THE INTERACTION OF INSECTICIDES AND THE SOIL MICROFLORA

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ABSTRACT

Three organophosphorus insecticides, malathion, sumithion and menazon stimulated the respiration of soil by varying amounts, and in two cases this was correlated with an increase in the bacterial count.

The insecticides had no effect on the anaerobic population, but malathion reduced the number of actinomycetes. Sumithion reduced the number of fungi and yeasts in the soil, but its hydrolysis product, 3-methyl-4-nitrophenol, was found to be the toxic compound. This compound also inhibited the growth of fungi and yeasts in pure culture and the germination of fungal spores. Malathion was found to be toxic at high concentrations to some Gram positive bacteria in pure culture.

The persistence of the insecticides in soil was determined using both colorimetric and bioassay techniques. Menazon is probably the most persistent, but using the radioactive compound, it was found that adsorption phenomena in the soil reduce the recovery by the usual techniques. Malathion was the least persistent, but chemical decomposition probably accounts for most of the loss from soil.

Isolates were obtained from the soil, which were apparently able to grow in the presence of the insecticides as the sole carbon source. However, malathion decomposes rapidly in solution and in this

case the isolates utilise the decomposition products. An actinomycete was isolated which could actively decompose malathion in pure culture, but the pathway was not investigated.

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INTRODUCTION

With the growing use of complex organic chemicals in Agriculture, it was almost inevitable that unpredicted effects would occur. The death of many forms of wildlife and the infertility of many birds has been attributed to the accumulation of the persistent chlorinated hydrocarbon insecticides within their tissues. It is not only wildlife that has been affected. At a Symposium held in the United States of America and organised by the National Academy of Sciences-National Research Council in 1966, it was stated that all Americans have between 4 and 7 parts per million of detectable DDT and related compounds in their tissues and are likely to receive more. Whilst literature such as 'Silent Spring' by Rachel Carson may be alarmist in outlook, it would be inadvisable to ignore completely the growing volume of reports in scientific literature of serious consequences caused directly by pesticides.

For practical purposes the term "pesticide" includes herbicides, fungicides, nematicides and insecticides and within each group the range of compounds is large. This thesis will therefore be entirely concerned with insecticides and the growth of literature on these alone shows the importance which is now being placed upon research into this important group of chemicals, in order to understand the implications of their unrestricted use.

The chemicals in use as insecticides range from the general toxicants such as hydrogen cyanide, to the highly selective systemic compounds such as menazon. The chemistry of the insecticides is beyond the scope of this thesis but reviews on the subject have been made by Brown (1951), Martin (1956), Dahm (1957), Spencer and O'Brien (1957), Fukuto (1961), Casida (1963) and Schrader (1963).

One of the main criteria for choosing an insecticide for general use is whether or not it is toxic to man, although until recently chemical companies did not place so much emphasis on this point. The mammalian toxicity of insecticides is frequently expressed as the LD_{50} for female albino rats being measured in milligrammes active ingredient per kilogram body weight. It has been calculated that an LD_{50} of less than 50 mg. a.i./kg. body wt. presents considerable risks to man (Edson, 1958). Unfortunately the sensitivity of animals from species to species tends to vary (Cramp, 1966) and this has contributed to the unpredicted effects on wildlife.

For many insecticides the phytotoxicity has been determined for the plants which are expected to be treated and effects upon other plants are rarely investigated.. A comparison of the toxicity of insecticides to animals and plants shows that it is impossible to predict one from a knowledge of the other. OMPA or schradan* with an LD_{50} of

*Full chemical names of all insecticides mentioned in the thesis have been placed in Appendix.

1.5 mg. a.i./kg. body wt. is considered to be non-phytotoxic at recommended rates of application, whereas dibrom with an LD_{50} of 430 mg. a.i./kg. body wt. is injurious to a wide variety of plants (Thomson, 1963). This necessitates the separate determination of the toxicity of insecticides to plants, and this must be extended to include microflora. However, since soils vary considerably in relation to such factors as pH and organic matter content, generalisation becomes even more difficult. Concern has been expressed by many microbiologists that the use of insecticides might affect the fertility of the soil and, in particular, emphasis has been placed upon the possible effects on microbial nitrogen fixation and the process of ammonification. The view has been expressed by some agriculturalists that disruption of the soil microflora need not give rise to concern, since it is thought that chemical fertilisers could substitute for the products of their activities. However McCalla (1946) outlined the ways in which the soil micro-organisms, as a dynamic population, aid in keeping the soil stabilised and able to support the growth of higher plants by maintaining good aeration and drainage of the soil.

The importance of soil micro-organisms is generally accepted by microbiologists and a large volume of studies have been carried out to ensure that the soil is not affected detrimentally by insecticides. The techniques of study have varied, making some of the results obtained difficult to evaluate.

In particular field trials with insecticides can give misleading results, due to the inconsistancy of the weather, and the mode of application may result in inadequate mixing with the soil. This means that even when great care is taken over sampling, the results may not indicate the true pattern of behaviour. Robinson and Mesmer (1958) admitted that their field trials with DDT and BHC gave such variable results that no conclusions could be drawn.

However, to obtain a complete picture, study of the action of insecticides under practical conditions is as important as studying their effect under laboratory conditions. On taking the combined results of all studies it is possible to give a reasonable account of the effects of many insecticides.

It is not proposed to review the literature relating to all insecticides, since the older ones which are common toxicants and the plant products such as pyrethrin, rotenone and nicotine are gradually being replaced by the synthetic compounds. Mention must be made of some of the chlorinated hydrocarbons which constitute the bulk of the literature and were the first of the synthetic chemicals to be used on a large scale.

ACTION OF INSECTICIDES UPON THE SOIL MICROFLORA

There have been some reviews on this topic mainly covering the earlier research. The review by Lochhead (1952) gives

a short account of the earlier work with the chlorinated hydrocarbons. Eno (1958) and Bollen (1961) give some more recent results including work with organophosphorus compounds. Domsch (1963) gives in his long review, on pesticides in general, a good account of the effect of a large range of compounds.

Effect on total numbers of organisms and soil respiration:-

The general effect of insecticides has received the attention of many workers, and there is much evidence that in the case of chlorinated hydrocarbons there is little effect at normal application rates. Wilson and Choudhri (1946) found that DDT at 5% dry wt. soil had no effect on many processes. Eno and Everett (1958) showed that at 100 ppm a number of chlorinated hydrocarbons had little effect after one or even 16 months.

However, stimulation has been shown to occur. Bollen, Morrison and Crowell (1954) showed that the gamma-isomer of BHC increased the total number of soil organisms and the other isomers also gave small increases. This was confirmed by Fletcher and Bollen (1954) who also showed that aldrin at 200 and 1000ppm stimulated aerobic spore forming bacteria. Eno and Everett (1958) found that carbon dioxide production was increased by toxaphene, dieldrin, TDE, DDT and BHC.

Toxic effects of the chlorinated hydrocarbons have however also been reported. Smith and Wenzel (1947) showed that 400 lb./acre of DDT caused temporary damage followed by

recovery of the organisms.

In general the organophosphorus insecticides tend to stimulate the total numbers of soil micro-organisms. Naumann (1960) showed that parathion up to 1% increased numbers. Sideropoulos, Adams, Laygo and Schulz (1963) reported that while 5 - 25 lb./acre of systox, thimet and malathion had no effect in the field, in the laboratory at 40 - 150 ppm, all of them promoted an increase in the population and an increase in carbon dioxide production. Diazinon which was also examined, had an even greater effect in the laboratory studies but also stimulated an increase in population of bacteria in the field, at a level of 10 lb./acre.

Of all the organophosphorus insecticides, only parathion has so far been reported to be toxic to bacteria and this occurred at rates of 250 ppm (Bollen, 1961) and at 100 lb./acre (Eno, 1958).

Effect on specific groups of micro-organisms:-

The effect of insecticides on specific groups of organisms enables one to make a more accurate assessment than the general picture obtained from viable counts on a single medium, which necessarily limits the organisms studied. The research reported below was probably carried out with this in mind.

a) Fungi:-

Smith and Wenzel (1947) showed that BHC at 100 and 500 lb./acre was fungicidal and Bollen et al (1954) that it was the alpha- and beta-isomers that were toxic, although the delta- and gamma-isomers increased the number of moulds. In the following paper they reported that DDT and BHC in several types of soil reduced the mould population although aldrin was toxic in only one trial. Eno (1958) reported that chlordane at 50 ppm would also reduce the number of fungi.

There has been evidence of increases in the numbers of fungi due to insecticides. Bollen et al (1954) showed that toxaphene significantly increased the numbers of fungi, which was also reported by Fletcher and Bollen (1954). Dieldrin also increases the number of fungi in the soil (Eno and Everett, 1958)

Of the organophosphorus compounds only thimet, malathion, systox and diazinon appear to have been studied with regard to their effect on the fungi and these have no significant action (Sideropoulos et al, 1963).

b) Actinomycetes:-

Bollen et al (1954) made a study of the effect of various chlorinated hydrocarbon insecticides on the actinomycetes in the soil. They found that while gamma-BHC caused a depression in numbers, other insecticides studied had little effect.

Organophosphorus compounds have received little study in relation to actinomycete populations, but Sideropoulos et al (1963) showed that in field trials at levels of 5 - 25 lb./acre and in the laboratory at 5 - 1500 ppm, malathion, thimet, systox and diazinon had no observable effect.

c) Nitrifying organisms:-

The effect of insecticides upon nitrification is generally determined by adding an inorganic ammonium compound to the soil and measuring the rate at which nitrite and nitrate are formed in the presence and absence of the insecticide.

Brown (1954) showed that a wide range of chlorinated hydrocarbon insecticides are toxic to nitrifying bacteria at concentrations greater than 0.5% and this was confirmed by Fletcher and Bollen (1954) using BHC and chlordane, although 0.05% BHC was toxic in solution but not in the soil. Jones (1956) found that many chlorinated hydrocarbon insecticides become toxic at levels of 0.1% or above, but Eno and Everett (1958) found that with heptachlor, lindane and BHC, the toxic level was lower i.e. 100 ppm (0.01%).

In contrast to the above results, Bollen et al (1954) and in a separate paper Fletcher and Bollen (1954) reported that gamma-BHC at 137.5 ppm in field studies increases the rate of nitrification, which was decreased in the laboratory. This was confirmed by Gray (1954). Wilson and Choudhri (1946)

using DDT at 0.5% found little effect on nitrification. Concentrations less than this were used by Fletcher and Bollen (1954), and Shaw and Brooks (1960) who also found that at 50 ppm aldrin, dieldrin, chlordane and heptachlor had little effect.

No results have been published of harmful effects of organophosphorus compounds on nitrification, and schradan at 3000ppm was reported to increase the number of Azotobacter in the soil (Bollen, 1961).

d) Ammonifying organisms:-

Ammonification in the soil is generally determined by adding a proteinaceous material such as peptone or blood to the soil and determining the ammonia produced. Jones (1956) found evidence that DDT, chlordane and BHC became inhibitory at concentrations of 1000 ppm; the inhibition being greater after one year than at the end of one week, but endrin only became toxic at levels above 1000 ppm. Eno and Everett (1958) showed that heptachlor, lindane and BHC become toxic at levels up to 100 ppm.

Wilson and Choudhri (1946) could find no effect of DDT on ammonification at 5000 ppm and Fletcher and Bollen (1954) confirmed this at levels up to 1000 ppm. Jones (1956) found that methoxychlor, dieldrin and aldrin at a level of 10,000 ppm caused no observable effects.

Bollen et al (1954) showed that the delta- and gamma-isomers of BHC increased the rate of ammonification of peptone, and Eno and Everett (1958) reported that toxaphene, TDE and DDT up to 100 ppm also increases the rate.

No harmful effects caused by organophosphorus insecticides have been recorded.

e) Effect on other micro-organisms:-

Wilson and Choudhri (1946) found no observable effect of DDT at up to 2% on nodulation in leguminous plants, and isolated nodule bacteria grew in the presence of the insecticide. Appleman and Sears (1946) also showed that DDT was not toxic at 100 lb./acre. Payne and Fults (1947) however, noted that use of 103 lb./acre of DDT reduced by more than half the numbers of nodules on bean roots. Chlordane at 10 lb./acre had no effect on nodulation (Simkover and Shenefelt, 1951). Brakel (1963) showed that isolated organisms from nodules of Trifolium, Medicago, Phaseolus and Pisium species exhibited different sensitivity towards lindane, aldrin and parathion.

DDT at up to 0.1% was found to have no effect on sulphur oxidising bacteria (Fletcher and Bollen, 1954), and Jones (1956) noted no effect after three years at 0.5% in soils with a high organic matter content. In sandy soils 0.1% was shown to be toxic, although BHC and chlordane were found to be toxic at 0.01% in all the soils used.

Work by Gray (1954) indicates that BHC reduces the hydrolysis of urea by mixed and pure cultures of bacteria in liquid culture but not in the soil. In a study of heterotrophic bacteria Gray also reported that BHC inhibited the growth of many organisms in pure culture, which confirmed the work of Fromageot and Confino (1948).

Mayhew (1965) studied the effect of malathion and menazon at 50 and 100 ppm on various organisms in pure culture. Malathion and menazon both increased the growth of Azotobacter vinelandii in liquid culture. Malathion caused a delay in the degradation of cellulose by Sporocytophaga sp. in liquid culture, but menazon had no effect. Malathion increased the rate of nitrite formation by a species of Nitrosomonas, isolated from the soil, in liquid culture. Menazon had no effect at 30°C. but at 25°C. it increased nitrite production.

PERSISTENCE OF INSECTICIDES IN SOIL

The chlorinated hydrocarbon insecticides have become notorious for their persistence, which although giving residual activity, is also the cause of the toxic side effects to wildlife and the build up of residues in the body of man.

A number of estimates have been made of the persistence of the chlorinated hydrocarbon insecticides using a variety of methods, and DDT has been found to be probably the most persistent. The relative persistance of some of the more

common insecticides compared with DDT, which is estimated to persist for 20 - 40 years, is as follows:-
DDT > dieldrin > lindane > chlordane > heptachlor > aldrin (Wheatley, 1965). The lack of persistence of aldrin is due to its conversion to dieldrin in the soil (Lichtenstein and Schulz, 1959 and 1960), where it may persist for 20 - 30 years (Wheatley, 1965). Terriere and Ingalsbe (1953) reported that toxaphene, BHC and chlordane persist for at least 3 years in a sandy soil. Kiigemagi, Morrison, Roberts and Bollen (1958) showed that heptachlor at 5 ppm would remain in the soil for 6 years.

The organophosphorus compounds have been regarded as being of short persistence compared with the chlorinated hydrocarbon insecticides, being decomposed by physical and chemical means, which is dependent on the stability of the individual insecticides. Parathion was shown to have little activity in the soil after 3 months, as determined by bioassay (Mulla, 1960), and Lichtenstein and Schulz (1964) demonstrated that only 3.0% of the applied insecticide remained after 90 days using both bioassay and colorimetric techniques. Parker and Dewey (1965) showed that in the laboratory 50% of phorate was lost from the soil in one week, although up to 10% remained for one month, as estimated by a bioassay technique. In the field the loss was even faster although dimethoate persisted slightly longer. Malathion disappears rapidly from the soil,

the results by both bioassay and colorimetric determinations indicating that none remains active 3 years after application of 108.6 lb./acre (Roberts, Chisholm and Koblitsky, 1962). Lichtenstein and Schulz (1964) observed that 85% of the applied malathion, at 5 lb./acre, disappeared in 3 days and only 3.1% remained after 8 days. However, this is in contrast to the work of Ruppel and Mok Yun (1965) who sprayed the soil with emulsifiable concentrate and showed 24% mortality, after 20 days, of the cereal leaf beetle which was used for the assay. At 100 mg./ft.^{2} malathion sprayed on ply-wood panels remained active against mosquitoes for 15 - 17 months (Spiller, 1961).

Mulla (1960) reported little activity of guthion, sevin, thiodan and delnav after 3 months, although Roberts et al (1962) showed that 0.84% of the applied guthion remained after 3 years. Mulla (1964) estimated that chlorthion had disappeared completely after 22 months and in the case of sumithion, only 2% of that applied remained, as determined by a bioassay technique. Menn, Patchett and Batchelder (1960) concluded that micro-organisms decompose trithion because it was found that it persisted longer in soils autoclaved or fumigated with vapam than in untreated soil, although the time taken for 50% to disappear in three soils was 200, 100 and 170 days respectively. Since 50% disappeared from aqueous solution in 115 days, the effect of sterilisation may be different than the obvious one of destroying the soil microflora.

Way and Scopes (1965) using a bioassay technique showed that 90% of the applied phorate disappeared in 18 months. They showed that menazon was slightly more and thionazin less persistent.

ADSORPTION OF INSECTICIDES IN SOIL

The adsorption of insecticides in soil is an important factor affecting their persistence and also their insecticidal activity. Adsorption may be responsible for errors in estimating persistence, since the first stage in most methods is their extraction from the soil with solvents. Bailey and White (1964) have reviewed the factors involved in adsorption in the soil which are:- organic matter and clay content of the soil, the solubility of the insecticide, the functional groups of the insecticide, the pH of the soil, the soil moisture content, temperature and the formulation of the insecticide.

Lichtenstein and Schulz (1959) showed that there was a greater recovery of DDT, aldrin and lindane from a muck soil than a Miami silt loam after $3\frac{1}{2}$ years, although the higher the temperature the faster they disappeared. Getzin and Chapman (1959) found that phosdrin was bound in the soil in proportion to the organic matter content. The quantity of phosdrin adsorbed onto 100 g. aliquots of soil was 127 µg. in a peat soil, 10 - 27 µg. in a heavy loam and 3 - 8 µg. in a sandy soil. Getzin and Chapman (1960) reported a similar effect with ^{32}P-labelled phorate.

The phorate apparently disappeared rapidly from the soil but they found that 25% was firmly bound in a sandy soil and 82% in a muck soil; the bound fraction was not recoverable by normal procedures. Bowman, Schechter and Carter (1965) demonstrated that leaching, volatilisation and degradation of many chlorinated hydrocarbon insecticides were less in soils having a high organic matter content. Harris (1964) showed that in dry soil insecticides are less toxic due to adsorption. Hadaway and Barlow (1951) and Barlow and Hadaway (1958) found that sorption of insecticide vapour takes place in dry mud. Menn, McBain, Adelson and Patchett (1965) found that imidan persisted longer in dry soil, which is similar to the findings of Bohn (1964) who reported that dimethoate had a half life of 4 days in dry soil and $2\frac{1}{2}$ days in wet soil.

DECOMPOSITION OF INSECTICIDES

Although the metabolism of insecticides in animals and higher plants has been studied, the mechanism of the decomposition of insecticides in soil has not been investigated thoroughly. Many insecticides decompose under the influence of physical factors such as sunlight, for example DDT decomposes under ultraviolet light (Fleck, 1947). There is, however, little conclusive proof that micro-organisms play a large part in the decomposition of insecticides in the soil. Metabolism of chlorinated hydrocarbon insecticides by resistant insects has been demonstrated. Sternburg, Kearns and Bruce (1950) first

showed the presence of DDT-dehydrochlorinase in resistant insects, but this enzyme has not been demonstrated in micro-organisms. More important in the decomposition of DDT in the soil is the iron content. Downs, Bardas and Navarro (1951) showed that iron oxide was the most active catalyst and it was calculated that 1 molecule of iron catalyses the decomposition of 160 molecules of DDT.

Lichtenstein and Schulz (1959 and 1960) showed that aldrin was lost from the soil due to oxidation to dieldrin which was greater in a Carrington loam than a muck soil. They also showed that the conversion was apparently correlated with the numbers of organisms in the soil. Lichtenstein, Schulz and Cowley (1963) showed that a number of synergists delayed the conversion. Sesamex was the most efficient synergist and it was noted that it also reduced the microbial population of the soil. This evidence that micro-organisms might be involved was reinforced by Ayers and Allen (1953) who found that 4 actinomycetes and 3 soil-aldrin infusions could grow in liquid culture and on silica gel media containing aldrin as the sole carbon source. This was confirmed by Jönsson and Fåhraeus (1960) who also used a bacterial isolate in liquid culture. They showed that decomposition was incomplete as the ring structure was not attacked.

Although the organophosphorus insecticides seem to disappear rapidly from the soil and often to stimulate the growth and metabolism of soil micro-organisms, very few cases have been reported where micro-organisms have been found to be the direct cause of the disappearance. The main evidence for microbial decomposition is the effect of sterilising the soil on the persistence. Apart from the example already quoted under the heading of Persistence, Naumann (1960) demonstrated that parathion persisted longer in partially sterile soil. This was confirmed by Lichtenstein and Schulz (1964). Imidan also persisted longer in partially sterilised soil (Menn et al, 1965). Hall and Yun-Pei Sun (1965) showed that steam sterilised soil slowed the rate of decomposition of bidrin. Corey (1965) found that air-dry soil had the same effect on extending the persistence as steam sterilisation and that chloropicrin at 500 ppm also improved persistence indicating general inhibition of micro-organisms. It was also found that sesamex, an enzyme inhibitor, also slowed the rate of decomposition.

Organophosphorus compounds are metabolised by animals and higher plants and reviews on this subject have been made by Arthur (1962) and Casida (1962). Little work has been done on the metabolism of insecticides by micro-organisms. Lichtenstein and Schulz (1964) showed that it was a yeast which was primarily responsible for the reduction of parathion to amino-parathion in the soil, and bacteria had little effect. Ahmed and Casida (1958)

found that Pseudomonas fluorescens, Thiobacillus thiooxidans, Torulopsis utilis and Chlorella all had an effect on phorate. Ps. fluorescens hydrolysed 58% of phorate in 8 days in liquid culture although no oxidation products were formed. The Thiobacillus hydrolysed 75% of the phorate, but was not able to utilise the sulphur in the molecule. Torulopsis utilis and Chlorella also hydrolysed phorate but in addition oxidised the sulphides to sulphoxides. Chlorella, which was the most efficient for oxidising the phorate, oxidised poorly parathion, dimefox and schradan.

In higher plants phorate is also oxidised to the sulphone and sulphoxide (Bowman and Casida, 1957). The oxidative products formed in the soil have been found to be more toxic to insects (Dewey and Parker, 1965). Getzin and Chapman (1960) suspected that the oxidation of phorate in the soil was due to natural causes rather than the microbiological, since partially sterile soil did not reduce the rate of oxidation.

Malathion is an example of a relatively unstable compound, being decomposed in the presence of iron and at elevated temperatures. It is hydrolysed in slightly acid or alkaline media and by solid diluents which have a positive alcoholic adsorption index, such as Bentonite (Spiller, 1961). It is readily metabolised by rat tissue preparations (Seume and O'Brien, 1960) and by lactating cows after oral administration (O'Brien, Dauterman and Niedermeier, 1961).

THE SCOPE OF THE THESIS

In the development of new insecticides, the standard screening procedure includes toxicity tests against animals and plants, but little detailed work is done to determine the effect on micro-organisms. The persistence in the soil may be studied but this does not indicate the part played by micro-organisms in the degradation, and the effect the insecticide may be having on the soil microflora.

The object of this investigation was to elucidate the part played by micro-organisms in the degradation of three organophosphorus insecticides, namely menazon, malathion and sumithion, in the soil, and further to see how these compounds affect the microflora of the soil. It was felt that it might then be possible to correlate the activities of the soil micro-organisms with the persistence of the insecticides and to obtain organisms which would decompose the insecticides in pure culture. It would then be possible to study the pathway of degradation.

Studies have therefore been made on the effect of the three insecticides on respiration of the soil microflora and, at the same time, population studies were made to detect any changes which might occur in the various types of micro-organisms. Counts on the bacteria, yeast, actinomycete and mould populations were therefore undertaken in the presence of the insecticides.

The effect of the insecticides on selected organisms was studied in pure culture and sumithion was studied in greater detail for its effect on fungi.

The persistence of the insecticides in soil was determined using both chemical methods and bioassay techniques. Investigations were also carried out in order to determine the relationship between the soil microflora and the persistence of the insecticides. Further evidence was obtained where possible by the use of ^{14}C-labelled menazon.

Enrichment cultures were made and isolates were obtained which were potentially capable of decomposing the insecticides. The isolates were tested for their activity for degradation and utilisation of the insecticides as the sole-carbon source. The degradation of the insecticides in liquid culture was followed in order to confirm the activity of the isolates and, in the case of menazon, one of the possible degradation products was measured to see if it accumulated. It was hoped from these studies to determine the role played by micro-organisms in the decomposition of insecticides in the soil and if possible the degradation pathway.

From the results obtained, it was thought possible that some general conclusions might be drawn of the effects of organophosphorus insecticides on the soil microflora, and the role played by micro-organisms in the decomposition of the insecticides in the soil.

PART I. ACTION OF THE INSECTICIDES ON THE SOIL MICROFLORA

MATERIALS AND METHODS

Sources of the Insecticides

Menazon was supplied as a crystalline powder by Plant Protection Ltd., Bracknell, Berkshire, and malathion as a liquid by the Murphy Chemical Co. Ltd. Sumithion, manufactured by the Sumitomo Chemical Co., Osaka, Japan, was supplied as a liquid by Plant Protection Ltd, DDT as a white powder also by Plant Protection Ltd. Samples of aldrin and dieldrin were obtained as crystalline products from the Shell Chemical Co. All the insecticides were of the Technical Grade; containing at least 95% of the active ingredient. Some important physical and chemical properties of these insecticides are shown in Table 1.

Sources of the Soils

Four soils with different characteristics were used. The Chelsea loam, with which most of the work was done, was obtained from the Chelsea Physic Gardens, London. A clay soil was obtained from a private garden in Oxted, Surrey, the chalk soil came from the North Downs at Hollingbourne in Kent, and a peat soil from Chobham Common in Surrey.

TABLE 1. Chemical, Physical and Toxicological properties of the insecticides [a]

	Solubility in water	Volatility mm. Hg.	Mammalian acute oral toxicity. LD_{50} for rats (single dose)	Chemical Stability
Malathion	145 ppm	4×10^{-5} at 30°C	480-5800mg./kg.	Hydrolysed in alkaline or mildly acid conditions[b]
Sumithion	20 ppm	-	673.3 mg./kg.[c]	Stable in acids but hydrolysed in alkaline conditions[c]
Menazon	240 ppm	Negligable	1950 mg./kg.	Hydrolysed by acids
DDT	Negligable	1.9×10^{-7} at 20°C	113 mg./kg.	Decomposed by alkalis
Aldrin	0.1 ppm[d]	6×10^{-6} at 25°C	550 mg./kg.	Stable in alkalis and in acids above pH3.0[d]
Dieldrin	0.1 ppm[d]	1.8×10^{-7} at 25°C	100 mg./kg.	Identical to aldrin[d]

(a) Figures obtained from Insecticide and Fungicide Handbook 1963 - H. Martin.

(b) Spiller (1961).

(c) Technical bulletin on Sumithion, Sumitomo Chemical Co., Japan.

(d) Details from Shell booklet of Information on the technical materials.

Classification of the Soils

Determination of the pH

Although many methods are used for determining the pH of the soil, including the use of indicators, it is generally accepted that an electric pH meter with an electrode placed in a soil suspension gives a reasonable approximation to the true pH. Satisfactory results were obtained by shaking a weighed sample of soil with an equal weight of distilled water in a screw capped bottle until the soil particles were evenly dispersed. The soil suspension was transferred to a beaker and the pH determined using a pH meter (Model 23A, Electronic Instruments Ltd., Richmond, Surrey) and a glass electrode.

Mechanical analysis of the soil

a) Pipette method

The pipette method was used initially, since at that time a Bouyoucos Hydrometer was not available. The pipette method is more accurate, but it is time consuming.

20 g. of air-dried soil was weighed into a beaker. 60 ml. of 6% hydrogen peroxide was added, to decompose the organic matter, and the mixture warmed gently. After frothing had ceased, a further 20 ml. of hydrogen peroxide was added and

the mixture heated once again. When effervescence had ceased 150 ml. of distilled water was added to the beaker, followed by 10 ml. of 5% sodium hexametaphosphate. The mixture was boiled to destroy excess hydrogen peroxide and after cooling, it was transferred to a 1 litre flask. Distilled water was added to give approximately 800 ml. of suspension, which was stirred mechanically overnight. The dispersed suspension was passed through a 100 mesh sieve (0.2 mm. diameter) into a 1 litre measuring cylinder, the residue being washed thoroughly until only coarse sand remained. The sieve containing the coarse sand fraction was dried in an oven at 105°C and then the sand was weighed. The weight of coarse sand was expressed as a percentage of the oven-dry fine-earth. The suspension in the cylinder was made up to 1 litre with distilled water and allowed to equilibrate at room temperature. The cylinder was inverted several times to suspend all fractions and then the particles allowed to settle for 4 minutes 48 seconds. A 20 ml. pipette, fitted with a rubber bung, was adjusted so that the tip of the pipette was 10 cm. below the surface of the suspension when the rubber bung rested on the top of the cylinder. 20 seconds before the time specified, the pipette was placed carefully into the cylinder and at the correct moment 20 ml. of the suspension was withdrawn and transferred to a weighed evaporating basin. The basin, containing $\frac{1}{50}$ of the total

silt and clay, was placed in an oven and dried to constant weight at 105°C. The combined silt and clay fractions were calculated as a percentage of oven-dry fine-earth. The clay fraction was determined by resuspending the soil and allowing it to settle for 8 hours. 20 ml. of the suspension were withdrawn 10 cm. below the new level, transferred to a weighed evaporating basin and the sample was dried and reweighed as above. The clay fraction alone was calculated as a percentage of oven-dry fine-earth, and by difference the silt fraction was determined.

After sampling the clay fraction, the bulk of the supernatent was poured away and the sediment, containing mainly the fine sand fraction, was transferred to a 400 ml. beaker. A mark was made 10 cm. from the bottom of the beaker and water was added to the suspension up to the mark. The contents, after stirring thoroughly, were allowed to settle for 4 minutes 48 seconds and the supernatent was poured away. This procedure was repeated until the supernatent was clear, and then the fine sand fraction was dried and weighed. From the weight, the percentage of fine sand in oven-dried fine-earth was calculated.

Since the organic matter was destroyed, the sum of the percentages did not total 100%, therefore the results were recalculated as percentages of the mineral matter alone.

b) Bouyoucos Hydrometer method

The hydrometer method was similar in principal to the pipette method, relying upon the differing sedimentation rates of the fractions. 50 g. of air-dry soil was mixed with 200 ml. water, followed by 15 ml. of 0.5N sodium oxalate, which disperses the organic matter. The mixture was allowed to stand overnight and then mixed thoroughly using a mechanical stirrer and baffle. The suspension was then transferred to a 1 litre measuring cylinder and the volume made up to the 1 litre mark. The cylinder was shaken and the suspension allowed to stand for 5 minutes. The hydrometer was carefully placed into the cylinder 20 seconds before the reading was to be taken and the clay and silt fractions were read directly from the hydrometer. The temperature of the suspension was also recorded. The soil suspension was then resuspended, allowed to settle for 5 hours and then the clay fraction alone determined as above. A blank reading was taken with the hydrometer in 1 litre of distilled water.

The results were corrected for the blank reading and for temperature, the silt and clay fractions being calculated as percentages of air-dry fine-earth. The total sand was found by difference and expressed as a percentage of air-dry fine-earth.

The method was modified for the chalk soil, since the calcium carbonate would precipitate the oxalate. The soil was first treated with concentrated hydrochloric acid until effervescence ceased. The suspension was then filtered through Whatman no. 5 filter paper in a Buchner funnel under vacuum. The soil was washed with distilled water to leach all the calcium chloride formed. The soil was air dried and 50 g. analysed as above.

Textural Classification

From the percentages of the mineral fractions present, the soils were classified on the International scale of size limits using a triangular soil texture diagram.

The main characteristics of the soils used are shown in Table 2.

TABLE 2. Characteristics of the soils used in the investigations

Soil	Mineral Fractions %			Classification on International size limits	pH
	Sand	Silt	Clay		
Chelsea Loam+	71.8	12.3	15.9	Loamy Sand	7.2
Clay *	32.0	39.4	28.6	Silty Clay Loam	5.4
Chalk *	78.3	13.5	8.2	Loamy Sand	7.6
Peat *	92.0	5.4	2.6	Sand	3.1

+ Expressed as a percentage oven-dry fine-earth

* Expressed as a percentage air-dry fine-earth

Preparation of Soils

The soils were passed through a 3 mm. sieve to remove the larger stones and then through a 2 mm. sieve to remove coarse particles. The fine-soil was stored in air-tight jars in the dark until required. Since the soil organisms did not seem to be greatly altered on storage, portions of the Chelsea loam were used for over a year.

Adjustment of the water content of soil

All experiments were carried out at 50% of the maximum water holding capacity of the soil; an arbitory level commonly used in soil studies. In order to calculate the amount of water required by a soil to bring it to the optimum level, the water content and the maximum water holding capacity were determined.

Determination of the water content

Three samples of the soil (approximately 5g.) were weighed into weighing bottles. The soil was dried in an oven at 105°C to constant weight. The water content was calculated for each sample, from the difference between the weights of soil before and after drying and the average of the three samples was taken. The results were expressed as a percentage of the oven-dried soil.

Determination of the maximum water holding capacity

Hilgard's method was used (Fred and Waksman, 1928). Three perforated, copper vessels were weighed and filled with approximately 15 g. soil and reweighed to determine the weight of soil. The vessels were then placed in a trough containing water and the level adjusted so that it just touched the bottom of the copper vessels. Water was taken up by capillarity attraction until the soil was saturated. The soil was left in contact with the water for 20 minutes and then removed, the outsides of the vessels were blotted dry and reweighed. The weight of water taken up was then calculated, and with the water content of the soil at the start, expressed as a percentage of the oven-dried soil. The average of the three samples was used as the final figure.

Measurement of Soil Respiration

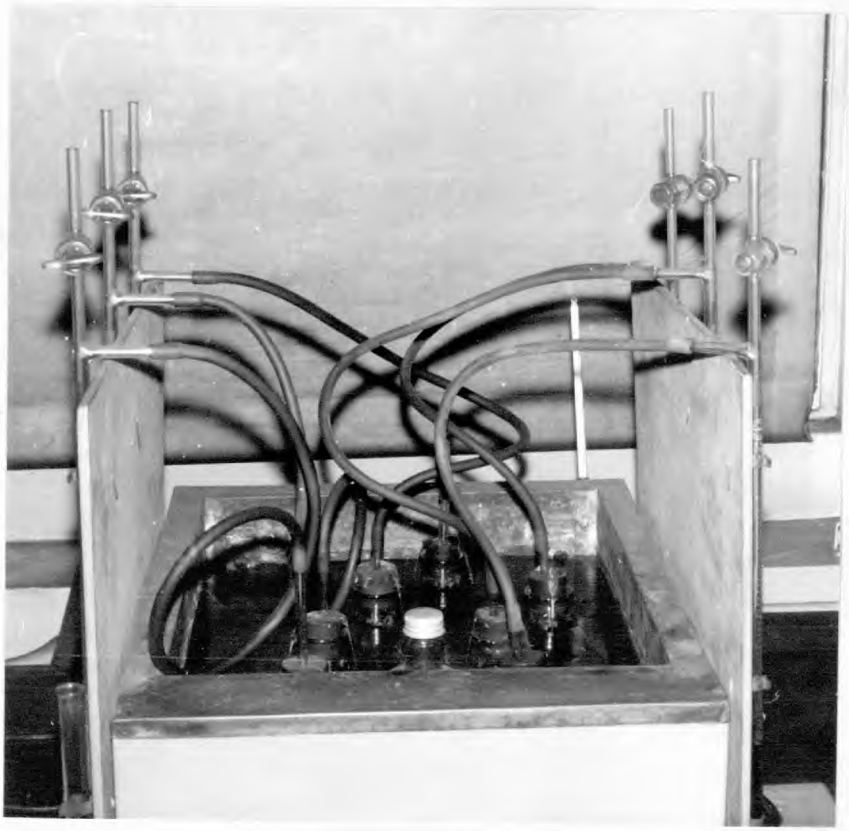
There have been a number of methods used to measure the respiration of micro-organisms in the soil, either based upon measurement of carbon dioxide produced or uptake of oxygen. It was decided to measure the uptake of oxygen and although the Warburg apparatus has been used for this, it is impracticable for use with insecticides because of their insolubility and the desire to measure the uptake over long periods.

The apparatus illustrated in Plates 1 and 2 was designed to enable large volumes of soil to be used (approximately 100 g.) and to run the experiments over a number of days. One arm of the manometer was made from a 5 ml. graduated pipette onto which a glass tap was fused, the other arm being wide bore glass tubing. The manometers were connected to the flask by $\frac{1}{4}$" diameter rubber tubing. The soil was placed into sterile 250 ml. conical flasks sealed with a rubber bung and connected to the rubber tubing by an air-tight glass tube through the bung. The soil temperature was kept constant by submerging the flasks up to the neck in a water bath. It was found better to fill the manometer with Krebs manometric liquid and have a tube of 40% potassium hydroxide solution in the flask, rather than to have the potassium hydroxide in the manometer, as in the Gamong respirometer. Initial experiments showed that 1.5 ml. of 40 % potassium hydroxide solution (Theoretically capable of absorbing 146 ml. carbon dioxide) was capable of giving an accurate measurement over 5 days, at normal rates of respiration. At the start of an experiment or after replenishing the potassium hydroxide, the flasks were allowed to equilibrate for 10 minutes. The levels of the manometric liquid were adjusted until equal at the point taken as zero. The taps of the manometers were closed and the time noted. Readings of the manometers were taken once or twice a day depending on the rate of respiration, adjusting back to

PLATE 1. Apparatus for Measuring the Respiration of Soil Micro-organisms

The Manometers

PLATE 2. Apparatus for Measuring the Respiration of Soil Micro-organisms



The manometers attached to flasks in the water bath.

zero if necessary. No results were obtained during the week-end, when the manometers were left open. The readings were taken by adjusting the movable arm of the manometer until the levels of the manometric liquid in each arm were at the same point. The volume of oxygen taken up was read directly from the graduated arm of the manometer. On occasions the level of manometric fluid rose too high for the necessary adjustment to be made. By experimentation it was found that the height at which the level of manometric fluid reached above zero, before adjustment, was directly proportional to the actual uptake of oxygen. The readings taken before adjustment were converted by a factor of 2.15 to give the uptake of oxygen. The results were corrected to 100 g. soil and for readings of the Thermobarometer which was attached to a flask containing 100 ml. of water and the final reading recorded as ml. of oxygen taken up at a specified temperature at atmospheric pressure per 100 g. soil. Graphs of the results were prepared and the rates of uptake were calculated from the curve obtained, being expressed as ml.O_2 taken up in 24 hours, measured from noon to noon, by 100 g. soil.

Soil Population Studies

The effect of insecticides on the population of soil micro-organisms was studied in general and more important groups were studied in detail. In all cases the level of

insecticide used was that which had made a significant difference in the rate of respiration of the soil micro-organisms.

The soil, in 250 ml. conical flasks, plugged with non-absorbent cotton wool, was incubated at 30°C. Samples of the soil, which were taken to determine the population, were weighed by two different methods. In the early studies duplicate 1 g. samples of soil at 50% maximum water holding capacity were weighed into specimen tubes and then transferred to dilution bottles containing sterile tap water. This method was found to give satisfactory results when studying a mixed flora and had the advantage that plate counts referred directly to 1 g. of soil. However, in later studies, to reduce the chances of contamination a sample of soil was added directly to a weighed dilution bottle. The bottle plus the soil was then reweighed and the weight of soil found by difference. Dilutions were made in sterile tap water and replicate plates were prepared of the appropriate dilution. For the pour-plate technique, 0.1 ml. or 1.0 ml. samples of the dilution were placed in sterile Petri dishes and after the culture medium was added, the Petri dishes were rotated to mix the sample. For some organisms, where surface colonies were desired, 0.1 ml. of the appropriate dilution was placed on the surface of the medium, which had been partially dried in an incubator at 37°C. The inoculum was spread over the surface of the

medium with a sterile glass spreader. The surface was allowed to dry and then the plates were inverted and incubated.

All results were calculated to give the number of organisms per gram of soil at 50% maximum water holding capacity.

Culture media

Thornton's medium

For general population studies, Thornton's medium was found to give satisfactory growth of a wide range of organisms. Best results were obtained by incubating at 25°C for ten days.

Constituents	g./litre
$K_2H\ PO_4$	1.0
KNO_3	0.5
Asparagine	0.5
$Mg\ SO_4 \cdot 7H_2O$	0.2
$Ca\ Cl_2$	0.1
$Na\ Cl$	0.1
$Fe\ Cl_3$	0.002 (0.2 ml. of 1% aqueous solution)

The constituents were dissolved separately in a small volume of water and then mixed. The medium was made up to 1 l. and

15 g. agar added and allowed to stand for 15 minutes. The agar was dissolved by heating in an autoclave and then allowed to cool to 60°C. 1 g. mannitol was added and the pH of the medium adjusted to 7.4 with N/10 sodium hydroxide. The medium was dispensed into bottles and sterilised for 15 minutes at 15 lb. pressure in an autoclave.

Sabouraud's Maltose medium

This medium was used for estimating the number of fungi in the soil.

Constituents	g./litre
Maltose	20
Mycological Peptone	10
Agar	15

The constituents were dissolved in water at 60°C and the pH adjusted to 5.6 The agar was melted in an autoclave and after dispensing into bottles, the medium was sterilised by autoclaving at 15 lb. pressure for 15 minutes. Maltose was used in preference to glucose because it could be sterilised with the complete medium.

Glycerine-Asparaginate Agar

This medium, devised by Conn (1921), was used to estimate the numbers of actinomycetes in soil.

Constituents	g/litre
Sodium asparaginate (or asparagine neutralised with Na OH)	1.0
$K_2H\ PO_4$	1.0
Agar	15

The constituents were dissolved in water and 10 ml. of glycerol were added. The medium was made up to 1 litre and the agar dissolved in an autoclave. The medium was dispensed into 100 ml. aliquots which were sterilised by heating in an autoclave at 15 lb. pressure for 15 minutes.

Selective medium for Yeasts

The medium used suppresses the growth of most bacteria and fungi, allowing the growth of visible colonies of the yeast from soil. The medium was basically nutrient agar (Oxoid) which was reinforced with glucose and the pH adjusted to 3.5 - 4.0 with tartaric acid. A 50% glucose, 5% tartaric acid solution was prepared and sterilised in an autoclave at 15 lb. pressure for 15 minutes. 10 ml. of this solution was added to 100 ml. sterile nutrient agar and mixed thoroughly before pouring the medium. 1 ml. of a 1% diphenyl solution in ethanol, which was considered to be self-sterilising, was also added to inhibit the growth of moulds. The medium was incubated at 25°C to prevent the too rapid volatilisation of the diphenyl, but if the growth of

fungi appeared to be too rapid, a further addition of diphenyl solution was made into the lid of the inverted Petri dish.

Reinforced Clostridial medium

This medium was obtained from Oxoid as a granular powder which was added to distilled water and sterilised. It was used to estimate the numbers of anaerobic bacteria in the soil, with the pour-plate technique. When the medium had solidified, it was dried at 37°C for 2 hours, because any surface moisture allowed spreading-colonies to form. The Petri dishes were placed in a Brewer anaerobic jar with a tube of indicator (4g. glucose, 2g. sodium orthoarsenate, 0.003g. methylene blue in 100 ml. water) boiled to decolourise it. The jar was evacuated with a water vacuum pump and then flushed through with hydrogen for 30 seconds. A palladium catalyst in the jar converted any residual oxygen to water.

EXPERIMENTAL AND RESULTS

A. Respiration of Treated and Untreated Soils

These studies were undertaken to give some indication of the overall effect of the insecticides on the soil micro-organisms. It was considered that an increase or decrease in the rate of respiration of the soil following the addition of an insecticide, would indicate that the insecticide was having some effect on the soil micro-organisms and that this could be inhibitory or stimulatory. Further, this might give some indication as to the persistence of the insecticides in relation to microbial degradation.

a) Preliminary Studies

During the development of the technique described in the materials and methods, which was finally used to measure the respiration of the soil, several experiments were carried out to enable to most efficient apparatus to be designed. Further, it was felt necessary to determine the effect of temperature and water content of the soil on respiration, so that conditions would be near the optimum for the majority of the soil micro-organisms.

(i) Manometer capacity

The first requirement for measuring the uptake of oxygen over a period of days was that the manometers should be capable of measuring the changes in volume of gases in the flasks with a reasonable degree of accuracy and with a minimum of inspection, so

that the apparatus could be left running for at least 12 hours without attention. A comparison was made between 5 ml. and 10 ml. pipettes, used as the measuring arm of the manometer, in order to determine the most useful capacity. Although the 10 ml. pipettes required less supervision, the accuracy of the 5 ml. ones gave them a distinct advantage. In fact the 10 ml. pipette manometer arms would have been more advantageous with the high rate of respiration experienced in some investigations, but where only small changes were measured their accuracy would not have been sufficient. The ideal manometer arm would have been of 10 ml. capacity, but with the same diameter bore and graduations as the 5 ml. pipettes, but this equipment was not available.

(ii) Absorption of carbon dioxide

In measuring the oxygen uptake during respiration, the carbon dioxide given off must be absorbed and this was done with a 40% solution of potassium hydroxide. An experiment was undertaken to compare the efficiency of absorbing the carbon dioxide with the potassium hydroxide contained in the manometer arms, as in the Ganong respirometer, or in a separate container inside the flask using Krebs manometric liquid in the manometer arms, as in the Warburg apparatus. The respiration was measured with both techniques for carbon dioxide absorption, using 100 g. of soil in each flask. It was thought that this was the maximum amount of soil that could be used in 250 ml. capacity flasks without imparing diffusion through the soil, which was approximately 1 inch in depth.

The soil was maintained at 30°C. in a water bath, this temperature being chosen as one giving a high respiration rate in untreated soil (see Table 4.) The uptake of oxygen with respect to time is shown graphically in Fig. 1, which also shows how the rate of respiration was determined in this and later experiments. It will be seen that the rate is obtained from the graph by measuring the oxygen taken up in 24 hours, measured from noon on one day to noon on the next. The rates of respiration calculated from the graph are shown in Table 3. The experiment demonstrated that with potassium hydroxide solution in the flask, absorption of carbon dioxide was carried out more efficiently. The probable reason for this is that the distance required for diffusion of the carbon dioxide to the potassium hydroxide in the manometer was much greater than to the absorbent in a test-tube in the flask. Also, since the potassium hydroxide in the manometer is in continual contact with the atmosphere, its efficiency probably diminishes over a period of time. The larger bore of the test-tube (7.0 mm. compared with 5.5 mm. of the pipette) also gives a greater surface area over which absorption could take place.

The method of calculating the rate of respiration from the graph was used for convenience, but it did introduce slight errors in the initial rates. As can be seen from Fig. 1, when the experiment was started after noon, the initial rate during the first 24 hours had to be calculated by extrapolation. Also, when an experiment was started before noon, the oxygen taken up in

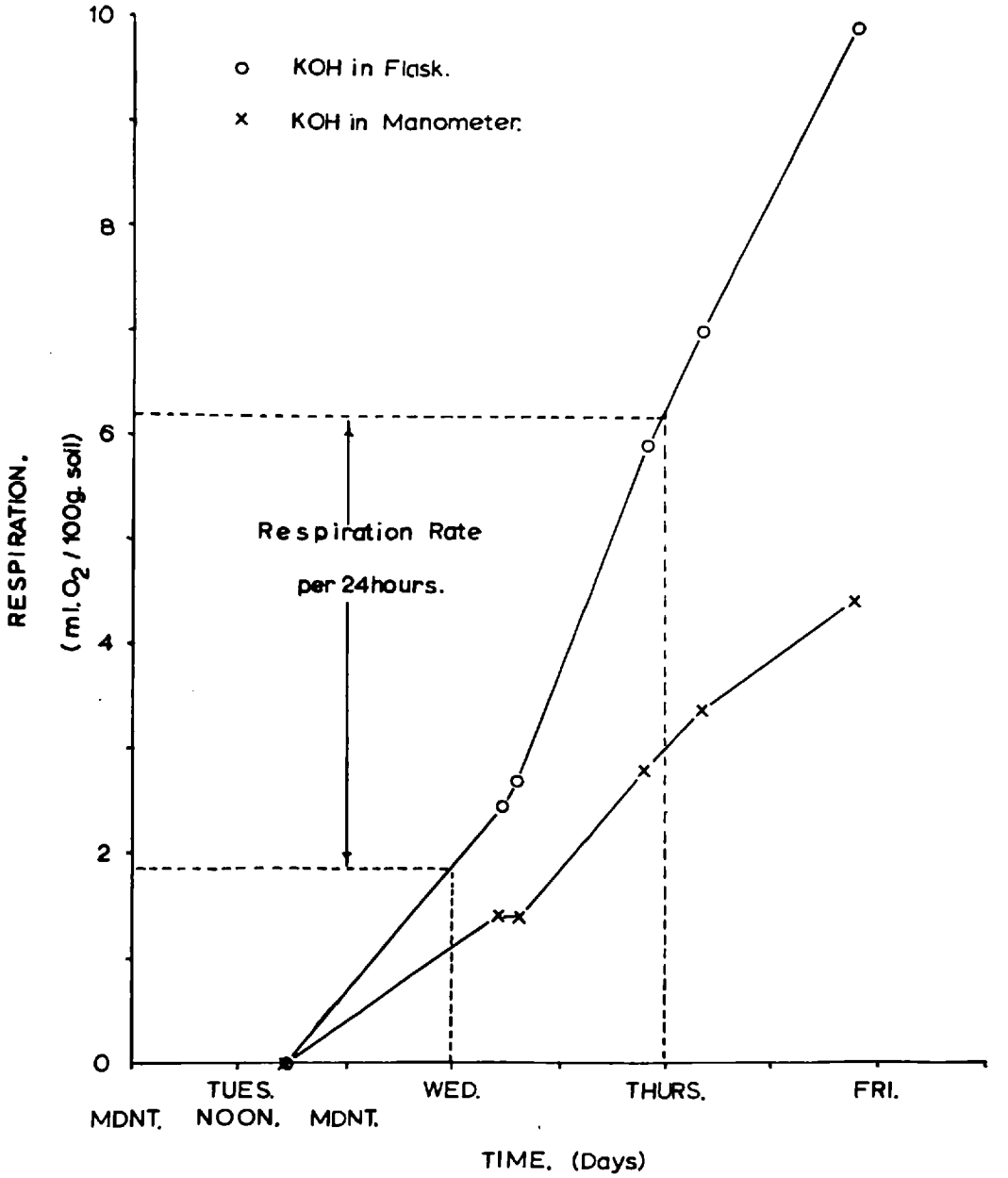
Table 3. Effect of the location of the carbon dioxide absorbent on the measurement of the respiration rate of Chelsea loam.

	Rate of Respiration (ml.O_2/24 hours/100 g. soil)	
	Location of CO_2 absorbent	
Time (days)	Manometer	Flask
1	1.4	2.45
2	1.6	4.25
3	1.8	3.85

Table 4. Effect of different temperatures on the respiration of Chelsea loam, untreated and containing malathion.

	Rate of Respiration (ml.O_2/24 hours/100 g. soil)			
	Temperature of incubation			
	25°C.		30°C.	
Time (days)	Untreated Soil	Soil + Malathion	Untreated Soil	Soil + Malathion
1	2.55	6.0	4.05	9.6
2	2.15	5.1	3.6	6.6
3	2.15	3.65	2.8	4.7
4	1.7	2.8	2.55	4.7
7	1.5	2.15	2.15	3.4
8	1.4	2.15	2.15	3.2

Fig. 1. The effect of the location of the carbon dioxide absorbent on the measurement of the respiration of Chelsea loam.



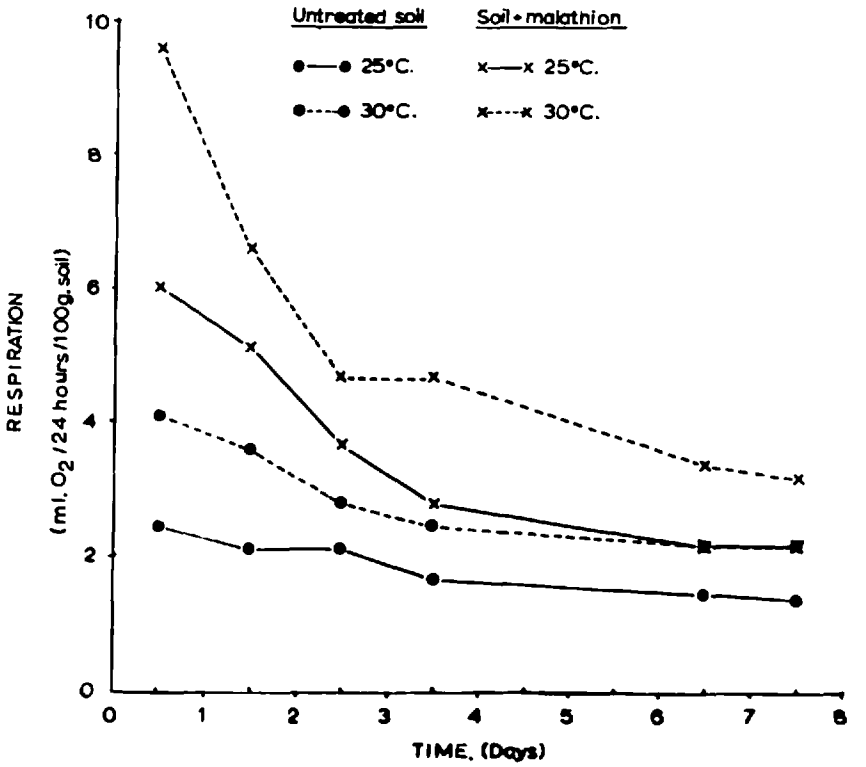
the first hour or two was ignored. Since in most experiments the start was within two hours before or after noon, it was thought that the error introduced would be small with respect to the total oxygen taken up in the first 24 hours.

(iii) Effect of temperature

100 g. soil was placed into each of the four flasks and the water content adjusted with sterile distilled water to 50% of the maximum water holding capacity. Two flasks were placed in a water-bath at 25°C. and the remaining two flasks in a water-bath at 30°C. Four similar flasks were prepared, containing 100 g. soil, to which malathion was added, as an emulsion, to give a concentration in the soil of 1000 ppm. Two of these flasks were then placed into each of the water baths. The rate of respiration of the untreated soils at the two temperatures was observed for a period of 8 days to determine the effect of temperature on respiration. The results were compared with the rates obtained with the malathion treated soil, to see if the presence of the insecticide had any effect on the response to temperature. The results are shown in Table 4, and graphically in Fig. 2.

The results show that the rate of respiration of both treated and untreated soil was higher at 30°C. than at 25°C. In each case the rate of respiration was highest in the first 24 hours, this being followed by a decline in the rates, which finally reached a constant level. The most marked decline in the rate of respiration

Fig. 2. The effect of different temperatures on the respiration of Chelsea loam, untreated and containing 1000 ppm malathion.



was observed in those flasks giving the highest initial rate of respiration i.e. those containing malathion treated soil. The rate of respiration of soil treated with malathion did not drop to the level of the control at either temperature.

As the main effect of elevating the temperature was to increase the overall rate of respiration and since the rate of respiration in the presence of the insecticide was not adversely affected, this temperature was used for all further studies.

(iv) Effect of water content

100 g. soil was placed into each of six flasks. The water required to adjust the water content of the soil to 35%, 50% and 65% of the maximum water holding capacity of the soil was calculated. Water was added to the soil to bring duplicate flasks to each of these levels. The flasks were incubated at 30°C. in a water-bath and the rate of respiration of each soil determined. The results are shown in Table 5 and graphically in Fig. 3.

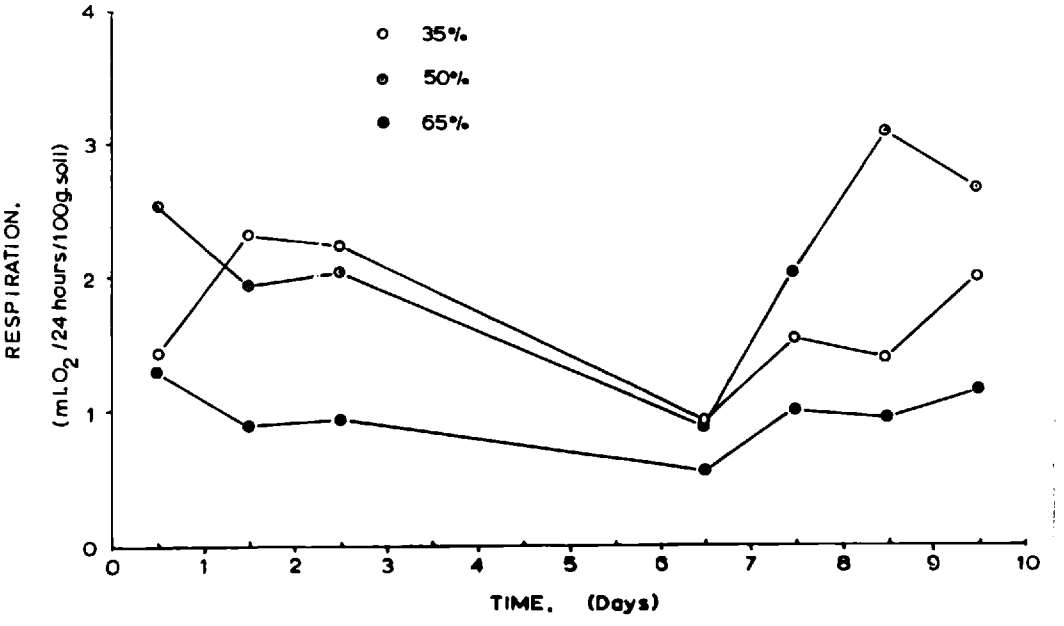
The results show that with soil at 65% of the maximum water holding capacity, the rate of respiration was fairly constant, but lower than at the other two levels. Initially, the highest rate was observed at a level of 50%, but an increase in the rate of respiration of soil at 35% on the two following days caused there to be little difference between these levels. However, when the potassium hydroxide was renewed after incubation for six days,

Table 5. The influence of water content on the rate of respiration of micro-organisms in Chelsea loam

Time (days)	Respiration Rate (ml.O_2/24 hours/100 g. soil) Water content of soil *		
	35%	50%	65%
1	1.45	2.55	1.3
2	2.35	1.95	0.9
3	2.25	2.05	0.95
7	0.95	0.9	0.55
8	1.55	2.05	1.0
9	1.4	3.1	0.95
10	2.0	2.65	1.15

* Water content as a percentage of the maximum water holding capacity.

Fig. 3. The influence of water content on the rate of respiration of micro-organisms in Chelsea loam.



the rate of respiration of the soil at 50% maximum water holding capacity gradually increased until it was higher than at both the other levels. Although the rate at 50% fluctuated, it was decided to use this level in later experiments, since it did give an overall greater rate of respiration than at the other levels.

b) The Effect of Insecticides at Various Levels on the Rate of Respiration.

The effect of malathion, sumithion and menazon on soil respiration was determined, using the technique described in the materials and methods.

(i) Effect of malathion

An experiment was carried out to test the apparatus using a commercial formulation of malathion, a 4% dust (Murphy Chemical Co.), since the technical grade was not available at the time. 5 g. of the dust was added to each of two flasks containing 100 g. soil and mixed thoroughly to give a final concentration of approximately 2000 ppm a.i. in the soil. This was the maximum concentration that it was considered advisable to use to avoid side effects from the presence of the solid diluent. The high concentration of insecticide used, which would only be found under exceptional circumstances, was thought desirable to magnify any possible effect which might occur. Two similar flasks containing untreated soil were used as controls. The water content of the soil

was adjusted to 50% maximum water holding capacity and the flasks were incubated at 30°C., connected to the manometers. The results are shown in Table 6 and graphically in Fig. 4.

From the results it will be seen that the malathion dust stimulated the initial rate of respiration of the soil to approximately 4 times that of the control. However, since the exact nature of the solid diluent was not known, and this may have affected the results, it was necessary to confirm this result by repeating the experiment with the technical product at the same concentration.

When using the small quantities of malathion required, it was found more convenient to pipette a known volume into a portion of the water required to adjust the water content of the soil than to weigh it. 0.2 ml. malathion and 6.0 ml. water were emulsified by sucking up the mixture and blowing out from a pipette a number of times. The emulsion was then pipetted into the soil to give approximately 2000 ppm and was mixed thoroughly with a spatula. Duplicate flasks were prepared of the soil plus the insecticide and untreated control soils. The flasks were connected to the manometers and incubated at 30°C. The results are shown in Table 7 and graphically in Fig. 5.

Table 6. Influence of Malathion (4% dust) at 2000 ppm on the rate of respiration of micro-organisms in Chelsea loam.

Time (days)	Rate of Respiration ($ml.O_2$/24 hours/100g.soil)	
	Untreated Soil	Soil with malathion (2000ppm)
1	2.25	12.2
2	3.65	13.8
3	3.65	9.45
4	2.0	11.4
5	2.65	3.65
6	2.65	3.25
7	2.25	1.6

Table 7. The effect of Technical malathion at 2000 ppm on the rate of respiration of micro-organisms in the Chelsea loam.

Time (days)	Rate o` Respiration ($ml.O_2$/24 hours/100g.soil)	
	Untreated Soil	Soil with malathion (2000 ppm)
1	3.25	13.2
2	3.25	13.0
5	2.65	7.3
6	2.25	4.05
7	2.65	5.3
8	2.45	4.05

Fig. 4. The Influence of malathion (4% dust) at 2000 ppm on the rate of respiration of micro-organisms in Chelsea loam.

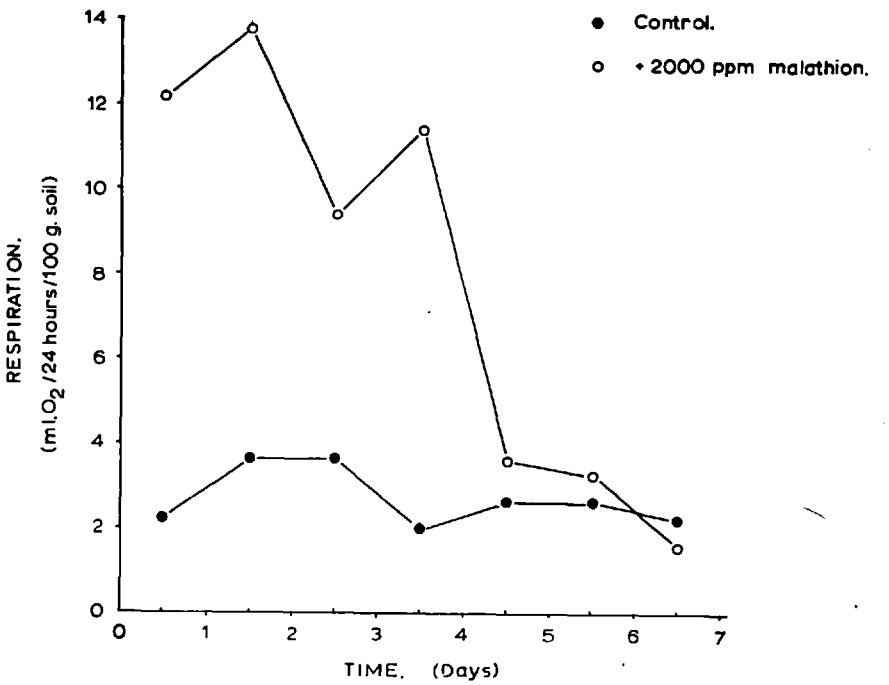
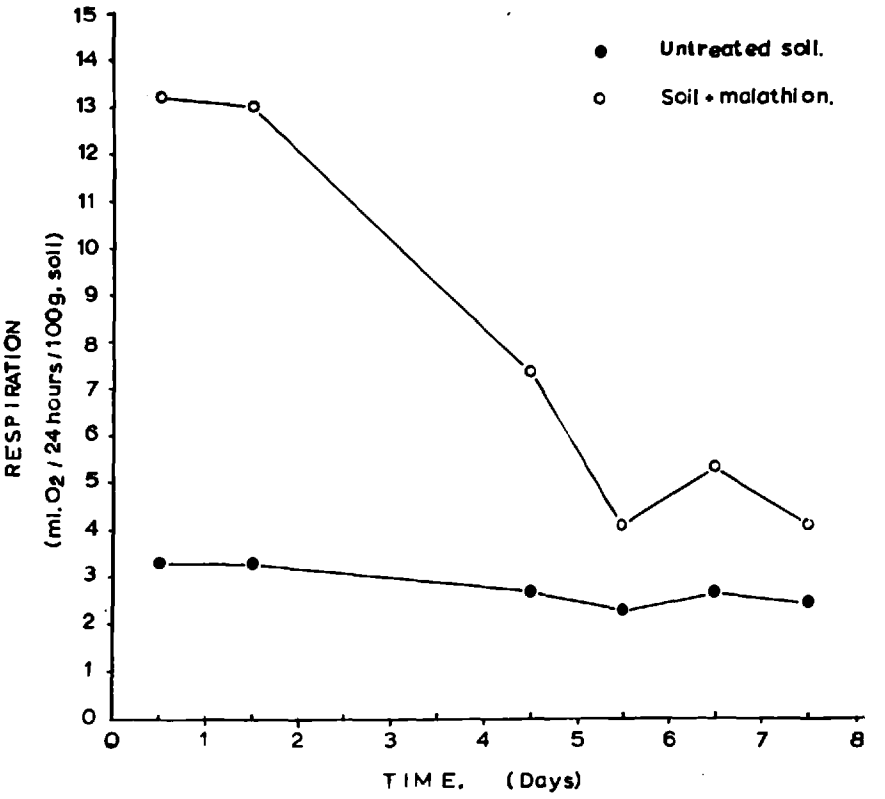


Fig. 5. The effect of Technical malathion at 2000 ppm on the rate of respiration of micro-organisms in Chelsea loam.



The rate of respiration was again stimulated in the presence of the malathion and was comparable in magnitude to that found with the commercial formulation. This indicated that it was in fact the malathion and not the diluent which was causing the increase in respiration. As a result of this observation it was decided to run, concurrently with the respiration experiments, population studies to see if there was any correlation between the rate of respiration and the number of viable micro-organisms in the soil. The results of these studies are given later.

After the experiments already described, the effect of malathion at 50, 100 and 1000 ppm on the rate of respiration of soil micro-organisms was determined. The experimental method was as already described. The results are shown in Table 8 and graphically in Fig. 6.

Stimulation of the respiration was apparently correlated with the concentration of the malathion which suggested that the soil micro-organisms were able to metabolise the insecticide or that the malathion is unstable in the soil and the micro-organisms were utilising its decomposition products.

(ii) The effect of sumithion

Sumithion was added to the soil to give concentrations of 50 and 2000 ppm and was incorporated in the same manner as given for malathion. The results are shown in Table 9 and graphically in Fig. 7.

Table 8. The effect of malathion at 50, 100 and 1000 ppm on the rate of respiration of micro-organisms in Chelsea loam.

Time (days)	Rate of Respiration (ml.O_2/24 hours/100 g. soil)					
	Soil with various concentrations of malathion					
	50 ppm		100 ppm		1000 ppm	
	Untreated soil	Soil plus malathion	Untreated soil	Soil plus malathion	Untreated soil	Soil plus malathion
1	4.2	5.9	3.0	5.2	4.05	9.6
2	2.6	4.1	3.4	4.0	3.6	6.6
3	3.0	4.5	2.2	3.2	2.8	4.7
4	2.6	2.8	1.8	2.8	2.55	4.7
7	2.4	2.6	2.0	2.2	-	-
8	2.3	3.8	2.0	2.2	2.15	3.4
9	2.0	-	1.8	2.6	2.15	3.2
10	2.1	-	2.4	2.1	-	-

Fig. 6. The effect of malathion at 50, 100 and 1000 ppm on the rate of respiration of micro-organisms in Chelsea loam.

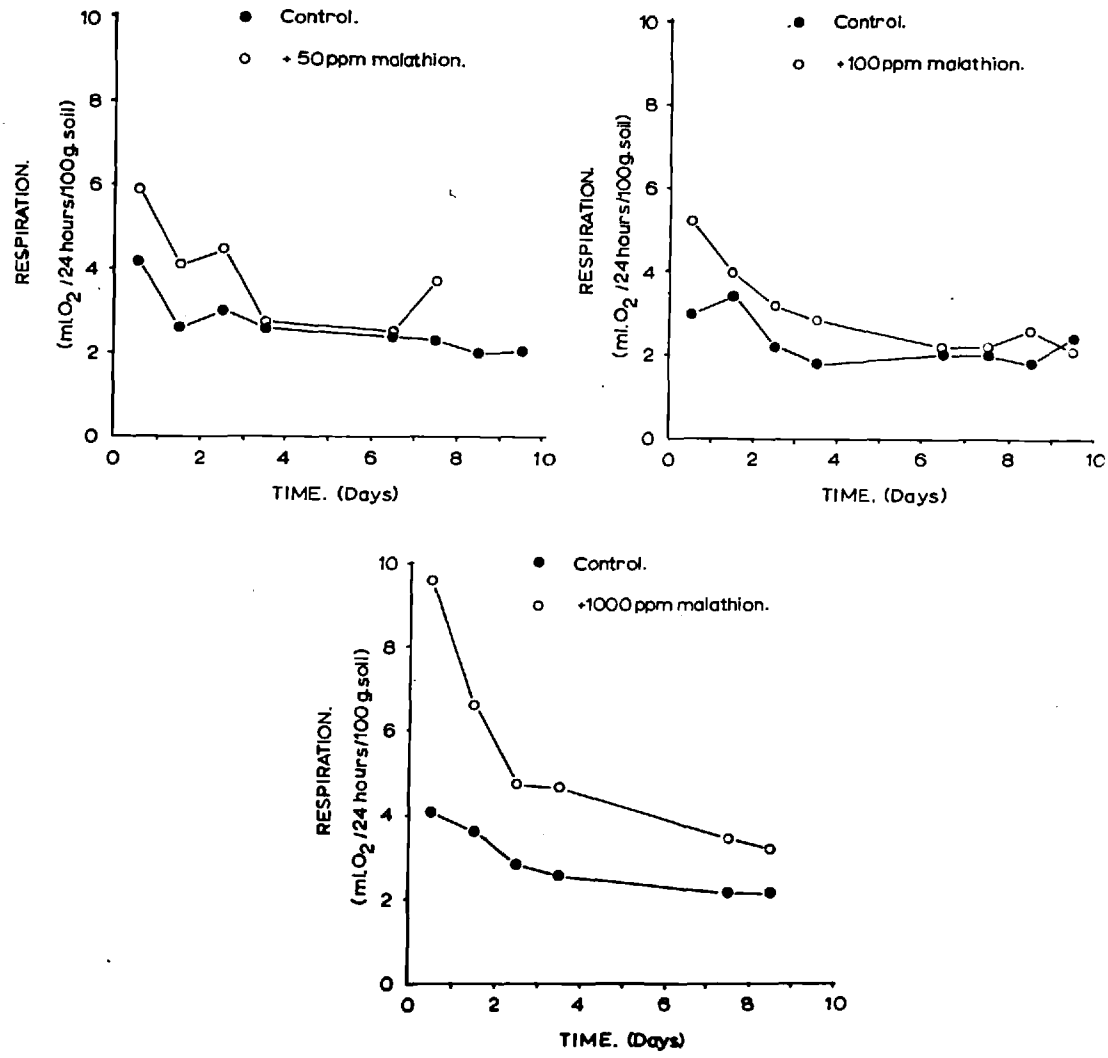
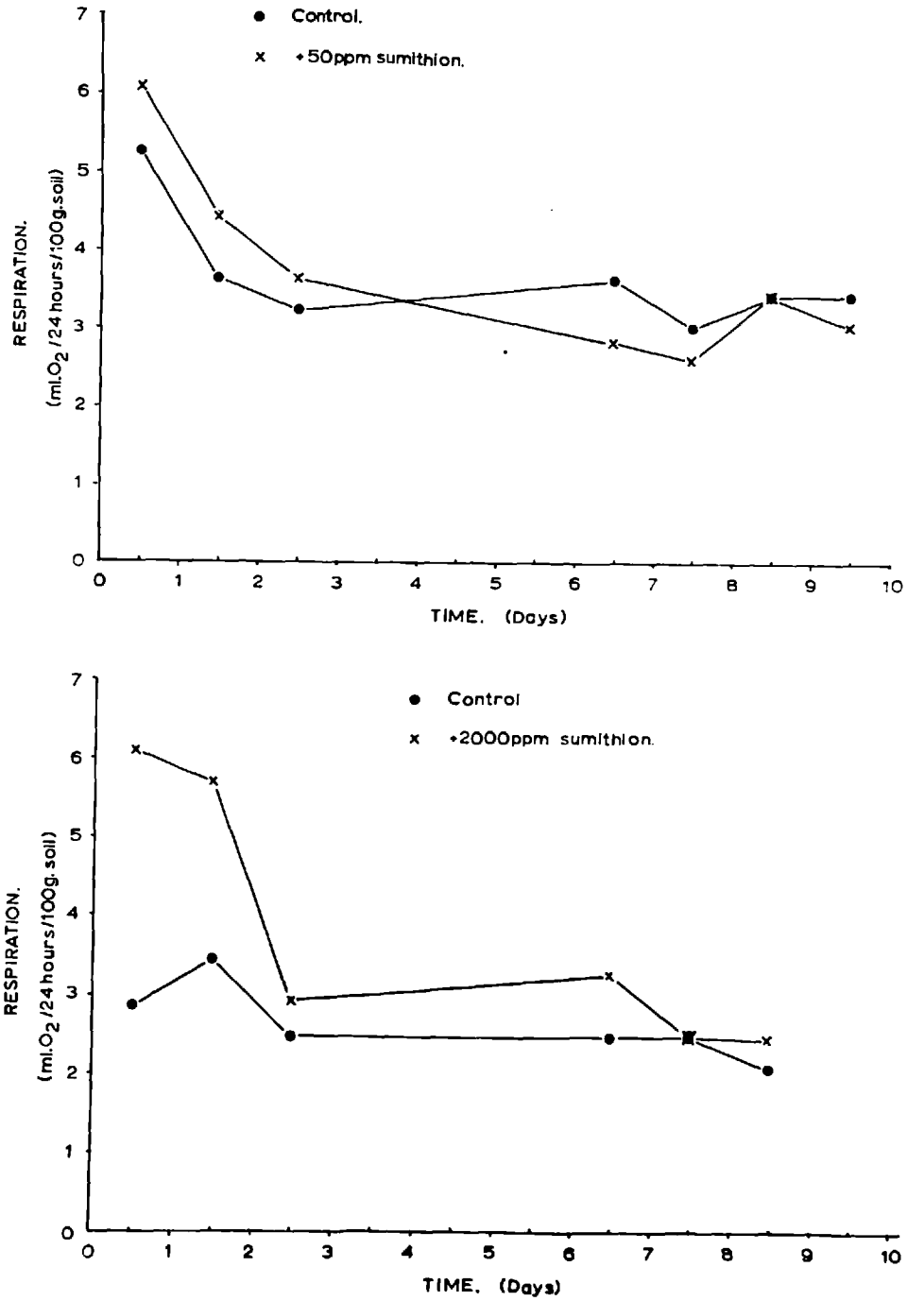


Table 9. The effect of sumithion at 50 and 2000 ppm on the rate of respiration of micro-organisms in Chelsea loam.

Time (days)	Rate of Respiration (ml.O_2/24 hours/100 g. soil)			
	Soil with and without two concentrations of sumithion			
	50 ppm		2000 ppm	
	Untreated soil	Soil with sumithion	Untreated soil	Soil with sumithion
1	5.3	6.1	2.85	6.1
2	3.65	4.45	3.45	5.7
3	3.25	3.65	2.45	2.85
7	3.65	2.85	2.45	3.25
8	3.05	2.65	2.45	2.45
9	3.45	3.45	2.05	2.45
10	3.45	3.05	-	-

Fig. 7. The effect of sumithion at 50 and 2000 ppm on the rate of respiration of micro-organisms in the Chelsea loam.



The results with sumithion indicate that stimulation of respiration occurs in its presence, but the degree of stimulation was less than that obtained with malathion. Although the initial rate of respiration in the presence of the sumithion at 50 and 2000 ppm were the same, the stimulation at the higher level was greater than at the lower level when compared with the controls. Also, slight variations in the initial rates of the controls may have masked the true pattern of the stimulation. It was noted that there was a slight depression in the rate of respiration of the soil containing 50 ppm sumithion after approximately 6 days, but since this effect was not seen at 2000 ppm, it may have been caused by normal variations in soil respiration.

(iii) The effect of menazon

The effect of menazon at 20, 50 and 10000 ppm on the rate of respiration of the Chelsea loam at 30°C. was determined. For the higher concentration, menazon was incorporated into the soil as the solid. The required amount was weighed and added to 100 g. of soil and mixed by shaking thoroughly. The water content of the soil was adjusted and then the soil was mixed with a spatula to ensure an even distribution. For the lower concentrations, menazon was dissolved in distilled water using a mechanical stirrer and heating gently. The required volume of the solution was added to 100 g. of soil to bring it to the required concentration and the soil was then mixed with a spatula to obtain an even distribution.

Duplicate flasks of untreated soil acting as controls were also prepared and incubated similarly in a water bath at 30°C. The results are shown in Table 10, but only the effect of 10000 ppm is shown graphically in Fig. 8, since the other concentrations did not produce an effect significantly different from the controls.

The results show that only at 10000 ppm was there a significant increase in the rate of respiration and no large depression was noted at any concentration. There was a small increase in the initial rate at 2000 ppm but there was apparently no stimulation at 20 and 50 ppm. However, 20 ppm caused a slight depression compared with the control but since this was not shown at any other level, it was thought to be due to variations in the soil.

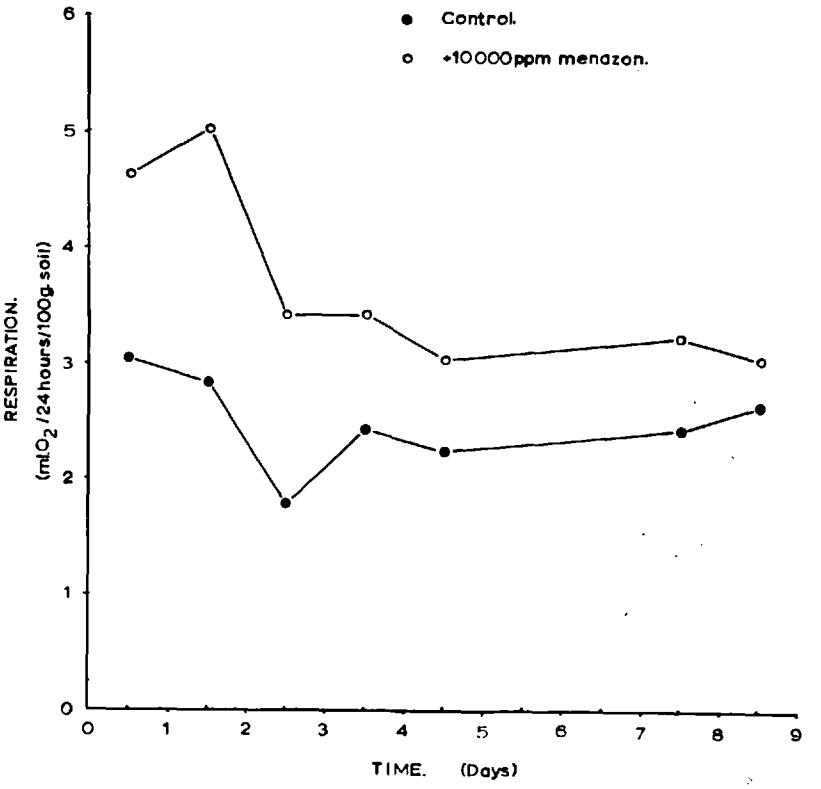
The results suggested that menazon or its decomposition products were more resistant to microbial degradation or were unavailable to the micro-organisms, but also that they were not inhibitory to respiration.

The results with the insecticides show a definite pattern which is probably associated with their chemical individuality. The first conclusion that may be drawn from the studies is that the insecticides do not appear to be toxic to the soil microflora as a whole, since on no occasion was the rate of respiration significantly depressed.

Table 10. The effect of menazon at 20, 50, 2000 and 10000 ppm on the rate of respiration of micro-organisms in Chelsea loam.

Time (days)	Rate of respiration (ml. O_2/24 hours/100 g. soil) Soil containing various concentrations of menazon							
	20 ppm		50 ppm		2000 ppm		10000 ppm	
	Untreated soil	Treated soil	Untreated soil	Treated soil	Untreated soil	Treated soil	Untreated soil	Treated soil
1	3.2	1.95	3.5	3.55	5.5	6.85	3.05	4.65
2	1.4	1.95	2.95	3.05	2.4	3.0	2.85	5.05
3	2.75	2.95	-	-	2.2	2.8	1.8	3.45
4	2.75	2.75	-	-	2.2	1.8	2.45	3.45
5	-	-	-	-	-	-	2.25	3.05
6	-	-	2.15	2.55	-	-	-	-
7	2.35	2.15	2.85	2.65	-	-	-	-
8	2.35	2.15	2.45	2.75	2.0	1.6	2.45	3.25
9	2.75	2.15	2.45	2.25	2.0	1.6	2.65	3.05
10	1.95	1.95	-	-	1.8	1.9	-	-

Fig. 8. The effect of menazon at 10000 ppm on the rate of respiration of micro-organisms in the Chelsea loam.



The stimulation of respiration with each insecticide suggests that the soil microflora is able to decompose the insecticides and utilise the products. The degree of stimulation might be associated with the ease with which the insecticides are decomposed and hence with their persistence. This would indicate a very short persistence for malathion in the soil and a longer persistence for menazon with sumithion falling between these two. However, physical phenomena such as adsorption could be obscuring the results, and chemical decomposition might play a relatively large role in the breakdown of the insecticides.

It must also be stressed that whilst the results have been recorded in a quantitative manner, too much reliance must not be placed upon individual figures. As can be seem from the untreated soils, the rate of respiration fluctuates due to the heterogeneous nature of the soil. Therefore, one can only compare trends in the rate of respiration of treated soils with their particular controls.

c) <u>The effect of formulation of the insecticides on the respiration rate.</u>

Whilst it was thought that various additives to the insecticides might give false results, it was thought desirable to obtain the most even distribution of the insecticides throughout the soil, so that concentration gradients were not produced and the insecticides were available to all the soil micro-organisms.

Investigations were undertaken to determine the rate of respiration using bentonite as a carrier for malathion and menazon, and Triton X-100 as an emulsifying agent for malathion.

(i) Bentonite as a carrier for the insecticides

Eight flasks were prepared containing 100 g. soil. 0.1 g. malathion was weighed onto 10 g. bentonite and mixed thoroughly. 1 g. of the malathion/bentonite mixture was added to each of two flasks, to give approximately 50 ppm a.i. and an equivalent amount of malathion was emulsified in water and added to two further flasks. 1 g. of bentonite was added to duplicate flasks and the remaining two flasks acted as untreated controls. The water content was adjusted to 50% of the maximum water holding capacity and the flasks were incubated in a water bath at 30°C.

In the case of menazon, it was thought that a more even distribution would be obtained in the bentonite if the menazon was added as a solution in methanol. 2.0 g. menazon were dissolved in 50 ml. methanol and 4 g. bentonite added. The methanol was evaporated off on a water bath at 90°C. and the menazon/bentonite mixture ground to a powder with a pestle and mortar. 3 g. of the mixture were added to each of two flasks containing 100 g. soil to give approximately 10000 ppm a.i. 1 g. menazon was added to duplicate flasks and further controls were prepared as for the malathion, but the bentonite added to the soil was treated with methanol in the same

manner as described above. The results for malathion and menazon are shown in Table 11 and graphically in Fig. 9

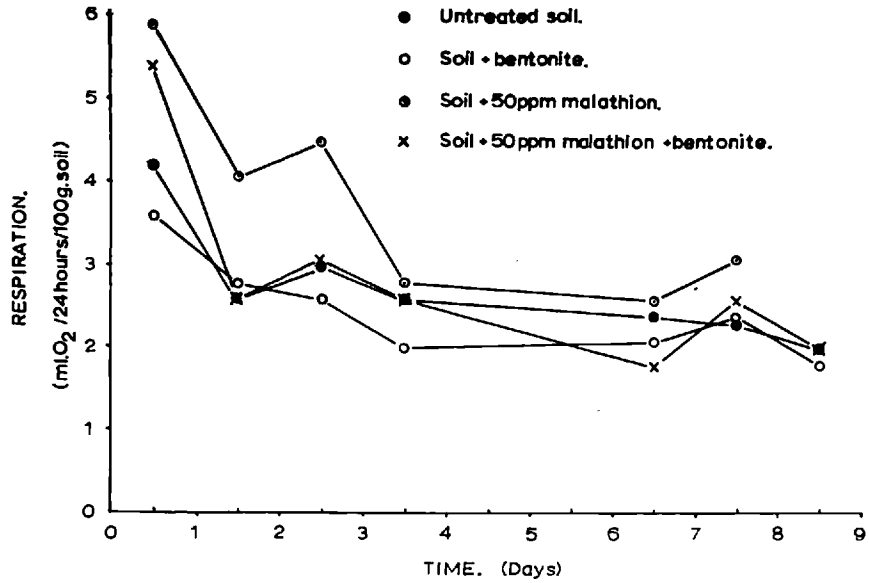
From the results it would appear that using bentonite as a carrier does not increase the stimulation due to the insecticides although side effects reduced the possibility of any clear result. The bentonite in the malathion experiment had a slight depressing effect on respiration in the presence and absence of malathion, possibly caused by a reduction of the free water content in the soil. It was learned later from the literature that malathion is unstable in bentonite, which would also make it unsuitable as a carrier for malathion.

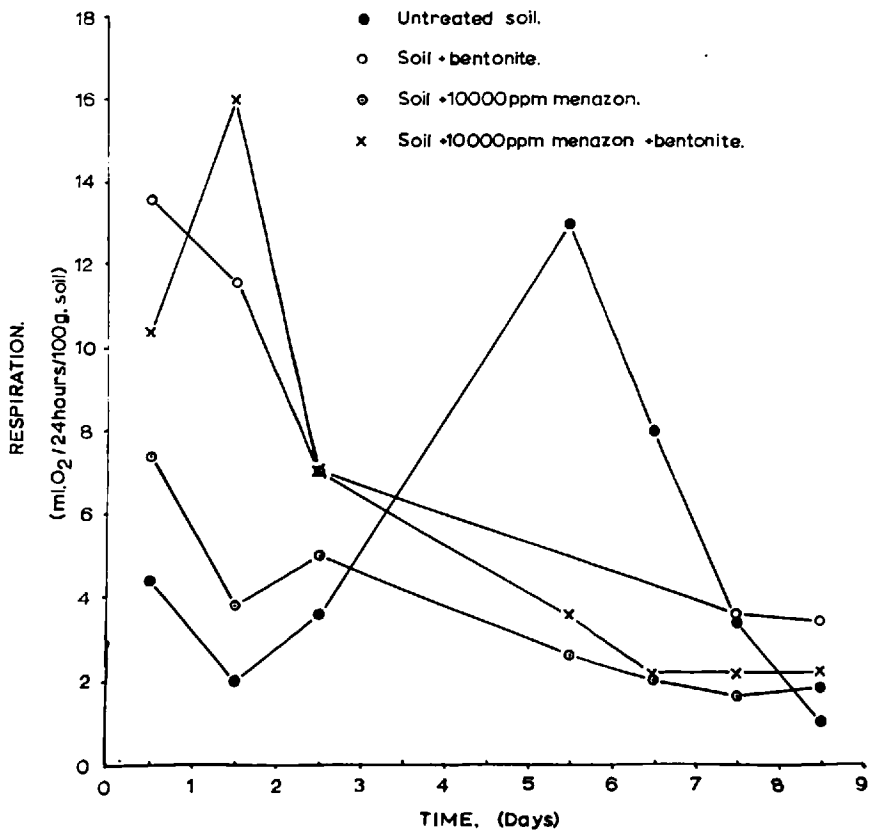
The bentonite treated with methanol in the menazon experiment gave an exceptional result. A large increase in respiration was observed, and this can only be explained by assuming that residual traces of methanol were being utilised by the microflora. This stimulation was reduced in the presence of menazon, indicating slight inhibition of the organisms utilising the residues of methanol. The control of untreated soil in the menazon experiment behaved in an atypical manner. A large increase in respiration was observed on renewing the potassium hydroxide solution in the flasks, after incubation for five days.

Table 11. The effect of Bentonite as a carrier for Malathion and Menazon on the rate of respiration of micro-organisms in the Chelsea loam.

Time (days)	Rate of Respiration (ml.O_2/24 hours/100 g. soil) Soil with Bentonite as the carrier of							
	Malathion (50 ppm)				Menazon (10000 ppm)			
	Untreated Soil	Soil with bentonite	Soil with malathion	Soil with bentonite & malathion	Untreated Soil	Soil with bentonite	Soil with menazon	Soil with bentonite & menazon
1	4.2	3.6	5.9	5.4	4.4	13.6	7.4	10.4
2	2.6	2.8	4.1	2.6	2.0	11.6	3.8	16.0
3	3.0	2.6	4.5	3.1	3.6	7.0	5.0	7.0
4	2.6	2.0	2.8	2.6	-	-	-	-
6	-	-	-	-	13.0	-	2.6	3.6
7	2.4	2.1	2.6	1.8	8.0	-	2.0	2.2
8	2.3	2.4	3.1	2.6	3.4	3.6	1.6	2.2
9	2.0	1.8	-	2.0	1.0	3.4	1.8	2.2

Fig. 9. The effect of Bentonite as a carrier for malathion at 50 ppm and menazon at 10000 ppm on the rate of respiration of micro-organisms in Chelsea loam.





The experiment with menazon was repeated using chloroform as the solvent, since it was hoped that the more volatile liquid would be more easily removed from the bentonite. The result of the experiment, however, was similar in form to that using methanol but the stimulation was even greater. The bentonite treated with chloroform gave an increase in the rate of respiration which could not be measured in the apparatus used, although in the presence of menazon this was again reduced. It became clear that the use of bentonite was impracticable as a carrier for malathion and menazon and the method was discontinued.

(ii) Triton X-100 as an emulsifying agent for malathion

As an alternative to the use of a solid diluent for the liquid insecticides, it was decided to try a liquid formulation using Triton X-100 (Lennig Chemicals Ltd.) as an emulsifying agent, although it was not known what effect it might itself have on the microflora. A 2% v/v solution of the Triton X-100 was prepared, and 0.1 ml. malathion was emulsified in 100 ml. of the solution, by vigorous shaking. Eight flasks were prepared containing 100 g. soil and 10 ml. of the emulsion were pipetted into each of two flasks, to give a final concentration of approximately 100 ppm malathion. 10 ml. of 2% Triton X-100 solution were added to a further two flasks and similarly 10 ml. of 0.1% v/v emulsion of malathion in distilled water to two further flasks. Duplicate flasks of untreated soil were used as controls, and after adjusting the water content of

the soils to 50% maximum water holding capacity, the flasks were incubated at 30°C. in a water bath. The results are shown in Table 12 and graphically in Fig. 10.

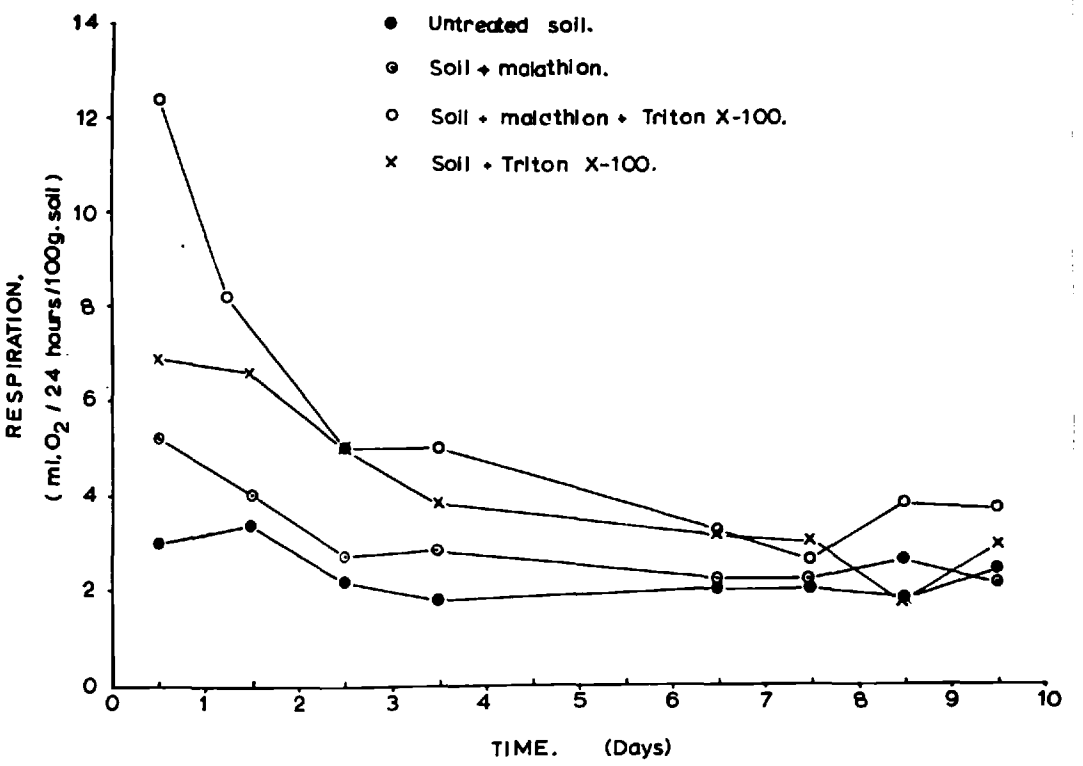
The results show that there is a marked increase in the respiration rate in the presence of Triton X-100, apparently due to the decomposition of this material by the soil micro-organisms. However, the rate of respiration of soil containing the malathion/ Triton X-100 mixture was generally higher than soil containing Triton X-100 alone, suggesting that even in the presence of the emulsifying agent, the malathion was still being metabolised.

However, by adding together the rate of respiration recorded for soil containing Triton X-100 and the rate of respiration of soil containing malathion only, correcting each for the rate in untreated soil, it will be seen that the figure obtained is less than that for soil containing both Triton X-100 and malathion, i.e. in the first 24 hours it was 3.9 and 2.2 compared with 9.4 ml.O_2/ 100 g. soil. This suggests either that the emulsifying agent was in fact enabling a more even distribution of the malathion to be obtained, or that it was assisting the soil microflora to act upon the malathion by altering the physical conditions within the soil. A small error might have been introduced in measuring the effect of malathion alone at 50 ppm, due to the difficulty of incorporating such a small quantity in the soil.

Table 12. The effect of Triton X-100 used for the incorporation of 100 ppm malathion into Chelsea loam, on the respiration of the micro-organisms.

Time (days)	Rate of Respiration (ml.O_2/24 hours/100 g. soil)			
	Untreated Soil	Soil with Triton X-100	Soil with malathion (100 ppm)	Soil with Triton X-100 & malathion (100 ppm)
1	3.0	6.9	5.2	12.4
2	3.4	6.6	4.0	8.2
3	2.2	5.5	3.2	5.0
4	1.8	3.8	2.8	5.0
7	2.0	3.1	2.2	4.2
8	2.0	3.0	2.2	2.6
9	1.8	2.3	2.6	3.8
10	2.4	2.9	2.1	3.7

Fig. 10. The effect of Triton X-100 used for the incorporation of 100 ppm malathion into the Chelsea loam, on the respiration of the micro-organisms.



Although Triton X-100 assists in the even distribution of low levels of malathion in the soil, it was felt that in view of the large increase in respiration which resulted, it might mask the true effect of the insecticide on the soil microflora, and the method was not used in later studies.

d) The effect of addition of a microbial nutrient to the soil on the respiration rate in the presence of menazon.

The addition of menazon to soil, even at 10000 ppm, brought about very little change in the rate of respiration of the soil micro-organisms, which indicated that either the microflora were unable to decompose the insecticide and utilise the products or that it was unavailable for microbial degradation through, for example, adsorption or chemical change. In order to test the former hypothesis, it was decided to stimulate growth and metabolic activity of a section of the soil population in order to determine if this enabled them to decompose menazon. This might be shown by an additional increase in the rate of respiration of the soil micro-organisms above that due to the nutrient alone.

Menazon at 10000 ppm was incorporated into 100 g. of soil in four flasks as previously described. To each of two of the flasks 1.0 g. of urea was added, the remaining two flasks acting as controls. Further controls of untreated soil and soil containing 1 g. urea/ 100 g. soil were also prepared in duplicate. The water content of

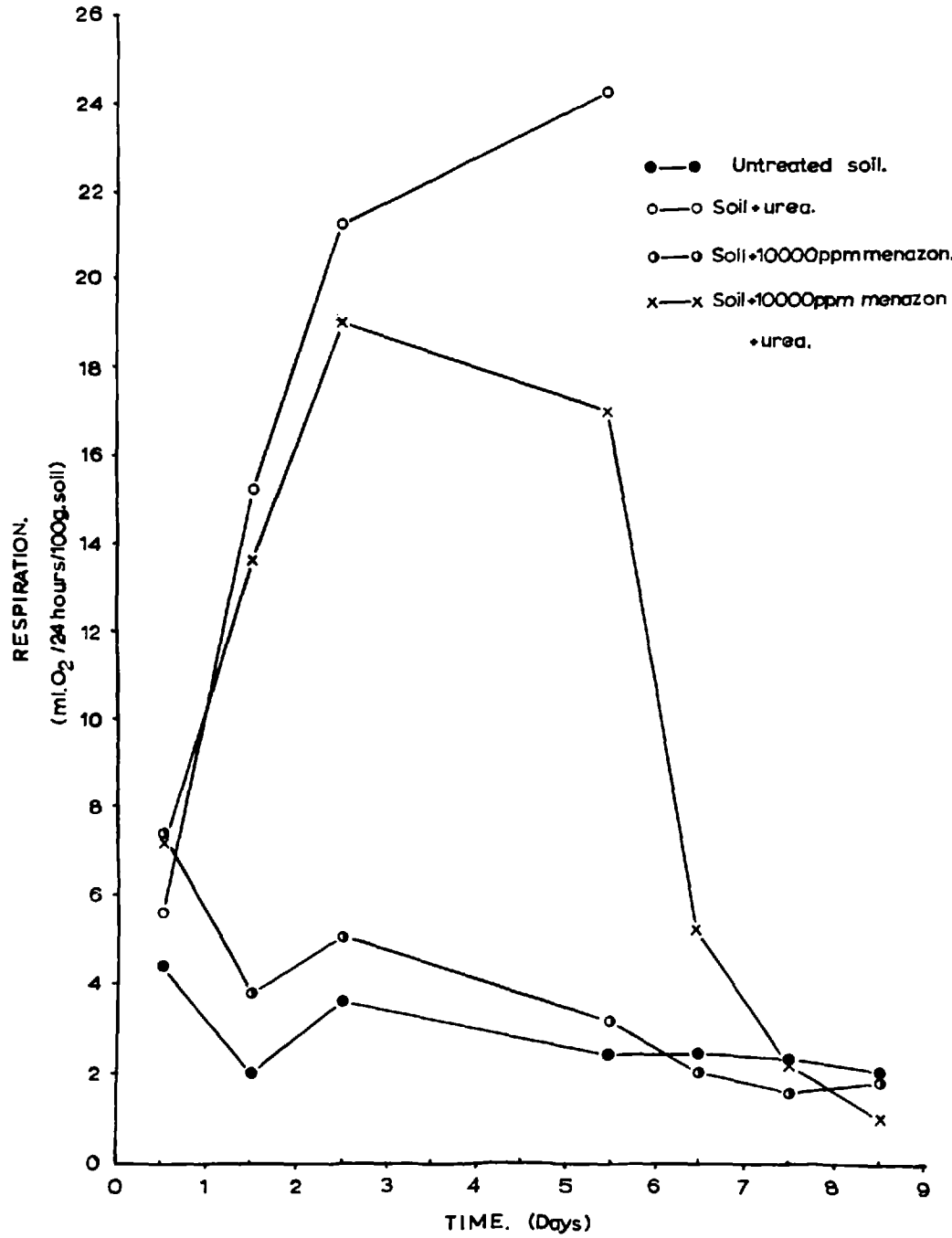
the soil was adjusted to 50 % maximum water holding capacity and the flasks incubated in a water bath at 30 ^{o}C. The results are shown in Table 13 and graphically in Fig. 11.

The results indicated that the addition of urea to menazon treated soil does not increase the degradation of the insecticide, i.e. the rate of respiration of soil treated with menazon plus urea was never higher than the sum of the rates of soil treated separately with urea and menazon. An important feature of the rates of respiration in the first 24 hours was that menazon stimulated respiration greater than the urea, although the rate in the presence of urea gradually increased up to six days, and after the sixth day, the rate of respiration was too high to measure with the apparatus used. During the same period, the stimulation caused by menazon decreased, until it was approximately the same as the untreated soil, on the ninth day. The greater initial stimulation by menazon over that by urea indicates that menazon or contaminating material in the technical product may be more readily metabolised by a larger proportion of the soil micro-flora than urea. The results show also that some days elapsed before the soil population reached a peak of assimilating the urea, which is what one might expect if the initial population of urea-decomposing micro-organisms was low, and were stimulated to grow in the presence of the urea. The accumulation of ammonia or nitrate produced from the urea might also have increased the rate of respiration of the general population. However, the rate of respiration of the soil

Table 13. The effect of the addition of urea on the rate of respiration of micro-organisms in Chelsea loam in the presence and absence of menazon.

Time (days)	Rate of respiration (ml.O_2/24 hours/100 g. soil) Soil with and without 10000 ppm menazon in the presence and absence of 1% urea			
	Untreated Soil	Soil with urea	Soil with menazon	Soil with urea plus menazon
1	4.4	5.6	7.4	7.2
2	2.0	15.2	3.8	13.6
3	3.6	21.2	5.0	19.0
6	2.4	24.2	2.6	17.0
7	2.4	-	2.0	5.2
8	2.3	-	1.6	2.2
9	2.0	-	1.8	1.0

Fig. 11. The effect of addition of urea on the rate of respiration of micro-organisms in Chelsea loam in the presence and absence of menazon.



treated with urea and menazon indicated that the menazon was depressing the activity of the urea-decomposing organisms. Initially, there was little difference between the soils containing menazon alone and menazon plus urea; but as the rate of respiration increased, with continued incubation in the soil containing urea, a reduced stimulation was observed in the soil containing urea plus menazon. If the organisms capable of decomposing urea were proliferating, it indicates that menazon must be affecting the rate of growth of these organisms. The rapid decline in the rate of respiration in the presence of menazon and urea after 6 days may be due to the toxicity of menazon, but since results were not obtained for the effect of urea alone, it might be that the urea was completely utilised and the respiration rate was returning to normal. The experiment therefore was repeated using 0.1% urea added to the soil, and this showed a similar pattern to the previous experiment. It was observed, however, that the rate of respiration, due to the urea, reached a peak after 7 days, after which it declined and fell below that brought about by menazon and urea together. This suggests that the menazon was exerting some inhibitory influence on the utilisation of urea in the soil. In order to confirm this, it would have been desirable to determine the effect in pure culture, but time did not permit this further investigation. The exact nature of the interaction of menazon and urea in the soil cannot be determined using the respiration apparatus. The stimulation of the population by urea might have caused a more rapid decomposition of menazon, but this could be shown more clearly

by following the persistence of menazon in urea-treated soil.

e) The effect of the insecticides on the rate of respiration of different soil types

The effect of the insecticides on the respiration of different soil types was studied to determine how such factors as pH, organic matter content and associated micro-organisms influence the results obtained. A clay soil, a peat soil and a chalky soil were chosen as representative of the wide variety of soils found in nature. Details of the soils are given in the materials and methods.

The method employed was identical to that used for Chelsea loam and the insecticides were incorporated in the same manner as that previously described for Chelsea loam and at the following levels; malathion 2000 ppm, sumithion and menazon 10,000 ppm. The results are given in Tables 14-16 and graphically in Figs. 12-14.

The results in general indicate a similar pattern of stimulation as in Chelsea loam. The rate of respiration of untreated clay and chalk soil was higher than Chelsea loam, but that of the peat soil was lower. The rate of respiration was highest in the first 24 hours as it was with Chelsea loam, and this was followed by a decline. Malathion stimulated the initial rate of each soil more than the other two insecticides, but there were differences in the degree of stimulation with each insecticide. The chalk soil was

Table 14. The effect of malathion, sumithion and menazon on the rate of respiration of micro-organisms in a clay soil.

Time (days)	Rate of Respiration (ml.O_2/24 Hours/100 g. soil)			
	Untreated Soil	Soil with malathion (2000ppm)	Soil with sumithion (10000 ppm)	Soil with menazon (10000 ppm)
1	5.9	11.4	7.35	7.0
2	4.6	8.3	5.5	5.15
3	4.2	6.45	4.4	4.2
4	3.7	-	3.7	3.5
6	-	5.15	-	-
7	-	4.4	-	-
8	2.4	4.8	0.4	3.1
9	2.2	3.7	-	2.4

Table 15. The effect of malathion, sumithion and menazon on the rate of respiration of micro-organisms in a chalk soil.

Time (days)	Rate of Respiration (ml.O_2/24 hours/100 g. soil)			
	Untreated Soil	Soil with malathion (2000ppm)	Soil with sumithion (10000 ppm)	Soil with menazon (10000 ppm)
1	5.05	28.0	10.1	8.85
2	3.4	14.3	11.5	9.7
3	3.8	14.3	8.5	7.6
4	5.05	8.3	8.75	8.4
Final pH	7.6	7.5	7.55	7.45

Fig. 12. The effect of malathion, sumithion and menazon on the rate of respiration of micro-organisms in a clay soil.

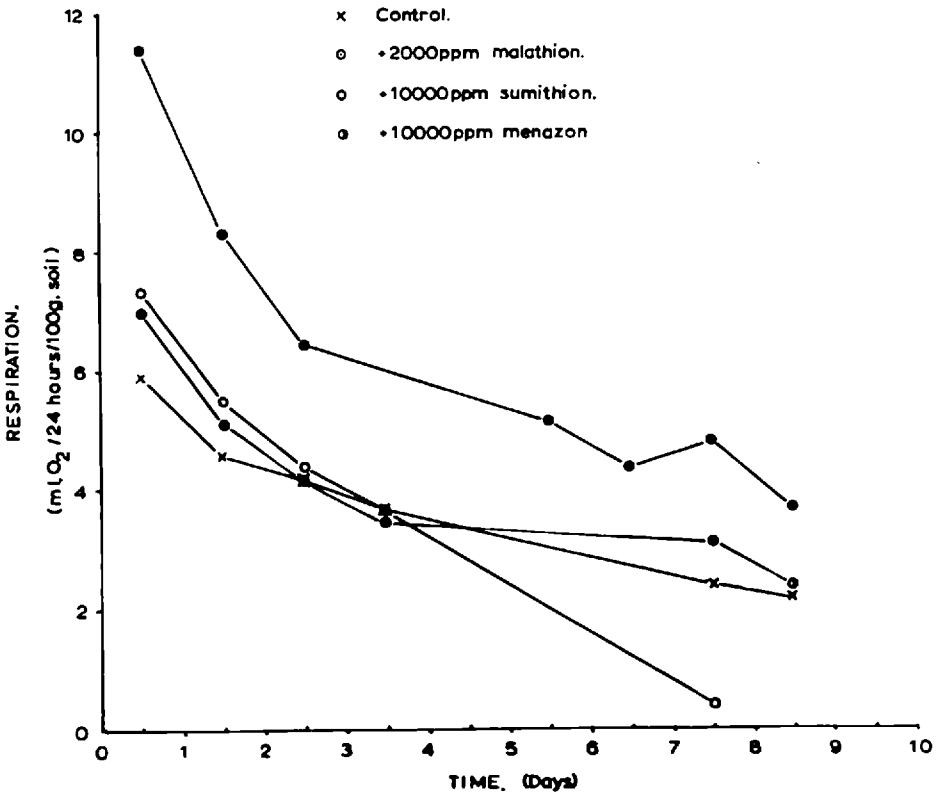


Fig. 13. The effect of malathion, sumithion and menazon on the rate of respiration of micro-organisms in a chalk soil.

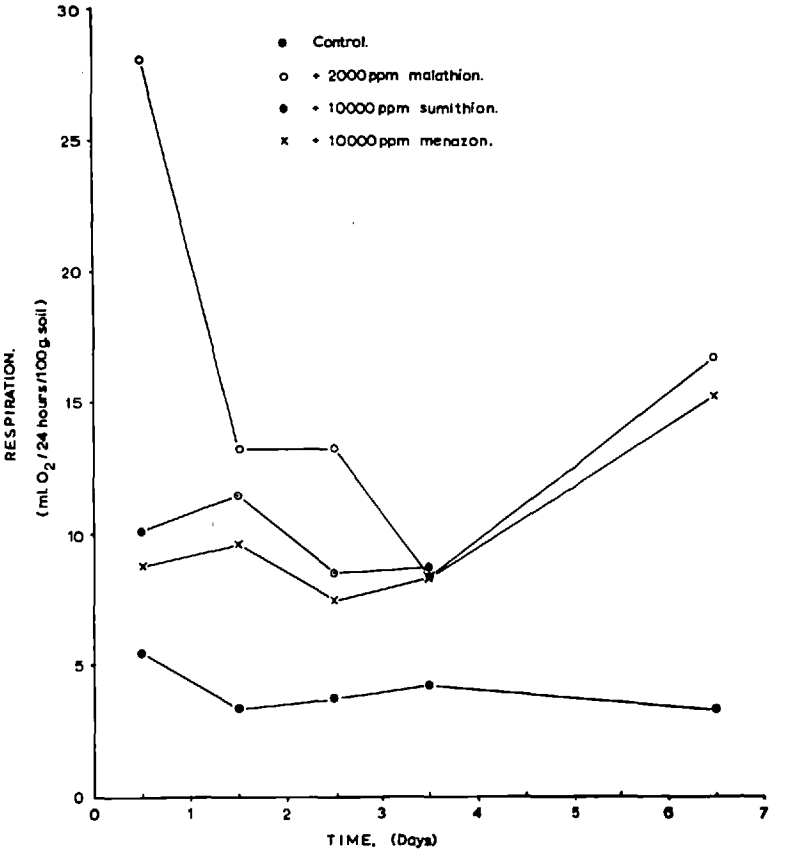
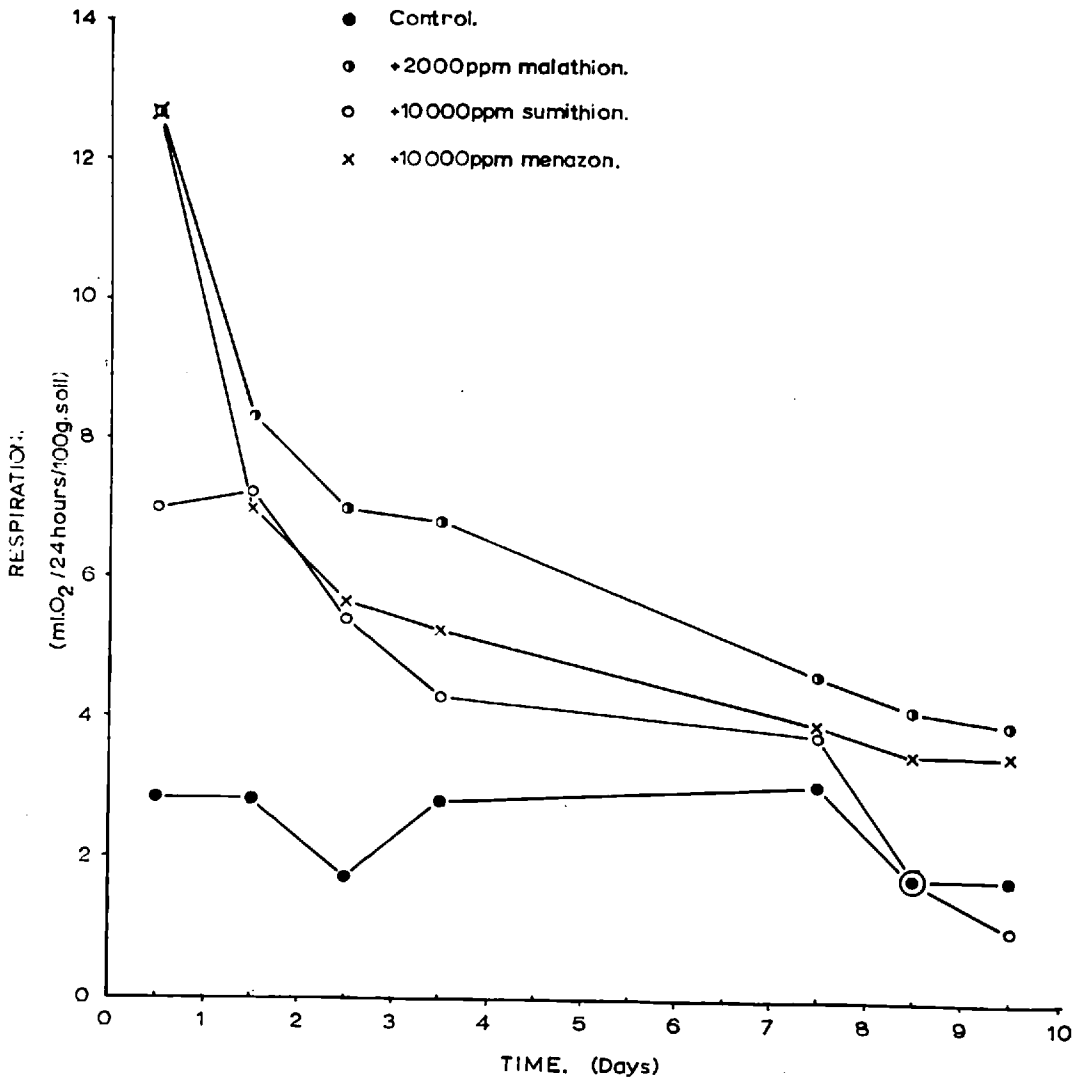


Table 16. The effect of malathion, sumithion and menazon on the rate of respiration of micro-organisms in a peat soil.

Time (days)	Rate of Respiration (ml.O_2/24 hours/100 g. soil)			
	Untreated Soil	Soil with malathion (2000 ppm)	Soil with sumithion (10000 ppm)	Soil with menazon (10000 ppm)
1	2.85	12.7	7.0	12.7
2	2.85	8.3	7.25	7.0
3	1.75	7.0	5.5	5.7
4	2.85	6.8	4.25	5.25
8	3.05	4.6	3.75	3.95
9	1.75	4.15	1.75	3.5
10	1.75	3.95	1.0	3.5
Final pH	3.35	3.15	3.4	3.05

Fig. 14. The effect of malathion, sumithion and menazon on the rate of respiration of micro-organisms in a peat soil.



stimulated by malathion to over five times that of the control, the peat soil was stimulated approximately four times and the clay soil only double that of the control. Sumithion stimulated the largest increase in the rate of respiration in the peat soil, when it was over twice that of the control. It also stimulated the rate in the chalk soil to approximately twice that of the control, but there was only a small increase in the rate of respiration in the clay soil.

Menazon apparently stimulated the rate of respiration of the peat soil to a similar level as malathion, but only caused slight stimulation in the rate of the other two soils.

The untreated clay soil had the highest rate of respiration of the three soils used, but the stimulation of the rate by the insecticides was lowest in this soil. It is probable that adsorption in the clay soil reduced the availability of the insecticides or their products to the soil microflora. Although one may also expect adsorption phenomena to affect the action of the insecticides in the peat soil due to the high organic matter content, it was found that the degree of stimulation was higher than in the clay soil.

The most surprising result occurred with menazon in the peat soil which apparently stimulated the rate of respiration to the

same extent as malathion. However, it was noted when the potassium hydroxide solution was being changed, that the flask contained hydrogen sulphide. Since this would also react with potassium hydroxide, it was concluded that false results were being obtained. In order to elucidate this effect, population studies were made on peat soils treated with menazon, in order to determine if the increase in the rate of respiration was associated with an increase in the soil population. The results of this investigation are fully described in the next section (P. 104).

The decomposition of menazon in peat soil with the evolution of hydrogen sulphide may have been entirely due to chemical action, especially as the pH of this soil was low. The problem therefore arose that the other insecticides as well as menazon may have been decomposed giving off volatile products. Chemical hydrolysis of sumithion almost certainly took place when sumithion was added to the chalk soil. When the pH of the soil was being determined, the water phase was coloured yellow which indicated accumulation in the soil of 3-methyl-4-nitrophenol, one of the hydrolysis products of sumithion.

Unfortunately, there was no way of determining how much the apparent oxygen uptake was due to chemical means, since it was found impracticable to add the insecticides to sterile soil and maintain sterility over a period of days.

f) The effect of chlorinated hydrocarbon insecticides on the rate of respiration of Chelsea loam

The chlorinated hydrocarbon insecticides DDT, aldrin and dieldrin were studied with respect to their effect on the respiration rate of Chelsea loam. These insecticides, and especially DDT, are known to persist in the soil for a relatively long time. It was hoped that by comparing the results obtained with the chlorinated hydrocarbon insecticides with those obtained with the organophosphorus compounds, that an estimation of their relative persistence might be made. If the stimulation of respiration caused by the organophosphorus compounds was entirely due to their decomposition, then little stimulation would be expected in the presence of the chlorinated hydrocarbon insecticides.

0.2 g. DDT was added to 100 g. soil to give a final concentration of approximately 2000 ppm, and its effect was compared with malathion and menazon also at 2000 ppm. The effect of aldrin and dieldrin at 5000 ppm was investigated in a separate experiment. The chlorinated hydrocarbon insecticides, as solids, were weighed and added to flasks containing 100 g. of soil. Duplicate flasks were set up for each insecticide at each concentration. Duplicate flasks of untreated soil as a control were prepared for the experiment with DDT, malathion and menazon, and separate controls were used for the experiment with aldrin and dieldrin. The water content of the flasks was adjusted and they were incubated in a water bath at 30°C.

The results which are shown in Table 17 demonstrate that the chlorinated hydrocarbon insecticides have little effect on the rate of respiration, indicating that they are not toxic to soil micro-organisms, and are not completely metabolised by the soil microflora. Since the untreated soils from each part of the investigation differ, it is difficult to compare the effect of DDT with that of aldrin and dieldrin directly. However, DDT did stimulate the rate of respiration of Chelsea loam in the first 24 hours almost as much as menazon, whereas aldrin and dieldrin had a negligable effect. The stimulation caused by DDT is very small compared to that of malathion, but does indicate that even if DDT itself is not being metabolised, then some contaminating material in the technical product is probably being utilised by the soil microflora.

The fact that the chlorinated hydrocarbon insecticides, which are known to be persistent in the soil, have little effect on soil respiration, tends to confirm the previous deductions that malathion and sumithion are readily decomposed in the soil, but that menazon may persist much longer.

Table 17. The effect of DDT compared with malathion and menazon, and aldrin and dieldrin, on the rate of respiration of micro-organisms in Chelsea loam.

Time (days)	Rate of Respiration (ml.O_2/24 hours/100 g. soil) Soil with and without insecticide at two concentrations						
	2000 ppm				5000 ppm		
	Untreated	DDT	malathion	menazon	Untreated	aldrin	dieldrin
1	5.5	6.5	15.6	6.85	4.2	4.2	4.8
2	2.4	2.6	5.0	3.0	3.6	3.6	3.8
3	2.2	2.4	4.9	2.8	3.4	3.3	3.5
4	2.2	2.2	2.6	1.8	3.4	3.0	3.6
7	-	-	-	-	3.2	2.7	3.0
8	2.0	2.2	3.2	1.6	3.1	3.4	3.0
9	2.0	2.0	3.6	1.6	3.2	2.0	2.9
10	1.8	1.2	-	1.9	3.2	2.3	3.0

B. Microbial Population of Treated and Untreated Soil

The effect of the organophosphorus insecticides on soil respiration indicated that the population of soil micro-organisms was being stimulated in some manner. The increase in oxygen uptake might have been a reflection of the increased respiration of a static population, or it might have indicated utilisation and proliferation of a section of or all the soil micro-organisms. Population studies were therefore undertaken to determine the effect of the insecticides on the total number of viable micro-organisms in the soil, and further, on the number of specific types of micro-organisms in the soil.

a) The effect of insecticides on the general population in Chelsea loam

The general population of the soil was studied in order to correlate the effect of insecticides on the rate of respiration with the numbers of viable organisms in the soil. The Chelsea loam was used for these studies and the insecticides were incorporated as in the respiration studies. The three organophosphorus insecticides were used, malathion at 200 and 2000 ppm, sumithion at 2000 and 10000 ppm and menazon at 2000 and 10000 ppm. Aldrin, dieldrin and DDT at 5000 ppm were used to confirm the respiration studies and to compare their effect with the organophosphorus compounds.

A single 250 ml. flask containing 100 g. soil was prepared for each insecticide concentration and the insecticide was incorporated either as the solid or as an emulsion. A flask containing untreated soil was also prepared to act as a control. The flasks were plugged with non-absorbent cotton wool; the water content of the soil was adjusted to 50% maximum water holding capacity, and the soil mixed thoroughly with a spatula to prevent the formation of lumps. The weight of the flasks plus the soil was determined after each sampling. Before the following samples were taken, the weight was redetermined and the loss in weight calculated. This quantity of sterile distilled water was then added to restore the correct water content. The method of sampling the soil was as described in the materials and methods. 1 g. samples in duplicate were taken from the organophosphorus insecticide-treated soil, and from the chlorinated hydrocarbon insecticide-treated soil, duplicate samples were weighed directly into dilution bottles.

Dilutions were made in sterile tap-water and 1 ml. aliquots from each of the appropriate dilutions pipetted into each of three Petri dishes. Thornton's agar, which was used to culture the organisms, was cooled to 42°C., poured into the Petri dishes and mixed with the soil suspension. When the agar had set, the plates were incubated inverted at 25°C. After 10 days, the number of viable organisms on the countable plates was recorded. An average of the three plates of the dilution chosen for counting, and of the

duplicate samples, was taken as the final result. The results of the effect of the organophosphorus compounds are given in Table 18 and graphically in Fig. 15 a) and b) and the effect of the chlorinated hydrocarbon insecticide in Table 19.

The results with the organophosphorus insecticides show that malathion at 2000 ppm greatly increases the number of viable organisms and only sumithion at 10000 ppm gave an increase in numbers which was comparable to the increase produced by malathion. The number of viable organisms in the untreated soil stayed approximately constant throughout the investigation, variations being caused by inaccuracies in the method. It was realised that variations in the number of micro-organisms in the soil would introduce errors, with the limited number of samples and replicate plates used. However, it was felt that the nature of the studies did not require absolute values, since only large changes were considered to be significant and these would be shown with the method used.

A significant feature of the results with malathion and sumithion was that the increase in the viable population depended on the concentration of the insecticides, which is similar to the results obtained in the respiration experiments. At the highest concentration used for malathion and sumithion, the population increased to a greater extent than at the lower concentrations.

Table 18. The effect of malathion, sumithion and menazon on the number of viable micro-organisms in Chelsea loam.

Time (days)	Number of viable micro-organisms X 10^6/g. soil						
	Soil in the presence and absence of three insecticides at various concentrations						
	Untreated Soil	Malathion		Sumithion		Menazon	
		200 ppm	2000 ppm	2000 ppm	10000 ppm	2000 ppm	10000 ppm
0	7.0	23.8	11.0	12.0	20.3	8.0	15.7
1	14.0	15.2	118.0	18.0	24.3	12.0	13.5
2	11.0	23.9	183.0	18.0	61.6		14.7
3	-	-	-	-	-	15.5	-
4	12.0	28.8	83.0	24.0	31.3		17.3
6	7.0	-	60.0	14.0	-	10.5	-
8	12.0	25.0	75.0	12.5	38.3	8.5	21.1
10	-	-	-	-	-	8.0	-
11	7.7	-	42.4	12.8	-	-	-
13	6.65	-	93.3	10.85	-	-	-
15	8.15	-	64.8	13.0	-	-	-

Fig. 15. The effect of malathion, sumithion and menazon on the number of viable micro-organisms in the Chelsea loam.

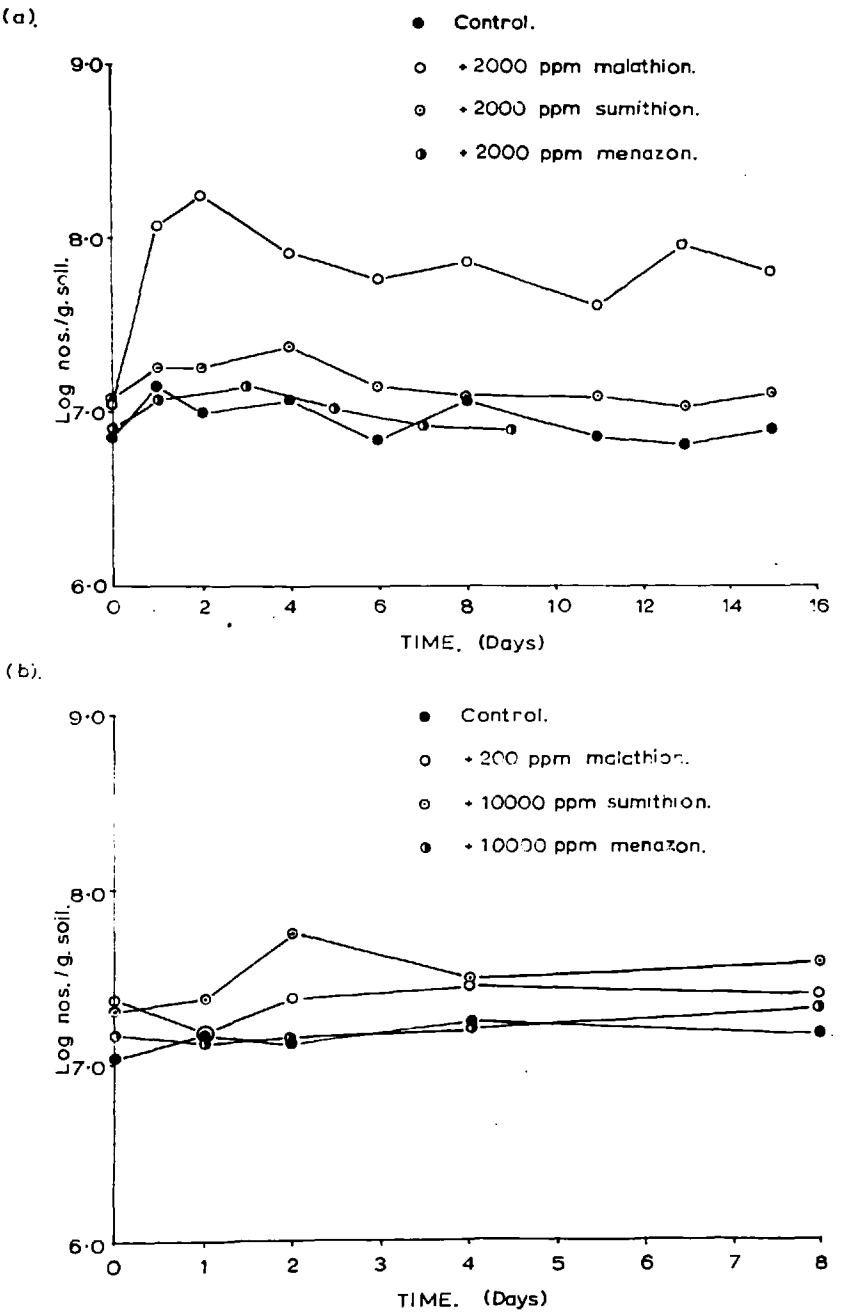


Table 19. The effect of DDT, Aldrin and Dieldrin on the number of viable micro-organisms in Chelsea loam.

Time (days)	Number of viable micro-organisms X 10^6/g. soil Soil with and without three insecticides at 5000 ppm			
	Untreated	DDT	Aldrin	Dieldrin
0	2.61	5.13	3.16	3.26
1	10.45	10.6	13.8	21.4
2	8.7	13.9	10.0	10.8
4	13.2	10.5	9.1	9.2
6	12.0	10.3	9.9	9.0
8	11.7	9.2	9.5	8.4

This indicates that the increase in respiration and the proliferation of the soil micro-organisms are associated with the utilisation of the insecticides. It was noted that, although the greatest rate of respiration in the presence of malathion and sumithion occured in the first 24 hours (see Tables 7 and 9), the highest viable population was not reached until the second day at the higher concentrations and the fourth day at the lower concentrations. This further confirms the previous deductions that the stimulation of respiration was caused by the proliferation of the soil microflora.

Menazon at 2000 ppm caused little effect on the soil population but at 10000 ppm there may have been a slight increase in the number of viable organisms up to the eighth day, but unfortunately counts were not continued beyond this time. The increase in respiration caused by menazon at 10000 ppm does not seem to be correlated with any change in the viable population, which indicates that the stimulation was caused by contaminating material in the technical product or by some mechanism which is unknown.

The results obtained with the chlorinated hydrocarbon insecticides tend to confirm the conclusions drawn from the respiration studies. The initial population in all soils was low but increased in the first 24 hours and then remained constant.

Only dieldrin showed any significant departure from this pattern. On the first day the population was approximately twice that of the control, but the viable count returned to normal on the following day. These results confirm the findings of other workers, who found that these chlorinated hydrocarbon insecticides are relatively inert in the soil.

b) The effect of menazon on the viable population of the peat soil

The effect of menazon at 10000 ppm on the viable population of peat soil was determined in order to investigate the atypical result obtained in the respiration studies (Table 16). Menazon in Chelsea loam is relatively inert and the result with the peat soil suggested that at the pH of this soil the microflora might be able to decompose the insecticide. If the microflora were able to decompose menazon, one might expect an increase in the number of viable organisms in a similar manner to that found with malathion and sumithion in Chelsea loam. However, if the increase in respiration was caused by the evolution of hydrogen sulphide, then there would not be any significant increase in the viable count.

The menazon was added as a solid to 100 g. of peat soil in a similar manner to that use for the investigation with Chelsea loam. A control of untreated soil was also used and the flasks were incubated in a water bath at 30°C. The results are shown in

Table 20 and graphically in Fig. 16, from which it will be seen that the total population in the peat soil being predominantly fungi, is lower than in the Chelsea loam, probably due to the low pH of the soil. The menazon did not increase the number of viable micro-organisms in the soil, in fact there was a decrease in numbers towards the end of the experiment. This indicates that the hydrogen sulphide evolved in the presence of the menazon not only gave a false rate of respiration, but was probably toxic to the organisms in the soil.

c) The effect of organophosphorus insecticides on the proportions of Gram positive and negative bacteria in Chelsea loam

An investigation was carried out to determine whether the increase in the number of viable organisms in Chelsea loam was due to any major group of bacteria, which might be indicated by changes in the proportion of organisms with a particular Gram reaction.

The method was essentially the same as for the population studies, except that the dilutions in sterile tap water were inoculated onto the surface of the Thornton's agar, so that colonies formed could be picked off, to determine their Gram reaction. The same concentration of insecticides was used as before, i.e. malathion 2000 ppm, sumithion and menazon 10000 ppm, and they were compared with an untreated soil control. Duplicate samples were taken at intervals and the appropriate dilutions inoculated onto Thornton's agar using

Table 20. The effect of menazon on the number of viable micro-organisms in peat soil.

Time (days)	Number of viable micro-organisms X 10^3/g. soil	
	Untreated Soil	Soil with menazon (10000 ppm)
0	199	264
1	338	181
2	308	285
3	271	304
7	355	151
9	460	162

Fig. 16. The effect of menazon on the number of viable micro-organisms in peat soil.

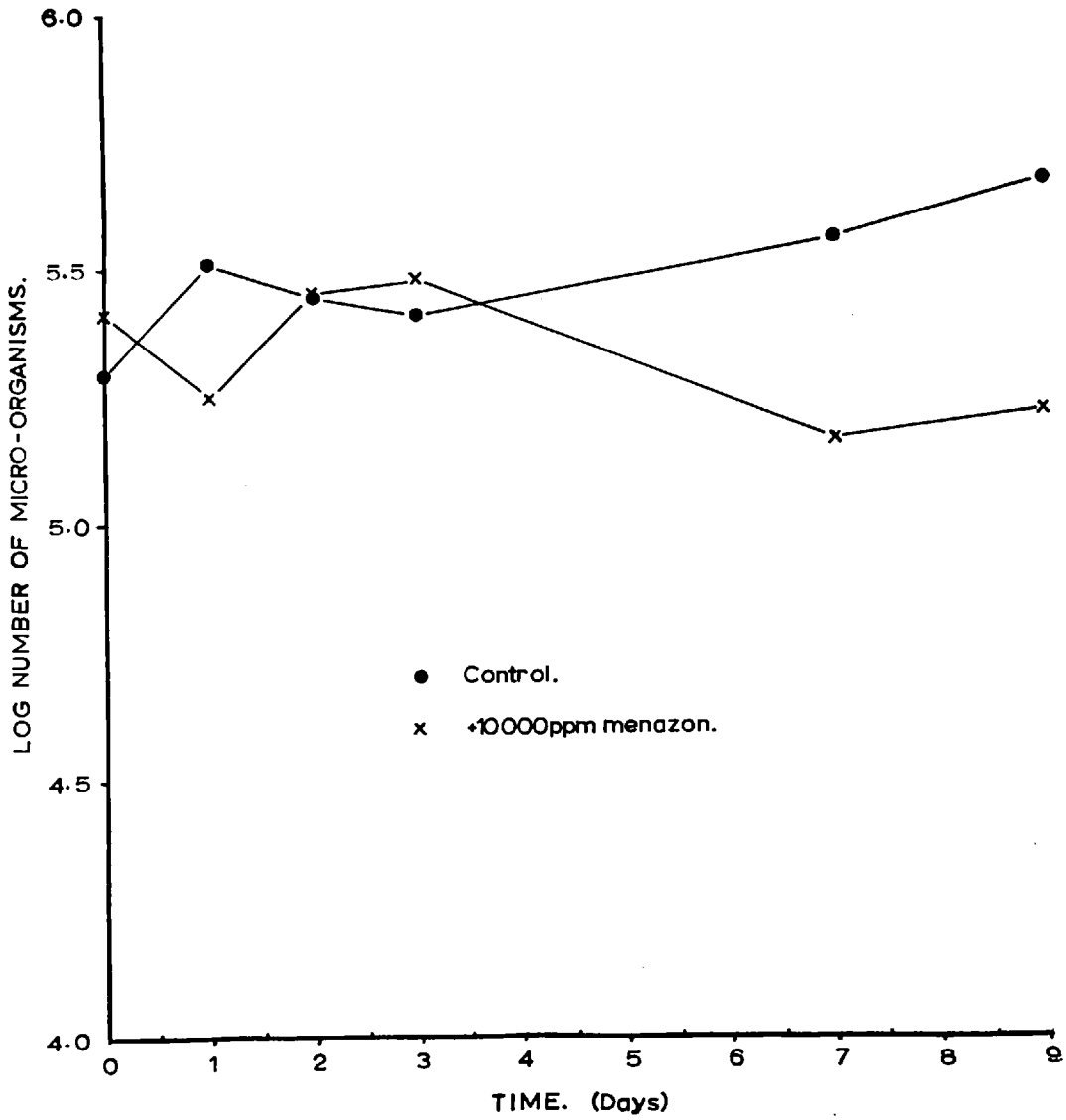
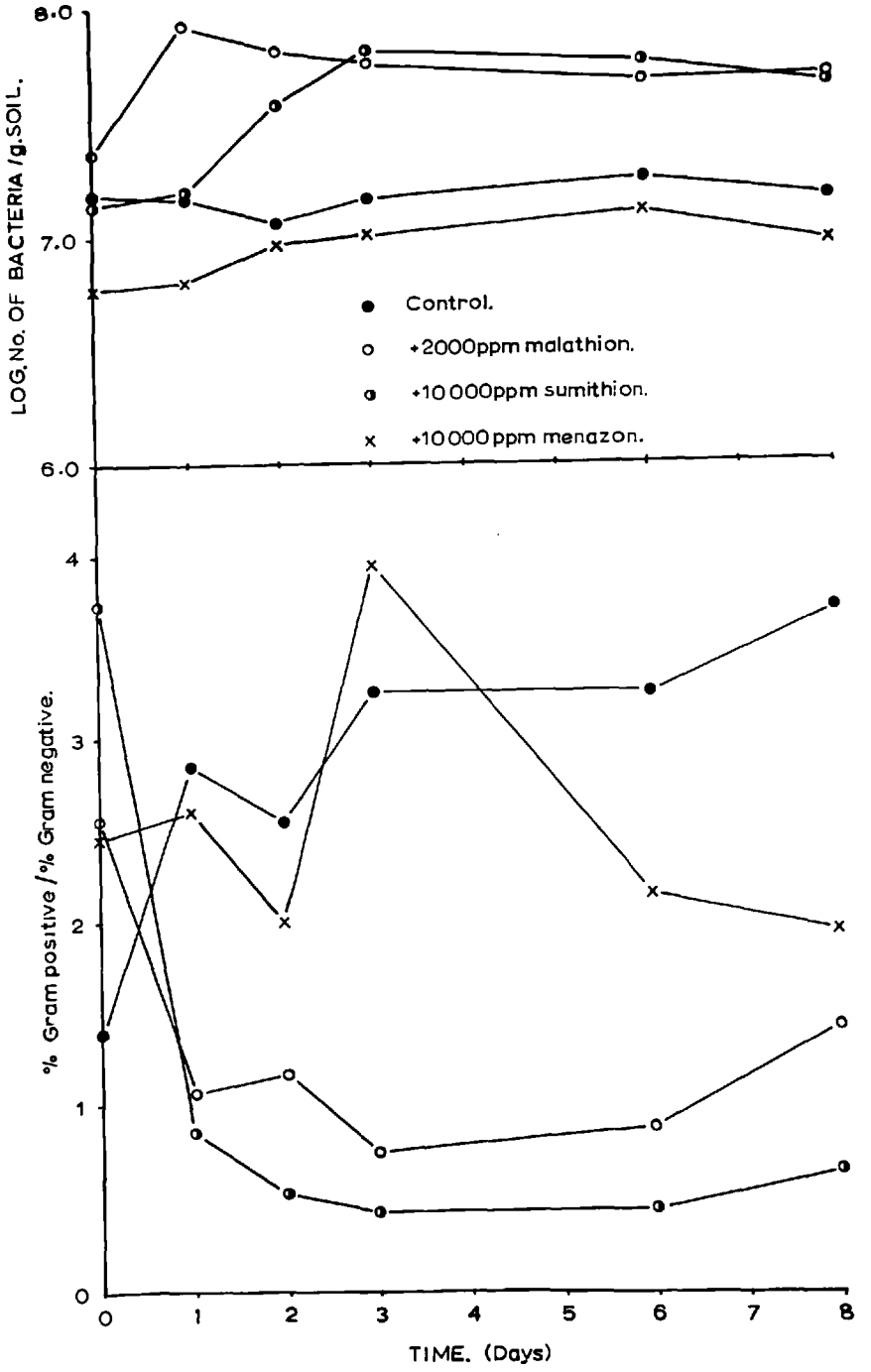


Table 21. The effect of malathion, sumithion and menazon on the proportions of Gram positive and negative bacteria in Chelsea loam.

Time (days)	Soil with and without three insecticides at various concentrations							
	Index of Gram +ve/Gram -ve *				Number of viable micro-organisms X 10^6 /g. soil			
	Untreated	malathion 2000 ppm	sumithion 10000 ppm	menazon 10000 ppm	Untreated	malathion 2000 ppm	sumithion 10000 ppm	menazon 10000 ppm
0	1.4	2.56	3.71	2.45	15.6	23.4	13.8	5.8
1	2.86	1.07	0.86	2.61	14.6	82.4	15.8	6.3
2	2.54	1.17	0.52	2.0	11.2	66.6	38.6	9.13
3	3.25	0.74	0.43	3.94	14.7	57.4	63.8	11.5
6	3.24	0.88	0.44	2.15	18.0	48.0	57.7	12.9
8	3.71	1.42	0.66	1.95	14.8	50.1	48.3	9.4

* Index calculated as the percentage of Gram positive bacteria/percentage of Gram negative bacteria.

Fig. 17. The effect of malathion, sumithion and menazon on the proportions of Gram positive and negative bacteria in Chelsea loam.



3 'plates' for each dilution. At least 50% of the colonies developing on two of the plates of each dilution were picked and their Gram reactions determined. The percentage of Gram positive and negative colonies was calculated. The percentage of Gram positive organisms was divided by the percentage of Gram negative organisms to give an index of the proportions present, i.e. Gram positive/Gram negative = 1 when the proportions are 50%/50%. The total number of colonies on the plates was also recorded to determine the number of viable organisms in the soil. The results are shown in Table 21 and graphically in Fig. 17.

The results show that the changes in the number of viable micro-organisms in the soil were similar to the results given in Table 18. Since the total number of viable organisms behaved in a similar manner, it was assumed that changes in the proportions of Gram positive and negative bacteria would, therefore, also be comparable.

Although the number of organisms in the untreated soil stayed relatively constant, there was apparently a slight increase in the proportion of Gram positive bacteria in the soil. The soil treated with menazon behaved almost identically to the control but towards the end of the investigation there was an increase in the proportion of Gram negative bacteria. The increase in the number of viable organisms in the presence of malathion and sumithion was

associated with an increase in the proportion of Gram negative bacteria until they were more numerous than Gram positive bacteria, shown by the index figures which are less than unity. However, there was a difference in the action of malathion and sumithion. The increase in the number of viable organisms in the presence of malathion occurred in 24 hours but the proportion of Gram positive and negative bacteria was approximately constant, indicating proliferation of both Gram positive and Gram negative types. After three days when the total number of viable organisms had started to decline the proportion of Gram negative bacteria increased which suggests that the number of Gram positive bacteria was declining faster than the number of Gram negative bacteria. This is in contrast to the effect of sumithion where the increase in the viable population was followed exactly by an increase in the proportion of Gram negative bacteria. This suggested that only the Gram negative bacteria were proliferating in the presence of sumithion and that the Gram positive bacteria were unaffected. At the end of the investigation on the eighth day, there was an indication that the number of Gram negative bacteria was declining in the soils treated with malathion and sumithion and that the proportion of Gram positive to negative bacteria was returning to normal.

It may be concluded from this investigation that menazon or its products cannot be utilised by soil micro-organisms

but that malathion and sumithion are readily assimilated. It would appear that both Gram positive and negative bacteria are able to utilise the degradation products of malathion but it is mainly the Gram negative bacteria which are able to utilise the products of sumithion.

d) The effect of organophosphorus insecticides on the anaerobic bacteria in Chelsea loam

The anaerobic bacteria are often neglected when studies are made of the soil microflora and since it is only under an anaerobic conditions that they become the dominant group, their importance is relatively small. However, since the aerobic organisms were stimulated by malathion and sumithion it was thought that there might be some change in the population of anaerobic micro-organisms. The method of culturing the anaerobes is described in the materials and methods. The insecticides were incorporated into 100g. aliquots of soil in 250 ml. flasks to give the following concentrations: malathion 2000ppm, sumithion and menazon 10000 ppm. A control of untreated soil was also included. The water content of the soils was adjusted and the flasks incubated aerobically at 30°C. Soil samples were taken using the second technique of weighing directly into the dilution bottle. Reinforced Clostridial Medium was used to culture the organisms using the pour-plate technique. The plates were incubated for one week at 30°C. in Brewer anaerobic jars.

Because there was limited space for incubating the plates, the control and each insecticide were studied separately, and only one dilution could be plated in triplicate for each of the duplicate samples. The results are shown in Table 22.

Care must be taken over the interpretation of the results in view of limiting factors such as the single dilution used, and differences in the soil over the period of the investigation, which lasted three months. The results show that the population of anaerobes did not change substantially throughout the periods of study, although there were variations in the soil used for each insecticide. Although in most cases the population doubled over the course of the experiment, this was also observed in the control. The investigation with sumithion had to be repeated, (hence the two sets of figures) since the anaerobe jar, containing the last two sets of plates (Expt. a), was found to be leaking and the plates had to be discarded. The initial population , in the second determination (b), was different from the first, but little change in numbers was shown over the period studied.

It was concluded that the insecticides did not affect the anaerobic population in the soil.

Table 22. The effect of malathion, sumithion and menazon on the number of anaerobic bacteria in Chelsea loam.

Time (days)	Number of viable anaerobic bacteria x 10^6/g. soil Soil with and without insecticides at various concentrations				
	Untreated	malathion 2000 ppm	sumithion 10000 ppm		menazon 10000 ppm
			a	b	
0	4.64	4.9	1.58	4.29	2.34
1	7.71	5.63	1.59	-	3.49
2	6.75	8.7	1.93	-	3.58
3	6.73	8.5	-	5.46	4.65
6	9.4	-	-	-	-
7	-	10.45	-	5.31	4.44

e) The effect of the organophosphorus insecticides on the numbers of actinomycetes in the soil

The numbers of viable actinomycetes in the soil was studied in a similar manner to that used for the other population studies. The insecticides were added to 100 g. soil at the following concentrations: malathion 2000 ppm, sumithion and menazon 10000 ppm. An untreated soil was included as a control. 1 g. samples of the soil were taken in duplicate and the actinomycetes cultured on Conn's glycerine-asparaginate agar, using 3 plates for each dilution. The plates were incubated at 25°C. for 10 days and then examined.

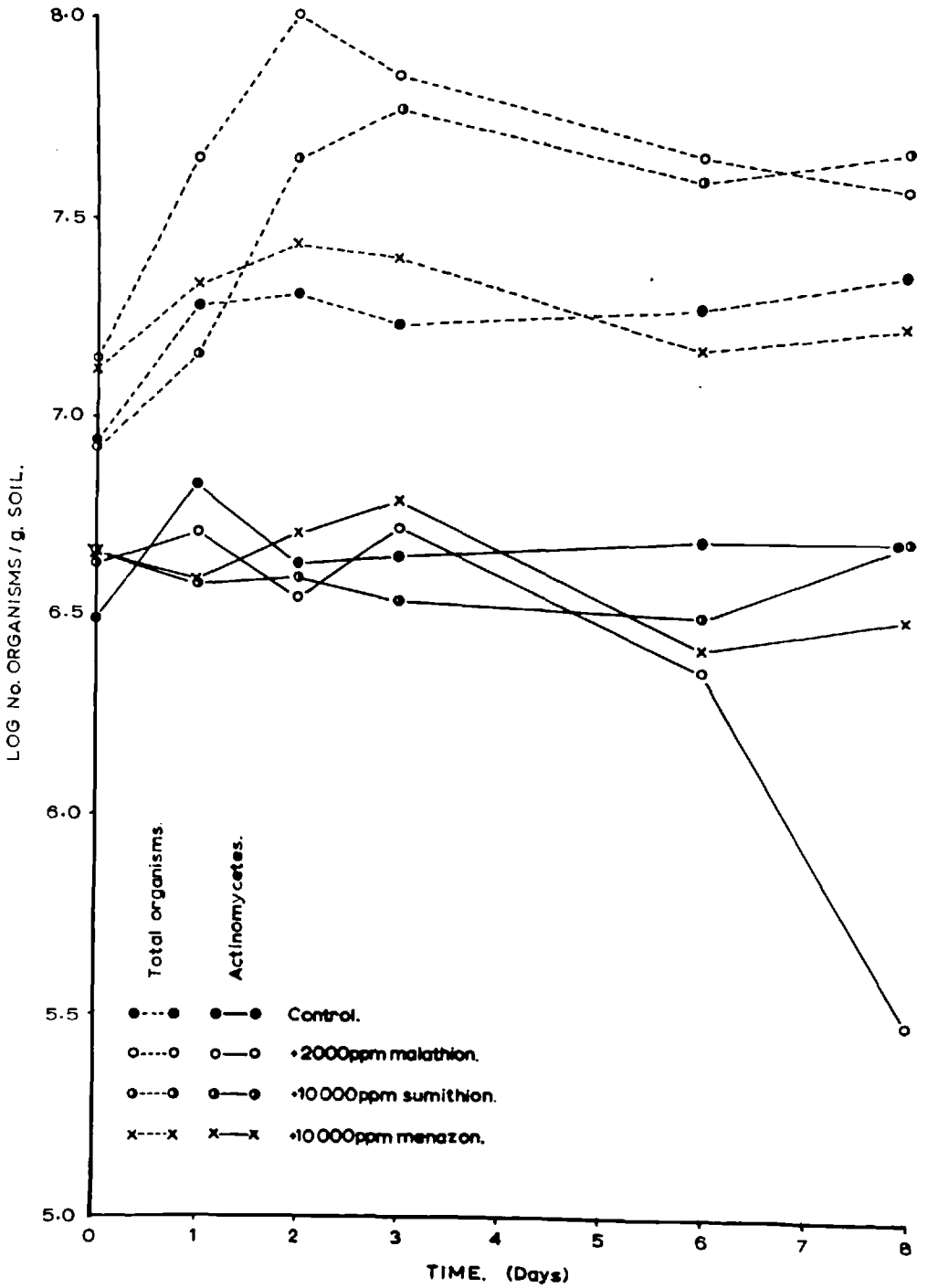
The actinomycetes were identified by the appearance of the colonies and if there was any doubt, the colonies were checked under the low power objective of a microscope. The total number of organisms developing on the plates was also recorded. The results are given in Table 23 and graphically in Fig. 18.

The results show that the total populations reacted as found in previous investigations, but that there was little effect on the number of viable actinomycetes. Only malathion appeared to cause any change and this was a depression in the number of actinomycetes. This depression is probably correlated with the drop in the proportion of Gram positive organisms described on Page 105 .

Table 23. The effect of malathion, sumithion and menazon on the number of viable actinomycetes in Chelsea loam.

Time (days)	Number of viable organisms x 10^6/g. soil Soil with and without three insecticides at various concentrations							
	Untreated		malathion 2000 ppm		sumithion 10000 ppm		menazon 10000 ppm	
	Total no.	Actino-mycetes	Total no.	Actino-mycetes	Total no.	Actino-mycetes	Total no.	Actino-mycetes
0	8.65	3.15	14.0	4.3	8.3	4.6	13.3	4.65
1	19.0	6.8	46.3	5.15	14.15	3.8	21.95	3.95
2	20.3	4.3	125.8	3.5	45.1	3.95	27.8	5.15
3	17.45	6.45	73.3	5.3	59.65	3.45	25.6	6.3
6	18.75	4.9	45.15	2.3	38.8	3.15	14.95	2.6
8	22.45	4.8	36.15	0.3	45.3	4.8	16.6	2.0

Fig. 18. The effect of malathion, sumithion and menazon on the number of actinomycetes in Chelsea loam.



When colonies of actinomycetes were picked and their Gram reaction determined, they were counted as being positive. The decline in the number of viable actinomycetes in the soil treated with malathion after three days would therefore increase the proportion of Gram negative bacteria and this was the result primarily observed.

The reason for the apparent toxicity of malathion to actinomycetes was not investigated, but since the number of actinomycetes in the soil was not affected until three days after the application of the malathion, it was probably a decomposition product, rather than the insecticide itself, which was having an effect.

f) The effect of the organophosphorus insecticides on the population of fungi in Chelsea loam

The effect of the insecticides on the population of the fungi in soil was determined, since they play an important role in cellulolytic decomposition and aid in maintaining the soil structure.

The method of estimating the population was similar to the previous studies. In an initial experiment, the insecticides were added to 100 g. aliquots of soil at the following concentrations: malathion 2000 ppm, sumithion and menazon 10000 ppm, and compared with an untreated soil as a control. The soil in flasks was

incubated at 30°C. and duplicate 1 g. samples were taken periodically. The fungi were cultured on Sabouraud's maltose agar using 3 plates for each dilution, and the plates were incubated at 25°C. for 4 days. Because the size of the colonies was so large, few colonies could be counted and plates from a high dilution had to be used. The results are shown in Table 23 and graphically in Fig. 19 (a).

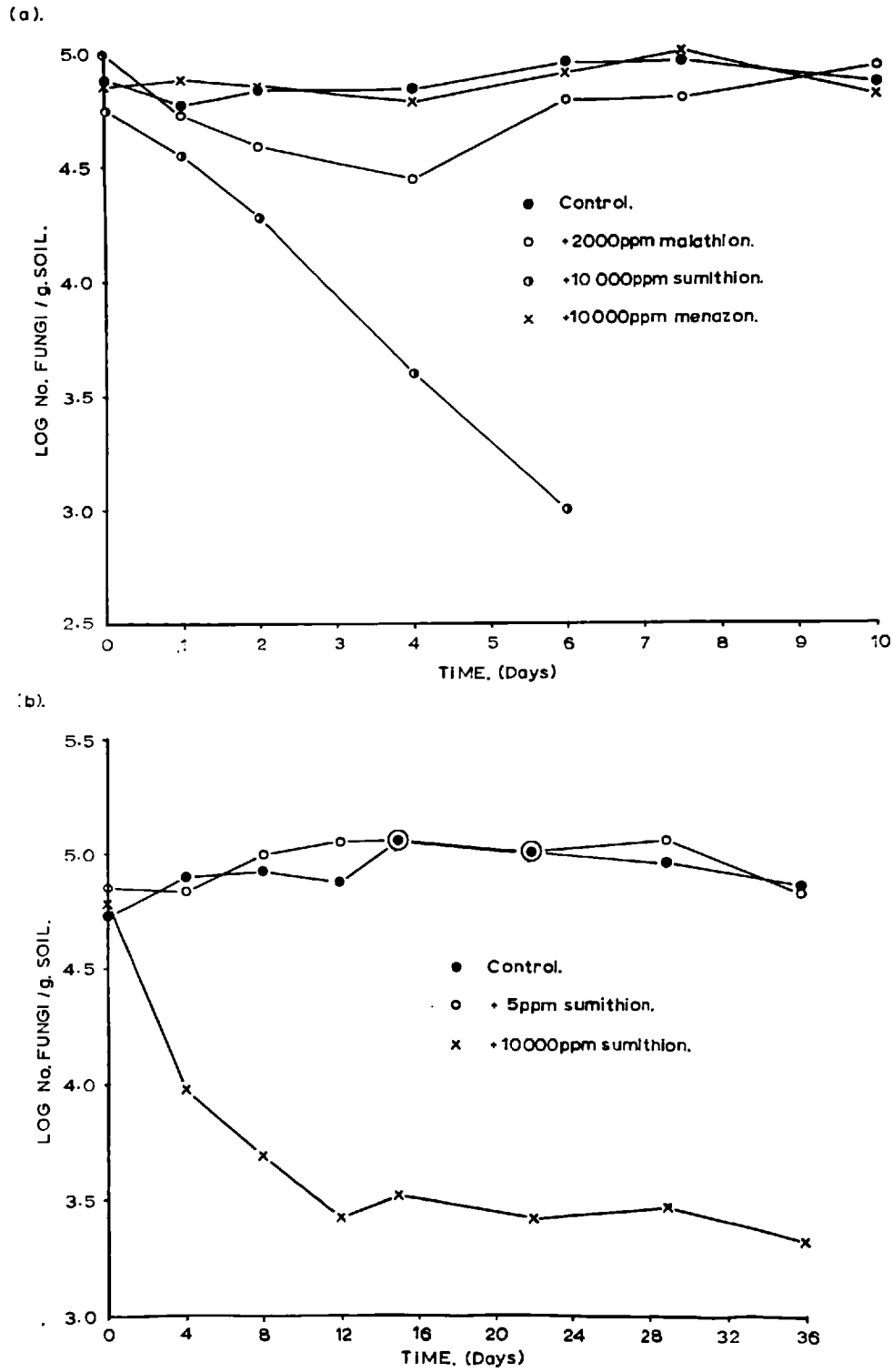
The results show that sumithion depressed the number of fungi in the soil to a level which could not be estimated at the dilutions used. Malathion also depressed the numbers slightly, but they soon recovered. Menazon had no apparent effect on the number of viable fungi.

The action of sumithion was so marked on the fungi that it was decided to see if the depression of the fungi continued until they had completely disappeared, and also to determine the effect of sumithion at 5 ppm, which is a level which could be found under field conditions. The sumithion at 5 ppm was incorporated into the soil using sand as a diluent. 0.01 g. sumithion was weighed into 20 g. acid-washed silver sand and mixed thoroughly. 1 g. of the mixture was added to 100 g. soil giving approximately 5 ppm. Since the number of bacteria developing on Sabouraud's maltose agar became too great at the dilutions plated, the media for counting yeasts, described in the materials and methods, was used without the diphenyl. At the low pH of the medium no bacteria develop,

Table 23. The effect of malathion, sumithion and menazon on the number of viable fungi in Chelsea loam

Time (days)	Number of viable fungi x 10^3/ g. soil Soil with and without three insecticides at various concentrations			
	Untreated	malathion 2000 ppm	sumithion 10000 ppm	menazon 10000 ppm
0	77.0	118.5	57.0	74.0
1	59.0	53.5	36.0	77.0
2	69.0	39.0	19.0	70.0
4	71.0	29.0	4.0	63.0
6	90.0	61.0	1.0	80.0
8	90.0	63.0	< 0.2	100.0
10	69.0	83.0	< 0.2	62.0

Fig. 19. The effect of malathion, sumithion and menazon on the number of fungi in the Chelsea loam



but the growth of fungi was luxuriant. The plates were incubated at 25°C. for 4 days and to increase the efficiency of the count, five replicate plates were used for each dilution. The results are shown in Table 23 and graphically in Fig. 19 (b).

The results again show a depression of the fungi with sumithion at 10000 ppm as in the previous experiment. However, the depression appears to reach a constant level which did not return to normal in 36 days. The sumithion at 5 ppm had no effect on the fungi when compared with the untreated soil.

The effect of the sumithion at the higher level indicated that sumithion or a decomposition product was toxic to the fungi and a more detailed investigation was carried out with fungi isolated from the soil. This will be reported fully in the next section, where the effect of the insecticides on micro-organisms in pure culture has been studied (Page 133).

The results obtained by studying the persistence of sumithion indicated that one of the hydrolysis products of sumithion, 3-methyl-4-nitrophenol, was accumulating in the soil. The pure compound was obtained (Aldrich Chemical Co. Inc.) and its effect on the population of fungi and bacteria in the soil studied. 3-methyl-4-nitrophenol was added to 100 g. soil to give a concentration equivalent to that which would be formed by the hydrolysis

Table 23. The effect of sumithion at 5 and 10000 ppm on the number of viable fungi in Chelsea loam.

Time (days)	Number of viable fungi x 10^3/g. soil Soil with and without sumithion at two concentrations		
	Untreated	5 ppm	10000 ppm
0	54.6	74.5	61.5
4	80.5	68.5	9.5
8	83.5	98.5	4.9
12	74.8	115.0	2.7
15	115.0	113.5	3.3
22	102.0	103.3	2.7
29	91.8	113.0	3.0
36	73.7	66.8	2.1

of 10000 ppm sumithion. (1g. sumithion is equivalent to 0.55 g. 3-methyl-4-nitrophenol). 0.55 g. of the solid 3-methyl-4-nitrophenol was weighed and added to 100 g. soil in a 250 ml. flask. The water content of the soil was adjusted and the flask incubated at 30°C. Duplicate samples were taken periodically and the number of fungi and bacteria in the soil determined. The counts of fungi were made on the nutrient agar/glucose/tartaric acid medium and total viable counts made on Thornton's agar. The plates were incubated at 25°C., the fungi being counted after 4 days and the general population after 10 days incubation. The results are shown in Table 24 and graphically in Fig. 20.

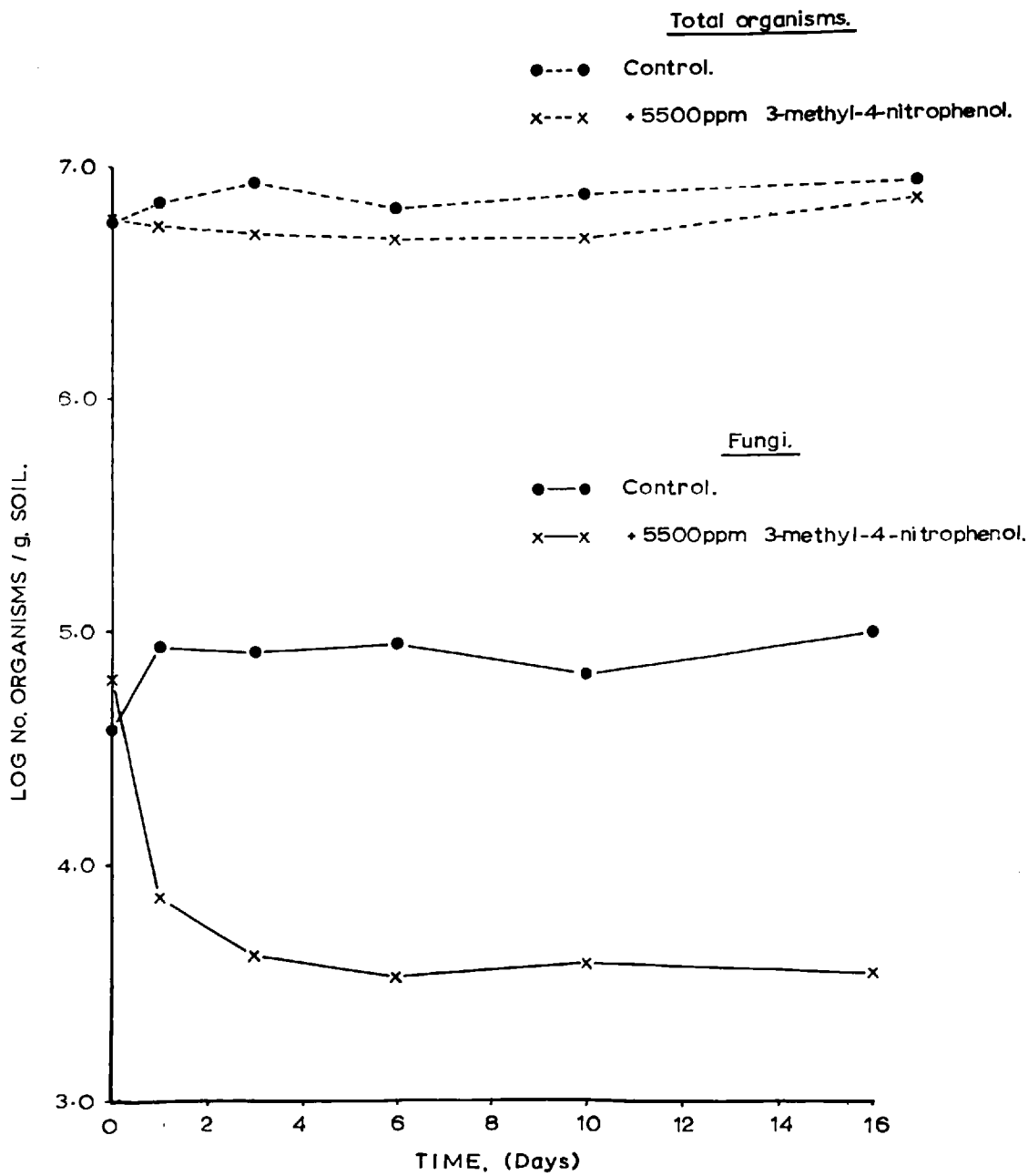
The results indicate that 3-methyl-4-nitrophenol has little or no effect on the total viable count in the soil, which suggests that the bacteria, which form the bulk of the count, are not susceptible to it. It also suggests that it is probably the other hydrolysis product, 0,0-dimethyl phosphorothioate, which brings about the stimulation of bacteria in soil treated with sumithion, noted in Tables 18 and 21.

The depression of the fungi with 3-methyl-4-nitrophenol was very similar to that of the sumithion except that the fall in numbers occurred at a faster rate. This tends to confirm the hypothesis that it is the 3-methyl-4-nitrophenol which is really toxic to fungi, since in the soil sumithion is not hydrolysed

Table 24. The effect of 3-methyl-4-nitrophenol on the number of viable fungi and total organisms in Chelsea loam

Time (days)	Soil with and without 3-methyl-4-nitrophenol at 550 ppm			
	Number of viable fungi x 10^3/g. soil		Total number of viable micro-organisms x 10^6/g. soil	
	Untreated	Treated	Untreated	Treated
0	37.9	61.7	5.78	5.88
1	85.8	6.98	7.08	5.53
3	80.2	4.09	8.41	5.06
6	87.5	3.28	6.45	4.76
10	64.5	3.79	7.42	3.92
16	98.5	3.45	8.04	6.8

Fig. 20. The effect of 3-methyl-4-nitrophenol on the number of fungi and total viable micro-organisms in Chelsea loam



immediately but over a period of days (c.f. persistence of sumithion in soil). Further work will be described (Page 133) using 3-methyl-4-nitrophenol in pure culture, which indicates that this compound is far more toxic than sumithion to a wide range of filamentous fungi.

g) The effect of organophosphorus insecticides on the population of yeasts in the soil

The population of yeasts in most soils is comparatively small, but because they are closely related to the filamentous fungi, it was decided to compare the effect of the insecticides upon them with the results obtained for the filamentous fungi. The insecticides were used at the following concentrations: malathion 2000 ppm, sumithion and menazon at 10000 ppm. A control of untreated soil was also prepared. The experimental procedure was identical to that used for the studies on the filamentous fungi in the soil, except for the medium, which was the nutrient agar/glucose/tartaric acid/diphenyl formulation. The medium, described in the materials and methods, supresses the growth of filamentous fungi but allows yeast colonies to develop. The plates were incubated at 25°C. for 4 days and yeast colonies were identified under the low power objective of a microscope. Since yeast colonies developing mycelia could not be distinguished from filamentous fungi, only the non-filamentous yeasts were counted. The results are given in Table 25.

Table 25. The effect of malathion, sumithion and menazon on the number of viable yeasts in Chelsea loam

Time (days)	Number of viable yeasts x 10^2/g. soil Soil with and without three insecticides			
	Untreated	malathion 2000 ppm	sumithion 10000 ppm	menazon 10000 ppm
0	10.0	3.5	3.5	3.5
2	67.0	66.5	< 1.5	78.5
4	23.95	12.68	< 1.5	22.1
6	52.8	26.4	< 1.5	32.75
10	17.65	20.35	< 1.5	8.45

The results were rather variable, due to the low number of colonies that developed on the plates. Although the medium reduced the rate of growth of fungi, it did not inhibit it completely, which prevented the counting of a lower dilution to increase the accuracy. However, it was clear that the sumithion depressed the number of yeasts in the soil in a similar manner to the fungi, but there was no effect with the other insecticides. The toxic action of sumithion and 3-methyl-4-nitrophenol to the yeasts was also investigated further, and is described later (P.142).

C. Action of insecticides on micro-organisms in pure culture

From the previous investigations it was concluded that the insecticides were not toxic to micro-organisms, with the exception of sumithion towards the fungi and possibly malathion towards the actinomycetes. However, the soil is a complex environment and the interaction of such factors as adsorption and pH makes it difficult to determine the true toxicity of compounds. Fletcher and Bollen (1954) and Gray (1954) have shown that BHC is toxic to a wide variety of organisms in pure culture but not in the soil. The action of the three organophosphorus compounds on various micro-organisms was, therefore, carried out to see if the insecticides were toxic in pure culture.

a) The effect of insecticides on bacteria

The organisms used were obtained from the stock culture collection; six Gram positive and five Gram negative bacteria being chosen at random. The organisms were plated out to check their purity and were then grown in nutrient broth to provide a turbid suspension. The suspensions were then flooded onto separate nutrient agar plates, previously dried at 37°C., the excess suspension was removed, and the plates allowed to stand until dry. Small paper discs cut from blotting paper were sterilised in Petri dishes in an oven at 165°C. for 2 hours. The malathion and sumithion were sterilised by passage through sintered glass filters (Pyrex, porosity grade No. 5), and drops of the insecticides were placed aseptically onto the paper discs. One disc impregnated with malathion and another impregnated with sumithion were placed on each seeded agar plate. A small pile of menazon was also placed onto each plate in the manner used for auxanography. Two of the organisms were seeded on to duplicate plates and after the discs and menazon had been placed in position, one plate from each duplicate was placed in the refrigerator. This slowed the rate of growth of the organisms but allowed the diffusion of the insecticides to take place. The plates were removed from the refrigerator after 24 hours and incubated with the other plates at 30°C. for 24 hours. The plates were examined for inhibition of growth or stimulation around the insecticides.

The results which are shown in Table 26 indicate that the three organophosphorus insecticides have little effect on the organisms used. The small effects noted all occurred with the Gram positive bacteria although menazon had no effect whatsoever. The inhibition noted was very small, producing a clear zone round the edge of the discs at a concentration which must have approached that on the disc itself. The stimulation noted with Bacillus subtilis was also round the edge of the discs. The plates which had been placed in the refrigerator for 24 hours showed similar results to those incubated immediately, which is what one would expect, since the lower concentrations after diffusion would not have an effect.

It was decided to investigate further the effect of malathion on Staphylococcus aureus, Bacillus mycoides and Bacillus megatherium, since although at the lower concentrations growth was not inhibited, it was felt that there might be some effect on the normal growth curve. The effect on the growth of Bacillus subtilis was also investigated.

0.01 ml. malathion was dissolved in 100 ml. nutrient broth (Oxoid), the solution filter sterilised and dispensed aseptically in 10 ml. aliquots into sterile test-tubes with metal caps. Control tubes of broth were sterilised by autoclaving at 15 lb. pressure for 15 minutes. Four control tubes and four tubes containing the insecticide solution were placed into a water bath

Table 26. The effect of malathion, sumithion and menazon on the growth of bacteria on nutrient agar

	Growth of bacteria in the presence of three insecticides *		
Gram positive bacteria	Malathion	Sumithion	Menazon
Staphylococcus aureus	+	-	-
Sarcina lutea	-	-	-
Streptococcus fecalis	-	-	-
Bacillus mycoides (Strain 16)	++	-	-
Bacillus subtilis (Marburg Strain)	--	--	-
Bacillus megatherium	++	+	-
Gram negative bacteria			
Eschericia coli (Type 1)	-	-	-
Serratia marcescens	-	-	-
Aerobacter aerogenes	-	-	-
Pseudomonas pyocyaneus	-	-	-
Proteus vulgaris	-	-	-

* ++ inhibition of growth
+ slight inhibition of growth
- no effect
-- stimulation of growth

at 30°C. to equilibrate. The organisms were grown at 30°C. for 24 hours in nutrient broth and 0.1 ml. of a suspension of each organism was placed into a tube containing nutrient broth and also into a tube containing the solution of malathion in nutrient broth. The growth of the organisms at 30°C. was followed using a nephelometer with a tube of sterile nutrient broth as a zero reference blank. The initial reading of each tube was subtracted from subsequent readings. The results are shown in Table 27 and graphically in Fig. 21.

The results show that the malathion did have a depressing effect on the growth of Staphylococcus aureus and Bacillus mycoides, although there was little effect on Bacillus megatherium. The malathion failed to stimulate Bacillus subtilis, as it had apparently done on the agar, and in fact slowed the growth rate slightly.

The results indicate that at very high concentrations malathion might be inhibitory to Gram positive bacteria, and at lower concentrations reduce their rate of growth.

b) The effect of sumithion on fungi

(i) Growth on agar media

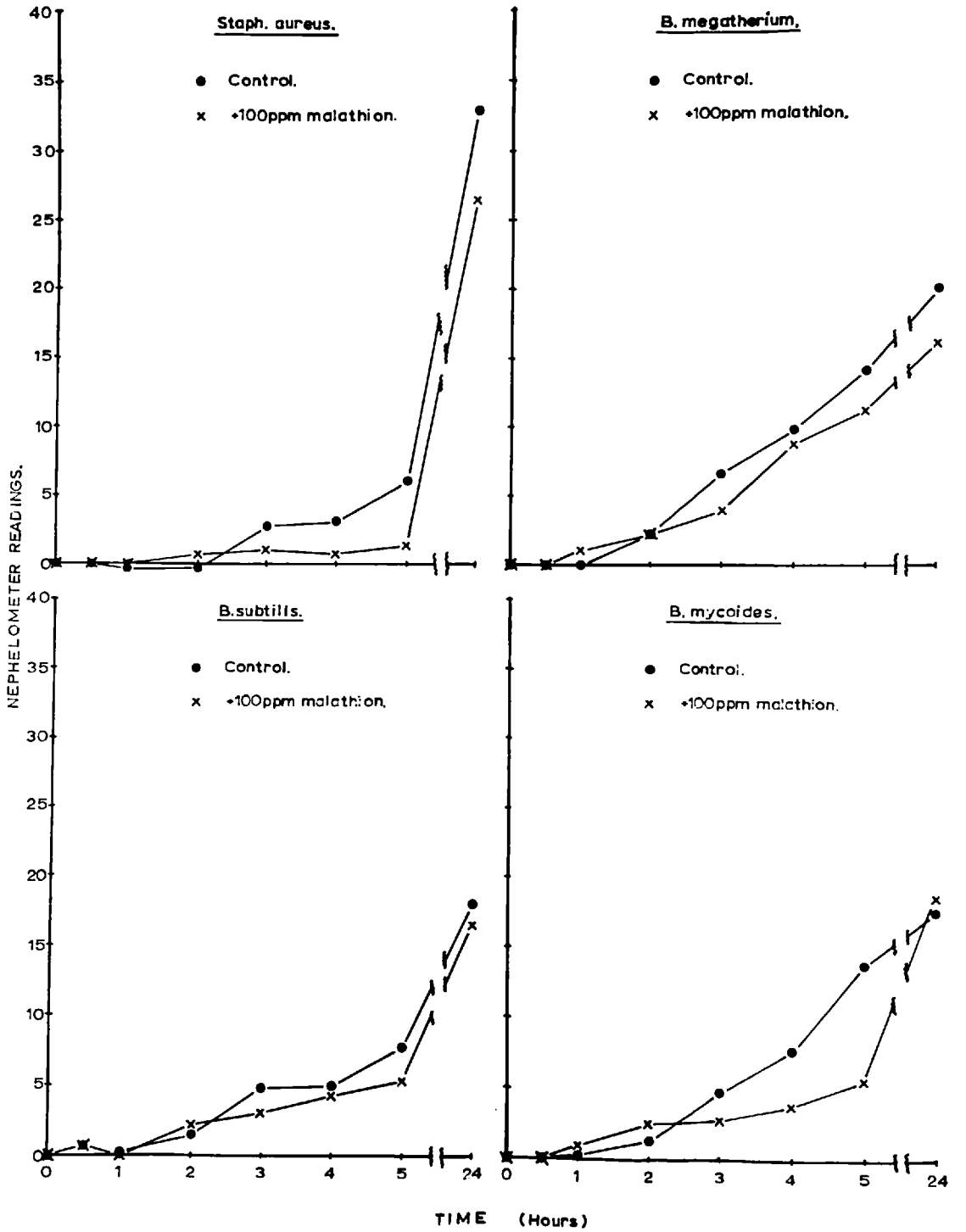
From the population studies it was concluded that sumithion, with one or more of its decomposition products, was toxic to filamentous fungi. An investigation was carried out with 8 fungi

Table 27. The effect of malathion on the growth of bacteria in nutrient broth

Time (hours)	Nephelometer readings * Growth of four bacteria in the presence and absence of malathion at 100 ppm							
	Staph. aureus		B. megatherium		B. subtilis		B. mycoides	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
0	0	0	0	0	0	0	0	0
½	0	0	0	0	0.7	0.7	0	0
1	-0.3	0	0	1.0	0.2	0	0.3	1.0
2	-0.2	0.7	2.2	2.1	1.4	2.2	1.3	2.5
3	2.7	1.0	6.5	3.9	4.7	3.3	4.7	2.7
4	5.2	0.7	9.8	8.7	4.9	4.2	7.7	3.7
5	6.0	1.4	14.0	11.1	7.9	5.2	13.7	5.5
24	32.9	26.5	20.0	16.1	17.9	16.4	17.5	18.5

* All readings corrected for initial readings

Fig. 21. The effect of malathion at 100 ppm on the growth of four species of bacteria in nutrient broth



isolated from the soil and 8 fungi which were aerial contaminants, on agar plates to determine the toxicity of sumithion in pure culture. 1.0 ml. of sumithion was added to 100 ml. of melted Thornton's agar, shaken to form an emulsion and then poured into sterile Petri dishes. Plates of Thornton's agar without sumithion were also prepared to act as controls. The plates were inoculated at a point source with the fungi and were incubated at 25°C. for 7 days, when growth was observed. The results shown in Table 28, compare the growth of the fungi on agar with and without sumithion.

The results indicated that although the amount of growth of the fungi was reduced in its presence, sumithion only completely inhibited two of the fungi. The soil isolates appeared to be more sensitive to sumithion than the aerial contaminants. It was noticed that some fungi which grew in the presence of sumithion apparently lost their pigmentation producing white or pink colonies. This was most commonly observed in penicillium and aspergillus species, although it was not always found even with these two genera.

(ii) Pigmentation on agar media

Three fungi were chosen which were blue or green on Thornton's agar, but white or pink on Thornton's agar plus 1.0% v/v sumithion. Microscopial examination showed them to be two aspergillus colonies and one penicillium.

Table 28. The effect of sumithion on the growth of fungi on Thornton's agar

Isolate	Growth of fungi from two sources, in the presence and absence of 1% sumithion, on Thornton's agar *			
	Aerial contaminants		Soil isolates	
	Control	Agar plus sumithion	Control	Agar plus sumithion
1	+++	++	+++	++
2	+++	++	-	-
3	+++	++	+++	+
4	+++	++	+++	-
5	+++	++	+++	++
6	+++	++	+++	++
7	+++	++	-	-
8	+++	++	+++	-

* After incubation for 1 week at 25°C.

+++ Good growth

++ Growth

\+ Little growth

\- No growth

The colonies from both media were reinoculated onto fresh media with and without 1.0% sumithion. It was hoped to determine if the fungi which had lost their pigment on subculturing onto the agar plus insecticide would do so again, and if those colonies which had lost their pigment would revert to normal when growing on medium without the insecticide.

The fungi were incubated at 25°C. for one week and observed for their pigmentation. Slide preparations were made to see if the fungi were pure cultures.

The results indicated that although some contamination was found, the fungi growing in the presence of the sumithion lost their normal pigmentation. The fungi growing as white or pink colonies in the presence of the sumithion reverted to their normal pigmentation when growing on fresh media without the insecticide.

The loss of pigmentation in the presence of sumithion might have been caused partly by reducing the amount of sporulation, but time did not permit a thorough investigation of the phenomenon.

(iii) The effect of sumithion and 3-methyl-4-nitrophenol on growth

The investigation described on Page 122 had already shown the toxicity of 3-methyl-4-nitrophenol to fungi in the soil,

and it was decided to investigate the action of this compound on them in pure culture compared with sumithion. It was felt necessary to do this since 3-methyl-4-nitrophenol is a chemical contaminant of technical sumithion and on addition of sumithion to Thornton's agar a yellow colour was produced indicating the presence of the hydrolysis product. This suggested that the effect of sumithion on fungi might be due solely to the presence of the contaminating material.

The method used was essentially the same as that described above, except that stock laboratory cultures of fungi were used. The six organisms chosen at random were compared with two soil isolates, which were apparently inhibited by sumithion. Each organism was inoculated into Thornton's agar untreated, with 1.0% v/v sumithion or 0.5% w/v 3-methyl-4-nitrophenol (The approximate concentration equivalent to 1.0% sumithion). The plates were incubated at 25°C. and observed after seven days. The results are shown in Table 29. From the results it was clear that 3-methyl-4-nitrophenol was far more toxic than sumithion to a wide range of fungi and traces in technical sumithion might account for the toxicity of the insecticide. Aspergillus flavus which did not grow in the presence of the sumithion, retained its normal pigment, which tends to contradict the previous observations, although Botrytis cinerea which had a brown pigment on Thornton's agar, was white when growing in the presence of sumithion.

Table 29. The effect of sumithion and 3-methyl-4-nitrophenol on the growth of fungi on Thornton's agar

Fungi	Growth of various fungi on Thornton's agar in the presence and absence of sumithion or 3-methyl-4-nitrophenol *		
	Untreated	with sumithion (1%)	with 3-methyl-4-nitrophenol (0.5%)
Mucor hiemalis (minus strain)	+	+	-
Aspergillus niger	+	-	-
Aspergillus flavus	+	+	-
Cephalothecium roseum	+	-	-
Botrytis cinerea	+	+	-
Fusarium sp.	+	+	-
Isolate 1.	+	-	-
Isolate 2.	+	-	-

* Growth after 7 days incubation at 25°C

\+ Good growth

\- No growth

(iv) Germination of spores

Although the toxicity of sumithion and 3-methyl-4-nitrophenol could account for the drop in the number of fungi in the soil, it was possible that their action was mainly exerted on the spores. In order to determine the effect of these compounds on spores, a germination experiment was carried out, using the slide culture technique. The two soil isolates used in the previous experiment were grown on Potato dextrose agar slopes until sporulation had taken place. A spore suspension of each isolate was made by washing off the spores from the culture with a sterile 0.001% v/v solution of Tween 80. Glass slides and cover-slips placed on a filter paper were sterilised in Petri dishes in an oven at 165°C. for 2 hours. The germination of the spores was carried out on Thornton's agar alone and in the presence of 1% v/v sumithion or 0.5% w/v 3-methyl-4-nitrophenol. The media was prepared and one drop of each medium was placed on each of two slides and a cover-slip placed on the drop to form a thin film. When the agar had set, the cover-slips were removed and 0.05 ml. of spore suspension of each of the isolates was placed onto separate slides of each medium. The cover-slips were replaced and the filter paper in the Petri dishes moistened to prevent the films of agar drying. The slides were removed from the Petri dishes periodically and the percentage of germinated spores determined. It was noted after two days of incubation at 25°C., that on the control slides germination was

far greater round the edges of the film than in the centre, probably due to lack of oxygen in the centre portion of the film. The cover-slips were, therefore, removed so that all the spores were maintained under aerobic conditions.

The results indicated that sumithion delayed the germination of both types of spore used, whilst 3-methyl-4-nitrophenol inhibits germination completely. After incubation for one day, from 40% to 50% of the spores had germinated on the Thornton's agar, compared with less than 10% in the presence of sumithion. After incubation for six days there was almost complete germination in the presence and absence of sumithion but no spores were observed to have germinated in the presence of 3-methyl-4-nitrophenol.

In order to determine whether the inhibition of germination by the 3-methyl-4-nitrophenol was reversable, a micromanipulator was used to remove some of the spores from the film and to inoculate them onto fresh Thornton's agar. No growth took place on the fresh medium indicating that the 3-methyl-4-nitrophenol was sporicidal and could, therefore, account for the decrease in the viable fungi population in sumithion treated soil.

c) The effect of sumithion on yeasts

Since it was shown that sumithion had reduced the population of yeasts in the soil in a similar manner to that found with the

filamentous fungi, it was decided to see if it was also toxic in agar media. A similar method was used as for the fungi. The six yeasts used were obtained from the stock culture collection and were streaked onto untreated Thornton's agar and agar containing either 1% sumithion or 0.5% 3-methyl-4-nitrophenol. The plates were incubated at 25°C. for 7 days.

The results showed that the growth of the yeasts was sparse on the control plates of Thornton's agar and the experiment was, therefore, repeated using malt agar as the culture medium. In this case the plates were incubated at 30°C. for 48 hours. The results for both media are shown in Table 30.

The results show that 3-methyl-4-nitrophenol is more toxic than sumithion to the growth of yeasts. The growth of the three yeasts on malt agar in the presence of 3-methyl-4-nitrophenol took place only where the inoculum was heavy, which indicates that the toxic action of this compound depends on the size of the inoculum. The culture medium also has an effect, since the yeasts were able to tolerate sumithion more easily on malt agar than on the Thornton's agar. It was also noted that the pigmentation of Rhodotorula growing on Thornton's agar plus sumithion was less strong that on the control plate. Although the growth of Rhodotorula was luxuriant on Thornton's agar, with and without sumithion, there was more in the absence of sumithion. Since the number of cells was

Table 30. The effect of sumithion and 3-methyl-4-nitrophenol on the growth of yeasts on Thornton's and malt agar.

Yeast	Growth of various yeasts on two different media in the presence and absence of 1% sumithion or 0.5% 3-methyl-4-nitrophenol *					
	Thornton's agar **			Malt agar ***		
	Untreated	with sumithion	with 3-methyl-4-nitro-phenol	Untreated	with sumithion	with 3-methyl-4-nitro-phenol
Trichosporon cutaneum	++	++	-	++	++	-
Schizosaccharomyces octosporus	+	-	-	++	-	++
Saccharomyces cerevisiae (DCL.535)	+	-	-	++	++	-
Saccharomyces cerevisiae (DCL.Asci.)	+	+	-	++	++	++
Candida tropicalis	+	-	-	++	++	-
Rhodotorula	+	+	+	++	++	++

* ++ Good growth; + sparse growth; - no growth

** Incubated at 25°C. for 7 days

*** Incubated at 30°C. for 2 days

less in the presence of the sumithion, this could account for the reduced pigmentation. This suggests that although sumithion or 3-methyl-4-nitrophenol may be inhibiting pigment formation in Rhodotorula and in the filamentous fungi, secondary effects play an equally large role, i.e. a reduction in sporulation or growth.

PART II. PERSISTENCE AND DECOMPOSITION OF INSECTICIDES

MATERIALS AND METHODS

Source of Insecticides

Details relating to malathion, sumithion and menazon may be found in Part I, Materials and Methods. The oxygen analogue of menazon was supplied as the pure crystalline solid by Plant Protection Ltd.

Source of Soil

Details of the Chelsea loam, which was used for all studies, may be found in Part I.

Methods of sterilising soil

Dry Heat:- 50 g. of soil in a 250 ml. conical flask plugged with cotton wool, was sterilised by heating at 165°C for 3 hours on two consecutive days. To test for sterility, a sample of the soil was taken aseptically and mixed with 9 ml. of sterile tap water. After mixing thoroughly, 1 ml. portions were inoculated into Thornton's medium. It was found that initially the soil was sterile, but during the course of the experiment a population of micro-organisms developed in the soil, probably due to aerial contamination.

Wet Heat:- As an alternative method, to reduce contamination, 5 g. aliquots of soil in 100 ml. conical flasks, plugged with cotton wool, were sterilised by autoclaving at 15 lb. pressure for 3 hours. The soil was tested for sterility as above, and it was found that control soils stayed sterile over the course of the experiments.

Estimation of Insecticides

Menazon

a) Colorimetric estimation of Menazon

The colorimetric estimation of menazon was based on the method of Plant Protection Ltd., Standard Method 170, Addendum 1, for measuring residues in soil

Extraction from soil. 5 g. samples of soil were shaken with 50 ml. of methanol in glass-stoppered 250 ml. conical flasks for 30 minutes and then filtered through Whatman No. 5 filter paper in a Buchner funnel under vacuum. The flask was washed with 40 ml. of methanol which was passed through the filter. The combined filtrate was transferred to a 100 ml. measuring cylinder, the volume made up to 100 ml. with distilled water and then transferred to a 500 ml. glass-stoppered conical flask. 100 ml. of 0.04N hydrochloric acid was added and the acidified extract was filtered through

Hyflo-Supercel (Johns Manville Co. Ltd.) in a Buchner funnel under vacuum. The clarified extract was purified by passing through a cation exchange column. The column was prepared by placing 4 g. of Zeo Carb 225, 1% cross-linked resin (Permutit Co. Ltd.) into a 25 ml. burette, containing a pad of glass wool. The column was charged by passing through it successively 50 ml. 2N hydrochloric acid and 50 ml. distilled water. The acidified extract was passed through the column at the rate of 4 ml. per minute and then the column was washed with 25 ml. distilled water, 50 ml. 0.5N hydrochloric acid and finally with 25 ml. distilled water. The menazon was eluted from the column with 75 ml. 1M ammonium acetate. At this stage the eluate could conveniently be left overnight in the refrigerator at 4°C. The menazon was extracted from the buffer by shaking three times with 50 ml. aliquots of chloroform, the extracts being collected in a clean, dry flask. The chloroform was distilled off by heating in a water bath at 90°C. until approximately 4 ml. remained in the flask. The concentrated extract was transferred to an acid-washed test-tube and the remaining chloroform evaporated off at 90°C. in a 400 ml. beaker of water acting as a water bath. The flask was rinsed twice with 4 ml. chloroform, the washings added to the test-tube and evaporated off.

Determination of Menazon. The menazon was estimated by digesting it with acid and measuring the inorganic phosphate released.

0.5 ml. of digestion mixture (4 parts analar perchloric acid:1 part conc. sulphuric acid) was added to the residue in the test-tube and boiled on an electric microkjeldahl stand for 30 minutes. After cooling, 1.5 ml. 4.7 N sodium hydroxide solution was added to neutralise the acid and the mixture was allowed to cool again. 2ml. of ascorbic acid - ammonium molybdate reagent (5ml. 5% aqueous ascorbic acid soln. + 20 ml. 0.625% aqueous ammonium molybdate solution containing 4.2 ml. conc. hydrochloric acid) was added and mixed immediately. The colour was developed at 37°C. in an incubator for 1 hour and measured at a wavelength of 820 mμ. in a spectrophotometer (SP.600, Unicam Instruments, Cambridge) using the red photocells.

A calibration curve was prepared using a standard methanol solution of menazon containing 20 μg./ml. The appropriate volume of methanolic menazon solution was added to a test-tube and the methanol evaporated off at 90°C. in a water bath. The menazon residue was digested and the phosphate determined as above.

b) Colorimetric estimation of the Thiolate

The method devised by Plant Protection Ltd. employs the same extraction procedure as used for menazon, but requires some changes for the clean-up procedure.

The cation exchange resin used was a Permutit Zeo-Carb 225, 8%, cross-linked resin, on which the thiolate is retained. The column was prepared as for the 1% cross-linked resin, but after passing through the acidified extract, 0.02 N hydrochloric acid was used to wash the resin. 200 ml. of 1M ammonium acetate buffer (pH 5.2) was used to elute the thiolate. The buffer was saturated with sodium chloride (approx. 30%) before extraction with 4 x 100 ml. chloroform. The final stages were identical to those used for menazon. The method measures both menazon and its thiolate, therefore for mixtures of the two analogues, a separate determination of the menazon had to be performed and the thiolate calculated by difference. A standard solution of thiolate in methanol containing 20 µg./ml. was used for the preparation of a calibration curve.

c) Polarographic estimation of Menazon

Some estimations of ^{14}C-labelled menazon were made by a Polarographic method using a Southern Analytical Differential Polarograph (Type A1660, Davis) with a dropping mercury electrode. The apparatus was calibrated using standard menazon solutions (in 1M ammonium acetate buffer) at concentrations just greater than expected, in order that as large a peak as possible could be obtained on the Cathode Ray screen with the unknown sample.

The menazon solution to be measured was placed in a clean glass polarographic cell to which had been added a few millilitres of mercury. The polarographic cell was maintained at 25°C. in the water bath of the electrode stand and the nitrogen gas supply connected. The rate of mercury drops was adjusted to 5 - 6 drops per minute by raising or lowering the mercury reservoir. The electrodes were then lowered into the polarographic cell, so that the cathode was touching the mercury in the bottom and the glass shield surrounding the anode was sealed by the water in the bath. Nitrogen was then bubbled through the menazon solution to deoxygenate it and the electrodes were connected to the synchronous device which produced a drop of mercury at intervals of 7 seconds.

The menazon peak was at 6.5 volts and the starting potential adjusted to between 0.5 - 1.5 volts so that, by scanning, the peak could be set on a vertical axis of the grid on the cathode ray screen. A straight base line was produced by adjusting the slope compensator and the maximum practical peak obtained by adjusting the shunt scale factor and amp scale factor. A record was made of the shunt scale factor and the number of units above the base line at which the peak was recorded. After calibrating the instrument with the standard menazon solution, the

procedure was carried out for the unknown sample. Since the concentration of menazon was proportional to the product of the shunt scale factor and the height of the peak, the strength of the unknown solution could be determined as µg./ml. At concentrations of about 10 µg./ml. there was interference from the soil extract and the polarograph could not be used for levels below this.

Preparation of soil extracts for measurement. Soil extracts were made as for the colorimetric estimation and then passed through the ion exchange column to purify and concentrate. To ensure that contamination did not occur from the ion exchange column, it was washed with 50 ml. 1M ammonium acetate buffer before charging with the 2N hydrochloric acid. The menazon was measured in the 1M ammonium acetate buffer used to elute the menazon from the column.

d) Aphid Bioassay

Aphids (Aphis fabae, Scop.) were used for the assay of menazon, because it has little activity against other insects. The method and apparatus (Plate 3) were devised by Galley (1964)*; the apparatus was reproduced from $\frac{1}{4}$" plastic from the original design.

* Private communication

Plate 3. Apparatus for the Aphid Bioassay of Menazon.

Apparatus in various stages of preparation

The wick supplying water to the leaf discs was prepared from dish-cloth cotton by boiling with detergent and rinsing with tap water. Before use, the wick was soaked in water to remove air and placed on the apparatus with the free ends in water to maintain saturation. The cross-pieces were laid across the main wick and the free ends teased around the sides of the recesses. The wick was then sealed with plastic-backed adhesive tape. 1"-diameter discs cut from broad bean leaves were placed in the recesses with the ventral side up and the main vein in contact with the wick. The plastic rings were coated on their inner surface with Fluon (I.C.I.) and, when they were dry, placed in the recesses above the leaf discs.

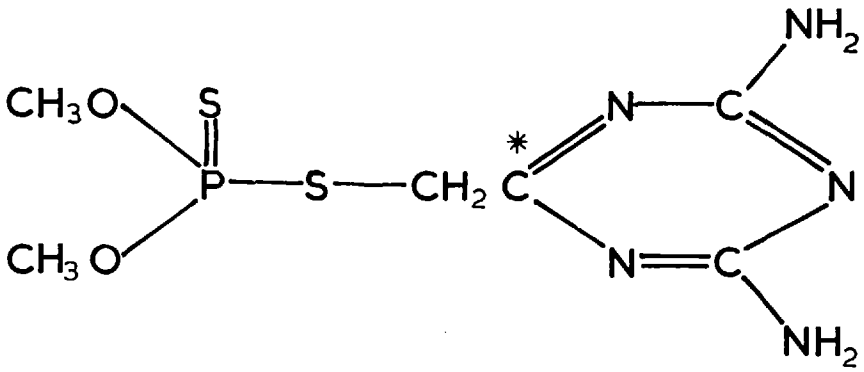
The aphids were cultured on broad bean plants in a greenhouse with a minimum of 12 hours light, supplemented with fluorescent tubes. The aphids were collected in a specimen tube using a camel-hair brush, being careful not to damage their mouth-parts when removing them from the plant. About 20 to 25 aphids were transferred to each leaf disc and allowed to resume feeding. The methanolic insecticide solution was then added to the wicks leading to the discs using a micropipette (Drummond Scientific Co., U.S.A.) and the apparatus was placed in an incubator at 15 - 20°C. The main wicks were placed in a Petri dish, which was fed with water

from a reservoir. The incubator was illuminated through the glass door by two 18" fluorescent tubes. The mortality of the aphids was determined after 24 hours.

Extraction of menazon from the soil. 5g. soil samples were shaken with 20 ml. methanol in 250 ml. glass-stoppered conical flasks for 30 minutes and filtered through Whatman No. 5 filter paper in a Buchner funnel under vacuum. The flask was washed with 10 ml. methanol which was passed through the filter. The filtrate was transferred to a clean 250 ml. round-bottomed flask and the methanol was distilled off under reduced pressure. The residue was redissolved in 4 ml. of methanol, the solution transferred to a test-tube and the methanol evaporated off in a water bath at 90°C. The flask was washed twice with 4 ml. methanol and the washings transferred to the test-tube and the methanol evaporated off. The residue in the test-tube was redissolved in 0.5 ml. methanol and 4 replicates of 5 µl. were applied to the wick. Standard solutions of menazon were prepared in methanol and 1 µl. aliquots applied to the wick. A log. dose probit curve was prepared from the mortality of the aphids and hence the concentration of menazon in the soil extracts was calculated from the graph.

e) Studies with ^{14}C-labelled Menazon

The ^{14}C-labelled menazon was supplied by I.C.I., Plant Protection Ltd., Research Laboratories, Bracknell, Berks. The carbon atom labelled was in the triazine ring:-



The active menazon was dissolved in distilled water to give 0.024% solution and the specific activity was determined, the result being expressed as μcuries/μmole.

The active menazon solution was added to soil to investigate the persistence and recovery. The menazon was extracted by the same procedure as for the colorimetric analysis. The volume of the methanolic extract was determined and 2 ml. were added to 8 ml. scintillator liquid (Scintillation medium NE 572 - Nuclear Enterprises (G.B.) Ltd., Edinburgh, in dioxane, B.D.H.) in glass vials. The vials were kept in the dark for 15 minutes before determining the activity. Background counts were made with 2 ml. methanol and 8 ml. scintillator liquid. Counts were made for 1000 seconds or the time determined for 4000 emissions, to obtain statistically significant results. The activity was measured in a liquid

* Active carbon atom (^{14}C)

scintillation counter 2022 with Scaler 1700 (Isotopes Developments Ltd.). The efficiency of the counting was determined by adding 10 µl. ^{14}C-labelled glycine solution, with a known activity, to each vial counted. The vials were then kept in the dark for 5 minutes and the activity measured. The activity before and after adding the glycine was determined and the difference calculated as a percentage of the known activity of the glycine. All counts made were corrected for the background and for the efficiency of counting. From the activity of the soil extracts, the menazon and/or decomposition products could be calculated from the specific activity. Similar determinations were made using a different scintillator liquid (6g./l. 2,5-diphenyloxazole + 0.1g./l. 1,4-bis-(2-(5-phenyloxazolyl)-benzene), Nuclear Enterprises (G.B.) Ltd. in toluene), counts being made on a Nuclear Chicago scintillator counter (720 series) and the efficiency determined using ^{14}C-glucose.

Direct measurement of activity in the soil

The ^{14}C-labelled menazon and degradation products remaining in the soil after extraction with methanol were measured by making a suspension of air-dried soil in a thixotrophic scintillation medium (4g. Thixin - Nuclear Enterprises (G.B.) Ltd. in 100 ml. scintillator liquid; NE 572 in xylene). The efficiency was determined as before.

Preparation of Autoradiograms

Methanolic extracts of the soil containing ^{14}C-labelled menazon were evaporated to dryness in a rotary-evaporator (Rotovapor "R", Büchi) at 30°C. The residue was redissolved in 0.5 ml. methanol. The solution was applied as a streak to Whatman No. 1 chromatography paper and the methanol evaporated with a hair-drier. The chromatogram was developed with an ascending solvent mixture of butanol:acetic acid:water (12:3:5). The chromatogram was developed for 24 hours and then removed from the tank and dried at room temperature in a fume cupboard. The chromatograms were pinned to Ilford Industrial G, X-ray film in a dark room and left in contact with the film for 14 days. The chromatograms were removed from the film which was then developed in Phen-X for 10 minutes, washed in 3% acetic acid and fixed (10 parts Amfix : 1 part Hardener "S"-type : 30 parts water, May and Baker Ltd.). The film was washed under running water and then dried. The chromatogram was then replaced on the film and the positions of the active components relative to the origin determined.

MALATHION

a) Colorimetric estimation of Malathion

The estimation of malathion was based on the method of Conroy (1957).

Extraction from soil. A 5 g. sample of soil was shaken with 20 ml. of methanol in a 250 ml. glass-stoppered conical flask for 30 minutes. The suspension was then filtered through Whatman No. 5 filter paper in a Buchner funnel under vacuum. The flask was washed with 5 ml. of methanol which was passed through the same filter. The combined extracts were transferred to a 50 ml. measuring cylinder and the volume made up to 50 ml. with distilled water. The extract was then transferred to a 1 l. separating funnel and extracted twice with 50 ml. of carbon tetrachloride, which was collected in a clean flask. The separating funnel was washed with tap water and then distilled water, and the carbon tetrachloride replaced into it. The carbon tetrachloride was then washed by shaking with 200 ml. of distilled water for 1 minute. The carbon tetrachloride layer was then transferred to a clean 250 ml. separating funnel.

Determination of the Malathion. To the carbon tetrachloride, 1 ml. of carbon disulphide reagent (1 ml. CS_2 in 200 ml. CCl_4) and 25 ml. absolute alcohol (A.R. ethanol) was added. After mixing, 75 ml. of sodium sulphate solution (9% aqueous anhydrous

Na_2SO_4 soln.) acidified with 2.5 ml., 0.5 N hydrochloric acid was added and shaken for 1 minute. The carbon tetrachloride layer was run off, filtered through a Whatman No. 5 filter paper and collected in a clean, dry flask. The separating funnel was washed and dried and the carbon tetrachloride replaced into it. 25 ml. of absolute alcohol was added to the separating funnel, mixed and then 1 ml. of 6N sodium hydroxide was added and shaken for 1 minute. Immediately 75 ml. of sodium sulphate solution, cooled to 15°C., was added and shaken for 1 minute. After separating, the carbon tetrachloride layer was run off and discarded. The sodium sulphate was washed with 25 ml. of carbon tetrachloride by shaking for 30 seconds, separating and discarding the lower layer. A further 25 ml. carbon tetrachloride was added to the separating funnel followed by 2 drops of phenolphthalein, and then 6N hydrochloric acid was added drop by drop until the pink colour disappeared. 1 ml. of ferric chloride solution (5 g. $FeCl_3.6H_2O$ in 100 ml. 1N HCl) was added and the funnel shaken for 30 seconds. After separating, the carbon tetrachloride was run off and discarded. The sodium sulphate was then extracted with successive portions of 25 ml. carbon tetrachloride until the extracts were colourless. Finally 25 ml. carbon tetrachloride, accurately measured, was added to the separating funnel followed by 1 ml. copper sulphate solution (3.5% $CuSO_4.5H_2O$). The funnel was shaken for 1 minute and the layers allowed to separate. The carbon tetrachloride layer was filtered through a cotton wool

pledget into a 1 cm. glass cell and the colour (stable 5 - 10 minutes) was measured at a wavelength of 418 mμ. in the Unicam spectrophotometer, using carbon tetrachloride in the reference cell. A calibration curve was prepared using standard solutions of malathion in methanol containing 40 μg./ml. and the procedure followed as above.

b) Mosquito Bioassay

The method was based on that for the routine determination of the susceptibility of mosquito larvae to insecticides as specified by the World Health Organisation : Technical Report Series (1963, No.265). The mosquitoes (Aëdes aegypti (L.)) were reared after the method outlined in the monograph by Sir Rickard Christophers (1960). Eggs were obtained from the School of Hygiene and Tropical Medicine and were hatched in ½" tap water in a plastic bowl (20" x 12" x 6"), which was kept in a constant temperature room at 30°C and 80% relative humidity. The water was aerated with a vibrator pump and the larvae were fed on ground liver powder. The larvae were used at the third to fourth instar stage or left to pupate in order to restock the adult mosquitoes. The pupae were collected and placed in a dish of water inside a muslin cage, also kept in the constant temperature room. The adults on emergence were maintained on 10% sucrose solution in a dispenser until eggs were required. To promote ovulation a meal of blood was provided from a guinea-pig

anaesthetised with Nembutal (Abbott Laboratories Ltd., Kent) at a dose of 1ml./5 lb. body weight, administered intraperitoneally. The abdomen of the guinea-pig was shaved and when immobilised it was layed on its back in the mosquito cage, where the mosquitoes were allowed to feed for 1 hour. A cone of filter paper in a dish of water was provided for the mosquitoes, on which they could lay eggs. The eggs were conditioned and stored at 30°C.

The bioassay was carried out in acid-washed 400 ml. beakers, into which 225 ml. tap water were placed and allowed to equilibrate at 30°C. Groups of 20 larvae were placed into 25 ml. water in 100 ml. beakers. 1 ml. of ethanolic solution of the insecticide or the soil extract was placed into each of the assay beakers, mixed and allowed to equilibrate. The larvae were then added and the mortality of the mosquitoes was determined after 18 hours.

The malathion standards in duplicate contained 0.25, 0.125 and 0.0625 µg./ml. ethanol final concentration, and controls of ethanol were prepared. A log. dose probit curve was prepared and, from the mortality of the mosquito larvae in contact with the soil extract, the concentration of the malathion was determined.

Extraction of Malathion from soil. The soil was incubated in 10 g. quantities in 100 ml. conical flasks plugged with cotton wool. 20 ml. ethanol were added to each flask, which was sealed

with Parafilm. The flasks were shaken mechanically for 30 minutes and the soil suspension was then filtered through Whatman No. 5 filter paper in a Buchner funnel under vacuum. The flasks were mixed with 20 ml. ethanol, the washings passed through the filter, and the final volume was made up to 50 ml. Samples of the extracts were assayed as above.

SUMITHION

a) Estimation of Sumithion

No published method of estimating sumithion in the soil was found, therefore the method below was devised. The sumithion was extracted from a 5 g. sample of the soil using 25 ml. methanol in the same manner as used for the other insecticides. The extract was filtered through a Hyflo-Supercel pad as described for menazon. The flask in which the extraction was carried out was rinsed with 25 ml. methanol which was passed through the filter. The extract was decolourised by shaking with 0.2 g. activated charcoal and then filtered through a Whatman No. 40 filter paper. The sumithion in the decolourised filtrate was then measured in an ultraviolet spectrophotometer (Uvispec MK 9, Hilgar and Watts Ltd.) at a wavelength of 269.5 mμ. Methanol was used as the reference and measurements were carried out in 1 cm. silica cells. Standards of sumithion in methanol

were prepared to give 0 - 40 µg./ml. and a calibration curve was prepared. The sumithion in the soil extracts was read from the calibration curve and corrected to 1 g. soil. The method used not only measures the sumithion but also decomposition products which absorb at the wavelength used.

b) Chromatographic techniques with Sumithion

In order to determine the nature of the absorbing material in the extracts from soil containing sumithion, reversed-phase chromatography was used. Whatman No. 1 chromatography paper was treated with 33% sodium silicate solution followed by 6N hydrochloric acid and then washed thoroughly in water and dried. The chromatogram was developed using ascending solvent (upper layer of n-hexane 100 parts, glacial acetic acid 40 parts and water 10 parts). The locations of the compounds were found using an ultraviolet light. In this system sumithion has an Rf. of 0.81. In order to purify the sumithion, the extract was run as a band and the portion of the chromatogram which contained sumithion was cut out and eluted with methanol.

A second system was used to separate the water soluble decomposition products of sumithion. Whatman No. 1 chromatography paper was impregnated with 5% silicone fluid (MS 550, Hopkin and Williams Ltd.) in cyclohexane and dried.

The chromatogram was developed using an ascending solvent (chloroform 2 parts, ethanol 38 parts and water 60 parts). Sumithion had an Rf. of 0.14 in this system.

ISOLATION OF MICRO-ORGANISMS FROM THE SOIL

Enrichment Culture. The insecticides were added to the soil at levels which were shown to produce an increase in the soil population. The water content of the soil was adjusted and the soil incubated at 30°C. Samples of the soil were taken and suspended in sterile tap water. Isolation of organisms capable of decomposing insecticides was them tried in a variety of ways.

Isolation on agar media. The insecticides were incorporated into melted Thornton's agar by either making an emulsion with malathion and sumithion or a suspension with menazon. The medium was poured into sterile Petri dishes, allowed to solidify and then inoculated with the soil suspension.

Isolation on silica gel media. The method used for the preparation of the silica gel media was that of Funk and Krulwich (1964).* A 10% solution of silicic acid was made in 7% analar potassium hydroxide and sterilised by autoclaving for 15 minutes at 15 lb. pressure. A double strength salts solution was prepared of the ingredients of Thornton's medium without the phosphate:-

* Funk, H.B. and Krulwich, T.A. (1964), J. Bact., 88, 1200

Analar Ingredients	g./litre
KNO_3	1.0
$MgSO_4 \cdot 7H_2O$	0.4
NaCl	0.2
$CaCl_2$	0.2
$FeCl_3$	0.04 (0.4 ml. 1% solution)

The solution was dispensed into 5 ml. quantities and was sterilised by autoclaving at 15 lb. pressure for 15 minutes. A 22% phosphoric acid solution was prepared and sterilised as above.

To prepare the medium, 5 ml. of the silicic acid solution was added aseptically to 5 ml. of the salts solution. The sterile insecticides as the sole-carbon source were then added and shaken thoroughly. 1 ml. of phosphoric acid solution was added aseptically, mixed and immediately poured into a sterile Petri dish. The final mixture was neutral and rapidly formed a gel. Syneresis took place and the surface moisture formed was dried in an incubator at 37°C. The Petri dishes were incubated for 24 hours normally and then placed into polythene bags which were sealed to prevent further loss of water from the medium.

Isolation by growth in liquid culture. An enrichment culture in soil was made and a suspension in sterile tap water prepared.

1 ml. suspension was inoculated into 30 ml. sterile salts solution in a 250 ml. conical flask containing 1% insecticide as a two-phase system. The salts solution was single strength of that used for the silica gel medium, plus 0.2% K_2HPO_4. The flasks were placed in a shaking incubator at 30°C. After turbidity was seen in the medium, 1 ml. was transferred to a fresh sterile flask of medium and was incubated as before. After growth had taken place, the organisms were isolated by inoculation onto Thornton's agar containing insecticide.

Isolates were purified by the streak-plate method and tested for their ability to decompose the insecticide by inoculating into insecticide-salts solution. Growth was followed in a Nephelometer (EEL Nephelometer head and Unigalvo Type 20) or by viable counts using the pour-plate method.

EXPERIMENTAL AND RESULTS

A. The Persistence of Insecticides in the soil

The persistence of an insecticide in the soil is governed by a number of factors including the chemical stability of the compound, the ease with which it is degraded by micro-organisms and the characteristics of the soil. A knowledge of the persistence of an insecticide in soil may help in determining whether or not micro-organisms are involved in the degradation. Studies were therefore undertaken to measure the persistence of malathion, sumithion and menazon and to correlate these studies with the results obtained in Part I of this thesis.

Two techniques frequently used for estimating residues of insecticides are chemical estimations and bioassay techniques, both of which it was thought desirable to use, so that loss of biological activity could be correlated with loss or alteration of the insecticides as shown by chemical estimation. For convenience, the work with each of the three insecticides studied will be considered separately.

a) Persistence of Malathion

From observations of the effect of this compound on the respiration of soil micro-organisms and from population studies,

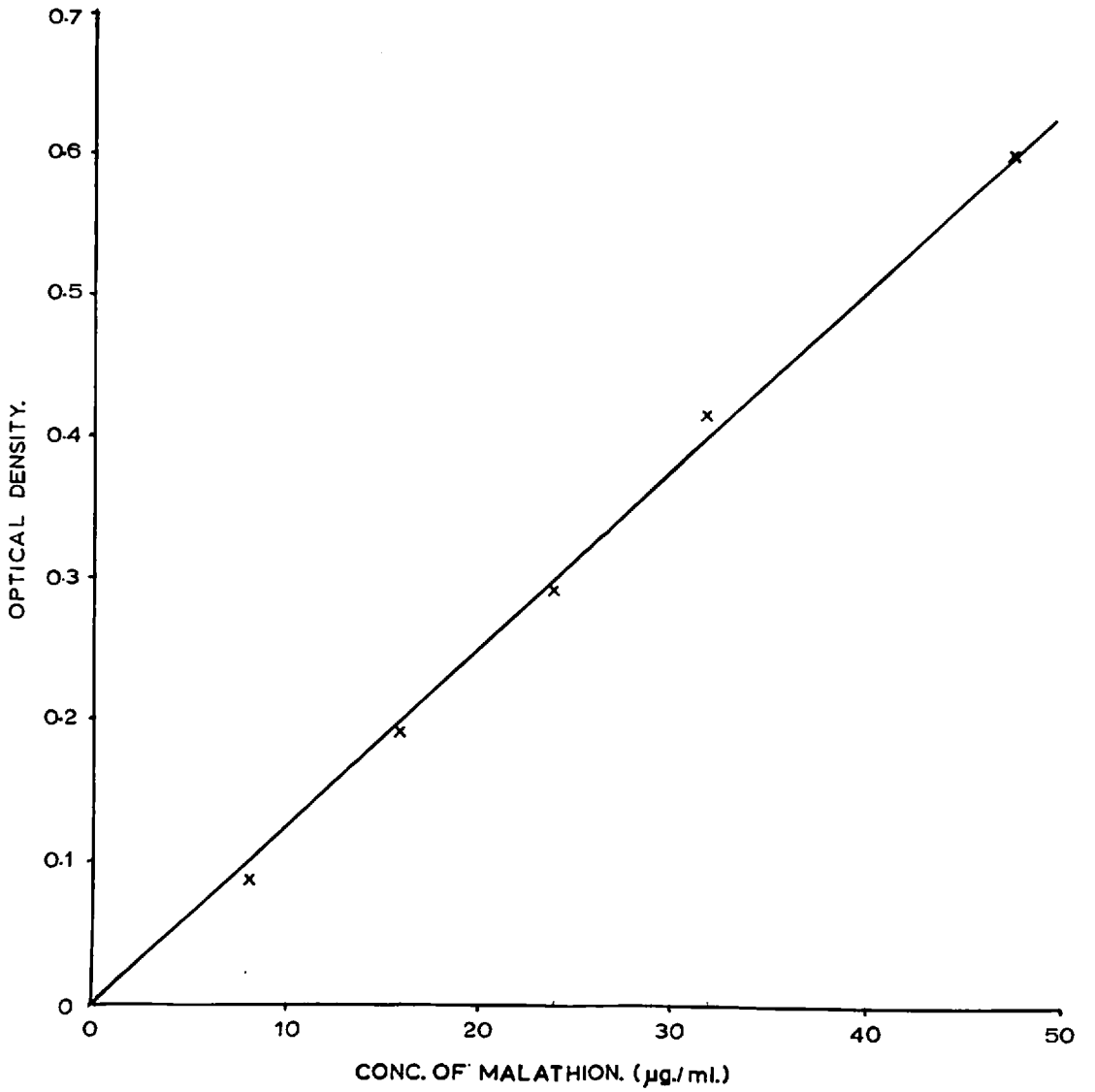
it would appear to be the one most readily decomposed by micro-organisms in the soil, the decomposition products being used for microbial proliferation.

(i) The persistence of malathion in soil as estimated by a colorimetric technique

Preliminary investigations showed that using the technique, described in the materials and methods, it was possible to estimate from 0 to 80 µg. malathion, as the final coloured complex, per ml. of carbon tetrachloride. Recovery of malathion from the soil was in the order of 80% and extracts from untreated soil did not interfere with the colour reaction. A calibration curve (Fig. 22) was prepared from standard solutions of malathion in methanol.

Persistence in normal soil: Malathion was added to the soil using acid-washed silver sand as a carrier. 0.1 g. malathion was added to 5 g. sand, mixed thoroughly and 1 g. of the mixture was incorporated into 100 g. of Chelsea loam in a 250 ml. conical flask, which was plugged with non-absorbent cotton wool. A similar flask was prepared with untreated soil to act as a control. However, the control was discontinued, since extracts under experimental conditions gave an optical density of zero. The water content of soil was adjusted with distilled water and the flasks were incubated at 30°C. in an incubator. Water lost during incubation was replaced with sterile distilled water.

Fig. 22. Calibration curve for the colorimetric estimation of malathion.



The final concentration of the malathion in the soil was 175 µg./g. soil. Duplicate 5 g. samples of soil were taken at intervals and extracted with methanol. The procedure was then followed as described in the materials and methods, the concentration of malathion being determined from the calibration curve. Two malathion standards were estimated with the samples to check the calibration curve. The results of the experiment are shown in Table 31 and graphically in Fig. 23.

From the results it is obvious that recovery of malathion from soil becomes difficult after incubation for 2 days at 30°C., only 2.2% of the applied insecticide being recoverable at this time. Assuming that inability to recover means that the insecticide has been decomposed, these results support the previous conclusion that the insecticide is degraded by micro-organisms in the soil.

In order to confirm the role played by micro-organisms, the persistence of malathion in sterile soil was determined.

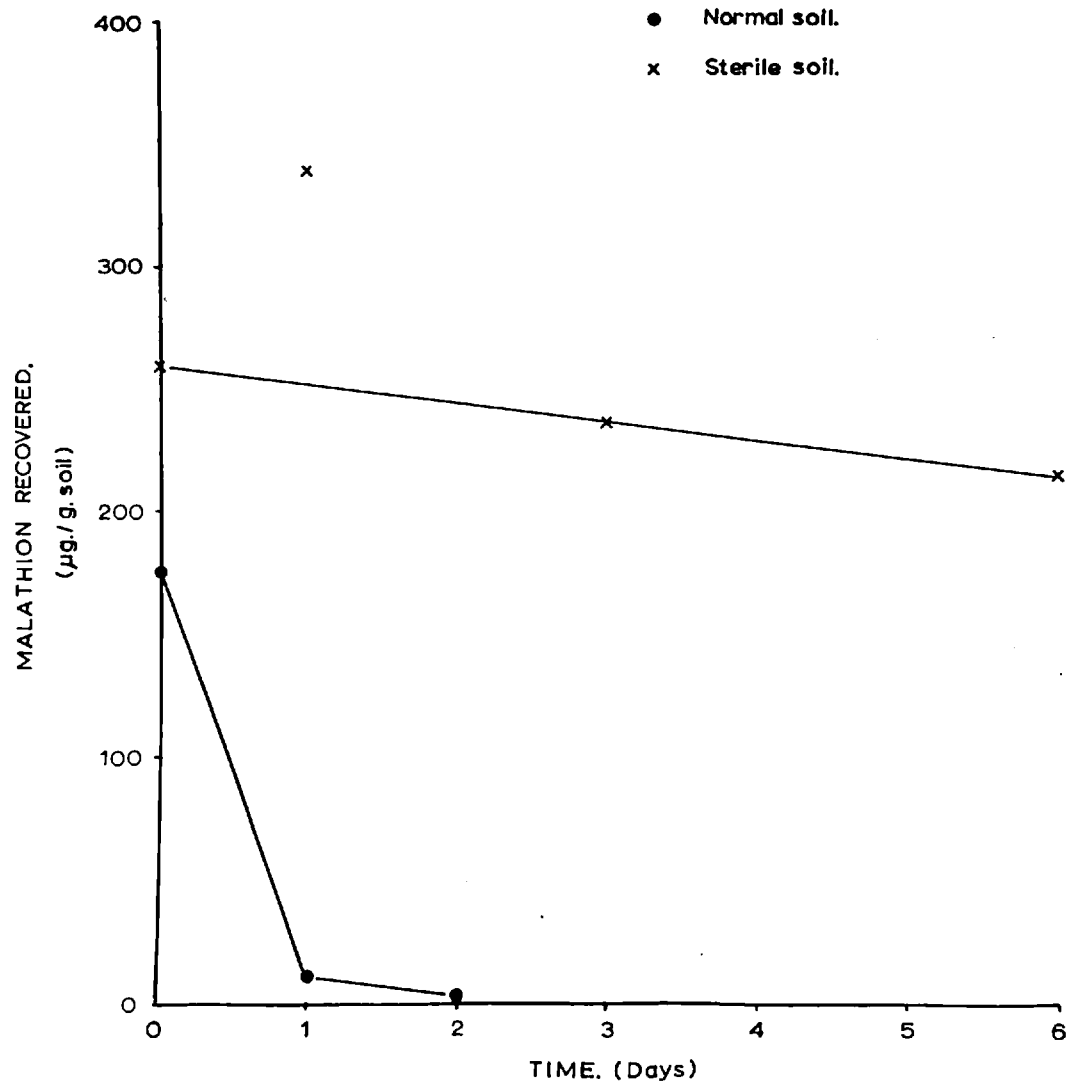
Persistence in sterile soil: 5 g. aliquots of Chelsea loam were sterilised in 100 ml. conical flasks as described in the materials and methods. The malathion was prepared as a methanolic solution at a concentration of 2 mg./ml. and was sterilised by passage through a sintered glass filter (Pyrex Porosity grade No. 5). 0.65 ml. of this solution was added aseptically to each of 8 flasks to give a final concentration of 260 µg./g. soil. The concentration of the

Table 31. The recovery of malathion at 175ppm from Chelsea loam as determined by colorimetric estimation

Time (days)	Recovery of malathion (µg./g.soil)*	
	Total	Range
0	175.0	±15.2
1	10.9	± 3.3
2	3.8	± 0.0

* The results are the average of duplicate samples corrected to 100% from an initial recovery of 79%.

Fig.23 The recovery of malathion at 175 ppm from normal Chelsea loam and at 260 ppm from sterile Chelsea loam.



malathion in the soil was increased, compared with the previous experiment, to prolong its possible persistence in the soil. 0.65 ml. of sterile distilled water was added to 4 flasks, which were used to test the efficiency of sterilisation. The flasks were incubated at 30°C. and samples were taken at intervals. To estimate the malathion and check sterility, 3 flasks were removed from the incubator; 2 containing soil with insecticide and the third containing untreated soil. 25 ml. methanol were added to each of the flasks containing soil with insecticide, and extracts made. The procedure for the estimation of malathion was then carried out as before. A sample of the untreated soil from the third flask was tested for sterility as described in the materials and methods. The results are shown in Table 32 and graphically in Fig. 23, and are compared in the graph with the persistence in normal soil.

The results show that sterilisation of the soil prolongs the persistence of malathion, 82% of the applied insecticide being recovered after 6 days. This increase in persistence tends to confirm that micro-organisms are actively involved in the decomposition of malathion in the soil. However, it was not known how the application of the insecticide as a methanolic solution to the soil might have affected the persistence and to what extent the water content of the soil was involved. In order to prevent aerial contamination on additions of water to the soil, the water content was not continually maintained at 50% maximum water holding capacity, and it is probable

Table 32. The recovery of malathion at 260 ppm from sterile Chelsea loam, as determined by colorimetric estimation.

Time (days)	Recovery of malathion* (µg./g. soil)		Number of viable organisms/g. soil
	Total	Range	
0	260.0	± 22.1	1.6
1	340.0	± 44.1	-
3	236.0	± 10.5	2.7
5	214.0	± 0.0	0

* The results are the average of duplicate samples, corrected to 100% from an initial recovery of 90.5%.

that as the soil became dry, the persistence of malathion was increased.

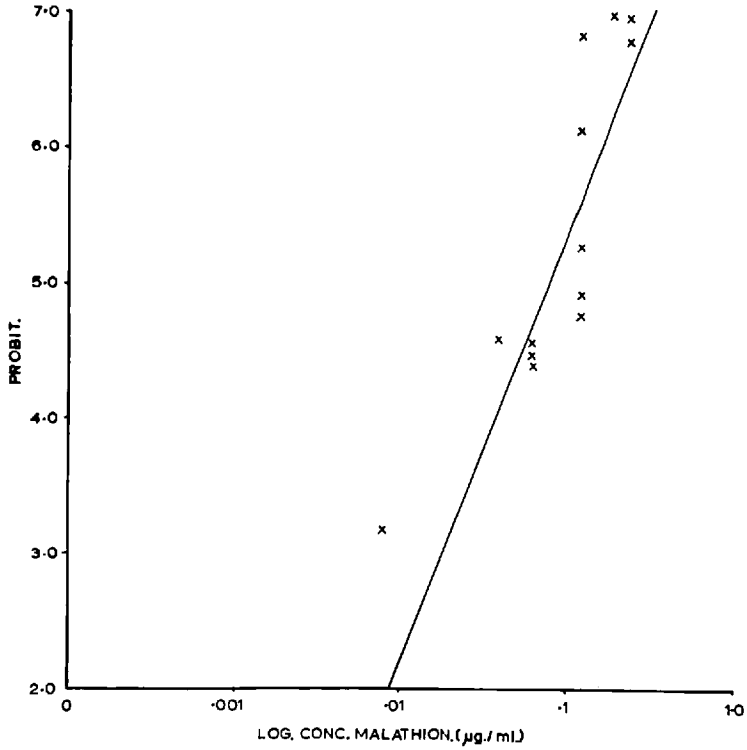
(ii) The persistence of malathion in soil as determined by bioassay

Bioassay using mosquito larvae

The method of rearing the mosquito larvae and of estimating the malathion was as described in the materials and methods. Preliminary experiments showed that the method was capable of estimating malathion in the final concentration range 0.008 to 0.25 µg./ml. Extracts from untreated soil were shown not to be toxic to the larvae. A calibration curve was prepared using standards of malathion in ethanol, and standards were used to check the curve when each estimation was made. The calibration curve is shown in Fig. 24, the probit being taken from the statistical tables of Fisher and Yates (1948), using the average percentage mortality of the mosquito larvae in duplicate beakers. For the purpose of estimating the percentage mortality, it was found better to count larvae which showed symptoms of insecticide poisoning, as half dead rather than completely dead, since recovery from the effects of the insecticide was sometimes noticed when counts were made over a long period.

The supply of eggs, from which the larvae were reared, was not unlimited and hence sufficient numbers were not obtained to provide larvae for estimations on consecutive days. The experiment

Fig. 24. Calibration curve for the bioassay of malathion with mosquito larvae.



was therefore designed so that when larvae were obtained at the right stage of development, samples could be taken from treated soil and assayed immediately. The soil was prepared to coincide with the growth of the larvae, so that it was not necessary to store extracts until larvae were available. 0.0125 g. malathion was weighed directly into each of two 100 ml. conical flasks and the water, required to bring 10 g. of Chelsea loam to 50% maximum water holding capacity, was added. The insecticide was mixed with the water, 10 g. soil placed in each flask and mixed thoroughly with a spatula to obtain an even distribution. The final concentration of malathion in the soil was 1250 ppm. The flasks were plugged with non-absorbent cotton wool and were incubated at 30°C., the water lost by evaporation being replaced by sterile distilled water. The malathion was extracted from the Chelsea loam with 20 ml. ethanol and the assay carried out with the filtered extract. The mortality of the mosquitoes was recorded after 18 hours and the concentration of the malathion in the soil extracts determined from the calibration curve. The results of the experiment are shown in Table 33 and graphically in Fig. 25.

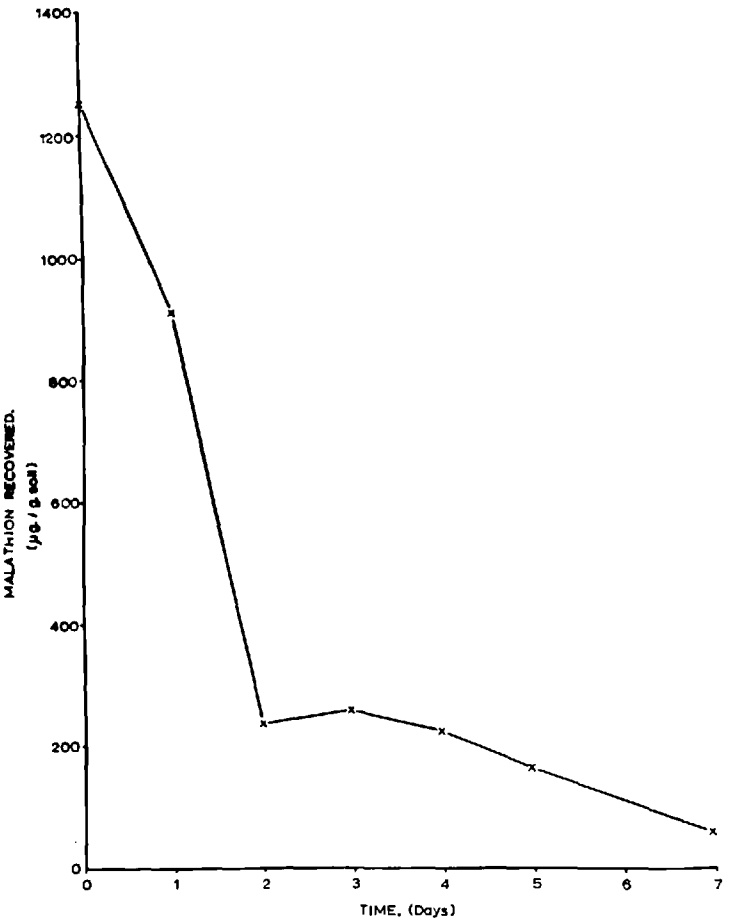
The results show that malathion disappears rapidly from the soil, only 4.1% of the applied insecticide being active after 7 days. Some of the results obtained had a wide range between duplicate samples, but this is not uncommon with bioassay techniques where many variables are involved.

Table 33. The recovery of malathion at 1250 ppm from Chelsea loam, as determined by mosquito larvae bioassay.

Time (Days)	Malathion recovered* (µg./g. soil)	
	Total	Range
0	1250.0	±186.5
1	912.0	±100.5
2	236.0	± 18.7
3	255.5	± 66.0
4	219.5	±117.5
5	160.5	± 17.2
7	52.0	± 5.3

*The results are the average of duplicate samples corrected to 100% from an initial recovery of 87.0%.

Fig. 25. Persistence of malathion at 1250 ppm in Chelsea loam, as determined by mosquito larvae bioassay.



The decline in the recovery of malathion from the soil is very similar to that observed using the colorimetric technique. The time taken for 50% of the malathion to disappear, using the bioassay technique, was just over one day compared with under one day using the colorimetric estimation. This difference is very small for independent methods of estimation and therefore confirms the previous results, although it would have been desirable to estimate the persistence of malathion at 1250 ppm using the colorimetric technique.

Bioassay using Fruit flies (Drosophila melanogaster, Meig.)

Although the results using the mosquito larvae gave satisfactory results, the method of rearing the mosquitoes and maintaining a supply of eggs was laborious. It was also felt that by extracting the insecticide from the soil before assaying the compound, errors might be introduced due to variations in the recovery. Consequently, an investigation was carried out with wild-type fruit flies in order to determine the practicability of using them as insects for bioassay. Fruit flies have been used in bioassay techniques for insecticides in soil by addition of the soil to fruit pulp. To avoid the dilution effect, a method was investigated using the insects in direct contact with the soil.

5 g. aliquots of soil were placed into 3" x 1" specimen tubes and 1 ml. from a series of standard solutions of malathion in

ether was placed into separate tubes to give 0, 16, 80, 400 and 2000 µg./g. soil. The ether was evaporated off and 20 flies were placed into each specimen tube, which were plugged with cotton wool. The tubes were left at room temperature and the percentage mortality determined at intervals. The results are shown in Table 34.

From the initial experiment it was concluded that the method might be used but it was obvious that when the concentration dropped to a certain level the contact time would have to be extended to a minimum of 18 hours. This required that the percentage mortality in the controls be reduced to less than 5% for an accurate assessment.

The experiment was repeated using lower concentrations of malathion in the soil, i.e. 0, 0.08, 0.4, 2.0 and 10.0 µg./g. soil, and after the ether was evaporated off, 1 ml. distilled water was added to the soil to provide a humid atmosphere, which it was hoped would reduce the mortality of the controls. The percentage mortality was determined after 1 and 2 days incubation at room temperature. The results are shown in Table 35.

At the lower concentrations of malathion, the assay did not give accurate results. The mortality at the range of concentrations chosen was variable and could not be used to prepare a calibration curve. Since it was in this particular range that measurements would be made, the method could not be used. The high mortality in the controls also emphasised the limitations of the method.

Table 34. Mortality of fruit flies in contact with soil containing malathion

% mortality of fruit flies in contact with soil containing various concentrations of malathion (µg./g.soil)

Time	0	16	80	400	2000
5 min.	0	0	0	36.0	66.0
15 min.	0	0	0	70.6	100.0
30 min.	0	0	3.15	81.5	-
45 min.	0	0	15.7	96.1	-
60 min.	0	0	28.1	96.1	-
75 min.	0	0	40.6	100	-
18 hr.15 min.	43	93.0	100	-	-

Table 35. Mortality of fruit flies in contact with soil containing malathion

% mortality of fruit flies in contact with soil containing various concentrations of malathion (µg./g. soil)

Time	0	0.08	0.4	2.0	10.0
25½ hours	14.4	12.8	27.0	21.9	25.5
43½ hours	44.0	54.0	60.5	39.0	56.0

One of the sources of error in the method was the limited surface area over which contact could be made and that the contact was probably accidental. In order to attract the flies to the soil, and to increase the surface area, a method was investigated in which 1 g. of the soil was suspended in 10 ml. 1% agar + 5% sucrose. A film of soil/agar mixture was formed round the inside of the specimen tube, and when the agar had set, the flies were introduced into the tubes and mortality counts made as before.

The method prolonged the life of the flies in contact with untreated soil, but there was no mortality after 48 hours in contact with soil containing 10 µg./g. soil.

Using the methods above, it was clear that Drosphila could not be used as an insect for bioassay of the malathion. Unfortunately, time did not permit the investigation of the technique using fruit pulp as a carrier of the soil for the assay.

b) Persistence of Sumithion

The method of estimating sumithion in preliminary investigations was based on the method used for determining residues in tomatoes and cocoa beans (Plant Protection Ltd., Standard method no. 227 and Addendum 1). The method relies upon the extraction of the sumithion with chloroform, which is then decolourised with activated charcoal. The chloroform is evaporated off and the sumithion residue converted to its amine. This is then diazotised to form a coloured compound,

the colour being measured in a spectrophotometer. The method gave good results with standard solutions of sumithion, but there was interference from amines and nitro compounds in extracts from untreated soil. Also in order to extract the sumithion from soil with chloroform, it was necessary to air-dry the soil. This reduced the recovery, which with the high blanks made the method impracticable.

(i) The persistence of sumithion as determined by ultraviolet light absorption

Since sumithion absorbs ultraviolet light with a peak at 269 - 270 mμ., it was decided to base the estimation upon this property. Chloroform extracts from the soil gave very low recoveries but methanol, which could be used to extract moist soil, gave a better recovery. It was found that the recommended level of 4 g. activated charcoal was removing most of the sumithion from solution. By reducing the quantity of charcoal to 0.2 g., when shaken with 50 ml. solution, the recovery was increased to approximately 60%. In order to see if the recovery was constant, 0.2 g. activated charcoal was shaken with 50 ml. of solutions of sumithion in methanol at various concentrations and the recovery determined from a calibration curve (Fig. 26) prepared from standard solutions of sumithion in ether. (The calibration curves of sumithion in ether and methanol are identical.) The results are shown in Table 36. The results show a relatively constant recovery from the charcoal and although the recovery was not as great as would be desired, it was hoped that it would enable the persistence of sumithion in soil to be determined. At a level of 0.2 g. the activated

Fig.26. Calibration curves for the estimation of sumithion and 3-methyl-4-nitrophenol, measuring their absorption at different wavelengths.

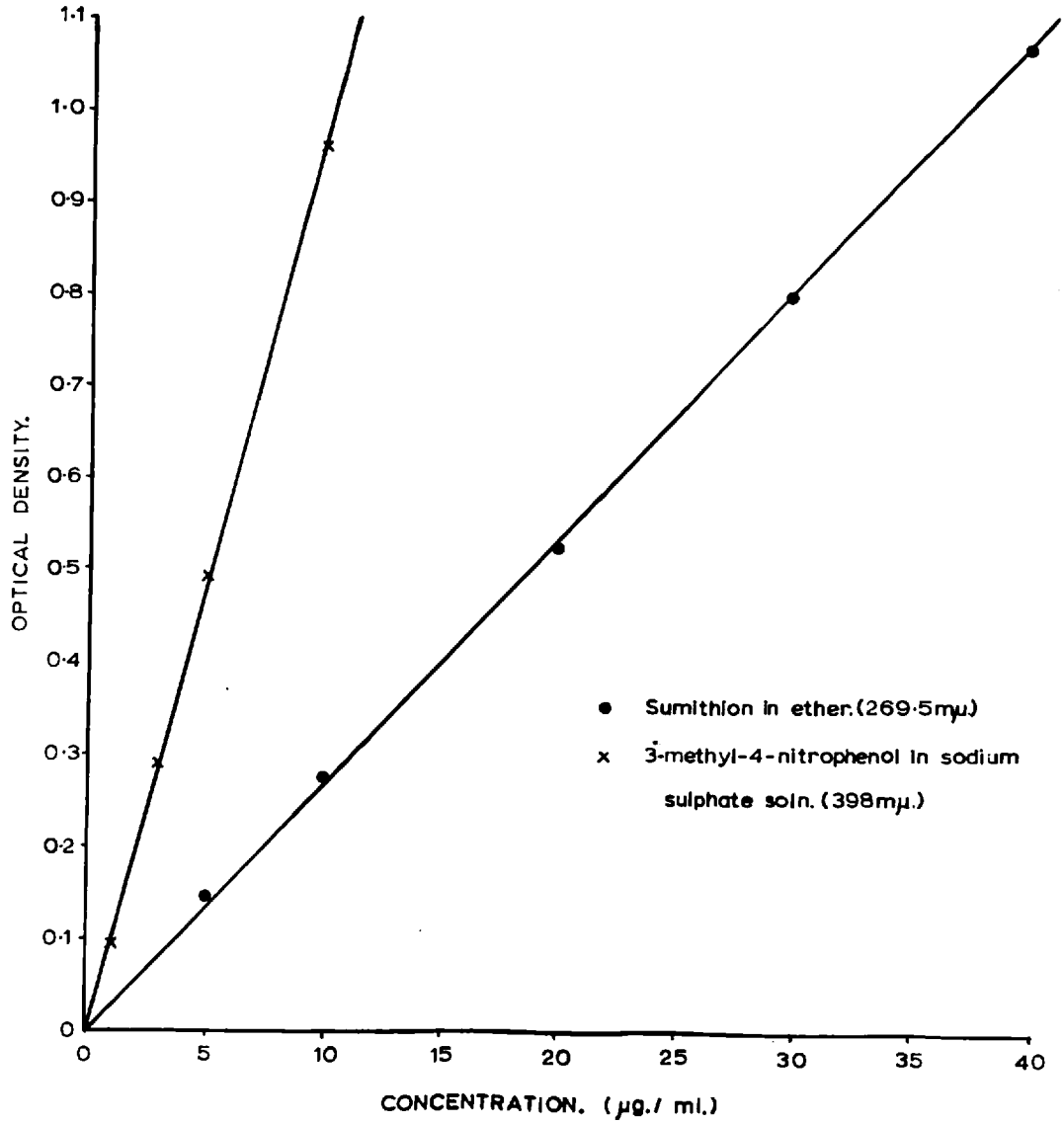


Table 36. The recovery of sumithion from solution after treatment with activated charcoal.

	Recovery of sumithion from standard methanolic solutions at various concentrations (µg./ml.) treated with 0.2g. activated charcoal.			
	16.0	24.0	32.0	40.0
µg./ml.:	10.25	15.25	20.25	24.75
% recovered:	64.0	63.5	63.5	62.0

Table 37. The recovery of sumithion from Chelsea loam as determined by ultraviolet absorption at 269.5mµ.

Time (Days)	Sumithion recovered (µg./g. soil)*	
	Total	Range
0	340.0	± 9.2
1	265.5	± 8.5
2	234.0	± 5.71
5	177.0	-
14	143.0	-
21	80.0	-

* The results were corrected to 100% from an initial recovery of 43.8%. Duplicate samples were taken only on the first three days.

charcoal reduced the content of ultraviolet light absorbing material in extracts from untreated soils to negligible proportions. Using this method, which is fully described in the materials and methods, the persistence of sumithion was determined.

Sumithion was added to the soil using acid-washed silver sand as a carrier. 2 g. sand containing 0.04 g. sumithion were added to 100 g. soil in a 250 ml. conical flask to give a final concentration of 340 µg./g. soil. A similar flask of untreated soil was also prepared and the water content of the soil in both flasks was adjusted to 50% of the maximum water holding capacity. The flasks were incubated at 30 °C. and initially duplicate 5 g. samples of soil were taken at intervals and the sumithion estimated. Because duplicate samples gave such good agreement in the results, only one sample was taken from each flask in later estimations. The absorption of ultraviolet light produced by the untreated soil was used to correct the readings found with the treated soil and the concentration of sumithion determined from the calibration curve, shown in Fig. 26. The results are shown in Table 37.

The results indicated that sumithion was more persistent than malathion, 23.5% of the applied sumithion remaining after 3 weeks. However, it was not known what effect the decomposition products of sumithion would have on the measurement of sumithion. A solution of 3-methyl-4-nitrophenol, one of the hydrolysis products of sumithion, in methanol was prepared and it was found that this compound absorbed

strongly at the wavelength used for estimating sumithion. If sumithion was hydrolysed in the soil, 3-methyl-4-nitrophenol would interfere with the measurement of the insecticide. However, it was possible that the hydrolysis products would be absorbed by the charcoal and removed from solution and this possibility was investigated. 50 ml. of a solution of 3-methyl-4-nitrophenol in methanol, at a concentration of 22 µg./ml., were shaken with 0.2 g. activated charcoal and then the mixture was filtered. The absorption of the solution before and after treatment with charcoal was determined at the wavelength 269.5 mµ.

It was found that 61.5% of the 3-methyl-4-nitrophenol was recovered from the charcoal, which was almost identical to the recovery of sumithion. Therefore it was obvious that the hydrolysis product would interfere with the measurement of sumithion. In order to determine whether hydrolysis did in fact take place in the soil, the previous experiment, to measure the persistence of sumithion in soil, was repeated. The experiment was essentially the same as before, but after measuring the ultraviolet absorption of the soil extracts, the methanolic solution was transferred to a 250 ml. round-bottomed flask and the methanol evaporated off under reduced pressure at 30°C. The residue was redisolved in 0.5 ml. methanol, applied as a streak to chromatography paper and developed as described in the materials and methods. The positions of sumithion and 3-methyl-4-nitrophenol were found by placing the chromatogram under ultraviolet light, which they absorb, and were identified by using the pure compounds

as markers. After incubation for six days, it became obvious that hydrolysis was occurring in the soil; the approximate concentration of the sumithion was estimated by eluting the sumithion band from the chromatogram with methanol, and by measuring the absorption of the solution. The results are shown in Table 38.

The results showed a marked difference in the concentration of sumithion before and after chromatography, indicating that the decomposition products gave a false estimation, and in particular 3-methyl-4-nitrophenol was the major compound to interfere with the estimation. Although the results, using the chromatographic procedure for purifying the sumithion, indicate that sumithion is unrecoverable after 19 days, the difficulty in loading the whole residue onto the chromatography paper may cause errors in the final estimation.

The purification of sumithion, described in the technical manual on sumithion (Sumitomo Chemical Co., Japan), is carried out using 1% sodium carbonate solution. 3-methyl-4-nitrophenol is extracted into the solution and the yellow colour formed may be used to estimate the compound, measuring the absorption as a wavelength of 398 mμ. The previous method of estimating sumithion was modified to incorporate this procedure, which enabled not only the sumithion but also its hydrolysis product to be measured.

The methanolic extract of the soil was decolourised as before, but the solution was then evaporated to dryness under reduced

Table 38. Recovery of sumithion from the Chelsea loam as determined by the absorption of ultraviolet light.

Time(Days)	Recovery of sumithion (µg./g. soil) *	
	Before Chromatography	After Chrcmatography
0	340.0	-
3	200.0	-
6	200.0	78.0
10	189.0	44.5
13	131.0	26.3
19	154.0	0.0

*The results are corrected to 100% from an initial recovery of 43.7%. Estimations of the sumithion, eluted from the chromatogams, were made on samples taken after incubation for six days.

pressure at 30°C. The residue was redissolved in 50 ml. ether and transferred to a separating funnel. 25 ml. 1% solution of sodium carbonate were added and the separating funnel was shaken for one minute. The layers were allowed to separate and the absorption of the lower layer containing 3-methyl-4-nitrophenol was measured at 398 mμ in a spectrophotometer using distilled water as the reference blank. The concentration was determined from a calibration curve (Fig. 26) prepared from standard solutions in ether, extracted with 25 ml. aliquots of a 1% solution of sodium carbonate. The upper layer of ether containing sumithion was measured at 269.5 mμ. in the Hilger ultraviolet spectrophotometer, using 1 cm. silica cells with ether as the reference blank.

The above modifications were used to determine the persistence of sumithion in Chelsea loam and also the accumulation of 3-methyl-4-nitrophenol. The experiment was carried out with sterile soil at the same time in order to investigate the effect of micro-organisms on the persistence. The soil was terilised in 5 g. aliquots in 100 ml. conical flasks as described in the materials and methods. The sumithion was added to the sterile soil as a solution in methanol. 0.5 g. sumithion was dissolved in 100 ml. methanol and sterilised by passage through sintered glass filter (Pyrex, Porosity Grage No. 5). 0.5 ml. of the sumithion solution was added to each of 5 flasks, giving a final concentration of 443 μg./g. soil, and 0.5 ml. sterile distilled water was added to each of 10 more flasks. 5 of the flasks containing soil without insecticide were used to determine the background

absorption and the other 5 flasks were used to check the efficiency of the sterilisation by autoclaving. Extracts were made of treated and untreated soil and the sumithion and 3-methyl-4-nitrophenol determined at intervals. Sterility was determined as described in the materials and methods. The results obtained with the normal soil are shown in Table 39 and graphically in Fig. 27. The results obtained with the sterile soil are only shown in the table.

Samples taken for sterility testing showed that no organisms developed in the flasks from which samples were taken, which indicated that at the start of the experiment no viable organisms were present in the soil. The results with the normal soil show that the recovery of sumithion declines fairly rapidly, only 14.2% of the applied sumithion being recovered after 16 days. The decline in sumithion was correlated with a rise in the level of 3-methyl-4-nitrophenol, the level found after 16 days being equivalent to the hydrolysis of 205 µg. sumithion/g. soil. Assuming that the percentage recovery of sumithion and 3-methyl-4-nitrophenol was constant, the results indicate that either 3-methyl-4-nitrophenol is decomposed in the soil or that other decomposition products of sumithion are formed. This would account for the discrepancy between the sumithion applied and that recovered in an unchanged form, plus that in the form of the hydrolysis products.

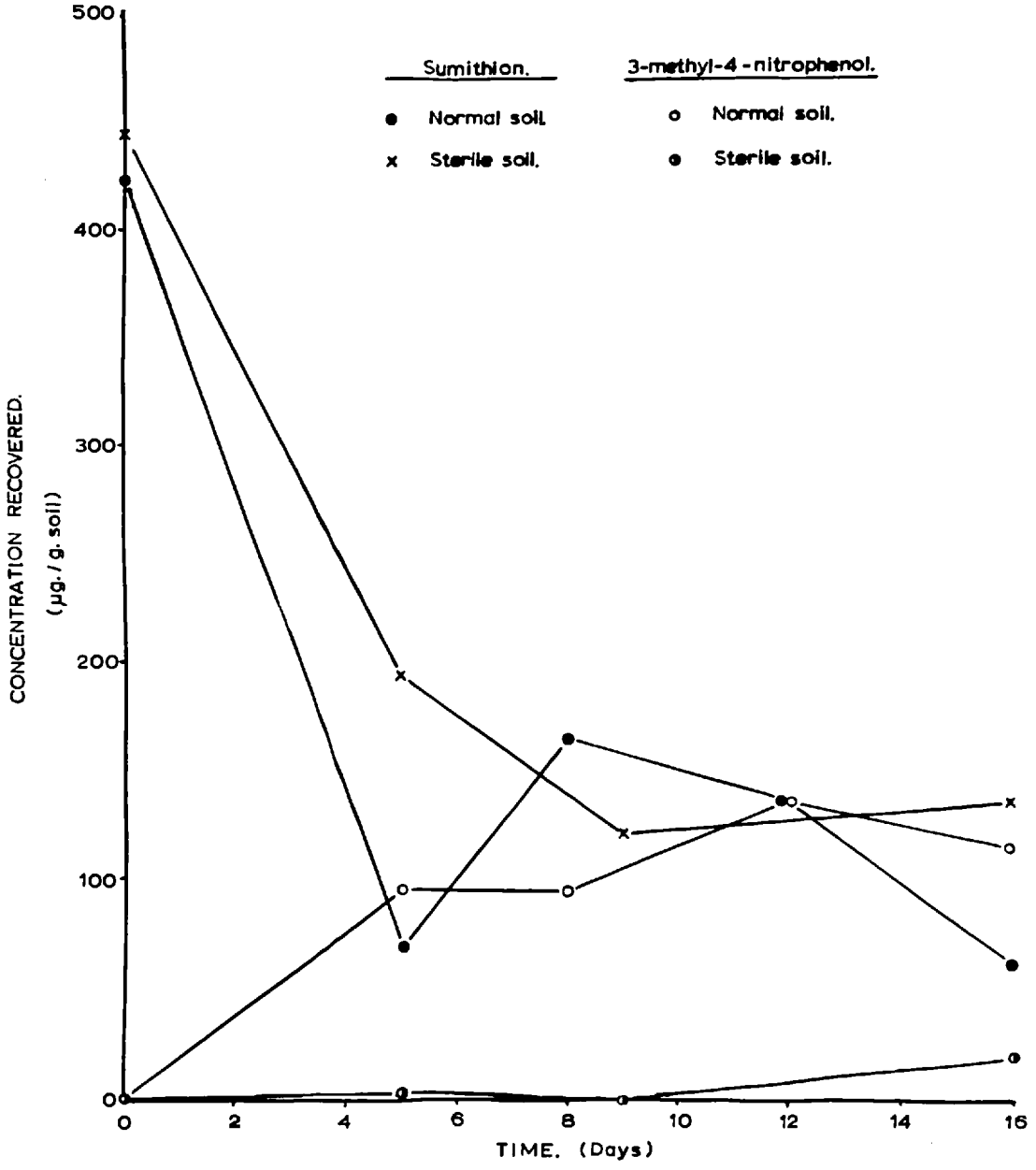
The results from the normal and sterile soils tended to be variable, which made the results difficult to interpret. Although initially the decline in the recovery of sumithion from the sterile soil was slower than in normal soil, the estimations on the eighth

Table 39. The recovery of sumithion and 3-methyl-4-nitrophenol from normal and sterile Chelsea loam

	Recovery (µg./g. soil) *			
	Normal Soil		Sterile Soil	
Time (days)	Sumithion	3-methyl-4-nitrophenol	Sumithion	3-methyl-4-nitrophenol
0	423.0	0.925	443.0	0
5	69.5	94.2	356.0	8.7
8	163.0	93.5	158.0	-
12	135.0	135.0	149.0	9.6
16	60.1	113.0	302.0	11.5

* Results corrected to 100% from an initial recovery of 54% in the normal soil and from 46% in the sterile soil.

Fig. 27. The recovery of sumithion and 3-methyl-4-nitrophenol from normal and sterile Chelsea loam.



and twelfth days indicated that there was little difference between the soils. However, after 16 days, 68.5% of the applied sumithion was recovered and this with the low level of 3-methyl-4-nitrophenol formed suggested that the loss from sterile soil was slower than from normal soil.

The results in some cases were found to be low because the ether used in the reference cell was different to that used to redissolve the extract residues. This produced negative results with extracts from untreated soils, which made correction of the extracts containing sumithion impossible.

Since the results with the sterile soil were unsatisfactory, the experiment was repeated as before, except that the sterile soil was kept moist by additions of sterile distilled water, so that decomposition due to chemical hydrolysis would not be inhibited. The results are shown in Table 40 and graphically in Fig. 27 compared with normal soil.

The results show that the recovery of sumithion from the sterile soil declines at a similar rate as in normal soil, although after 16 days incubation only 14.4% of the initial concentration was recovered from the normal soil compared with 30.3% from the sterile soil.

The findings are similar to the previous experiment with sterile soil, indicating that a large proportion of the decomposition of sumithion is not caused by micro-organisms. The accumulation of 3-methyl-4-nitrophenol, however, was higher in normal than sterile soil,

Table 40. The recovery of sumithion and 3-methyl-4-nitrophenol from sterile Chelsea loam

Time (days)	Recovery (µg./g. soil) *	
	Sumithion	3-methyl-4-nitrophenol
0	443.0	0
5	193.0	2.9
9	120.0	0
16	134.0	18.2

* Results corrected to 100% from an initial recovery of 61.0%

which may indicate that micro-organisms are primarily responsible for the hydrolysis of sumithion. Since only a small amount of 3-methyl-4-nitrophenol was formed in sterile soil, even in the presence of water, most of the sumithion must be either decomposed by some other mechanism, or be unrecoverable.

Identification of the water-soluble decomposition products of sumithion

The studies with normal and sterile soil indicated that other pathways might exist for the decomposition of sumithion other than hydrolysis and, therefore, an experiment was carried out to identify any water-soluble products which might be found in normal soil.

The experiment was carried out in a similar manner to the previous studies. Sumithion was added to the soil, mixed with sand, to give a final concentration of 340 µg./g. soil. The soil was sampled and extracted with methanol as described previously. The extract was evaporated to dryness and then redissolved in 0.5 ml. methanol. The concentrated extract was then applied as a streak to chromatography paper, which was impregnated with silicone fluid, and developed as described in the materials and methods. In this technique, sumithion remains near the origin and the water soluble products are separated, in particular the oxygen-analogue and des-methyl sumithion run close to the solvent front with Rf. values of 0.82 and 0.94 respectively. Since the ring structure is not changed in these compounds, it was hoped to see if they were present, using their absorption of ultra-violet light to identify them.

The results obtained using this technique showed that there was apparently no formation of the thiolate or des-methyl sumithion in normal soil, although they may have been formed at a level below which they were detectable. The major product formed was 3-methyl-4-nitrophenol, which was identified using a marker of the pure compound. In only one instance, after 11 days incubation, was there any other absorbing material present which had separated from the hydrolysis product. The unidentified compound had an Rf. of 0.44 compared with an Rf. of 0.74 for 3-methyl-4-nitrophenol. However, the compound produced a yellow colour on the chromatogram, in a similar manner to 3-methyl-4-nitrophenol, which indicated that the compound was closely related to the hydrolysis product and might have been formed when the extract was concentrated. This seems probable since it was noticed on only one chromatogram from a series extending over 16 days.

The results suggest that in normal soil, hydrolysis of free sumithion is due to the action of micro-organisms, causing the accumulation of 3-methyl-4-nitrophenol.

The stability of sumithion in solution

Although the previous experiments suggested that sumithion was hydrolysed by micro-organisms in the soil, there was the possibility that the sumithion was hydrolytically unstable. Because sumithion is relatively insoluble in water, its stability in a 50% aqueous solution of methanol was determined. It was presumed that if sumithion was unstable in the presence of water, then it should decompose in this

aqueous solvent. Sumithion was dissolved in aqueous methanol to give a concentration of 400 µg./ml. 100 ml. portions of the solution were placed into two 250 ml. glass-stoppered, conical flasks. One flask was placed in an incubator at 25°C. and the other at 30°C. 5 ml. samples were taken at intervals and evaporated to dryness in round-bottomed flasks under reduced pressure. The residue was redissolved in 50 ml. of ether and the sumithion and 3-methyl-4-nitrophenol were estimated as described previously.

The flasks were incubated for 23 days, but only a small quantity of the sumithion was not recovered after this time. The incubation at the different temperatures had little effect; after 23 days 95.5% of the original sumithion was recovered after incubation at 25°C. and 91.0% recovered at 30°C. The amounts of 3-methyl-4-nitrophenol formed at 25°C. and 30 °C. were 2.5 and 5.0 µg./ml. respectively. This indicated that the hydrolysis in the soil was due to biological activity, although it was probable that the catalytic action of normal soil might aid in the hydrolysis. The stimulation of the population and respiration in the soil in the presence of sumithion was probably due to the utilisation of O,O-dimethyl phosphorothioate, also formed on the hydrolysis of sumithion, since 3-methyl-4-nitrophenol added to the soil had no stimulating effect on the general population (c.f. Table 24).

(ii) The persistence of sumithion as determined by bioassay

It was found by experimentation that sumithion might have been estimated using the mosquito larvae bioassay, since both

malathion and sumithion produced 100% mortality of the larvae at a final concentration of 1 µg./ml. However, since the mosquito larvae assay was so time consuming and had the disadvantage of having to extract the insecticide before the assay, it was decided to investigate the possible use of an insect, which inhabits the soil.

The Collembolon, Folsomia candida Willem (1902) is reared easily in the laboratory on a diet of yeast, and when placed on the soil actively migrates between the soil particles. The most useful characteristic of the insect was that all live individuals could be recovered from the soil by drying it, since the spring-tails move with the water gradient.

After an initial experiment had shown that the spring-tails could be recovered, an investigation of the toxicity of sumithion to them was undertaken. Sumithion was added to the soil as a solution in ether at a concentration of 5000 µg./ml. 0.5 ml. of the sumithion solution was added to each of two specimen tubes containing 5 g. soil. The ether was evaporated off and the soil was transferred to 3" diameter glass funnels, resting in 3" x 1" specimen tubes containing a drop of water. Similar funnels containing untreated soil were also prepared. The soil was moistened and 15 spring-tails were added to each funnel. The funnels were then covered with Petri dish lids to maintain a humid atmosphere. After incubation at room temperature for 24 hours the Petri dish lids were removed and the soil dried by placing a lamp above the funnels. The live spring-tails migrated through the soil and fell down the neck of the funnel into the specimen tube where counts of the viable insects were made.

At the high concentration of sumithion used, all the spring-tails were apparently killed since none could be recovered after 24 hours, whilst 100% recovery was obtained from untreated soil. The experiment was repeated using sumithion at concentrations of 3.5, 35 and 350 µg./g. soil, but it was found that many spring-tails had migrated into the specimen tube before the soil was dried, indicating avoidance of the insecticide.

The assay was modified by applying the insecticide to 4.5 g. soil in a specimen tube and after evaporating off the ether, 0.5 ml. water was added to give 5 g. soil at approximately 50% of the maximum water holding capacity. The spring-tails were added to the specimen tube, where they were obliged to remain in contact with the soil. After 24 or 48 hours incubation, the soil was transferred to the funnels, into the necks of which small coils of stainless steel wire were placed to prevent the soil blocking the passage of the insects. The funnels were placed onto 100 ml. conical flasks containing a drop of water and the soil dried. Counts were made as before.

It was found that the use of a contact time of 48 hours improved the assay and it was decided to try to prepare a calibration curve for sumithion. The insecticide was applied to duplicate soil aliquots at concentrations of 0.16, 0.8, 4.0 and 20.0 µg./g. soil. 25 spring-tails were added to each specimen tube and the live insects recovered after a contact time of 48 hours. The results are shown in Table 41.

Table 41. The mortality of Spring-tails in Chelsea loam containing various concentrations of sumithion.

Concentration of sumithion (µg./g. soil)	% mortality of Spring-tails
0	0
0.16	0
0.8	0
4.0	88.0
20.0	100.0

* The results are the average of duplicate samples, after incubation at room temperature for 48 hours.

The results show that the level of concentrations over which the spring-tails are killed is relatively narrow, 88% being killed at 4.0 µg./g. soil but none being killed at $^1/_5$ of this concentration. This makes the use of spring-tails impracticable for the bioassay of sumithion and the method had to be discontinued. The persistence of sumithion as determined by bioassay was, therefore, not carried out.

c) The persistence of menazon

Preliminary investigations with the colorimetric estimation of menazon indicated that technical difficulties might make it unreliable. An alternative method was investigated using a chromatographic technique. It was hoped that an approximate estimation might be made from the intensity of the colour reaction of menazon on the chromatogram. Soil extracts in methanol were placed on Whatman No. 1 chromatography paper previously washed in EDTA solution and developed with a solvent mixture of n-butanol 12 parts, glacial acetic acid 3 parts and water 5 parts. The menazon was converted to a pink compound by dipping the chromatogram in a 2% solution of 2:6 dibromoquinone chloroimide in cyclohexane and heating the paper at 100°C. for 1 hour. The menazon was identified using a marker of the pure insecticide. It was found that the range of concentrations of menazon that could be measured in this way was limited. An alternative method was used relying upon the fact that menazon absorbs ultraviolet light. The developed chromatogram was placed upon photographic paper and exposed to ultraviolet light. On developing the paper, white unexposed areas

due to menazon appeared on a dark grey background. However, soil extracts containing menazon produced fluorescence which masked the white areas of the insecticide, and this made the method impracticable.

(i) Persistence of menazon in the soil as determined by colorimetric estimation

The method finally used, was as described in the materials and methods. Few modifications were required to the original method although difficulty was experienced in obtaining low optical densities for the reagent blanks, due to phosphate contamination. It was found necessary to clean all glassware with concentrated nitric acid and acid baths were used for this purpose. The glassware was then washed thoroughly with tap water and distilled water before use. Although the original method stated that the optical density of reagent blanks should be below 0.05, in practice it was found impossible to achieve this with the chemicals used. But since the blanks remained consistently in the range 0.15 - 0.2, it was decided to use the method correcting the results for the optical density obtained with the blanks.

Persistence in Chelsea loam

Since the estimation of menazon was so sensitive, the insecticide was added to the soil as an aqueous solution. A 0.01% solution of menazon in distilled water was prepared and 10 ml. was added to 100 g. of Chelsea loam in a 250 ml. conical flask. This gave a final concentration of 10.0 µg./g. soil. A similar flask containing untreated soil was prepared and the water content of both flasks was

adjusted to 50% of the maximum water holding capacity. This effectively reduced the menazon content to 8.85 µg./g. soil The flasks were incubated at 30°C. and water lost by evaporation was replenished by additions of distilled water. 5 g. samples of soil were taken at intervals and the menazon estimated. A calibration curve was prepared from standard solutions of menazon in methanol (Fig. 28) plotting the optical density produced against the concentration of menazon in µg./l. final solution. The concentration of menazon in the soil extracts was determined from the graph. Standards were estimated with each sample to check the calibration curve. The results are shown in Table 42 and graphically in Fig. 29.

From the results it will be seen that the recovery of menazon declines rapidly, only 1.6% being recovered after 9 days. The failure to recover most of the menazon after 9 days suggested that the insecticide was being rapidly decomposed in the soil or that it was not being extracted by the methanol. If the menazon were being decomposed it was possible that micro-organisms were involved, although the results obtained from the respiration and population studies indicated that the degradation products were not utilised to the same extent as those of malathion and sumithion.

Persistence of menazon in Chelsea loam after a repeated addition

An experiment was carried out with Chelsea loam to which a second addition of menazon was made, after the majority of the first addition had disappeared. It was thought that if micro-organisms were

Fig. 28. Calibration curve for the colorimetric estimation of menazon.

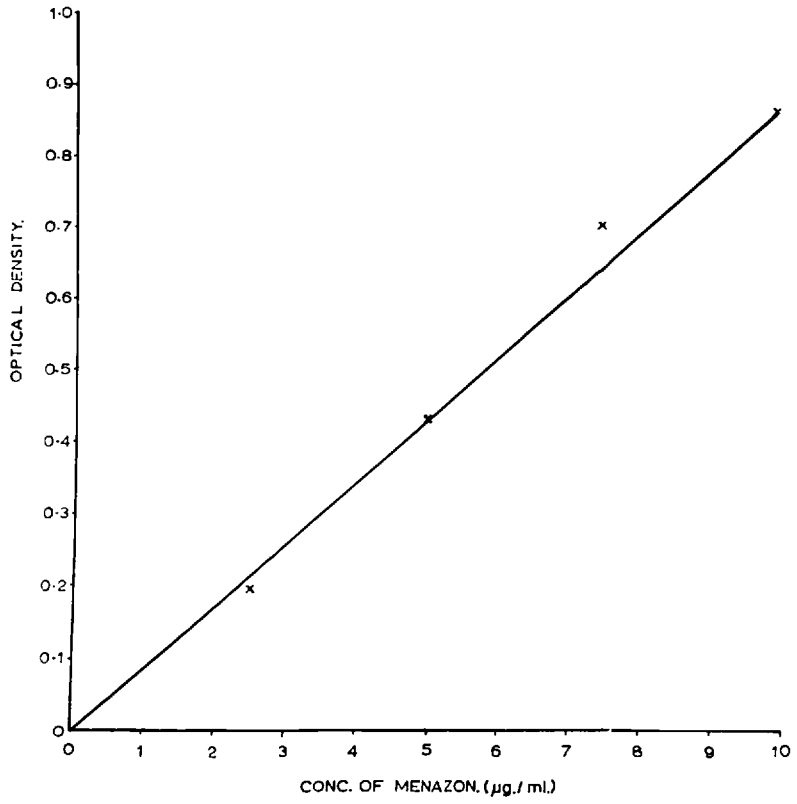
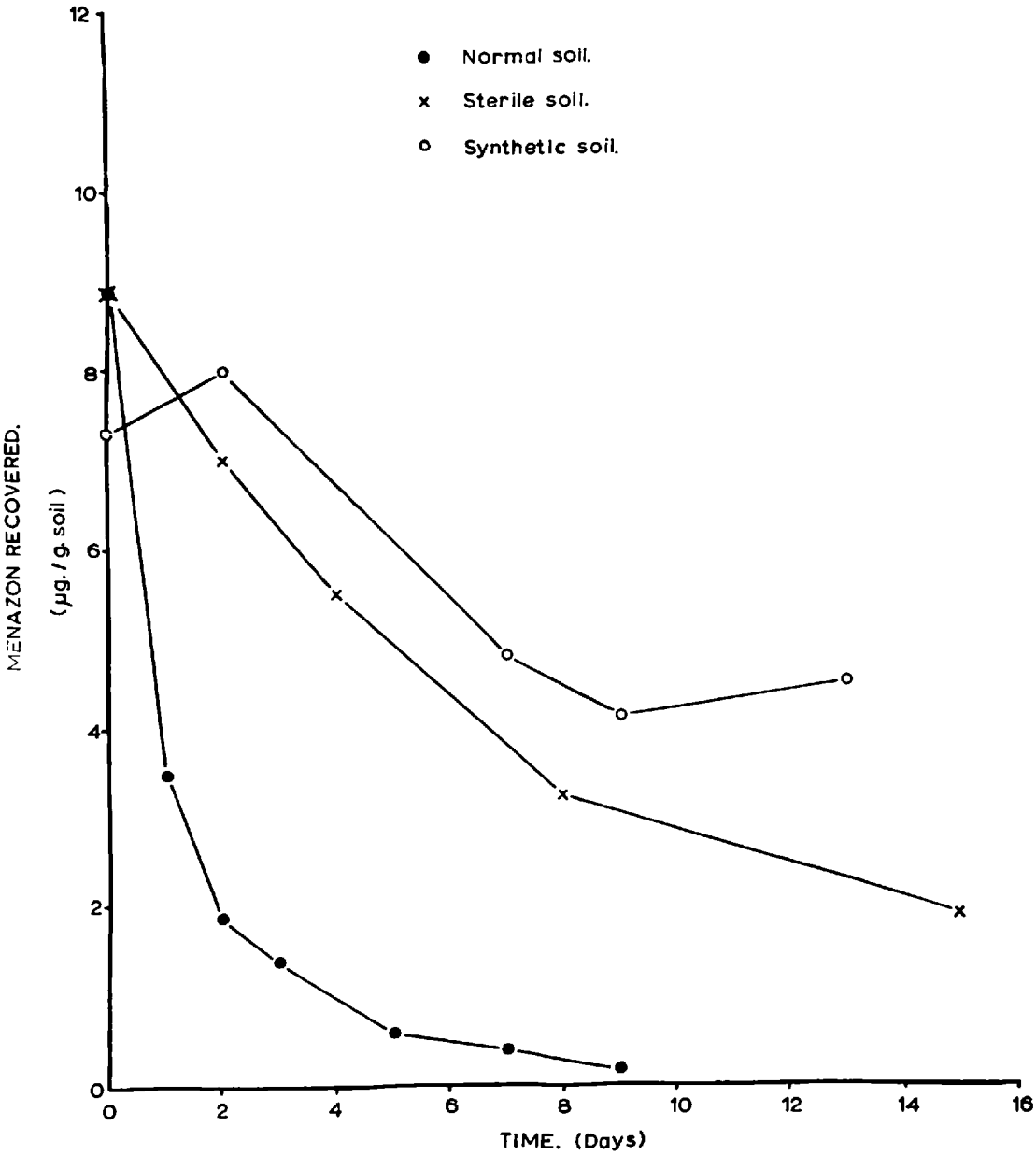


Table 42. The recovery of menazon from Chelsea loam as determined by colorimetric estimation.

Time (Days)	Menazon recovered * (µg./g. soil)
0	8.85
1	3.5
2	1.89
3	1.4
5	0.61
7	0.39
9	0.14

*Results corrected to 100% from an initial recovery of 114%.

Fig. 29. The persistence of menazon in normal and sterile Chelsea loam and a synthetic soil, as determined by colorimetry.



adapting to the decomposition of menazon, then the second addition might be degraded faster. The soil population was also measured to see if changes took place in the total number, indicating utilisation of the degradation products.

A saturated solution of menazon in distilled water was prepared giving a concentration of 240 µg./ml. All the water required to bring 100 g. soil to 50% of the maximum water holding capacity was replaced with the menazon solution giving a final concentration of 28.6 µg./g. soil. After two weeks incubation at 30°C. the water lost by evaporation was replaced with a freshly prepared saturated solution of menazon, and the persistence of the new concentration determined. Duplicate 5 g. samples of soil were taken at intervals and the menazon concentration determined. Further samples were taken to determine the number of viable organisms on Thornton's agar, using the pour-plate technique. The results are shown in Table 43 and graphically in Fig.30.

The results show that the recovery of menazon, added after two weeks, declined at a similar rate to the first addition. After incubation for one week, 13.3% of the initial concentration was recovered. Similarly after incubation for one week 13.6% of the concentration after the second addition was recovered.

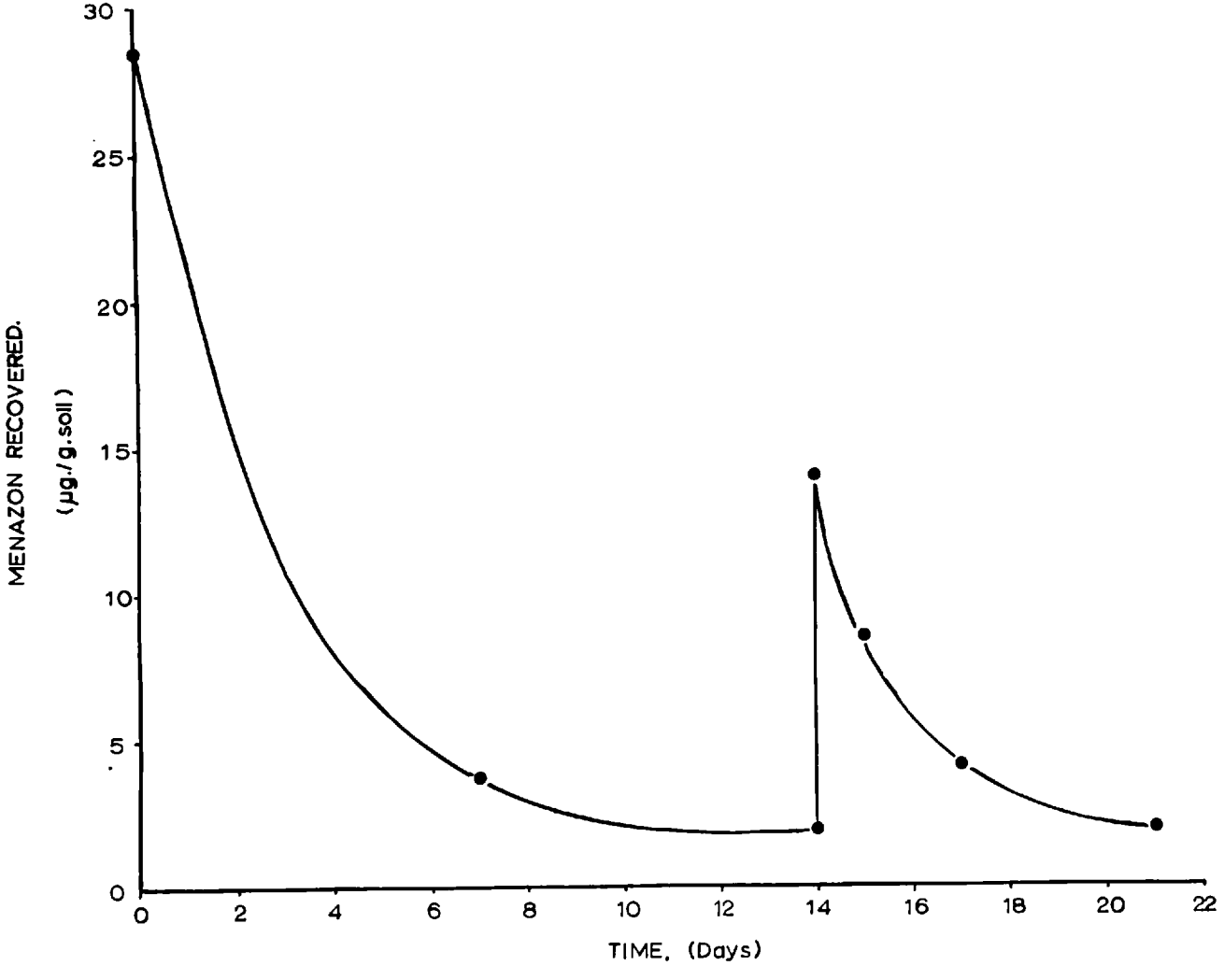
Although the viable soil population increased over the course of the experiment, there was no increase in the rate of decline of the second addition of menazon in the soil. The results suggested that if the menazon was being decomposed then the majority of the insecticide was being lost by chemical decomposition, which is reinforced by the

Table 43. The recovery of menazon, before and after a second addition of insecticide, from the Chelsea loam, as determined by colorimetric estimation.

Time (days)		Recovery of menazon * (µg./g. soil)		Number of organisms x 10^6/g.
		Total	Range	
0		28.6	±3.4	9.6
7		3.81	±0.5	8.05
14	Before addition	1.96	±0.66	13.78
14	After addition	14.0	±0.0	-
15		8.54	±0.49	14.2
17		4.12	±0.32	18.65
21		1.9	±0.06	26.4

* Results corrected to 100% from an initial recovery of 76.5%

Fig. 30. The effect of a second addition of menazon on its persistence and the number of micro-organisms in the Chelsea loam.



rapid disappearance of the first addition. However, it is possible that the second addition was made after the peak of activity of the microflora had been reached and when the population had declined to its normal proportion.

Persistence of menazon in sterile Chelsea loam and a synthetic soil

In order to determine the role played by micro-organisms in the apparent decomposition of menazon, the persistence in sterile soil was determined. 50 g. soil were sterilised in duplicate 250 ml. flasks, as described in the materials and methods. A solution of menazon in distilled water was prepared at a concentration of 0.01% and was sterilised by passage through a sintered glass filter. 5 ml. of the sterile solution was added to the soil in one of the flasks aseptically and the water content of both flasks of soil adjusted with sterile distilled water, assuming all water was lost on sterilisation. The water contents were also maintained with sterile distilled water. 5 g. samples were taken aseptically at suitable intervals and the menazon content estimated. Results obtained from soil containing menazon were corrected for the blanks. Samples were also taken to check the sterility of the soil. In spite of the precautions taken contamination of the sterile Chelsea loam occurred and, therefore, to reduce the possibility of the contaminants developing, the experiment was repeated using a synthetic soil. The soil was prepared to contain exactly the same proportion of mineral fractions as in Chelsea loam, previously determined by the mechanical analysis, described in the materials and methods. The fractions were prepared

using coarse sand, acid-washed silver sand, kaolin and bentonite, and the water content and maximum water holding capacity determined. 50 g. of the synthetic soil were sterilised in duplicated flasks and menazon added as described above, but because the water capacity was different to Chelsea loam, the concentration was reduced to 7.3 µg./g. soil at 50% of the maximum water holding capacity.
The same experimental procedure was then followed as described for the sterile Chelsea loam. The results obtained with sterile Chelsea loam and the synthetic soil are shown in Table 44 and graphically in Fig. 29 compared with Chelsea loam.

The results show that in approximately one week, the recovery of menazon from the sterile soil dropped to 50% of the initial concentration, whereas over 50% was recoverable from the synthetic soil after 13 days. This shows a great increase in the recovery over the normal Chelsea loam in which less than 50% was recovered after one day. Despite the fact that viable organisms were found in the flasks the results indicate that, by reducing the population of micro-organisms in the soil, the persistence of menazon can be increased. The results suggest that some of the menazon is decomposed by micro-organisms but that they are unable to utilise the products. This is consistent with the slight increase in respiration without proliferation in the presence of menazon, described in PartI.

The fact that menazon disappeared from sterile Chelsea loam and the synthetic soil tends to confirm the previous conclusions that the majority of the degradation is due to chemical means or failure to

Table 44. The recovery of menazon from sterile Chelsea loam and a synthetic soil, as determined by colorimetric analysis

Time (days)	Recovery of menazon (µg./g. soil)	
	Sterile Chelsea loam *	Sterile synthetic soil **
0	8.85	7.3
2	7.0	8.0
4	5.5	-
7	-	4.8
8	3.24	-
9	-	4.16
13	-	4.5
15	1.88	-

* The results were corrected to 100% after an initial recovery of 133%

** The results were corrected to 100% after an initial recovery of 74.5%

recover the menazon. It was noted that the apparent rate of disappearance of menazon was greater in the sterile Chelsea loam than in the synthetic soil. Since the main difference between these two soils was that the sterile Chelsea loam contained organic matter, it is possible that presence of organic matter increases the rate of decomposition of menazon.

The persistence of menazon under anaerobic conditions

An experiment was prepared to determine the persistence of menazon under anaerobic conditions, in order to investigate the influence of anaerobic micro-organisms on the persistence.

The menazon was added as a saturated solution in distilled water to 100 g. of Chelsea loam, giving a final concentration of 28.2 µg./g. soil. Duplicate flasks were prepared, one of them being incubated aerobically and the other in an atmosphere of hydrogen in a Brewer anaerobic jar. Duplicate 5 g. samples were taken at weekly intervals from both flasks and the menazon content determined. The number of viable anaerobic bacteria in both flasks was estimated on Reinforced Clostridial medium using the pour-plate technique. It was hoped that if the anaerobic organisms were active in the decomposition of menazon, this would be reflected by an increase in number. The results are shown in Table 45.

The results show that there is little difference in the ability to recover menazon from both soils, but anaerobic conditions

Table 45. The recovery of menazon from Chelsea loam incubated at 30°C., aerobically and anaerobically, as determined by colorimetric estimation

Time (days)	Recovery of menazon * (µg./g. soil)		Number of anaerobic bacteria x 10^6/g.soil	
	Aerobic soil	Anaerobic soil	Aerobic soil	Anaerobic soil
0	28.2 ±2.7	28.2 ±5.65	2.3	2.3
7	2.84 ±0.14	5.66 ±0.47	2.14	3.09
14	0.63 ±0.09	1.11 ±0.094	3.5	2.3

* Results corrected to 100% from an initial recovery of 111.0% from soil incubated aerobically, and 106.0% recovery from soil incubated anaerobically.

do increase the persistence slightly. After 7 and 14 days, almost twice as much menazon was recovered from the soil incubated anaerobically than from the soil under aerobic conditions. The decline in the recovery of the menazon in the anaerobic soil was faster than in sterile soil, which suggests that the anaerobic organisms were partly responsible for the degradation. However, the number of viable anaerobic bacteria did not change significantly, which is similar to the results obtained with the aerobic population. The results also indicate that aerobic conditions are in part responsible for the decline as shown by the difference in the rates of disappearance in the soils incubated under aerobic and anaerobic conditions.

The estimation of the accumulation of the oxygen-analogue of menazon

In the metabolism of organophosphorus insecticides, oxidation to the oxygen-analogue or thiolate is the reaction producing the more toxic compound. It was thought possible that either conversion by micro-organisms or catalytic oxidation might be forming the thiolate in the soil, and this would reduce the menazon content of the soil as determined colorimetrically without reducing biological activity. This would not be shown by the method used for estimating menazon, since it is specific for the sulphur-analogue. The modifications described in the materials and methods do, however, enable the thiolate to be estimated. An experiment was carried out with menazon at 8.85 µg./g. soil, added as a 0.01% aqueous solution to 100 g. of Chelsea loam, to determine the possible accumulation of the thiolate. Since at the time pure thiolate was not available, it was not possible to

test the method or determine the recovery of thiolate from soil. But it was considered probable that if the thiolate accumulated to any great extent this could be easily demonstrated. The flasks containing treated and untreated soil were incubated at 30°C. and duplicate 5 g. samples were taken at intervals, the menazon and the menazon plus thiolate being determined separately. The results are shown in Table 46.

The results show little difference between the recovery of menazon alone and of menazon plus thiolate, indicating that little if any thiolate accumulates in the soil. However, as will be seen, the initial recovery of the menazon plus thiolate was low and therefore a sample of the pure thiolate was obtained in order that the recovery of menazon and its thiolate from the ion exchange colomns could be investigated.

Solutions of menazon and its thiolate were prepared in methanol to give concentrations of 20 µg./ml. 2 ml. of each solution in duplicate were placed into separate flasks, and also 1 ml. of each solution were mixed in duplicate flasks. The solutions were diluted with distilled water to 100 ml. and 100 ml. 0.04N hydrochloric acid added to each flask. One acidified solution of each duplicate was then passed through a 1% cross-linked and the other through an 8% cross-linked ion-exchange resin and the compounds estimated colorimetrically. The final concentration for each solution was 10 µg./ml. A calibration curve for each compound was prepared using standards in methanol and the recovery from the columns determined from the graphs. The results are shown in Table 47.

Table 46. The recovery of menazon and its thiolate from Chelsea loam as determined colorimetrically.

Time (days)	Recovery (µg./g. soil) *	
	Menazon	Menazon plus thiolate
0	8.85	8.85
2	2.40	2.80
6	0.40	0.20

* Results corrected to 100% from an initial recovery of 75.0% of the menazon, and 47.4% of the menazon plus thiolate.

Table 47. The recovery of menazon and its thiolate from 1% and 8% cross-linked ion exchange resins.

	Menazon and Thiolate recovered								
	Menazon (10 ppm)			Thiolate (10 ppm)			Menazon plus Thiolate (5 ppm + 5 ppm)		
	µg./ml.		%	µg./ml.		%	µg./ml.		%
1% cross-linked	8.08	±1.72	80.8	1.0	±0.0	10.0	3.17	±0.17	63.65
8% cross-linked	4.56	±0.44	45.6	8.69	±0.19	86.9	7.25	-	72.5

The results show that more menazon is recovered from the 1% resin than from the 8% resin, whilst the opposite is true for the thiolate. The fact that 10.0% of the thiolate was recovered from the 1% resin was surprising, since it means that the original method is not completely specific for menazon alone. This makes the estimation of mixtures of menazon and thiolate more difficult, since it is necessary to know the exact concentration of menazon in order to determine the concentration of the thiolate by difference. This is shown by the recovery of the mixture from both columns. Assuming that only the menazon is recovered from the 1% cross-linked resin, the amount of thiolate recovered from the 8% cross-linked resin is 7.25 - 3.17 = 4.08 µg./ml. This represents a recovery of 81.5% of the thiolate and is similar to the recovery of thiolate alone from the 8% resin. However, since there is an error due to the recovery of thiolate from the 1% resin, the exact concentration cannot be determined. Despite the error involved, it is clear that the method enables an approximation to be made of the concentration of the thiolate in mixtures of the two analogues. This confirms that there was no measurable accumulation of thiolate in the soil, as determined in the previous experiment.

The estimation of the adsorption in, and recovery of menazon from Chelsea loam

An experiment was undertaken to determine if menazon was adsorbed in Chelsea loam and if so, to investigate the recovery of adsorbed menazon by the normal procedures.

The main evidence for the action of micro-organisms on the decomposition of menazon in the soil was obtained from the increased persistence in sterile soil. Other investigations suggested that micro-organisms were not involved and even the loss from normal Chelsea loam could be accounted for by chemical decomposition or adsorption. If menazon is bound in the soil which makes it unrecoverable, an explanation for most of the observed facts could be found.

5 g. soil were placed into each of two MacCartney bottles and 5 ml. of 0.02½% solution of menazon added. The bottles were then shaken mechanically for one hour. The soil was then spun down in a centrifuge to form a pellet, the supernatents were removed and the menazon content determined colorimetrically. The soil from each bottle was transferred to separate 250 ml. conical flasks and to one 50 ml. distilled water and to the other 50 ml. methanol were added. The flasks were shaken mechanically for 30 minutes and the soil suspensions were filtered using the same method as for normal extractions of menazon from the soil. The menazon content of the filtrates was then determined colorimetrically. A sample of the original aqueous solution was also estimated to determine the actual content of menazon. The results are shown in Table 48.

The results show that 5 g. soil removed 57.5% of the menazon from aqueous solution, although this was completely recovered by extraction with water and methanol. This experiment indicated that adsorption phenomena do remove menazon from solutions placed in the soil.

Table 48. The adsorption in and recovery of menazon from Chelsea loam as determined colorimetrically.

	Theoretical concentration of menazon	Menazon recovered	
	µg./ml.	µg./ml.	percentage
Aqueous solution before treatment	240	188.5	78.5
Aqueous solution after treatment	240	80.0	33.3
50 ml. methanol extract from soil	5.4	6.4	118.0
50 ml. aqueous extract from soil	5.4	5.7	106.0

However, as was found in practice, all the applied menazon can be recovered from the soil before it is incubated. This suggested that if menazon was not decomposed in the soil, the mechanism by which it was found was not reversable adsorption but chemical binding.

(ii) Persistence of menazon in the soil as determined by the activity of ^{14}C-labelled menazon, compared by means of colorimetric, chromatographic and polarographic techniques.

The use of ^{14}C-labelled menazon enabled the simple determination of the persistence in, and recovery of menazon from the soil, and also the determination of the accumulation of the possible degradation products of menazon. It was thought that if menazon were being decomposed in the soil colorimetric, polarographic and chromatographic techniques would show the disappearance, but the total radioactivity extracted from the soil might remain the same, indicating recovery of the degradation products. If, however, the radioactivity extracted were also reduced, this would indicate adsorption of menazon itself and/or its degradation products.

The experiment was essentially the same as in the earlier studies. A saturated aqueous solution of ^{14}C-labelled menazon was prepared and added to duplicate flasks containing 100 g. Chelsea loam to give 28.2 µg./g. soil. One flask was incubated aerobically and the other in an atmosphere of hydrogen in an anaerobic jar, both at 30°C. It was hoped that a comparison of any degradation products formed in the soils might indicate whether or not micro-organisms were involved.

Duplicate 5 g. samples were taken at intervals from each flask and the menazon content determined colorimetrically or polarographically, as described in the materials and methods. A further 5 g. sample from each flask was taken and extracted with 50 ml. methanol to determine the recovery of the activity from the soil. 2 ml. of the methanolic extracts were used to determine the activity using a liquid scintillation counter and from the specific activity determined from the original aqueous solution, the theoretical concentration of menazon could be determined. However, since some of the activity in the extract would be accounted for by degradation products, autoradiograms were prepared which would show the presence of these products. The extracts remaining after the determination of the total activity were concentrated and developed on a chromatogram; the method being described in full in the materials and methods. The results are shown in Table 49. The autoradiograms shown in Plates 4, 5 and 6 have the base lines, marked with crosses, and the solvent fronts drawn in.

From the results it appeared that either menazon and/or its degradation products were being firmly bound in the soil. Although it was intended to use the polarographic method of estimation throughout, at the lower concentrations of menazon, contaminating material from the soil interfered with the peak obtained on the cathode-ray screen. Therefore, the colorimetric estimation was used for confirming the results obtained after 7 days and alone after 14 days. The measurement using the total activity indicated that there were few decomposition products recovered from normal soil and this was confirmed by the autoradiograms.

Table 49. The recovery of ^{14}C-labelled menazon from the Chelsea loam incubated aerobically and anaerobically, as determined by various methods.

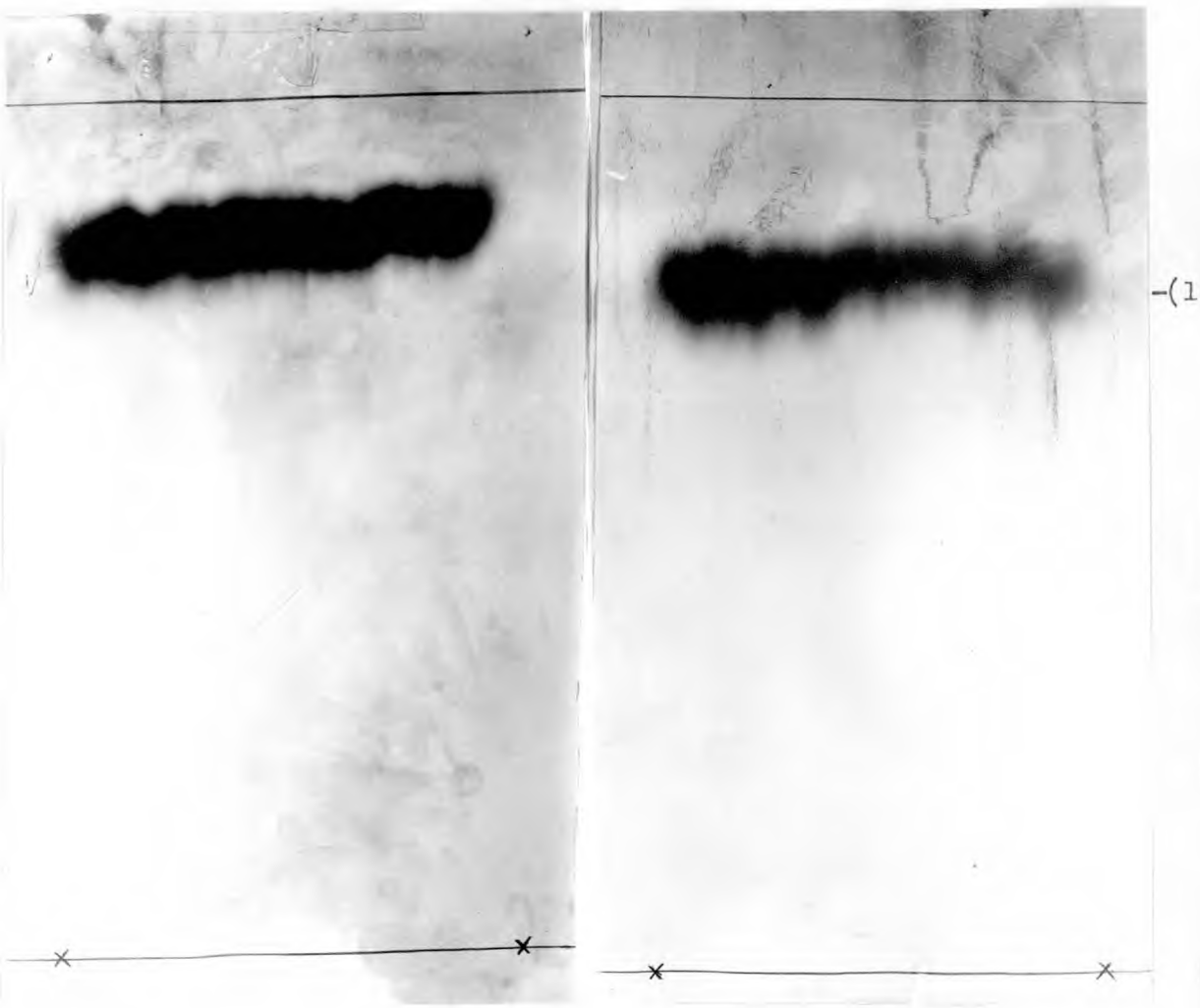
Time (days)	Recovery of menazon (µg./g. soil) from the Chelsea loam					
	Incubated aerobically			Incubated anaerobically		
	Colorimetric estimation	Polarographic estimation	Radioactivity	Colorimetric estimation	Polarographic estimation	Radioactivity
0	-	31.1 ±3.2	26.0	-	29.12 ±2.08	29.3
7	2.03 ±0.13	1.27 ±0.63	3.58	3.7 ±0.55	4.17 ±0.46	14.8
14	0.75 ±0.05	-	1.43 ±0.01	0.65 ±0.05	-	1.36 ±0.1

Plate 4. Autoradiograms of soil extracts from soil containing ^{14}C-labelled menazon incubated aerobically and anaerobically.

Exracts at zero time.

Aerobic soil

Anaerobic soil



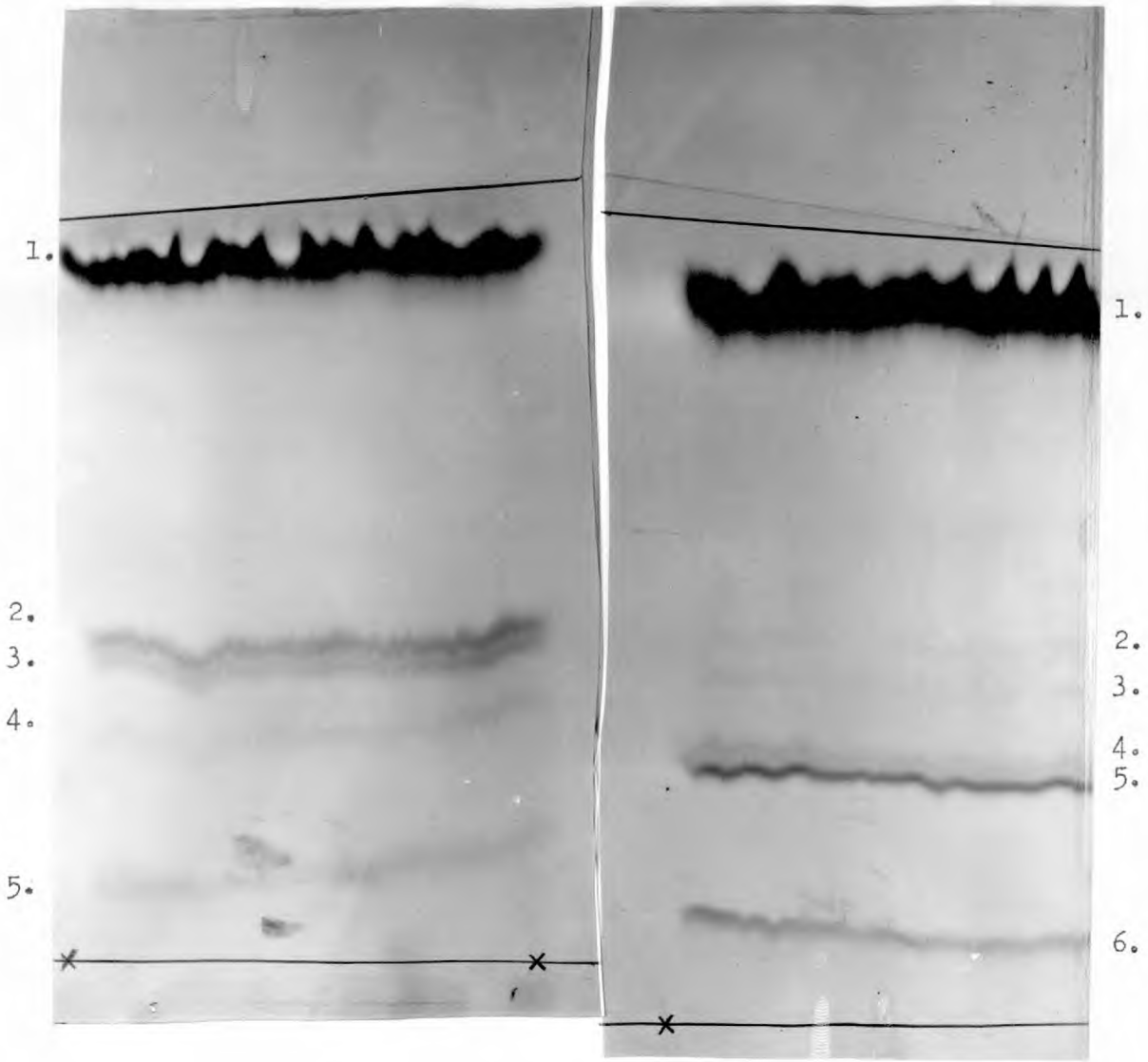
1. Menazon band.

Plate 5. Autoradiograms of soil extracts from soil containing ^{14}C-labelled menazon incubated aerobically and anaerobically.

Extracts after 7 days incubation.

Aerobic soil

Anaerobic soil

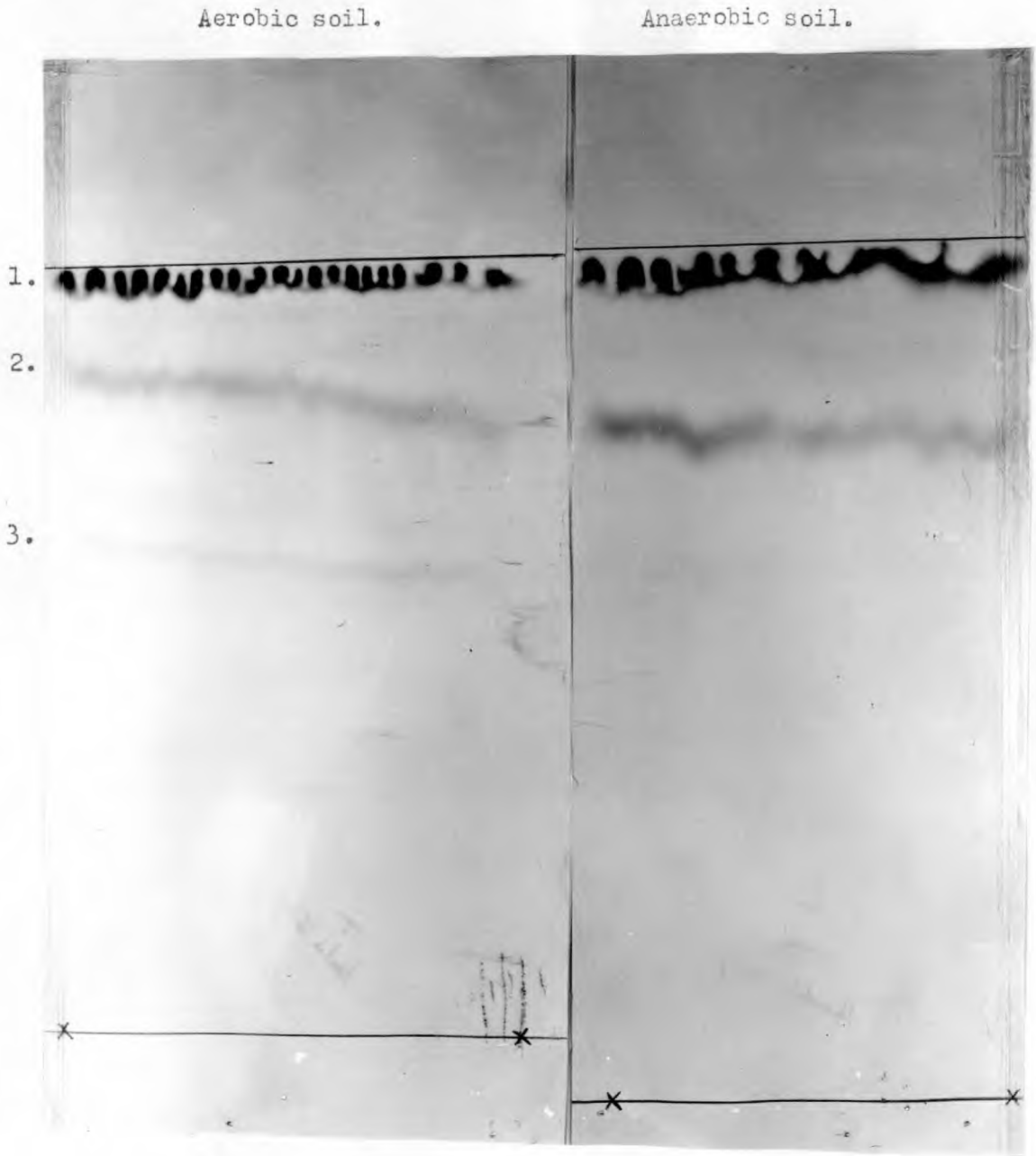


1. Menazon band

2-6. Unidentified decomposition products.

Plate 6. Autoradiograms of soil extracts from soil containing ^{14}C-labelled menazon incubated aerobically and anaerobically.

Extracts after 14 days incubation.



1. Menazon band.

2-3. Unidentified decomposition products.

The fact that decomposition products were formed shows that menazon is degraded, but since more bands were present after 7 days than 14 days, on the autoradiograms, it suggests that they are probably bound to the soil. Unfortunately, one cannot show from the experiment whether menazon is completely decomposed before being bound to the soil or if both menazon and its products are being bound. The results seem to suggest that much of the menazon was decomposed before being adsorbed, when under anaerobic conditions. Since the equivalent of 14.8 µg. menazon was extracted, as determined by the total activity recovered after 7 days incubation, and only approximately 4.0 µg. of this was unchanged menazon, it indicates that 73.0% was in the form of degradation products. This means that even if the unrecovered activity was in the form of unchanged menazon, almost 40% of the applied menazon would have been decomposed. Unfortunately the results do not indicate how the menazon was decomposed. Either chemical or biological action could be responsible in the soil incubated aerobically or anaerobically. The increased rate of disappearance in the aerobic soil might indicate that aerobic organisms are involved, but it is possible that the chemical decomposition takes place faster in the presence of oxygen.

Measurement of the radioactivity bound in the soil

Although it was most likely that the decline in the recovery of the radioactivity was due to binding in the soil, it was possible that the soil micro-organisms were slowly metabolising menazon and the activity was lost as $^{14}CO_2$. In order to see if the activity was still in the soil, direct measurements were made in a thixotrophic medium,

as described in the materials and methods. After taking the 14 day samples, the soil remaining on the filter paper, after filtering the soil extracts, was recovered, The soil was air-dried and the caked soil was ground to a fine consistency with a pestle and mortar. 1 g. of soil was added to 10 ml. of thixotrophic medium. This suspension was too opaque to measure and therefore 0.1 ml. and 1.0 ml. were pipetted into clean vials. The thixotrophic medium was made up to 10 ml. and shaken thoroughly to mix the soil. The dilution of the original suspension improved the efficiency of the counting and the activity of the soil was recorded. The results were corrected to µg. menazon/g. soil at 50% maximum water holding capacity, using the specific activity of the menazon and assuming that no water remained in the soil after drying.

The efficiency of the counting was determined and found to be reduced to 2.16% in the presence of 0.1 g. soil and to 19.8% in the presence of 0.01 g. soil. The activity measured was also extremely variable, but after correcting the counts obtained, it would appear that the soil probably contained all the activity not recovered by the methanol extraction, although in what form the experiment does not indicate.

The recovery of radioactivity bound in the soil

Since the activity not recovered by the methanol extract was still in the soil, it was decided to try to recover it using various solvents and if possible to try and determine what fraction remained

as unchanged menazon.

A further sample of 5 g. soil, deposited on a filter paper during the filtration of a soil extract, was transferred to a 250 ml. conical flask. The soil was then extracted once more with 50 ml. methanol and the activity in the extract determined.

In a separate experiment, duplicate 1 g. samples of soil were taken from both the soils incubated aerobically and anaerobically, and placed into 100 ml. conical flasks. 10 ml. methanol were added to each flask and the flasks were shaken mechanically for 30 minutes. The soil suspensions were then transferred to centrifuge tubes and the soil spun down to pellets. The supernatents were removed and their activity determined. One pellet of each soil treatment was then resuspended in 10 ml. 1M ammonium acetate buffer and the remaining two in 10 ml. 0.2N acetic acid. The suspensions were transferred to clean 100 ml. conical flasks and shaken mechanically for 30 minutes. The soil suspensions were then centrifuged to pellets as described above and the activity in the supernatents determined. The ammonium acetate formed an emulsion in the scintillator liquid, which was cleared on the addition of 1 ml. methanol. The activity recovered by the various solvents is shown in Table 50, an average of the duplicates being taken as the final result. For convenience the activity was converted to µg. menazon using the specific activity.

The results show that the solvents recovered only a small fraction of the applied menazon either as unchanged menazon or as its

Table 50. The recovery of radioactivity bound to the soil using various solvents.

Treatment	Recovery of menazon from the Chelsea loam	
	µg./g. soil	Percentage of total applied
Second extraction with methanol	0.4	1.5
First extraction of 1 g. soil with methanol	1.19	4.2
Second extraction with ammonium acetate buffer	1.93	6.85
Second extraction with dilute acetic acid	0.92	3.28

decomposition products. The most efficient of the solvents tried was the ammonium acetate buffer, but the recovery with that obtained plus the methanol extract, amounted to approximately 11% of the applied menazon. From this low recovery, it was impossible to determine what fraction of the bound activity remained as unchanged menazon.

In order to investigate the possibility that the activity was firmly bound to the organic matter in the soil, an experiment was carried out to destroy the organic matter with hydrogen peroxide. It was realised that the method would probably decompose the menazon, but it was thought that if the experiment helped to determine the site of binding in the soil, this would aid in the finding of a method of recovery.

A 1 g. sample of the soil incubated under aerobic conditions and later stored in the refrigerator was treated with 4 ml. of 6% hydrogen peroxide. The mixture was warmed gently until effervescence had ceased and the gases evolved were passed through potassium hydroxide solution to absorb any $^{14}CO_2$ evolved. A further 3 ml. of hydrogen peroxide were added to destroy the remaining organic matter, and when the evolution of oxygen had ceased, the mixture was boiled to destroy excess hydrogen peroxide. The suspension was transferred into a centrifuge tube, and the sides of the flask were washed with a little distilled water. The soil was spun down to a pellet in a centrifuge, the supernatent removed and placed into a clean, glass-stoppered flask. The supernatent was then evaporated to dryness under reduced pressure at 30°C. and the

residue redissolved in 10 ml. methanol. The activity in the methanol was then measured using the second technique described in the materials and methods.

The method recovered the equivalent of 4.04 µg. menazon per gram soil or 14.35% of the total activity present. The increase in recovery was not significantly higher than when the other solvents were used, and indicated that the menazon or its degradation products were not bound to the organic matter in the soil. Since such extreme measures were unable to recover the menazon, it was doubtful if any method could be found which would recover the activity without decomposing the menazon. The only method capable of measuring the menazon was thought to be the determination of the aphicidal activity of plants growing in the active soil.

Estimation of ^{14}C-labelled menazon in the soil using an aphid bioassay

The bioassay technique of estimating menazon, described later, relies upon the extraction of the menazon from the soil. Since it was obvious that this would not give a true estimation of the menazon, an alternative method was devised. As there was little soil remaining from the experiment with the ^{14}C-labelled menazon, the method was carried out with 5 g. samples. Duplicate samples from the soils incubated under aerobic and anaerobic conditions were placed into 1" x 1" specimen tubes. Duplicate standards in similar tubes were prepared at concentrations of 10, 1.0 and 0.1 µg. menazon/g. soil by addition of

aqueous solutions to 5 g. aliquots of soil. Peas were soaked overnight, and one was placed into each tube and grown at 25°C. until the plants were approximately 3" high. 25 aphids (Aphis fabae Scop.) were then placed on each plant and the mortality determined after 48 and 72 hours. A circle of filter paper was placed round the stem to prevent the aphids falling onto the soil and each plant was placed onto a Petri dish lid to catch the dead aphids. The results are shown in Table 51.

The results obtained were not very conclusive, but there appeared to be some aphicidal activity in one of the plants grown in soil treated with ^{14}C-labelled menazon, which had been incubated under aerobic conditions. The method used was completely untried and therefore it was not surprising that difficulties were found in carrying out the investigation. The method of growing the pea plants in such a small quantity of soil was found to be difficult, since water was lost rapidly causing the death of some of the plants by wilting. Also, since leaking might have reduced the concentration of the menazon in the soil, the specimen tubes were not perforated and when too much water was applied, damping off occurred reducing the plants available for the assay.

The method was obviously impracticable for a quantitative assay, but by modifying the technique it should be possible to determine the persistence of menazon in the soil using a similar assay. Unfortunately time did not permit the development of such a technique.

Table 51. The mortality of aphids on pea plants grown in Chelsea loam containing various concentrations of menazon.

Time (hours)	Percentage mortality of aphids on pea plants						
	Soil standards with menazon (µg./g.soil)			Soil from aerobic culture		Soil from anaerobic culture	
	0.1	1.0	10.0	a	b	a	b
48	36.0	-	87.0	57.0	-	10.0	-
72	50	50	95.5	64.0	-	-	-

Table 52. The mortality of mosquito larvae at various concentrations of menazon.

Final concentration menazon (µg./ml.)	Percentage mortality
0	0
2	1.5
4	69.75
6	96.4
8	100
10	100

(iii) The persistence of menazon in Chelsea loam as determined by a bioassay technique

A preliminary investigation was carried out at the same time as malathion and sumithion for the assay of menazon with mosquito larvae. It was found that a minimum final concentration of 2 µg./ml. was necessary to produce any mortality. A range of concentrations from 0 to 10 µg./ml., final concentration, was prepared and the mortality of the larvae determined after 48 hours. The results are shown in Table 52.

The results show a very narrow range of concentrations over which the larvae are killed, which makes the method impracticable for a bioassay technique, and it was therefore discontinued.

Aphid bioassay technique

Since menazon is an aphicide, the only practical bioassay technique is with aphids. The method using Aphis fabae Scop. was as described in the materials and methods. A preliminary investigation showed that mortality of the aphids occurred over a wide range of concentrations of menazon and soil extract did not interfere with the toxicity of menazon. It was also found that there was no difference between adding 1 or 5 µl. methanol to the wicks leading to the leaf discs.

Menazon was added to soil as a saturated aqueous solution (0.024%) to give a concentration in the soil of 21.2 µg./g. Duplicate 5 g. samples of soil were extracted with methanol and prepared for the

assay as described in the materials and methods. The flasks were incubated at 30°C. and were prepared to coincide with the growth of the aphids, so that there could be enough to carry out the assay at the required time.

5 µl. of each concentrated soil extract were placed onto the wicks leading to four leaf discs, and similarly using 1 µl. of menazon standards in methanol. The apparatus was incubated at 17 - 20°C. in an incubator and the mortality of the aphids determined after 24 hours. A log. dose/probit curve (Fig. 31) was prepared from the standards and the concentration of the menazon in the soil extracts determined from the graph, using the average mortality on the four leaf discs. The results were corrected to µg./g. soil, the average of the duplicate saomples being taken as the final result. After the studies with the ^{14}C-labelled menazon, it was realised that the method was not actually measuring the true concentration of menazon in the soil and therefore a detailed investigation was not carried out. The results of the menazon recovered are shown in Table 53.

The results show a similar decline in the recovery of menazon as found using the colorimetric technique, which confirms the previous results. The wide range in the results may be accounted for by the difficulty experienced in maintaining uniform conditions for rearing the aphids, and during the assays. However, it must be remembered that, as previously shown, the extraction procedure does not recover all the menazon and its degradation products from soil and the results for this type of bioassay must therefore give a false impression of

Fig. 31 Calibration curve for the bioassay of menazon using aphids (Aphis fabae Scop.)

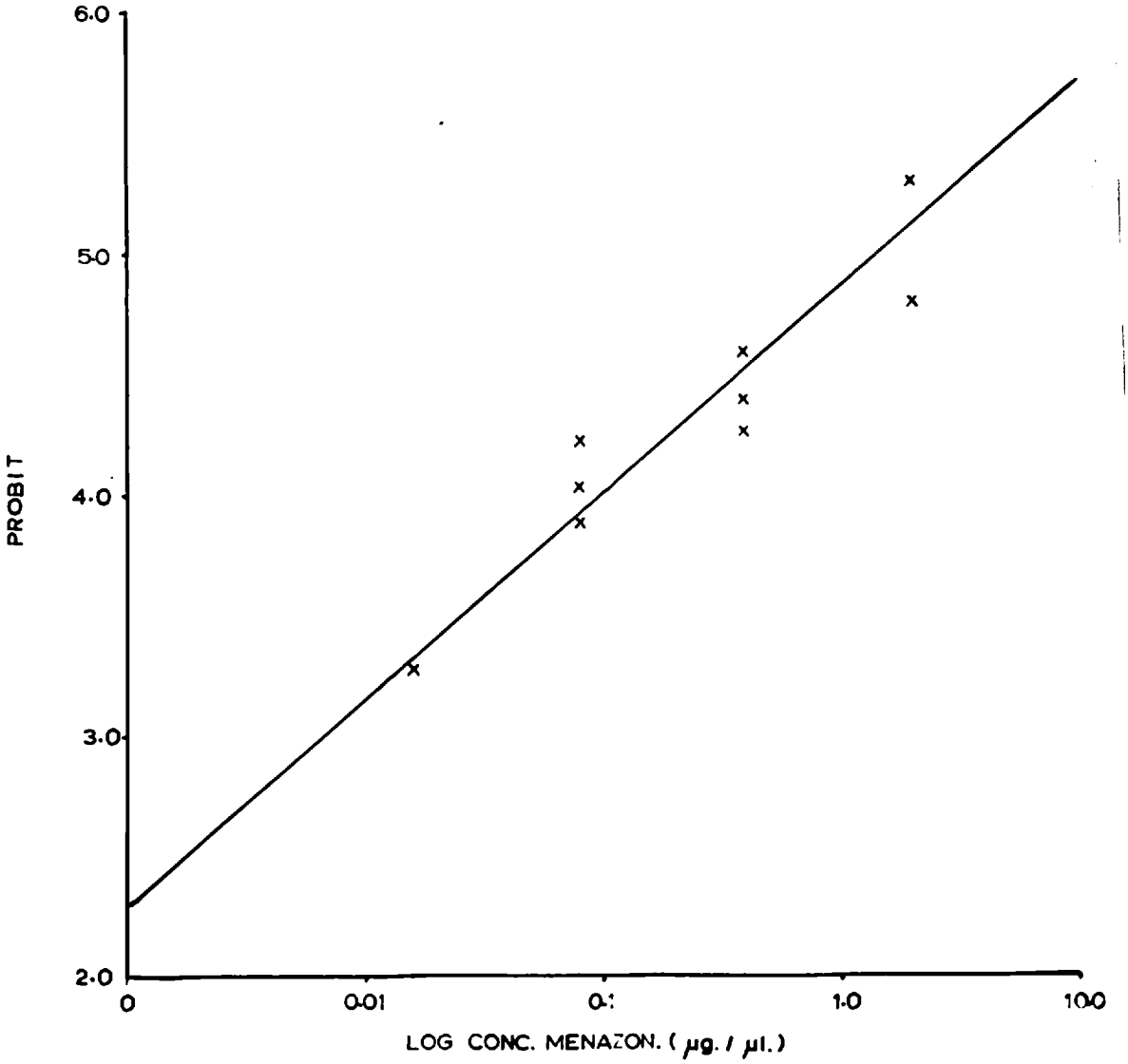


Table 53. The recovery of menazon from the Chelsea loam as determined by the Aphid bioassay technique.

Time (days)	Menazon recovered * (µg./g. soil)	
	Total	Range
0	21.2	-
1	7.2	±3.5
2	1.6	±1.20
7	0.58	±0.37

* The results are corrected to 100% from an initial recovery of 75%.

the persistence. As already mentioned, the only satisfactory assay would be to add menazon to aliquots of soil and at intervals grow plants in some of the aliquots. After growth of the plants, aphids could be placed on the leaves and the aphicidal activity in the plants determined.

B. The Isolation of micro-organisms capable of degrading the insecticides

The three organophosphorus insecticides all showed some evidence that micro-organisms were able to decompose them in the soil. In order to confirm this and possibly to determine the pathway of decomposition, it was necessary to isolate the organisms concerned from the soil and to carry out the investigation in pure culture.

a) Preliminary studies

Since malathion was the most likely insecticide to be decomposed by micro-organisms, the technique of isolation was tried first using this compound. An enrichment culture in the soil was prepared, as described in the materials and methods. Dilutions of the soil were made in sterile tap water and samples were plated onto Thornton's agar containing 0.1% and 1.0% v/v malathion as an emulsion. After incubation at 25°C., two main types of colony predominated, both of which were Gram negative rods. It was expected that the insecticides would be toxic to the growth of many bacteria, but the indications were that the organisms growing were able to tolerate high concentrations of the

insecticides. In order to reduce the growth of organisms able to tolerate the insecticide, Thornton's agar was prepared without the mannitol or asparagine (basic salts medium). Since little growth was expected with the insecticide as the sole carbon source, ammonium sulphate at 0.05% as an extra nitrogen source and mannitol at 0.1% were added separately or combined, to aliquots of the salts medium. The salts medium without the additions was also used. The malathion was added at a concentration of 0.5% v/v before the medium was poured. Three organisms isolated from the previous experiment and a soil suspension were inoculated onto the media. The plates were incubated at 25°C. Growth occurred on all the media, even without the added ammonium salt and mannitol. Since control media were not used, the experiment was repeated using media without the insecticide or mannitol. The temperature of incubation was raised to 30°C. and the plates observed at intervals for 23 days.

It was found that growth still occurred and was better on the control plates without any added insecticide than in the presence of the insecticide. This showed that greater care had to be taken in the preparation of the media to eliminate alternative carbon sources, but also that the isolates were inhibited at the concentration of insecticide used and were, therefore, probably not able to utilise it as a carbon source.

b) Isolation of insecticide decomposing organisms on agar media

Although no positive results had been obtained with the preliminary studies, the method was investigated further to try and improve the procedure. The basic salts medium was prepared using analytical grade chemicals and ion-agar, to prevent contamination of the medium with an alternative carbon source. Malathion, sumithion and menazon were added to separate portions of the medium at concentrations of 0, 0.1, 0.5 and 1.0%. The media were inoculated with a dilute soil suspension and the plates were incubated at 30°C. for 17 days. The results again showed good growth on control plates without the insecticides, but reduced growth in their presence, being completely inhibited with 1% malathion.

It was clear that using a solid medium containing agar would continually provide an alternative carbon source and that a method of isolation, based on growth in liquid culture or on a silica gel medium, should be investigated.

c) Isolation of insecticide decomposing organisms on silica gel media

Since the use of agar apparently supplied enough organic nutrients to grow micro-organisms, it was hoped to decrease the chance of contamination using a mineral salts medium. The silica gel plates were prepared as described in the materials and methods and the insecticides were incorporated in a similar manner to that used for the agar media. It was found that if the insecticides were autoclaved with the salts

solution, decomposition took place with the formation of hydrogen sulphide. Therefore, the insecticides malathion and sumithion were filter sterilised and a saturated solution of menazon was made in double strength salts solution which was also filter sterilised. A preliminary experiment showed that the previous isolates failed to grow on the medium, indicating that they were unable to utilise the insecticides for proliferation. Fresh enrichment cultures were made in the soil and portions of soil suspension were plated onto the silica gel media containing the insecticides, with or without added 0.01% mannitol to provide minimal growth. However, colonies developed in the absence of mannitol and they were replated onto fresh media containing the insecticides, but only micro-colonies developed after incubation at 30°C. for 10 days.

It was concluded from these results that soil micro-organisms could not readily adapt themselves to utilise the organophosphorus insecticides as the sole carbon source, since there was little evidence that the isolates could grow in their presence. It was also concluded that solid media would probably not enable the isolation of insecticide decomposing organisms, although it would be useful to obtain pure cultures for testing in some other way.

d) Isolation of insecticide decomposing organisms using liquid culture

A preliminary investigation showed that malathion and sumithion added to a salts solution would support the growth of organisms, although the insecticides were immiscible.

A number of isolates were obtained, using the solid medium, by streaking a soil suspension from enrichment cultures, and replating the fastest growing organisms onto salts medium plus the appropriate insecticide. Four organisms were chosen for malathion and sumithion and their ability to grow in liquid culture investigated. In order to follow the growth in a nephelometer, it was necessary to obtain an homogenous mixture with malathion and sumithion. It was therefore decided to use Triton X-100 as an emulsifying agent for this purpose. A 2% solution of Triton X-100 was made in the salts solution and sterilised by autoclaving at 15 lb. pressure for 15 minutes. The malathion and sumithion were filter sterilised and added to the medium at a concentration of 0.1% v/v. At this level the insecticides dispersed completely, giving a clear solution with a pH of approximately 7.0. 10 ml. of each insecticide solution were placed into separate sterile test-tubes and placed into a water bath at 30°C. The tubes were inoculated with the isolates and growth was followed, using a nephelometer, for 19 days.

The readings obtained showed that there was probably a change in the medium, causing the readings to fall rather than increase as expected. Since nephelometry was impracticable to measure the growth, it was decided to make viable counts using the pour-plate method.

20 ml. sterile salts solution containing 2% Triton X-100 were placed into each of four sterile 250 ml. conical flasks plugged with cotton wool. Malathion or sumithion were added to the flasks at concentrations of 0.1% and 0.005% v/v and emulsified by shaking.

One isolate for each insecticide was inoculated with a loop into each concentration and the flasks were incubated at 30°C. 1 ml. samples were taken periodically to follow the growth, dilutions were made in sterile tap water and counts were made in nutrient agar using the pour-plate technique. The results are shown in Table 54.

The results show that growth took place in each flask, but was better at 1000 ppm malathion and 50 ppm sumithion. It was decided that the method would probably be adequate to screen isolates, since the two chosen were apparently able to utilise the insecticides. However, it was necessary to check the growth in media without a carbon source before conclusions could be drown. For this reason it was decided to dispense with the use of the Triton X-100, since it had caused an increase in respiration in the soil, and might act as an alternative carbon source in solution.

In the modified technique, 25 ml. sterile salts solution were placed into each of ten sterile 250 ml. conical flasks. Into two flasks 0.1 ml. sterile malathion was placed and 0.1 ml, sterile sumithion into a further two. Menazon was dissolved in salts solution to give a saturated solution and was then filter sterilised, 25 ml. being placed into each of two sterile 250 ml. flasks. The remaining flasks of sterile salts solution acted as controls without a carbon source. The isolates were suspended in 10 ml., and in a second experiment in 100 ml., aliquots of sterile distilled water, and 0.1 ml. of each suspension was inoculated into a control flask and one containing insecticide/salts solution. The flasks were

Table 54. Growth of isolates in liquid culture containing malathion and sumithion at two concentrations.

Time (days)	Number of organisms x 10^6/ml.			
	Salts solution with malathion		Salts solution with sumithion	
	50 ppm	1000 ppm	50 ppm	1000 ppm
0	0.9	0.36	4.06	2.6
2	1.53	20.0	10.0	20.0
6	1.5	64.0	25.5	19.5
9	10.5	61.0	54.5	30.5
Final pH	7.0	7.0	7.0	7.0

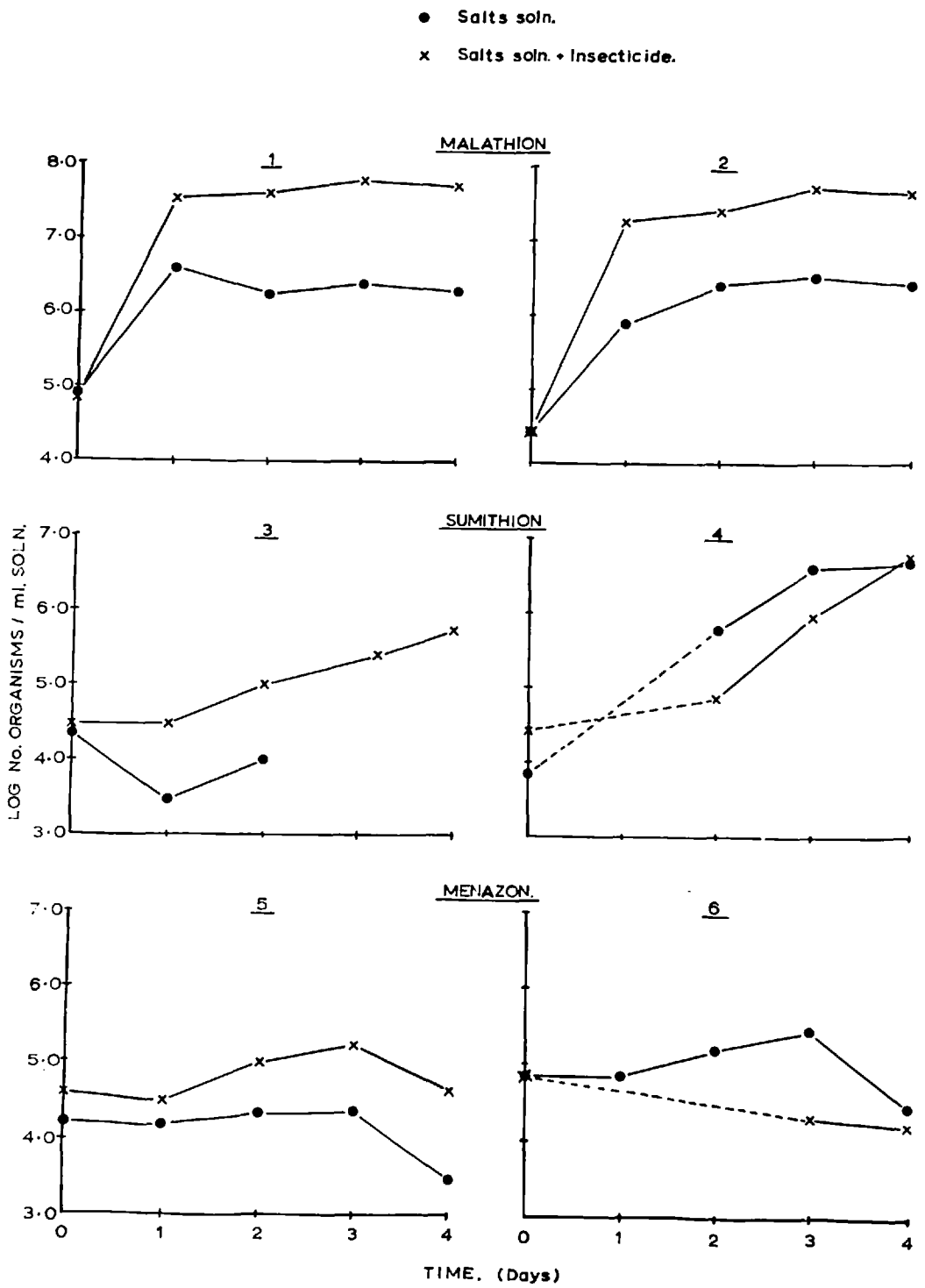
incubated at 30°C. in a reciprocal shaker incubator. 1 ml. samples were removed from the flasks and the growth followed using nutrient agar as the culture medium with the pour-plate technique. The results using the inocula at two concentrations were similar, and therefore only the growth curves of the second experiment are illustrated in Fig. 32.

The growth curves obtained with the more concentrated inoculum showed more growth in the controls than was thought desirable, and the concentration was, therefore, reduced. In the second experiment the higher dilution inoculated not only reduced the number or organisms added, but also the concentration of any carbon compounds carried over with the organisms.

The curves obtained in both experiments showed similar features, the most striking being the growth of the isolates designated 1 and 2 in the presence of malathion. Also remarkable was the growth of these organisms without any added carbon source. However, the growth in the presence of the insecticide was much better, which indicated utilisation of malathion or its products.

The curves obtained with the other isolates were not conclusive of decomposition and utilisation of the insecticides. Only isolate 3 increased significantly in number in the presence of sumithion to suggest that it could utilise the compound.

Fig. 32 Growth curves of isolates in salts solution with and without malathion, sumithion or menazon.



The strict measures taken in the second experiment to eliminate an alternative carbon source, although apparently not entirely successful, made distinguishable the true utilisation of the insecticides. However, the poor growth shown by the isolates in the presence of sumithion and menazon might have been partly caused by the lack of the compounds in solution. The saturated solution of menazon provides only 240 µg./ml. and sumithion considerably less, although excess sumithion was present as finely divided globules, and both should have provided a sufficient concentration for some growth. The results indicate that three of the organisms isolated were able to decompose the insecticides and utilise some, if not all, of the products for proliferation. However, it was necessary to confirm this by measuring the degradation of the insecticides, since the organisms could be utilising contaminating material added with the insecticides. It was also possible that degradation was a chemical phenomenon followed by growth of the organisms on the products.

C. The decomposition of the insecticides in liquid culture

Investigations were undertaken to follow the decomposition of the insecticides, using the colorimetric technique of estimating malathion and menazon. The technique available for sumithion did not permit such studies, since in order to get the sumithion into solution at the level required, the use of Triton X-100 would have been necessary. The emulsifying agent absorbed ultraviolet light at the

wavelength used for sumithion, making a direct reading impossible, and the extraction of the sumithion from the solution with solvents was also found to be impracticable in the presence of the emulsifying agent. Since no other method was available, the investigation with sumithion was not carried out.

a) The decomposition of malathion in liquid culture

(i) The effect of a soil isolate on the decomposition of malathion

The method was essentially the same as that used for the study of the growth of isolates in liquid culture and malathion was estimated using the colorimetric technique used for the soil studies. Since it was necessary to have an homogeneous medium, the malathion was dissolved in the salts solution at a concentration of 100 µg./ml. using a mechanical stirrer. The solution was sterilised by passage through a sintered glass filter and 100 ml. aliquots were pipetted aseptically into three 500 ml. sterile conical flasks. The two isolates used in the growth studies were suspended in sterile distilled water and 1 ml. of each suspension was inoculated into a flask, the third flask acting as an uninoculated control. The flasks were incubated at 30°C. in a shaker incubator and 10 ml. samples were taken at intervals and the malathion content estimated. The samples were diluted with distilled water to 50 ml. and then the extraction and estimation were as described in the materials and methods. 1 ml. samples were also taken to follow the growth of the isolates, counts

being made on nutrient agar. The results are shown in Table 55.

The results show that the isolates grew in the presence of the insecticides, although growth in the uninoculated control showed that the solution was contaminated. However, the high density of organisms present in the inoculated flasks did not affect the rate of decomposition of the malathion, since the rate was almost exactly the same in the control flask . This indicated that the isolate was not in fact decomposing the insecticide, but was able to utilise the degradation products.

(ii) The effect of ferric ions on the decomposition of malathion

Since ferric ions are known to decompose malathion, it was thought possible that the low level in the salts solution might be catalysing the reaction. It was also thought possible that the malathion might be deposited on the sides of the flasks during incubation, since a ring of sediment was noticed when the samples were taken.

Malathion was dissolved in distilled water at a concentration of 100 µg./ml. and the solution was filter sterilised. 100 ml. aliquots were pipetted aseptically into four sterile, 500 ml. conical flasks. A 0.1% solution of ferric chloride was prepared and sterilised by autoclaving and then 0.2 ml. of the solution was added to two of the flasks containing the malathion solution. One flask containing

Table 55. The decomposition of malathion in liquid culture by soil isolates as determined by colorimetric estimation.

Time (days)	Malathion recovered (µg./ml.)*			Number of organisms/ml.		
	Control	Isolate 1	Isolate 2	Control	Isolate 1	Isolate 2
0	100	100	100	1.3	1.77×10^5	4.52×10^5
2	43.0	43.2	48.9	1.07×10^2	2.87×10^7	5.0×10^6
5	23.8	26.1	27.3	-	2.87×10^7	4.87×10^7

* The results were corrected to 100% from an initial recovery of 88%

added ferric chloride and one without were incubated on the shaker incubator at 30°C. and the remaining two incubated without shaking at 30°C. 10 ml. samples were taken at intervals and the malathion estimated. The results are shown in Table 56.

The results show that shaking the flasks while incubating affects the recovery of the malathion. Over twice as much malathion was recovered from the flasks incubated without shaking than from the flasks incubated with shaking. Since no ring of sediment was formed, the increase in decomposition is probably correlated with the degree of aeration of the medium. The ferric chloride had little effect on the decomposition, the difference found after 5 days incubated with shaking could be due to experimental error, since the effect was not observed in the other flasks.

From the results obtained, it was decided to incubate the flasks without shaking in all later experiments, in order to slow down the rate of chemical decomposition.

(iii) The influence of kaolin as an adsorbent on the decomposition of malathion by soil isolates

Since the isolates obtained from soil had no effect on the decomposition of malathion in liquid culture, it was decided to add an adsorbing material to the solution which might provide conditions approximating to those found in the soil, and kaolin was used for this purpose.

Table 56. The effect of ferric chloride and method of incubation on the decomposition of malathion in aqueous solution at 30°C.

	Malathion recovered (µg./ml.) *			
	Incubated with shaking		Incubated unshaken	
Time (days)	Malathion (100ppm)	Malathion (100ppm) with ferric chloride	Malathion (100 ppm)	Malathion (100ppm) with ferric chloride
0	100	100	100	100
2	42.3	49.1	86.8	84.5
5	18.3	33.1	73.1	73.1

* The results were corrected to 100% from an average initial recovery of 87.5%.

0.1 g. kaolin was added to each of eight 50 ml. conical flasks and sterilisedby heating in an oven at 165°C. for 2 hours. Eight similar flasks were prepared without the kaolin. A solution of malathion in the salts medium was prepared at a concentration of 100 µg./ml. and filter sterilised, and then 15 ml. of the sterile solution were added aseptically to each flask. A suspension of isolate 2, which had grown better in the previous study, was made in sterile distilled water and 0.1 ml. was placed into 4 flasks containing kaolin and 4 flasks without. The remaining flasks acted as uninoculated controls. It was felt necessary to carry out the incubation in separate flasks, since if adsorption took place onto the kaolin, samples taken from a single flask would not remove a representative portion of the malathion.

The flasks were incubated at 30°C. and samples were taken at intervals to determine the malathion. 10 ml. solution were removed from each of two flasks which contained malathion/salts solution, one of which had been inoculated and the other which was sterile. The samples were diluted to 50 ml. with distilled water and extracted as usual. When sampling the flasks containing kaolin, the contents of inoculated and uninoculated flasks were transferred to separate MacCartney bottles. The kaolin was then spun down into a pellet in a centrifuge and 10 ml. from each bottle pipetted into separating funnels. The remaining supernatent was then dissolved. In order to recover any malathion adsorbed onto the kaolin, 20 ml. methanol were added to each bottle and the pellets resuspended.

The bottles were shaken to obtain an even suspension, the kaolin was spun down to a pellet once more, and the supernatents were transferred to the appropriate separating funnels. The pellet was washed once more with 20 ml. distilled water in the manner used for the methanol. The combined extracts were then shaken with carbon tetrachloride, as described in the materials and methods. 1 ml. samples were taken from the inoculated flasks in order to follow the growth of the isolates. The results are shown in Table 57.

The results show almost identical declines in the recovery of malathion in the presence and absence of kaolin, inoculated or sterile. It can only be concluded that the kaolin does not provide conditions for the decomposition of the malathion by the isolate. The growth of the isolate again indicates utilisation of the decomposition products, and was slightly better in the presence of kaolin.

(iv) The effect of a mixed flora on the decomposition of malathion in liquid culture

Since a single organism was unable to decompose the malathion, it was thought possible that a mixed flora with its interactions might be able to decompose the insecticide faster than its observed chemical breakdown.

The experiment with the kaolin was repeated using as an inoculum 0.1 ml. of a soil suspension, containing the clay and silt fractions with suspended micro-organisms, which was presumed would contain a mixed culture of soil organisms. The results are shown in Table 58.

Table 57. The influence of kaolin on the decomposition of malathion in the presence of a soil isolate.

Time (days)	Malathion recovered from salts solution with and without Kaolin, in the presence and absence of a soil isolate *					
	With Kaolin			Without Kaolin		
	Malathion recovered (µg./ml.)		No. of organisms /ml.	Malathion recovered (µg./ml.)		No. of organisms /ml.
	Sterile	Inoculated		Sterile	Inoculated	
0	100	100	2.97×10^7	100	100	2.83×10^5
2	67.0	70.5	2.57×10^7	72.0	69.5	1.55×10^7
5	34.1	36.4	3.83×10^7	36.0	36.0	1.42×10^7

* The results were corrected to 100% from an initial recovery of 88% with kaolin and 83.5% without kaolin.

Table 58. The effect of a mixture of soil micro-organisms on the decomposition of malathion in liquid culture.

Time (days)	Recovery of malathion from solutions with and without kaolin, in the presence and absence of a mixture of soil micro-organisms *							
	With Kaolin				Without Kaolin			
	Malathion recovered (µg./ml.)		Number of organisms/ml.		Malathion recovered (µg./ml.)		Number of organisms/ml.	
	Sterile	Inoculated	Sterile	Inoculated	Sterile	Inoculated	Sterile	Inoculated
0	100.0	100.0	0	1.0×10^4	100.0	100.0	0	7.0×10^3
2	05.0	66.2	27.0	1.25×10^7	71.8	63.3	0	7.5×10^6
5	30.8	37.3	7.0	1.03×10^7	37.3	35.0	3.0	1.63×10^7

* Results corrected to 100% from an initial recovery of 84.0% in the presence of kaolin and 85.0% in the absence of kaolin.

The results show that there was little effect of the micro-organisms on the decline in recovery of malathion, even in the presence of kaolin. This indicated that the experimental conditions did not permit the action of the organisms on the insecticide.

Although the mixed culture used above should have contained enough organisms so that those able to decompose malathion would be able to exert their influence, it was decided to try using a soil suspension from an enrichment culture. It was presumed that if the micro-organisms were decomposing the malathion in the soil, this fraction would increase in numbers and predominate in the soil suspension.

However, the results were almost identical to those found with the normal mixed flora, which suggested that the increase in numbers of bacteria in the soil is primarily caused by the utilisation of the degradation products of malathion.

(v) The effect of an actinomycete isolated from the soil on the decomposition of malathion

Although the results indicated that the oxidation or hydrolysis of malathion was not brought about by bacteria, it was possible that other micro-organisms were capable of these reactions. It was noticed on plating a soil suspension on Thornton's agar containing malathion, that an actinomycete growing on the agar had caused a clearing of the opaque medium around the colony, and when

replated onto fresh medium it produced the same effect. Since some actinomycetes tend to turn the medium alkaline, it was thought that chemical hydrolysis might have occurred. But drops of indicator solutions placed onto the medium showed that the clear zones had apparently the same pH as the surrounding medium. It appeared that some extracellular compound was decomposing the malathion and it was decided to see if the actinomycete was able to decompose malathion in liquid culture, using a very large inoculum.

The actinomycete was grown in nutrient broth at 30°C. in a shaker incubator for 4 days. The pellets of organism formed were harvested by spinning down in a centrifuge tube, and were washed twice with Ringer's solution, spinning down to a pellet each time and discarding the supernatent. The pellet was then resuspended in a little of the malathion/salts solution, containing 100 µg. malathion /ml., which was then transferred to a 500 ml. flask containing the bulk of the solution, giving a final volume of 50 ml. A similar flask was prepared, which was not inoculated, to act as a sterile control. The flasks were incubated at 30°C. and 10 ml. samples were taken at intervals and the malathion content estimated. Viable counts were not made, due to the formation of pellets by the organism, but the suspension was observed at the end of the experiment to determine the possible existence of contaminants. The results are shown in Table 59.

The results show that in the presence of the actinomycete, the decomposition of malathion was faster than in the uninoculated

Table 59. The effect of an actinomycete isolated from the soil on the decomposition of malathion in liquid culture.

Time (days)	Malathion recovered (µg./ml.)* from salts solution in the presence and absence of an actinomycete			
	Uninoculated Control		Actinomycete culture	
	Total	Range	Total	Range
0	100.0	-	100.0	-
1	87.0	-	70.5	-
2	70.0	±1.0	46.0	±3.0

* The results are corrected to 100% from an initial recovery of 88.3%. Duplicate samples were only made after incubation for two days.

control, indicating that the organism was able to decompose the insecticide. Since strict aseptic conditions were not followed in the harvesting of the actinomycete, the flask was contaminated, but the overwhelming density of the actinomycete in the flask must indicate that it was this organism which was decomposing the malathion.

The exact nature of the pathway of the decomposition was not investigated because the identification of the decomposition products by chromatography requires the use of the possible products in pure form to act as markers and these were not available. Alternatively it is desirable to use isotope labelled insecticide which was also unavailable.

b) The decomposition of menazon in liquid culture

(i) The decomposition by a soil isolate

The technique used to investigate the action of a soil isolate on the decomposition of menazon was similar to that used for malathion. The organism used was isolated as described fully in the materials and methods, by growing a soil suspension in menazon/salts solution with repeated subculturing. A preliminary growth curve indicated that the isolate was able to grow slowly in the menazon/salts solution.

A saturated solution of the insecticide was prepared in a salts solution, giving a concentration of 240 µg./ml. The solution

was filter sterilised and 50 ml. of the solution pipetted aseptically into a sterile 500 ml. flask. A similar flask, containing salts solution alone was also prepared to act as a control, since the medium contained phosphate which might have interfered with the colorimetric estimation. 1 ml. of a suspension of the isolate in sterile distilled water was added to each flask and they were incubated at 30°C. in a shaker incubator. The original inoculum was so small that a further addition was made after incubation for 2 days. Two 0.4 ml. samples were taken from each flask, diluted to 200 ml. with distilled water and divided into equal portions. The menazon content was determined in one portion from each sample and the menazon plus thiolate in the remaining portions. The results were corrected for the readings obtained from the salts solution, but since these were consistently negligible, it was decided to take duplicate samples of the menazon solution and samples of the control were discontinued. Standard solutions of menazon in methanol were estimated with the samples to check the calibration curve (Fig. 28), and the concentration of menazon determined from the gr ph. The concentration of thiolate was not strictly quantitative, due to the reasons given in the soil studies. The figures quoted were calculated by taking the difference between the results obtained from the 1% and 8% cross-linked resins. Any excess obtained from the 8% resin was then corrected to 100% recovery from 85%, which is the approximate recovery of the thiolate from the column.

1 ml. samples were also taken to follow the growth of the isolate, nutrient agar being used to culture the organisms. The results are shown in Table 60.

The results obtained showed conflicting evidence of the decomposition of menazon in the presence of the isolate. The figures obtained for menazon vary considerably, but after 15 days there was still approximately 94% of the original menazon in solution. However, there was an increase in the thiolate in solution, as shown by the recovery from the 8% cross-linked resin, but these results when added to those of menazon, indicated an increase in the total insecticide concentration.

The variations in the results were probably caused by inaccuracies due to the size of the sample and possibly to variations in the recovery from the columns.

However, the concentration of menazon recovered after 15 days indicated that under the conditions of the experiment, the isolate was not able to decompose the insecticide. In order to increase the population density of the isolate in solution, it was decided to add an alternative carbon source and mannitol was used for this purpose. The menazon/salts solution was prepared as before and 0.1% mannitol was added. The solution was sterilised and dispensed as before, using a single flask, from which duplicate samples were removed at intervals. The method was then identical to the previous experiment. The results are shown in Table 61.

Table 60. The effect of a soil isolate on the recovery of menazon and its thiolate from liquid culture.

Time (days)	Menazon and Thiolate recovered (µg./ml.)*						Number of organisms/ml.**	
	Menazon		Menazon plus thiolate		Thiolate		Control	Soln. plus menazon
	Total	Range	Total	Range	Total	Range		
0	240	-	240	-	0	-	$<3.0\times10^{2}$	3.0×10^{2}
2	194	-	173	-	0	-	2.6×10^{5}	5.97×10^{5}
6	246	-	404	-	101	-	6.5×10^{4}	1.88×10^{6}
9	138	-	288	-	95	-	3.3×10^{6}	2.11×10^{6}
13	197	±55.9	295	±26.4	58.5	±12.3	-	1.36×10^{6}
15	225	±22.0	386.5	±40.5	95.5	±24.3	-	7.0×10^{4}

* The results are corrected to 100% from an initial recovery of 85% of the menazon and 52% of the menazon plus thiolate.

** A second inoculation with the isolate was made after 2 days incubation.

Table 61. The effect of a soil isolate on the recovery of menazon and its thiolate from liquid culture containing mannitol.

Time (days)	Concentration recovered (µg./ml.) *						Viable organism per ml.
	Menazon		Menazon plus thiolate		Thiolate		
	Total	Range	Total	Range	Total	Range	
0	240	±3.1	240	±4.0	0	-	1.7×10^3
2	240	±6.2	206	±2.0	0	-	4.3×10^7
4	188	±29.5	224	-	26.3	-	5.3×10^7
7	216	±16.2	300	±10.0	60.2	±7.2	2.33×10^7

* The results were corrected to 100% from an initial recovery of 80.2% for the menazon alone and 62.5% for the menazon with its thiolate.

The results, although less variable, show a similar pattern as in the previous experiment. There was 90% of the original menazon remaining after incubation for 7 days, whereas the apparent concentration of thiolate formed indicated more decomposition than could be accounted for. Despite this discrepancy, it was clear that even at a level of over 10^7 organisms/ml., the isolate failed to decompose the insecticide; the thiolate formed could have been produced by chemical means.

(ii) The effect of a mixed soil population on the decomposition of menazon

Since the soil isolate was apparently unable to decompose menazon, it was decided to try the effect of a mixed culture obtained from a soil suspension. The suspension was made by shaking 1 g. soil with 100 ml. sterile distilled water and allowing the coarser particles to settle. Two flasks were prepared containing 40 ml. sterile menazon/salts solution, one of which contained 0.1% mannitol as an alternative carbon source. The solutions were prepared as previously described. To each flask 10 ml. of the soil suspension were added, to give a final concentration of menazon of 192 µg./ml. Duplicate samples were taken as before, but only the menazon was estimated. The results are shown in Table 62.

The results show that in the presence of mannitol, there was a small drop in the concentration of menazon in solution, 88% being recovered after 14 days. This rate of decomposition would not account

Table 62. The effect of a mixture of soil micro-organisms on the recovery of menazon from liquid culture, in the presence and absence of mannitol.

Time (days)	Menazon recovered (µg./ml.) *				Viable organisms/ml.	
	Soln. with mannitol		Soln. without mannitol		Soln. with mannitol	Soln. without mannitol
	Total	Range	Total	Range		
0	192.0	±9.4	192.0	±14.9	1.03×10^5	8.3×10^4
7	191.9	±4.4	242.0	±14.9	2.25×10^8	6.0×10^6
14	169.0	±7.5	236.0	± 1.2	-	-

* The results are corrected to 100% from an initial recovery of 67.0% in the presence of mannitol and 65.05% in the absence of mannitol.

for the loss of menazon from the soil. The evidence suggests that if soil micro-organisms are able to decompose menazon, then they are unable to do so under the conditions of the experiment.

However, the results obtained in the absence of mannitol after 7 and 14 days suggested that there was an increase in recovery over the initial recovery of 65.05%, which was lower than found in previous experiments. If this increase in recovery was obtained in the presence as well as the absence of mannitol, then the drop in menazon concentration measured would be greater than the figures suggest. Despite this slight error, the action of the mixed flora in liquid culture could not account for the loss of menazon from the soil, which suggests that most of the decomposition is chemical. Menazon was apparently quite stable under the conditions of the experiment, but in the soil many other factors may cause the decomposition of the insecticide, with little action by the soil microflora.

DISCUSSION

The interaction of insecticides and the soil microflora has been shown to be more complex than was, perhaps, originally thought. So many factors are involved in the study of the soil, that it is difficult to determine and interpret the results. The laboratory studies described in this thesis have been made under constant conditions, in order to eliminate external factors such as the weather, which must effect the results obtained in field experiments.

In the studies with the organophosphorus insecticides, the primary concern was that they might be toxic to the soil microflora. However, the results obtained indicate that at normal application rates the compounds will not have a detrimental effect.

This is the conclusion arrived at by many workers using both chlorinated hydrocarbon and organophosphorus insecticides. However, many results have been contradictory, due to the experimental methods employed and the variable nature of soil. Also, when the range of compounds available is considered, comparatively little work has been published of investigations carried out with organophosphorus compounds. It is not surprising, therefore, that toxic effects have not been recorded by other workers, although it has been shown in this thesis that each of the insecticides may be toxic to microorganisms under certain experimental conditions.

Malathion at 2000 ppm, or its decomposition products, were shown to reduce the viable population of actinomycetes in the soil, although Siderapoulos et al (1964) noticed no similar effect. The exact nature of the toxicity is unknown, but since the population did not decline until after incubation for some days, when most of the malathion would have been decomposed, it seems probable that it was a decomposition product which was toxic. Since the decomposition products of malathion were shown to stimulate micro-organisms in general, it might be that the toxicity was caused by metabolic products arising through the growth of other organisms. It is notable that from the actinomycetes, which are apparently susceptible to malathion or its degradation products, an isolate was obtained that could decompose the insecticide.

Malathion was also shown to be inhibitory to the growth of some Gram positive bacteria when treated on agar media and in liquid culture. This suggests that the insecticide may affect directly the metabolic processes of certain organisms, possibly by blocking esterases, as it does in insects.

There was a little evidence that menazon might also reduce the growth of micro-organisms in a similar manner to that found with malathion. In the respiration studies when the soil microflora was stimulated by urea, methanol or chloroform, the amount of increase in respiration over the control was less in the presence of menazon. This indicates that although menazon is not toxic to a static population, it might affect a section if not all of the dividing micro-organisms.

Sumithion showed similar toxic effects to Gram positive organisms as malathion, but was most notable for its toxicity to filamentous fungi and yeasts, both in the soil and in pure culture. An important feature of these studies was the identification of 3-methyl-4-nitrophenol as the compound responsible for the toxicity. It was probably due to traces of this compound in the technical sumithion which caused the toxicity, and possibly the decolourising effect, in agar media. Christensen and Daly (1951) stated that modifications to morphology and pigment are commonly produced when fungi are growing on differential media, presumably when the normal metabolism is upset, as it might be in the presence of 3-methyl-4-nitrophenol. The similarity of 3-methyl-4-nitrophenol to a common toxicant was even more marked by its dependence on the nutritional status of the medium and the size of the inoculum.

The hydrolysis product of sumithion is similar in structure to 2-methyl-4, 6-dinitrophenol (DNOC), which is a powerful toxicant and commonly used as a soil fungicide. Although it is mainly the relative positions of the two nitro-groups which confer the toxicity of DNOC, it is probable that 3-methyl-4-nitrophenol obtains some of its activity from its relationship to the fungicide.

When sumithion was added to soil, at concentrations likely to be found in practice, there was no effect on the fungi; but if the hydrolysis product was persistent, it might cause permanent damage to the fungi when in conjunction with other compounds.

It is surprising that no other investigations have been carried out on the possibility of the accumulation of toxic degradation products of organophosphorus insecticides. A brief study of the structure of many compounds indicates that it is highly probable that toxic products would be formed. Parathion, upon which the structure of sumithion was based, would release p-nitrophenol on hydrolysis. This compound might be toxic under similar conditions to those when toxicity was shown by 3-methyl-4-nitrophenol. While these products might be degraded further, some residues could remain in the soil for a considerable time.

It is probable that the negative results obtained by most workers is due to the methods employed and the level of insecticides used. It is obviously impossible to carry out detailed investigations with each insecticide produced, but more attention must be paid to facets of the problem which hitherto have been largely ignored.

No work has been done on the effect of mixtures of insecticides in the soil and yet it has been shown that they are more toxic to mammals than when used separately. (Dubois, 1961 and Frawley, 1964). The phenomenon is known as 'potentiation', and it is possible that decomposition products might interact in a similar manner.

One problem, which has received little attention due to the difficulty in assessing results, is the possibility that insecticides might have mutagenic or carcinogenic effects. Carson (1965) and Nelson (1965), on discussing this subject, indicated how little is

known about the direct or indirect influences of compounds on the living cell, although they may appear to be harmless.

A more immediate concern is the possible effect of the formulation of insecticides on the action of compounds on the soil. The formulations are designed to increase persistence, and often toxicity, and yet insecticides are rarely studied in the form in which they are most commonly used. Lichtenstein (1966) has shown that emulsifying agents increased the persistence of parathion and diazinon. He believes that the persistence was increased by allowing closer contact of the insecticides with the soil particles, and presumably acts in a similar manner to adsorption. Unfortunately, any factor which increased the persistence of the insecticides also increases the potential danger of the compounds, as was found with the chlorinated hydrocarbon insecticides.

Although it is possible to speculate on the factors which might cause the organophosphorus insecticides to become toxic to the soil microflora, the evidence obtained has shown that it is only under extreme circumstances that harmful effects will be produced. In fact, it is considered that the action of the microflora on insecticides will aid in ridding the soil of residues of these compounds.

There is evidence that the soil microflora is able to decompose organophosphorus insecticides, but the majority is rather circumstantial. As was found with the three compounds used in the work presented above, when added to a variety of soils, stimulation

of respiration occurs and this was associated with an increase in population in the presence of malathion and sumithion. This stimulation was proportional to the concentration of the insecticides, which indicated that the compounds were being used as substrates for metabolism and growth. This phenomenon has been recorded for many compounds and suggests microbial degradation of the insecticides. However, as was found in the case of malathion, and as is probably true for other compounds, the degradation is basically chemical, with the soil microflora utilising the products of decomposition. It is believed that the soil can act as a catalyst for the degradation of relatively ustable compounds, and ferric ions amongst others are thought to be active in this respect. Also, the pH of soil must affect the persistence of organophosphorus insecticides, since many are hydrolysed in mildly acid or alkaline solutions.

The degree of stimulation caused by the three insecticides was also associated with the persistence of compounds. Malathion, which stimulated the microflora more that sumithion or menazon, was also the least persistence of the compounds. Malathion almost completely disappeared from Chelsea loam in two days after application of 175 ppm, and at a similar rate when applied at 1250 ppm, as determined by bioassay. Lichtenstein and Schulz (1964) also found that it disappeared rapidly. It was not surprising therefore, that Roberts et al (1962) found no residues of malathion at the end of the third year, after three annual applications of malathion emulsion, giving a total application of 108.6 lb. a.i./acre (54.3 ppm).

Sumithion is more persistent in soil than malathion, and this is probably due to its greater chemical stability. The figures published by the World Health Organisation of the persistence of sumithion vary considerably in different soils, but are probably not very different to those of the persistence on the surface of fruit and vegetables. This is strange since the evidence obtained indicated that microbial hydrolysis of sumithion was occuring in Chelsea loam. However, the method devised to estimate the sumithion indicated a discrepancy between the disappeareance of the insecticide and the accumulation of 3-methyl-4-nitrophenol. This could mean that the hydrolysis product was being decomposed or that the insecticide was unrecoverable from the soil, as was found with menazon.

From the short persistence of menazon, as determined colorimetrically, one might expect it to be decomposed by micro-organisms. However, the studies with menazon raised a fundamental problem of actually measuring the compound. Estimations by I.C.I. Plant Protection Ltd. indicate that it persists in various soils for 3 - 6 weeks. In the Chelsea loam the persistence measured was even less with a half life of approximately 2 days. However, menazon was designed to give protection for a whole season when applied as a seed dressing and Way and Scopes (1965) found that it was active 18 months after application when estimated with a bioassay.

The experiments with ^{14}C-labelled menazon seem to indicate that the estimations based on the bioassay technique might be more reliable that the colorimetric estimation. Most methods of estimating

insecticide residues in soils require that they are first extracted with a solvent, whereas bioassay techniques can be carried out directly on the soil. If, as was found with menazon, the insecticide is not completely recovered in any form then the assay of soil extracts must give a false estimation of the persistence. However, it is possible that in a bound state, the toxicity to insects would also be reduced thereby rendering the bioassay erroneous (Harris,1964).

Because ^{14}C-labelled menazon is unrecoverable after incubation by normal techniques, and since it is impossible to determine in what form the activity is present, a concise estimation of its persistence is impossible.

Menazon is not only adsorbed into soil, as was shown, but because it contains two amine groups on the triazine ring, it has basic properties. These groups enable it to be separated from contaminating material on an ion-exchange column, but may hold it firmly in the soil by chemical combination. Even on hydrolysis of labelled menazon, the ^{14}C-atom would remain in the triazine ring, which would still retain its basic properties. This could account for the binding of the degradation products of menazon as shown in the autoradiograms.

The binding of menazon and its products in soil could also explain why the soil microflora were apparently unable to decompose it and utilise the products. It is believed that adsorbed insecticides persist longer in soil, but bacteria are also supposed to be bound to

clay particles. This should bring them into close proximity and enable the organisms to degrade the compounds, unless the bacteria are unable to remove the compounds from the bound state.

The insecticides adsorbed or bound chemically in soil are protected from volatilisation or leaching, which must increase the persistence. Bowman et al (1965) showed that soils with a high organic matter content increased the persistence due to adsorption, and Harris (1964) found that the moisture content governed not only the strength but the site of adsorption.

The failure to recover insecticides from soil has been observed with other insecticides and may be more common than is realised. Unless the recovery of an insecticide or its products has been checked using an isotope-labelled compound, it cannot be assumed that the persistence as measured by other methods is correct. A sharp distinction must be drawn between failure to recover an insecticide, and the actual persistence.

This distinction must be born in mind when evaluating the results obtained by the most common method of determining the role played by micro-organisms in the decomposition of insecticides. It is considered that if an insecticide is more persistent in sterile soil, then micro-organisms must be involved in the degradation in normal soil.

This is a logical approach, but little concern is shown about the effect of sterilisation on the chemical nature of the soil. When soil is sterilised, Wakesman (1931) reported that the colloidal structure of soil is changed and therefore the adsorption properties of the soil must alter. Since sterilisation does not completely stop degradation of insecticides, one cannot conclude that an increase in persistence is due to the reduction in the viable population of micro-organisms alone. The results obtained with menazon show that in "partially sterile" soil, the persistence was longer, but since in normal soil adsorption phenomena were reducing the amount of menazon measured, it is possible that in the partially sterile soil the effect was caused by a greater recovery of the insecticide.

In experiments with malathion it was concluded that the water content of the sterile soil had as much influence on increasing the persistence as the reduction in numbers of micro-organisms, i.e. it was shown that malathion was more stable in dry soil than aqueous solution. Also, as other workers have shown, some insecticides including sumithion decompose at a similar rate in normal and sterile soil, when the water content is the same.

Hall and Yin Pei-Sun (1965) solved the problem in the soil by adding microbial inhibitors and showed that the persistence of bidrin was increased. However, it is not known what effect the addition of the chemicals had on the chemical and physical properties

of the soil, which might affect the stability of bidrin. It is possible that ions capable of catalysing the decomposition of insecticides were inactivated in the presence of the inhibitors.

Ultimately the only proof of microbial degradation is to study soil isolates in pure culture, and even this will only indicate whether the decomposition is possible rather than if it actually takes place in the soil.

This aspect has largely been ignored, since the mechanism of degradation is of little importance to agriculturalists, and the techniques of isolation of insecticide-decomposing micro-organisms and their study present difficulties. The method which yielded the malathion-decomposing actinomycete should, however, enable the isolation of organisms capable of decomposing other insecticides.

Results presented in this thesis indicate that a probable first stage in the degradation of organophosphorus insecticides is hydrolysis. The hydrolysis is probably preceded by oxidation of the insecticide to the oxygen-analogue, which is generally the more toxic product. In mammals and plants it is this compound which is more easily hydrolysed. Micro-organisms contain esterases and it should be possible for them to carry out this reaction.

A product of hydrolysis which is common to all organophosphorus insecticides is either diethyl of dimethyl phosphorothiolate. It is probable that the rapid stimulation of respiration

of the soil microflora when an organophosphorus compound is applied to soil is due to the mineralisation of organic phosphate released on hydrolysis, with traces found as contaminating material. This was also the conclusion of Verona and Picci (1953).

Little evidence was obtained that the phosphate-ester link of malathion is hydrolysed, although the actinomycete isolated from the soil was clearly able to decompose the insecticide. The fact that malathion decomposed faster when incubated with shaking indicates that chemical oxidation is the first step in chemical degradation, and then hydrolysis would follow.

On the hydrolysis of malathion, the other product formed with the organic phosphate is diethyl succinate, and this could account for the stimulation of respiration and growth observed.

Sumithion was shown to be hydrolysed in the soil, and it was concluded that this was mainly microbiological. Unlike the hydrolysis products of malathion, 3-methyl-4-nitrophenol cannot readily be utilised by micro-organisms, which is confirmed by its accumulation in the soil. However, Simpson and Evans (1953) showed that a species of Pseudomonas was able to utilise o-nitrophenol and p-nitrophenol, which was confirmed by Lichtenstein and Schulz (1964), who showed that the p-nitrophenol released on the hydrolysis of parathion was decomposed. This suggests that the hydrolysis products of sumithion would also be decomposed further.

Little evidence was obtained that menazon could be decomposed by micro-organisms even by mixed cultures. Respiration studies and population studies indicated that menazon was resistant to degradation and it was surprising, therefore, to discover how many decomposition products were formed in the soil from the ^{14}C-labelled compound. It seems probable that some of these products were the result of microbiological action and a Rhizobium sp. has been obtained by Plant Protection Ltd., which can apparently decompose menazon in liquid culture. In this experiment and in the work of Ahmed and Casida (1958) the organisms were unable to utilise the insecticides as the sole-carbon source. It was found that bacteria mainly hydrolysed the compounds but that a yeast and an alga oxidised Thimet to the sulphone and sulphoxide. Unfortunately uninoculated controls were not used and therefore the oxidation and hydrolysis might have been due to chemical means as was found with malathion.

Des-methyl menazon was formed in the presence of the Rhizobium which suggested that the organism was able to dealkylate menazon. This reaction is commonly found in the metabolism of organophophorus insecticides by both plants and animals and is a possible pathway in the decomposition by micro-organisms.

The evidence which has accumulated in the past few years indicates that although dangers exist from the indiscriminate use of organophosphorus insecticides, these compounds are preferable to the chlorinated hydrocarbon insecticides in that problems caused by long

persistence are not involved. Those compounds which do not decompose chemically in soil will probably be hydrolysed by micro-organisms and the products broken down further. The dangers described previously, however, cannot be ignored and research into all facets of the problem must be continued.

SUMMARY

1. An apparatus was developed to measure the respiration of soil and optimum conditions for respiration were determined. These were found to be a temperature of 30°C. and a moisture content of 50% of the maximum water holding capacity.

2. Malathion added to soil at 50 - 2000 ppm increased respiration and this was proportional to concentration. Sumithion at 50 - 10000 ppm was similar in effect to malathion, but the increase was of a lower order. Menazon at 20 - 10000 ppm produced only a small increase and only at the highest concentration.

3. Bentonite when used as a carrier for malathion depressed the respiration of soil in the presence and absence of the insecticide, by an unknown mechanism. Menazon was added to the bentonite as a solution, but residues of the two solvents used stimulated the respiration of soil, although this was reduced in the presence of menazon. Malathion, added to soil as an emulsion in 2% Triton X-100, apparently increased the ease with which the insecticide was decomposed, but the emulsifying agent also stimulated the microflora which may have masked the true effect.

4. Urea at 0.1% and 1.0% added with menazon to soil did not increase the ability of the microflora to decompose the insecticide. Menazon appeared to inhibit the growth of urea-decomposing organisms.

5. Soil type had little influence on the effect of the three insecticides, in respiration studies. However, menazon in peat soil gave a false stimulation due to decomposition of the insecticide with the evolution of hydrogen sulphide.

6. The chlorinated hydrocarbon insecticides investigated had little effect on soil respiration.

7. Malathion and sumithion increased the number of viable micro-organisms in soil at various concentrations, but menazon had no effect at 10000 ppm in Chelsea loam or peat soil.

8. Malathion added to Chelsea loam stimulated the growth of Gram positive and negative bacteria, whereas sumithion stimulated only Gram negative bacteria. Menazon had no observable effect.

9. The organophosphorus insecticides had no effect on the viable population of anaerobic bacteria in Chelsea loam, incubated aerobically.

10. Sumithion and menazon had little effect on the number of viable actinomycetes in Chelsea loam, but malathion significantly reduced the population.

11. Malathion and menazon had little effect on the viable fungi in Chelsea loam, but sumithion caused a significant reduction in numbers. This occurred at 10000 ppm but not at 5 ppm.

12. 3-methyl-4-nitrophenol at a concentration equivalent to 10000 ppm sumithion caused a similar reduction of the filamentous fungi, but had no effect on the general population.

13. Sumithion also reduced the number of non-filamentous yeasts in the soil, but malathion and menazon had no effect.

14. Malathion-impregnated paper discs, when added to seeded agar plates, caused zones of inhibition of three Gram positive bacteria and sumithion inhibited one. Menazon as the solid had no effect.

15. Malathion at 100 ppm slowed the growth rate of Gram positive bacteria in liquid culture as determined by nephelometry.

16. Sumithion reduced the growth of various fungi on agar, but only two fungi were completely inhibited. 3-methyl-4-nitrophenol was more toxic than sumithion to the growth of fungi and also more inhibitory to the germination of fungal spores.

17. Fungi growing in the presence of sumithion often changed their pigmentation, which was partly the result of less sporulation.

18. Sumithion and to a greater extent 3-methyl-4-nitrophenol were toxic to the growth of yeasts on Thornton's agar and malt agar. A species of Rhodotorula produced less pigment when growing in the presence of sumithion.

19. Colorimetric estimation showed that malathion disappears rapidly from Chelsea loam, only 2.2% of the 175 ppm applied being recovered after 2 days. Sterile soil increased the persistence, 82% of the 260 ppm applied being recovered after 6 days.

20. Mosquito bioassay of malathion indicated a similar rate of decline of 1250 ppm in Chelsea loam as determined colorimetrically.

21. Drosophila melanogaster Meig. was found to be impracticable for the bioassay of malathion.

22. Sumithion was estimated using its absorption of ultraviolet light. The technique was developed and it was estimated that sumithion persisted longer than malathion, 14.2% of the 423 ppm applied being recovered after 16 days. In sterile Chelsea loam the persistence was similar, but little 3-methyl-4-nitrophenol accumulated, whereas in normal soil the equivalent of 205 µg. sumithion was found after 16 days.

23. Only hydrolysis products of sumithion were formed in soil, although it was found to be stable in a 50% aqueous solution of methanol.

24. The use of spring-tails (Folsomia candida Willem.) was found to be impracticable for the bioassay of sumithion.

25. After incubation for 9 days, it was found difficult to recover any menazon from soil after application of 8.85 ppm.

26. A second addition of menazon to soil disappeared at the same rate as the first application.

27. Menazon persisted longer in a sterile soil and a synthetic soil than in normal soil. Incubating the soil in an atmosphere of hydrogen also increased the persistence.

28. The oxygen-analogue of menazon did not accumulate in soil, as measured by a colorimetric technique.

29. Menazon was adsorbed onto soil particles, but was recovered by extraction with water and methanol.

30. Only 5% of the radioactivity, as ^{14}C-menazon and/or its degradation products, were recovered from soil after incubation for 14 days.

31. The unrecovered radioactivity was found to be in the soil, and could not be extracted by the usual procedures.

32. An aphid bioassay was not able to determine if the bound activity was in the form of menazon.

33. A mosquito larvae bioassay was found to be impracticable for menazon, and an aphid assay, although estimating the menazon recovered, did not measure the persistence of the insecticide in soil.

34. Isolation of micro-organisms capable of decomposing insecticides on agar and silica gel media was found to be impracticable.

35. Isolates from the soil were found to grow best in liquid culture with malathion as the sole carbon source. There was little growth of isolates in the presence of sumithion or menazon.

36. The decomposition of malathion in liquid culture was found to be chemical, an isolate having no effect.

37. The decomposition of malathion was increased by shaking the cultures, but ferric chloride had no effect.

38. Kaolin as an adsorbent did not increase the ability of a pure or mixed soil culture to decompose malathion.

39. An actinomycete was isolated which was able to decompose malathion in liquid culture.

40. A soil isolate was unable to utilise menazon in liquid culture or to decompose it, even in the presence of mannitol.

41. A mixed soil population was also unable to decompose menazon in liquid culture, in the presence of mannitol, at a rate sufficient to account for the loss from soil.

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APPENDIX

Common or other name used	Chemical names or definitions	Other names
Aldrin	1,2,3,4,10,10-hexachloro-1:4,5:8-diendo-methano-1,4,4a,5,8,8a-hexahydro-naphthalene	
BHC	mixture of the isomers of 1,2,3,4,5,6-hexachlorocyclohexane	HCH
Bidrin	3-hydroxy-N,N-dimethyl-cis-croton-amide dimethyl phosphate	
Chlordane	1,2,4,5,6,7,8,8-octachloro-4,7-methano-3a,4,7,7a-tetrahydroindane	heptachlor + hexachlor
Chlorthion	O,O-dimethyl O - 3-chloro-4-nitrophenyl phosphorothioate	
Delnav	2,3-p-dioxane S ,S-bis(O,O-diethyl phosphorodithioate)	dioxathion
DDT	1,1-di(p-chlorophenyl)2,2,2-trichloro-ethane	
Diazinon	O,O-diethyl O-2-isopropyl-4-methyl-6-pyrimidinyl phosphorothioate	
Dibrom	dimethyl 1,2-dibromo-2,2-dichloroethyl phosphate	naled
Dieldrin	1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1:4,5:8-dimethanonaphthalene	
Dimefox	bis(dimethylamino)fluorophosphine oxide	Hanane
Dimethoate	O,O-dimethyl S-methylcarbamoylmethyl phosphorodithioate	Rogor
Endrin	1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-exo-1,4-exo-5,8-dimethanonaphthalene	
Guthion	O,O-dimethyl S-(4-oxo-benzotrazino-3-methyl) phosphorodithioate	azinphos-methyl

Common or other name used	Chemical names or definitions	Other names
Heptachlor	1 or 3a,4,5,6,7,8,8-heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoindane	
Imidan	0,0-dimethyl S-phthalimidomethyl phosphorodithioate	
Lindane	gamma -isomer of BHC	
Malathion	0,0-dimethyl S-(1,2-di(ethoxycarbonyl) ethyl) phosphorodithioate	
Menazon	0,0-dimethyl S-(4,6-diamino-1,3,5-triazin-2-yl)-methyl phosphorodithioate	
Methoxychlor	2,2-bis(p-methoxy-phenyl)-1,1,1-trichloro-ethane	
OMPA	Octamethyl pyrophosphoramide	Schradan, Pestox III
Parathion	0,0-diethyl O-p-nitrophenyl phosphoro-thioate	
Phosdrin	dimethyl 2-methoxycarbonyl-1-methylvinyl phosphate	mevinphos
Sevin	1-naphthyl N-methylcarbamate	carbaryl
Sumithion	0,0-dimethyl-O-(3-methyl-4-nitrophenyl) phosphorothioate	folithion, fenitrothion
Systox	mixture of 0,0-diethyl S-2-(ethylthio) ethyl phosphorothioate and 0,0-diethyl O-2-(ethylthio) ethyl phosphorothioate	Demeton
TDE	1,1-di(p-chlorophenyl)-2,2-dichloroethane	DDD, Rhothane
Thimet	0,0-diethyl S-ethylthiomethyl phosphoro-dithioate	Phorate
Thiodan	6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexa-hydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide	endosulphan
Thionazin	0,0-diethyl O-2-pyrazinyl phosphorothioate	Zinophos

Common or other name used	Chemical names or definitions	Other names
Toxaphene	Chlorinated camphene having a chlorine content of 67-69%	
Trithion	O,O-diethyl S-p-chlorophenylthiomethyl phosphorodithioate	carbophenothion