BACTERIAL DEGRADATION OF THE ACARICIDE AMITRAZ

by

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ABSTRACT

This thesis describes dip tank field trials and laboratory investigations on the acaricide Amitraz. Amitraz is a triazapenta-diene compound which is relatively unstable in fouled dip washes. The field trials were conducted on the farm Sea View according to the "Total Replacement Method" and on the farm Sea Ways according to the "Lime Stabilization Method" of dipping. The results of these trials showed that Amitraz was stable in clean dip washes, and under conditions of high pH resulting from the addition of slaked lime to the dip wash. Using mixed bacterial populations optimum conditions for degradation of Amitraz in the laboratory were determined. Bacterial cultures degraded Amitraz most efficiently in media supplemented with yeast extract or with a high content of sterile cattle faeces. Amitraz concentrations were determined by gas chromatography. A culture, efficient at degrading Amitraz, was enriched from a dip tank sludge inoculum. From this culture ten bacterial isolates were identified; nine of these were of the genus *Pseudomonas* and one was an *Achromobacter* sp. Experiments with both mixed and pure cultures demonstrated that

bacterial degradation of Amitraz was by the process of co-metabolism. The existence of four degradation products was shown using thin layer chromatography. Tentative identification of two of the products was made.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

CHAPTER I

GENERAL INTRODUCTION

1.1 Chemical control of cattle ticks

Unless otherwise indicated the information in this discussion related to the chemical control of cattle ticks is taken from comprehensive review articles by Wharton and Roulston (1970) and Harrison et al. (1973).

Most countries between latitudes 35°N and 35°S support one or more species of cattle tick, but it is in the important cattle – rearing areas of South Africa, Australia and South America that cattle ticks have become a problem of major economic significance. Ticks have a four-fold effect on their bovine hosts: (a) They are voracious blood feeders — it is estimated that a female tick requires 1 to 3 ml of host blood to complete its life cycle. These blood sucking activities can cause anaemia. (b) The hides of cattle may be damaged by the puncture wounds made by the mouth parts of the tick. Localized tissue reactions may occur resulting in irritation which causes the animal to lick and scratch the wound. Often such wounds become septic and may also

become fly blown. (c) Ticks transmit certain protozoal diseases, e.g., Babesiosis, Anaplasmosis and Theileriosis, and rickettsial diseases, e.g., Heartwater. To non-immunized stock these diseases may prove fatal. (d) Ticks can inject toxins via their saliva, e.g., in the case of *Hyalomma* spp., the toxin produces sweating sickness and in the case of *Ixodes* spp. it can cause paralysis. In view of these effects, the control of ticks on cattle has long been a matter of prime importance to stock owners over most of the intensive natural grazing areas in the Southern Hemisphere.

It has been found that the only practicable method of dealing with the cattle tick problem in the short term is by treating the infected bovine host with acaricidal compounds, i.e., by chemical control. Acaricides[a] are generally applied either by plunging cattle into a dip tank containing aqueous suspensions or emulsions of the acaricide or by spraying them with dip suspensions in a spray race.

Effective chemical control of ticks began in 1893 when arsenical solutions containing the equivalent of 0,16% As_2O_3 were introduced as cattle dips in South

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[a] Commonly used synonyms for acaricide are insecticide, tickicide and ixodicide.

Africa. These dips gave effective control of ticks until 1941 when a strain of *Boophilus decoloratus* resistant to sodium arsenite was found. The number of resistant strains increased rapidly and they soon became widespread throughout the cattle-rearing areas of the eastern coast of South Africa. In Australia and in several South American countries, where arsenical dips were used, ticks also developed resistance to arsenite after its continuous use for several decades.

In 1949 0,005% gamma-benzene hexachloride (γ-BHC or Lindane) was used effectively against arsenite resistant strains of *B. decoloratus* in South Africa. However, after only 18 months of use γ-BHC-resistant tick populations were found in those areas where there had originally been arsenite-resistant populations. These populations also showed cross-resistance to other chlorinated insecticides, e.g., Camphechlor, Chlordane, Dieldrin and Aldrin, even though Dieldrin and Aldrin had not been used to control ticks in the field. Similar results regarding γ-BHC resistance were found in Australia and South America.

In South Africa, DDT preparations were introduced as a substitute for γ-BHC, but after approximately 5 years ticks resistant to DDT were reported. These ticks were also resistant to arsenite and γ-BHC.

The next acaricides to be developed were the organophosphorus compounds, e.g., Dioxathion, Diazinon and Ethion. These compounds were available in Australia in 1962 at the time when DDT and other chlorinated hydrocarbons were banned for use on cattle. However, in 1963 a strain of *Boophilus microplus* (called the Ridgelands strain) was shown to be resistant to all organophosphorus acaricides in common use and also to carbamate compounds. Since 1966 four more organophosphorus-resistant *B. microplus* strains have been found in eastern Queensland. These strains are the Biarra, the Mackay, the Gracemere and the Mount Alford strains. All are resistant to some organophosphorus and carbamate acaricides, but they show different resistance spectra. Resistance to organophosphorus compounds in South Africa developed more slowly; the first report of an organophosphorus-resistant tick was a strain of *B. decoloratus* described by Shaw et al. (1967). Resistance to organophosphorus compounds has now become a matter of great concern in intensive cattle-rearing areas of South Africa. Organophosphorus-resistant strains of *B. microplus* in Venezuela and Brazil were reported by Shaw et al. (1968).

Other methods of tick control have been directed against the free-living stages of the tick. Burning-off

of grassland and "pasture-spelling" (removal of bovine hosts in attempts to starve the free-living stages) have not proved practicable or successful. Tick eradication campaigns have been attempted with varying degrees of success. In the southern United States of America a campaign against *Boophilus annulatus* using arsenic dips was mounted in 1911 and by 1940 eradication had been achieved. An unsuccessful campaign using DDT against *B. microplus* was mounted in New South Wales, Australia in 1956. In Argentina a campaign against *B. microplus* was started in 1938 and still continues. As a result of this campaign approximately 20 million hectares are tick-free but there are a further 70 million hectares still infested.

The need for a different chemical type of acaricide to control cattle ticks is therefore obvious. A new compound, 1,5-di-(2,4-dimethylphenyl)-3-methyl-1,3,5-triazapenta-1,4-diene (also known by its IUPAC name: 1,3-di-(2,4-dimethylphenylimine)-2-methyl-2-azapropane and its common name: Amitraz), was developed by the Boots Company Ltd., Nottingham, England, and found to show high acaricidal activity. Palmer et al. (1971) performed laboratory and calf experiments to demonstrate the activity of Amitraz against 3 strains of *B.microplus*, viz.

the Biarra strain, the Mackay strain and the Yeerongpilly strain, which is an organophosphorus-sensitive strain. Amitraz was found to be very effective against these strains. Harrison et al. (1972) reported that, in laboratory tests, Amitraz was highly active against a variety of commercially important cattle tick species, e.g., *B.decoloratus* (the blue tick), *Rhipicephalus appendiculatus* (the brown ear tick) and *Amblyomma hebraeum* (the bont tick).

Excellent results were obtained with Amitraz in field trials in which tick infested cattle were hand sprayed (Baker et al., 1973). Among the useful acaricidal features of Amitraz were the following:

(a) It gives good tick control in the field within the concentration range 0,001% to 0,05%.

(b) It has a significant ovicidal effect on gravid females which is of value in reducing the tick infestation on farms.

(c) It rapidly expels attached ticks. This reduces the chances of disease transmission and promotes faster healing of tick damage.

(d) It has a partial systemic effect and thereby overcomes problems caused by poor wetting, e.g., of the

ears of the cattle, which may occur during conventional dipping or spraying procedures.

In addition to these properties Amitraz has extremely low mammalian toxicity.

In August 1972 personnel from Kwanyanga Research Station began a dip tank field trial with Amitraz on the farm Wiltonside, near East London. The methods used in this trial were similar to those described in Chapter II. Since Wiltonside dip tank was managed according to the Coopers "Total Replacement Method" (see Section 2,1), 3,15 kg of Amitraz (formulated as a wettable powder) was added before dipping each week. (3,15 kg in the volume of the dip tank gave a concentration of 0,005% active ingredient.)

Since Amitraz was added every week it was expected that the concentration of active ingredient in the tank would rise. Fig. 1 shows that this happened for the first 8 weeks of the trial. However a rapid and unexpected loss of Amitraz activity occurred after week 10. From week 14 to week 26 the Amitraz concentrations determined in samples taken at the end of dipping remained at 0,005% even though 0,005% active ingredient was added each week. The level of fouling (see Section 2,23) in the tank rose from 0,8% at week 1 to 2,4% at week 14 and

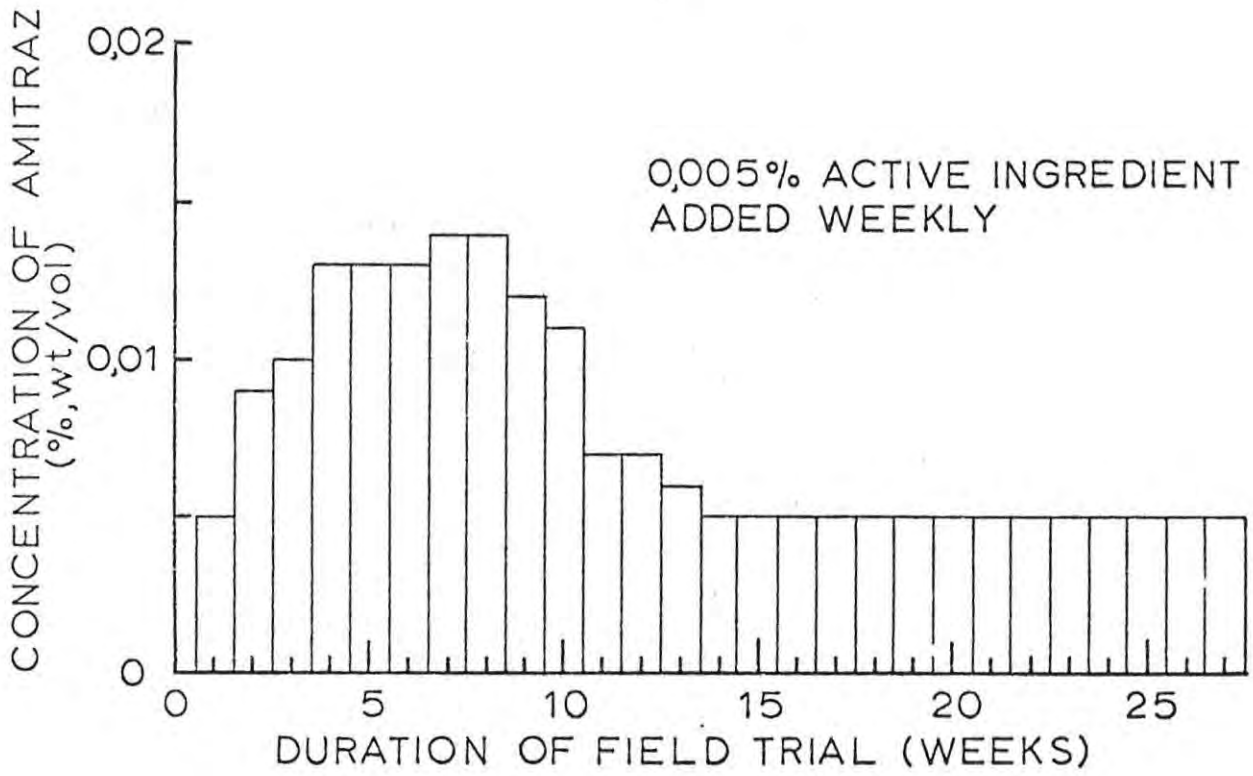


Fig.1. Weekly Amitraz concentrations during the field trial at Wiltonside dip tank.

reached 4,2% by week 26. Laboratory studies of artificially fouled washes (Matthewson and Child, 1971) showed that Amitraz was relatively stable in 0,5% fouled washes, but it was highly unstable in washes fouled to 3,0% and 6,0%. In 4 other field trials conducted by personnel from Kwanyanga Research Station the unexpected loss of Amitraz, first observed in Wiltonside, occurred. In each case this was linked to increasing fouling in the tank. In laboratory experiments on artificially fouled washes Griffiths and Palmer (1972) found that bacteria present in cattle faeces were involved in Amitraz degradation. These results indicate microbial involvement in the sudden loss of Amitraz experienced at Wiltonside.

1.2 Microbial transformation of pesticides

Biological transformation of pesticides in a soil, freshwater or estuarine ecosystem appears to be caused chiefly by bacteria, actinomycetes and fungi. If a pesticide is exposed to a microbial species the organism can transform or inactivate it by the following four mechanisms: (a) the pesticide can serve as a growth and energy substrate; (b) the entire pesticide molecule or one of its intermediates can be conjugated with naturally

occurring compounds; (c) the pesticide can accumulate within the organism; and (d) the microorganism can transform the pesticide but cannot derive energy for growth from it, i.e., the pesticide can be co-metabolized (Bollag, 1974). During exposure of a pesticide to a single organism or to a whole microflora under natural conditions it may be transformed by one or a combination of the above mechanisms.

(a) Pesticide as growth substrate

If a microorganism can use a pesticide as its sole carbon source, it decomposes the pesticide into compounds that can be channelled into known oxidative cycles, e.g., the tricarboxylic acid cycle, and thus it derives the energy necessary for growth. The experimental approach to determining biodegradability by this mechanism entails the elective culture method (Payne et al., 1970). After isolating the enriched organisms pure culture studies on pesticide degradation follow. By this method microbial species which can utilize pesticides as sole carbon and energy sources have been isolated (Sethunathan, 1972a; Wong and Kaiser, 1975). In certain cases a pesticide can be degraded only in the presence of two microbial species (Gunner and Zuckerman, 1968).

(b) Formation of conjugates

Conjugation reactions are syntheses whereby a pesticide, or any of its metabolites, is combined with naturally occurring compounds, e.g., amino acids and carbohydrates. Although conjugations of pesticides are common reactions in higher organisms, they have been reported to a much lesser extent in microorganisms (Bollag, 1974). Conjugation reactions imply only temporary removal of a toxic compound from the environment.

(c) Microbial accumulation of pesticides

Ko and Lockwood (1968a) found that if soils containing Dieldrin, DDT and PCNB were inoculated with mycelia of actinomycetes and fungi the compounds were accumulated at levels above ambient concentrations. Voerman and Tammes (1969) showed that boiled cells of *Saccharomyces cerevisiae* had a greater capacity to adsorb Lindane and Dieldrin than did untreated cells. Since both live and boiled cells showed uptake of pesticides a metabolic factor is probably not involved in the accumulation of pesticides. Accumulation of pesticides by microorganisms results in only temporary removal of the toxic compounds from the environment.

(d) Co-metabolism

The phenomenon that a microorganism can transform a chemical without deriving energy to support its growth is a relatively recent observation. Leadbetter and Foster (1959) found that, when growing at the expense of methane, a culture of *Pseudomonas methanica* (an obligate methane utilizing organism) converted ethane to acetic acid and acetaldehyde, propane to propionic acid and acetone and *n*-butane to *n*-butyric acid and 2-butanone. No volatile organic products were detected in methane cultures unsupplemented with a second hydrocarbon. Davis and Raymond (1961) observed a similar phenomenon when a *Nocardia* sp. was found to produce cumic acid from *p*-cymene when growing on *n*-alkanes. Similarly Raymond et al. (1967) reported oxidation of *p*-xylene to *p*-toluic acid when *Nocardia corallina* A-6 was grown on *n*-hexadecane (*p*-xylene alone would not support the growth of this bacterium). To describe this process Foster (1962) suggested the term "co-oxidation". Jensen (1963) proposed the more general term "co-metabolism" to include the dehalogenation reactions of pesticides by bacteria even though they are unable to use these halogen-substituted compounds as carbon sources. Ruiz-Herrera and Starkey (1969) found that soil fungi, which lacked

the ability to grow at the expense of methionine, would deaminate and demethiolate this amino acid in the presence of growth-supporting organic compounds, e.g., glucose. With replacement cultures, using pre-grown mycelium, methionine was deaminated in the absence of glucose, but a source of energy was needed for demethiolation. This process was designated "co-dissimilation". The terms "co-oxidation", "co-metabolism" and "co-dissimilation" are used to express the same concept; however it appears that co-metabolism is the more general term (Bollag, 1974) and it will be used in this thesis.

Many microbial transformations of pesticides that could not be understood because the microbes derived neither energy nor compounds essential for growth from the transformation are interpretable by co-metabolism. Horvath (1972a) points out that care must be taken not to overlook the occurrence of co-metabolic transformations. He cites several examples where authors failed to consider co-metabolism as the mechanism by which pesticides are degraded. Co-metabolic degradation of a pesticide is indicated if the concentration of pesticide decreases in the presence of microorganisms but not in their absence, and if no microorganism capable of utilizing the

pesticide as a sole carbon and energy source can be isolated.

Horvath (1972a) compiled a list of 16 genera of microorganisms known to be widespread in natural ecosystems, in which a co-metabolic type of metabolism has been demonstrated in the laboratory. He also lists 22 organic compounds subject to co-metabolic attack and the products which accumulate during co-metabolism of these compounds. Co-metabolism of a pesticide molecule does not generally result in complete mineralization of the compound, but many compounds which were described as recalcitrant (Alexander, 1965) have been shown to be degraded co-metabolically in laboratory experiments. In addition, it has been demonstrated that considerable degradation of certain pesticides occurs by co-metabolic attack by more than one microorganism. Horvath (1971a, b) showed that the herbicides 2,4,5-trichlorophenoxyacetate (a molecule regarded as recalcitrant because the elective culture method did not result in an isolate capable of growing at the expense of 2,4,5-T (Alexander, 1964)) and 2,3,6-trichlorobenzoate (2,3,6-TBA) were degraded by a co-metabolic oxidation to 3,5 - dichlorocatechol by a *Brevibacterium* sp. This chlorocatechol was found to be a product of degradation of 2,4-dichlorophenoxyacetic

acid by an *Arthrobacter* (Loos et al., 1967) and to be completely metabolized by this bacterium. In addition, Horvath (1970) isolated an *Achromobacter* sp. which co-metabolized the 3,5-dichlorocatechol to 3,5-dichloro-2-hydroxymuconic semialdehyde.

The pesticide DDT, which is highly persistent in soil, lost the trichloromethyl moeity by co-metabolic degradation by *Aerobacter aerogenes* (Wedemeyer, 1967a,b). One of the degradation products was *p,p'*-dichlorodiphenylmethane (DDM). Focht and Alexander (1971) isolated a *Hydrogenomonas* sp. from sewage effluent which co-metabolized DDM to *p*-chlorophenylacetic acid; this compound was then co-metabolized through fission of the benzene ring. Although the *Hydrogenomonas* cleaved both benzene rings of DDM it was unable to dehalogenate both ring fission products. The chlorinated fragment formed from DDM degradation was probably a 3-chloro-substituted acid. Focht (1972) reported the isolation of a fungus of the family *Moniliaceae* which was capable of growing on these chlorinated bacterial degradation products and thus converting them to H_2O, CO_2 and HCl.

The above results demonstrate that it is likely that pesticides may be completely degraded in mixed culture by the action of more than one microbial species,

by a series of co-metabolic reactions or by the processes of co-metabolism and metabolism combined.

Horvath (1972b), using the technique of analogue enrichment, found that the addition of a biodegradable analogue of 2,3,6-TBA (benzoic acid) to unsterile lake water contaminated with 2,3,6-TBA increased both the rate of degradation and the total degradation of the herbicide by the mixed microbial population present in the water. In contrast, Pfaender and Alexander (1973) found that the addition of diphenylmethane, a biodegradable analogue of DDT, to sewage did not selectively stimulate the growth of co-metabolizing heterotrophs and therefore did not enhance degradation of DDT.

Laboratory experiments have shown that, under the correct environmental conditions, pesticides previously regarded as recalcitrant can be degraded by the process of co-metabolism by microbes isolated from natural habitats. It is, however, important to be aware of the difficulty in extrapolating results obtained *in vitro* to the complex environment of a natural ecosystem. No direct means are known for testing the significance of co-metabolism as a mechanism for the microbial transformation of pesticides in the natural environment (Pfaender and Alexander, 1973). Sethunathan and Pathak (1971) found that Diazinon degraded rapidly when in-

cubated with water from a rice paddy that had had several applications of Diazinon and that it persisted if incubated with water from untreated fields. Therefore, Diazinon treatment had resulted in a selection of a Diazinon-degrading bacterial population. An *Arthrobacter* sp. which had high Diazinon degrading activity was isolated from water from treated fields. However, this isolate metabolized Diazinon only in the presence of additional carbon and energy sources (ethanol or glucose). Thus there is indirect evidence for co-metabolic degradation of a pesticide under natural conditions.

CHAPTER II

DIP TANK FIELD TRIALS

2.1 Introduction

Research personnel from Kwanyanga Research Station, near East London, observed the rapid and unexpected loss of Amitraz described in the previous chapter during five field trials run in the East London area. Dip tank field trials were started in two dip tanks near to Rhodes University, Grahamstown, to facilitate investigations of this loss of activity. Sethunathan and Pathak (1971, 1972) demonstrated the selection of Diazinon degrading populations of microorganisms in the field after repeated applications of Diazinon. It was envisaged that during the course of the field trials it would be possible to isolate, from the dip tanks, bacterial populations which had been similarly enriched to degrade Amitraz. These populations would be useful to study Amitraz degradation in the laboratory.

One of the dip tanks is situated on the farm Sea Ways near Port Alfred and the second is on the farm Sea View near Kleinemonde (see Fig. 2). Sea Ways dip tank

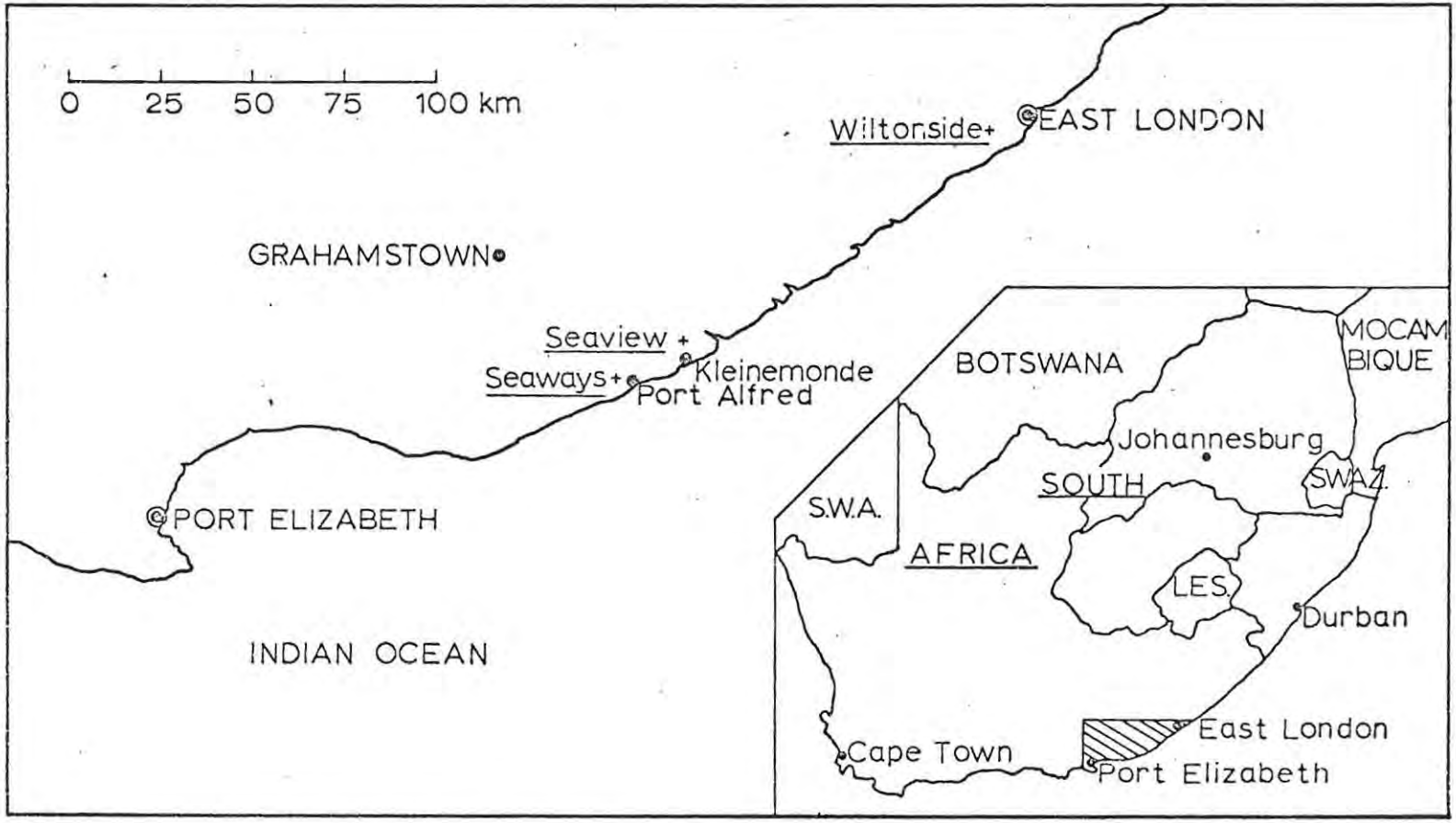


Fig.2. Map of Eastern Cape coastal region showing the locations of Sea Ways, Sea View and Wiltonside dip tanks.

was managed according to the Coopers "Lime Stabilization Method". Harrison et al. (1973) found that raising the pH to 12,2 by the addition of slaked lime to fouled dip washes stabilized the Amitraz. In view of this, a field trial was run at Sea Ways farm during which the addition of slaked lime to the dip wash maintained its pH at 12. Sea View dip tank was managed in accordance with the Coopers "Total Replacement Method". This method involves the addition of a specified amount of Amitraz wettable powder at each dipping session (in this case each week). This ensures that the concentration of Amitraz does not fall below that recommended for tick control.

2.11 Structure of dip tanks

The construction of a typical dip tank is shown in Fig. 3. At the entrance to the tank, leading from the pen where the cattle are held before dipping, there is a concrete slab to reduce the amount of filth that the cattle carry into the tank. This slab is usually cleaned before dipping begins. The passage of large numbers of animals through a dip tank introduces foreign matter which accumulates, and the dip wash may become extremely foul. It is possible that cattle may emerge from a

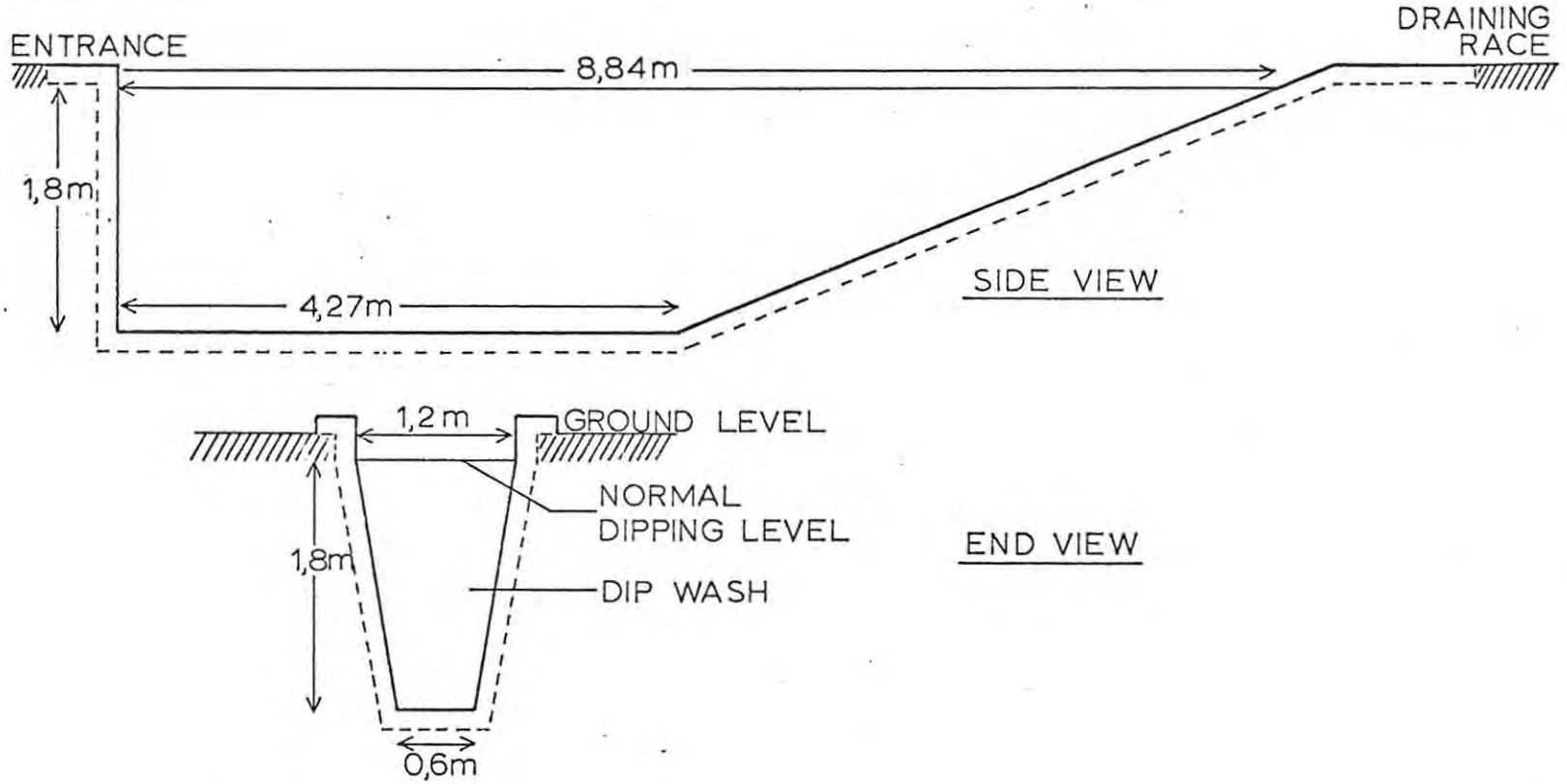


Fig.3. Plan of the Sea Ways dip tank, illustrating the construction of a typical dip tank.

fouled dip tank dirtier than when they entered it. Eventually an equilibrium state of loss and gain of filth is reached. The presence of severe fouling can cause chemical degradation of acaracides. If an acaricide is formulated as a wettable powder, fouling can reduce its biological efficacy (Fiedler, 1969). The cattle plunge into the dip tank at the entrance, where the depth is commonly 2m to ensure complete immersion of the beasts; they swim through the tank and emerge at the shallow end which has a gently sloping floor. After passing through the tank the cattle stand in the draining race — a pen with a concrete floor sloping back towards the dip tank. The cattle are usually held in the draining race until they are no longer dripping and so the volume of dip wash removed by them is reduced.

The dip tanks at Sea Ways and Sea View farms are roofed with corrugated iron, the roof being about 2m above the level of the dip wash. From the entrance to a point about half way along the length of the tank the walls are built up to the roof to reduce loss of dip wash by splashing as the cattle plunge into the dip.

2.12 Calibration of dip tanks

The Sea Ways dip tank was built by the present

owner of the farm and consequently a plan is available and is shown in Fig. 3. Its total capacity is 10965 ℓ, which is taken as 11000 ℓ when doing calculations. Since the exact dimensions of the tank are known a precise calibration of its volume has been made.

The exact plan of the dip tank on Sea View farm is not available since this tank was built by the previous owner of the farm. The walls of the tank are all vertical for the first 15 cm below the full mark and the surface area of the tank at the full mark is known. It has therefore been calculated that each 2,5 cm below the full mark (for the first 15 cm) represents a volume of 450 ℓ of dip wash. The total volume of the tank was determined by the owner of the farm to be 13830 ℓ.

2.2 Methods used in field trials

The field trial at Sea Ways farm was run from 8 July 1974 to 10 February 1975 and that at Sea View farm from 8 April 1974 to 10 February 1975.

2.21 Charging the tanks

At the initiation of the Sea Ways field trial the dip tank was charged with 11 kg of Amitraz wettable powder

containing approximately 25% active ingredient. This gave a concentration of approximately 0,02% active ingredient in the dip wash. After the first week's dipping 50 kg of slaked lime containing approximately 85% $Ca(OH)_2$ were added to the wash to yield a $Ca(OH)_2$ concentration of approximately 0,4% and a pH of 12.

For Sea View dip tank it was calculated that the mass of Amitraz wettable powder (containing approximately 25% active ingredient) to be added each week was 3 kg. At the initiation of the trial the tank was charged with this mass of formulated Amitraz to give a level of about 0,005% active ingredient in the dip wash. This is an effective level for tick control (Baker et al., 1973).

2.22 Replenishing the tanks

Cattle emerging from a dip tank remove some dip wash from it and thus the level in the tank drops. When water is added to the tank to restore the volume of dip wash to its original level acaricide must also be added to maintain the concentration in the dip wash at a level effective for tick control.

Sea Ways dip tank was topped up with water when the wash level dropped too low for effective dipping, i.e.,

when the volume reached 8000 ℓ. On topping up the tank a suitable amount of formulated dip powder was added to restore the dip wash to approximately the original Amitraz concentration.

Sea View dip tank was topped up with water to the full mark and the required mass (3 kg) of formulated dip powder was added each week. The wettable powder was mixed with water in a plastic bin (capacity 40 ℓ) and thoroughly stirred. The resulting suspension was added in small volumes to the tank starting about half way along the length and working back towards the entrance of the tank. The bin was thoroughly rinsed out into the dip wash once all the dip suspension had been added.

2.23 Routine dipping methods

Temperature. The temperatures at the top and bottom of the dip wash were measured at the entrance to the tank before dipping. After dipping the temperature of the wash was measured at the same place.

Dip wash volumes. The level of the dip wash below the full mark was measured and recorded. A comparison of this level with the level at the end of the previous week's dipping indicated whether evaporation or flooding

by rain had occurred. At Sea View farm, if flooding had occurred, the dip wash was pumped out of the tank until the full mark was reached. It is important to note that any liquid pumped out was assayed and found to contain no active ingredient. This is not unexpected since the wettable powder settles to the bottom of the tank in the interval between the weekly dipping sessions.

After dipping the level of the wash below the full mark was measured once the wash had settled. By comparing the volumes before and after dipping each week the volume of dip wash removed by the cattle was determined.

Dipping and sampling procedures. Half the herd was dipped and 2 samples were taken in sterile glass bottles (300 ml) mounted on a sampling stick (Fig. 4). Each week the samples were taken from near the bottom of the wash at a position about half way along the length of the tank. The second half of the herd was dipped and a further 2 samples were taken immediately after the last animal had been through the tank. Of the 2 pairs of samples one of each pair was used for bacteriological analysis and the other was used for the determination of Amitraz concentration. To the latter samples $Ca(OH)_2$ was immediately added to a concentration of 1%(wt/vol) to ensure that no breakdown of Amitraz occurred before

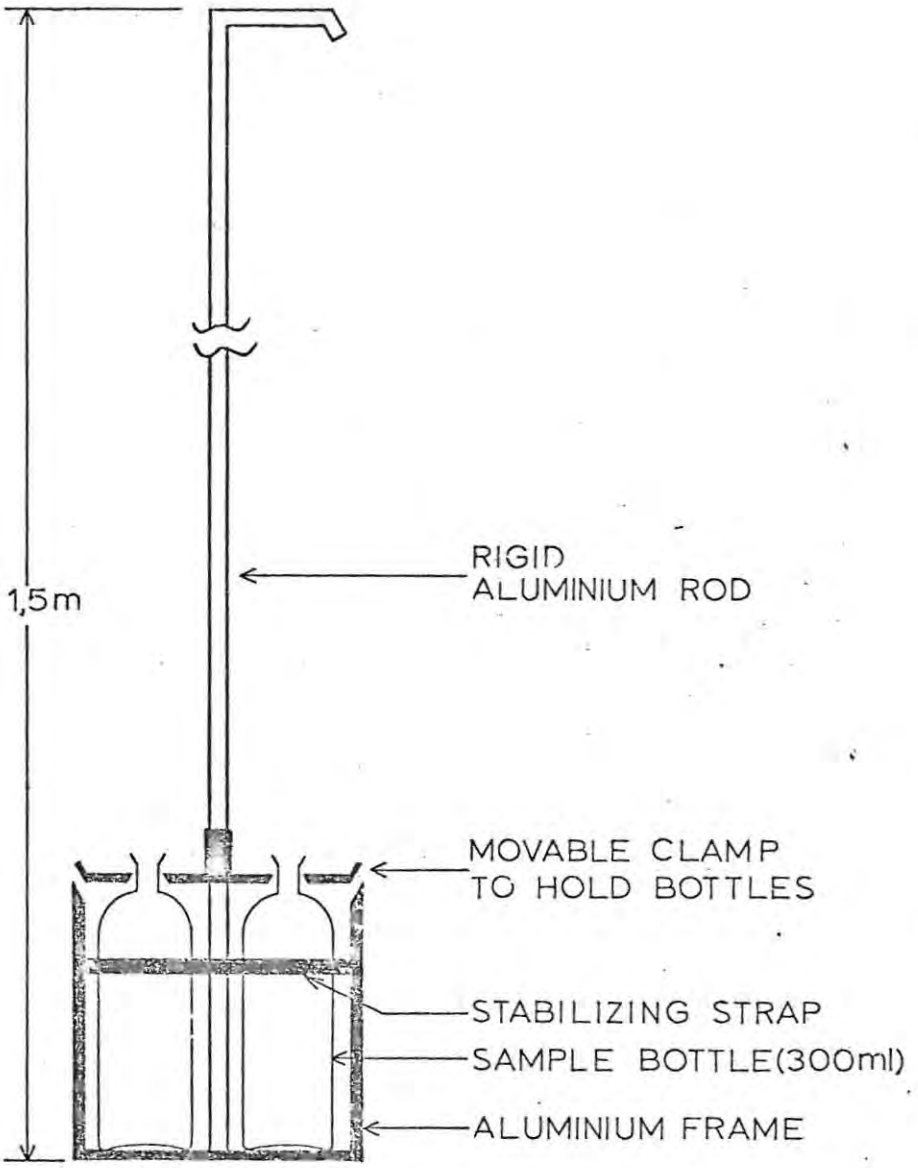


Fig.4. Sketch of apparatus for taking wash samples from dip tanks.

its concentration was determined in the laboratory.

Determination of Amitraz concentration and bacterial numbers. Amitraz concentrations were determined by gas chromatography. Details of the analysis are given in Appendix A. Numbers of bacteria present in the dip wash samples were determined on nutrient agar (Difco) plates, buffered at pH 7 and pH 11, using the spread plate technique. Incubation was at 30°C for 48h.

Measurement of pH. The pH at the surface of the dip was measured before and after dipping each week using Merck pH indicator sticks.

Tank-side method for the assay of $Ca(OH)_2$. The following method was used to determine the mass of slaked lime required to be added to Sea Ways dip tank to maintain the $Ca(OH)_2$ level at 0,4%(wt/vol). This mass of slaked lime was added to Sea Ways dip tank as soon after dipping as possible.

The method for determining the $Ca(OH)_2$ concentration of the wash at the dip tank was developed by B.H. Palmer of the Thurgarton Research Station, Nottingham, England. It is essentially a volumetric titration of 0,1 M HCl against the $Ca(OH)_2$ contained in the dip wash. The equation of the reaction is:

$$2HCl + Ca(OH)_2 \rightarrow 2H_2O + CaCl_2$$

Because 73g of HCl are equivalent to 74g of $Ca(OH)_2$, 1ml of 0,1 M HCl is equivalent to 3,7mg of $Ca(OH)_2$. Thymolphthlein is the indicator used since the colour change is from blue to colourless in the pH range of 10,5 to 9,3, i.e., before the HCl begins to attack the $CaCl_2$ invariably present in commercially available slaked lime.

A 25ml aliquot is removed from a well-shaken dip wash sample taken immediately after dipping. A drop from the 25ml aliquot is removed and spotted onto a small piece of thymolphthlein indicator paper. If the indicator paper remains white then it is concluded that, for all practical purposes, there is no $Ca(OH)_2$ in the sample. If, however, the indicator paper turns blue 2ml of 0,1 M HCl are injected into the sample using a disposable plastic syringe. After stirring for a few seconds a drop of the sample is removed and spotted onto a fresh piece of indicator paper. The titration is continued in 2ml amounts until the indicator change from blue to white is obtained. The total volume of acid added to the sample that gave the last blue colour is taken as the titration reading. This ensures that the error in the method favours a slight excess of $Ca(OH)_2$ being added to the tank. The concentration of $Ca(OH)_2$

is obtained from Table 1 which correlates the volume of HCl with the theoretical percentage $Ca(OH)_2$.

The mass M of $Ca(OH)_2$ to be added to the dip tank in order to restore the concentration to 0,4% is calculated from the formula:

$$M = \frac{V(0.4 - C)}{100},$$

where V = volume in litres of the dip wash at the end of dipping

C = the assayed percentage $Ca(OH)_2$ in the dip wash sample.

The purity of slaked lime is known to vary in terms of its $Ca(OH)_2$ content, both with its source of supply and its age. During this field trial Cape Lime Company Hydrated Plastic Pressure Lime was used and, although obtained from the same commercial source, its percentage $Ca(OH)_2$ was found to vary from 89% to 54%. It was therefore necessary to determine the percentage $Ca(OH)_2$ in each batch before adding the lime to the tank. Once the purity had been established the mass of slaked lime to be added could be calculated in order to achieve the required concentration of $Ca(OH)_2$.

This titration method was found to be a quick and

Table 1. Correlation of titration volume of HCl with $Ca(OH)_2$ concentration in wash samples.

Volume of 0,1 M HCl (ml)	Theoretical % $Ca(OH)_2$
2	0,029
4	0,059
6	0,089
8	0,118
10	0,148
12	0,178
14	0,207
16	0,237
18	0,266
20	0,296
22	0,326
24	0,355
26	0,385
28	0,414
30	0,444
32	0,474
34	0,503
36	0,533
38	0,562
40	0,592

convenient method for establishing $Ca(OH)_2$ concentrations at the dip tank. Although adequate, the accuracy of this method is limited by the fact that a relatively large volume, i.e., 2ml of HCl was added at each stage of the titration.

Determination of fouling. To measure the levels of fouling in Sea View dip tank the following method was used: 100g of well-shaken dip wash taken at the end of dipping was kept at 40°C for 48h until dry. The dry residue was weighed. This mass represents the amount of solids in the original 100g of dip wash and gives the percentage fouling of the tank.

2.3 Results of field trials

2.31 Temperature

At Sea Ways farm the temperature of the dip wash ranged from a minimum of just over 10°C to a maximum of 22°C (see Table 2). In Sea View dip tank the range was 12°C to 22°C (see Table 3). The rise in temperature in summer occurred later in these dip tanks, which are roofed, than in Wiltonside dip tank, which is open (Kwanyanga Research Station, private communication). A comparison

of the temperatures taken at the top and bottom of the dip washes shows that there is not a marked or consistent temperature gradient between the top and bottom of the tanks. During each dipping session, both at Sea Ways and Sea View, the temperature of the wash rose between 0,5 and 1°C. This result is similar to that observed in Wiltonside dip tank.

2.32 Removal of dip wash by cattle

At Sea Ways farm the average volume of dip wash removed per beast was 2,2 ℓ. This is comparable to the figure determined by Gretsy (1971, cited in Harrison et al., 1973), who found that each adult beast removes about 9 ℓ of dip wash as it emerges from the tank and about 6,5 ℓ drain off and return to the tank while the beast is standing in the draining race. On passing through the hair of the beast the acaricide is stripped from the dip wash so that the concentration in the drained fluid is less than that in the original dip wash. Consequently, after several weeks of dipping a loss of volume of dip wash and also a reduction in acaricide concentration occurs. The tanks are replenished at intervals to compensate for these losses (see Section 2.22). As the number of cattle being dipped at Sea Ways each week increased (see Table 4) the frequency of replenish-

ment increased (see Fig. 5).

The average volume of dip wash removed per beast at Sea View farm was 4,6 ℓ per week (see Table 5). This is twice the volume removed per head at Sea Ways farm and reflects the fact that the cattle do not stand in the draining race for an appreciable length of time at Sea View.

2.33 Amitraz concentrations

A histogram of the weekly concentrations of Amitraz active ingredient in Sea Ways dip tank at the end of dipping is given in Fig. 5. In addition the figure shows the times at which the tank was replenished. The graph illustrates the effect of stripping in the dip tank, i.e., the Amitraz concentration drops between replenishments. The loss of Amitraz between replenishments could be accounted for entirely by stripping, which indicates that the Amitraz was not degrading in this tank.

The weekly Amitraz concentrations at Sea View dip tank are given in Fig. 6. Since this dip tank was run according to the "Total Replacement Method", 3kg of formulated dip powder (equivalent to approximately 750g of active ingredient) should have been added before dipping each week. However, because of occasional delays

Table 2. Weekly values of temperature, pH and $Ca(OH)_2$ concentration in Sea Ways dip tank.

Week	Temperature (°C)			pH[a]		Theoretical % $Ca(OH)_2$ at end of dipping[b]	Mass of slaked lime added (kg)
	Before dipping		After dipping	Before dipping	After dipping		
	bottom	top					
1	14,0	14,3	15,1	7	7	—	50
2	14,1	14,4	15,6	9	12	0,237	20
3	13,1	13,6	14,0	12	12	0,444	0
4	—	14,5	15,0	12	13	0,414	10
5	—	13,5	14,5	12	13	0,444	10
6	11,5	12,0	12,9	12	13	0,562	0
7	13,3	14,5	15,4	13	13	0,444	0
8	12,0	13,0	13,5	12	13	0,414	10
9	10,0	10,5	10,5	12	12	0,355	7,5
10	11,0	10,5	11,5	12	13	0,355	2,5
11	12,7	13,5	14,5	12	13	0,326	10
12[c]	—	—	—	—	—	—	—
13	—	16,0	17,0	12	12	—	10
14	14,5	15,0	15,4	12	12	0,266	20
15	15,5	16,0	16,6	11	12	0,326	10
16	14,0	14,5	15,5	12	12	—	10
17	14,5	14,5	15,9	12	12	0,326	10
18	16,9	17,4	18,2	12	12	0,296	12,5
19	17,4	18,0	18,8	12	12	0,237	20
20	16,8	16,4	17,1	12	12	0,237	20
21	17,6	17,1	18,3	12	12	0,296	12,5
22	17,5	18,1	19,4	12	12	0,266	15
23	18,5	19,5	20,5	12	12	0,296	12,5
24	19,5	19,0	20,0	12	12	—	12,5
25	19,5	20,0	20,5	12	12	—	12,5
26	17,0	17,6	18,0	12	12	—	12,5
27	17,0	17,5	18,0	12	12	—	12,5
28	19,0	18,5	20,4	12	12	0,237	15
29	20,0	19,8	21,0	12	12	0,207	20
30	20,5	20,7	21,8	11	12	0,178	25
31	21,5	22,0	22,5	11	12	—	25
32	20,0	20,5	21,5	11	12	—	12,5

[a] Measured with pH paper

[b] This is the theoretical % $Ca(OH)_2$ determined at the end of dipping before adding slaked lime to the required concentration

[c] There was no dipping due to rain

Table 3. Weekly values of temperature, pH and percentage fouling in Sea View dip tank.

Week	Temperature (°C)			pH[a]		% Fouling
	Before dipping		After dipping	Before dipping	After dipping	
	bottom	top				
1	17,5	20,0	20,0	7,5	8,0	0,34
2	20,0	20,0	20,0	7,5	8,0	0,34
3	17,0	18,0	18,0	7,5-8,0	8,0	0,41
4	18,0	18,0	19,0	8,0	8,0	0,47
5	17,5	17,5	18,5	8,0	8,0	0,55
6	17,0	17,0	18,0	8,0	8,0	0,60
7	15,0	15,8	16,5	8,0	8,0	0,57
8	16,0	16,0	16,5	8,0	8,0	0,60
9	15,0	15,0	16,0	8,0	8,0	0,67
10	15,0	15,0	16,0	8,0	8,0	0,67
11	14,5	15,0	16,0	7,5-8,0	8,0	0,69
12	15,0	15,0	16,0	7,5	8,0	0,79
13	14,0	14,0	15,0	7,5	8,0	0,80
14	14,5	14,0	15,0	7,5-8,0	8,0	—
15	14,5	15,0	15,5	7,5-8,0	8,0	—
16	14,0	14,5	14,5	8,0	8,0	0,90
17	14,0	15,0	16,0	—	8,0	0,92
18	14,0	14,0	15,0	—	8,0	1,05
19	12,0	13,0	14,0	7,5	8,0	0,96
20	14,0	15,0	16,0	7,5	8,0	0,94
21	12,0	13,0	14,0	8,0	8,0	0,98
22[b]	—	—	—	—	—	—
23	11,5	12,0	13,1	7,5-8,0	8,0	1,02
24	13,5	14,3	15,1	7,5	8,0	0,92
25[b]	—	—	—	—	—	—
26	15,0	16,0	16,9	8,0	8,0	0,87
27	15,0	16,0	16,4	7,5-8,0	8,0	0,90
28	16,0	16,5	17,3	7,5	8,0	0,97
29	15,0	15,0	15,5	7,5	8,0	—
30	15,6	16,1	16,8	8,0	8,0	0,87
31	15,6	16,1	16,8	7,5	8,0	0,94
32	17,6	18,1	18,5	7,5-8,0	8,0	0,96
33	18,5	18,5	19,3	7,5-8,0	8,0	0,99
34	17,4	17,0	18,1	7,5	8,0	1,01
35	18,1	17,9	18,8	7,5-8,0	8,0	1,03
36	19,1	18,7	19,5	7,5	8,0	0,98
37	19,0	20,0	20,6	8,0	8,0	—
38	19,5	19,0	20,5	8,0	8,0	0,92
39	18,5	19,0	20,5	8,0	8,0	0,88
40	18,0	17,5	19,0	7,5	8,0	1,14
41	19,5	19,0	20,5	8,0	8,0	0,99
42	19,0	19,0	20,0	8,0	8,0	1,22
43	20,0	20,1	20,8	8,0	8,0	1,17
44	20,8	21,4	22,0	7,5	8,0	1,17
45	21,0	22,0	22,5	7,5	8,0	1,19

a Measured with pH paper
b There was no dipping due to rain

Table 4. Weekly records of dip wash volumes, number of cattle dipped and volume of wash removed at Sea Ways farm.

Week	Volume of dip wash (ℓ)		Number of cattle dipped	Volume of wash removed (ℓ)	
	Before dipping	After dipping		Total	Per head
1	11000	10500	118	500	4,2
2	10500	10300	119	200	1,7
3	10300	9900	113	400	3,1
4	9675	9425	130	250	1,9
5	9500	9300	134	200	1,5
6	9125	9000	134	125	0,9
7	11000	10500	134	500	3,7
8	11000	10765	127	235	1,9
9	10500	10250	130	250	1,9
10	10250	9625	150	625	4,2
11	9625	9425	171	200	1,2
12	—	—	—	—	—
13	10400	10100	154	300	1,9
14	10300	9625	152	675	4,4
15	9625	9350	152	275	1,8
16	9425	9300	152	125	0,8
17	9300	9000	177	300	1,7
18	9000	8175	189	825	4,4
19	11000	10650	192	350	1,8
20	10530	9900	192	630	3,3
21	9900	9600	198	300	1,5
22	9600	9300	198	300	1,5
23	9300	9000	198	300	1,5
24	11000	10775	203	225	1,1
25	10775	10300	203	475	2,3
26	10300	9900	205	400	2,0
27	9900	9425	201	475	2,4
28	9300	9000	206	300	1,5
29	9000	8175	206	825	4,0
30	11000	10775	206	225	1,1
31	10775	10400	207	375	1,8
32	10500	9900	207	500	2,4

Table 5. Weekly records of dip wash volumes, number of cattle dipped, volume of wash removed and mass of Amitraz added at Sea View farm.

Week	Volume of dip wash (ℓ)		Number of cattle dipped	Volume of wash removed (ℓ)		Mass of wettable powder added (kg)	% active ingredient in wettable powder
	Before dipping	After dipping		Total	Per head		
1	13830	13280	196	550	2,8	3	≈25
2	13830	13280	197	550	2,8	3	≈25
3	13830	12930	196	900	4,6	3	≈25
4	13830	13030	199	800	4,0	3	≈25
5	13830	13130	193	750	3,9	3	≈25
6	13830	12630	197	1200	6,0	3	≈25
7	13830	12930	203	900	4,4	3	≈25
8	13830	12830	195	1000	5,1	3	≈25
9	13830	12930	203	900	4,5	3	≈25
10	13830	13400	201	430	2,1	1,5	≈25
11	13830	12930	209	900	4,3	1,5	≈25
12	13830	12930	199	900	4,5	1,5	≈25
13	13830	12930	199	900	4,5	3	≈25
14	13830	12930	203	900	4,4	3	≈25
15	13830	12930	203	900	4,4	3	≈25
16	13830	12930	207	900	4,4	3	≈25
17	13830	12930	208	900	4,3	3	≈25
18	13830	12750	205	1080	4,4	2	≈25
19	13830	12530	203	1300	6,4	3	≈25
20	13830	12930	203	900	4,4	3	≈25
21	13830	13380	207	450	2,2	3	≈25
22	—	—	—	—	—	—	—
23	13830	12915	193	1125	5,8	3	≈25
24	13830	12530	194	1300	6,7	3	≈25
25	—	—	—	—	—	—	—
26	13830	12930	198	900	4,6	3	≈25
27	13830	12705	197	1125	5,7	3	≈25
28	13830	12705	196	1125	5,7	3	≈25
29	13830	12930	196	900	4,6	3	≈25
30	13830	12930	185	900	4,9	3	≈25
31	13830	12930	186	900	4,9	3	≈25
32	13830	12930	193	900	4,7	3	≈25
33	13830	12705	202	1125	5,6	1,8	20,7
34	13830	12930	205	900	4,4	1,8	20,7
35	13830	12930	218	900	4,1	3	20,7
36	13830	12930	217	900	4,2	3	20,7
37	13830	12530	205	1300	6,3	3	20,7
38	13830	12705	203	1125	5,5	3	20,7
39	13830	12930	206	900	4,4	3	20,7
40	13830	12930	205	900	4,4	3	20,7
41	13830	12705	207	1125	5,4	6	11,7
42	13830	12930	206	900	4,4	6	11,7
43	13830	12930	212	900	4,3	3	24,8
44	13830	12930	214	900	4,2	3	24,8
45	13830	12705	214	1125	5,3	3	24,8

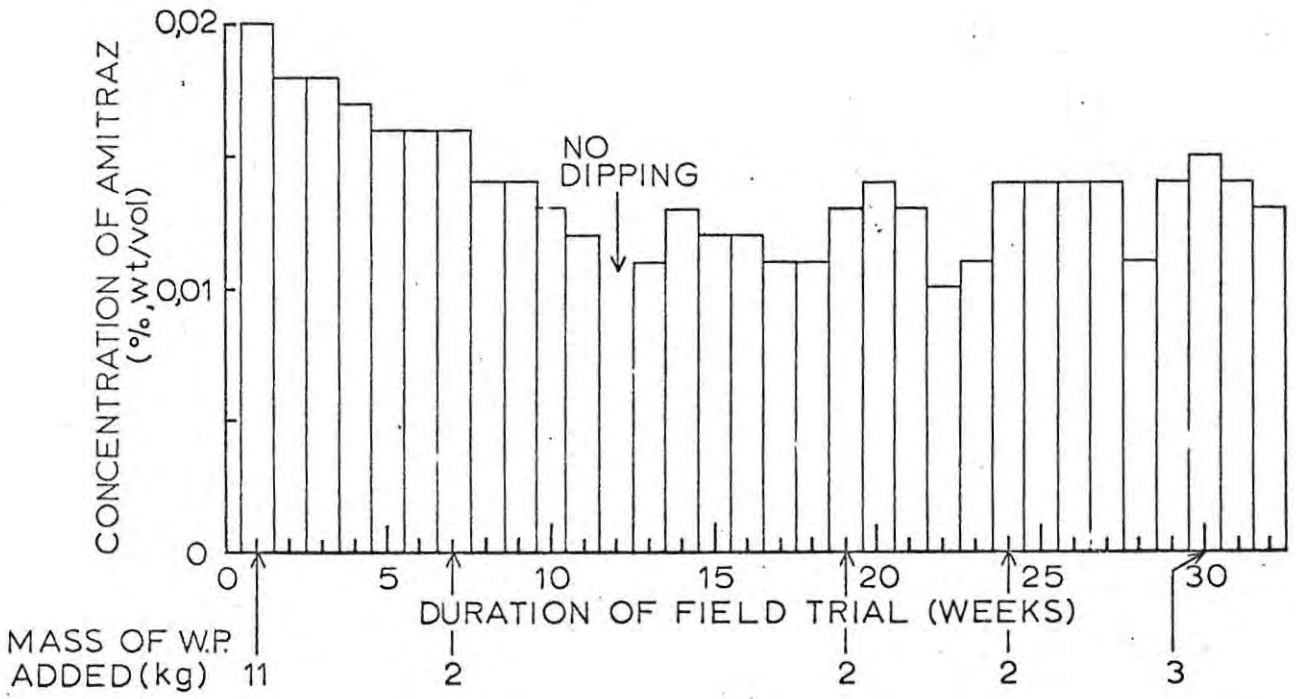


Fig.5. Weekly Amitraz concentrations during the field trial at Sea Ways dip tank. Replenishments of Amitraz wettable powder are indicated by arrows.

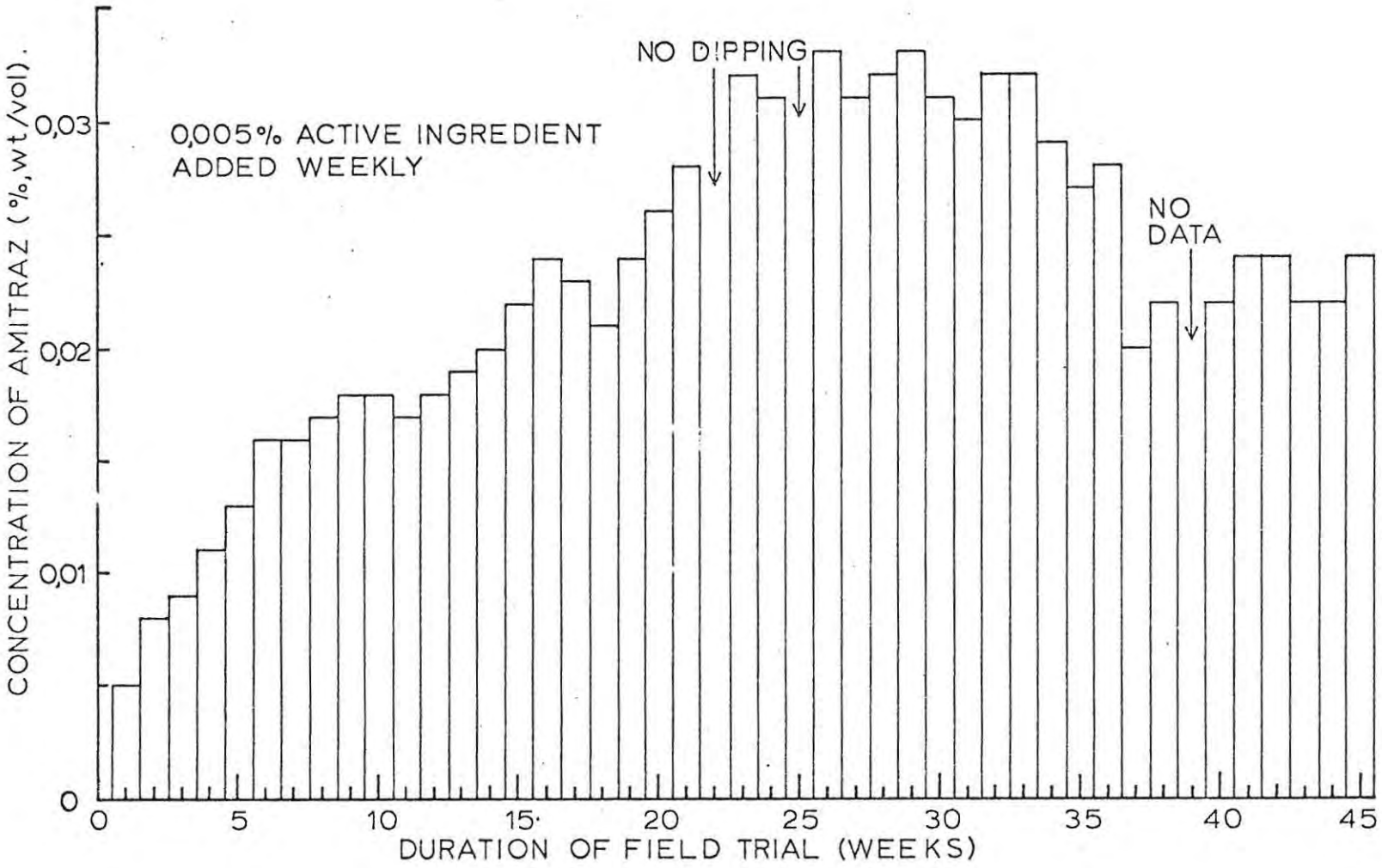


Fig.6. Weekly Amitraz concentrations during the field trial at Sea View dip tank.

in the supply of Amitraz, the full amount was not always added. Table 5 shows the mass of wettable powder added each week. Clearly, if less active ingredient is added at certain times the level of Amitraz falls; this is reflected on the histogram, e.g., the concentration falls between weeks 10 and 11 and during this time only half the required amount of Amitraz was added.

From the histogram it is evident that after rising slowly for 23 weeks, the Amitraz level plateaued between weeks 23 and 32, and then began to drop slowly from week 33 onwards. The dip analyses from weeks 32 to 45 were done by the Research Department at African Explosives and Chemical Industries Ltd., Modderfontein, Transvaal. The standard used for these analyses was 100% pure active ingredient, whereas the standard used at Rhodes University was technical Amitraz, i.e., not 100% pure active ingredient. This may explain the initial drop at week 32. Stripping alone does not account for the total loss of Amitraz. Some degradation of active ingredient must have taken place, but the disappearance of Amitraz from Sea View dip tank is not nearly as marked as that observed in Wiltonside dip tank (see Section 1.1 and Fig. 1).

2.34 Bacterial counts

Numbers of bacteria in the Sea Ways dip wash are shown in Table 6. After a week of exposure to a high pH the bacteria in the dip tank were unable to grow at high pH in the laboratory. However with increasing time of exposure to pH 12 in the dip tank the bacteria, both aerobes and anaerobes, adapted to grow at high pH. By week 17 the aerobic bacterial count averaged $3x10^4$ bacteria per ml at pH 7 and $5x10^4$ bacteria per ml at pH 11 and the anaerobic count averaged $5x10^2$ bacteria per ml at pH 7 and $3x10^4$ bacteria per ml at pH 11. From weeks 18 to 32 the numbers of bacteria growing at both pH 7 and pH 11 increased to reach a level of approximately 10^5 bacteria (both aerobes and anaerobes) per ml. It therefore appears that a population of alkali-tolerant bacteria was selected.

The colony morphology of the aerobic bacteria growing on medium at pH 7 differed markedly from the morphology of those growing at pH 11. At pH 7 there were many cream-coloured spreading colonies and those that did not spread tended to be large (5-10 mm in diameter) and coloured cream, yellow, pink or orange. In contrast, at pH 11 the colonies were small (0,5-1,5 mm in diameter) and most were transluscent. After

Table 6. Bacterial counts determined from weekly samples taken from Sea Ways dip tank.

Week	Bacterial count (Bacteria/ml)							
	Half way through dipping				At end of dipping			
	Aerobes		Anaerobes		Aerobes		Anaerobes	
	pH 7[a]	pH 11[a]	pH 7[a]	pH 11[a]	pH 7[a]	pH 11[a]	pH 7[a]	pH 11[a]
1	$1,3x10^7$	–	$4,7x10^5$	–	$1,5x10^7$	–	$7,5x10^5$	–
2	$1,2x10^3$	$<10^2$	$<10^2$	$<10^2$	$1,2x10^3$	$<10^2$	$1,3x10^2$	$<10^2$
3	$<10^2$	$1,1x10^3$	$<10^2$	$1,4x10^2$	$<10^2$	$1,2x10^4$	$<10^2$	$1,0x10^3$
4	–	–	–	–	$<10^2$	$5,9x10^5$	$<10^2$	$4,9x10^5$
5	–	–	–	–	$<10^2$	$2,5x10^4$	$<10^2$	$2,0x10^3$
6	$3,7x10^3$	$8,5x10^2$	$5,0x10^2$	$2,0x10^2$	$4,9x10^3$	$9,0x10^2$	$8,0x10^2$	$4,0x10^2$
7	$2,2x10^5$	$2,5x10^3$	$2,0x10^3$	$5,0x10^2$	$2,9x10^5$	$3,0x10^3$	$2,0x10^3$	$3,0x10^3$
8	$1,2x10^3$	$1,1x10^3$	$<10^2$	$<10^2$	$5,3x10^3$	$2,2x10^3$	$<10^2$	$<10^2$
9	$1,8x10^3$	$2,0x10^3$	$<10^2$	$<10^2$	$4,1x10^3$	$3,2x10^3$	$1,0x10^2$	$<10^2$
10	$3,0x10^3$	$5,0x10^2$	$1,0x10^2$	$1,5x10^2$	$3,1x10^3$	$5,0x10^2$	$1,0x10^2$	$1,0x10^2$
11	$4,2x10^3$	$<10^2$	$1,0x10^2$	$<10^2$	$5,2x10^3$	$<10^2$	$2,0x10^2$	$<10^2$
12	–	–	–	–	–	–	–	–
13	–	–	–	–	$1,4x10^4$	$2,0x10^4$	$2,6x10^2$	$1,2x10^2$
14	$5,2x10^3$	$1,5x10^3$	$1,1x10^3$	$7,5x10^2$	$9,5x10^3$	$2,4x10^3$	$1,5x10^3$	$1,0x10^3$
15	$3,8x10^3$	$2,0x10^2$	$1,4x10^3$	$2,0x10^2$	$1,6x10^4$	$8,3x10^3$	$6,5x10^2$	$3,8x10^3$
16	$7,7x10^4$	$5,7x10^4$	$1,0x10^2$	$<10^2$	$8,5x10^4$	$4,9x10^4$	$8,0x10^2$	$<10^2$
17	$5,1x10^4$	$2,3x10^4$	$8,5x10^2$	$2,5x10^2$	$6,6x10^4$	$3,7x10^4$	$1,1x10^3$	$9,0x10^2$
18	$5,7x10^4$	$6,4x10^4$	$<10^2$	$1,6x10^3$	$6,5x10^4$	$9,3x10^4$	$<10^2$	$9,8x10^3$
19	$7,1x10^4$	$1,7x10^5$	$8,8x10^3$	$1,1x10^5$	$1,1x10^5$	$3,2x10^5$	$1,0x10^3$	$1,7x10^5$
20	$5,6x10^3$	$6,5x10^2$	$5,5x10^2$	$4,5x10^2$	$5,9x10^3$	$7,0x10^2$	$6,0x10^2$	$1,5x10^2$
21	$1,0x10^4$	$9,6x10^3$	$1,1x10^3$	$5,0x10^2$	$1,3x10^4$	$9,0x10^3$	$1,9x10^3$	$6,0x10^2$
22	$1,3x10^4$	$1,9x10^4$	$1,7x10^3$	$5,1x10^2$	$1,2x10^4$	$9,8x10^3$	$1,7x10^3$	$6,8x10^2$
23	$1,3x10^4$	$2,4x10^5$	$1,7x10^3$	$4,5x10^3$	$1,8x10^4$	$9,9x10^4$	$1,9x10^3$	$4,0x10^3$
24	$4,4x10^4$	$8,8x10^4$	$2,0x10^3$	$9,1x10^2$	$2,2x10^4$	$9,0x10^4$	$2,4x10^3$	$2,1x10^3$
25	$5,6x10^4$	$1,2x10^5$	$1,8x10^3$	$1,1x10^4$	$7,8x10^4$	$1,0x10^5$	$2,1x10^3$	$1,0x10^4$
26	$2,8x10^4$	$1,9x10^5$	$2,2x10^3$	$1,9x10^4$	$3,0x10^4$	$1,2x10^5$	$5,8x10^3$	$2,2x10^4$
27	$5,6x10^4$	$2,8x10^5$	$2,5x10^3$	$2,2x10^4$	$5,8x10^4$	$3,2x10^5$	$7,2x10^3$	$2,3x10^4$
28	$7,1x10^4$	$1,2x10^5$	$3,2x10^3$	$9,2x10^4$	$8,1x10^4$	$1,9x10^5$	$4,4x10^3$	$2,2x10^5$
29	$7,8x10^4$	$1,1x10^5$	$2,8x10^3$	$1,0x10^5$	$1,0x10^5$	$2,4x10^5$	$8,6x10^3$	$8,6x10^4$
30	$8,1x10^4$	$2,1x10^5$	$2,3x10^3$	$1,5x10^5$	$1,5x10^5$	$2,9x10^5$	$2,9x10^4$	$2,7x10^5$
31	$1,7x10^5$	$1,7x10^5$	$5,1x10^4$	$1,0x10^5$	$1,8x10^5$	$2,1x10^5$	$5,7x10^4$	$1,5x10^5$
32	$1,8x10^5$	$1,6x10^5$	$9,4x10^4$	$9,9x10^4$	$2,1x10^5$	$2,1x10^5$	$3,8x10^4$	$9,8x10^4$

[a] This pH is that of the nutrient agar assay medium

anaerobic incubation there was no apparent difference in colony morphology between bacteria growing at neutral pH and those growing at high pH.

An experiment to test the difference between bacteria growing at pH 7 and those growing at pH 11 showed that 98% of bacteria tested would grow at both pH values. It was found that for each strain growth at pH 11 was slower than at pH 7 and the difference in colony morphology described above was apparent.

The results of bacterial counts determined in samples taken from Sea View dip tank are shown in Table 7. Over the 45 week period there was very little difference between the numbers of bacteria (both aerobes and anaerobes) isolated from the sample taken half way through dipping and that taken at the end. During the first 7 weeks of the trial the counts of both aerobes and anaerobes were of the order of 10^6 bacteria per ml. By the end of the trial the number of both aerobes and anaerobes had increased approximately 10-fold. Over the entire period of the trial the average aerobic count was 4×10^7 bacteria per ml and the average anaerobic count was 6×10^6 bacteria per ml. It was found that 100% of all anaerobic clones tested continued to grow when incubated aerobically, i.e., they were facultative anaerobes. Any

Table 7. Bacterial counts determined from weekly samples taken from Sea View dip tank.

Week	Bacterial count[a]			
	Half way through dipping		At end of dipping	
	Aerobes/ml x10^7	Anaerobes/ml x10^6	Aerobes/ml x10^7	Anaerobes/ml x10^6
1	1,2	—	3,3	—
2	0,7	2,4	0,7	6,6
3	0,6	1,7	0,5	3,2
4	0,6	1,6	0,6	5,0
5	0,3	2,4	0,7	2,3
6	0,9	8,8	1,7	12,4
7	0,3	2,5	0,3	5,0
8	1,8	1,8	3,0	3,6
9	2,0	1,9	5,0	3,2
10	2,2	*	2,0	*
11	2,9	*	2,6	*
12	2,3	*	3,2	*
13	6,4	0,8	5,7	0,9
14	4,8	1,0	5,3	0,8
15	2,5	0,8	3,2	1,1
16	2,8	0,6	2,9	0,8
17	—	—	3,3	1,2
18	—	—	2,7	0,9
19	4,5	2,0	5,0	1,6
20	5,4	3,2	5,7	4,0
21	—	—	6,6	9,4
22	—	—	—	—
23	6,0	2,8	8,1	3,1
24	2,8	1,3	4,0	2,0
25	—	—	—	—
26	—	—	3,5	1,6
27	8,1	5,5	6,1	6,4
28	4,0	6,0	5,4	6,2
29	5,2	4,5	6,3	6,0
30	5,4	4,5	6,6	4,9
31	2,5	2,0	2,5	2,0
32	2,2	1,1	3,8	1,0
33	2,2	5,0	2,1	5,0
34	8,0	9,0	8,8	8,0
35	4,2	10,1	4,1	11,1
36	4,4	8,4	3,6	8,1
37	6,0	10,2	6,1	11,0
38	8,7	6,0	8,8	9,0
39	8,4	10,0	7,7	8,8
40	5,1	11,2	5,9	11,6
41	4,7	11,2	6,2	14,0
42	5,8	8,3	6,3	15,1
43	5,7	9,1	6,0	30,0
44	4,8	17,2	6,2	39,6
45	6,1	21,4	6,2	22,0

* These counts are less than 10^2 bacteria/ml.

a Bacterial counts determined on nutrient agar plates at pH7.

obligate anaerobes present would not have survived during sampling.

2.35 pH and $Ca(OH)_2$ concentration

Table 2 shows the weekly pH levels and $Ca(OH)_2$ concentrations in Sea Ways dip tank. The pH reading taken at the end of dipping remained at or above pH 12 after week 1 of the trial; therefore the pH of the tank favoured stabilization of the Amitraz.

After determining the theoretical percentage of $Ca(OH)_2$ in the tank at the end of dipping the amount of slaked lime required to maintain a $Ca(OH)_2$ level of 0,4% was calculated and added to the tank (see Table 2). When the percentage $Ca(OH)_2$ had not been determined an estimate of the amount of lime to be added was made.

The pH of Sea View dip wash remained at pH 8 throughout the trial (see Table 3). Since Amitraz is rapidly hydrolysed in acid conditions, the fact that the dip wash has a pH of 8 contributes towards its stability (Matthewson and Child, 1971; McCarthy, 1972). This slightly alkaline pH is generally found in most dip tanks run on conventional methods (Kwanyanga Research Station, private communication). The fact that Amitraz active ingredient is mixed with $CaCO_3$ when formulated as

a wettable powder contributes to the maintainance of alkalinity.

2.36 Fouling in Sea View dip tank

The percentage fouling of Sea View dip tank rose steadily from a value of 0,34% at week 1 to a maximum of 1,19% by week 45 (see Table 3). This represents a very low level of fouling — levels of fouling commonly found in dip tanks are 4% or more (Kwanyanga Research Station, private communication). The low level of fouling at Sea View is attributed to the fact that most of the farm and the entire area around the dip tank are covered with grass. In addition, a relatively small number of cattle were dipped each week. At Wiltonside dip tank there is no grass cover around the tank and 550 head of cattle were dipped weekly; consequently large amounts of filth were carried into tank by the cattle, thus causing bad fouling (4,2%).

2.4 Discussion

The results obtained from the field trial run at Sea Ways farm confirm the observation of Harrison et al. (1973) that the addition of slaked lime to fouled dip washes stabilizes Amitraz. It is interesting to note

that the problem of instability in dip tanks of Chlorphenamidine was overcome by keeping tanks acid (pH 5,2 to 5,6) by the addition of superphosphate (Roulston et al., 1971). Over the 32 week period of the trial a population of alkali-tolerant bacteria was selected. This showed that Amitraz was not being stabilized by inhibition of bacterial growth. The bacterial numbers determined in samples from Sea Ways dip tank were characterized by their variability from week to week. This result is in direct contrast to the bacterial numbers found is Sea View dip wash samples. In these samples the bacterial counts remained at a constant level of approximately 10^7 aerobes per ml and 10^6 anaerobes per ml.

At Sea View dip tank the results obtained were unexpected in that the sudden disappearance of Amitraz experienced at Wiltonside and other dip tanks in the East London area did not occur. There was some small loss of Amitraz which could not be accounted for by stripping. The excellent tick control achieved throughout this trial and the trial at Sea Ways farm demonstrates that there was no reduction in biological efficacy of the Amitraz. These results show that Amitraz is suitable for use in the field.

The difference in results obtained at Sea View (in the Grahamstown area) and at Wiltonside (in the East London area) is surprising since the distance between them is only about 100 km and they are in the same climatic region. It is unlikely that completely different bacterial populations develop in the two tanks; therefore the presence or absence of degradation is probably not due to the presence or absence of particular bacterial strains. It is possible that Amitraz degradation is linked to fouling in the tanks. This would imply that degradation of Amitraz occurs by a co-metabolic process; the necessary co-metabolite being present in the fouled Wiltonside dip wash and absent in the clean Sea View dip wash. The Sea View trial demonstrates that the "Total Replacement Method" of dipping using Amitraz is suitable for tick control and that lime stabilization is not required in clean tanks.

CHAPTER III

OPTIMUM CONDITIONS FOR AMITRAZ DEGRADATION IN THE LABORATORY USING MIXED BACTERIAL CULTURES

3.1 Introduction

The stability of Amitraz was tested under simulated field conditions by Matthewson and Child (1971). They determined the stability of Amitraz dispersable powder in clean and artificially fouled washes and found that the degree of degradation of active ingredient was directly related to the degree of pollution of the wash. Griffiths and Palmer (1972) began experiments to determine the extent of microbial involvement in the degradation of Amitraz in fouled washes as described above. They found that an inoculum of unsterile cattle faeces into nutrient broth containing Amitraz resulted in degradation of active ingredient. When the nutrient broth was replaced by non-sterile tap water there was no Amitraz degradation. They concluded that bacteria, which were present in faeces, were involved in Amitraz degra-

dation. However, for degradation to occur, not only was an inoculum of the relevant organism (or organisms) required but also a suitably nutritious medium.

In view of the fact that microbial involvement in Amitraz degradation had been indicated as shown by the above discussion, laboratory experiments to study this further were initiated. In the laboratory factors influencing degradation, e.g., temperature, pH, fouling, etc., can be tailored to allow investigation of a single factor. Clearly, this is not possible during a dip tank field trial as described in Chapter II.

In this thesis the term "degradation" is used to indicate loss of Amitraz active ingredient. A quantitative measure of this loss is "percentage degradation" (% degradation), which is the ratio of concentration of active ingredient at a specific time to the initial concentration of active ingredient. The method used to evaluate degradation was a "die-away" test (Payne et al., 1970) using gas chromatographic analysis of the active ingredient. Details of this analysis are given in Appendix A. The analysis allowed quantitative measurement of Amitraz degradation but degradation products of Amitraz were not detected on the chromatograms. In order to detect degradation products a thin layer chromatography

method was used. A discussion of this method is given in Chapter V.

Amitraz active ingredient is prepared in two forms, a wettable powder (W.P.) and a miscible oil (M.O.). At the start of this study only the W.P. formulation was available and so initial experiments were done using W.P. A number of problems were encountered when using W.P. (see Section 3.31) and later experiments were done using the M.O. formulation. In the M.O. the active ingredient is formulated with an emulsifier and a solvent.

3.2 Methods and materials

3.21 Media

The term percentage (%) to denote concentration in media refers to a weight per volume (wt/vol) concentration unless otherwise stated. The following media were used in the experiments described in this chapter:

Ye,P medium. This broth medium contained yeast extract (Difco) 0,5% + peptone (Difco) 0,5% in distilled water. Its pH was adjusted to pH 7 by the addition of NaOH. Ye,P agar medium was made by adding 1,5% agar (Oxoid No. 3) to Ye,P broth.

Ye,P medium + M.O. Ye,P broth or Ye,P agar was supplemented with 0,2% M.O. to give an active ingredient concentration of 0,02%. When supplementing medium with M.O. it was cooled to at least 50°C before adding M.O. Heating M.O. to 50°C for 10 min. does not inactivate the active ingredient.

Minimal salts medium (MM). The minimal salts medium contained (g/ℓ): NH_4Cl, 40,0; NH_4NO_3, 8,0; anhydrous Na_2SO_4, 16,0; K_2HPO_4, 24,0; KH_2PO_4, 8,0 and $MgSO_4.7H_2O$, 0,8. This medium was supplemented with 0,2% M.O. to give a concentration of 0,02% active ingredient. For minimal agar medium 1,5% Ion agar (Oxoid No. 2) was added to the minimal salts solution.

3.22 Determination of Amitraz concentrations, bacterial counts and pH values

Amitraz concentrations were determined by gas chromatography (see Appendix A). Numbers of bacteria in samples were determined on various agar media using the spread plate technique. Measurement of pH was with Merck pH indicator sticks. These determinations were done at the beginning of each experiment (day 0) and then at various time intervals during the experiment. Incubation of samples was at 30°C unless otherwise stated.

3.23 Degradation of Amitraz wettable powder

The basal medium used was autoclaved dip wash taken from the surface of the dip tank before dipping at week 45 of the Sea View field trial. After autoclaving, the dip wash showed no Amitraz activity. Four hundred millilitre aliquots of sterile dip wash, each containing 0,02% active ingredient of Amitraz W.P. and supplemented as shown in Table 8, were inoculated with 10 ml of non-sterile dip wash from Sea View dip tank. Each flask in a parallel series was inoculated with 10 ml of dip wash from Wiltonside dip tank. All flasks were incubated for 15 days before determining bacterial counts and Amitraz concentrations.

3.24 Comparison between degradation of miscible oil and wettable powder formulations of Amitraz

Ye,P medium + 0,015% glucose was used. Instead of using a large culture as in the previous experiment (see Section 3.23), 100 ml amounts of medium containing 0,1g W.P. or 0,2 ml M.O., i.e., 0,02% active ingredient, were inoculated with 2% (wet wt/vol) of sludge from the bottom of Wiltonside dip tank. Samples were incubated and assayed after 4 and 8 days.

3.25 Enrichment culture technique

Enrichment culture at pH 7. Using Ye,P broth + M.O., a sludge inoculum from Wiltonside was enriched for the bacteria most efficient at degrading Amitraz. Each stage in the enrichment process involved transferring 1 ml of the culture into 100 ml fresh medium containing Amitraz and incubating at 30°C for 3 weeks.

Enrichment cultures at pH 10,0 and pH 11,5. Once the pH 7 enrichment culture was established an inoculum from it was inoculated into Ye,P broths + M.O. buffered at pH 10,0 and pH 11,5 to enrich for bacteria able to degrade Amitraz at high pH. The bacterial counts and Amitraz levels were monitored for 18 days.

Preparation of inoculum from the enrichment culture. Bacteria from the enrichment culture (pH 7) were used to study the effect of various factors on degradation. The inoculum used in these experiments was prepared as follows: 50 ml of the enrichment culture were centrifuged at 4000 rpm; the cells that were harvested were washed twice with sterile 0,85% saline and then resuspended in 25 ml of sterile 0,85% saline. This saline suspension of bacteria was added to media at a dilution of 1 in 100.

3.26 Effect of yeast extract on degradation

To investigate the role of yeast extract in degradation the following media at pH 7 were used:

(a) 0,5% yeast extract + 0,5% peptone + 0,015% glucose

(b) 0,5% yeast extract + 0,5% peptone

(c) 0,5% yeast extract + 0,015% glucose

(d) 0,5% peptone + 0,015% glucose

(e) 0,5% yeast extract

(f) 0,5% peptone

(g) 0,015% glucose

Each medium was supplemented with M.O. (0,02% active ingredient) and inoculated with Wiltonside sludge (2%). The pH values were measured daily and the Amitraz concentrations on days 0, 3 and 6.

To determine the effect of the concentration of yeast extract on degradation, a basal medium of minimal salts medium (MM) containing 0,5% peptone and 0,02% active ingredient was supplemented with concentrations of yeast extract ranging from 0,001 to 0,5% (see Table 11). Samples were inoculated with an inoculum from the pH 7 enrichment culture. In addition MM + M.O. and MM + 0,5% P + M.O. were similarly inoculated. The control to detect possible non-microbial degradation consisted of

sterile Ye,P medium containing 0,2% M.O. Bacterial numbers, pH values and Amitraz concentrations of all samples were determined on days 0, 3 and 6.

3.27 Factors influencing Amitraz degradation

Effect of temperature on degradation. Ye,P broths + M.O. were inoculated with Wiltonside sludge (2%). Samples were assayed for Amitraz activity on days 0, 3, 6 and 9 after incubation at 10, 15, 20, 25, 30 and 37°C.

Effect of aeration on degradation. Ye,P broths + M.O. were inoculated with an enrichment culture inoculum and incubated anaerobically, standing and aerated by bubbling with a stream of filtered air. To obtain anaerobic conditions the bottles were sealed with rubber bungs and perfused with a mixture of CO_2 and H_2 for 60 min. Methylene blue (1%) was added as an indicator since it decolourizes in anaerobic conditions. Amitraz levels, pH values and bacterial counts (on Ye,P agar + M.O.) were determined on days 0, 3 and 6. The bacterial numbers in the anaerobic cultures were determined by plating aerobically and incubating the plates in anaerobic jars.

Effect of fouling on degradation. The source of fouling was freshly collected cattle faeces which had been autoclaved at 121°C for 30 min. A basal medium of 0,5%

peptone at pH 7 containing 0,02% active ingredient was fouled with the following percentages (wet wt/vol) of cattle faeces: 0,1; 0,5; 1,0; 2,0; 3,0; 4,0 and 8,0%. Samples were inoculated with an inoculum from the enrichment culture (pH 7). In addition Ye,P broth + M.O. and broth containing 0,5% peptone + M.O. were similarly inoculated. The control comprised 0,02% Amitraz in sterile 0,5% peptone.

Effect of pH on degradation. Ye,P broth + M.O. was buffered at the following pH values 7,0; 8,0; 9,0; 10,0; 11,0 and 11,5 and inoculated with bacteria from the enrichment culture. For pH 7-8 0,2 M phosphate buffer was used; for pH 9-10 glycine-NaOH buffer was used and for pH 11-11,5 hydroxide-chloride buffer was used (Dawson et al., 1969). Controls comprised sterile medium at each pH. Amitraz levels, pH values and bacterial counts were determined on days 0, 4 and 7.

Effect of $Ca(OH)_2$ on degradation. Ye,P broths + M.O. were supplemented with the following $Ca(OH)_2$ (Analar grade) concentrations: 0,4; 0,3; 0,2; 0,1; 0,05 and 0,01%. The bacterial inoculum was taken from the enrichment culture at pH 10 and was prepared as described in Section 3.25. Bacterial numbers were determined on days 0, 6 and 12 on Ye,P agar medium buffered at pH 7,0

and pH 10,5 and on the same media containing M.O. Amitraz concentrations and pH values were determined at the same time intervals.

3.3 Results

3.31 Degradation of Amitraz wettable powder

As described in Appendix A, Amitraz activity was usually determined after extracting the active ingredient from a 100 ml sample into xylene. However, it was found that there was a strong tendency for the wettable powder to stick to the glass of the flasks even when chemically clean glassware was used. In addition to this, in flasks where there had been considerable bacterial growth, a pellicle formed and on shaking the flasks the pellicle produced lumps in which the white dip powder was trapped. Consequently, even after the flasks had been shaken for 4h, the contents were not homogeneous and so a truly representative 100 ml sample could not be taken for assaying the Amitraz activity. Therefore the whole 400 ml sample was extracted and assayed after a sample for bacterial analysis had been taken.

The results in Table 8 show that the greatest de-

Table 8. Degradation of Amitraz wettable powder by bacteria from Sea View and Wiltonside dip tanks in supplemented sterile dip wash.

Inoculum	Supplement	Bacterial count		% Amitraz remaining on day 15
		Day 0 (Bacteria/ml x 10^5)	Day 15 (Bacteria/ml x 10^8)	
None[a]	None	0	0	94
SV[b]	None	3,0	1,7	93
SV	0,15% tryptone + 0,015% glucose	1,4	1,5	72
SV	0,5% yeast extract + 0,015% glucose	2,7	3,1	36
SV	0,5% NaCl + 0,5% glucose	3,2	2,8	85
W[c]	None	1,1	1,1	78
W	0,15% tryptone + 0,015% glucose	1,6	2,5	71
W	0,5% yeast extract + 0,015 glucose	1,4	2,8	21
W	0,5% NaCl + 0,5% glucose	3,1	3,0	77

[a] Sterile control

[b] SV = 10 ml of inoculum from Sea View dip tank

[c] W = 10 ml of inoculum from Wiltonside dip tank

gradation occurred in the two media which had been supplemented with 0,5% yeast extract + 0,015% glucose. It is interesting to note that both populations of bacteria, i.e., from Wiltonside and from Sea View dip tanks, were able to degrade Amitraz rapidly in the laboratory although very little degradation occurred in the field at Sea View dip tank. There was much less degradation in the other media which were supplemented with glucose, but not with yeast extract; therefore it is concluded that the presence of yeast extract in some way facilitates degradation. Since the bacteria in the media containing yeast extract did not reach markedly higher numbers than those growing without yeast extract the increase in degradation in the medium containing 0,5% yeast extract + 0,015% glucose is not caused by enhanced bacterial growth.

3.32 Comparison between degradation of the two Amitraz formulations

The results of this comparison are shown in Fig. 7. Each value is the mean of two samples assayed by gas chromatography. The two values obtained from samples containing W.P. were not highly reproducible, whereas there was good agreement in values obtained from samples containing M.O. The M.O. formulation is more susceptible

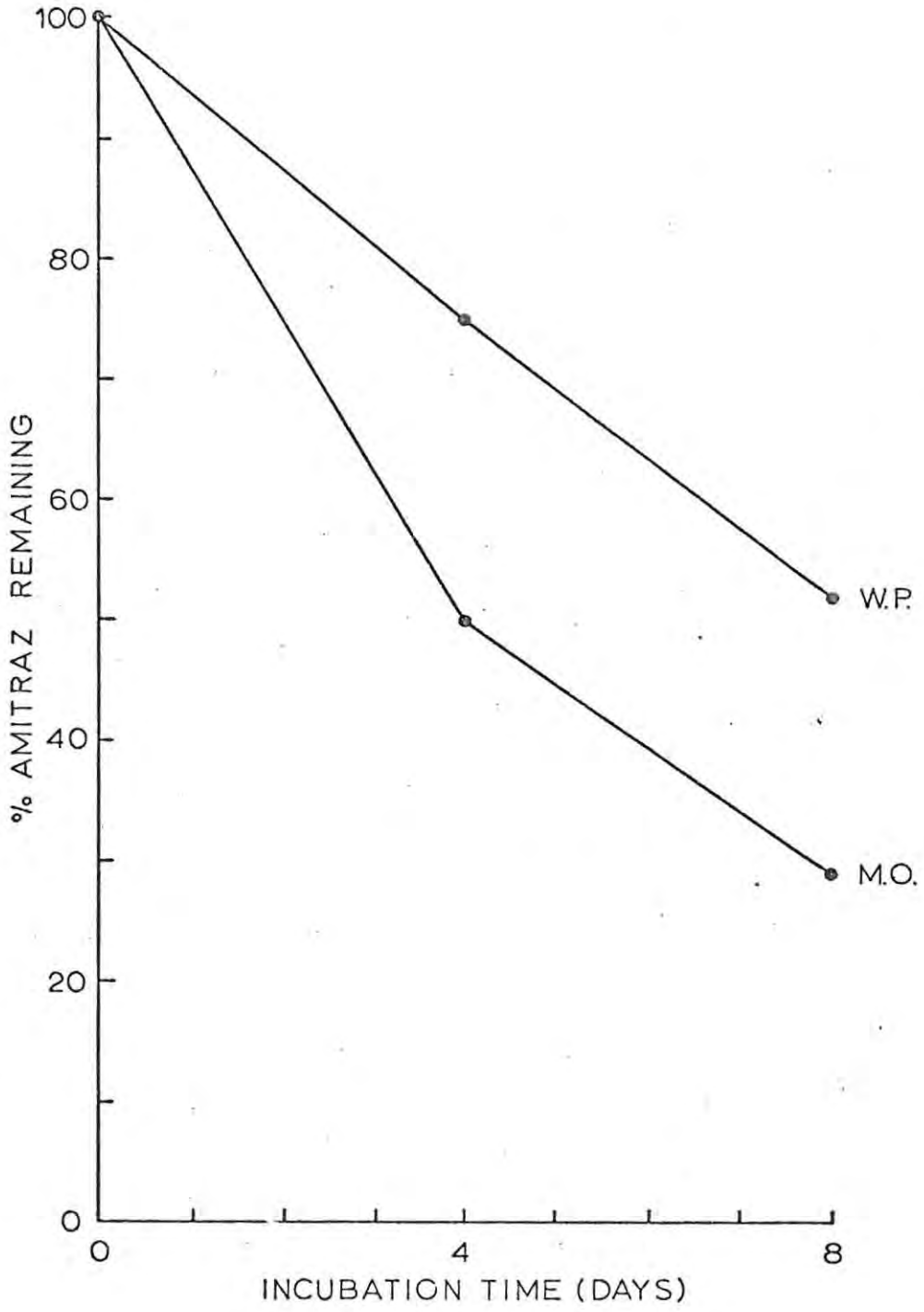


Fig.7. Comparison between degradation of miscible oil (M.O.) and wettable powder (W.P.) formulations of Amitraz in broth media containing Wiltonside sludge inocula.

to degradation than the W.P. formulation. Since this is so, and since it gives more reproducible results the rest of the work described in this thesis was done using the Amitraz M.O. formulation.

3.33 Enrichment cultures

Neutral pH enrichment. The enrichment process at pH 7 resulted in a bacterial population which was able to degrade, in 6 days, approximately 70% of the available Amitraz.

High pH enrichment. Amitraz levels in the media at pH 10,0 and pH 11,5 are shown in Fig. 8. There was minimal degradation at pH 11,5. The rate and degree of degradation at pH 10,0 were much less than those at pH 7. The pH values and bacterial counts of the samples are given in Table 9. In both cases the original pH of the growth medium dropped by about 1 pH unit. Approximately $4x10^3$ bacteria per ml (assayed on plates with and without M.O. at pH 10) were inoculated into the medium at pH 11,5. However, even though 0,3 ml of the undiluted sample was plated on each agar medium on days 6, 12 and 18, no bacterial growth was observed (see Table 9). Therefore the bacteria had been killed in this medium. The bacteria inoculated into the medium at pH 10,0 reached a

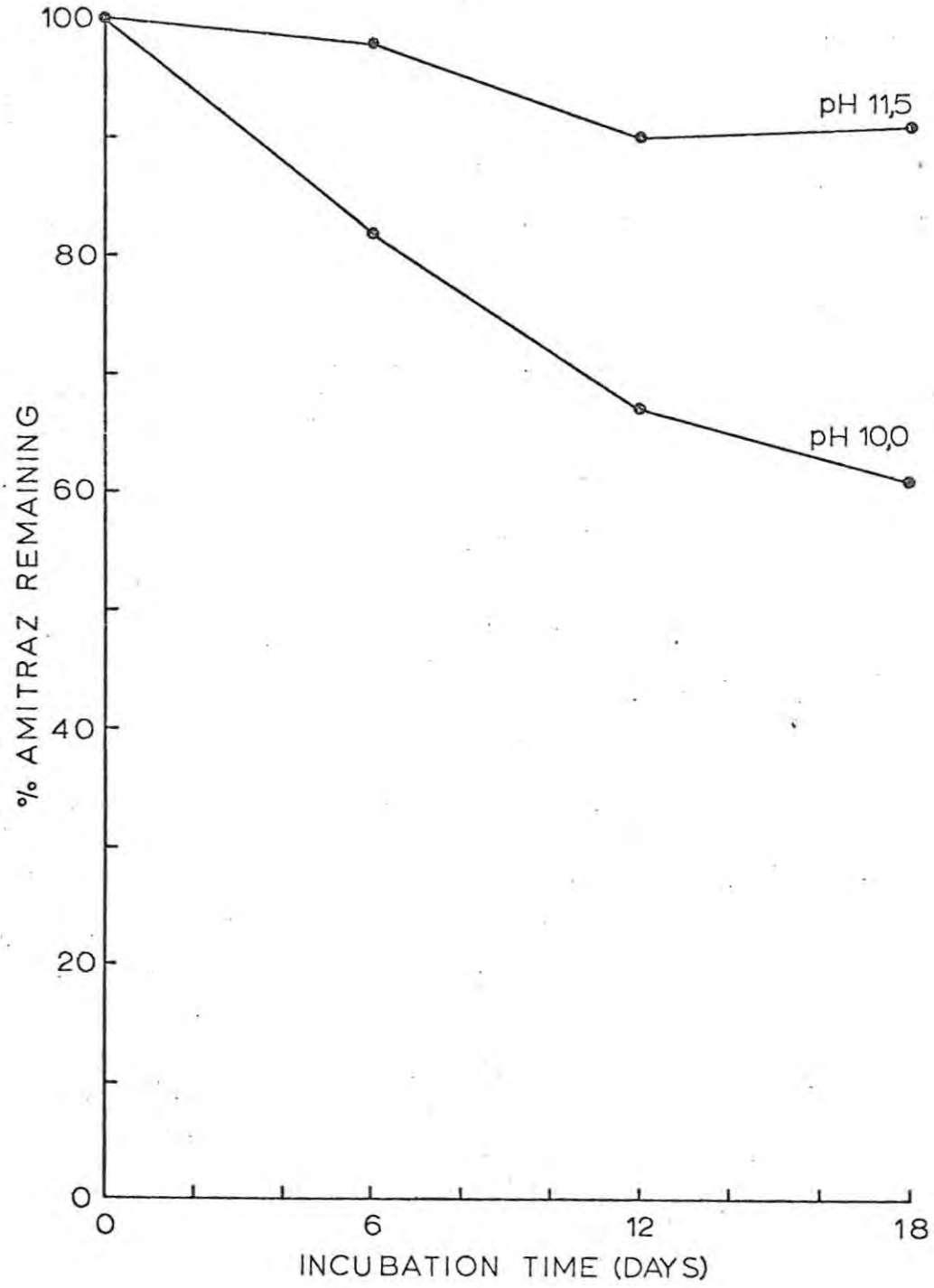


Fig.8. Degradation of Amitraz M.O. by bacteria from the pH 7 enrichment culture in Ye,P broths buffered at pH 11,5 and pH 10,0.

Table 9. High pH enrichment cultures. Bacterial counts determined on various Ye,P agar media after growth of inocula from the enrichment culture at pH 7 in Ye,P broths + M.O. initially at pH 10,0 and pH 11,0.

pH of growth medium				Ye,P agar assay medium	Bacterial count (Bacteria/ml)			
Day 0	Day 6	Day 12	Day 18		Day 0	Day 6	Day 12	Day 18
10,0	9,5	9,5	9,0	pH 7	$1,9x10^4$	$2,0x10^7$	$2,4x10^5$	$1,2x10^7$
10,0	9,5	9,5	9,0	pH 7 +M.O.	$1,4x10^4$	$8,0x10^7$	$1,0x10^3$	$1,6x10^4$
10,0	9,5	9,5	9,0	pH 10	$4,4x10^3$	$1,3x10^7$	$7,0x10^4$	$5,2x10^6$
10,0	9,5	9,5	9,0	pH 10+M.O.	$4,1x10^3$	$9,2x10^6$	$3,4x10^4$	$1,8x10^6$
11,5	11,0	10,5	10,5	pH 7	$1,9x10^4$	0	0	0
11,5	11,0	10,5	10,5	pH 7 +M.O.	$1,4x10^4$	0	0	0
11,5	11,0	10,5	10,5	pH 10	$4,4x10^3$	0	0	0
11,5	11,0	10,5	10,5	pH 10+M.O.	$4,1x10^3$	0	0	0

level of $1,2x10^7$ bacteria per ml (assayed on Ye,P agar at pH 7 by day 18. However, when assayed on Ye,P agar + M.O. at pH 7, the bacterial count of the same sample was 750-fold less. This phenomenon was not observed when the bacteria were plated onto media at pH 10,0, although the count determined in the presence of M.O. is slightly less than that determined in its absence. These results indicate that under certain conditions M.O. inhibits bacterial growth. This inhibition is discussed in Appendix B.

After 3 transfers of bacteria growing at pH 10,0 to medium at pH 11,5, Amitraz determinations and bacterial counts showed a similar result to that discussed above. No bacterial population has been enriched to degrade Amitraz at pH 11,5.

3.34 Effect of yeast extract on degradation

Role of yeast extract in degradation. From the graphs in Fig. 9 it is clear that the greatest Amitraz degradation (approximately 50%) occurred in all the media containing 0,5% yeast extract, i.e., in samples (a), (b), (c) and (e). There was approximately 25% degradation in the media which contained 0,5% peptone + 0,015% glucose and 0,5% peptone, i.e., in samples (d) and (f).

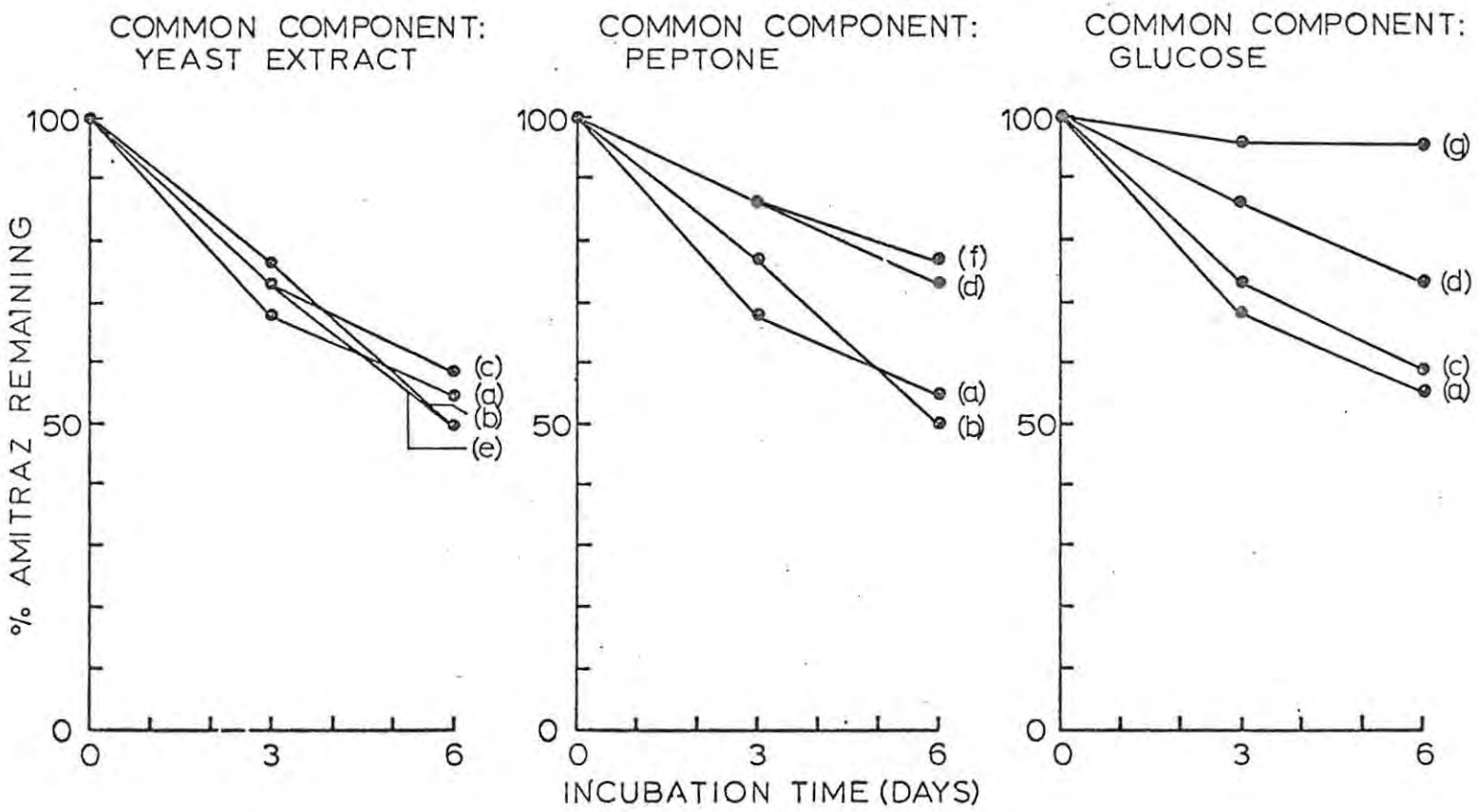


Fig.9. The effect of media containing various combinations of yeast extract, peptone and glucose on degradation of Amitraz M.O. by bacteria from Wiltonside sludge. The media (a)-(g) are listed in Section 3.26.

There was minimal degradation in medium (g) which contained 0,015% glucose only.

Table 10 shows that in all the media containing 0,5% yeast extract there was a drop in pH by day 1. The largest drop was in medium (a), whose pH fell to 5,5 on day 1 and remained below pH 7 until day 5. The smallest drop was in medium (b), whose pH dropped to 6,7 on day 1 but increased to pH 7 on day 2 and pH 8,0 on day 6. McCarthy (1972) showed that Amitraz was unstable in acid conditions and so in the case of medium (a) Amitraz could have undergone chemical degradation as a result of the production of acid conditions in the medium. However, this is unlikely since the conditions in medium (g) remained acid for the duration of the experiment and negligible degradation was detected in this medium. To minimize degradation that may occur due to the production of acid, medium (b), i.e., 0,5% yeast extract + 0,5% peptone (Ye,P medium) was chosen as the medium to be used for future experiments since less acid was produced in this medium than in the others.

Effect of concentration of yeast extract on degradation. The Amitraz concentrations are illustrated in Fig. 10 and the bacterial counts and pH values are given in

Table 10. Daily measurements of pH in various media supplemented with yeast extract, peptone and glucose and inoculated with Wiltonside sludge.

Day	pH values of media[a]						
	(a)	(b)	(c)	(d)	(e)	(f)	(g)
0	7,0	7,0	7,0	7,0	7,0	7,0	7,0
1	5,5	6,7	6,0	7,0	6,5	7,0	6,0
2	5,5	7,0	6,5	7,0	6,7	7,5	5,5
3	6,0	7,5	7,0	7,5	7,5	7,5	5,5
4	6,5	8,0	7,5	7,7	8,0	7,5	5,5
5	7,0	8,5	8,0	8,0	8,2	8,0	5,5
6	7,5	8,5	8,0	8,0	8,5	8,0	5,5

[a] The media (a)-(g) are listed in Section 3.26

Table 11. In the sterile control there was minimal degradation, the pH did not change and the control remained sterile throughout the experiment. Therefore any degradation which occurred in the inoculated samples was caused by bacteria. In the minimal salts medium there was minimal degradation and no change in pH although the bacterial count had increased approximately 100-fold by day 3, with no further increase by day 6. In MM + 0,5% peptone and MM + 0,5% peptone + 0,001% yeast extract there was 74% and 78% Amitraz remaining, respectively. The count of bacteria assayed on Ye,P agar and Ye,P agar + M.O. increased approximately 1000-fold and the count assayed on MM agar + M.O. increased approximately 100-fold by day 3. The counts decreased slightly after day 3.

As the yeast extract concentration in the medium increased from 0,005% to 0,5% its presence showed an increasing effect on degradation. The maximum degradation occurred in medium containing 0,5% yeast extract. In media containing from 0,005% to 0,5% yeast extract the bacterial counts increased approximately 1000-fold in the first 3 days with only a slight increase in the following three days. Although the bacterial count in

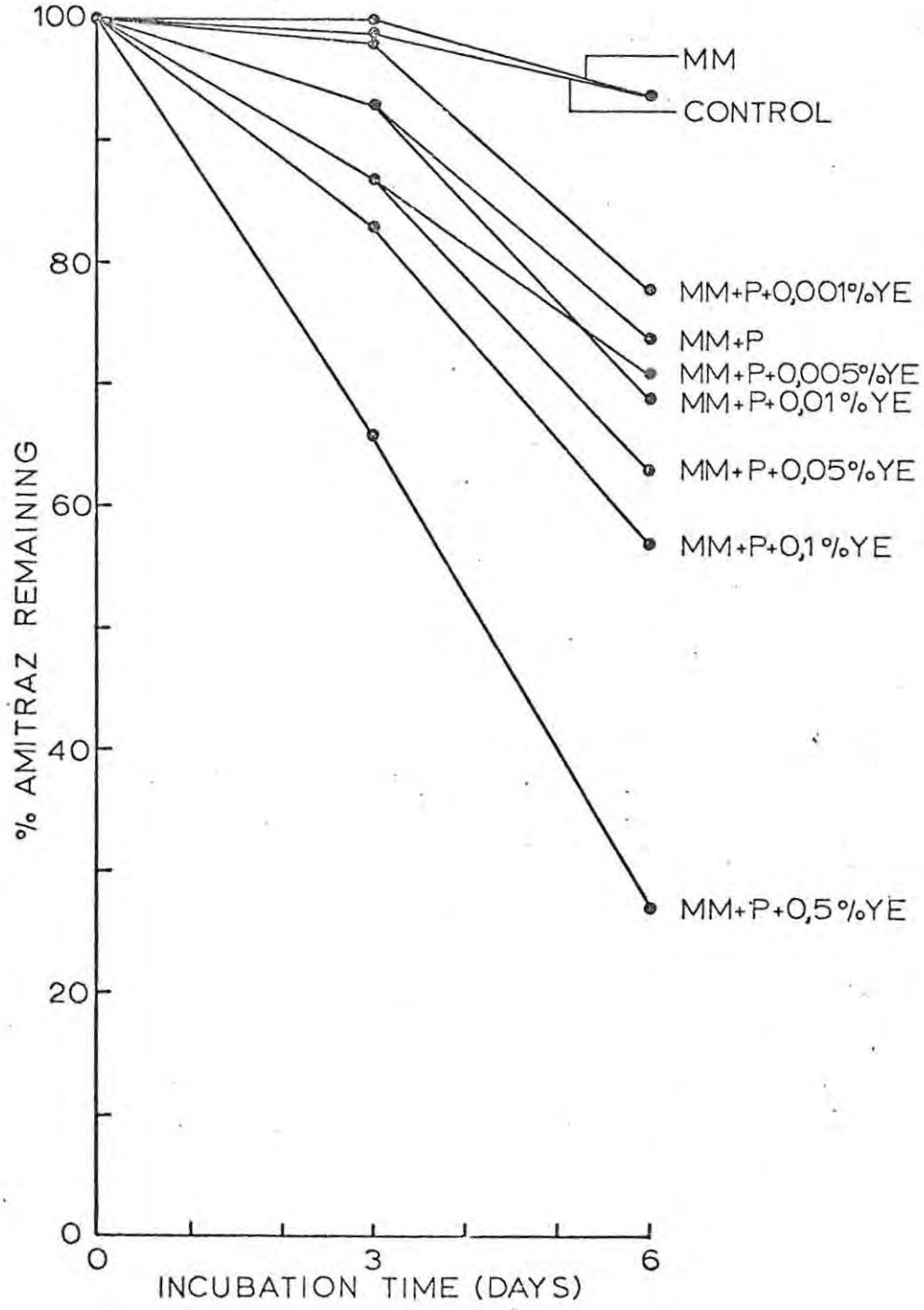


Fig.10. Effect of increasing concentrations of yeast extract (YE) on degradation of Amitraz M.O. by bacteria from the pH 7 enrichment culture in minimal medium (MM) + 0,5% peptone (P).

Table 11. Bacterial counts and pH values determined after growth of bacteria from the enrichment culture (pH 7) in minimal medium, minimal medium + 0,5% peptone and minimal medium + 0,5% peptone supplemented with various concentrations of yeast extract.

Medium	Bacterial count									pH		
	Ye,P agar			Ye,P agar + M.O.			MM agar + M.O.					
	Day 0 (Bacteria/ $ml \times 10^4$)	Day 3 (Bacteria/ $ml \times 10^7$)	Day 6 (Bacteria/ $ml \times 10^7$)	Day 0 (Bacteria/ $ml \times 10^4$)	Day 3 (Bacteria/ $ml \times 10^7$)	Day 6 (Bacteria/ $ml \times 10^7$)	Day 0 (Bacteria/ $ml \times 10^4$)	Day 3 (Bacteria/ $ml \times 10^7$)	Day 6 (Bacteria/ $ml \times 10^7$)	Day 0	Day 3	Day 6
MM[a]	6,9	1,5	0,6	1,5	0,8	0,5	2,0	0,3	0,1	7,0	7,0	7,0
MM+0,5% P[b]	6,9	5,1	7,9	1,5	3,6	2,6	2,0	0,6	0,1	7,0	7,5	7,5
MM+0,5% P+ 0,001% Ye[c]	6,9	5,2	5,7	1,5	3,7	1,7	2,0	0,7	0,1	7,0	7,5	7,5
MM+0,5% P+ 0,005% Ye	6,9	5,2	7,5	1,5	2,3	1,7	2,0	1,8	1,7	7,0	7,5	7,5
MM+0,5% P+ 0,01% Ye	6,9	5,5	6,8	1,5	2,4	1,7	2,0	1,1	1,2	7,0	7,5	8,0
MM+0,5% P+ 0,05% Ye	6,9	5,2	7,2	1,5	1,9	2,0	2,0	1,2	1,2	7,0	7,5	8,0
MM+0,5% P+ 0,1% Ye	6,9	7,9	9,2	1,5	1,9	1,9	2,0	1,6	1,3	7,0	7,5	8,0
MM+0,5% P+ 0,5% Ye	6,9	9,0	9,5	1,5	5,7	6,3	2,0	1,0	1,0	7,0	7,5	8,5
MM+0,5% P+ 0,5% Ye (Sterile control)	0	0	0	0	0	0	0	0	0	7,0	7,0	7,0

[a] MM = minimal salts medium

[b] P = peptone

[c] Ye = yeast extract

the medium containing 0,5% yeast extract was slightly higher than in the other media, this small increase in numbers is unlikely to account for the much greater degree of degradation in this medium.

3.35 Factors influencing Amitraz degradation

Temperature. As the temperature of incubation increased the Amitraz degraded faster (see Fig. 11). At 37°C the rate of degradation was fastest during the first 3 days. During the last 3 days there was little change in Amitraz activity. At 30°C degradation occurred fastest between days 3 and 6 and slowed down after day 6. At 25°C there was an initial lag in which degradation was slow; from days 3 to 9 Amitraz activity was lost at a faster rate. At 20, 15 and 10°C the lag period in which degradation was slow was 6 days; from days 6 to 9 the rate of degradation increased. Lowering the temperature did not, therefore, prevent degradation although it did influence the lag period before degradation began and the rate of degradation. The maximum rate of degradation at 10°C was approximately 3 times less than the maximum rate at 37°C.

Aeration. Throughout the experiment the sterile control remained uncontaminated and showed minimal loss of Amitraz activity (see Table 12 and Fig. 12). Under anaerobic

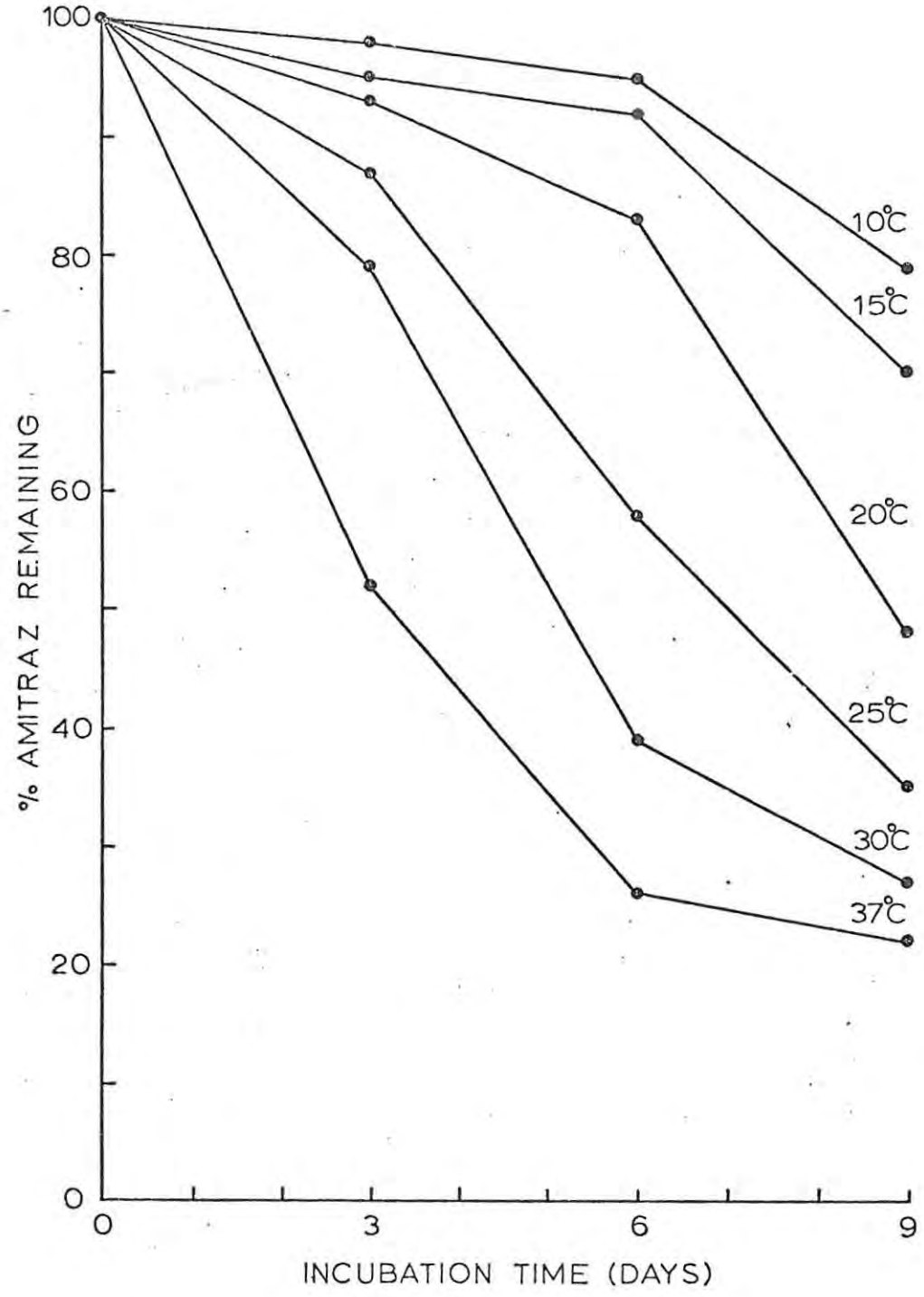


Fig.11. Effect of temperature on degradation of Amitraz M.O. by bacteria from Wiltonside sludge.

Table 12. Bacterial counts and pH values determined after growth of pH 7 enrichment culture inocula in Ye, P broths + M.O. incubated anaerobically, standing and aerated.

Incubation conditions	Bacterial count (Bacteria/ml)			pH		
	Day 0	Day 3	Day 6	Day 0	Day 3	Day 6
Anaerobic	$9,8x10^4$	$7,1x10^5$	$3,4x10^5$	7,0	6,0	6,0
Standing	$1,1x10^6$	$4,0x10^7$	$1,4x10^7$	7,0	7,5	8,5
Bubbled	$1,1x10^6$	$8,9x10^8$	$2,6x10^9$	7,0	8,5	9,0
Sterile control	0	0	0	7,0	7,0	7,0

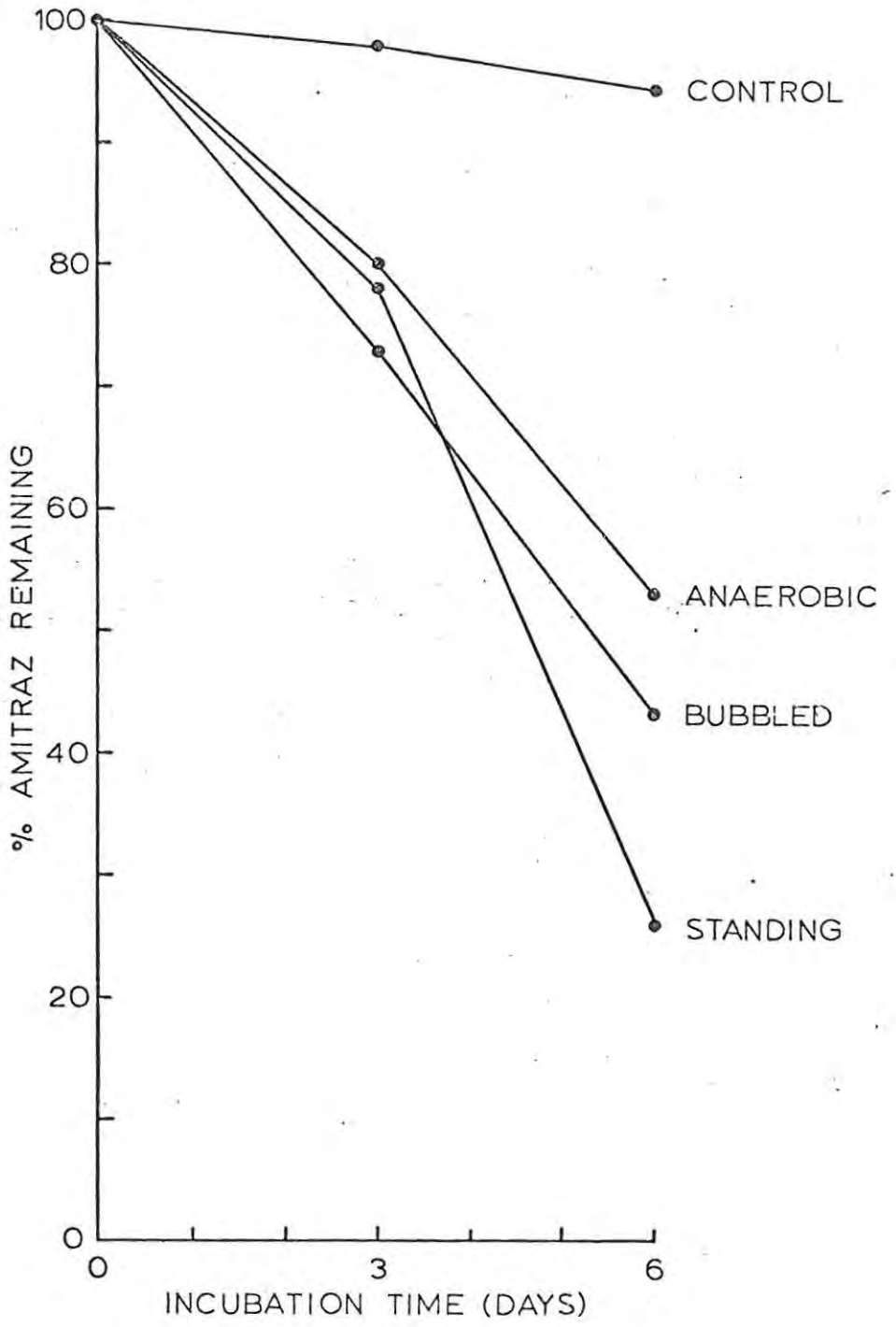


Fig.12. Effect of aeration on degradation of Amitraz M.O. by bacteria from the enrichment culture at pH 7.

conditions 47% of Amitraz activity had been lost and the pH had dropped to 6,0 by day 6. There was approximately a 7-fold increase in bacterial numbers by day 3 and by day 6 there was a slight drop. The standing culture showed a similar initial rate of degradation, but from days 3 to 6 there was much more rapid degradation resulting in only 26% of the Amitraz remaining on day 6. The bacterial count of the standing culture increased approximately 40-fold by day 3 and then dropped slightly by day 6. The pH of the standing culture increased to pH 8,5 on day 6. The Amitraz in the bubbled culture degraded at the same rate throughout the experiment and by day 6 43% of it remained. The pH of this culture rose to pH 9,0 on day 6. In contrast to the anaerobic and standing cultures there was a much sharper rise in bacterial numbers in the bubbled culture; by day 3 the count had increased almost 1000-fold and it continued to increase from day 3 to day 6 to reach a final level of $2,6 \times 10^9$ bacteria per ml. Despite the fact that there was much more vigorous bacterial growth in the bubbled culture than in the standing culture, the Amitraz degraded to a lower level in the standing culture.

Effect of Fouling. The results of the experiment are summarized in Table 13 and Fig. 13; the following obser-

Table 13. Bacterial counts and pH values determined after growth of pH 7 enrichment culture inocula in 0,5% peptone, 0,5% peptone supplemented with various concentrations of sterile cattle faeces and 0,5% peptone + 0,5% yeast extract.

Medium	Bacterial count						pH		
	Ye,P agar			Ye,P agar + M.O.					
	Day 0 (Bacteria/ $mlx10^5$)	Day 3 (Bacteria/ $mlx10^7$)	Day 6 (Bacteria/ $mlx10^7$)	Day 0 (Bacteria/ $mlx10^5$)	Day 3 (Bacteria/ $mlx10^7$)	Day 6 (Bacteria/ $mlx10^7$)	Day 0	Day 3	Day 6
0,5% P	1,6	5,2	2,6	1,1	2,9	0,6	7,0	7,5	7,5
0,5% P + 0,1% faeces	1,6	7,0	2,1	1,1	3,2	1,1	7,0	7,5	7,5
0,5% P + 0,5% faeces	1,6	7,5	3,9	1,1	2,0	1,5	7,0	7,5	7,5
0,5% P + 1,0% faeces	1,6	7,5	5,3	1,1	3,9	2,2	7,0	7,5	8,0
0,5% P + 2,0% faeces	1,6	6,1	4,8	1,1	4,2	2,1	7,0	7,5	8,0
0,5% P + 3,0% faeces	1,6	6,8	5,2	1,1	4,1	2,4	7,0	7,5	8,0
0,5% P + 4,0% faeces	1,6	15,0	2,6	1,1	7,2	2,0	7,0	7,5	8,0
0,5% P + 8,0% faeces	1,6	25,0	5,9	1,1	17,0	2,9	7,0	7,5	8,0
0,5% P + 0,5% yeast extract	1,6	17,0	2,7	1,1	13,0	1,0	7,0	7,5	8,5
0,5% P (Sterile control)	0	0	0	0	0	0	7,0	7,0	7,0

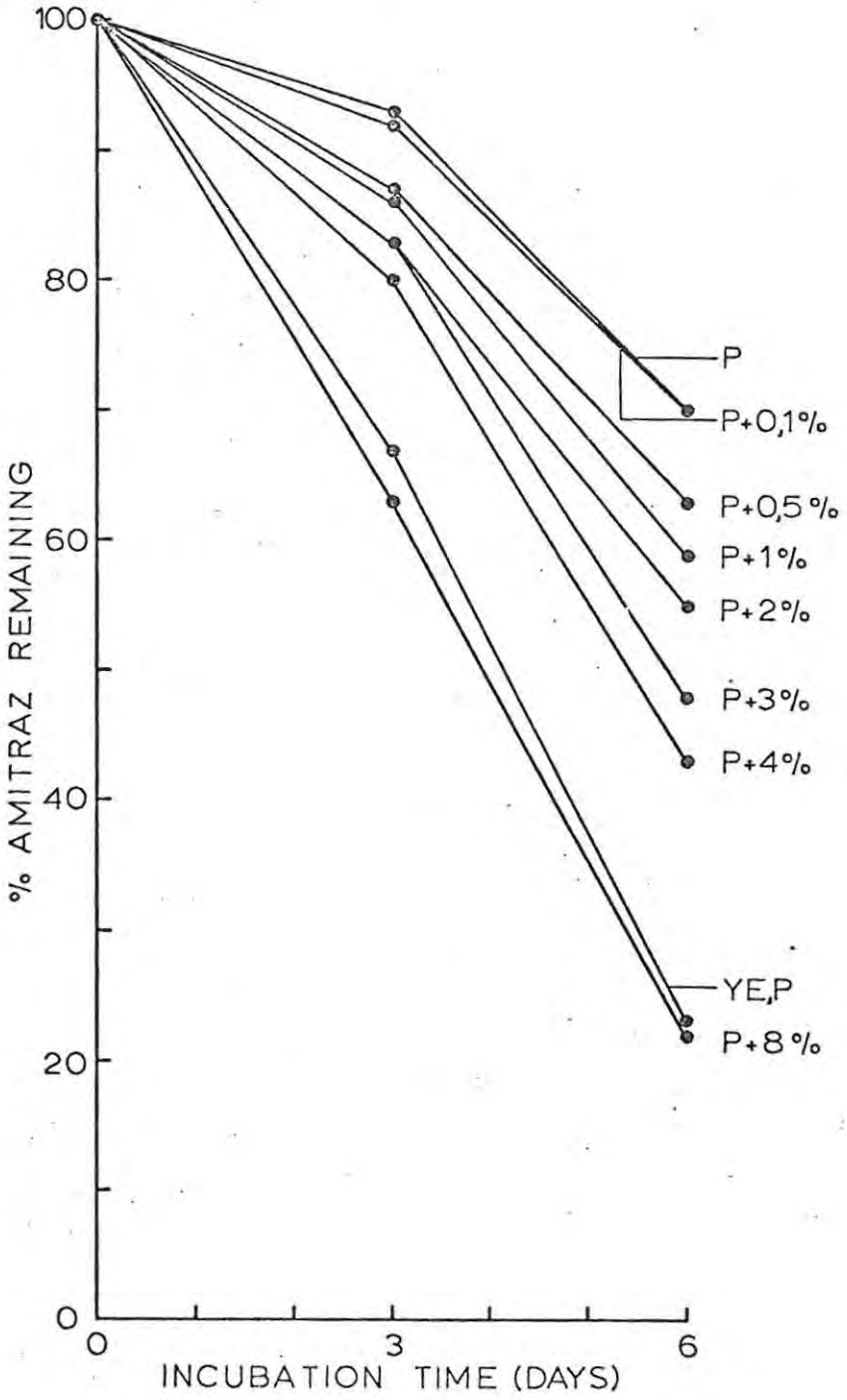


Fig.13. Effect of increasing levels of fouling on Amitraz degradation by bacteria from the pH 7 enrichment culture in 0,5% peptone (P). The source of fouling was sterile cattle faeces.

vations are noteworthy: With increase in fouling there was an increase in Amitraz degradation. In the medium fouled with 8% faeces there was almost identical degradation to that in the medium supplemented with 0,5% yeast extract. In these media degradation occurred at the same rate throughout the experiment. In contrast to this, in all the other samples there was a slower rate of degradation during the first 3 days, although the rate increased during the last 3 days to become approximately the same as that in the 8% fouled medium. In the medium which was 0,1% fouled the degradation was the same as that in medium containing 0,5% peptone only.

The marked increase in degradation in the 8% fouled medium and in the medium containing 0,5% yeast extract was not accompanied by a marked increase in bacterial growth.

Effect of pH. Since there was minimal degradation in the sterile controls Amitraz was stable in the buffers used. The bacteria from the enrichment culture did not survive when inoculated into medium at pH 11,5 (see Table 14) and there was no Amitraz degradation (see Fig. 14). By day 7 the pH of this medium had dropped to 11,0, as had the pH of the sterile control. The bacterial count in the medium initially at pH 11,0 had decreased

Table 14. Growth of bacteria from the pH 7 enrichment culture in Ye,P + M.O. media buffered at various pH values and the changes in pH values in inoculated and sterile samples.

Original pH of Ye,P medium	Bacterial count (Bacteria/ml)			pH		
	Day 0	Day 4	Day 7	Day 0	Day 4	Day 7
11,5 (Inoculated)	$8,7x10^5$	0	0	11,5	11,5	11,0
11,5 (Sterile)	0	0	0	11,5	11,5	11,0
11,0 (Inoculated)	$8,7x10^5$	$2,1x10^5$	$3,4x10^7$	11,0	10,5	9,5
11,0 (Sterile)	0	0	0	11,0	11,0	10,5
10,0 (Inoculated)	$8,7x10^5$	$2,4x10^7$	$2,1x10^7$	10,0	10,0	10,0
10,0 (Sterile)	0	0	0	10,0	10,0	10,0
9,0 (Inoculated)	$8,7x10^5$	$3,0x10^7$	$2,2x10^7$	9,0	9,0	9,0
9,0 (Sterile)	0	0	0	9,0	9,0	9,0
8,0 (Inoculated)	$8,7x10^5$	$2,3x10^7$	$1,5x10^7$	8,0	8,0	8,5
8,0 (Sterile)	0	0	0	8,0	8,0	8,0
7,0 (Inoculated)	$8,7x10^5$	$3,0x10^7$	$2,2x10^7$	7,0	7,0	7,5
7,0 (Sterile)	0	0	0	7,0	7,0	7,0

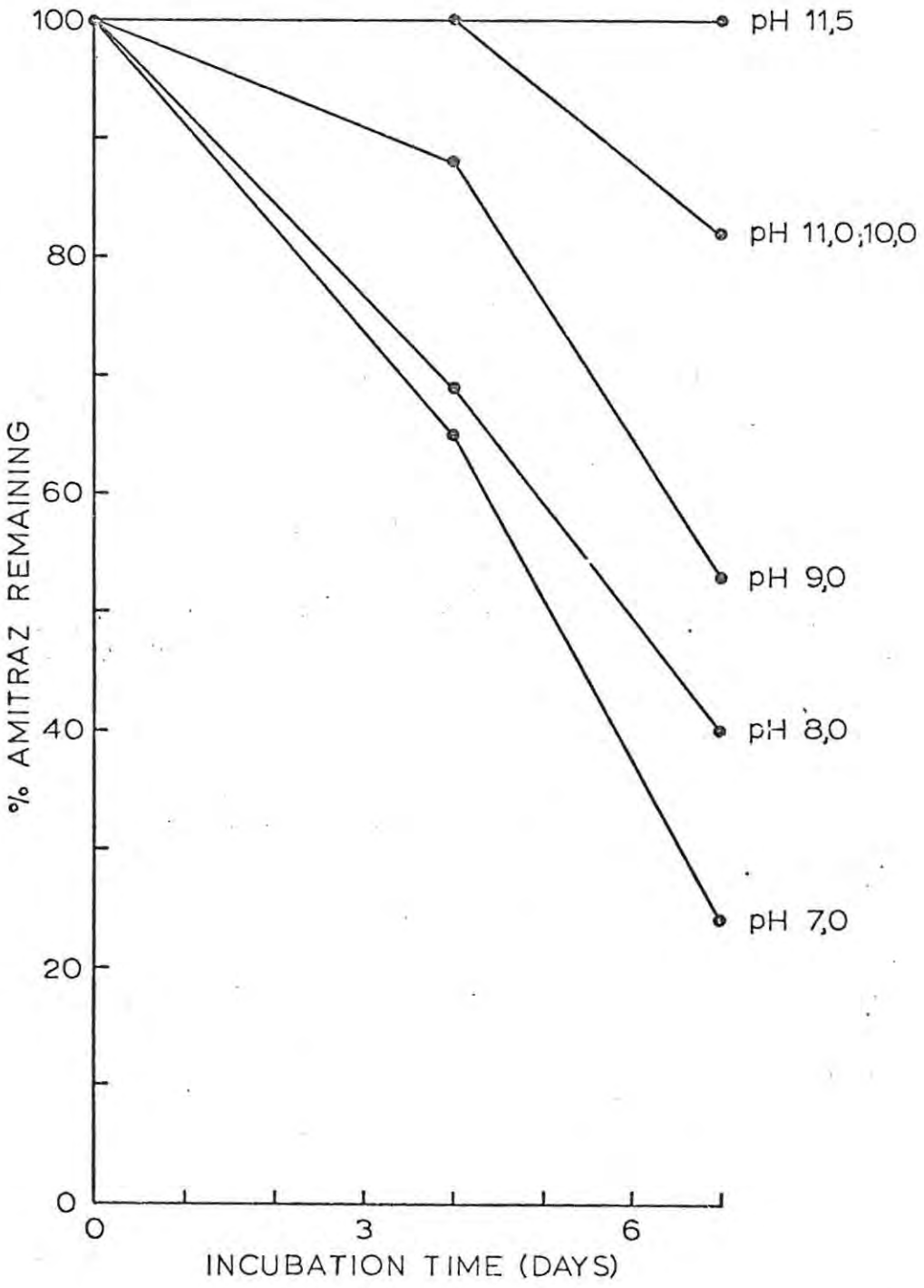


Fig.14. Degradation of Amitraz M.O. by bacteria from the enrichment culture (pH 7) in Ye,P media buffered at the following pH values: 11,5; 11,0; 10,0; 9,0; 8,0 and 7,0.

approximately 4-fold by day 4 and there had been no Amitraz degradation. From day 4 to day 7 the pH of the medium dropped to 9,5 and there was 100-fold increase in bacterial count; during this time 18% of the Amitraz degraded. It is possible that the bacterial growth in this medium could have resulted in the production of acid, thus lowering the pH to 9,5. At pH values of 10,0; 9,0; 8,0 and 7,0 there was rapid bacterial growth during the first 4 days, resulting in approximately 100-fold increase in count; from day 4 there was a slight drop. The pH values of the sterile controls did not change over this period. At pH 10,0 there was no Amitraz degradation for the first 4 days, but by day 7 it had degraded to the same level as in the medium originally at pH 11,0. In the medium at pH 9,0 the initial rate of degradation was slow but by day 7 approximately 50% of Amitraz activity had been lost. At pH 8,0 and pH 7,0 degradation rates were approximately the same throughout the 7 day period, more degradation occurring at pH 7,0.

The rate of degradation increased with decrease in pH; the optimum pH for degradation being pH 7,0. Since Amitraz is unstable in acid conditions (McCarthy, 1972) this experiment was not done at pH values below neutral pH.

Effect of $Ca(OH)_2$. There was minimal Amitraz degradation in the samples containing 0,4; 0,3; 0,2 and 0,1% $Ca(OH)_2$ and in the sterile control (see Table 15). In the medium containing 0,05% $Ca(OH)_2$ there was no degradation by day 6, but rapid loss of Amitraz activity occurred between days 6 and 12. In the medium containing 0,01% $Ca(OH)_2$ there was maximum degradation and this occurred at approximately the same rate throughout the 12 day period.

Concentrations of $Ca(OH)_2$ from 0,2-0,4% apparently caused the death of the inoculated bacteria (see Table 16). Whether this resulted in total sterilization of the medium is uncertain since only 0,1 ml samples were assayed. To determine whether or not total sterilization occurred larger samples need to be assayed. The sampling technique used explains why the culture containing 0,1% $Ca(OH)_2$ showed no bacterial contamination on day 6 but on day 12 a count of $3,3x10^2$ bacteria per ml was recorded.

3.4 Discussion

As shown in Section 3.32, the M.O. formulation of Amitraz was found to be preferable to the W.P. formula-

Table 15. Effect of $Ca(OH)_2$ concentration on pH and on Amitraz degradation in Ye,P media + M.O. inoculated with bacteria from the pH 10 enrichment culture.

% $Ca(OH)_2$ in growth medium	% Amitraz remaining			pH		
	Day 0	Day 6	Day 12	Day 0	Day 6	Day 12
0,4	100	100	100	12,5	12,5	12,5
0,3	100	100	93	12,0	12,0	12,0
0,2	100	100	95	12,0	10,5	10,5
0,1	100	100	93	9,5	9,0	9,0
0,05	100	100	36	8,5	8,5	9,0
0,01	100	69	31	7,5	8,0	8,5
0 (Sterile control)	100	94	94	7,0	7,0	7,0

Table 16. Effect of $Ca(OH)_2$ concentration on growth of bacteria from the pH 10 enrichment culture in Ye,P media + M.O. Bacterial counts were determined on various Ye,P agar assay media.

% $Ca(OH)_2$ in growth medium	Ye,P agar assay medium	Bacterial count (Bacteria/ml)		
		Day 0	Day 6	Day 12
0,2; 0,3; 0,4	pH 7	$1,6x10^5$	0	0
	pH 7 + M.O.	$1,0x10^3$	0	0
	pH 10	$3,0x10^5$	0	0
	pH 10 + M.O.	$2,7x10^5$	0	0
0,1	pH 7	$1,6x10^5$	0	$3,3x10^2$
	pH 7 + M.O.	$1,0x10^3$	0	$1,4x10^2$
	pH 10	$3,0x10^5$	0	0
	pH 10 + M.O.	$2,7x10^5$	0	0
0,05	pH 7	$1,6x10^5$	$9,6x10^6$	$7,6x10^5$
	pH 7 + M.O.	$1,0x10^3$	$6,4x10^6$	$2,3x10^5$
	pH 10	$3,0x10^5$	0	0
	pH 10 + M.O.	$2,7x10^5$	0	0
0,01	pH 7	$1,6x10^5$	$3,5x10^6$	$4,3x10^6$
	pH 7 + M.O.	$1,0x10^3$	$1,9x10^6$	$2,8x10^6$
	pH 10	$3,0x10^5$	$3,8x10^6$	$2,3x10^6$
	pH 10 + M.O.	$2,7x10^5$	$1,5x10^6$	$3,4x10^6$

tion for laboratory experiments since it was easier to work with, gave more reproducible results and degraded more rapidly. In the field, W.P. is generally used in dip tanks and M.O. is used in spray races. When using a spray race the dip wash is usually made up just before spraying begins and so the instability of the M.O. does not limit its use.

Initial experiments with W.P. indicated that the presence of 0,5% yeast extract + 0,015% glucose enhanced Amitraz degradation. Using the M.O. formulation it was found that maximum degradation occurred in media containing 0,5% yeast extract and the presence or absence of 0,015% glucose in the medium did not affect the degradation of Amitraz. There was no loss of Amitraz activity in a sterile control incubated under the same conditions, therefore the degradation which occurred in unsterile media was due to the presence of bacteria.

In MM + M.O., inoculated with bacteria from the pH 7 enrichment culture, there was minimal loss of active ingredient although there was 100-fold increase in bacterial numbers. Hence the bacteria did not use the active ingredient as a carbon source; they must have utilized the emulsifier and solvent components of the M.O. for growth. It was impossible to test the ability of the bacteria to

utilize pure active ingredient as their sole carbon source since the active ingredient is totally insoluble in water. The fact that the addition of increasing amounts of yeast extract to the minimal medium resulted in greater Amitraz degradation indicates that a co-metabolic process is involved in this system. This requirement for an additional nutrient source parallels observations reported by Bernade et al. (1965), Sethunathan (1972b) and Sagardia et al. (1975). Bernade et al. found that the addition of glucose to lake water containing alkyl benzene sulphonate resulted in an approximately 2-fold increase in the rate of degradation over that obtained if no glucose were present. Sethunathan reported that the addition of yeast extract to the incubation mixture enhanced anaerobic degradation of Lindane by a *Clostridium* sp. Sagardia et al. observed that Benzothiophene, which did not support the growth of *Pseudomonas aeruginosa* PRG-1, was degraded if yeast extract were added as a growth substrate.

Experiments to determine the effect of fouling on Amitraz degradation showed that with increasing concentrations of organic matter, in this case cattle faeces, in the medium there was enhanced degradation. Castro and Yoshida (1971) found that the rate of degradation of DDT

in soil depends on the content or organic matter in the soil. Ko and Lockwood (1968b) reported that the addition of alfalfa and barley residues greatly increased the rate of anaerobic conversion of DDT to DDD. Alfalfa residues were more effective than barley straw in promoting this conversion, possibly because the former contains larger amounts of soluble carbohydrates and amino acids. They showed that enhanced degradation was also brought about by the addition of a mixture of glucose and peptone. Sethunathan (1973) found that faster degradation of Parathion occurred in soils with a high content of organic matter.

Since Amitraz degraded at the same rate in peptone medium containing 0,5% yeast extract and 8% (wet wt/vol) cattle faeces it is likely that the co-metabolite present in yeast extract is also present in autoclaved, freshly collected cattle faeces. Griffiths and Palmer (1972) found that Amitraz degraded in nutrient broth containing bacteria present in cattle faeces but it did not degrade if the same bacteria were inoculated into tap water. Nutrient broth (Difco) contains peptone (0,5%) and beef extract (0,3%) and so it is likely that the co-metabolite required for Amitraz degradation is present in beef extract.

In the field, rapid Amitraz degradation occurred in Wiltonside dip tank which was fouled to a level of 4,2%. In contrast, in Sea View dip tank which was relatively clean (1,2% fouled), the rapid loss of Amitraz activity did not occur. This observation can be accounted for by the laboratory results obtained in the study of the effect of fouling on degradation.

The rate of degradation was found to increase with increasing temperature. In Sea View dip tank the temperature is generally lower than that in Wiltonside dip tank (Kwanyanga Research Station, private communication) and the lower temperatures at Sea View probably result in inhibition, but not in prevention of degradation.

When examining the effect of aeration on degradation it was found that, although bubbling the cultures resulted in much more vigorous growth than that in standing cultures, it did not result in greater degradation. Anaerobic conditions did not inhibit degradation. The standing cultures most closely approximate the situation in the field where the contents of the dip tank are actively aerated only once a week when the cattle swim through the tanks.

In buffered Ye,P media + M.O. degradation occurred

at pH values up to pH 10-10,5 although there was a lag before it began. However, when $Ca(OH)_2$ was added to Ye,P broth + M.O. to a level of 0,1% there was very little Amitraz degradation although the pH was only raised to pH 9-9,5. These results indicate that $Ca(OH)_2$ does not prevent degradation only by raising the pH of the medium. In the field trial at Sea Ways dip tank slaked lime was added to the dip wash to give a $Ca(OH)_2$ concentration of 0,4%; this results in a pH of 12-12,5. In the laboratory, in experiments using buffered media no degradation was observed at pH 11,5 even after attempts had been made to enrich a culture to degrade Amitraz at this pH.

It is apparent from the experiments described in this chapter that the fastest rate of degradation occurs when the bacteria are not actively growing, i.e., when they have reached stationary phase. There is frequently a lag before degradation begins and during this lag the bacteria are actively growing. All microbial transformations are caused by enzymes and many of the enzymes catalysing the reactions must be induced since pesticides are foreign materials which have a molecular structure which may not occur in the natural environment. This

may cause an initial lag period until metabolic activity can be determined. The transformations that the enzymes catalyse are usually reactions also required to metabolize natural substances (Bollag, 1974).

CHAPTER IV

STUDIES ON AMITRAZ DEGRADATION USING PURE BACTERIAL CULTURES

4.1 Introduction

In the previous chapter it was shown that Amitraz degraded in Ye,P broth medium but not in minimal medium when the same mixed bacterial population was inoculated into each medium. Pure culture experiments were initiated using 10 strains isolated from the pH 7 enrichment culture and 6 bacterial strains commonly stocked in laboratories. The purpose of this study was to determine whether the acaricide can be decomposed by a single microbial strain or whether two or more different strains are required. Horvath and Alexander (1970) elucidated the mechanism of degradation of *m*-chlorobenzoate by an *Arthrobacter* sp. A *Flavobacterium* sp. which decomposed Diazinon as its sole carbon source was isolated by Sethunathan and Yoshida (1973). In contrast to these reports, Gunner and Zuckerman (1968) found that an *Arthrobacter* sp. and a *Streptomyces* sp. degraded the

pyrimidyl ring of Diazinon by synergistic microbial action but neither could achieve this degradation by itself. Similarly, Schwartz et al. (1974) present evidence that 5 strains of *Aeromonas*, *Pseudomonas* and *Vibrio* spp. could not be individually cultured on *n*-hexadecane salts medium but in combination they exhibit metabolic synergism and degrade the hydrocarbon.

4.2 Methods and materials

4.21 Isolation and identification of strains

Seven bacterial colonies (numbered 1-7) were isolated from the enrichment culture and purified by cloning on Ye,P agar + M.O. In addition, 3 colonies (labelled A, B and C) were isolated and purified from the enrichment culture by plating onto minimal salts agar + M.O. These 10 pure bacterial strains were characterized using methods obtained chiefly from the following texts: *Manuel de techniques bactériologiques* (Buttiaux et al., 1974), *Identification Methods for Microbiologists* Part B (Gibbs and Shapton, 1968) and *Laboratory Methods for Microbiologists* (Harrigan and McCance, 1966).

4.22 Amitraz degradation by pure cultures

From Ye,P + M.O. plate cultures of strains 1-7 saline suspensions containing approximately 10^8 bacteria per ml were prepared. A mixture containing approximately equal concentrations of cells of all 7 types was prepared. From Ye,P plate cultures of *Escherichia coli*, *Serratia marcescens*, *Bacillus subtilis*, *Klebsiella aerogenes*, *Proteus vulgaris* and *Pseudomonas fluorescens* similar saline suspensions, containing each strain individually and the 6 strains mixed, were prepared.

All the saline suspensions were inoculated into Ye,P broths + M.O. at pH 7 to test the ability of the strains to degrade Amitraz. In addition, degradation by an inoculum from the enrichment culture in the same medium was determined. The control was sterile Ye,P broth + M.O.

Strains A, B and C were grown up overnight in Ye,P broth cultures. The cells were harvested by centrifugation, washed twice with 0,85% saline and resuspended in saline at a level of approximately 10^8 bacteria per ml. A suspension containing all 3 strains mixed in approximately equal concentrations was prepared. These bacterial suspensions were inoculated into MM + M.O. and

into Ye,P broths + M.O. Sterile MM + M.O. and sterile Ye,P broth + M.O. were controls.

4.23 Growth of pure cultures on agar medium containing miscible oil and its components

Minimal salts agar was autoclaved and cooled to 50°C before the following supplements were added:

(a) 0,2% (vol/vol) M.O. (to give 0,02% active ingredient)

(b) 0,2% (vol/vol) emulsifier

(c) 0,2% (vol/vol) solvent

(d) 0,1% (vol/vol) emulsifier + 0,1% (vol/vol) solvent.

Plates were poured immediately after adding the supplements. In addition, Ye,P agar plates and Ye,P agar + M.O. plates were poured. The pH of all plates was 7. Overnight Ye,P broth cultures of the 16 pure strains were grown up and inoculated onto the agar media. The plates were incubated at 30°C for 4 days before determining whether there had been bacterial growth.

4.3 Results

4.31 Identification of isolates

The characteristics of the bacteria are listed in Table 17. Electron micrographs of strains 4 and 5 are

Table 17. Characteristics of bacteria isolated from the pH 7 enrichment culture.

Characteristic	Isolate									
	1	2	3	4	5	6	7	A	B	C
Motility in hanging drop	+	+	+	+	+	+	+	+	+	+
Flagella	2-8 polar	2-8 polar	4-8 polar	Lateral	2-10 polar	2-6 polar	2-12 polar	4-10 polar	1 polar	4-10 polar
Gram stain	-	-	-	-	-	-	-	-	-	-
Capsule stain	-	-	-	-	-	-	-	-	-	-
Spore stain	-	-	-	-	-	-	-	-	-	-
Growth in Hugh and Liefson's medium (glucose)	Oxida-tive	Oxida-tive	Oxida-tive	Fermen-tative	Oxida-tive	Oxida-tive	Oxida-tive	Oxida-tive	Oxida-tive	Oxida-tive
Growth in thioglycollate medium	Aero-bic	Aero-bic	Aero-bic	Facul-tative	Aero-bic	Aero-bic	Aero-bic	Aero-bic	Facul-tative	Aero-bic
Oxidase production	+	+	+	weakly +	+	+	+	+	+	+
Catalase production	+	+	+	+	+	+	+	+	+	+
Urease production	-	-	-	+	-	-	-	-	-	-
Reduction of nitrate to nitrite	-	-	-	-	-	-	-	-	+	-
Denitrification	-	-	-	-	-	-	-	-	+	-
Arginine hydrolysis	+	+	+	+	+	+	+	+	+	+
Starch hydrolysis	-	-	-	-	-	-	-	-	-	-
Gelatin hydrolysis	-	-	-	-	-	-	-	-	-	-
Citrate utilization	+	+	+	+	+	+	+	+	-	+
Pigmentation on King's medium A	Green	Green	Green	White	Green	Green	Green	Green	Cream	Green
Pigmentation on King's medium B	Green	Green	Green	White	Green	Green	Green	Green	Cream	Green

given in Figs. 15 and 16. Using the 8th edition of *Bergey's Manual of Determinative Bacteriology* (Buchanan and Gibbons, 1974), isolates 1, 2, 3, 5, 7, A and C were identified as *Pseudomonas* spp. and isolate B was identified as *Pseudomonas mendocina*. Since isolate 4 could not be identified using the characteristics listed in Table 17, additional characteristics were determined (see Table 18). Isolate 4 was assigned to the genus *Achromobacter* using the 7th edition of *Bergey's Manual of Determinative Bacteriology* (Breed et al., 1957), but using the 8th edition it was found to be of uncertain taxonomic position.

4.32 Amitraz degradation by pure cultures

From table 19 the following important points are evident: There was minimal Amitraz degradation in the sterile control. All the isolates tested could degrade Amitraz with the exception of *B.subtilis*, which was killed when inoculated into Ye,P medium + M.O. The greatest degradation by individual strains was by *Achromobacter* sp., *S.marcescens* and *Ps.fluorescens*. In these 3 cultures there was approximately 50% degradation after 6 days. In media containing the other cultures there was from 29-44% degradation. When the medium was

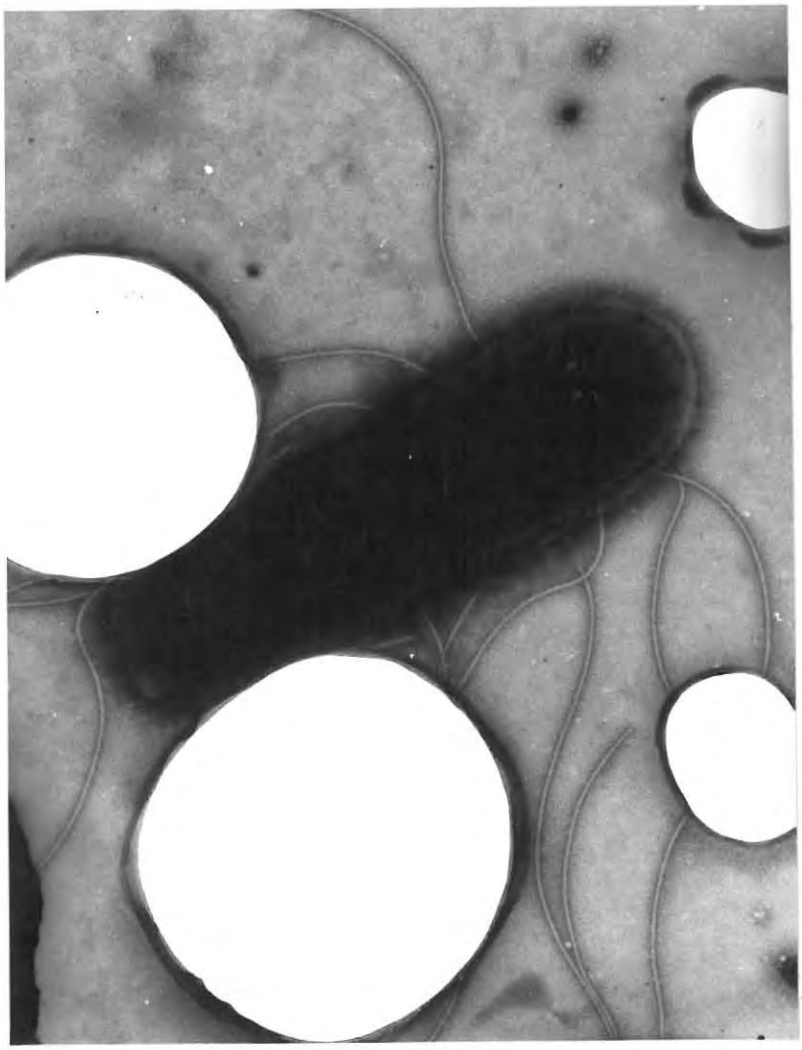


Fig.15. Electron micrograph (negatively stained with 2% phosphotungstic acid) of isolate 4 (*Achromobacter* sp.) showing lateral flagella. Magnification x 28500.

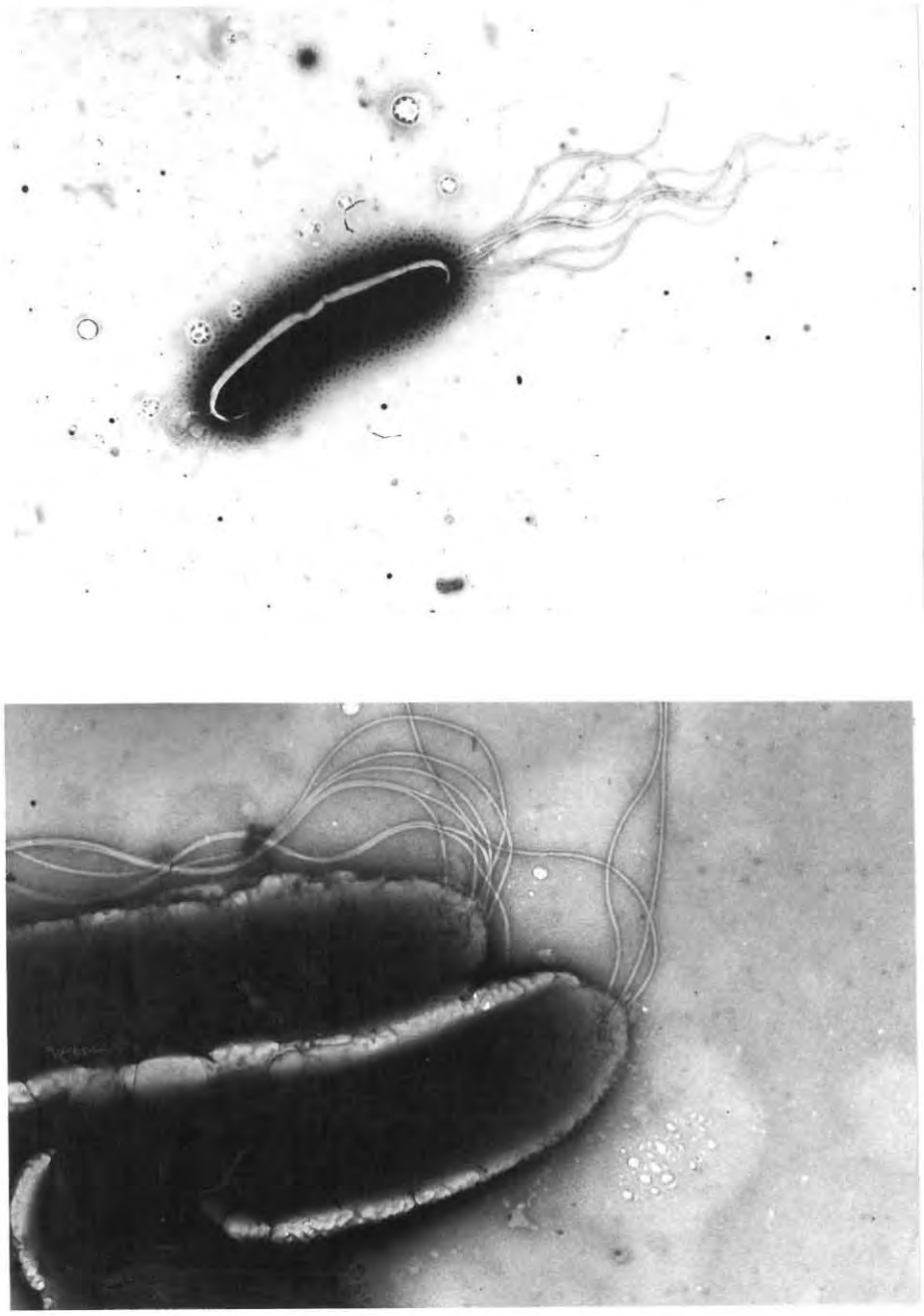


Fig. 16. Electron micrographs (negatively stained with 2% phosphotungstic acid) of isolate 5 (*Pseudomonas* sp.) showing polar flagella. Magnification x 6700 (upper) and x 28500 (lower).

Table 18. Utilization of sugars by isolate 4.

Sugar	Production of acid
Glucose	+
Lactose	*
Maltose	+
Galactose	+
Mannitol	+
L-Arabinose	+
Raffinose	-
D-Xylose	+
Sucrose	+

* Lactose was weakly utilized but the reaction was masked by the production of ammonia

Table 19. Amitraz degradation and bacterial growth after inoculation of pure cultures into Ye,P broth containing M.O.

Inoculum	% Amitraz remaining			Bacterial count		
	Day 0	Day 3	Day 6	Day 0	Day 3	Day 6
				(Bacteria/ $ml \times 10^5$)	(Bacteria/ $ml \times 10^7$)	(Bacteria/ $ml \times 10^7$)
Enrichment culture	100	63	28	6,8	5,9	5,7
Pseudomonas strain 1	100	100	57	7,3	7,9	6,9
Pseudomonas strain 2	100	94	69	6,4	5,9	5,3
Pseudomonas strain 3	100	94	56	8,2	7,8	7,6
Achromobacter sp.	100	84	51	8,8	7,6	6,8
Pseudomonas strain 5	100	100	64	6,3	5,9	6,0
Pseudomonas strain 6	100	100	68	6,1	7,7	7,1
Pseudomonas strain 7	100	95	67	6,7	6,8	6,0
Mixture of above strains	100	72	34	6,8	6,0	5,7
Escherichia coli	100	81	67	7,2	8,1	7,8
Serratia marcescens	100	82	52	6,4	8,4	7,9
Bacillus subtilis	100	100	90	6,9	0	0
Klebsiella aerogenes	100	89	71	8,3	7,4	7,0
Proteus vulgaris	100	95	69	5,9	4,8	5,2
Pseudomonas fluorescens	100	90	50	7,4	6,3	5,7
Mixture of laboratory spp.	100	60	35	7,9	5,8	5,1
None (sterile control)	100	94	94	0	0	0

inoculated with bacteria from the enrichment culture or with a mixture of the 7 isolates from the enrichment culture or with a mixture of the 6 laboratory strains there was much greater degradation. In all cultures, except *B.subtilis*, there was approximately 100-fold increase in bacterial numbers by day 3 with very little change by day 6; the pH increased from 7,0 to 8-8,5. Therefore the greater degradation which occurred in some of the cultures was not accompanied by marked bacterial growth in these cultures.

In the minimal medium cultures there was approximately a 10-fold increase in bacterial count when assayed on MM + M.O. (see Table 20). However Table 21 shows that even after incubation at 30°C for 18 days there was minimal loss of Amitraz activity (in all cultures the Amitraz concentration fell to the same level as that in the sterile control). In contrast, in Ye,P medium + M.O. *Pseudomonas* strain A and *Pseudomonas* strain C caused 58% and 52% degradation respectively. In Ye,P medium inoculated with *Ps. mendocina* there was only 27% degradation. There was approximately 100-fold increase in bacterial count when these 3 *Pseudomonas* strains were inoculated together into Ye,P medium + M.O. Again it is apparent that a mixture of bacteria caused greater degradation than each individually.

Table 20. Bacterial counts determined after inoculation of three strains of *Pseudomonas* into minimal medium containing M.O. and Ye,P broth containing M.O.

Bacterial inoculum	Agar assay medium	Bacterial count					
		MM Culture (Bacteria/ml x 10^5)				Ye,P Culture (Bacteria/ml x 10^7)	
		Day 0	Day 6	Day 12	Day 18	Day 3	Day 6
Pseudomonas strain A	MM+M.O.	0,2	7,6	9,8	8,1	1,4	1,3
	Ye,P+M.O.	0,2	3,3	1,9	4,7	1,1	0,8
Ps. mendocina	MM+M.O.	0,9	9,6	9,2	8,7	1,3	1,3
	Ye,P+M.O.	1,2	6,4	8,0	10,0	1,9	1,7
Pseudomonas strain C	MM+M.O.	0,4	9,8	6,3	8,8	1,8	1,2
	Ye,P+M.O.	0,1	7,9	9,5	5,5	1,1	0,9
Mixture of above strains	MM+M.O.	1,6	9,1	15,0	10,0	1,9	2,1
	Ye,P+M.O.	1,7	1,1	7,7	10,0	0,9	1,0

Table 21. Degradation of Amitraz by three *Pseudomonas* strains, both individually and when mixed, after inoculation into Ye,P broth containing M.O. and minimal medium containing M.O.

Bacterial inoculum	% Amitraz remaining							
	Ye,P Culture			MM Culture				
	Day 0	Day 3	Day 6	Day 0	Day 3	Day 6	Day 12	Day 18
Pseudomonas strain A	100	85	42	100	100	100	86	90
Ps. mendocina	100	90	73	100	100	98	95	87
Pseudomonas strain C	100	85	48	100	100	96	91	87
Mixture of above strains	100	69	35	100	100	100	91	87
None (sterile control)	100	97	91	100	100	95	89	88

4.33 Growth of pure cultures on M.O., emulsifier and solvent

Table 22 shows the results of this study. Of the 10 isolates from the enrichment culture, all except *Achromobacter* sp. were able to use M.O. and its constituents as carbon sources for growth. The *Achromobacter* sp. grew well on Ye,P agar and on Ye,P agar + M.O., therefore the M.O. did not inhibit its growth. However, the M.O. and the emulsifier and solvent components were unable to support its growth. On these media *E. coli* and *K.aerogenes* showed similar growth patterns to the *Achromobacter* sp. On Ye,P agar and on Ye,P agar + M.O. *S.marcescens* and *P.vulgaris* grew well, but they grew weakly on MM containing M.O., emulsifier and emulsifier + solvent. They did not grow on MM containing solvent. This indicates that growth of *S.marcescens* and *P.vulgaris* in MM containing M.O. is at the expense of the emulsifier. All the *Pseudomonas* strains were able to grow well on all media tested, this indicates that they can utilize both the solvent and the emulsifier as growth substrates.

4.4 Discussion

Of the 10 clones isolated from the enrichment culture 9 were identified as *Pseudomonas* spp. and 1 as an

Table 22. Growth of pure cultures on Ye,P and Ye,P + M.O. agar and on minimal salts agar supplemented with M.O., emulsifier and solvent.

Inoculum	Agar plate assay medium					
	Ye,P	Ye,P+M.O.	MM+M.O.	MM+E[a]	MM+S[b]	MM+E+S
Pseudomonas strain 1	++[c]	++	++	++	++	++
Pseudomonas strain 2	++	++	++	++	++	++
Pseudomonas strain 3	++	++	++	++	++	++
Achromobacter sp.	++	++	-[d]	-	-	-
Pseudomonas strain 5	++	++	++	++	++	++
Pseudomonas strain 6	++	++	++	++	++	++
Pseudomonas strain 7	++	++	++	++	++	++
Pseudomonas strain A	++	++	++	++	++	++
Ps. mendocina	++	++	++	++	++	++
Pseudomonas strain C	++	++	++	++	++	++
E. coli	++	++	-	-	-	-
S. marcescens	++	++	+[e]	+	-	+
B. subtilis	++	-	-	-	-	-
K. aerogenes	++	++	-	-	-	-
P. vulgaris	++	++	+	+	-	+
Ps. fluorescens	++	++	+	+	+	+

[a] E = emulsifier

[b] S = solvent

[c] ++ = good growth

[d] - = no growth

[e] + = weak growth

Achromobacter sp. These identifications were not unexpected since the enrichment culture came originally from an inoculum of sludge from Wiltonside dip tank and members of the genera *Pseudomonas* and *Achromobacter* are common inhabitants of soil and fresh water environments. In their natural habitat members of the genus *Pseudomonas* are important in the mineralization of organic matter.

Experiments with these 10 isolates and with 6 bacterial types stocked in the laboratory showed that 15 of the 16 bacteria tested could degrade Amitraz when inoculated individually into Ye,P medium containing Amitraz. It was found that mixtures of the bacterial strains resulted in greater degradation than pure cultures. Enhanced degradation was not accompanied by exceptionally vigorous bacterial growth. Thus it appears that the presence of more than one bacterial strain aids the degradation of Amitraz.

A notable exception found among the bacteria tested was *B. subtilis* which was killed by the presence of M.O. in the medium. Since *B. subtilis* was the only gram-positive organism tested in this experiment, the ability of two other gram-positive organisms, *Bacillus cereus* and *Staphylococcus aureus*, to grow in the presence of M.O. was examined. The growth of *B. cereus* was inhibited by the

presence of M.O. Two strains of *S. aureus*, one sensitive and one resistant to penicillin, showed different responses to M.O. The resistant strain was inhibited by it and the sensitive one was unaffected. The mechanism of action of this inhibition is unknown but these results indicate that the inhibition does not affect all gram-positive bacteria.

It was found that the *Achromobacter* sp. could degrade 49% of the Amitraz in Ye,P medium but it could not grow on minimal salts agar containing M.O. as the sole carbon source. This is evidence that the degradation of Amitraz occurs by co-metabolism. Further evidence comes from the results of experiments done using *Pseudomonas* strain A, *Ps. mendocina* and *Pseudomonas* strain C. These strains did not degrade Amitraz active ingredient when M.O. was the sole carbon source but if an additional nutrient source was added to the medium loss of Amitraz activity occurred. Although they did not degrade Amitraz in minimal medium, the bacterial count of these three strains increased; they can, however, utilize the emulsifier and solvent components of the M.O. for growth.

CHAPTER V

STUDIES OF DEGRADATION PRODUCTS OF AMITRAZ USING THIN LAYER CHROMATOGRAPHY

5.1 Introduction

Using a thin layer chromatography (TLC) technique developed by Dr. D.K. Lewis (cited by Griffiths and Palmer, 1972) it is possible to separate and identify Amitraz and its degradation products. This technique is especially useful since, under the conditions used in the gas chromatographic analysis of Amitraz (see Appendix A), degradation products are not separated. For example, one degradation product, 2,4-dimethyl formanilide, would appear between the xylene and Sumithion peaks (see Fig. A 1) only at a column temperature of 190°C and this temperature is too low for quantitative gas chromatographic analysis of Amitraz.

The Boots Company Ltd., Nottingham, England supplied a degradation pathway for Amitraz (I.R. Harrison, personal communication) as given in Fig. 17. In animals Amitraz is rapidly converted to BTS 28 369 (see Fig. 17) and is

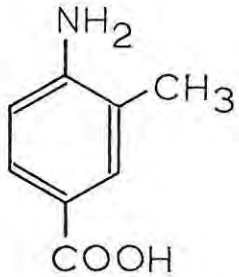
Boots designation		Chemical designation
BTS 27 419	ArN:CH.NMe.CH:NAr	Amitraz
	↓	
BTS 27 271	ArN:CH.NHMe	*N*-2,4-dimethylphenyl-*N'*-methyl formamidine
	+ ↓	
BTS 27 919	ArNHCHO	2,4-dimethyl formanilide
	↓	
BTS 24 868	$ArNH_2$	2,4-dimethyl aniline
	↓	
BTS 28 369		3-methyl 4-amino benzoic acid
	↓	
	Conjugates	

Fig.17. Degradation pathway for Amitraz supplied by the Boots Company Ltd. Ar represents 2,4-dimethylphenyl.

excreted in the urine, mainly in the form of conjugates. In plants the major metabolites are BTS 27 271 and BTS 27 919 and only the latter has been identified in soil. Further degradation is slow and no products accumulate to identifiable levels. The presumptive intermediate BTS 24 868 has never been found in plants or animals. appreciable concentrations have, however, been identified in fouled dip washes.

TLC was used to study the rate of formation of degradation products when Amitraz M.O. was incubated with bacteria from the pH 7 enrichment culture under conditions that favour degradation. In addition it was used to make tentative identification of some of the products of the degradation of Amitraz.

5.2 Methods and materials

5.21 Extraction of Amitraz and its degradation products from aqueous samples

Amitraz active ingredient and its degradation products were extracted from aqueous samples by the following method:

One hundred millilitres of the well-mixed sample were transferred to a 250 ml separating funnel and 10g of

NaCl were added. After the NaCl had been dissolved by shaking, the contents of the separating funnel were allowed to settle for 5 min. After the addition of 50 ml of chloroform (Analar grade) the separating funnel was shaken gently but thoroughly. The active ingredient and degradation products were now contained in the chloroform layer which was run off into a glass-stoppered flask.

5.22 Thin layer chromatography technique

The plates used for the thin layer chromatography were TLC aluminium sheets (manufactured by E. Merck) with layer thickness of Silica Gel F_{254} (fast running) equal to 0,25 mm. Ten or 20 µl samples were spotted onto the plates which were developed to a height of approximately 100 mm in a solvent system of toluene-methanol (40:1). Plates were air dried, then exposed to NO_2 gas for 5 min and, finally, they were sprayed with a solution of 0,005% (wt/vol) naphthyl-ethylene-diamine-dihydrochloride in 50% methanol-water. After spraying, some of the spots became visible in white light (see Fig. 18). When the chromatograms were viewed in a dark room in light emitted by a mercury vapour lamp the spots showed up with greater clarity and, in addition, spots which could not be seen in white light became visible (see Fig. 19).

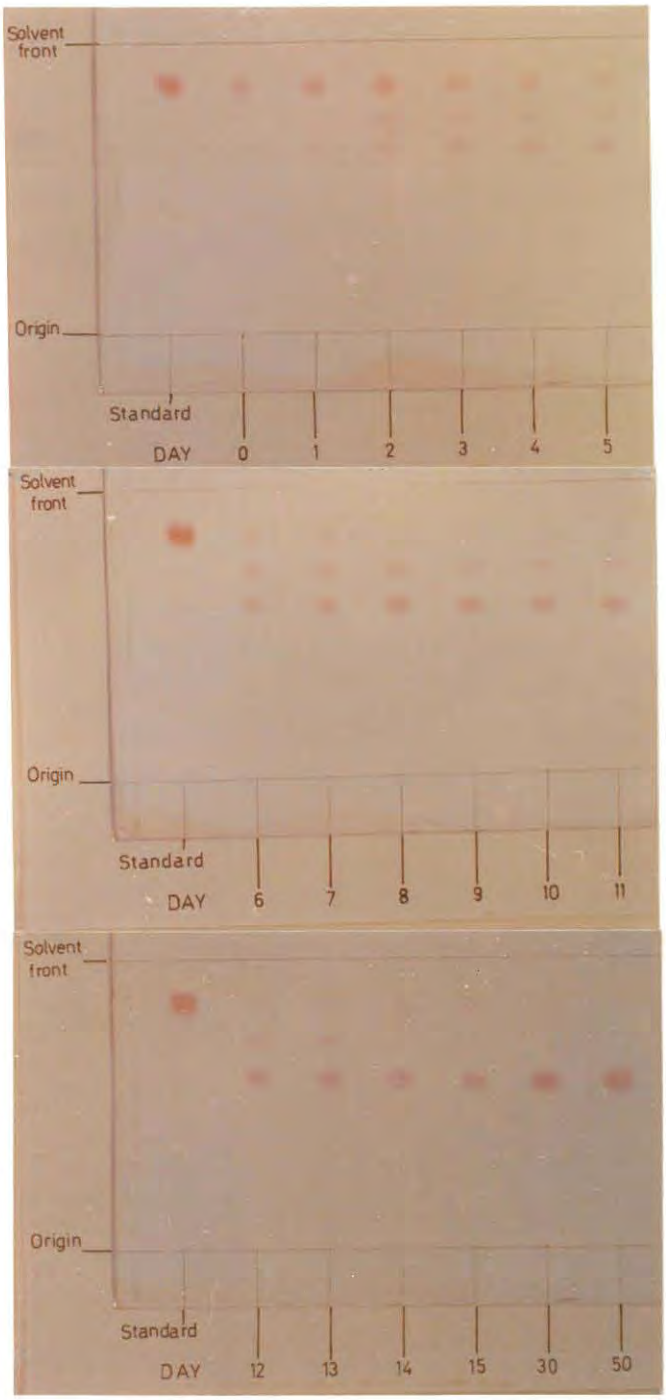


Fig.18. Thin layer chromatograms of Amitraz and its degradation products showing spots visible in white light. Degradation products resulted after incubating M.O. with bacteria in Ye,P broths for various time intervals.

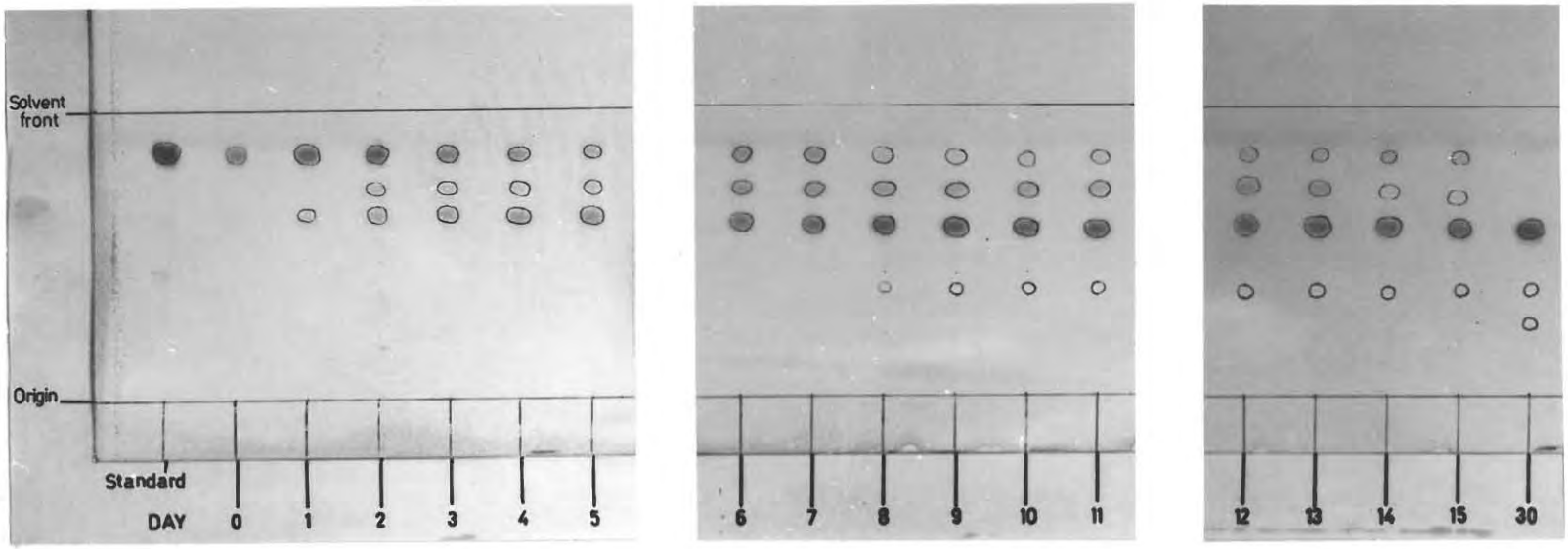


Fig. 19. Thin layer chromatograms of Amitraz and its degradation products showing spots visible in ultraviolet light. Spots were ringed while viewed in ultraviolet light.

5.23 Formation of degradation products after incubation of Amitraz with bacteria

To determine the rate of formation of degradation products when Amitraz was incubated with bacteria capable of degrading it, Ye,P broths containing M.O. were inoculated with bacteria from the pH 7 enrichment culture and incubated at 30°C for various time intervals (see Fig. 20). The active ingredient and degradation products were extracted from all the samples on the same day and analysed by TLC, together with a standard of 0,025% active ingredient (technical Amitraz) in chloroform.

Tentative identification of some of the compounds was made after TLC analysis of 99,7% pure Amitraz and some possible degradation products, viz. 2,4-dimethyl aniline (BTS 24 868), 2,4-dimethyl formanilide (BTS 27 919), *N*-2,4-dimethylphenyl-*N'*-methyl formamidine (BTS 27 271), and *N*-2,4-dimethylphenyl-*N'*-methyl formamidine hydrochloride (RC 28 428). These pure compounds were supplied by The Boots Company Ltd., Nottingham, England.

5.24 Investigation of possible intracellular accumulation of degradation products

Since radioactively labelled Amitraz was not available, TLC was used to give an indication of whether the

degradation of Amitraz by bacteria was extra- or intracellular. Bacteria from the enrichment culture were inoculated into Ye,P broths + M.O. After incubation for 8 days at 30°C the cells were harvested by centrifugation and washed 4 times with 0,85% saline. The active ingredient and degradation products were extracted from the supernatant and the washings as described in Section 5.21. Any active ingredient and degradation products that might have been inside the cells were extracted when the 50 ml of chloroform were added to the cells since chloroform lyses bacterial cell walls. Thin layer chromatograms of all the extracts were run together with an extract from a control comprising an 8-day-old culture which had not been centrifuged.

5.3 Results

5.31 Formation of degradation products by bacteria

The Amitraz (technical) standard and the day 0 sample (i.e., Ye,P broth containing M.O. extracted immediately after adding the M.O.) had R_f values of 0,83 (see Fig. 20). The first product (R_f = 0,60) appeared after 12h of incubation and the second (R_f = 0,71) after

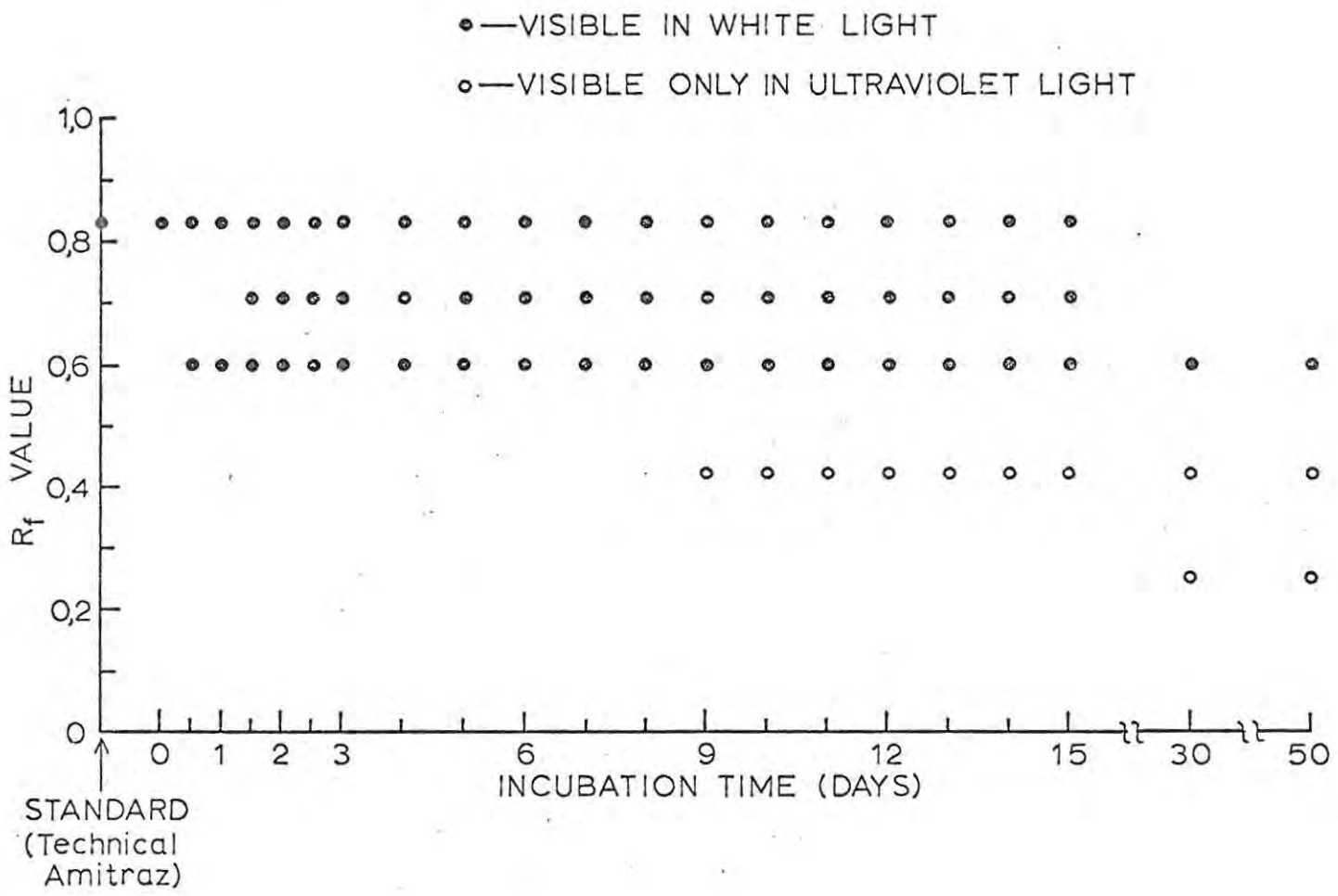


Fig.20. *Rf* values of Amitraz and the degradation products formed after incubation of M.O. with bacteria from the enrichment culture (pH 7) in Ye,P broths for various time intervals.

36h. A third product (R_f = 0,42) became visible after 8 days and a fourth (R_f = 0,25) between days 16 and 30.

The spot with an R_f value of 0,83 became progressively less intense as the time of incubation increased until, by day 30, it was no longer visible. The spot with an R_f value of 0,71 became progressively more intense, reaching maximum intensity on days 6 and 7 and then became lighter, being only faintly visible on days 14 and 15. It had disappeared after incubation for 30 days. The spot of the first product to appear (R_f = 0,60) became progressively more intense right up to day 50; as did the spots with R_f values of 0,42 and 0,25 (see Figs. 18 and 19).

On the basis of the R_f values obtained for pure compounds in only one solvent system (see Fig. 21) the first product to appear (R_f = 0,60) was tentatively identified as BTS 24 868 and the fourth spot to appear as BTS 27 919. The pure Amitraz active ingredient had the same R_f value as the technical material and the active ingredient extracted from M.O., i.e., from the day 0 samples. Of the other pure compounds analysed, BTS 26 839 had an R_f value of 0,07 and BTS 27 271 and RC 28 428 were not visible on the chromatograms.

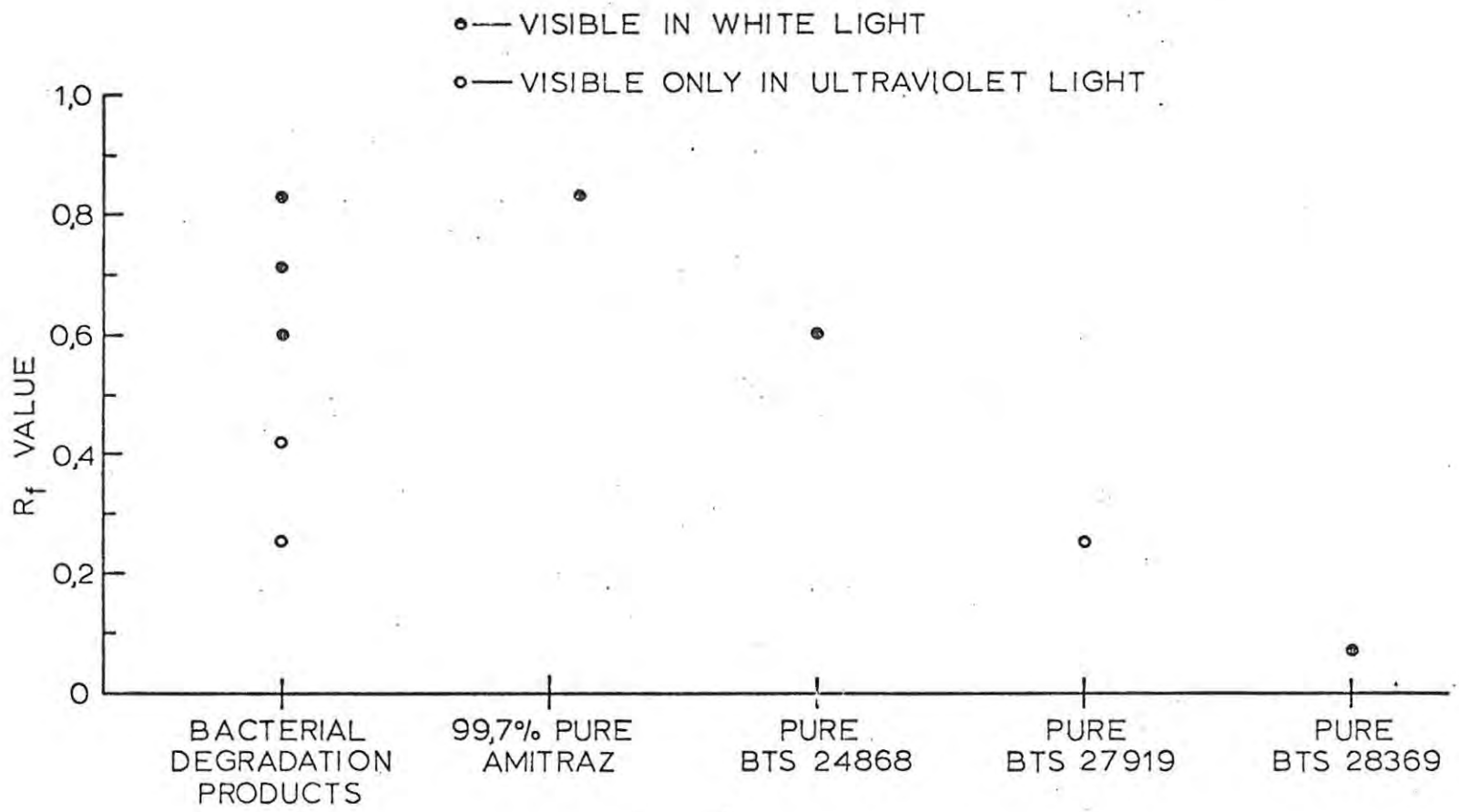


Fig.21. *Rf* values of pure Amitraz, its bacterial degradation products, pure BTS 24 868, BTS 27 919 and BTS 28 369.

5.32 Separation of degradation products from cells and supernatant by TLC

The extracts from the supernatant and the first washing gave spots with R_f values 0,83; 0,71; 0,60 and 0,42. The same result was found in the control. In the extract from the second washing faint spots with R_f values 0,83; 0,71 and 0,60 were visible and there were no spots visible in extracts from the third and fourth washings. There were no spots visible after TLC analysis of the chloroform extract from cells which had been harvested by centrifugation.

5.4 Discussion

Thin layer chromatography provided evidence for the formation of 4 degradation products of Amitraz by bacteria. Two of the products were tentatively identified in a single solvent system. R_f values should be obtained in two more solvent systems to provide more definitive evidence for the identity of these products. The first product to appear persisted throughout the 50 day period of the experiment. It was tentatively identified as 2,4-dimethyl aniline which is found in appreciable concentrations in fouled dip washes. A second product to

be tentatively identified was 2,4-dimethyl formanilide which has also been identified in soil (see Section 5.1). A product which has been identified in animals (3-methyl, 4-amino benzoic acid) was not identified as a product of bacterial degradation of Amitraz.

The results described in Section 5.32 indicate that the degradation of Amitraz by bacteria is probably extracellular, since none of the degradation products found in the control were found when washed cells were lysed with chloroform and the chloroform extract was analysed by TLC. These results do not provide proof of this — studies with radioactively labelled Amitraz should be done to confirm or contradict the above indication.

CHAPTER VI

CONCLUSIONS

During the field trial run at Sea Ways farm it was found that Amitraz was stabilized by the addition of slaked lime. In this trial slaked lime was added to give a concentration of 0,4% $Ca(OH)_2$ but laboratory experiments showed that the addition of only 0,1% $Ca(OH)_2$ resulted in minimal Amitraz degradation. Therefore the addition of less than half the amount of lime used in this field trial would have been sufficient for stabilization. The stabilization of Amitraz by lime is not only the result of raising the pH of the medium to pH 12; the addition of 0,1% $Ca(OH)_2$ raises the pH to pH 9,5 and stabilizes the Amitraz. In Sea Ways dip tank a population of alkali-tolerant bacteria built up rapidly indicating that prevention of Amitraz degradation was not a result of inhibition of bacterial growth.

From the trial at Sea View farm it was evident that lime stabilization is not required in clean dip tanks and that the "Total Replacement Method" of dipping with Amitraz is suitable for use in the field. During both

field trials excellent tick control was achieved. Degradation of Amitraz in dip tanks is apparently linked to fouling since it degraded rapidly in Wiltonside dip tank which was fouled to a level of 4,2% (dry wt/vol) and there was very little degradation in Sea View dip tank which was 1,2% fouled. Laboratory experiments showed that with increasing levels of fouling the degree of degradation increased.

In the laboratory it was found that Amitraz degradation occurred by the process of co-metabolism. Although growth of mixed bacterial populations occurred in minimal medium containing Amitraz M.O., this was not at the expense of active ingredient, i.e., there was minimal degradation. This result might indicate the absence of microbial involvement in degradation. However, the addition of yeast extract to minimal medium containing bacteria resulted in marked loss of Amitraz activity. Under the same conditions there was minimal degradation in sterile controls, thereby implicating microbial involvement. Three strains of *Pseudomonas* isolated from an enrichment culture were unable to degrade Amitraz in minimal medium although they were able to grow on minimal agar containing M.O.; they could, however, utilize the emulsifier and solvent components of the M.O. for growth. These

pseudomonads were able to degrade Amitraz in the presence of yeast extract. An *Achromobacter* sp. was isolated which, although unable to grow on minimal agar containing M.O., emulsifier or solvent, rapidly degraded Amitraz in medium containing yeast extract. The biochemical mechanism of this co-metabolic process is unknown and should be investigated with a view to characterizing the enzyme system responsible for degradation. Positive identification of the bacterial degradation products would facilitate this investigation. Laboratory experiments to study co-metabolism involved closed systems, in which depletion of nutrients and production of a toxic environment may occur; a constant flow system, e.g., in a chemostat, would approximate more closely the conditions in a natural system.

Mixed microbial populations degraded Amitraz more efficiently than did pure cultures. Complex control mechanisms affecting the regulation, growth and interactions of two or more microorganisms growing in close proximity must exist. Experiments involving controlled mixed-culture conditions to study pesticide metabolism may prove more useful than using pure cultures since mixed culture conditions prevail in natural ecosystems. The definition of the conditions under which bacterial degradation of Amitraz could be inhibited in fouled dip washes is of great practical and economic importance.

APPENDIX A

QUANTITATIVE ANALYSIS OF AMITRAZ BY GAS CHROMATOGRAPHY

A.1 Extraction of active ingredient from aqueous samples

Before the active ingredient can be subjected to analysis by gas chromatography it must be extracted from aqueous samples into a suitable solvent. The following method of extraction was routinely used:

One hundred millilitres of well-shaken aqueous sample were transferred to a 250 ml bottle fitted with a ground glass stopper. To this sample 25 ml of xylene (Analar grade) containing 0,1% (vol/vol) Sumithion (to serve as the internal standard) were added. The bottle was closed and shaken vigorously for 60 min. The ground glass stopper was replaced with a cork and the bottle was centrifuged at 2000 rpm for 15 min. Centrifugation separated the organic phase (xylene) from the aqueous phase. The active ingredient was now contained in the xylene and its concentration could be determined gas chromatographically.

A.2 Operating conditions for gas chromatograph

The instrument used was a Perkin-Elmer Model 990 Gas Chromatograph. The following "optimum" operating conditions were determined (Ettre, 1973; Research Department, African Explosives and Chemical Industries Ltd., private communication) and used for routine Amitraz determinations:

Column. A 0,9 m long glass column of internal diameter 2,0 mm and external diameter 6,25 mm was used. The column was packed, under slight pressure using a vacuum pump, with 11% (QF1 + OV17) on gas chrom Q (QF1 = trifluoropropyl (50%) methyl silicone; OV17 = phenyl (50%) methyl silicone). The column temperature used was 230°C (isothermal). New columns were conditioned at 240°C for 24h with a flow of 30 ml min^{-1} N_2 to remove any excess solvent or volatile materials from the stationary phase.

Sample injection. One microlitre samples of Amitraz active ingredient in xylene were introduced into the injector system using a 10µl Hamilton syringe fitted with a Chaney adaptor to fix the size of the injection. The temperature of the injector port was 270°C.

Sample detection. The gas chromatograph was equipped with flame ionization detectors. The temperature of the detector manifold was 280°C. The signal from the detector amplifier was recorded on a Hitachi chart recorder with a sensitivity of 1mV and a chart speed of 10mm min^{-1}.

Gas flow rates. A soap bubble flowmeter was used to measure gas flow rates. The following rates were optimum:

Carrier gas: High purity nitrogen was used at a flow rate of 40 ml min^{-1}.

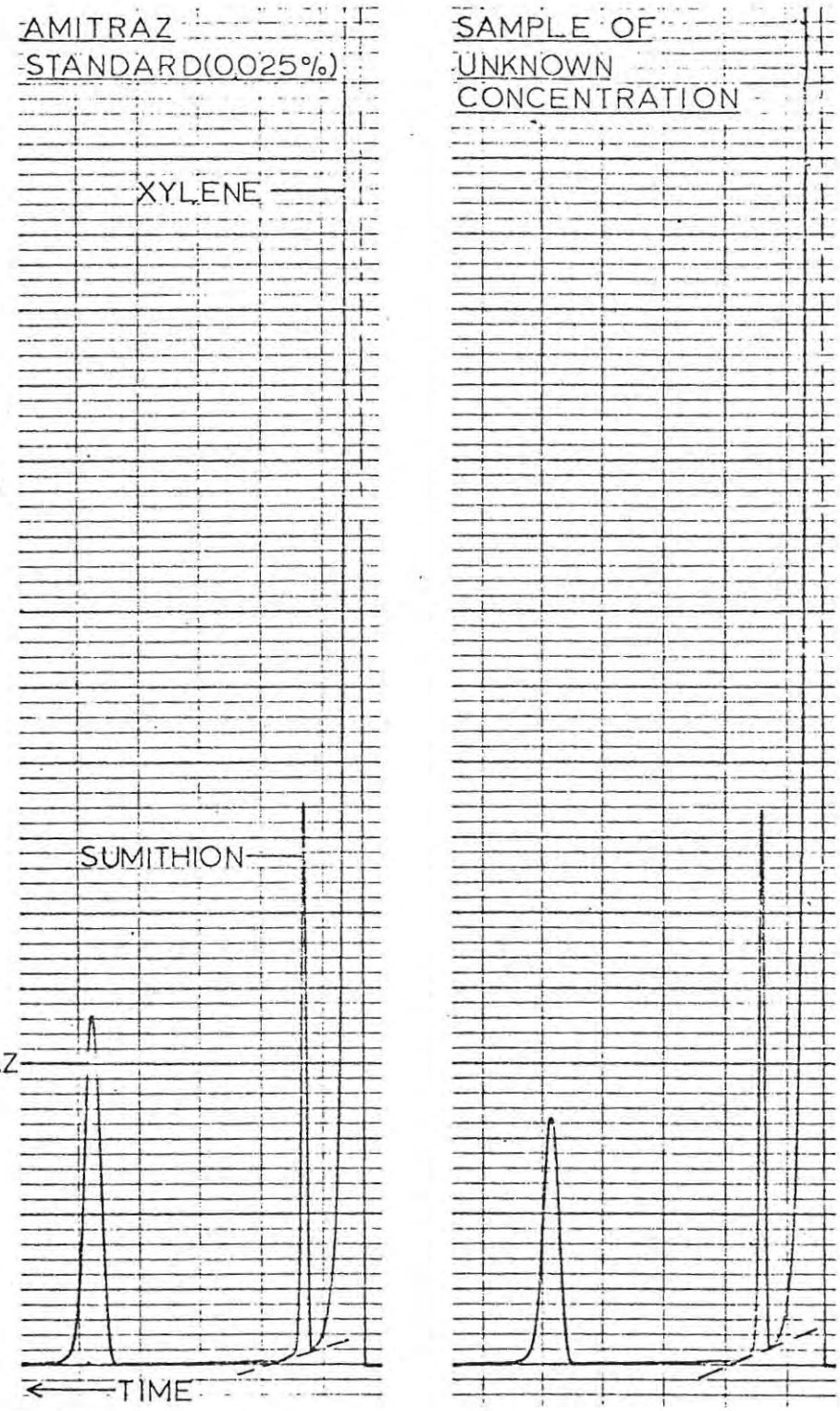
Hydrogen: High purity H_2 was used at a flow rate of 40 ml min^{-1}.

Air: Medical air was used at a flow rate of 500 ml min^{-1}.

In all three gas lines there was a filter dryer between the cylinder regulator and the instrument. These filters removed any water and organic contaminants.

A.3 Analysis of gas chromatograms

A typical gas chromatogram is given in Fig. A1. Quantitative evaluation of the peaks was based entirely on the measurement of peak heights. Before measuring the peak height a base line had to be drawn for each peak.



$$\frac{\text{Amitraz peak height}}{\text{Sumithion peak height}}: \quad 0{,}634 \qquad\qquad 0{,}454$$

$$\therefore \text{CONCENTRATION OF SAMPLE} = 0{,}025\,\frac{0{,}454}{0{,}634}$$

$$= 0{,}018\%$$

Fig. A1. Gas chromatograms of Amitraz standard (0,025%) and sample of unknown concentration. Internal standard: Sumithion. Amitraz concentration in sample is computed as shown.

This was done by drawing a tangent to the inflection points of the peak (see Fig. A1). Since measurement of peak height involves only two operations, viz. drawing the base line and measuring the height, the precision is inherently better than that in measuring peak area (Ball et al., 1967). Provided experimental conditions can be adequately controlled the peak height method gives the best precision of all manual methods for peak evaluation (Ball et al., 1967).

Sumithion was used as the internal standard for quantitative determinations of Amitraz concentrations. The concentration of Sumithion was fixed at 0,1% (vol/vol), which allowed analysis of both peaks at the same attenuation, and a calibration curve (see Fig. A2) was established in which the ratio of the peak heights (the Amitraz and the internal standard) was plotted against known concentrations of Amitraz technical material. The calibration curve was found to be linear over the range of Amitraz concentrations used. This range was wider than the range of Amitraz concentrations encountered in this study. The best straight line was drawn through the points using the three group method (Lyon, 1970). In practice, a standard solution of 0,0250% (wt/vol) technical Amitraz in xylene + 0,1% Sumithion was made and it was injected at the

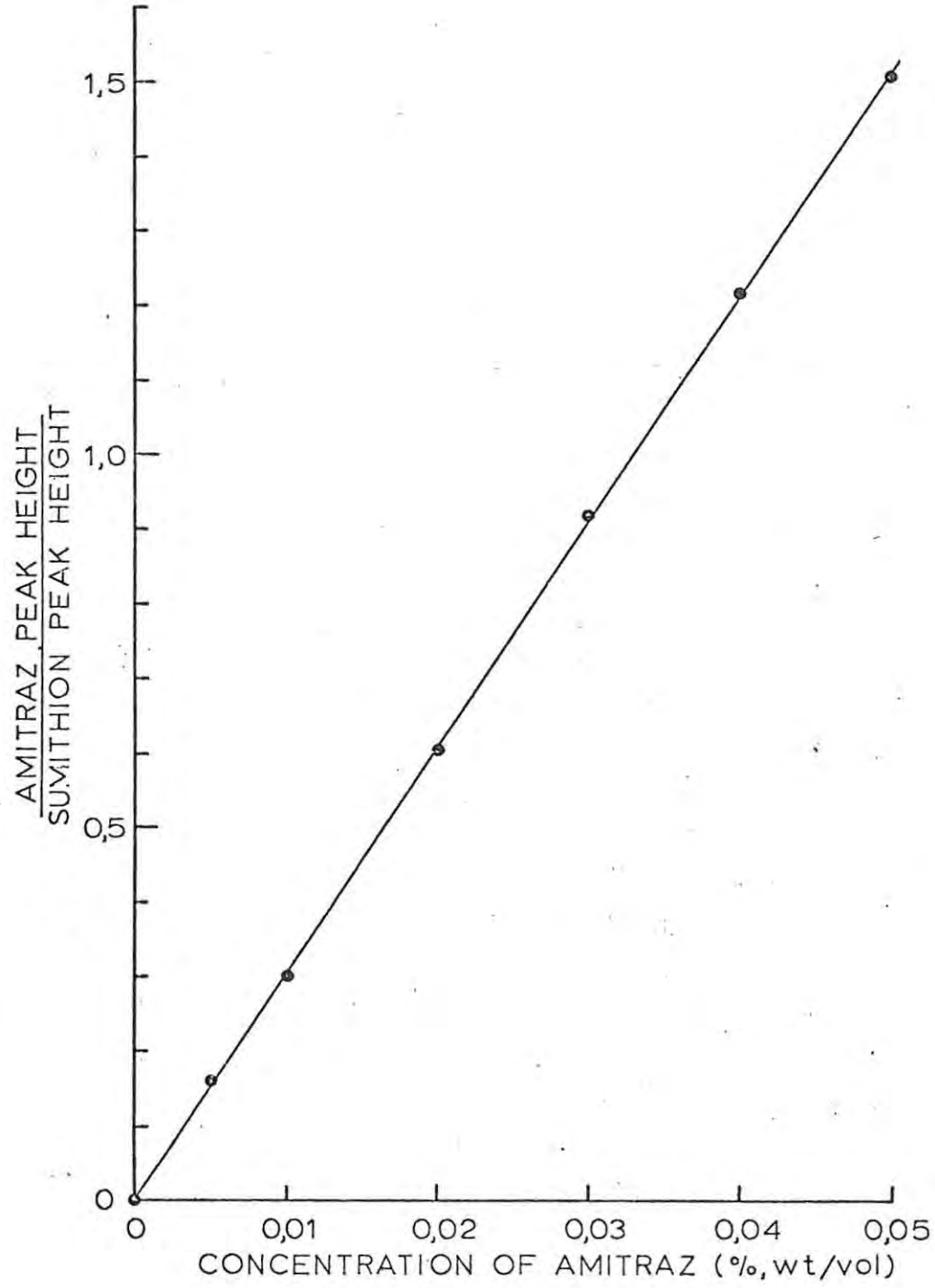


Fig. A2. Calibration curve relating the ratio of Amitraz and Sumithion peak heights to known concentrations of Amitraz. Internal standard: Sumithion.

beginning, in the middle and at the end of each set of analyses. The ratio of Amitraz to Sumithion peak heights in the standard Amitraz solution was used when determining the Amitraz concentration of samples instead of using the calibration curve since the concentration of Sumithion was not always exactly the same from experiment to experiment. A sample calculation is given in Fig. A1. The use of an internal standard effectively eliminates the effects of slight changes in column temperature and carrier gas flow rate on which peak heights depend.

APPENDIX B

EFFECT OF THE COMPONENTS OF MISCIBLE OIL ON THE GROWTH OF DIFFERENT BACTERIAL POPULATIONS ON AGAR MEDIA AT pH 7 AND pH 10

B.1 Introduction

In Section 3.33 it was observed that under certain conditions Amitraz M.O. inhibits bacterial growth. An inoculum from the enrichment culture (pH 7) was added to Ye,P broth + M.O. buffered at pH 10 and incubated at 30°C. The results of bacterial counts on days 0, 6, 12 and 18 are given in Table 9 and repeated in Table B1. From the table it is clear that when samples were plated onto media at pH 7 on day 0 there was very little difference in bacterial counts determined on plates with and without M.O. However, as the time of incubation increased the ratio of the counts determined on plates not containing M.O. to the counts determined on plates containing M.O. increased to reach a value of 750 by day 18. This ratio gives a measure of the inhibition of growth by the M.O. These results show that the M.O. contained in the agar medium

Table B1. Bacterial counts determined after inoculation of pH 7 enrichment culture into Ye,P broth + M.O. at pH 10,0. Counts assayed on various agar media illustrate the inhibition of bacterial growth under certain conditions.

Day	Bacterial count (Bacteria/ml)		Ratio: Count on Ye,P / Count on Ye,P + M.O.	Bacterial count (Bacteria/ml)		Ratio: Count on Ye,P / Count on Ye,P + M.O.
	Agar assay medium			Agar assay medium		
	Ye,P pH 7	Ye,P + M.O. pH 7		Ye,P pH 10	Ye,P + M.O. pH 10	
0	$1,9 \times 10^4$	$1,4 \times 10^4$	~1	$4,4 \times 10^3$	$4,1 \times 10^3$	1,1
6	$2,0 \times 10^7$	$8,0 \times 10^5$	25	$1,3 \times 10^7$	$9,2 \times 10^6$	1,4
12	$2,4 \times 10^5$	$1,0 \times 10^3$	240	$7,0 \times 10^4$	$3,4 \times 10^4$	2,1
18	$1,2 \times 10^7$	$1,6 \times 10^4$	750	$5,2 \times 10^6$	$1,8 \times 10^6$	2,9

at pH 7 in some way inhibited the growth of bacteria which had been growing in broth at pH 10. This effect was not observed when the same broth culture was plated onto agar media at pH 10; by day 18 the ratio had only increased from 1,1 to 2,9.

B.2 Method

In an attempt to find an explanation for this inhibition, different bacterial cultures were plated onto Ye,P media buffered at pH 7 and pH 10 supplemented with M.O. and its components as described in Section 4.23. The following cultures were plated:

(a) the enrichment culture at pH 7

(b) a culture in which an inoculum from (a) had been growing in Ye,P broth + M.O. at pH 10 for 28 days

(c) a sample taken from Sea Ways dip tank. This sample had a pH of 12.

The plates were incubated at 30°C for 4 days before counting bacterial colonies.

B.3 Results

The bacterial counts are given in Tables B2-B4.

From these tables it is evident that the 3 bacterial populations responded differently to M.O. and its components at high and low pH. There was very little inhibition by the M.O., the emulsifier or the solvent when bacteria from the pH 7 enrichment culture were inoculated onto plates at pH 7 and pH 10 supplemented with these compounds. The ratio of the count on unsupplemented medium to the count on medium containing supplement was between 1,3 and 2,6 for plates at pH 7 and between 0,7 and 3,1 for plates at pH 10 (see Table B2).

In contrast, when an inoculum which had been growing at high pH (inoculum (b)) was plated, it was found that although there was very little inhibition on media at pH 10, there was marked inhibition on media at pH 7. On medium containing 0,2% solvent the ratio was lowest indicating that the solvent caused least inhibition. When this inoculum was plated onto pH 7 medium containing 0,2% M.O. the count determined was 2745 times less than that determined on unsupplemented medium. The ratio was approximately the same when counts determined on unsupplemented medium are compared with those determined on medium containing 0,1% solvent + 0,1% emulsifier. The ratio obtained for medium containing 0,2% emulsifier was 5185; this is approximately twice the value obtained in

the media supplemented with M.O. and with emulsifier + solvent. These results indicate that the inhibition of growth of the culture which had been growing at pH 10 and then inoculated onto media at pH 7 was caused by the emulsifier component of the M.O. The reason that this inhibition does not occur at pH 10 is unknown.

Table B4 shows that bacteria which had been in an environment at pH 12 were totally inhibited by the M.O. and by its components when these bacteria were plated onto supplemented media at pH 10. There was a considerably smaller degree of inhibition by the M.O. and its components when the bacteria from the Sea Ways dip wash were plated onto media at pH 7. This effect is opposite to that described above.

B.4 Discussion

It is apparent that M.O. exerts an inhibitory effect on the growth of bacterial cultures under certain conditions. This effect depends on the interaction between the pH of the agar assay medium and the components of M.O. It is also dependent on the pH at which the culture was initially growing. The results described above do not

Table B2. Bacterial counts of the pH 7 enrichment culture when inoculated on Ye,P agar media supplemented with M.O., emulsifier and solvent.

pH of Ye,P agar medium	Supplement	Bacterial count (Bacteria/ml)	Ratio Count on Ye,P / Count on Ye,P + supplement
7,0	None	$1,5x10^6$	
7,0	M.O.	$5,7x10^5$	2,6
7,0	E[a]	$1,1x10^6$	1,4
7,0	S[b]	$7,3x10^5$	2,1
7,0	E+S	$1,2x10^6$	1,3
10,0	None	$1,1x10^5$	
10,0	M.O.	$8,6x10^4$	1,3
10,0	E	$1,1x10^5$	1,0
10,0	S	$1,5x10^5$	0,7
10,0	E+S	$3,5x10^4$	3,1

[a] E = emulsifier

[b] S = solvent

Table B3. Bacterial counts of a culture in which an inoculum from the pH 7 enrichment culture had been growing at pH 10 for 28 days. Counts were determined on Ye,P agar media supplemented with M.O.,emulsifier and solvent.

pH of Ye,P agar medium	Supplement	Bacterial count (Bacteria/ml)	Ratio Count on Ye,P / Count on Ye,P + supplement
7,0	None	$1,4 \times 10^7$	
7,0	M.O.	$5,1 \times 10^3$	2745
7,0	E[a]	$2,7 \times 10^3$	5185
7,0	S[b]	$9,3 \times 10^5$	15
7,0	E+S	$6,0 \times 10^3$	2333
10,0	None	$1,9 \times 10^7$	
10,0	M.O.	$1,7 \times 10^7$	1,1
10,0	E	$1,6 \times 10^7$	1,2
10,0	S	$2,0 \times 10^7$	1,0
10,0	E+S	$1,5 \times 10^7$	1,2

[a] E = emulsifier

[b] S = solvent

Table B4. Bacterial counts of a sample of Sea Ways dip wash when inoculated on Ye,P agar media supplemented with M.O., emulsifier and solvent.

pH of Ye,P agar medium	Supplement	Bacterial count (Bacteria/ml)	Ratio Count on Ye,P / Count on Ye,P + supplement
7,0	None	$5,4x10^4$	
7,0	M.O.	$4,6x10^3$	12
7,0	E[a]	$1,2x10^4$	5
7,0	S[b]	$5,3x10^3$	10
7,0	E+S	$9,4x10^3$	6
10,0	None	$7,3x10^3$	
10,0	M.O.	0	$\rightarrow \infty$[c]
10,0	E	0	$\rightarrow \infty$
10,0	S	0	$\rightarrow \infty$
10,0	E+S	0	$\rightarrow \infty$

[a] E = emulsifier

[b] S = solvent

[c] $\rightarrow \infty$: no colonies visible on supplemented Ye,P agar therefore the ratio was very large

give any explanation for this phenomenon but indicate that the interaction must be complex. The toxicity of the M.O. to bacteria is important and should be studied further to define the conditions under which it occurs.

APPENDIX C

PESTICIDES MENTIONED IN THE TEXT AND THEIR CHEMICAL DESIGNATIONS

Common or trade name	Chemical designation
Aldrin	1,2,3,4,10,10-Hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-*endo*, *exo*-5,8-dimethanonaphthalene
Amitraz	1,5-di-(2,4-dimethylphenyl)-3-methyl-1,3,5,-triazapenta-1,4-diene
Camphechlor	Chlorinated camphene — precise structure unknown but approximately $C_{10}H_{10}Cl_8$
Chlordane	1,2,4,5,6,7,10,10,-octachloro-4,7,8,9-tetrahydro-4,7-methyleneindane
Chlorphenamidine	*N*'-(4-chloro-*o*-tolyl)-*N*,*N*-dimethyl formamidine
DDD	2,2-Bis(*p*-chlorophenyl)-1,1-dichloroethane
DDM	*p*,*p*'-dichlorophenylmethane
DDT	2,2-Bis(*p*-chlorophenyl)-1,1,1-trichloroethane
Diazinon	*O*,*O*-Diethyl *O*-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate
Dieldrin	1,2,3,4,10,10-Hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-*endo*, *exo*-5,8-dimethanonaphthalene
Dioxathion	1,4-dioxan-2,3-ylidene bis (*O*,*O*-diethyl phosphorothiolothionate)

Common or trade name	Chemical designation
Ethion	tetraethyl *S*,*S*'-methylene bis (phosphorothiolothionate)
Lindane (γ-BHC)	γ-1,2,3,4,5,6-Hexachlorocyclohexane
Parathion	*O*,*O*-Diethyl *O*-*p*-nitrophenyl phosphorothioate
PCNB	Pentachloronitrobenzene
Sumithion	*O*,*O*-Dimethyl *O*-(3-methyl-4-nitrophenyl) phosphorothionate
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
2,3,6-TBA	2,3,6-Trichlorobenzoic acid

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