glycerol, lactate and ethanol) but not on fermentable sugars (glucose, galactose and fructose) when provided as energy sources, indicating that these insecticides do not inhibit the transport of substrates into the cell but inhibit the growth of *Saccharomyces cerevisiae* by interfering with oxidative metabolism. Clark (1977) proposed that DDVP (dichlorvos) inhibits some enzymes such as acetylcholinestrase or serine estrase in the slime mold *Polysphondylium violaceum,* since cultures of the slime mold on exposure to vapors of DDVP or eserine (a specific inhibitor of enzyme acetylcholinestrase) showed an increase in the number of aggregation centers and sorocarps. However, Clark (personal communication) does not now believe that the effect of DDVP on aggregation centers is mediated through acetylcholinestrase, because *Polysphondylium violaceum* produces a number of enzymes with estrase activity during development and while some of these enzymes are DDVP-inhibited, none of them is cholinestrase.

In *Bacillus subtilis,* malathion treatment accelerated starch and saccharose breakdown, but delayed the breakdown of sorbitol (Maleszewska 1974), suggesting the interference of malathion with catabolic enzymes. Enzymes of the bacterial respiratory cycle such as phosphatase and dehydrogenase have also been shown to be susceptible to the action of chlorfenvinphos (Rahmatullah et al. 1978). Some of the organophosphorus insecticides such as diazinon, phorate, and chlorpyrifos inhibited the ability of microorganisms to metabolize herbicides (Kaufman et al. 1970; Kaufman 1977; Anderson and Domsch 1980), thereby prolonging the herbicidal activity. This type of effect can be attributed to the competitive inhibition of enzymes (involved in the metabolism of herbicides) by organophosphorus insecticides.

There is a considerable interest in the regulation and properties of cytochrome P-450 system which is involved in a wide range of biological activities in higher organisms. It mediates the metabolism of numerous, structurally diverse substrates and catalyzes the metabolism, activation, and detoxification of many compounds of diverse nature. A variety of xenobiotics have been reported to increase the concentration of cytochrome P-450 in mammals. The induction of P-450 is dependent on the monooxygenase system in the endoplasmic reticulum of mammalian liver. Several species of yeasts have also been reported to contain cytochrome P-450 (Karenlampi et al. 1982). Recently, Karenlampi and his coworkers made an attempt to study whether such an induction of P-450 also occurs in microorganisms (Karenlampi et al. 1982). They found that lindane at 5, 10 or 50 ppm increased the cy-

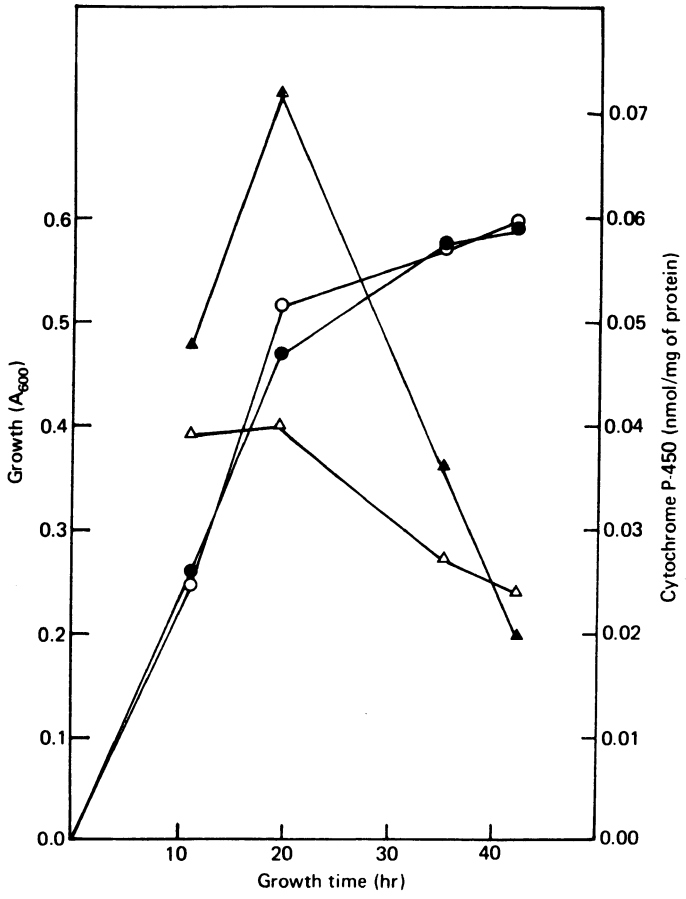


Fig. 4. Effect of lindane on growth and cytochrome P-450 in *Saccharomyces cerevisiae.* The yeast was grown on a medium containing 1% glucose as the main carbon source, with and without lindane. *Open circles* growth in the absence of lindane (A_{600}); *closed circles* growth in the presence of 10 μg lindane ml^{-1}; *open triangles* cytochrome P-450 from cells grown in the absence of lindane (nmol mg^{-1} protein); *closed triangles* cytochrome P-450 from cells grown in the presence of 10 μg lindane ml^{-1}. (Karenlampi et al. 1982)

tochrome P-450 content by 50% in *Saccharomyces cerevisiae* grown in 0.5% glucose medium with and without aeration (Fig. 4). However, with other insecticides such as mirex, Kepone and malathion no increase in cytochrome P-450 was observed. In another yeast *Torulopsis glabrata,* lindane increased cytochrome P-450 only by 25% while in *Kluyveromyces fragilis* lindane had no effect. Thus it was concluded that in contrast to mammals and other systems these insecticides are poor inducers of cytochrome P-450 in yeasts.

The inhibitory effect of malathion on citric acid production in *Aspergillus niger* was postulated as resulting from the direct action of insecticide on ATP production (Rahmatullah et al. 1978). To test this hypothesis ATP was added exogenously to growing cultures of fungi to which malathion had been added at 10, 50, and 90 ppm

and the citric acid contents were measured (Rahmatullah et al. 1979). Malathion under these conditions did not inhibit the production of citric acid. This indicates that malathion, by interfering with the oxidative metabolism in *Aspergillus niger,* reduced the production of cellular ATP, resulting in the decrease in citric acid production.

4 Nucleic Acids and Protein Synthesis

In an attempt to understand the mode of action of insecticides at the molecular level a number of investigations have been carried out to study the effects of these compounds on the synthesis of DNA, RNA, and proteins, the obvious reason being that any compound that would affect the synthesis of nucleic acids and proteins would also affect the growth of the microorganisms. However, the synthesis of nucleic acids and proteins does not appear to be the primary target for the action of most of the insecticides. The only significant compounds which seem to affect DNA primarily are DDVP and malathion. The mutagenic action of DDVP was reported for the first time in *Escherichia coli* (Lofroth et al. 1969) and was later confirmed in the same organism (Lofroth 1970; Bridges et al. 1973; Lawely et al. 1974; Wennerberg and Lofroth 1974; Green et al. 1974). DDVP has also been reported to be mutagenic in *Salmonella* and *Streptomyces* (Carere et al. 1978). As compared to DDVP, malathion is not a very potent mutagenic agent (McCann et al. 1975). Shiau et al. (1980) tested the DNA-damaging and mutagenic activity of malathion in *Bacillus subtilis* and *Salmonella typhimurium.* Malathion was found to be mutagenic and did not require metabolic activation. *Bacillus subtilis* was superior to *Salmonella typhimurium* for the detection of mutagenic activity.

The mutagenic action of DDVP and malathion may be attributed to the induction of DNA strand breaks (Green et al. 1974; Griffin and Walter 1978). Apart from this, DDVP (which is a potential methylating agent) also methylated isolated DNA and RNA from *Escherichia coli* (Lofroth et al. 1969). Bridges et al. (1973) showed that DDVP activity was identical to that of methyl methane sulfonate, a known methylating agent. However, attempt to localize the exact site of alkylation by Lawely et al. (1974) in DNA from DDVP-treated *Escherichia coli* cells, was not conclusive, though under in vitro conditions 3-methyl guanine was the methylating product. These findings were further confirmed by Wennerberg and Lofroth (1974). Since DDVP and methyl methane sulfonate do not differ in the type of strand breakage they cause the major DNA damage in microorganisms resulting from DDVP treatment seems to arise indirectly through alkylation and the consequent uncontrolled nuclease attack on DNA (Green et al. 1974). Carere et al. (1978) attributed the mutagenicity to the presence of the vinyliden chloride group in DDVP, contrary to the suggestion of Bridges et al. (1973) that mutagenecity results from the phosphoric moiety of the molecule.

The effects of most of the insecticides on the synthesis of nucleic acids and proteins appear to be secondary in nature, since the inhibition is induced only at very high concentrations. For instance, in *Crithidia fasciculata,* DDT at 425 ppm inhibited the synthesis of DNA and RNA (French and Roberts 1976) by affecting the up-

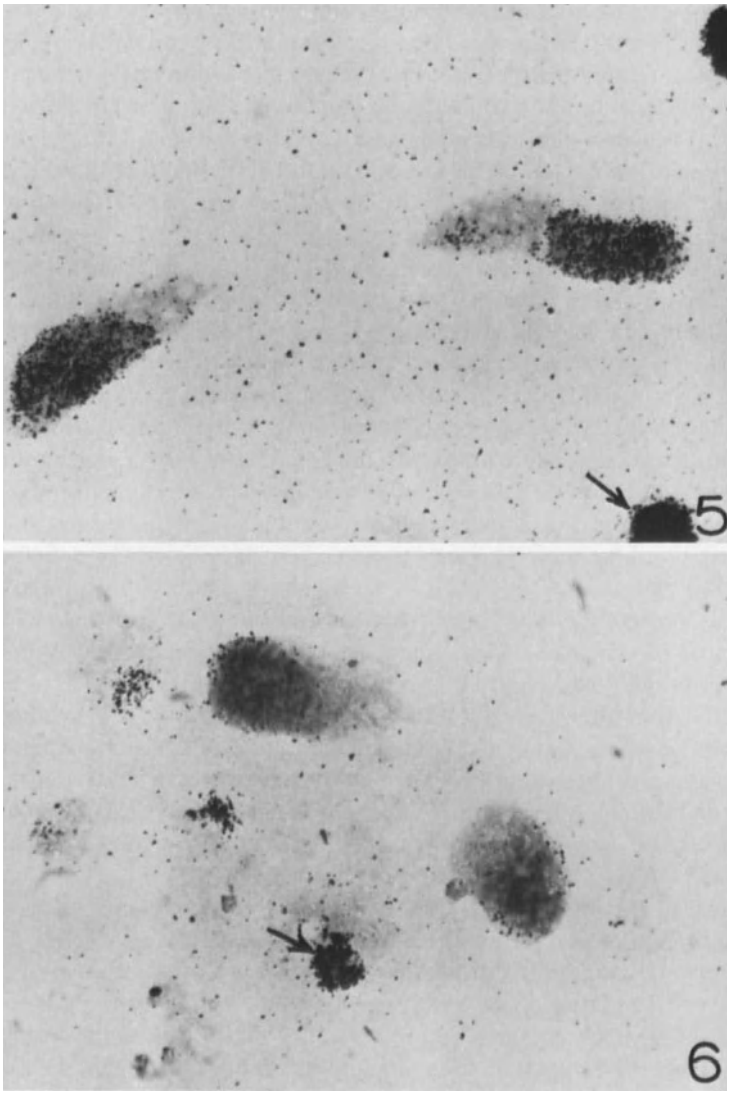


Fig. 5. Autoradiograph of untreated *Stylonychia notophora.* Animals were picked up in G_1 phase and provided with *Tetrahymena* prelabeled with ^{3}H-thymidine. After 6 h of feeding, animals were fixed and processed for autoradiography. At the time of fixation, the animals were in S-phase. The macronuclei clearly show incorporation of precursor. *Arrow* the food vacuole formed by ingested *Tetrahymena* ×450. (Lal and Saxena 1980 b)

Fig. 6. Autoradiography of *Stylonychia notophora.* Animals were picked up in S-phase and treated with 100 ppm DDT for 6 h. Simultaneously, they were provided with *Tetrahymena* prelabeled with ^{3}H-thymidine. At the end of 6 h of treatment and feeding, animals were fixed and processed for autoradiography. No incorporation of ^{3}H-thymidine is seen in the macronuclei (although the replication bands are present). *Arrow* ingested *Tetrahymena* ×450. (Lal and Saxena 1980 b)

take of precursors probably by interacting with the complex regulatory processes associated with the transport of precursors necessary for nucleic acid synthesis. In *Tetrahymena pyriformis,* treated with 50 ppm DDT, the incorporation of ^{3}H-thymidine was inhibited by 50% within 24 h of treatment and the inhibition was increased to 70% on the fifth day of treatment (Lal and Saxena 1979; Saxena et al. 1981). With 100 ppm DDT treatment the reduction in incorporation of ^{3}H-thymidine increased to 65% at the end of 24 h. The synthesis of RNA and proteins was also significantly inhibited. *Tetrahymena* treated with DDT when transferred to DDT-free medium after 3 days of treatment recovered from the effect of DDT within 24 h, suggesting that the damage caused by DDT is not permanent (Saxena et al. 1981). In another ciliate protozoan, *Stylonychia notophora,* 50 and 100 ppm DDT inhibited cell division completely in the G_1 and S phase (Lal and Saxena 1981) but the G_2 ciliates divided after a lag. In order to study the effect of DDT on DNA synthesis, *Stylonychia notophora* was provided with *Tetrahymena* prelabeled with ^{3}H-thymidine as the precursor source (as the ciliate was unable to take up the precursor directly) and simultaneously treated with 100 ppm DDT for 6 h and autoradiographed. The autoradiographs of such organisms did not show any incorporation of ^{3}H-thymidine; the macronuclei of corresponding controls were, however heavily labeled (Figs. 5, 6). *Stylonychia notophora* exposed to 100 ppm DDT also showed inhibition of RNA and protein synthesis throughout the cell cycle (Saxena and Lal 1981). The degree of inhibition of RNA and protein was dependent on the age of the ciliate and the time of treatment. Ciliates in the S phase were more susceptible to DDT than G_2 ciliates. Similarly, DDT at 100 ppm inhibited cell division, DNA, RNA, and protein synthesis in *Blepharisma intermedium* (Lal et al. 1981). The inhibition of DNA, RNA, and protein synthesis in these ciliates cannot be attributed to the effect of DDT on the transport of precursors through membrane because the treated ciliates *(Stylonychia notophora* and *Blepharisma intermedium)* took up the precursor through prelabeled *Tetrahymena,* but failed to incorporate it into DNA or RNA.

Lindane treatment in *Dunaliella bioculata* and *Amphidinium carteri,* also inhibited cell division and the synthesis of DNA and RNA (Jeanne-Levain 1974). The synthesis of DNA was strongly inhibited during the first cell cycle as compared to that of the second cell cycle. The latter effect was attributed to the adaptation of the organism to the toxicant and subsequent dilution and metabolism of lindane in the daughter cells. In *Dictyosteleum,* malathion at 10 ppm inhibited growth, DNA, RNA, and protein synthesis significantly (Bushway and Hanks 1976; Bushway 1978).

Lindane did not affect the growth of *Escherichia coli* and *Bacillus thuringiensis,* but inhibited growth in *Tetrahymena pyriformis* and *Euglena gracilis* (Puiseux-Dao et al. 1977). In *Acetabularia,* however, lindane slowed down the morphogenesis but DNA synthesis continued. It was also suggested that lindane acts on cellular membranes, as indicated by osmotic shocks in *Acetabularia,* and does not interact with cellular membranes of prokaryotes in order to explain why lindane affects eukaryotes but not prokaryotes.

Apart from organochlorine, organophosphorus, and carbamates which have been extensively used as insecticides, the aziridinyl group of alkylating agents have also been employed to check the growth of insect populations. In insects, aziridines such as tepa, thiotepa, and apholate block the synthesis of nucleic acids (Madhukar

et al. 1970; Gadallah and Nasar 1975). The possibility that such compounds may enter the environment and affect microorganisms cannot be ruled out, and studies on the cytological and biochemical effects of aziridinyl alkylating agents on microorganisms became necessary. The effects of metapa on cell division and the synthesis of nucleic acids and proteins were studied in the ciliate protozoans, *Stylonychia notophora, Blepharisma intermedium* and *Tetrahymena pyriformis* (Shivaji et al. 1975, 1978 a, b, c, 1979; Saxena et al. 1982). In all the three ciliates studied, concentrations of metapa above 100 ppm lysed the cells and at lower concentrations cell division was inhibited. At the macromolecular level, the primary target of action appeared to be the inhibition of the synthesis of DNA. In *Stylonychia* (Shivaji et al. 1975) and *Blepharisma* (Shivaji et al. 1978 b) metapa inhibited the G_1 cells from proceeding into the S phase and prevented the continuation of DNA synthesis in the S phase cells, thus implying that metapa inhibits both the initiation and continuation of DNA synthesis. However, metapa also inhibits the synthesis of RNA and proteins on these ciliates (Shivaji et al. 1978 a, c, d; Saxena et al. 1982) but this effect is apparently a manifestation of the effect of metapa on DNA synthesis. Recent studies have also indicated that metapa under in vitro conditions inhibited the DNA-dependent DNA polymerase synthesis of DNA by binding to the DNA template (Shivaji unpublished data).

5 Photosynthesis

The process of photosynthesis in which carbohydrates are synthesized from CO_2 and water is unique to green plants and autotrophic microorganisms. Photosynthesis consists of light and dark reactions. Adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide phosphate are synthesized in the light reaction, while CO_2 is reduced and incorporated into various organic compounds using ATP and reduced nicotinamide adenine dinucleotide phosphate during dark reaction. In algae, it appears that the detrimental effects of insecticides are probably mediated through photosynthesis. Insecticides can interfere with photosynthesis by affecting (1) the development and structural integrity of chloroplast, (2) the photochemical pathway involved in the conversion of radiant energy to chemical energy and (3) many biosynthetic pathways that are involved in the conversion of output products.

Earlier studies on the interaction of insecticides with algae have revealed that insecticides have some form of damaging effect on algae (Ukeles 1962). This has prompted further research on the effects of insecticides on photosynthesis in algae (Chacko et al. 1966; Wurster 1968; Bowes and Gee 1971; Bowes 1972; Mosser et al. 1972 a, b; Cole and Plapp 1974; Butler 1977). The effect of DDT on the morphology of the chloroplast in *Nitzschia delicatissima* indicated that even the lowest concentration (9.4 ppb) distorted the chloroplast, and at 100 and 1000 ppb the morphology of the chloroplast was completely destroyed (MacFarlane et al. 1971). Kopecek et al. (1976) reported that lindane at 10 ppm reduced the chlorophyll contents in *Ankistrodesmus braunnii* and *Anacystis nidulans,* whereas concentrations below 10 ppm were stimulatory. Malathion at 1 ppm has also been reported to inhibit photosynthesis by reducing chlorophyll production (Torres and O'Flaherty 1976). However,

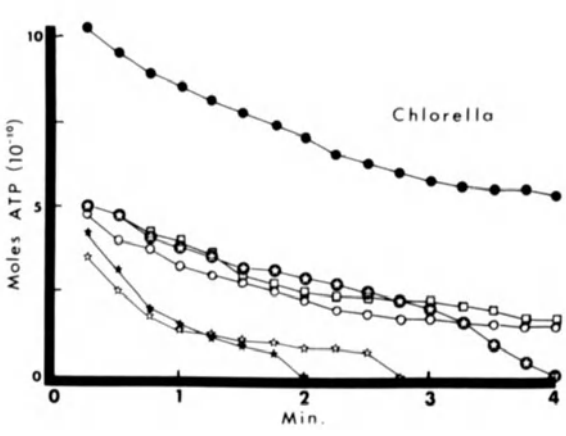


Fig. 7. ATP levels (measured after 4 min in luciferin luciferase bioluminescence test) in culture of *Chlorella ellipsoidea* after 3 days exposure to aldrin *(open squares),* dieldrin *(open circles),* DDT *(solid stars),* diazinon *(stars in circles),* chlordane *(open stars)* or no insecticide *(solid circles).* (Clegg and Koevenig 1974)

malathion did not inhibit chlorophyll production in *Chlorococcum hymnosphorum.* Lower concentrations of malathion (0.1 and 0.55 ppb) even stimulated chlorophyll production in *Chlorella, Tribonema,* and *Oscillatoria.* Torres and O'Flaherty (1976) also observed a synergistic interaction between two triazine herbicides and malathion on the effect of chlorophyll production in algae. Chlorophyll contents were reduced to a greater extent when treated with a combination of pesticides.

Clegg and Koevenig (1974) studied the effects of five insecticides (DDT, aldrin, chlordane, dieldrin, and diazinon) on the light reaction of photosynthesis (by measuring the production of ATP). Three species of algae *Chlorella ellipsoidea, Chlamydomonas* sp. and *Euglena gracilis* were exposed to 100 ppm of each of the insecticides separately for 3 days and then ATP was extracted and assayed. Exposure of *Chlorella* to any one of the above insecticides reduced by half or more the amount of ATP that was detected after 15 seconds of shaking (Fig. 7). However, exposure of *Chlamydomonas* culture to diazinon, aldrin, dieldrin chlordane and DDT reduced ATP levels by 20 to 25% (Fig. 8). Exposure of *Euglena* to chlordane reduced ATP levels by 40%, while aldrin and dieldrin were less effective (Fig. 9). DDT and diazinon had no significant effect after 15 s, but after 2 min the amount of ATP was significantly lowered. However, population levels of these algae were not

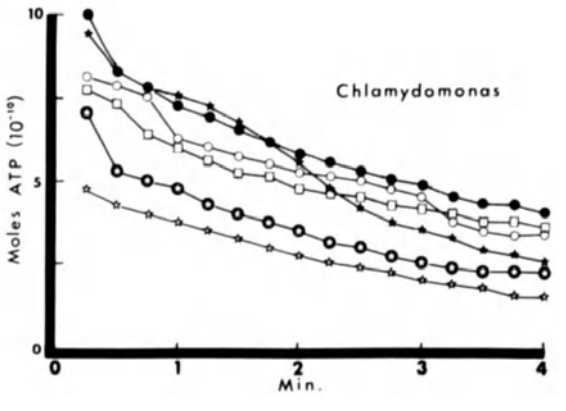


Fig. 8. ATP levels (measured over 4 min in luciferin luciferase bioluminescence test) in cultures of *Chlamydomonas* sp. after 3 days of exposure to aldrin *(open squares),* dieldrin *(open circles),* DDT *(solid stars),* diazinon *(stars in circles),* chlordane *(open stars),* or no insecticide *(solid circles).* (Clegg and Koevenig 1974)

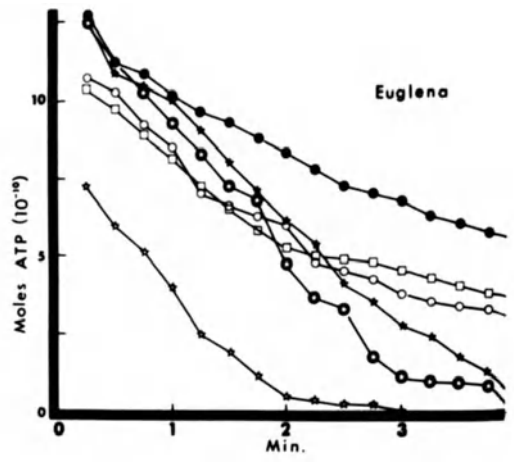


Fig. 9. ATP levels (measured over 4 min in luciferin luciferase bioluminescence test) in cultures of *Euglena elastica* after 3 days of exposure to aldrin *(open squares),* dieldrin *(open circles),* DDT *(solid stars),* diazinon *(stars in circles),* chlordane *(open stars),* or no insecticide *(solid circles).* (Clegg and Koevenig 1974)

affected by any of the insecticides, suggesting that these insecticides interfere with photophosphorylation during light reaction of photosynthesis.

As the insecticides interfere with the production of ATP due to their effect on photophosphorylation in the light reactions, this will obviously affect the CO_2 fixation, resulting in a decrease in the concentration of sugars. A few studies on the effects of insecticides on photosynthesis in algae have generally relied upon measurements of the rate of fixation of ^{14}C-labeled bicarbonate. Bowes and Gee (1971) showed that in marine algae, DDT and DDE altered the morphology of chloroplast and inhibited $^{14}CO_2$-fixation. Wurster (1968) found that fixation of $^{14}CO_2$ in four species of marine algae declined as the concentration of DDT was increased to 100 ppb. Menzel et al. (1970) observed that DDT affected $^{14}CO_2$-uptake differently in different marine algae, and suggested that this was due to differential penetration of insecticides through the cell walls and membranes in different organisms.

Inhibition of photosynthesis in algae treated with insecticide has also been reported on the basis of ^{14}C-uptake per unit volume culture (Wurster 1968; Menzel et al. 1970; Batterton et al. 1971; Luard 1973; MacFarlane et al. 1971) and per cell (MacFarlane 1971; Fisher 1975) and in some cases mechanisms have been proposed. However, it is not universally agreed that decrease in carbon fixation in treated cultures is necessarily the result of an interference with photosynthesis. Fisher (1975), observing a decrease in ^{14}C-uptake per cell in *Thallassiosira pseudonana,* suggested that reduced photosynthesis in DDT-treated (growth-inhibited) culture was only due to the presence of fewer photosynthesizing cells, implying that primary impact of the insecticide was on cell division rather than on photosynthesis. This has been further confirmed by Powers et al. (1979) in *Exuviella baltica.* However, Simonis and Lee-Kaden (1979) reported that in *Anacystis nidulans,* lindane affects $^{14}CO_2$-fixation primarily resulting in the suppression of protein synthesis caused by a depletion of intermediates of CO_2-fixation.

Lee et al. (1976) investigated the effect of DDT on photosynthesis in *Selanastrum capricormutum.* Their studies revealed that DDT concentrations (between 3.6 and 36 ppb) inhibited photosynthetic CO_2-fixation and that the inhibition was dose-dependent. The incorporation of ^{14}C from $^{14}CO_2$ indicated that DDT stimulated the incorporation of ^{14}C into glycolic acid, a major compound of photorespiration, and

caused the concomitant suppression of flow of ^{14}C into aspartic acid, a major component of the C_4-dicarboxylic acid pathway. This shift from an efficient pathway into a nonefficient pathway by DDT was also interpreted as being caused by interruption of cyclic photophosphorylation.

Derby and Ruber (1970), unlike in the other studies, used oxygen output as the variable to study the effect of 100 ppb fenthion and temephos on the photosynthetic activity in *Skeletonema, Dunaliella,* and *Phaeodactylum.* Inhibition of oxygen output after treatment with fenthion was maximum in *Skeletonema* followed by *Cyclotella* and *Dunaliella,* whereas temephos reduced oxygen output to maximum in *Phaeodactylum* followed by *Dunaliella, Skeletonema,* and *Cyclotella.* Glooschenko and Lott (1977), using oxygen output as a measure of photosynthesis, reported that chlordane at 0.1 to 100 ppb in *Scenedesmus quadricauda* and 0.1 to 50 ppb in *Chlamydomonas* sp. stimulated respiration rate, which increased with increasing concentration. Similar stimulatory effects were produced using 2,4 dinitrophenol (DNP), a known uncoupling agent of oxidative phosphorylation.

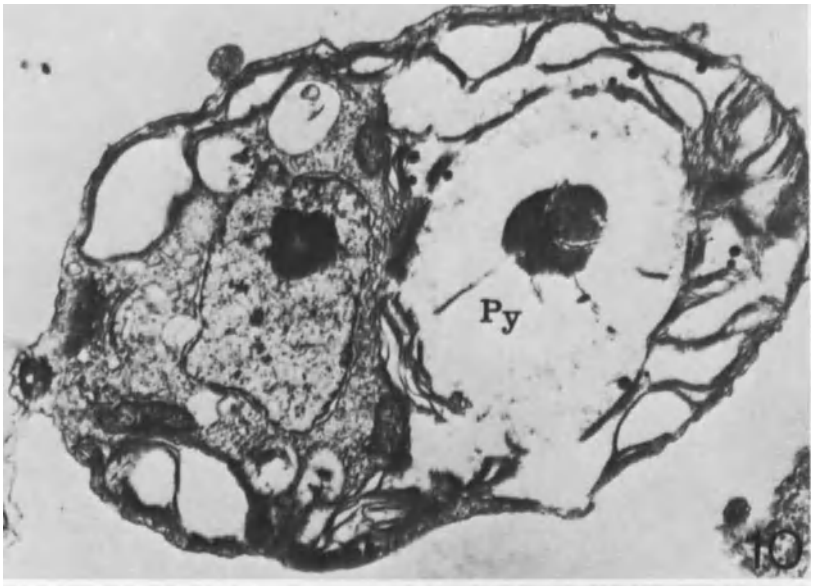
6 Cell Morphology

Protozoan *Stylonychia notophora* treated with 100 ppm DDT revealed several abnormalities in the nuclear apparatus (Lal and Saxena 1980 b). Continuous treatment of 100 ppm DDT for 9 h resulted in deep incisions in the macronuclei. The relationship between micro and macronuclei was also disturbed. Further treatment up to 18 h with 100 ppm DDT, showed nuclei with loose chromatin, while treatment beyond 18 h fragmented the macronuclei into spherical bodies. In *Crithidia fasciculata,* DDT at 425 ppm changed the architecture of plasma membrane and produced mitochondrial swelling (French and Roberts 1976).

In *Dunaliella bioculata,* lindane at 10 ppm altered the number of cellular organelles and caused degeneration of the nuclear apparatus (Jeanne-Levain 1979). In addition, in *Dunaliella bioculata* lindane at 10 ppm caused morphological changes such as enlarged cells, increase in the number of Golgi apparatus, endomultiplication of organelles (Figs. 10 and 11) and large vacuoles (Fig. 12). *Amphidinium carteri* treated with lindane at 3 ppm showed abnormal partitioning of the nucleus (Fig. 13) and decrease in the number of microtubules (Figs. 14 and 15). Earlier studies at the ultrastructural level in *Dunaliella* and *Amphidinium* revealed that lindane treatment markedly altered the structure of plastids (Puiseux-Dao et al. 1977). In *Acetabularia* the basal part of the plastids the lamellae were extended with one or several carbohydrate grains, whereas in the apical part small chloroplasts with numerous thalakoid and with small polysaccharide granules were observed (Borghi et al. 1973).

7 Conclusions and Future Prospects

Little information is available on the cytological and biochemical effects of insecticides in microorganisms, thus making it difficult to point out their modes of ac-



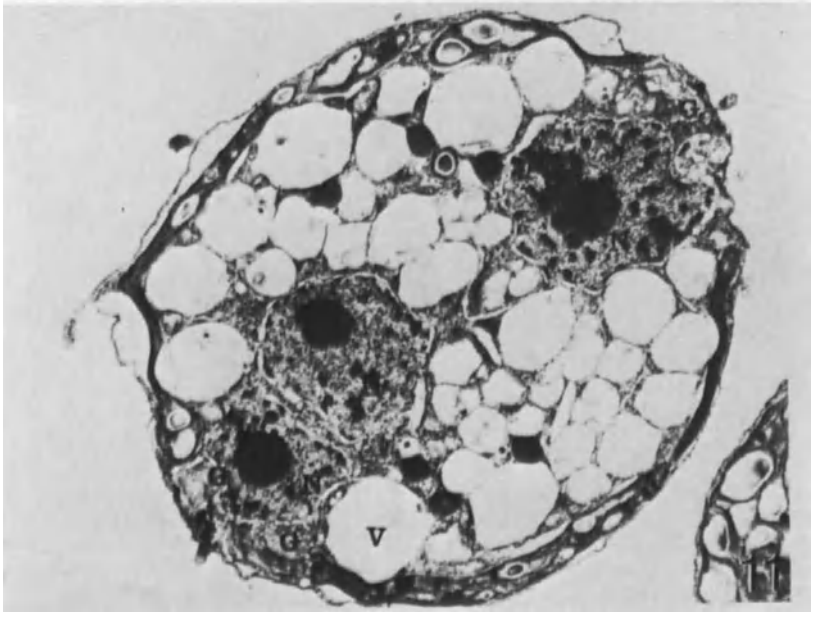


Fig. 10. *Dunaliella bioculata:* Control. *Py* pyrenoids. × 12,000. (Jeanne-Levain 1979)

Fig. 11. *Dunaliella bioculata:* Cell observed after 7 days treatment with lindane (10 μg ml^{-1}). *N* nucleus; *G* dictyosome; *V* vacuole. × 10,000. (Jeanne-Levain 1979)

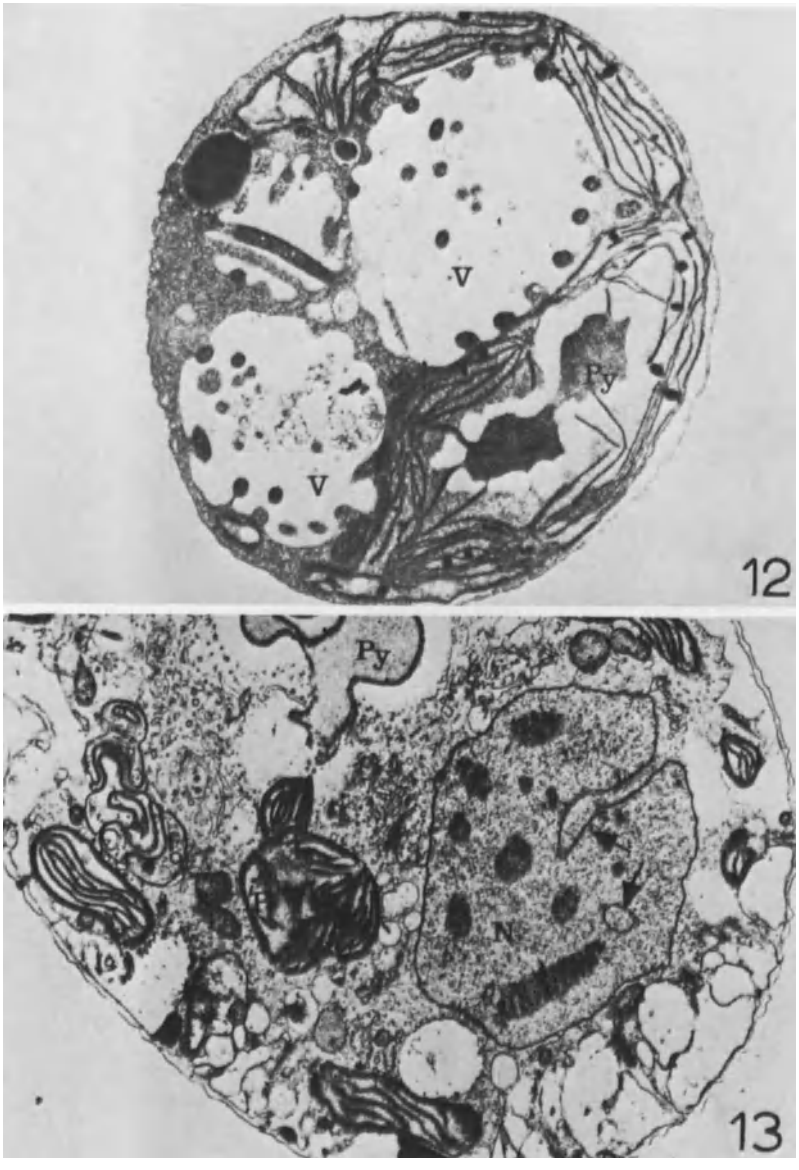


Fig. 12. Electron micrograph of *Dunaliella bioculata* treated with lindane (20 μg ml^{-1}) during 5 days. Note particularly the large vacuoles full of lytic particles that appear in the major part of the cell. *Py* pyrenoids; *V* vacuole. ×8000. (Jeanne-Levain 1979)

Fig. 13. Electron micrograph of *Amphidinium carteri* treated with lindane (0.3 μg ml^{-1}) during 3 days. The nucleus in dividing; this is marked by the two cytoplasmic channels which can be observed inside *(arrows). Py* pyrenoids; *N* nucleus. ×10,000. (Jeanne-Levain 1979)

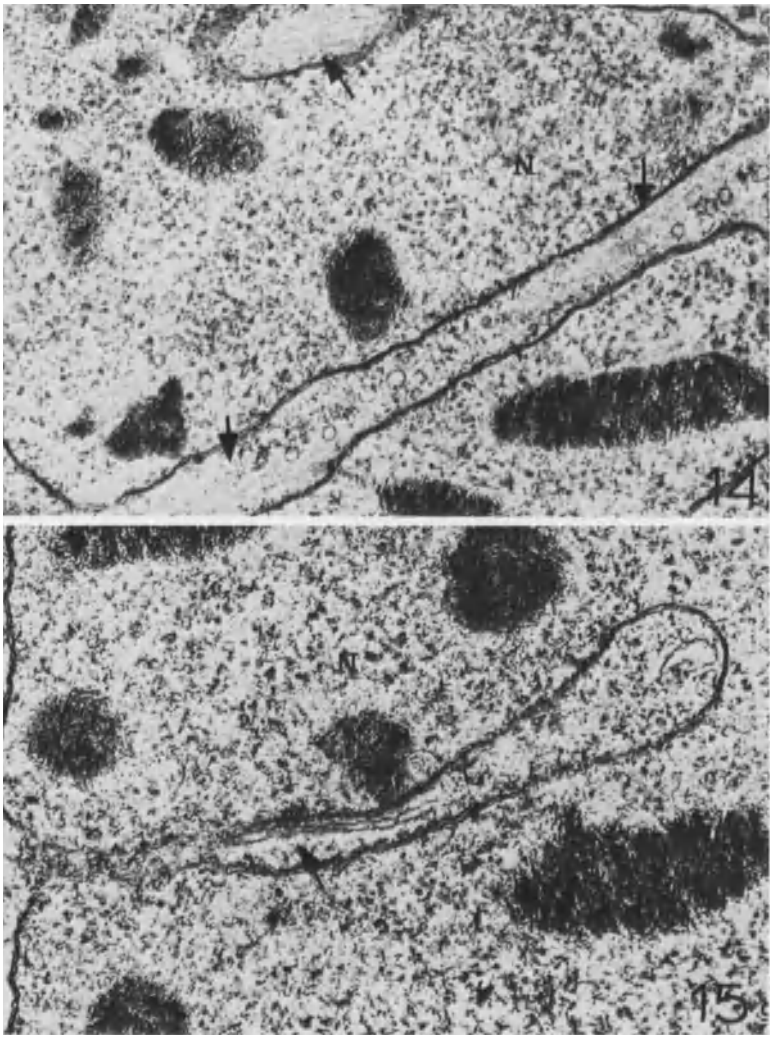


Fig. 14. Electron micrograph of nucleus (*N*) of *Amphidinium carteri* in a dividing control cell. Inside the channels, numerous microtubules *(arrows)* can be observed. ×35,000. (Jeanne-Levain 1979)

Fig. 15. Electron micrograph of a dividing nucleus (*N*) observed in *Amphidinium carteri* after 3-day treatment with lindane (0.3 μg ml^{-1}). Only a few microtubules *(arrow)* are observed in the channel. ×35,000. (Jeanne-Levain 1979)

tion, and it is only under certain fortuitous circumstances that generalization can be made. Further the physiological expressions of interaction between insecticides and microorganisms are very diverse and depend on the type and complexity of the organism. The effects at the level of plasma membrane, photosynthesis, mutations, DNA, RNA and protein synthesis, enzymes, and metabolic pathways are known. Most of the insecticides seem to alter primarily the structure and function of plasma

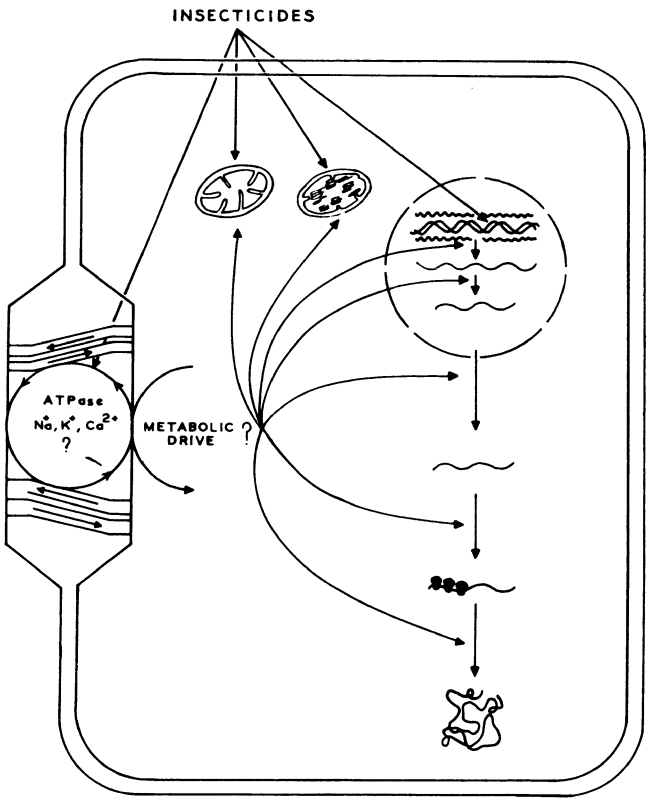


Fig. 16. Model illustrating possible targets for insecticide interaction at cellular level in microorganisms

membrane, which may lead to many changes of secondary nature such as change in structure of cellular organelles and synthesis of nucleic acids and proteins. Insecticides such as DDVP and malathion are also mutagenic and capable of alkylating DNA, which may alter the transcription and translation activities, leading to many morphological changes. However, the mutagenic effects of insecticides on microorganisms have not been explained due to the effect of insecticides on membranes. A generalized scheme for the action of insecticides on different targets in microorganisms is shown in Fig. 16.

In autotrophic organisms insecticides seem to affect the photophosphorylation in the light reaction of photosynthesis. The first effect observed after the application of photosynthesis inhibiting insecticide is a reduction in CO_2-fixation, resulting in a decrease in the concentration of soluble sugars. Subsequent changes may alter the chloroplast anatomy and morphology. From a survey of the literature, it is also evident that studies on the following parameters should be carried out in order to point out the primary site of action:

1. Identification of subcellular fractions accumulating maximum amount of insecticide. This can be accomplished by the use of both labeled and unlabeled insecticides and autoradiographic studies.

2. A concentration relationship should be established over the total effective range from initiation of symptoms to death. Time-course studies to follow the se-

quence of events that lead to death might prove helpful, since delay in the onset of inhibition may indicate that the applied insecticide is not the inhibitor but must be metabolized to the inhibitor in order to function.

3. Another aspect of time-course relation is the degree of reversibility as related to recovery. When an organism recovers, it suggests either a detoxification mechanism or that the insecticide is actually lost from the microorganisms by leakage.

4. Preliminary studies should also be designed to identify large cytological and biochemical areas affected. All this should be evaluated in the same organism. This should be followed by in-depth study into particular areas which might be suspected as the primary target.

5. Recognition of the symptoms should also be carried out. For example, if chlorosis is an early symptom it would indicate that chloroplast development and possibly chlorophyll synthesis has been blocked.

6. Finally, metabolic studies on purified in vitro systems and correlation between in vivo and in vitro systems may shed light on the primary mode of action of insecticides.

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