BACTERIAL DEGRADATION OF THE IXODICIDE AMITRAZ

by

Errol Ralph Allcock

A thesis submitted in partial fulfilment
of the requirements for the degree of
Master of Science
Rhodes University
Grahamstown

December 1977

RHODES UNIVERSITY
LIBRARY
Cl. No. TR 78-15
Acc. No. 78/282

ADDENDUM

1. Chemical Identification of Bacterial Degradation Products

This work was performed in collaboration with Professor D.E.A. Rivett who crystallized the products and carried out the melting point determinations and mass spectroscopy. The chemical analysis of Product 3 was performed by the C.S.I.R. Pretoria. Dr. P. Oxley, Boots Company, England and Mr. R. Sear, Wellcome Research Laboratories ($B_e$rkhamsted) England were also consulted.

2. Infra Red Spectra

The infra red spectrum of Product 1 was obtained from an oil type residue (c 25 mg) after elution from TLC plates. This residue was further purified and identified by conversion to the crystalline acetate form of 2,4-dimethylaniline (2,4-dimethylacetanilide). There was an approximately 90% conversion of the oil type residue to the crystalline acetate form and the remaining ± 10% presumably accounted for the extra IR peaks. The crystalline acetate form had a m.p. 127-128°C, which was undepressed in admixture with an authentic specimen. These identifications were confirmed by mass spectrometry. Since these identifications were considered sufficient and were accepted for publication by the Journal of Applied Bacteriology it was decided not to repeat the IR spectra but rather proceed with other aspects of the project. It was impossible to spike the bacterial cultures with the authentic material because of the very limited quantities available.

ACKNOWLEDGEMENTS

I express my sincere appreciation to Professor D.R. Woods, my research supervisor for his consistant and invaluable encouragement and advise throughout this project.

I wish to thank Professor D.E.A. Rivett who kindly performed the mass spectrometry and melting point determinations.

I am grateful to Mrs J. Webster for her interest and help during this project.

My grateful thanks are due to Coopers (South Africa) (Pty.) Limited for the generous research grant which supported this work.

I thank Mr. R. Cross of the electron microscopy unit for the electron micrographs.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	ii
LIST OF TABLES	vi
LIST OF ILLUSTRATIONS	viii
CHAPTER I	
GENERAL INTRODUCTION	1
1.1 Amitraz degradation and tick control	1
1.2 Identification of degradation products of ixodicides	4
CHAPTER II	
DEGRADATION OF AMITRAZ BY BACTERIAL POPULATIONS	9
2.1 Introduction	9
2.2 Materials and methods	10
2.3 Results	15
2.4 Discussion	24
CHAPTER III	
THE BACTERIAL DEGRADATION PRODUCTS OF AMITRAZ	32
3.1 Introduction	32
3.2 Materials and methods	33
3.3 Results	35

	Page
3.4 Discussion	43
CHAPTER IV	
MECHANISM OF AMITRAZ DEGRADATION BY BACTERIA	46
4.1 Introduction	46
4.2 Materials and methods	47
4.3 Results	53
4.4 Discussion	60
CHAPTER V	
DEVELOPMENT OF A LIQUID SCINTILLATION RADIOASSAY OF THIN-LAYER CHROMATOGRAMS	66
5.1 Introduction	66
5.2 Materials and methods	67
5.3 Results	68
5.4 Discussion	71
CHAPTER VI	
INHIBITION OF AMITRAZ DEGRADATION BY ENZYME INHIBITORS AND ANTISEPTIC COMPOUNDS	72
6.1 Introduction	72
6.2 Materials and methods	73
6.3 Results	75
6.4 Discussion	77

Page

CHAPTER VII

A BACTERIAL MODEL FOR DIP TANKS 80

7.1 Introduction 80

7.2 Materials and methods 81

7.3 Results 83

7.4 Discussion 103

CHAPTER VIII

SUMMARY AND CONCLUSIONS 108

APPENDIX A

QUANTITATIVE ANALYSIS OF AMITRAZ BY GAS CHROMATOGRAPHY 112

APPENDIX B

IXODICIDES MENTIONED IN THE TEXT AND THEIR CHEMICAL DESIGNATIONS 118

REFERENCES 119

LIST OF TABLES

Table		Page
1.	The effect of growth in complete medium on the degradation of amitraz in minimal medium.	17
2.	The effect of prewashing cells in saline on the degradation of amitraz.	18
3.	Production of ammonia by the Wiltonside and Carrig bacterial populations.	22
4.	Degradation of amitraz by cell free culture filtrates.	23
5.	Characteristics of bacteria isolated from the Carrig dip tank.	25
6.	Degradation of amitraz by pure and mixed isolates from the Carrig dip tank.	28
7.	T.l.c. of bacterial degradation products of amitraz and some standard compounds.	36
8.	Uptake of $[^{14}C]$amitraz by whole cells.	55
9.	Incorporation of $[^{14}C]$amitraz into TCA-precipitable material.	55
10.	The effect of sonication of the degradation of amitraz.	58
11.	Degradation of amitraz by osmotically shocked cells.	59
12.	Degradation of amitraz by osmotic shock fluids.	59
13.	Induction of the amitraz degradation system by amitraz and a related ixodicide.	61
14.	Utilization of M.O. as a sole source of nitrogen.	62
15.	Radioassay for the degradation of amitraz.	69

Table		Page
16.	Commercial and other antiseptics tested for their ability to inhibit the bacterial degradation of amitraz.	74
17.	Stabilization of amitraz by enzyme inhibitors.	76
18.	Inhibition of degradation by the addition of antiseptic compounds.	78
19.	Abbreviations used in the Identification Tables 20-29.	85
20-29.	Characteristics of bacteria isolated from the dip tanks.	86-100
30.	Distribution of bacterial populations in dip tanks.	101
31.	Degradation of amitraz by dip tank populations in Ye,P medium.	104
32.	Degradation of amitraz by dip tank populations in minimal medium.	105
33.	Degradation of amitraz by the test dip population in Ye,P medium.	106

LIST OF ILLUSTRATIONS

Figure		Page
1.	Degradation of amitraz in minimal medium by bacteria from the Carrig dip tank.	16
2.	The effect of growth in complete medium on the degradation of amitraz in minimal medium.	19
3.	Presence of inactivating agents in the cell free dipwash.	21
4.	Electron micrograph (negatively stained with 2% phosphotungstic acid) of isolate B (Pseudomonas sp.) showing polar flagellum.	26
5.	Electron micrograph (negatively stained with 2% phosphotungstic acid) of isolate 3 (Citrobacter sp.) showing peritrichous flagella.	27
6.	Infrared spectra of 2,4-dimethylformanilide and bacterial product 3.	38
7.	Infrared spectra of 2,4-dimethylaniline and bacterial product 1.	39
8.	Mass spectra of acetylated bacterial product 1 and 2,4-dimethylacetanilide.	41
9.	Mass spectra of bacterial product 3 and 2,4-dimethylformanilide.	42
10.	Postulated pathways for the degradation of amitraz by mixed bacterial cultures.	44
11.	Uptake by whole cells and incorporation of [^{3}H]uracil into TCA-precipitable material.	54
12.	Calibration curve relating peak areas to known concentrations of 2,4-dimethylaniline.	57

Figure		Page
13.	Thin-layer chromatography radioassay of amitraz.	70
14.	Map of Southern Africa showing location of the dip tanks and their geographical and climatic conditions.	84
15.	Distribution function for bacterial populations in dip tanks.	102
A1.	Hewlett Packard 5830 A gas chromatograph used for the analysis of amitraz.	114
A2.	A typical list of operating conditions, gas chromatogram and report.	116

CHAPTER I

GENERAL INTRODUCTION

1.1 Amitraz degradation and tick control

Information in this discussion related to the chemical control of cattle ticks was taken from review articles by Wharton & Roulston (1970) and Harrison *et al.* (1973).

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. The only practical method of dealing with the cattle tick problem in the short term is by treating the infected bovine host with ixodicides i.e. by chemical control. This can be achieved by either plunging the cattle into a dip tank containing aqueous suspensions or emulsions of the ixodicide or by spraying them with dip suspensions in a spray race.

Effective chemical control of ticks was initiated in 1893 with the use of arsenical solutions in South Africa. As the number of resistant strains of ticks

increased the arsenical solutions were replaced by the use of gamma-benzene hexachloride (γ-BHC or Lindane). Before long resistant strains appeared showing cross-resistance to other chlorinated insecticides as well. DDT was introduced and after approximately 5 years ticks resistant to DDT were reported. The organophosphorous compounds eg. Diazinon and Ethion became available in Australia at the time when DDT and other chlorinated hydrocarbons were banned for use on cattle. By 1968 resistance to organophosphorous compounds bacame a matter of great concern in intensive cattle-rearing areas of South Africa. A new compound, amitraz (1,5-di-(2,4-dimethylphenyl)-3-methyl-1,3,5-triazapenta-1,4-diene, Harrison et al., 1972) was developed by the Boots Company Limited, Nottingham, England, and was found to show high ixodicidal activity. Harrison et al. (1972) reported that in laboratory tests, amitraz was highly active against a variety of commercially important cattle tick species. Palmer et al. (1971) performed laboratory and calf experiments to demonstrate the activity of amitraz against 3 strains of cattle ticks. Baker et al. (1973) obtained excellent results with amitraz in field trials in which tick infested cattle were hand sprayed.

The discussion that follows is a review of the work done by Baker (1975) on the co-metabolism of amitraz.

She described dip tank field trials and laboratory investigations which 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. Laboratory experiments showed that the addition of 0,1% $Ca(OH)_2$ resulted in minimal amitraz degradation. The mechanism of stabilization of amitraz by the addition of slaked lime is not known. In the 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 results of the trials it was shown that lime stabilization was not required in clean dip tanks and that the "Total Replacement Method" of dipping with amitraz is suitable for use in the field. Under these conditions excellent tick control was achieved. It was found that the degradation of amitraz was linked to fouling in the dip tank and 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 (Baker & Woods, 1977) as defined by Bollag (1974) and Horvath (1972). Co-metabolic degradation of a pesticide is indicated if the concentration of a pesticide decreases in the presence of microorganisms but not in their absence, and if no

microorganism capable of utilizing the pesticide as an energy source can be isolated. Horvath (1972) 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 listed 22 organic compounds subject to co-metabolic attack and the products which accumulated during co-metabolism of these compounds. Baker (1975) found that the addition of yeast extract as the co-metabolite to a chemically defined medium simulated the effect of fouling in the dip tanks. Using mixed bacterial populations the optimum conditions for degradation of amitraz in the laboratory were determined. A culture, efficient at degrading amitraz, was enriched from a dip tank sludge inoculum. From this culture 10 bacterial isolates were identified: nine were of the genus *Pseudomonas* and one was an *Achromobacter sp.* The presence of four degradation products using thin layer chromatography was shown. A tentative identification of two of the products was made.

1.2 Identification of degradation products of ixodicides

The analysis, structure elucidation and identification of pesticides and their metabolites may be established by a number of methods. These include gas chromato-

graphy, thin-layer chromatography, nuclear magnetic resonance, mass spectrometry and ultraviolet and infrared spectroscopy. A survey aimed at promoting the standards of these methods for the measurement of biodegradability of chemical compounds by Blok (1975) revealed a number of problems eg. degradation not due to microorganisms and analytical restrictions. Consequent rules and regulations aimed at precision and obtaining reliable measurements were formulated.

Thin-layer chromatography (t.l.c.) is probably the most frequently used method for detection and separation of degradation products of ixodicides. In comparison to conventional paper and column chromatographic methods t.l.c. has a number of special advantages. These are:

1. The unusual speed of the method which is especially suitable for the analysis of unstable materials.

2. Noticeably sharper separation than can be obtained with paper or conventional column chromatography.

3. Sensitivity which is at least 10 times greater than paper chromatography.

4. The method requires limited sample quantities i.e. a sensitive ultra-micro method suited to trace analysis.

5. The method may be utilized for quantitative

analysis by elution and analysis of the separated products.

Eastin (1976) separated bifenox and its four degradation products using t.l.c. He found that optimum separation using t.l.c. was obtained with two solvent systems and two dimensional chromatography. In order to identify degradation products by t.l.c. it is necessary to characterise them in at least 3 different solvent systems. This eliminates the possibility of two different compounds having exactly the same R_f values in all 3 systems. Nelson & Hedrick (1976) used a t.l.c. procedure to semi-quantitatively determine residual concentrations of an experimental herbicide in bacterial and fungal soil cultures. Hsu & Camper (1976) using radioactively labelled ioxynil detected several degradation products of the ixodicide by t.l.c. and subsequent radioautography. Ambrosi & Helling (1977) compared the movement, on soil t.l.c. plates of [^{14}C]oxadiazon and [^{14}C]phosphalone with three standard pesticides.

High-performance, gas-liquid and gas chromatography are also frequently used to detect and separate ixodicides and their degradation products. Sparacino & Hines (1976) studied a number of carbamate pesticides using high-performance liquid chromatography. They determined conditions for the separation of 30 compounds as well as the ultraviolet spectroscopic characteristics of each compound. Takimoto _et al_. (1976) reported that they obtained

enhanced sensitivity with a potassium bromide thermionic detector over flame ionization and electron capture detectors in the gas chromatographic residue analysis of organophosphate pesticides. The analysis of gases produced as end-products of metabolism is also possible using gas chromatographic methods. Beard & Guenzi (1976) developed a rapid g.c. method for the detection of H_2, N_2, O_2 + Ar, CH_4, CO_2 and N_2O in soil from a single soil atmospheric sample using a thermal conductivity detector and paralell columns at 40°C. Richey, Bartley & Sheets (1977) were able to recover up to 82% of a ^{14}C labelled pesticide aldicarb as $^{14}CO_2$ after treatment with soil microorganisms and a method, based on quantitating $^{14}CO_2$ produced from [^{14}C]hexadecane, was developed by Seki (1976) for estimating the rate of hexadecane decomposition by microorganisms in sea water in Tokyo Bay.

Once the degradation products have been separated and eluted, a number of methods are available for the final identification. Nuclear magnetic resonance, mass spectrometry and infrared spectroscopy are the most frequently used. Horvath (1970) used u.v. and i.r. spectroscopy and mass spectrometry to identify the co-metabolic degradation products of methyl- and chloro-substituted catechols by an <u>Achromobacter sp</u>. Focht &

Alexander (1971) studied the aerobic co-metabolism of DDT analogues by a *Hydrogenomonas* sp. They used melting point analyses, i.r. spectroscopy and mass spectrometry to identify the degradation products of the DDT analogues.

CHAPTER II

DEGRADATION OF AMITRAZ BY BACTERIAL POPULATIONS

2.1 Introduction

Baker (1975) investigated the degradation of amitraz by a bacterial population enriched from the Wiltonside dip tank which was charged with amitraz wettable powder (W.P.). A second experimental dip tank, the Carrig tank was charged with the miscible oil formulation (M.O.). The Carrig dip tank was of special interest because of the signs of toxicity which developed in the cattle dipped in this tank. Furthermore the rate of degradation of amitraz in this tank was exceptionally rapid and within one week the amitraz concentration was reduced to almost zero. This contrasted with the relatively slow degradation of amitraz involving months in the Wiltonside tank (Baker 1975). Because of the rapid rate of degradation in the Carrig tank and the toxicity problems it was decided to enrich and study the bacterial population from this tank.

2.2 Materials and Methods

2.21 Media

All experiments were done using M.O. because a number of problems were encountered when the W.P. formulation was used (Baker, 1975). In the M.O. the active ingredient was formulated with an emulsifier and a solvent.

All percentage compositions are w/v and all nutrients are Difco unless stated otherwise.

Ye,P medium

The yeast extract peptone broth (Ye,P Baker, 1975) contained 0,5% yeast extract and 0,5% peptone and the 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 did not inactivate the active ingredient.

MM medium

The minimal salts medium (MM) contained (g/l) :

NH_4Cl, 4,0; NH_4NO_3, 0,8; anhydrous Na_2SO_4, 1,6; K_2HPO_4, 2,4; KH_2PO_4, 0,8 and $MgSO_4 \cdot 7H_2O$, 0,08. 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.

2.22 Determination of amitraz concentrations and bacterial numbers

Amitraz concentrations were determined by gas chromatography (see Appendix A). Bacterial viable counts were determined on various agar media using the spread plate technique. Incubation of samples was at 30°C unless stated otherwise.

2.23 Degradation of amitraz in minimal salts medium

Experiments were carried out to investigate the degradation of amitraz in liquid MM by the Carrig bacterial population and to determine whether this degradation was maintained on reinoculation. Sterile MM + M.O. samples (100ml) were inoculated with 1 ml samples of Carrig dipwash and the amitraz concentrations monitored over a period of 8 days. After this period inocula from these samples were transferred to sterile MM + M.O. samples and the amitraz concentrations

monitored for a further 8 days. A 1 ml inoculum of the Carrig dipwash in a Ye,P + M.O. sample was also monitored. Samples of Ye,P cultures and Carrig dipwash were also centrifuged to remove the cells. The pellets were washed 4 times in 0,85% saline, and 1 ml samples were inoculated into MM + M.O. media and the amitraz concentrations monitored over a period of 8 days.

2.24 The effect of growth in complete medium on the degradation of amitraz in minimal salts medium

The effect of growing bacteria on Ye,P and Ye,P + M.O. liquid and agar media prior to inoculation into liquid MM + M.O. was investigated. Samples (0,1 ml) of Carrig dipwash were inoculated into Ye,P and Ye,P + M.O. liquid or agar media. After incubation suspensions of the bacteria were added to sterile MM + M.O. and the amitraz concentrations monitored over a period of 5 days. Viable counts on Ye,P, Ye,P + M.O. and MM + M.O. were determined at different time intervals.

2.25 Presence of inactivating agents in the cell free dipwash and the effect of ammonia on the degradation of amitraz

Experiments were carried out to determine whether degradation by the Carrig inoculum was due to bacterial degradation or not. Two samples of Carrig dipwash were

sterilized by filtration through bacterial membrane filters. One sample was inoculated into MM + M.O. and the other into a Ye,P + M.O. medium. Unsterilized Carrig samples were inoculated into MM and Ye,P media + M.O. as controls. The amitraz concentrations were monitored over 6 days.

As a result of the facile reaction between amitraz and free ammonium ions it could be suggested that this reaction may account for the degradation of amitraz. This experiment was carried out to determine whether the production of ammonia by bacteria could account for the loss of amitraz in the dip tank. A comparative study was carried out to determine the amounts of ammonia produced by mixed bacterial populations from the Carrig and Wiltonside dip tanks in conjunction with the amount of amitraz degraded over a period of 7 days. Cultures (350 ml) containing various concentrations of peptone, yeast extract and tryptone were assayed before and after 7 days incubation. Ammonia concentrations were determined according to Methods for Chemical Analysis of Fresh Waters (Golterman, 1969).

Experiments were carried out to determine whether sterile culture filtrates were able to degrade amitraz. Aliquots (100 ml) of 3 day old Ye,P enrichment cultures were centrifuged to remove the cells. The supernatants

were cooled to $4^{o}C$, sterilized by filtration and supplemented with 0,2% M.O. The amitraz concentrations were determined after 2 days. Amitraz concentrations were also determined in 3 day old Ye,P + M.O. supernatants to which non-growing viable cells previously harvested from Ye,P + M.O. media had been added.

2.26 Isolation and identification of strains from the mixed Carrig population

Six bacterial strains (numbered 1- 6) were isolated from a Ye,P enrichment culture. These were purified by cloning on Ye,P + M.O. plates. Five bacterial strains (numbered A - E) were isolated from a MM enrichment culture and these were also purified by cloning on Ye,P + M.O. plates. The eleven pure bacterial isolates were characterised according to the following texts: Manual for the Identification of Medical Bacteria (Cowan and Steel, 1966); A Guide to the Identification of the Genera of Bacteria (Skerman, 1967) and the 8th edition of Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974). The eleven isolates were tested individually and in groups for their ability to degrade amitraz in Ye,P or MM media.

2.3 Results

2.31 Degradation of amitraz in minimal salts medium

Bacteria from the Carrig inoculum were able to degrade amitraz in MM and between 60 - 70% of the amitraz activity was lost in 8 days (Fig. 1). The amount and rate of degradation in MM was less than in complete medium where approximately 90% activity was lost in 4,5 days. However it is interesting to note that inocula from the MM samples after 8 days were unable to degrade amitraz in MM (Fig. 1). The bacteria were not killed by growth in MM (Table 1). Less than 10-fold increase in bacterial numbers occurred in MM. Prewashing the cells in 0,85% saline prevented degradation in MM (Table 2).

2.32 The effect of growth in complete medium on the degradation of amitraz in minimal salts medium

Growth of bacteria in Ye,P and Ye,P + M.O. media prior to inoculation into liquid MM + M.O. resulted in the degradation of amitraz (Fig. 2). The results in Table 1 indicate that in Ye,P + M.O. broth the bacterial numbers increased <u>c</u>. 10^1 - 10^3 fold over 3 days. There was relatively little difference between the bacterial numbers assayed on Ye,P and Ye,P + M.O. plates. The results of assaying the bacteria from the MM + M.O. cultures are interesting. In contrast to the Ye,P + M.O. cultures there was a 10^5 fold difference at day 3 between

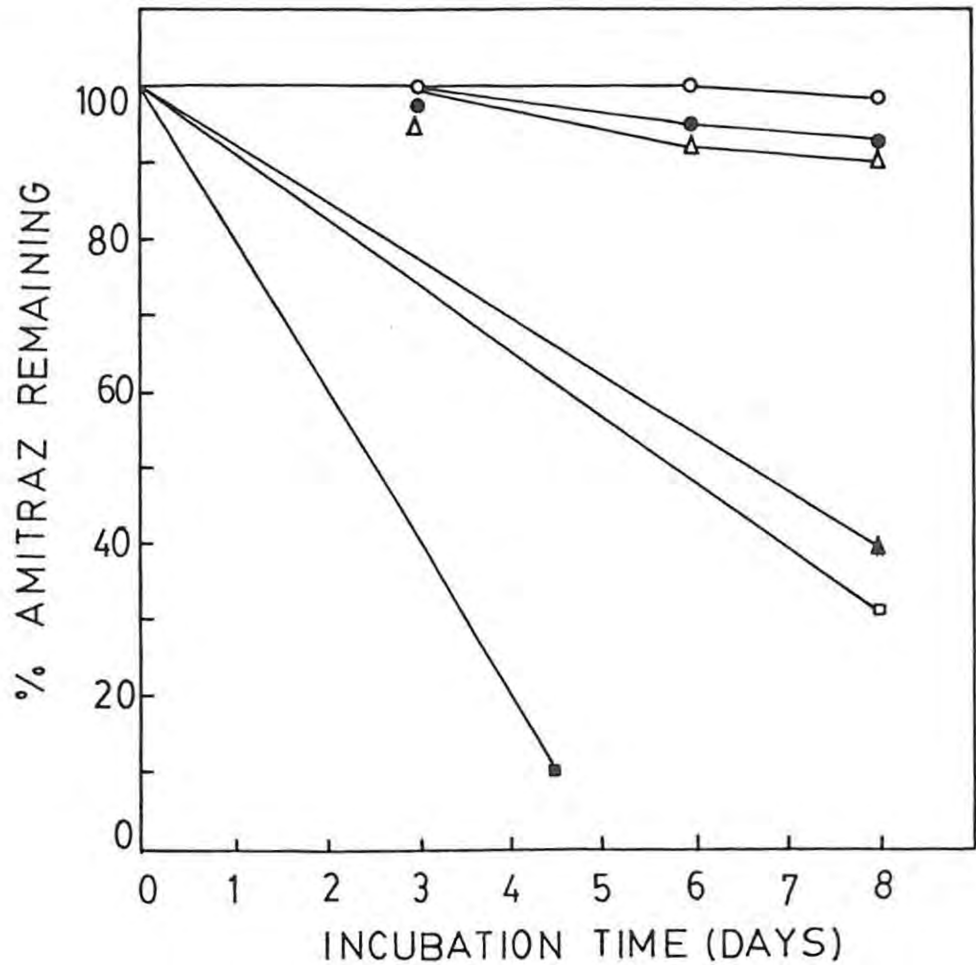


Fig. 1. Degradation of amitraz in minimal medium by bacteria from the Carrig dip tank. (○) uninoculated MM control; (▲, □) Carrig inocula in MM; (●, Δ) inocula from 8 day MM experiment; (■) Carrig inoculum in Ye,P medium.

Table 1. The effect of growth in complete medium on the degradation of amitraz in minimal medium.

Inoculum	Amitraz degradation medium	Assay medium	Bacterial no. ml^{-1} Day 0	Bacterial no. ml^{-1} Day 3
Carrig dip sample	Ye,P	Ye,P+M.O.	$4x10^{4}$	$1,3x10^{8}$
		Ye,P	$1x10^{6}$	$1x10^{8}$
Carrig enrichment in Ye,P	MM	Ye,P+M.O.	$2x10^{5}$	$3x10^{1}$
		Ye,P	$9x10^{5}$	$5x10^{6}$
Carrig enrichment in Ye,P+M.O.	MM	Ye,P+M.O.	$6x10^{3}$	$6x10^{1}$
		Ye,P	$1x10^{6}$	$9x10^{6}$
Carrig enrichment in Ye,P	Ye,P	Ye,P+M.O.	$9x10^{5}$	$3x10^{7}$
		Ye,P	$1x10^{6}$	$6x10^{7}$
Carrig enrichment in Ye,P+M.O.	Ye,P	Ye,P+M.O.	$9x10^{5}$	$4x10^{7}$
		Ye,P	$2x10^{6}$	$7x10^{7}$

Table 2. The effect of prewashing cells in saline on the degradation of amitraz.

Sample Origin	Degradation Medium	% amitraz Remaining
Ye,P Medium	MM + M.O.	88
Ye,P Medium	MM + M.O.	84
Carrig Dipwash	MM + M.O.	86
Carrig Dipwash	MM + M.O.	84
Uninoc. Control	MM + M.O.	90
Inoc. Control (unwashed inoculum)	MM + M.O.	38

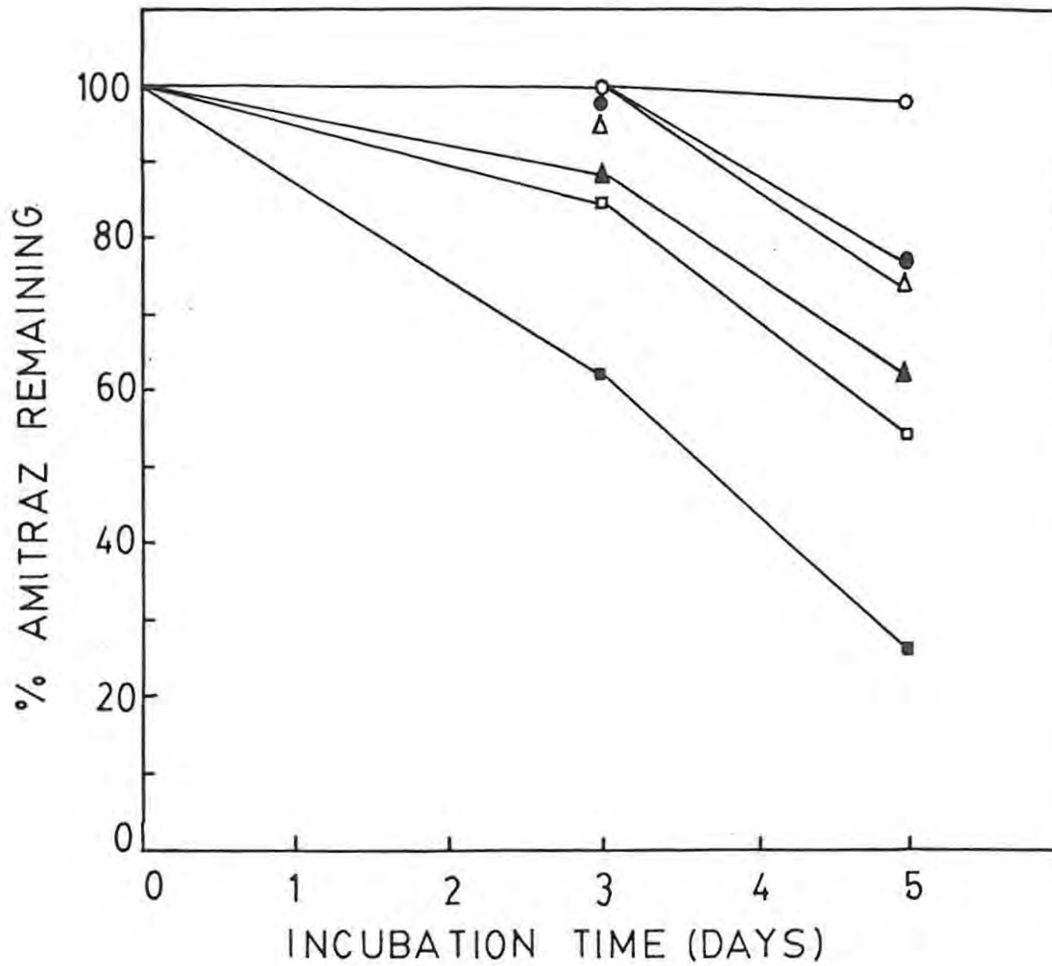


Fig. 2. The effect of growth in complete medium on the degradation of amitraz in minimal medium.
(o) Ye,P uninoculated control;
(●) degradation in MM after growth in Ye,P;
(Δ) degradation in MM after growth in Ye,P+M.O.;
(▲) degradation in Ye,P after growth in Ye,P;
(□) degradation in Ye,P after growth in Ye,P+M.O.;
(■) Carrig dip inoculum in Ye,P.

the numbers obtained on the Ye,P and the Ye,P + M.O. plates. The results of the Ye,P plates indicated that the bacterial numbers increased slightly (c. 10 fold) in the MM + M.O. cultures. No growth was obtained on MM + M.O. plates but the bacteria grew on MM + glucose plates

2.33 Presence of inactivating agents in the cell free dipwash and the effect of ammonia on the degradation of amitraz

Little or no degradation occurred in samples inoculated with the sterile filtrates of Carrig dipwash while substantial degradation occurred in those inoculated with unsterile dipwash (Fig. 3).

Although there was an increase in the amount of ammonia present in the media inoculated with the bacterial populations this increase did not correlate with the amount of degradation observed (Table 3). The highest ammonia concentration recorded was in the MM but little degradation occurred in these cultures. Sterile culture filtrates containing ammonia showed little degradation whereas the addition of amitraz to stationary phase cultures resulted in significant degradation (Table 4).

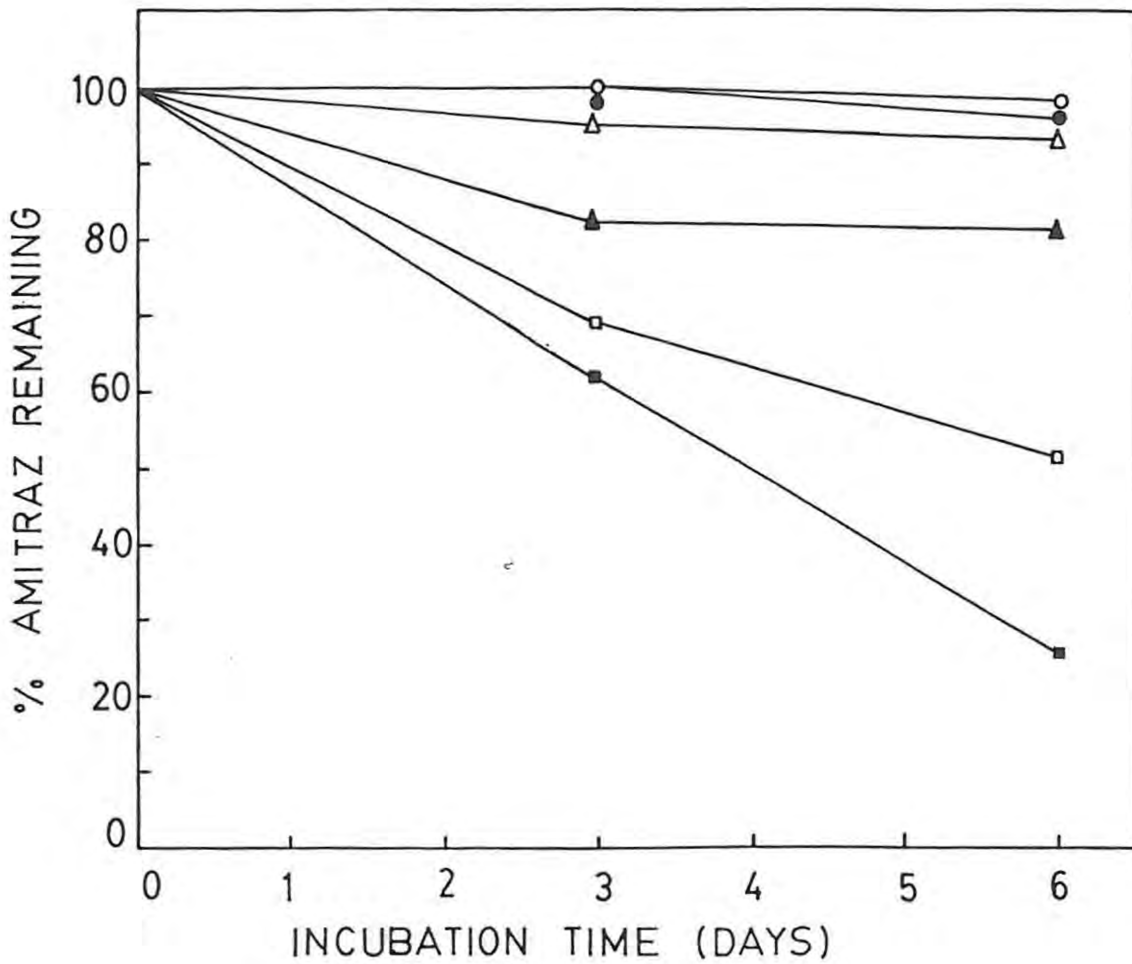


Fig. 3. Presence of inactivating agents in the cell free dipwash.
(o) Ye,P uninoculated control;
(●) MM uninoculated control;
(Δ) sterile dip sample in MM;
(▲) sterile dip sample in Ye,P;
(□) dip sample in MM;
(■) dip sample in Ye,P.

Table 3. Production of ammonia by the Wiltonside and Carrig bacterial populations.

Carrig bacterial population:

Medium tested	N g/l Day 0	N g/l Day 7	Increase in N g/l	% amitraz remaining
1,0% Tryptone	0,027	0,216	0,189	78
0,5% P+0,5% Ye	0,012	0,191	0,179	63
1,0% Peptone	0,012	0,176	0,164	85
0,5% P+0,5% T	0,012	0,160	0,148	85
0,5% P+0,05% Ye	0,012	0,101	0,089	78
0,5% P+0,001% Ye	0,011	0,070	0,059	84
0,5% Peptone	0,012	0,048	0,036	94
minimal medium	0,686	0,692	0,006	73
Ye,P uninoc. control	0,017	0,022	0,006	94
MM uninoc. control	0,602	0,605	0,003	98

Wiltonside bacterial population:

Medium tested	N g/l Day 0	N g/l Day 7	Increase in N g/l	% amitraz remaining
1,0% Tryptone	0,014	0,184	0,170	82
0,5% P+0,5% Ye	0,014	0,319	0,305	55
1,0% Peptone	0,014	0,126	0,112	98
0,5% P+0,5% T	0,015	0,168	0,153	93
0,5% P+0,05% Ye	0,014	0,300	0,286	93
0,5% P+0,00% Ye	0,014	0,154	0,140	98
0,5% Peptone	0,013	0,157	0,144	97
minimal medium	0,521	0,524	0,003	91
Ye,P uninoc. control	0,014	0,028	0,014	98
MM uninoc. control	0,523	0,535	0,003	99

Table 4. Degradation of amitraz by cell free culture filtrates.

Experiment No.	Sample	% amitraz remaining	
		Day 0	Day 2
1.	Uninoc. control	100	97
	Culture filtrate	100	100
	Stationary phase culture	100	76
2.	Uninoc. control	100	95
	Culture filtrate	100	89
	Stationary phase culture	100	72

2.34 Isolation and identification of strains from the mixed Carrig population

The characteristics of the bacteria isolated are listed in Table 5. Seven of the isolates (2,6,A-E) were identified as Pseudomonas sp. and the other four (1,3,4,5) as members of the family Enterobacteriaceae (No. 1 Escherechia sp., No. 3 Citrobacter sp., No. 4 Enterobacter sp., No. 5 Klebsiella sp.). Electron micrographs of strains 3 and B are given in Figures 4 and 5. Results of the degradation of amitraz by the 11 isolates in Ye,P and MM are listed in Table 6. It is evident from the results that degradation of amitraz occurred most rapidly in the mixture of all 11 isolates. There was relatively little degradation in most of the pure cultures. Strain No. 5 showed the most degradation in both Ye,P and MM. An interesting result was obtained with strain No. 1 where little degradation occurred in Ye,P but substantially more in MM.

2.4 Discussion

Degradation of amitraz by bacteria from the Carrig tank which caused toxicity problems was investigated and compared with that by bacteria from the Wiltonside tank. The results are interesting in that the Carrig population

Table 5. Characteristics of bacteria isolated from the Carrig dip tank.

CHARACTERISTIC	ISOLATE										
	1	2	3	4	5	6	A	B	C	D	E
Motility in Hanging Drop	+	+	+	+	-	+	+	+	+	+	+
Gram's Stain	-	-	-	-	-	-	-	-	-	-	-
Morphology	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Growth in Hugh and Liefson's Medium Glucose)	Ferm Acid+Gas	No Action	Ferm Acid+Gas	Ferm Acid+Gas	Ferm Acid+Gas	No Action	No Action	No Action	No Action	No Action	No Action
Growth in Thioglyidllate Medium	Facult	Aerobic	Facult	Facult	Facult	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic
Spore Stain	-	-	-	-	-	-	-	-	-	-	-
Oxidase Production	-	+	-	-	-	+	+	+	+	+	+
Catalase Production	+	+	+	-	-	+	+	+	+	+	+
Methyl Red Test	+	-	-	+	+	-	-	-	-	-	-
Urease Product	-	-	-	-	-	-	-	-	-	-	-
Citrate Utilization	-	+	+	+	+	+	+	+	+	+	+
Reduction of Nitrite	+	-	+	+	+	+	-	-	-	-	-
Arginine Hydrolysis	-	+	-	-	-	+	+	+	+	+	+
Gelatin Hydrolysis	-	+	-	-	-	+	+	+	+	+	+
Starch Hydrolysis	-	-	-	-	-	-	-	-	-	-	-
Nitrate Reduction	+	-	+	+	+	+	-	-	-	-	-
Pigmentation on King's Medium A	White	Orange	White	White	White	Yellow	Orange	Orange	Orange	Orange	Orange
Pigmentation on King's Medium B	White	Green	White	White	White	Green	Green	Green	Green	Green	Green
Flagelation	Polar	Polar	Peritri-chous	Polar	Non-Flag	Polar-Tufted	Polar	Polar	Polar	Polar	Polar
Indole Production	+	-	+	-	-	-	-	-	-	-	-
Size (IN U)	1,5x0,65	1,5x0,6	2,5x0,8	2,5x0,64	1,6x0,85	1,9x0,6	1,1x0,6	1,3x0,65	1,6x0,6	1,50x0,7	1,6x0,6
Sediment in Liquid Culture	Granualr	Flocculent	Granular	Flaky	Flaky	Viscid	Flaky	Flaky	Flaky	Flaky	Flaky

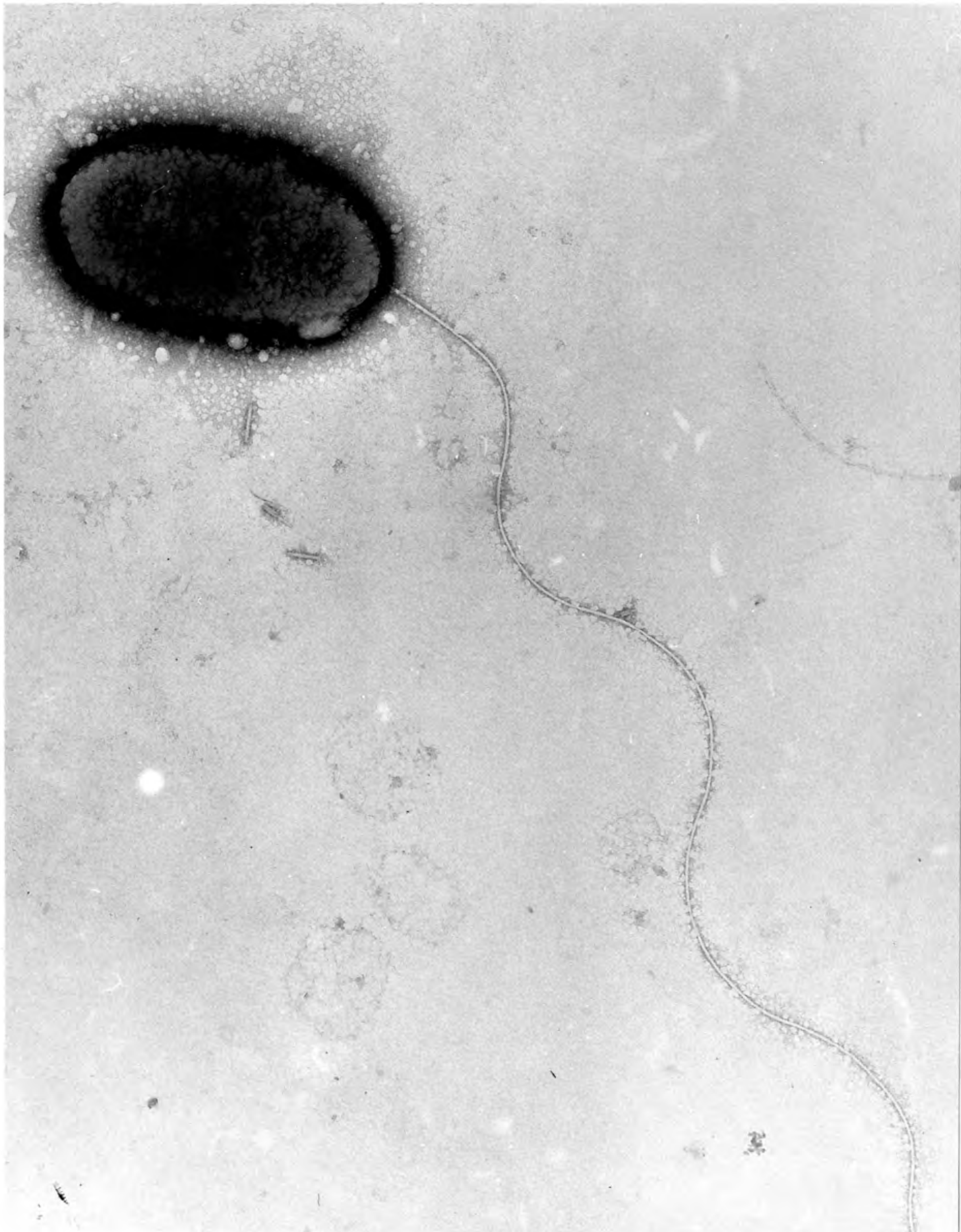


Fig. 4. Electron micrograph (negatively stained with 2% phosphotungstic acid) of isolate B (Pseudomonas sp.) showing polar flagellum. Magnification x 34,500.

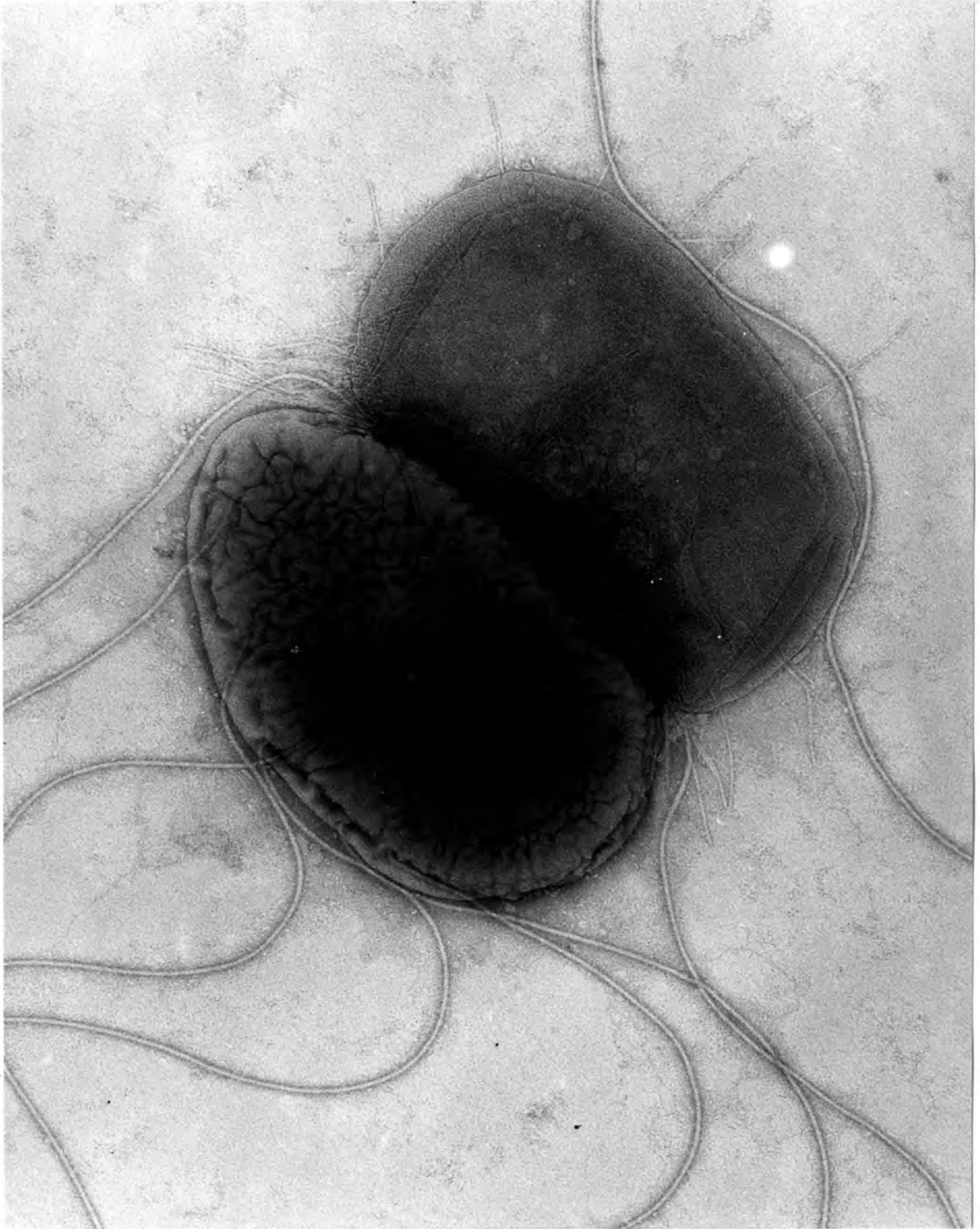


Fig. 5. Electron micrograph (negatively stained with 2% phosphotungstic acid) of isolate 3 (Citrobacter sp.) showing peritrichous flagella. Magnification x 55,500.

Table 6. Degradation of amitraz by pure and mixed isolates from the Carrig dip tank.

Isolate	% amitraz remaining		
	Day 0	Day 6	
		Ye,P	MM
1	100	90,6	76,5
2	100	83,7	84,0
3	100	93,0	90,0
4	100	85,0	91,2
5	100	64,4	77,8
6	100	84,0	89,3
A	100	90,0	88,8
B	100	85,0	88,5
C	100	86,5	90,4
D	100	82,7	88,2
E	100	70,6	91,4
Mixture of all 11	100	58,4	72,3
Mixture of N° 1-6	100	74,0	77,1
Mixture of N° A-E	100	73,1	85,7
Controls (uninoc.)	100	98,0	99,1

from the dip tank or grown in complete medium degraded amitraz in minimal medium (without the co-metabolite). However this ability to degrade amitraz in MM was transient and was lost after a single cycle in MM + M.O. This suggests that either the bacteria were killed by growth in MM or the co-metabolite carried over in the inoculum was used up and no further degradation could take place. The mechanism of the transient degradation in MM is not understood but amitraz, emulsifier and solvent were not acting as metabolites. The results could be explained by assuming that in the initial inoculum from the dip tank or Ye,P medium there was sufficient of the co-metabolite for amitraz to be degraded by co-metabolism. The co-metabolite was removed by repeated washings in saline as this prevented degradation in MM. The bacteria were not killed by growth in MM where a limited amount of growth took place (c. 10-fold increase in bacterial numbers in MM). In similar experiments using an inoculum of Wiltonside dipwash, Baker (1975) showed that no degradation occurred in the MM and that there was no change in the pH although the bacterial count increased c. 100-fold by day 3 with no further increase by day 6. On MM + M.O. plates growth of Carrig bacteria was curtailed to such an extent that no visible clones were obtained. However the bacteria grew on MM +

glucose plates. The bacteria were therefore not utilizing the solvent or emulsifier in the M.O. for growth. This contrasts with similar experiments done by Baker (1975) using Wiltonside bacteria where the bacteria grew on MM + M.O. plates utilizing the solvent and or emulsifier as substrates for growth. A new bacterial population has therefore evolved in the Carrig tank. It should be noted that the Wiltonside tank was charged with amitraz wettable powder whereas in the Carrig tank miscible oil was used. This could account for the different bacterial populations. It should also be noted that the degradation products obtained by co-metabolism with the Wiltonside and Carrig populations were identical (Chapter 3).

Exposure of the bacteria to MM + M.O. rendered the bacteria sensitive to amitraz because very low numbers of bacteria were obtained on the Ye,P + M.O. plates, as compared with the Ye,P plates ($10^{1}ml^{-1}$ as compared with $10^{6}ml^{-1}$). This interesting variation in sensitivity to amitraz depending on the nature of the previous growth medium was observed previously by Baker (1975) and should be studied further.

Experiments carried out to determine the presence of inactivating agents in the cell free dipwash showed that for degradation to occur the presence of cells was

essential. The production of ammonia by Carrig and Wiltonside bacterial populations indicated that although the amount of ammonia present in the media increased, the increase did not correlate with the amount of amitraz degradation observed. The reaction between amitraz and free ammonium ions may account for some of the degradation but it is not the major factor.

Eleven strains were isolated from the Carrig mixed population. Seven of these were classified as *Pseudomonas* sp. and the other four were members of the family Enterobacteriaceae. Degradation of amitraz by pure cultures and in groups showed a synergistic effect with the mixture of all 11 strains. There was a high degree of overlap between the predominant bacterial types from the Wiltonside and Carrig dip tanks and the conditions required for optimal degradation were identical for the two bacterial populations.

CHAPTER III

THE BACTERIAL DEGRADATION PRODUCTS OF AMITRAZ

3.1 Introduction

In order to fully understand the bacterial degradation of amitraz it is important that the nature and order of appearance of the bacterial degradation products be determined. This point is emphasised by the extremely rapid degradation and toxicity problems observed with the Carrig dip tank charged with M.O. As amitraz is non-toxic to cattle (Baker, 1975) one of the degradation products could be implicated.

Thin-layer chromatography (t.l.c.) involving 4 solvent systems was used to isolate and identify the bacterial degradation products of amitraz. These identifications were confirmed by infrared spectroscopy, melting point determinations and mass spectrometry. A pathway for the degradation of amitraz by bacteria is proposed.

3.2 Materials and Methods

Two mixed bacterial populations enriched from Carrig and Wiltonside dip tanks charged with M.O. and W.P. respectively were used.

The Ye,P broths were supplemented with 0,2% M.O. (v/v) to give an active amitraz concentration of 0,02%. The Ye,P + M.O. broths (100 ml) were inoculated with 1 ml of an enrichment culture and incubated at 30°C. At different time intervals samples were removed, extracted with chloroform and assayed for amitraz degradation products.

3.21 Identification of the degradation products

Four t.l.c. systems (A-D) involving Kieselgel 60 F 254 (Merck) plates (20 x 20 cm) were used for the initial identification of the amitraz degradation products. System A was developed in toluene-methanol (40 : 1 v/v); B in chloroform-isoamylalcohol (24 : 1 v/v); C in toluene-isoamylalcohol (40 : 1 v/v) and D in ether-triethylamine (19 : 1 v/v). In system D the lower edge of the plate was dipped immediately before use to a depth of 3,5 cm in a filtrate obtained by shaking together 100 ml formamide, 150 ml acetone and 2 g sodium bicarbonate. Any acetone was removed by standing

the plate in a stream of cold air for 1 min.

Compounds were detected by ultraviolet light or by colour development with a spray procedure which involved exposing dried plates to dense NO_2 fumes for 5 min prior to spraying with a naphthyl-ethylene-diamine-dihydrochloride reagent (0,5% (w/v) in 50% (v/v) methanol-water). In system D the plates were placed in a tank saturated with hydrochloric acid vapour to neutralize any residual triethylamine on the plates before transferring for 5 min to a tank filled with NO_2 fumes. The R_f values of the degradation spots obtained in the 4 systems were compared with technical grade amitraz supplied by Wellcome, Southern Africa and authentic samples of 2,4-dimethylaniline, N-2,4-dimethylphenyl-N'-methylformamidine and 2,4-dimethylformanilide supplied by the Boots Company Limited, Nottingham, England.

For preparative t.l.c. the chloroform extracts were concentrated by evaporation prior to spotting on t.l.c. plates. T.l.c. plates were prepared by mixing 60% Kieselgel (Merck) and 40% Kieselguhr (Merck) in 0,1N boric acid which was rapidly stirred to form an even slurry. This was poured onto clean glass plates and spread evenly over the surface using a t.l.c. spreader to a thickness of 0,5 mm.

The spots of the major degradation products were visualized under ultraviolet light, scraped off the plates and extracted with chloroform. The residues were identified by infrared spectroscopy on a Beckman model IR 10 infrared spectrophotometer, melting point determinations and mass spectrometry on an A.E.I. M 30 mass spectrometer at an inlet temperature of 90°C and a voltage of 70 eV.

3.3 Results

3.31 Identification of the degradation products

The Ye,P + M.O. degradation cultures inoculated with the two bacterial populations were extracted at different time intervals and analysed by the t.l.c. techniques. Two major degradation products (products 1 and 3) appeared on the four t.l.c. systems (Table 7). They were initially identified as 2,4-dimethylaniline and 2,4-dimethylformanilide respectively based on similar R_f values in the four t.l.c. systems.

A third degradation product (product 2) was present in very small amounts and was only detected in one t.l.c. system. This minor product was postulated to be N-2,4-dimethylphenyl-N'-methylformamidine based on an identical

Table 7. T.l.c. of bacterial degradation products of amitraz and some standard compounds.

Bacterial degradation product	Standard compound	R_f			
		System A	System B	System C	System D
	1,5-Di-(2,4-dimethylphenyl)-3-methyl-1,3,5-triazapenta-1,4-diene	0,83	0,79	0,34	0,86
1	2,4-dimethyl-aniline	0,57	0,64	0,23	0,72
2	N-2,4-dimethyl-phenyl-N'-methyl-formamidine	- *	-	-	0,58
3	2,4-dimethyl-formanilide	0,20	0,53	0,13	0,40

* Degradation product and N-2,4-dimethylphenyl-N'-methyl-formamidine not visible on t.l.c. plate.

R_f value in a t.l.c. system specifically designed to detect N-2,4-dimethylphenyl-N'-methylformamidine (Boots, 1973).

Identification by infrared spectroscopy

The infrared spectra of the authentic samples likely to be involved in the identification of the bacterial degradation products were determined. Infrared spectra of bacterial product 3 and 2,4-dimethylformanilide are shown in Fig. 6. These two spectra were identical in all respects. Spectra of bacterial product 1 and 2,4-dimethylaniline are shown in Fig. 7. It can be seen that these two spectra were similar in many respects but were not identical. The characteristic N-H stretch (3400 cm^{-1}) of the 2,4-dimethylaniline molecule was absent in the spectrum of product 1. The differences in the 2 spectra were not due to the elution procedure as authentic samples subjected to the elution procedure had spectra similar to the original compounds. The reason for the differences between the two spectra is unknown.

Identification by melting point determinations and mass spectrometry

Crystallization of the residue obtained from product 3 (c. 25 mg obtained from 500 ml of Ye,P culture) from benzene gave almost colourless crystals of 2,4-dimethyl-

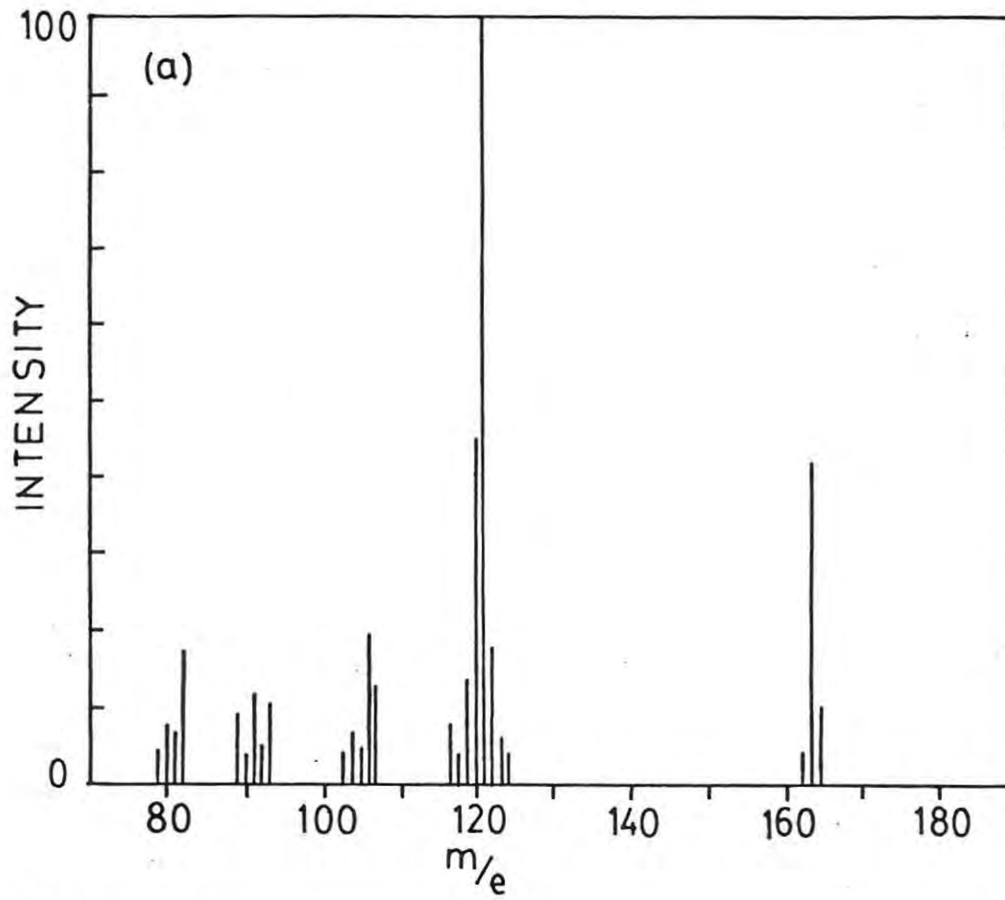
Fig. 6. Infrared spectra of (a) 2,4-dimethylformanilide and (b) bacterial product 3.

Fig. 7. Infrared spectra of (a) 2,4-dimethylaniline and (b) bacterial product 1.

formanilide, m.p. 113-115°C, which was undepressed in admixture with an authentic specimen. On chemical analysis product 3 was shown to contain : C, 72,6; H, 7,3%. On theoretical grounds 2,4-dimethylformanilide ($C_9H_{11}NO$) contains : C, 72,5; H, 7,4%.

The residue obtained from product 1 was acetylated by leaving overnight with acetic anhydride and pyridine and the product chromatographed on neutral alumina. The ethyl acetate eluate was crystallized from benzene-hexane to produce colourless flakes of 2,4-dimethylacetanilide, m.p. 127-128°C, which was undepressed in admixture with an authentic specimen. These identifications were confirmed by mass spectrometry. The mass spectra of acetylated product 1 and 2,4-dimethylacetanilide and product 3 and 2,4-dimethylformanilide are shown in Figs. 8 and 9 respectively.

Product 1 (2,4-dimethylaniline) was the first degradation product to appear and was detected after incubation of the amitraz cultures for 1 day. This product continued to accumulate and after 3 months incubation was the major product remaining in the cultures. Product 3 (2,4-dimethylformanilide) appeared after 3 days incubation and was slowly converted to 2,4-dimethylaniline. Trace amounts of the transient product 2 (N-2,4-dimethylphenyl-N'-methylformamidine) were detected between 3-19



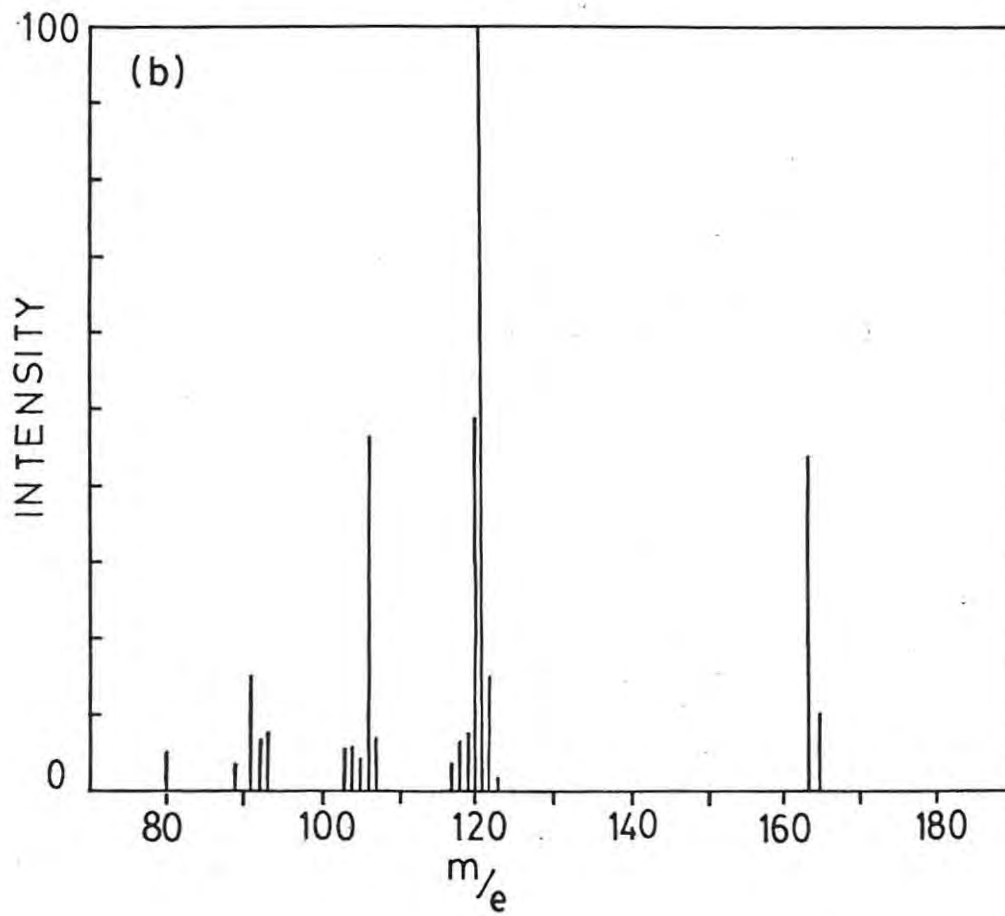
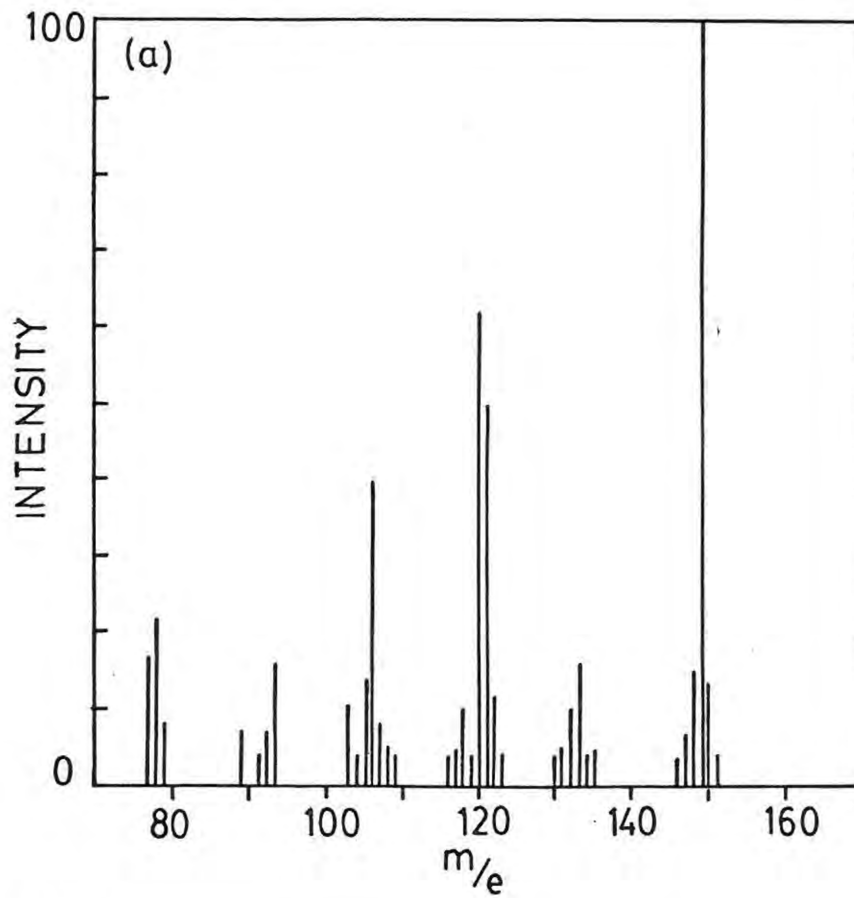


Fig. 8. Mass spectra of (a) acetylated bacterial product 1 and (b) 2,4-dimethylacetanilide.



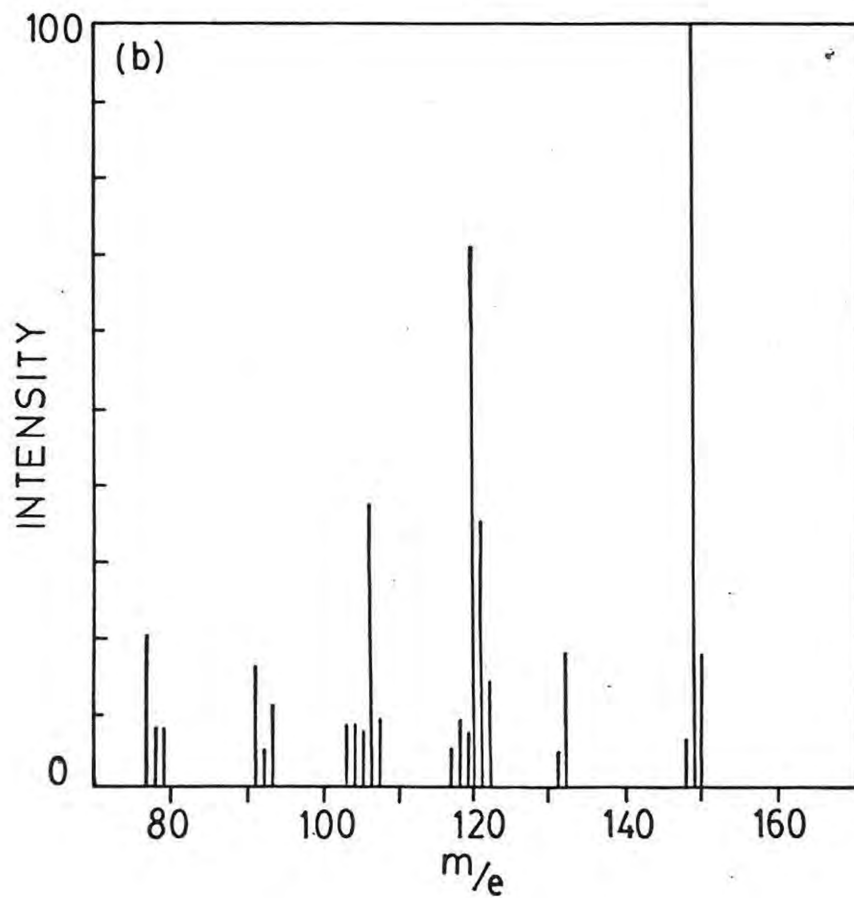


Fig. 9. Mass spectra of (a) bacterial product 3 and (b) 2,4-dimethylformanilide.

days incubation.

3.4 Discussion

Initial identification of the degradation products of amitraz showed that 3 degradation products were present (Allcock & Woods, 1977). Product 1, the major product was identified as 2,4-dimethylaniline, a transient minor product 2 as N-2,4-dimethylphenyl-N'-methylformamidine and product 3 as 2,4-dimethylformanilide. These identifications were confirmed by infrared spectroscopy, melting point determinations and mass spectrometry.

Amitraz is converted by bacteria directly to 2,4-dimethylaniline which is stable and accumulates in the bacterial cultures. This is the primary pathway for the bacterial degradation of amitraz (Fig. 10). A slower secondary pathway involves the conversion of amitraz to 2,4-dimethylaniline via the intermediates N-2,4-dimethylphenyl-N'-methylformamidine and 2,4-dimethylformanilide. The primary degradation pathway of amitraz to 2,4-dimethylaniline seems to involve a single step since no intermediates have been observed. The bacterial degradation pathway of amitraz differs from that in plants and animals. In plants it appears to be converted first to N-2,4-dimethylphenyl-N'-methylformamidine and then to 2,4-dimethylformanilide, whilst the terminal metabolite

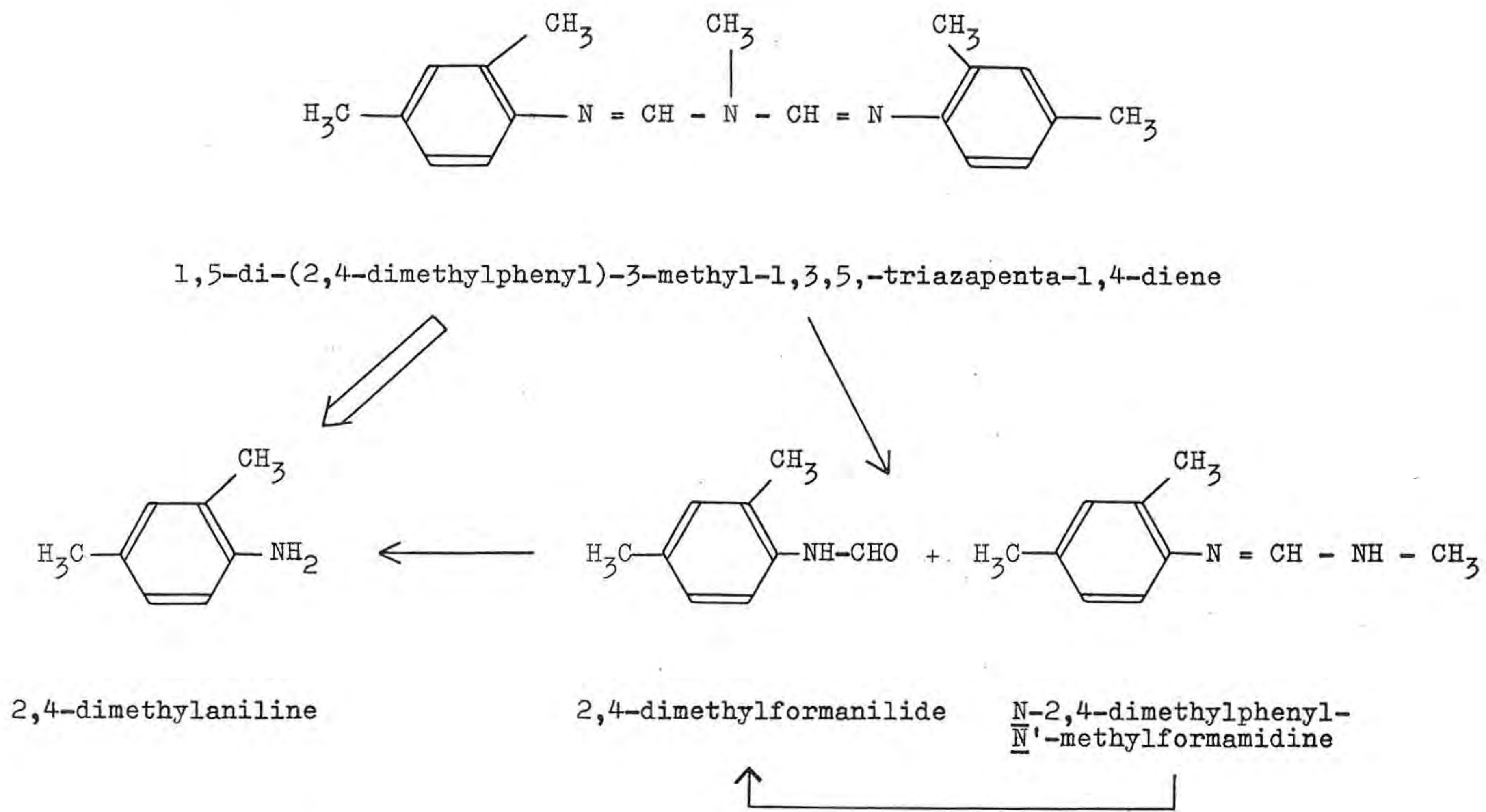


Fig. 10. Postulated pathways for the degradation of amitraz by mixed bacterial cultures.

excreted in the urine of animals is 4-amino-3-methylbenzoic acid (P.Oxley, private communication). Conversion of 2,4-dimethylformanilide to 4-amino-3-methylbenzoic acid probably goes through 2,4-dimethylaniline but this intermediate has never been identified in animals or plants. Of the degradation products identified only N-2,4-dimethylphenyl-N'-methylformamidine is known to be toxic (Coopers South Africa, private communication). The identification of this compound in the cultures suggests that it may be the cause of the toxicity problems experienced in the Carrig tank, but accumulation of solvent and emulsifier from the miscible oil may also be implicated. Cornish & Paterson (1977, private communication) on investigation of the Carrig dip wash reported that the toxicity was due to a build-up of the solvent Esso 200 arising from the miscible oil and a considerable amount of the toxic product N-2,4-dimethylphenyl-N'-methylformamidine in the tank. Further studies revealed that this toxic metabolite was pH sensitive and the addition of either lime or hydrochloric acid to the dipwash resulted in its disappearance. This fact may explain its transitory presence in laboratory cultures where ammonia produced by the bacteria resulting in an increased pH could have accounted for its disappearance.

CHAPTER IV

MECHANISM OF AMITRAZ DEGRADATION BY BACTERIA

4.1 Introduction

Co-metabolism is a phenomenon which has been observed so frequently that it appears to represent a very important type of microbial metabolism (Horvath, 1972). He reported a number of examples where enzymes required for complete metabolism of growth-supporting substrates could be induced by non-growth-supporting analogues. This indicated that the initial co-metabolic attack of the analogues involved the same enzyme or enzymes as that used for the metabolism of the growth substrate. Horvath also cited examples in which the substrate induced the formation of enzymes which co-metabolically metabolized the analogues, thus providing further evidence for an enzyme common to both initial metabolic attack of the substrate and co-metabolism of the analogues. It was previously shown (Chapter II) that in 3-day old sterile culture filtrates degradation of amitraz did not occur, however substantial degradation occurred when amitraz was

added to stationary phase cultures. This indicates that the co-metabolic degradation of amitraz by bacteria was not due to extra-cellular enzyme systems. Experiments were therefore carried out to determine the location and mechanism of action of the bacterial enzyme systems responsible for the co-metabolism of amitraz.

4.2 Materials and methods

4.21 Chemicals

[^{14}C]amitraz was supplied by the Boots Company Limited, Nottingham, England. [^{3}H]uracil was supplied by Amersham, England.

4.22 Determination of uptake of [^{3}H]uracil and [^{14}C]amitraz by whole cells

A sample (4 ml) of Ye,P medium containing unlabelled uracil (2 μg ml^{-1}) and [^{3}H]uracil (0,001 μg ml^{-1}, 0,4 μCi ml^{-1}) was inoculated with a 1 ml inoculum from an exponential Carrig culture. The sample was incubated at 30°C and 0,5 ml samples withdrawn after 0, 15, 30, 45 and 60 min, filtered through nucleopore membrane filters (0,2 μm pore size) and washed with 3 x 5 ml aliquots of warm Ye,P medium containing unlabelled uracil (2 μg ml^{-1}).

Similarly a 4 ml Ye,P sample containing 0,2% M.O. and [^{14}C]amitraz (0,62 mg ml^{-1}, 2,5 µCi ml^{-1}) was inoculated with a 1 ml inoculum from a 6 day Ye,P + M.O. Carrig culture. A control inoculum was 1 ml of the 6 day enrichment culture which had been boiled for 15 min. Incubation was at 30°C and samples were withdrawn after 0, 0,5, 1, 2, 3, 4, 5, 6, 7 and 8 h, filtered through a nucleopore membrane filter and washed with 3 x 5 ml aliquots of warm Ye,P medium containing 0,2% M.O. The membrane filters were dried, added to a Triton X - 100 + toluene scintillant and counted in a Beckman LS 3150 T scintillation counter.

4.23 Determination of incorporation of [^{3}H]uracil and [^{14}C]amitraz into trichloroacetic acid (TCA) precipitable material

The incorporation of [^{3}H]uracil and [^{14}C]amitraz into TCA-precipitable material (macromolecules including proteins and nucleic acids) was investigated by the method of Eichenlaub and Winkler (1974). Samples were prepared as described in section 4.22. Aliquots were withdrawn after similar time intervals and added to cold 0,5 ml aliquots of 10% TCA containing the unlabelled carrier. This was allowed to stand in an ice bath for 30 min after which it was filtered through a glass fibre filter pad.

The filter pad was washed with 2 x 5 ml aliquots of cold 5% TCA and 1 x 5 ml aliquot of 1% acetic acid. They were dried, placed into vials containing a Triton X - 100 + toluene scintillant and counted in a Beckman LS 3150 T scintillation counter.

4.24 Thin-layer chromatography assay for the degradation of amitraz

A t.l.c. assay for the degradation of amitraz was developed which involved assaying samples on glass-backed, silica-gel t.l.c. plates (layer thickness 0,5 mm). Samples (10 ml) to be analysed were extracted with 5 ml $CHCl_3$ and 10 µl aliquots were spotted onto the t.l.c. plates which were developed in a toluene-methanol (40 : 1 v/v) system. The plates were air dried and transferred to a tank filled with NO_2 fumes for 5 min prior to spraying with a N-naphthyl-ethylene-diamine-dihydrochloride spray reagent (0,5% (w/v) in 50% (v/v) methanol-water). Concentrations of the amitraz and 2,4-dimethylaniline spots were determined by densitometry. By assigning a value of 100 percent to the time zero amitraz spot the percentage remaining in the other amitraz spots was calculated. The t.l.c. plates were read perpendicular to the direction of migration using a Vitatron TLD 100 densitometer equipped with a power-driven plate scanner

and a variable response recorder which automatically charted the scanned densitometer values. For the 2,4-dimethylaniline spots integrated peak areas were plotted against known concentrations of 2,4-dimethylaniline to produce a reference curve for use in determining the concentration of the 2,4-dimethylaniline spots. The external standard used was 10 µl of a 0,05% (w/v) coomassie blue in ethanol solution.

4.25 The effect of sonication on the degradation of amitraz

A number of cell samples were prepared by harvesting cells from 400 ml samples of Ye,P cultures and resuspending the pellets in 5 ml aliquots of sterile MM. Experimental samples were cooled to $5^{o}C$ and sonicated for 3 x 1 min intervals at 25 KH_2 and maximum amplitude. The samples were reconstituted in 95 ml MM + M.O. and incubated at 30 and $37^{o}C$. The degradation of amitraz in the sonicated samples after 0,10 and 23 h was compared with unsonicated cultures and cell free controls by the t.l.c. assay described in section 4.24.

4.26 The release of enzymes by osmotic shock experiments

Osmotic shock experiments were carried out according to the method of Willis et al. (1974). They describ-

ed high yield growth procedures adaptable to most laboratories and a simplified osmotic shock procedure for the preparation of periplasmic, shock-releasable enzymes. Large scale osmotic shock experiments involved fermentations using 14 litres of Ye,P medium inoculated with a Carrig enrichment culture. The culture was allowed to stand in the fermenter for 6 days at a temperature of 30°C, aerated at 8 l min^{-1} and agitated at a speed of 200 rev.min^{-1}. The cells were collected in a Cepa model LE laboratory centrifuge at 45000 rev. min^{-1}. The resulting crude shock fluid after shocking was concentrated by ammonium sulphate precipitation (80%,Dixon & Webb, 1958), collected by centrifugation and dialysed against a phosphate buffer pH 7,6. The concentrated shock fluid was supplemented with 0,2% M.O. and the amitraz concentrations determined after 15 h incubation at 30°C.

4.27 Induction of the amitraz degradation system by amitraz and a related ixodicide

Experiments were carried out to determine whether amitraz acted as an inducer for the enzymes responsible for it's degradation or not. Samples (100 ml) of Ye,P + M.O. were inoculated with a Carrig inoculum and incubated at 30°C for 5 days. The amitraz concentrations of each were determined. Further samples (100 ml) of Ye,P

RHODES UNIVERSITY LIBRARY

were inoculated with a Carrig inoculum and incubated at 30°C for 4 days. After this time each was supplemented with 0,2% M.O. and incubated for a further 24 h before determining the amitraz concentrations. In a similar experiment designed to determine whether another ixodicide Delnav D.F.F. induced the degradation of amitraz, 100 ml Ye,P Carrig cultures were supplemented with 0,05% Delnav D.F.F. After 4 days incubation at 30°C 0,2% M.O. was added to each culture and the amitraz concentrations were determined after a further 24 h incubation. Uninoculated controls containing 0,05% Delnav D.F.F. and 0,2% M.O. were also monitored.

4.28 Utilization of M.O. as a sole source of nitrogen

These experiments were done to determine whether bacteria present in the dip samples were able to utilize a component of the M.O. as a sole source of nitrogen (Campacci, New and Tchan, 1977). Aliquots of dipwash from 10 dip tank samples (see Chapter 7, p 80) were centrifuged to remove the cells. The pellets were washed in 0,85% saline and 1 ml samples inoculated into 100 ml volumes of a basal salts medium containing 0,03 g K_2HPO_4, 0,01 g $MgSO_4 \cdot 7H_2O$ and 0,2% M.O. The medium was adjusted to pH 7,0 with NaOH. To prevent aerial contamination with combined nitrogen, each flask was sealed with a

rubber stopper connected with the exterior by a U-tube containing H_2SO_4. Under these conditions the bacteria were unable to fix molecular nitrogen. The flasks were incubated at 30°C for 12 weeks and assayed for bacterial numbers and degradation of amitraz. The washed inocula were also assayed for bacterial numbers.

4.3 Results

4.31 Uptake of [^{3}H]uracil and [^{14}C]amitraz by whole cells

[^{3}H]uracil was taken up by whole cells (Fig. 11) but no detectable uptake of [^{14}C]amitraz was observed (Table 8).

4.32 Determination of incorporation of [^{3}H]uracil and [^{14}C]amitraz into TCA-precipitable material

[^{3}H]uracil was incorporated into TCA-precipitable material (Fig. 11) but no incorporation of [^{14}C]amitraz occurred (Table 9).

4.33 Thin-layer chromatography assay for the degradation of amitraz

The calibration curve relating spot intensities to

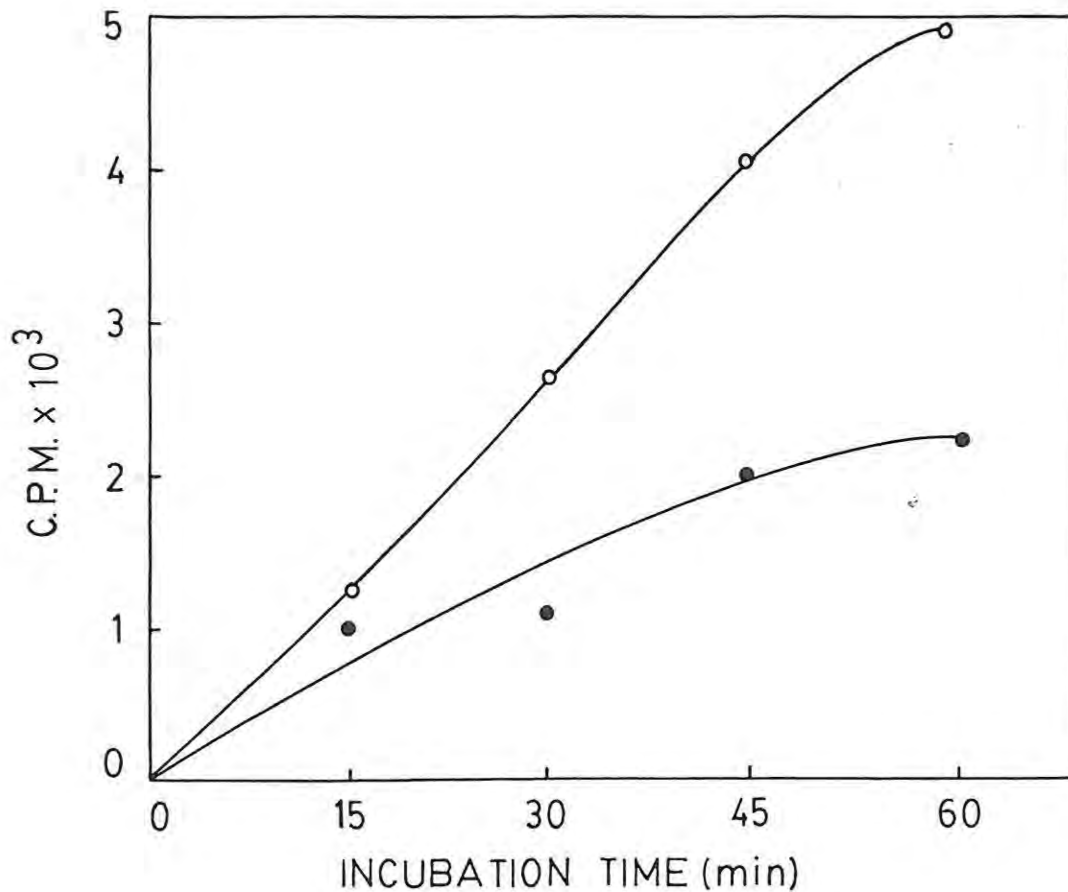


Fig. 11. Uptake by whole cells and incorporation of [^{3}H]uracil into TCA-precipitable material (o) uptake of [^{3}H]uracil by whole cells; (•) incorporation of [^{3}H]uracil into TCA-precipitable material; 0,1 ml [^{3}H]uracil medium contained 2,7 x 10^4 C.P.M.

Table 8. Uptake of [^{14}C]amitraz by whole cells.

Sampling Time (h)	C.P.M. Control (cells boiled for 15 min)	C.P.M. Experimental
0	51,2	66,7
0,5	51,9	53,7
1	53,9	70,4
2	60,7	62,9
3	87,1	69,1
4	68,9	73,4
5	61,2	53,8
6	61,3	53,0
7	49,0	63,6
8	52,0	52,9
0,5ml [^{14}C]amitraz medium	6300,0	6300,0

Table 9. Incorporation of [^{14}C]amitraz into TCA-precipitable material.

Sampling Time (h)	TCA-precipitate C.P.M.
0	101,2
0,25	88,2
0,5	79,1
0,75	68,3
1	75,6
1,5	63,9
2	59,1
3	110,9
4	76,4
5	71,2
0,5ml [^{14}C]amitraz medium	6300,0

known concentrations of 2,4-dimethylaniline is shown in Fig. 12. This assay is ideally suited to the analysis of degradation products and is a sensitive and quantitative technique.

4.34 The effect of sonication on the degradation of amitraz

No detectable differences in the rates of degradation between sonicated and whole cells at 30 or 37°C were observed (Table 10).

4.35 The release of enzymes by osmotic shock experiments

Osmotic shock experiments indicated that the shocked cells degraded amitraz less rapidly than unshocked cells in MM (Table 11). In Ye,P + M.O. medium recovery of the cells during the 24 h incubation period necessary to show significant differences in degradation resulted in little differences between shocked and unshocked cells. In large scale experiments no degradation of amitraz was observed in either the crude shock fluid or the ammonium sulphate concentrated sample (Table 12).

4.36 Induction of the amitraz degradation system by amitraz and a related ixodicide

Neither amitraz nor Delnav D.F.F. induced the

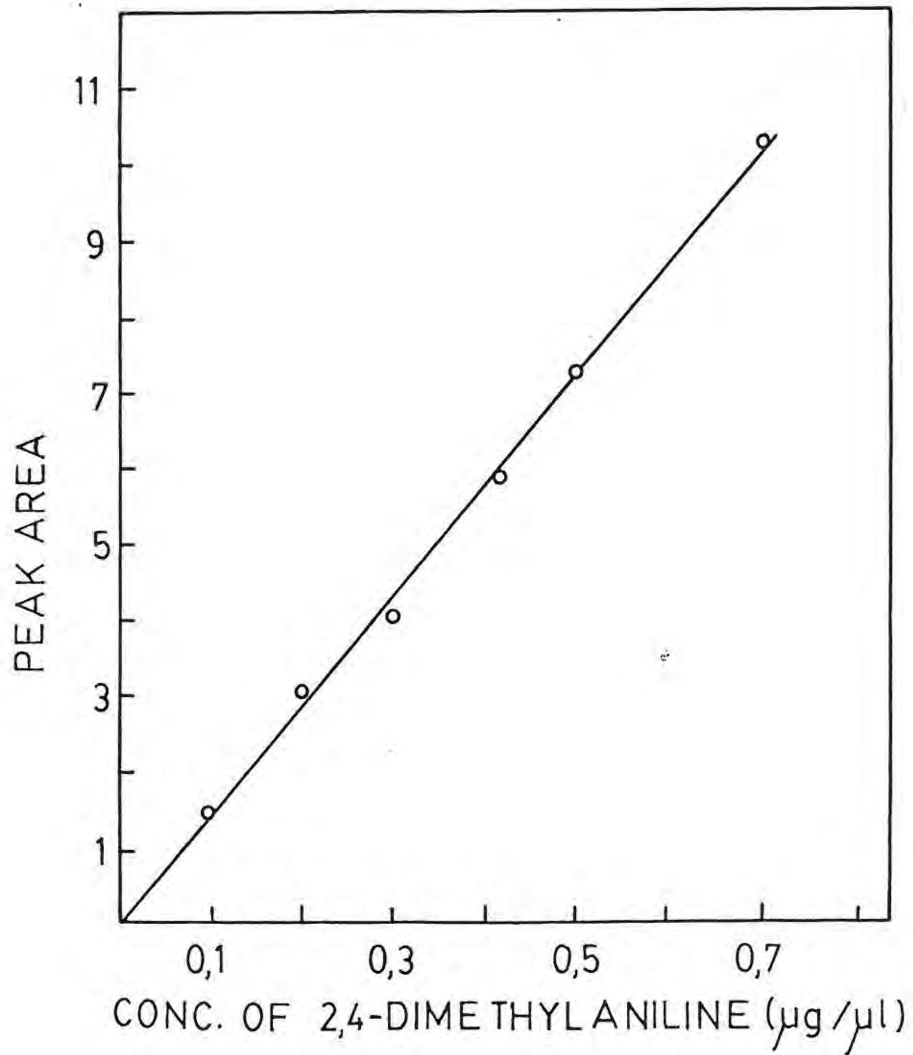


Fig. 12 Calibration curve relating peak areas to known concentrations of 2,4-dimethylaniline.

Table 10. The effect of sonication on the degradation of amitraz.

Amitraz spots

Time (h)	% amitraz remaining					
	30°C			37°C		
	Sonicated cells	Whole cells	Cell free control	Sonicated cells	Whole cells	Cell free control
0	100	100	100	100	100	100
10	67	71	94	70	67	100
23	44	43	94	40	44	100

2,4-dimethylaniline spots

Time (h)	2,4-dimethylaniline in $CHCl_3$ (µg/µl)					
	30°C			37°C		
	Sonicated cells	Whole cells	Cell free control	Sonicated cells	Whole cells	Cell free control
0	0,13	0,19	0,31	0,13	0,19	0,31
10	0,38	0,25	0,31	0,31	0,40	0,31
23	0,45	0,41	0,31	0,42	0,41	0,31

Table 11. Degradation of amitraz by osmotically shocked cells.

Recovery medium	% amitraz remaining		
	Time 0	8 h	24 h
shocked cells in MM	100	100	96
unshocked cells in MM	100	87	83
uninoculated control MM	100	100	98

Table 12. Degradation of amitraz by osmotic shock fluids.

Sample	% amitraz remaining			
	Experiment 1		Experiment 2	
	Time 0	15 h	Time 0	15 h
uninoculated control	100,0	100,0	100,0	98,7
1. crude shock fluid	100,0	96,3	100,0	95,1
2. crude shock fluid	100,0	94,7	100,0	93,0
3. crude shock fluid	100,0	97,1		
concentrated shock fluid	100,0	90,3	100,0	91,7

enzyme systems responsible for the degradation of amitraz (Table 13). Approximately 43% degradation due to bacteria was observed in the cultures containing amitraz for 5 days, while in the 4 day old cultures to which M.O. was added c. 40% degradation was observed in 24 hours. In the cultures containing Delnav D.F.F. only 30% degradation occurred. In the uninoculated control containing Delnav D.F.F. 20% degradation was obtained indicating that chemical degradation of amitraz had taken place.

4.37 Utilization of M.O. as a sole source of nitrogen

Viable bacteria were isolated from each of the bacterial populations tested (Table 14). Eight of the 10 populations tested were able to degrade amitraz in the basal salts medium after 12 weeks. Bacteria from the Rooipan and Carrig dip tanks were especially successful in degrading amitraz (c. 60% degradation). With the exception of the bacterial population from the Vryheid dip tank each population decreased 10 - 1000-fold in bacterial numbers during the 12 weeks.

4.4 Discussion

The [^{14}C]amitraz uptake experiments indicated that amitraz was not taken up by bacterial cells nor

Table 13. Induction of the amitraz degradation system by amitraz and a related ixodicide.

Sample	inducer	% amitraz remaining	% degradation	*Corrected % degradation
Ye,P uninoc. control	amitraz	87,8	c. 12	NIL
Ye,P+Carrig	amitraz	41,8	c. 55	c. 43
Ye,P+Carrig	amitraz	49,5		
Ye,P+Carrig	-	65,0	c. 40	c. 40
Ye,P+Carrig	-	60,6		
Ye,P uninoc. control	Delnav DFF	67,8	c. 30	c. 20
Ye,P+Carrig	Delnav DFF	58,3	c. 45	c. 30
Ye,P+Carrig	Delnav DFF	52,2		

*Corrected % degradation = degradation due to the action of bacteria only.

Table 14. Utilization of M.O. as a sole source of nitrogen.

Dip sample	Bacterial number ml^{-1} Initial	Final	% amitraz remaining after 12 weeks
Uninoculated control	NIL	NIL	86,3
Wiltonside	$1,3x10^7$	$3,3x10^4$	90,7
Carrig	$6,8x10^6$	$6,3x10^4$	44,5
Tayside	$8,1x10^6$	$9,3x10^4$	59,0
Nottingham road	$4,1x10^7$	$4,8x10^5$	64,3
Richmond	$5,0x10^6$	$6,9x10^5$	49,3
Ixopo*	$1,2x10^7$	$3,6x10^4$	65,2
Carrig**	$1,1x10^7$	$3,6x10^4$	39,6
Rooipan	$3,5x10^7$	$1,5x10^6$	26,4
Vryheid	$1,3x10^6$	$1,1x10^6$	57,7
Hluhluwe	$5,1x10^6$	$4,8x10^5$	97,4

Ixopo* - dip tank charged with Altik

Carrig** - dip tank charged with Delnav D.F.F.

incorporated into cellular macromolecules. Control experiments using [^{3}H]uracil showed uptake and incorporation of uracil indicating that the experimental protocol was suitable for uptake experiments. Amitraz was therefore not degraded intra-cellularly and previously it had been shown that no degradation occurred in cell-free culture filtrates but significant degradation occurred in the presence of stationary phase cells. This indicated that amitraz was not degraded by extra-cellular enzyme systems. However there was no detectable difference in the rates of degradation between sonicated and whole cells. Bacteria have a number of enzyme systems which are situated outside the cell membrane but remain associated with the cells envelope and are not released into the medium. These enzymes are located in the periplasmic space. Osmotic shock experiments designed to release enzymes from the periplasmic space indicated that shocked cells degraded amitraz less rapidly than unshocked cells in MM. No activity however was found in the shock fluids and it is therefore concluded that the enzyme or enzymes responsible for amitraz degradation are situated in the periplasmic space but are either inactivated in the osmotic shock procedure or are present in too low a concentration for detection. High yield growth procedures to provide shockable cells has been reviewed by Phares (1971). Culture densities as high as 70 g wet

cell paste litre^{-1} of culture medium have been obtained, however the osmotic shocking of cells from such cultures treated with antifoam agents (necessary at high cell densities) resulted in extreme cell lysis and lower overall yields of periplasmic enzymes. This may account for the low yields obtained as an antifoam agent was used to control foaming.

The amitraz degradation system was not induced by amitraz or Delnav D.F•F. and is therefore a constitutive system. Degradation of amitraz occurred to a similar extent in cultures containing amitraz for 5 days or 24 h. In the cultures containing Delnav D.F.F. less degradation of amitraz was observed in 24 h. Chemical degradation of amitraz occurred in the uninoculated sample containing Delnav D.F.F. due to a chemical reaction between the ixodicides.

Eight of the 10 bacterial populations tested were able to degrade amitraz in a basal salts medium with M.O. as the only source of nitrogen. Campacci, New and Tchan (1977) isolated bacteria capable of degrading the herbicide amitrole by using an enrichment medium supplemented with amitrole as a sole source of nitrogen rather than organic carbon. They also found that the decomposition of amitrole was inhibited in soil dilutions less than 1 in 1000 due to the preferential use of other nitrogen

sources present in the soil. The MM used in other experiments involving amitraz contained ample amounts of nitrogen which could have been used in preference to amitraz molecules. In the absence of any other source of nitrogen eight of the bacterial populations tested were able to degrade amitraz over a long period without the co-metabolite. It is interesting to note that a large percentage of the bacteria isolated from the basal salts cultures after 12 weeks produced diffuse pigments and odours characteristic of the genus *Pseudomonas*. This enrichment in basal salts + M.O. could have selected for bacteria of this type as they were present in most of the mixed dip populations.

CHAPTER V

DEVELOPMENT OF A LIQUID SCINTILLATION RADIOASSAY OF THIN-LAYER CHROMATOGRAMS

5.1 Introduction

The gas chromatography (g.c.) technique used in the amitraz assay is unsuitable for enzyme experiments because of the large volumes (100 ml) required for each analysis and the length of time between samplings (1-3 days) which is necessary for the detection of degradation. The development of an effective and rapid radioassay to replace the g.c. assay was undertaken.

Procedures available for the radioassay of thin-layer chromatograms (t.l.c.) have been reviewed by Bransome (1970). The methods include elution analysis, strip scanning, auto-radiography, zonal-profile analysis, beta camera detection and combustion analysis. Special scintillation solutions must be used in conjunction with the radioassay of t.l.c. layers, since problems in quantitation may be encountered. The solutions should contain either water for deactivation or gels for suspension of the

adsorbent particles. The polyvinyl alcohols and other organic binders and the thin support surfaces used in commercially available plates (plastic and aluminium sheeting) are not quenchers in the liquid scintillation systems used (Bransome, 1970). Zonal-profile analysis using aluminium chromatosheets was used in this analysis.

5.2 Materials and methods

5.21 Chemicals

The dioxane-water scintillation fluid used contained g/l : 2,5-diphenyloxazole, 7,0; p-bis-2-(4-methyl-5-phenyloxazolyl)benzene, 0,3; naphthalene, 10,0 in reagent grade dioxane. This solution was ready for use after it was mixed with water in the proportions of 15 : 3 (v/v).

5.22 Thin-layer chromatography assay

Bacteria from 100 ml Ye,P broth inoculated with 1 ml of the Carrig enrichment culture were resuspended in 10 ml of the used medium. This was supplemented with 20 µl of [^{14}C]amitraz (12 mg/ml; 48 µCi/ml) and 0,1% M.O. and incubated at 30°C. Samples (1 ml) were removed after 0, 2, 3, 4 and 22 h and extracted with 1 ml $CHCl_3$ by shaking gently for 5 min. The $CHCl_3$ layer was removed

and duplicate 20 µl samples spotted onto a t.l.c. plate (Kieselguhr F_{254}, Merck). The plate was developed in a toluene-methanol (40 : 1 v/v) system, air dried and cut in half longitudinally. One half of the plate was exposed to dense NO_2 fumes for 5 min prior to spraying with a N-naphthyl-ethylene-diamine-dihydrochloride spray reagent (0,5% (w/v) in 50% (v/v) methanol-water). The R_f values of amitraz and the degradation products were marked off and served as a guide for locating the degradation products on the unsprayed half. The relative zones were cut out of the plate and placed in scintillation vials containing 10 ml of the dioxane-water scintillation fluid and counted in a Beckman LS 3150 T scintillation counter.

5.3 Results

An increase in the C.P.M. with time was found in the two major degradation products (1,5 - 11,4% for 2,4-dimethylaniline and 29,6 - 39,2 for 2,4-dimethylformanilide). These increases coincided with a decrease in the C.P.M. in the amitraz spots (22,3 - 5,7%, Table 15 and Fig. 13). The percentage recovery of C.P.M. from each sample spotted onto the plate was c. 50%.

Table 15. Radioassay for the degradation of amitraz.

Sample spot	C.P.M.									
	Time 0	%	2 h	%	3 h	%	4 h	%	22 h	%
Amitraz	2058,4	(22,3)	1881,8	(20,4)	1631,0	(17,7)	1330,1	(14,4)	526,5	(5,7)
2,4-dimethyl-aniline	141,1	(1,5)	175,1	(1,9)	186,8	(2,0)	396,6	(4,3)	1055,1	(11,4)
2,4-dimethyl-formanilide	2739,6	(29,6)	2940,9	(31,9)	3141,4	(34,0)	3162,2	(34,3)	3613,1	(39,2)
Sum of amitraz and degradation products	4939,1	(53,5)	4997,8	(54,2)	4959,2	(53,7)	4888,9	(53,0)	5194,7	(56,3)

Total number of counts in a 20 µl sample was 9228,2
% = percentage of the total number of C.P.M. in a 20 µl sample.

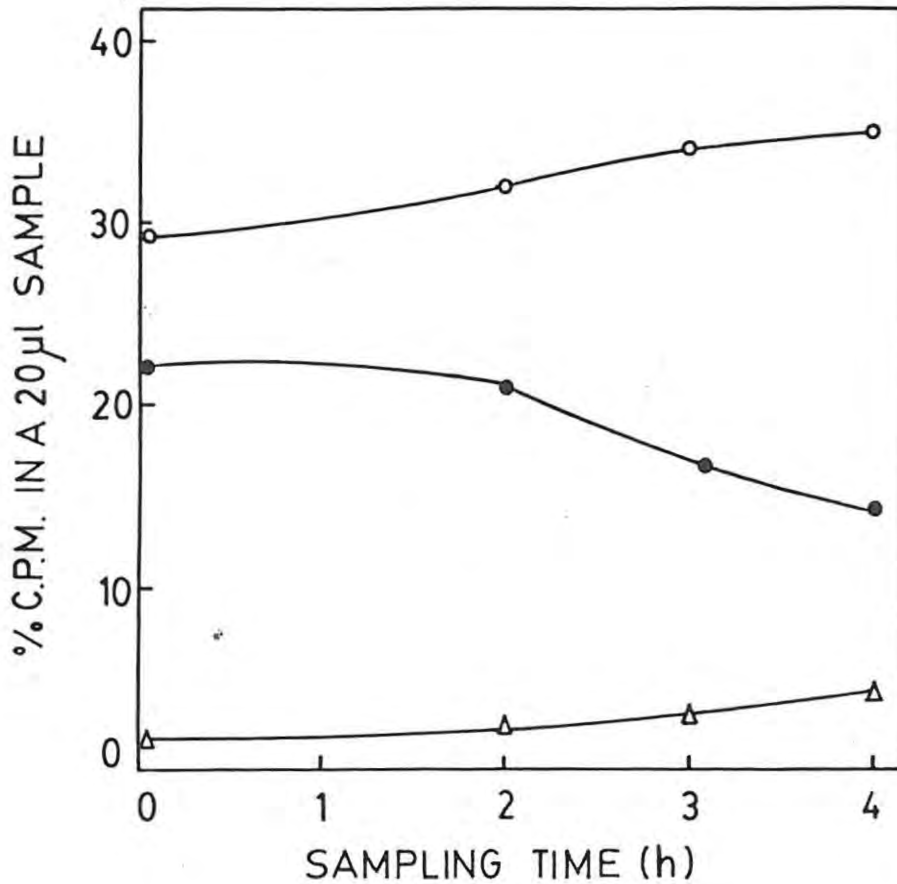


Fig. 13. Thin-layer chromatography radioassay of amitraz (•) [^{14}C]amitraz levels; (o) [^{14}C]2,4-dimethylformanilide levels; (Δ) [^{14}C]2,4-dimethylaniline levels; 20 µl of [^{14}C]amitraz medium contained 9,2 x 10^3 C.P.M.

5.4 Discussion

The results indicated that the total number of C.P.M. recovered from each spotting remained approximately the same. The increase in C.P.M. in the degradation products coincided with the decrease in the amitraz counts. However the large number of counts in the 2,4-dimethylformanilide spot at time zero indicated that a large percentage of the [^{14}C]amitraz had been chemically degraded. Losses in C.P.M. during development may be due to absorption of the labelled compound on the particles present on the chromatography plate. This sensitive and relatively rapid assay could be of value in experiments aimed at investigating the enzymes involved in the degradation of amitraz.

CHAPTER VI

INHIBITION OF AMITRAZ DEGRADATION BY ENZYME INHIBITORS AND ANTISEPTIC COMPOUNDS

6.1 Introduction

Harrison et al.(1973) found that raising the pH to 12,2 by the addition of slaked lime to fouled dip washes resulted in the stabilization of amitraz. The Cooper's "Lime Stabilization Method" involved adding a large amount of slaked lime containing approximately 85% $Ca(OH)_2$ after the first week's dipping to the wash to yield a $Ca(OH)_2$ concentration of approximately 0,4% and a pH of 12 (Baker, 1975). Other methods of stabilizing amitraz e.g. the addition of enzyme inhibitors and antiseptic compounds were investigated. Studies with enzyme inhibitors can also indicate the nature of the enzymes involved.

6.2 Materials and methods

6.21 Stabilization of amitraz by enzyme inhibitors

The enzyme inhibitors : N-ethylmaleimide, p-chloro-mercuro-benzoate (pCMB), dithiothriotol (Cleland's reagent), ethylenediamine-tetraacetic acid (E.D.T.A.) and 1,10-phenanthroline were tested for their ability to inhibit the bacterial degradation of amitraz. Different concentrations of the inhibitors in sterile water were added to 3 day old Carrig bacterial populations in Ye,P medium. Amitraz M.O. was added and the samples were analysed for degradation of amitraz and bacterial numbers after 0 and 5 days incubation at 30°C.

6.22 Stabilization of amitraz by the addition of antiseptic compounds

A range of commercial and other antiseptic compounds (Table 16) were tested for their ability to inhibit the bacterial degradation of amitraz. The antiseptics at a concentration of 0,01% were added to 3 day old Carrig bacterial populations in Ye,P medium. Amitraz M.O. was added and the samples were analysed for degradation of amitraz and bacterial numbers after 0 and 5 days incubation at 30°C. Five of the compounds

Table 16. Commercial and other antiseptics tested for their ability to inhibit the bacterial degradation of amitraz.

Merpin WS extra 40	Busan 80
Henkel BG 8427	Chembac 7
P.I.D. 121	Chembac 8
Busan 30-1	Cequartyl CTH
Preventol	C.R.L.
K.M.	Mystox Q.L.
K.M. 11	Fresh bark extract
Hyamine F.H.P.	Tannery wattle
Mystox S.N.	Garmin K
Kathon L.P.	Calgan
Vantoc 'CL'	Kromex
Anabac	Merpin T.K.E.
Nercolan F 24	P.I.D. 131
Formalin	K.M. 102
Vantocil	Busperse 49

(Vantoc 'CL', Garmin K, KM 102, Hyamine F.H.P. and Vantocil) which showed potential effectiveness were subjected to further tests at two concentrations (0,01 and 0,05%). The incubation time was increased to 10 days and the degradation compared with samples containing 0,4% $Ca(OH)_2$.

6.3 Results

6.31 Stabilization of amitraz by enzyme inhibitors

N-ethylmaleimide, pCMB and E.D.T.A. showed promising results (Table 17). In the N-ethylmaleimide samples c. 80% of the amitraz remained after 5 days. The most effective stabilization was obtained in the pCMB samples where 100% activity remained in the uninoculated control after 5 days. Decreasing concentrations of pCMB resulted in decreasing amounts of amitraz remaining. The E.D.T.A. samples were partially effective and c. 60% of the amitraz activity was maintained. Dithiothriotol and 1,10-phenanthroline proved unsuccessful in preventing the degradation of amitraz.

Table 17. Stabilization of amitraz by enzyme inhibitors.

Sample	Molar Conc.	% conc.	% amitraz remaining	Bacterial No ml^{-1}
1,10 Phenanthroline				
Uninoc. control	10^{-3}	0,02	94	NIL
Inoc. sample	10^{-3}	0,02	31	$1,2 \times 10^{3}$
Inoc. sample	10^{-4}	0,002	52	$4,2 \times 10^{2}$
Inoc. sample	10^{-5}	0,0002	57	$3,7 \times 10^{8}$
N-ethylmaleimide				
Uninoc. control	10^{-3}	0,012	79	NIL
Inoc. sample	10^{-3}	0,012	79	$9,6 \times 10^{6}$
Inoc. sample	10^{-4}	0,0012	79	$3,9 \times 10^{8}$
Inoc. sample	10^{-5}	0,00012	73	$3,9 \times 10^{8}$
Dithiothriotol				
Uninoc. control	10^{-3}	0,015	90	NIL
Inoc. sample	10^{-3}	0,015	36	$8,1 \times 10^{5}$
Inoc. sample	10^{-4}	0,0015	36	$9,9 \times 10^{5}$
Inoc. sample	10^{-5}	0,00015	52	$1,1 \times 10^{6}$
E.D.T.A.				
Uninoc. control	10^{-3}	0,037	90	NIL
Inoc. control	10^{-3}	0,037	68	$1,3 \times 10^{7}$
Inoc. control	10^{-4}	0,0037	52	$3,0 \times 10^{8}$
Inoc. control	10^{-5}	0,00037	57	$3,9 \times 10^{8}$
PCMB				
Uninoc. control	10^{-3}	0,036	100	NIL
Inoc. sample	10^{-3}	0,036	90	$5,4 \times 10^{3}$
Inoc. sample	10^{-4}	0,0036	84	$2,3 \times 10^{8}$
Inoc. sample	10^{-5}	0,00036	68	$6,6 \times 10^{8}$
Uninoc. control	No inhibitor		84	NIL
Inoc. control	No inhibitor		21	$1,4 \times 10^{9}$

Bacterial number at the time of addition of inhibitor = $7,8 \times 10^{7}$ ml^{-1}.

6.32 Stabilization of amitraz by the addition of antiseptic compounds

Hyamine F.H.P. and Vantoc 'CL' were partially effective in limiting the bacterial degradation of amitraz (Table 18). The most effective stabilization was obtained at the 0,01% level as chemical degradation was found to occur in the 0,05% samples. Most of the bacteria were killed by the addition of 0,05% Hyamine F.H.P., Vantoc 'CL' and Garmin K. KM 102, Garmin K and Vantocil were unsuccessful at either concentration in limiting the bacterial degradation of amitraz. Excellent stabilization was obtained in the samples containing $Ca(OH)_2$.

6.4 Discussion

Although pCMB, N-ethylmaleimide and E.D.T.A. showed promising results in stabilizing the degradation of amitraz they are unlikely to be of much practical value because of their toxicity. The fact that pCMB and N-ethylmaleimide inhibited bacterial degradation of amitraz suggests that the enzyme or enzymes involved contain thiol groups, as these inhibitors complex with thiol groups on enzymes and inhibit their activity. However Dithiothriotol which also affects thiol groups did not stabilize amitraz.

Table 18. Inhibition of degradation by the addition of antiseptic compounds.

Sample	% amitraz remaining	Bacterial No ml^{-1}
Uninoc. control	95,1	NIL
Inoc. control	19,4	$6,0 \times 10^6$
Hyamine F.H.P.		
0,01% uninoc. control	81,0	NIL
0,01% inoc. sample	65,0	$2,7 \times 10^3$
0,05% uninoc. control	75,6	NIL
0,05% inoc. sample	48,1	NIL
Vantoc 'CL'		
0,01% uninoc. control	66,3	NIL
0,01% inoc. sample	55,6	$3,3 \times 10^5$
0,05% uninoc. control	62,5	NIL
0,05% uninoc. sample	58,8	NIL
Garmin K		
0,01% uninoc. control	100,0	NIL
0,01% inoc. sample	15,6	$2,4 \times 10^6$
0,05% uninoc. control	97,5	NIL
0,05% inoc. sample	20,0	NIL
KM 102		
0,01% uninoc. control	100,0	NIL
0,01% inoc. sample	2,1	$2,1 \times 10^6$
0,05% uninoc. control	100,0	NIL
0,05% inoc. sample	7,5	$1,1 \times 10^5$
Vantocil		
0,01% uninoc. control	100,0	NIL
0,01% inoc. sample	6,2	$2,4 \times 10^6$
0,05% uninoc. control	100,0	NIL
0,05% inoc. sample	5,6	$6,3 \times 10^4$
$Ca(OH)_2$		
0,4% uninoc. control	100,0	NIL
0,4% inoc. sample	91,8	$1,2 \times 10^4$

Bacterial number at time of addition of antiseptic was $6,6 \times 10^{10}$ ml^{-1}.

Hyamine F.H.P. and Vantoc 'CL' were not as effective as $Ca(OH)_2$ employed in the Coopers' "Lime Stabilization Method" but these antiseptics were acting at a concentration <40 x that of the $Ca(OH)_2$. Although they were 50% less efficient, for a dip tank of volume 11 000 l they would effectively reduce the amount of stabilizer added from c. 50 kg of slaked lime to c. 1.25 kg of the antiseptic. The relative costs involved as well as stringent toxicity trials would have to be undertaken to ensure that the antiseptic used was harmless to cattle. Hyamine F.H.P. is formulated as a 50% concentrate in ethanol containing a selected blend of alkyl dimethyl benzyl ammonium chlorides. The formulation and active ingredient of Vantoc 'CL' was unavailable.

CHAPTER VII

A BACTERIAL MODEL FOR DIP TANKS

7.1 Introduction

The development of a laboratory scale model which simulates the bacteriology of a dip tank was initiated. Such a model would be most useful in the evaluation of the biodegradation properties of new ixodicides and could result in the elimination of lengthy field trials which are not ideally suited to biodegradation studies. Studies in this project to date have resulted in experience with the bacteriology of two dip tanks, Wiltonside and Carrig, charged with amitraz wettable powder and miscible oil respectively. A laboratory medium has been developed which is very similar to the field situation. The degree of fouling of a tank can be simulated by the addition of yeast extract to a chemically defined medium. The predominant bacteria in the Wiltonside and Carrig tanks have been identified and there is a high degree of overlap between the bacterial types from the two tanks. The conditions required for amitraz degradation are virtually identical for the two bacterial populations.

In order to establish whether this pattern of amitraz degradation by bacteria is a general pattern for all dip tanks, the bacterial populations from amitraz and other tanks located throughout Southern Africa were investigated. The bacterial populations were also preserved for future reference.

Because of the dip tank environment (i.e. input of bacteria from soil and faeces) it is predicted that a unifying model for the degradation of amitraz by dip tank bacteria could apply. If such a model for amitraz degradation is verified, its general applicability may be tested with other ixodicides which have already undergone extensive field trials and have been discarded because of their instability.

7.2 Materials and methods

7.21 Isolation and identification of strains

Identification studies on dip samples from a wide variety of total replacement amitraz tanks and tanks charged with other ixodicides throughout South Africa were carried out. Summer samples from 10 dip tanks were analysed and in order to determine whether a seasonal variation in bacterial types existed winter samples from

5 of the same dip tanks were analysed. Each dip was diluted and plated (by the spread plate method) onto Ye,P; Ye,P + M.O. and MacConkey agar. From the Ye,P plates of each dip each of the morphologically different colony types (between 10 - 16 per dip) were selected and purified by cloning on Ye,P agar. The pure bacterial strains were characterised using methods obtained from the following texts: Manual for the Identification of Medical Bacteria (Cowan & Steel, 1966); The Genera of Bacteria (Skerman, 1967), 8th edition of Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974) and using the Analytical Profile Index (API) 20 E system supplied by Ayerst Laboratories, South Africa. The API 20 E kit consists of a sterile preset battery of 23 biochemical tests for use in identifying 48 species and subspecies of Enterobacteriaceae and 30 species of other gram negative bacteria.

7.22 Amitraz degradation by dip tank populations

The abilities of the 10 summer dip tank populations and a test dip population consisting of the 5 most common genera isolated from the dip tanks, to degrade amitraz were determined. Inocula from each of the dips and the test dip population were washed in 0,85% saline and added

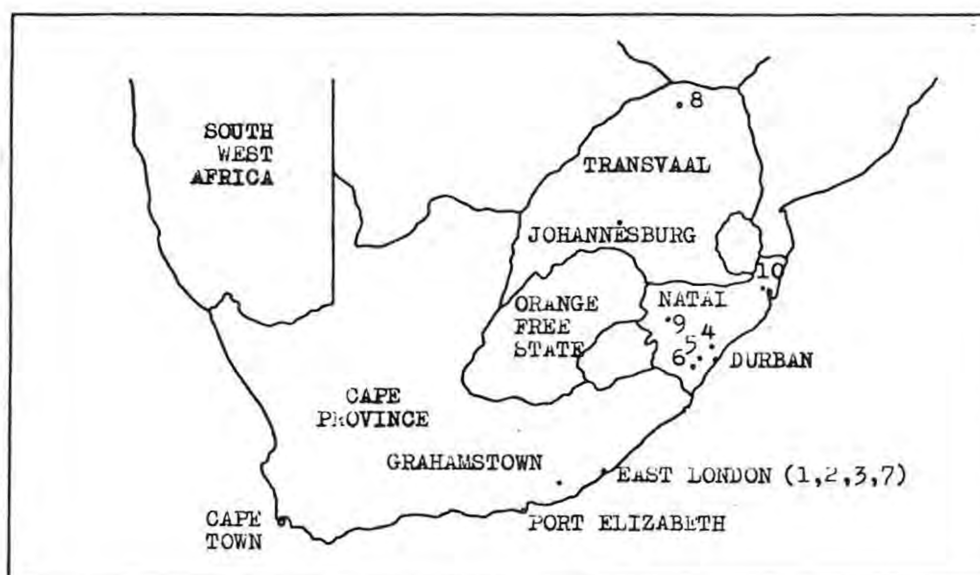
to Ye,P + M.O. and MM + M.O. broths at pH 7. Controls were sterile Ye,P + M.O. and MM + M.O. broths. Incubation was at 30°C and samples were analysed for amitraz after 3 and 6 days.

7.3 Results

7.31 Isolation and Identification of strains

The location of the dip tanks analysed and their geographical and climatic conditions (Weather Bureau Reports 19(1954) & 29(1960)) are shown in Fig. 14. The bacterial analysis of the dip samples from these tanks are listed in Tables 19-29. A total of 155 bacterial strains were isolated and identified from the 10 dip tanks. The percentage frequencies and distribution functions for the summer and winter bacterial populations in the dip tanks were calculated (Table 30; Fig. 15). The summer and winter bacterial populations were extremely similar (Fig. 15). Four predominant genera constituted 80% of both the summer and winter bacterial populations. These were: *Pseudomonas*; *Bacillus*; *Alcaligenes*; and *Escherichia coli*. The other 13 genera identified represented much smaller percentages and in some cases only 1 isolate of a bacterial genus was present. A test

No. on map	Dip tank	No. on map	Dip tank
1	Wiltonside	6	Ixopo
2	Carrig	7	Carrig (Delnav D.F.F.)
3	Tayside	8	Rooipan Tvl.
4	Nottingham road	9	Vryheid
5	Richmond	10	Hluhluwe



Dip tank No.	Closest town	Lat.	Long.	Alt. (m)	Monthly highest rainfall (mm)	Monthly lowest rainfall (mm)	Annual rainfall (mm)	Average daily temp.	Range (max-min)	Period over which data was collected (years)
1,2, 3,7	Cambridge (E.L.)	32° 59'	27° 55'	122	March 108,3	June 29,8	849,7	18,3	8,1	25
4	Nottingham Road	29° 22'	29° 59'	1438	Dec. 146,2	June 10,3	856,7	16,5	14,8	51
5	Richmond	29° 52'	30° 16'	856	Dec. 167,5	July 15,8	1094,3	18,9	13,9	63
6	Ixopo	30° 09'	30° 04'	992	Dec. 120,5	July 15,3	824,9	18,3	10,0	41
8	Pietersburg	23° 54'	29° 28'	1266	Dec. 94,6	June 3,7	488,4	17,3	12,3	56
9	Vryheid	27° 47'	30° 46'	1194	Dec./Jan. 157,7	June 12,2	890,3	17,6	13,5	34
10	Hluhluwe	28° 05'	32° 03	452	Dec. 147,5	July 21,3	981,8	21,2	10,8	30

Fig. 14. Map of Southern Africa showing location of the dip tanks and their geographical and climatic conditions.

Table 19. Abbreviations used in the Identification Tables 20-29.

g-b = gram -ve bacillus

g+b = gram +ve bacillus

g+c = gram +ve coccus

gvb = gram variable bacillus

pigmentation is on King's A medium

O/F = oxidative or fermentative

M-R = methyl-red test

w+ = weakly positive

For API 20 E system:

ONPG = β-galactosidase production

ADH = argenine dihydrolase production

LDC = lysine decarbolase production

ODC = ornithine decarbolase production

CIT = citrate utilization

H_2S = hydrogen sulphide production

URE = urease production

TDA = tryptophane deaminase production

IND = indole formation from tryptophane

VP = acetoin production from sodium pyruvate

GEL = production of proteolytic enzymes

GLU = glucose utilization

MAN = mannitol utilization

INO = inositol utilization

SOR = sorbitol utilization

RHA = rhamnose utilization

SAC = sucrose utilization

MEL = melibiose utilization

AMY = amygdalin utilization

ARA = (L+) arabinose utilization

OX = cytochrome oxidase production

NO_2 = reduction of nitrates to nitrites

N_2 = reduction of nitrates to N_2

MOB = motility

MAC = growth in MacConkeys medium

OF-O = oxidative utilization of carbohydrates

OF-F = fermentative utilization of carbohydrates

Table 20. Characteristics of isolated bacteria. Area: Wiltonside, East London.
Ixodicide : Amitraz. Season : summer.

No.	Gram.	Oxidase	Catalase	Motility	Pigment	O/F	Growth in Thiogly.	Urease Product	Citrate Utilizat.	Denitri-fication	Identification
1	g-b	+	+	2-8 Polar	Green	Oxid.	Aerob.	-	+	-	Pseudomonas spp.
2	g-b	+	+	2-8 Polar	"	Oxid.	Aerob.	-	+	-	Pseudomonas spp.
3	g-b	+	+	4-8 Polar	"	Oxid.	Aerob.	-	+	-	Pseudomonas spp.
4	g-b	Weakly +	+	Lateral	White	Ferm.	Facult.	+	+	-	Achromobacter spp.
5	g-b	+	+	2-10 Polar	Green	Oxid.	Aerob.	-	+	-	Pseudomonas spp.
6	g-b	+	+	2-6 Polar	"	Oxid.	Aerob.	-	+	-	Pseudomonas spp.
7	g-b	+	+	2-12 Polar	"	Oxid.	Aerob.	-	+	-	Pseudomonas spp.
A	g-b	+	+	4-10 Polar	"	Oxid.	Aerob.	-	+	-	Pseudomonas spp.
B	g-b	+	+	1 Polar	Cream	Oxid.	Facult.	-	-	+	Pseudomonas mendocina
C	g-b	+	+	4-10 Polar	Green	Oxid.	Aerob.	-	+	-	Pseudomonas spp.

For abbreviations see Table 19.

Table 21. Characteristics of isolated bacteria. Area : Carrig, East London.
Ixodicide : Amitraz. Season : summer.

No.	Gram.	Oxidase	Catalase	Motility	Pigment	O/F	Growth in Thiogly.	Urease Product	Citrate Utilizat.	Indole Product	Identification
1	g-b	-	+	Peritrichous	White	Ferm.	Facult.	-	-	+	Escherichia coli
2	g-b	+	+	Polar	Orange	No action	Aerob.	-	+	-	Pseudomonas spp.
3	g-b	-	+	Peritrichous	White	Ferm.	Facult.	-	+	+	Citrobacter spp.
4	g-b	-	-	Peritrichous	White	Ferm.	Facult.	-	+	-	Enterobacter spp.
5	g-b	-	-	non-Flagel.	White	Ferm.	Facult.	-	+	-	Klebsiella spp.
6	g-b	+	+	Polar-Tufted	Yellow	No action	Aerob.	-	+	-	Pseudomonas spp.
A	g-b	+	+	Polar	Orange	No action	Aerob.	-	+	-	Pseudomonas spp.
B	g-b	+	+	Polar	Orange	No action	Aerob.	-	+	-	Pseudomonas spp.
C	g-b	+	+	Polar	Orange	No action	Aerob.	-	+	-	Pseudomonas spp.
D	g-b	+	+	Polar	Orange	No action	Aerob.	-	+	-	Pseudomonas spp.
E	g-b	+	+	Polar	Orange	No action	Aerob.	-	+	-	Pseudomonas spp.

For abbreviations see Table 19.

Table 22a. Characteristics of isolated bacteria. Area : Tayside, East London.
Ixodicide : Amitraz. Season : Summer.

No.	Gram	Oxidase	Catalase	Motility	Pigment	O/F	Growth in Thiogly	Growth in MacConk.	Indole Product	Urease Product	Identification
A	g-b	-	+	-	-	Oxid.	Aerob.	-	-	-	Acinetobacter spp.
B	g+b	-	+	+	-	No action	Facult.	-	-	-	Bacillus spp.
C	g+b	÷	+	+	-	No action	Facult.	-	-	-	Bacillus spp.
D	g+b	W +	+	+	-	No action	Facult.	-	-	-	Bacillus spp.
E	g+b	-	+	+	-	No action	Facult.	-	-	-	Bacillus spp.
F	g+b	+	+	+	-	Ferm.	Facult.	-	-	-	Bacillus spp.
G	g+b	-	+	+	-	No action	Facult.	-	-	-	Bacillus spp.
H	g+b	+	+	+	-	Ferm.	Facult.	-	-	-	Bacillus spp.
I	g+b	-	+	+	-	Ferm.	Facult.	-	-	-	Bacillus spp.
J	g+b	-	+	+	-	No action	Aerob.	-	-	-	Bacillus spp.
P	g-b	W +	+	+	-	No action	Aerob.	+	-	-	Alcaligenes spp.
Q	g-b	W +	+	+	-	No action	Aerob.	+	-	-	Alcaligenes spp.
S	g-b	+	+	+	-	No action	Aerob.	+	-	-	Alcaligenes spp.
T	g-b	+	+	+	-	No action	Aerob.	+	-	-	Alcaligenes spp.

For abbreviations see Table 19.

Table 22b. Characteristics of isolated bacteria. Area : Tayside, East London.

Ixodicide : Amitraz. Season : Winter.

No.	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX	NO_2	N_2	MOB	MAC	OF-O	OF-F	IDENTIFICATION
a.	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	+	+	-	-	+	+	-	-	Pseudomonas spp.
b	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-	Alcaligenes spp.
d	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-	Pseudomonas spp.
f	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	Pseudomonas spp.
g.	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-	Pseudomonas spp.
i	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-	Pseudomonas spp.
j	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-	Alcaligenes spp.

No.	Gram	Oxidase	Catalase	Motility	Pigment	O/F	H_2S	Indole	Citrate	KCN	Urea	IDENTIFICATION
c	g + b	+	+	+	-	Ferm	-	-	+	+	-	Bacillus spp.
e	g + b	-	+	+	-	Ferm	-	-	+	+	+	Bacillus spp.
h	g + b	-	+	+	-	Ferm	-	-	w +	+	-	Bacillus spp.

For abbreviations see Table 19.

Table 23. Characteristics of isolated bacteria. Area : Nottingham Road, Natal.

Ixodicide : Amitraz. Season : Summer.

No.	Gram	Oxidase	Catalase	Motility	Pigment	O/F	Growth in Thiolgy	Growth in MacConk.	Indole	M-R	Identification
a	g-b	+	+	+	-	Oxid.	Aerob.	+	-		Pseudomonas spp.
b	g-b	-	+	+	-	Ferm.	Facult.	+	-		Yersinia spp.
c	g-b	+	+	+	-	No action	Aerob.	+	-		Alcaligenes spp.
d	g-b	+	+	+	-	No action	Aerob.	+	-		Alcaligenes spp.
e	g-b	+	+	+	diffuse green	Oxid.	Aerob.	+	-		Pseudomonas spp.
g	g+b	+	+	+	-	Ferm.	Facult.	-	-		Bacillus spp.
h	g-b	-	+	+	-	Ferm.	Facult.	a/g	+		Escherichia coli
i	g+b	-	+	+	-	No action	Facult.	-	-		Bacillus spp.
j	g+b	-	+	+	-	No action	Facult.	-	-		Bacillus spp.

For abbreviations see Table 19.

Table 24a. Characteristics of isolated bacteria. Area : Richmond, Natal.

Ixodicide : Amitraz. Season : Summer.

No.	Gram	Oxidase	Catalase	Motility	Pigment	O/F	Growth in Thiogly	Growth in MacConk.	Indole	M-R	Identification
a	g+b	-	+	+	-	No action	Facult.	-	-	+	Bacillus spp.
b	g-b	+	+	+	diffuse green	Oxid.	Aerob.	+	-	-	Pseudonomas spp.
c	g-b	+	W +	+	-	No action	Aerob.	+	-	-	Alcaligenes spp.
d	g+b	-	+	-	-	No action	Aerob.	-	-	-	Corynebacteria spp.
e	g+c	-	+	-	Yellow	No action	Facult.	-	-	-	Micrococcus spp.
f	gvb	-	+	+	-	No action	Facult.	-	-	-	Bacillus spp.
g	g-b	-	+	+	-	Ferm.	Facult.	A+G	+	-	Escherichia coli
h	g-b	+	+	+	-	Oxid.	Aerob.	+	-	-	Pseudomonas spp.
i	g-b	-	+	+	-	Ferm.	Facult.	A&G	+	+	Escherichia coli
j	g+b	-	+	+	-	Ferm.	Facult.	-	-	-	Bacillus spp.

For abbreviations see Table 19.

Table 24b. Characteristics of isolated bacteria. Area : Richmond, Natal.

Ixodicide : Amitraz. Season : Winter.

No.	CNPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX	NO_2	N_2	MOB	MAC	OF-O	OF-F	IDENTIFICATION
a	+	+	+	+	−	−	−	−	+	−	−	+	+	−	+	+	+	+	−	+	−							Escherichia coli
b	+	−	+	+	−	−	−	−	+	−	−	+	+	−	+	+	+	+	−	+	−							Escherichia coli
c	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	+	−	−	Alcaligenes spp.
d	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+	+	−	−	Pseudomonas spp.
g	+	−	+	−	−	−	−	−	+	−	−	+	+	−	+	+	−	+	−	+	−							Escherichia coli
h	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	+	−	−	Alcaligenes spp.
i	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−	Moraxella spp.

No.	Gram	Oxidase	Catalase	Motility	Pigment	O/F	H_2S	Indole	Citrate	KCN	Urea	IDENTIFICATION
e	g + b	−	+	+	yellow	Ferm	−	−	+	+	−	Bacillus spp.
f	g + c	−	+	−	pink	No Action	−	−	+	+	−	Micrococcus spp.
j	g + b	−	+	+	yellow	Ferm	−	−	+	+	−	Bacillus spp.
k	g + b	−	+	+	−	Ferm	−	−	+	+	−	Bacillus spp.

For abbreviations see Table 19.

Table 25a. Characteristics of isolated bacteria. Area : Glenkaize, Ixopo.

Ixodicide : Altik [R]. Season : Summer.

No.	Gram	Oxidase	Catalase	Motility	Pigment	O/F	Growth in Thiogly	Growth in MacConk.	Indole	M-R	Identification
a	g+b	-	+	+	-	No action	Facult.	-	-	-	Bacillus spp.
b	g+b	+	+	+	-	Ferm.	Facult.	-	-	-	Bacillus spp.
c	g-b	+	+	+	-	No action	Aerob.	+	-	-	Alcaligenes spp.
d	g-b	W +	+	-	-	No action	Aerob.	-	-	-	Moraxella spp.
e	g-b	-	+	+	-	Ferm.	Facult.	+	-	-	Yersinia spp.
* f	g-b	-	+	-	-	No action	Aerob.	-	-	-	Acinetobacter parapertussis
g	g+b	-	+	+	-	No action	Facult.	-	-	-	Bacillus spp.
h	g-b	+	+	-	Yellow non-diff.	Oxid.	Aerob.	-	-	-	Flavobacterium meningosepticum
i	g+b	-	+	+	-	No action	Facult.	-	-	-	Bacillus spp.
j	g+b	-	+	+	-	No action	Facult.	-	-	-	Bacillus spp.

For abbreviations see Table 19. * = species confirmed by + ve urease test

Table 25b. Characteristics of isolated bacteria. Area : Glenkaize, Ixopo.

Ixodicide : Altik[R]. Season : Winter.

No.	Gram	Oxidase	Catalase	Motility	Pigment	O/F	H_2S	Indole	Citrate	KCN	Urea	IDENTIFICATION
a	g - b	-	+	+	-	Ferm	+	-	+	+	w +	Proteus spp.
b	g - b	+	+	+	-	No Action	w +	-	+	+	-	Alcaligenes spp.
c	g - b	-	+	+	-	Ferm	+	+	+	+	+	Proteus spp.
d	g - b	+	+	+	-	Oxid	-	-	+	+	-	Pseudomonas spp.
e	g - b	+	+	+	-	Oxid	-	-	+	+	-	Pseudomonas spp.
f	g - b	+	+	+	-	Oxid	-	-	+	+	-	Pseudomonas spp.
g	g - b	+	+	+	pink non-diffuse	Oxid	-	-	+	+	w +	Pseudomonas spp.
h	g - b	+	+	+	-	Oxid	-	-	+	+	+	Pseudomonas spp.
i	g - b	+	+	+	-	Oxid	-	-	+	+	-	Pseudomonas spp.
j	g - b	-	+	+	-	Ferm	-	+	-	-	-	Escherichia coli

For abbreviations see Table 19.

Table 26. Characteristics of isolated bacteria. Area : Carrig, East London.

Ixodicide : Delnav D.F.F.[R] Season : Summer.

No.	Gram	Oxidase	Catalase	Motility	Pigment	O/F	Growth in Thiogly	Growth in MacConk.	Indole	M-R	Identification
a	g-b	+	W +	-	-	No action	Aerob.	-	-	-	Moraxella spp.
*b	g-b	-	+	+	-	Ferm.	Facult.	A+G	-	+	Citrobacter spp.
c	g-b	+	+	+	-	No action	Aerob.	+	-	-	Alcaligenes spp.
*d	g-b	-	+	+	-	Ferm.	Facult.	A+G	-	+	Citrobacter spp.
e	g-b	+	+	+	-	No action	Aerob.	+	-	-	Alcaligenes spp.
**f	g+b	-	+	-	Pink	No action	Facult.	-	-	+	Corynebacteria spp.
g	gvb	-	+	+	-	Ferm.	Facult.	-	-	-	Bacillus spp.
h	g-b	+	+	+	-	No action	Aerob.	+	-	-	Alcaligenes spp.
i	g-b	-	+	+	Yellow	No action	Aerob.	+	-	-	Pseudomonas spp.
*j	g-b	-	+	+	-	Ferm.	Facult.	A+G	-	+	Citrobacter spp.

For abbreviations see Table 19.

* = H_2S test +ve

** = gram showed diphthroid-like g+ve rods

Table 27a. Characteristics of isolated bacteria. Area : Rooipan, Transvaal.

Ixodicide : Amitraz. Season : Summer.

No.	Gram	Oxidase	Catalase	Motility	Pigment	O/F	Growth in Thiogly	Growth in MacConk.	Indole	M-R	Identification
a	g+b	-	+	+	-	Ferm.	Facult.	+	-	+	Bacillus spp.
* b	g+b	-	+	-	Pink	No action	Aerob.	+	-	-	Corynebacteria spp.
c	g-b	-	+	+	Yellow	No action	Aerob.	+	-	-	Pseudomonas spp.
d	g-b	+	+	+	diffuse green	Oxid.	Aerob.	+	-	-	Pseudomonas spp.
e	g-b	W +	+	+	-	No action	Aerob.	+	-	-	Alcaligenes spp.
f	g-b	W +	+	+	-	No action	Aerob.	+	-	-	Alcaligenes spp.
g	g-b	-	+	+	diffuse green	Oxid.	Aerob.	+	-	-	Pseudomonas spp.
h	g-b	-	+	+	-	Oxid.	Aerob.	+	-	-	Pseudomonas spp.
i	g-b	+	+	+	-	No action	Aerob.	+	-	-	Alcaligenes spp.
j	g-b	-	+	+	-	Ferm.	Facult.	A+G	+	+	Escherichia coli
k	g-b	-	+	+	-	Ferm.	Facult.	A+G	+	+	Escherichia coli

For abbreviations see Table 19. * = gram showed diphthroid-like g+ve rods.

Table 27b. Characteristics of isolated bacteria. Area : Rooipan, Transvaal.

Ixodicide : Amitraz Season : Winter.

No.	Gram	Oxidase	Catalase	Motility	Pigment	O/F	H_2S	Indole	Citrate	KCN	Urea	IDENTIFICATION
a	g - b	-	+	+	-	Ferm	-	+	+	+	w +	Providence spp.
b	g - b	+	+	+	-	Oxid	-	-	+	+	-	Pseudomonas spp.
c	g - b	+	+	+	-	No Action	-	-	-	+	-	Alcaligenes spp.
d	g - b	-	+	+	-	Ferm	+	+	-	+	+	Proteus spp.
e	g - b	+	+	+	-	Oxid	-	-	+	+	-	Pseudomonas spp.
f	g - b	+	+	+	-	No Action	-	-	+	+	w +	Alcaligenes spp.
g	g + b	-	+	+	-	Ferm	-	-	+	+	-	Bacillus spp.
h	g + b	-	+	+	-	Ferm	+	-	-	+	-	Bacillus spp.
i	g - b	+	+	+	-	No Action	-	-	+	+	-	Alcaligenes spp.

For abbreviations see Table 19.

Table 28. Characteristics of isolated bacteria. Area : Vryheid, Natal.

Ixodicide : Amitraz. Season : Summer.

No.	Gram	Oxidase	Catalase	Motility	Pigment	O/F	Growth in Thiogly	Growth in MacConk.	Indole	M-R	Identification
a	g+b	-	+	+	-	Ferm.	Facult.	-	-	-	Bacillus spp.
b	g-b	+	W +	+	-	Ferm.	Facult.	+	+	-	Aeromonas spp.
c	g-b	+	+	+	-	No action	Aerob.	+	-	-	Alcaligenes spp.
d	g-b	+	+	+	-	No action	Aerob.	+	-	-	Alcaligenes spp.
e	g-b	+	+	+	-	No action	Aerob.	+	-	-	Alcaligenes spp.
f	g-b	-	+	+	-	Ferm.	Facult.	+	-	-	Yersinia spp.
g	g-b	+	+	+	-	No action	Aerob.	+	-	-	Alcaligenes spp.
h	g-b	+	+	+	Yellow	No action	Aerob.	+	-	-	Pseudomonas spp.
i	g+b	-	+	+	+	Ferm.	Facult.	-	-	-	Bacillus spp.
j	g+b	-	+	+	-	Ferm.	Facult.	+	-	-	Bacillus spp.

For abbreviations see Table 19.

Table 29a. Characteristics of isolated bacteria. Area : Hluhluwe, Zululand.

Ixodicide : Amitraz. Season : Summer.

No.	Gram	Oxidase	Catalase	Motility	Pigment	O/F	Growth in Thiogly	Growth in MacConk.	Indole Product	M-R	Identification
a	g-b	-	+	+	-	Ferm.	Facult.	A+G	+	+	Escherichia coli
b	g-b	+	+	+	-	No action	Aerob.	+	-	-	Alcaligenes spp.
c	g-b	W +	+	+	Yellow diffuse	Oxid.	Aerob.	+	-	-	Pseudomonas spp.
d	g-b	-	+	+	-	Ferm.	Facult.	A+G	+	-	Escherichia coli
e	g-b	-	+	+	-	Ferm.	Facult.	A+G	+	+	Escherichia coli
f	g-b	+	+	+	Green diffuse	Oxid.	Aerob.	+	-	-	Pseudomonas spp.
g	g-b	-	+	+	-	Ferm.	Facult.	A+G	+	+	Escherichia coli
h	g-b	-	+	+	Yellow	Oxid.	Aerob.	+	-	-	Pseudomonas spp.
i	g+b	-	+	+	Yellow	No action	Facult.	-	-	-	Bacillus spp.
j	g-b	+	+	+	-	No action	Aerob.	+	-	-	Alcaligenes spp.
k	g+b	-	+	+	Pink	No action	Facult.	-	-	-	Bacillus spp.

For abbreviations see Table 19.

Table 29b. Characteristics of isolated bacteria. Area : Hluhluwe, Zululand.

Ixodicide : Amitraz. Season : Winter.

No.	Gram	Oxidase	Catalase	Motility	Pigment	O/F	H_2S	Indole	Citrate	KCN	Urea	IDENTIFICATION
a	g + b	-	+	+	-	Ferm	-	-	+	+	-	Bacillus spp.
b	g + b	w +	+	+	-	Ferm	-	-	w +	+	+	Bacillus spp.
c	g - b	-	+	-	-	No Action	-	-	-	-	-	Acinitobacter spp.
d	g - b	+	+	+	-	No Action	w +	-	+	+	-	Alcaligenes spp.
e	g - b	+	+	+	-	No Action	-	-	-	+	w +	Alcaligenes spp.
f	g - b	-	+	-	-	Oxid	-	-	-	-	+	Acinitobacter spp.
g	g - b	+	+	+	-	No Action	-	-	-	+	-	Alcaligenes spp.
h	g - b	-	+	-	-	No Action	-	-	-	-	-	Acinitobacter spp.
i	g - b	+	+	-	-	No Action	-	-	-	-	+	Moraxella spp.

For abbreviations see Table 19.

Table 30. Distribution of bacterial populations in dip tanks.

No.	Bacterial Genera	% Frequency	
		Summer	Winter
1	Pseudomonas	27,4	28,6
2	Bacillus	25,5	20,4
3	Alcaligenes	18,9	22,5
4	Escherichia coli	9,4	8,2
5	Citrobacter	3,8	NIL
6	Proteus	NIL	6,1
7	Yersinia	2,8	NIL
8	Corynebacterium	2,8	NIL
9	Acinitobacter	2,0	6,1
10	Moraxella	2,0	4,1
11	Achromobacter	0,9	NIL
12	Enterobacter	0,9	NIL
13	Klebsiella	0,9	NIL
14	Micrococcus	0,9	2,0
15	Flavobacterium	0,9	NIL
16	Aeromonas	0,9	NIL
17	Providence	NIL	2,0
Total No. of genera isolated =		106	49

Groups of bacterial genera	% Frequency	
1	27,4	28,6
1,2	52,9	49,0
1,2,3	71,8	71,5
1,2,3,4	81,2	79,7
1,2,3,4,5,6	85,0	85,8
1,2,3,4,5,6,7,8	90,6	85,8
1,2,3,4,5,6,7,8,9	92,6	91,9
1,2,3,4,5,6,7,8,9,10	94,6	96,0
1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17	100,0	100,0

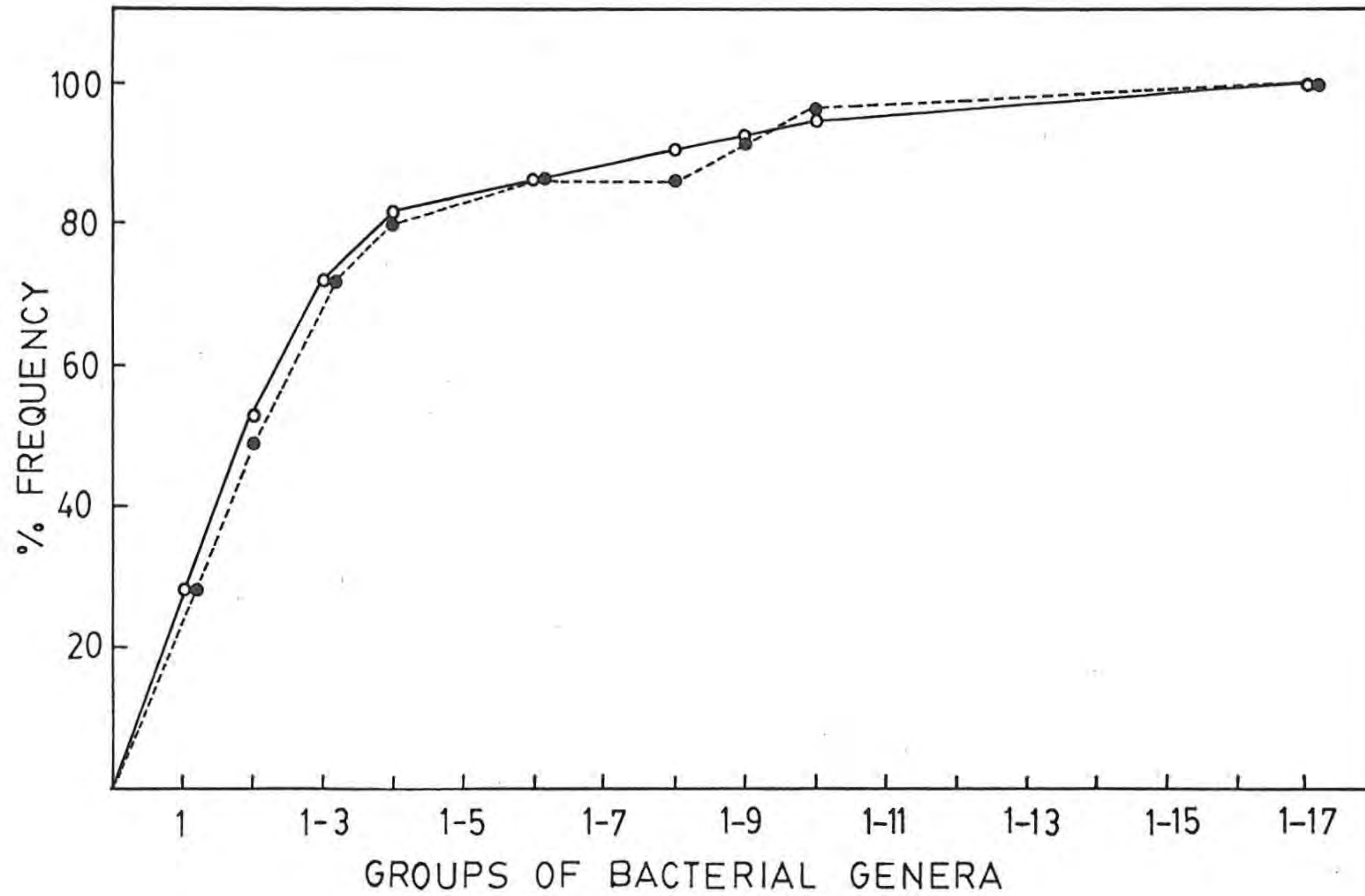


Fig. 15. Distribution function for bacterial populations in dip tanks, (o) Summer distribution (•) Winter distribution.

dip population consisting of strains from the 5 major genera present in the summer bacterial populations was made. The genera included Pseudomonas; Bacillus; Alcaligenes; Escherichia coli and Citrobacter and strains from these genera were mixed in the following respective ratios 7 : 6 : 5 : 2 : 1.

7.32 Amitraz degradation by dip tank populations

The washed bacterial populations from the 10 different dip tanks in Ye,P and MM cultures degraded amitraz by co-metabolism (Tables 31 and 32). There was little or no degradation in the MM cultures (without the co-metabolite yeast extract) but significant degradation in the Ye,P cultures. Degradation of amitraz by the test dip population in Ye,P showed that little difference was found between the abilities of the mixture of 1 strain, 20% of the strains or all of the strains from each of the 5 genera to degrade amitraz (Table 33). The average percentage degradation of the 10 dip tank populations was very similar to that of the test dip population in Ye,P medium (46% as opposed to 38%).

7.4 Discussion

Little variation was found between the bacterial types isolated from the summer and winter dip tank

Table 31. Degradation of amitraz by dip tank populations in Ye,P medium

Sample	% amitraz remaining			
	Day 3	Average	Day 6	Average
Uninoculated control	90	90	89	89
Wiltonside	86 90 90 90	89	63 63 58	61
Carrig	80 80 80	80	58 58 52	56
Tayside	87 95 90	90	48 73 68	63
Nottingham Road	80 70 70	73	20 5 47	24
Richmond	70 70 75	71	48 63 52	54
Altik, Ixopo	80 95 95	90	44 5 21	23
Delnav D.F.F. in Carrig	90 80 65	78	8 5 5	6
Rooipan, Tvl.	87 90 90	89	52 68 63	61
Vryheid	82 90 90	87	56 63 68	62
Hluhluwe	74 80 80	78	44 58 58	53
Average		82,5		46

Table 32. Degradation of amitraz by dip tank populations in MM.

Sample	% amitraz remaining			
	Day 3	Average	Day 6	Average
Uninoculated control	96	96	88	88
Wiltonside	90 90 93	91	87 92 90	89
Carrig	89 88 88	88	82 83 83	83
Tayside	90 93	91	86 90	88
Nottingham Road	91 91	91	87 89	88
Richmond	91 90	91	89 85	87
Altik, Ixopo	89 91	90	89 92	90
Delnav D.F.F. in Carrig	90 92	91	88 90	89
Rooipan, Tvl.	90 88	89	91 90	91
Vryheid	94 89	92	90 89	89
Hluhluwe	87 92	89	92 88	90
Average		90		88

Table 33. Degradation of amitraz by the test dip population in Ye,P medium.

Sample	% amitraz remaining			
	Day 3	Average	Day 6	Average
Uninoculated control	95	95	89	89
Mixture of 1 strain from each of the 5 genera (Total: 5 strains)	75 80	77	42 42	42
Mixture of 20% of the strains from each of the 5 genera (Total: 18 strains)	80 75	77	31 42	36
Mixture of all of the strains from each of the 5 genera (Total: 90 strains)	85 75 75 75 80 75 75 75 75 70	76	26 47 42 42 42 36 26 42 42 42	38

samples. The four major genera: Pseudomonas; Bacillus; Alcaligenes and Escherichia coli were present in similar percentages in both the summer and winter samples. From the samples analysed it appears that no major seasonal variation in bacterial types exist. It is interesting to note that bacterial isolates from 4 genera were isolated in 80% of the cases. The dip tank populations degraded amitraz by co-metabolism. An interesting feature was that both of the tanks charged with ixodicides other than amitraz i.e. Altik and Delnav D.F.F. were extremely virulent in degrading amitraz. The Carrig tank charged with Delnav D.F.F. was however previously charged with amitraz miscible oil. Results obtained with the test dip population showed that it was representative of the bacterial types isolated from the dip tanks and that it's average percentage degradation was similar to that of the 10 dip tank populations. The test dip population which is representative of the bacterial populations present in dip tanks may be very useful in the evaluation of the biodegradation properties of new ixodicides. The general applicability of such a test dip population may be verified by testing other ixodicides which have already undergone extensive field trials and have been discarded because of their instability.

CHAPTER VIII

SUMMARY AND CONCLUSIONS

The degradation of amitraz by bacterial populations was investigated. The bacterial population from the Carrig dip tank which experienced toxicity problems degraded amitraz rapidly by co-metabolism. Transient degradation in minimal medium occurred by carry-over of the co-metabolite. This ability was lost once the co-metabolite was metabolised. An interesting variation in sensitivity to amitraz depending on the nature of the previous growth medium was observed. Exposure of the bacterial population to the miscible oil in minimal salts medium rendered the bacteria sensitive to amitraz when they were grown on MM + M.O. plates. The existance of a reaction between amitraz and free ammonium ions suggested that this reaction may account for the degradation of amitraz. It was found that this reaction in the medium accounted for some of the degradation but was not the major factor. Eleven strains were isolated from the Carrig mixed population. Four of these were classified as members of the family Enterobacteriaceae and the other seven as *Pseudomonas* sp. Conditions required for optimal

degradation by the Wiltonside and Carrig bacterial populations were identical.

The nature and order of appearance of the degradation products of amitraz by mixed bacterial cultures were investigated. The primary degradation pathway involves the conversion of amitraz to 2,4-dimethylaniline. A slower secondary pathway involves the conversion of amitraz to 2,4-dimethylaniline via the intermediates N-2,4-dimethylphenyl-N'-methylformamidine and 2,4-dimethylformanilide. The bacterial degradation pathway of amitraz differs from that found in plants and animals. The toxicity problems experienced in the Carrig tank were due to a build-up of the solvent and the toxic metabolite N-2,4-dimethylphenyl-N'-methylformamidine. This toxic metabolite was found to be pH sensitive and this probably accounted for its transitory presence in laboratory cultures.

The mechanism of amitraz degradation by bacteria indicated that the enzymes responsible are situated in the periplasmic space which is situated outside the cell membrane but remains associated with the cell envelope. Osmotic shocking of cells however failed to yield useable amounts of the enzymes. The amitraz degradation system was found to be constitutive and was not induced by amitraz or a related ixodicide. Eight of the 10

bacterial populations tested were able to degrade amitraz in a basal salts medium with miscible oil as the sole source of nitrogen.

A sensitive and rapid t.l.c. radioassay was developed to replace the g.c. amitraz assay which is unsuitable for enzyme experiments. In the g.c. assay large volumes were required for each analysis and the length of time necessary for the detection of degradation was 1-3 days. Relatively small volumes (10 ml) were used in the radioassay which could be completed in 6 h.

Three enzyme inhibitors (pCMB, N-ethylmaleimide and E.D.T.A.) showed promising results in stabilizing the degradation of amitraz but they are unlikely to be of any practical value because of their toxicity. However their modes of action suggest that the enzymes involved in amitraz degradation contain thiol groups. Two of the antiseptic compounds tested (Hyamine F.H.P. and Vantoc 'CL') proved to be useful in preventing the bacterial degradation of amitraz. They were not as effective as $Ca(OH)_2$ employed in the Coopers' "Lime Stabilization Method" but were acting at a concentration < 40 x that of the $Ca(OH)_2$. Before use they would have to be tested for toxicity to the cattle and the relative costs involved would have to be investigated.

Four major genera were predominant in the bacterial populations isolated from dip tanks throughout South Africa. They were Pseudomonas; Bacillus; Alcaligenes and Escherichia coli and from the samples analysed it appears that no major seasonal variation in bacterial types exist. All of the dip tank populations tested degraded amitraz by co-metabolism. A test dip population was formulated and was representative of the bacterial populations present in the dip tanks. This test dip population may be of use in the evaluation of the biodegradation properties of new ixodicides.

APPENDIX A

QUANTITATIVE ANALYSIS OF AMITRAZ BY GAS CHROMATOGRAPHY

A.1 Extraction of amitraz from aqueous samples

Before amitraz can be subjected to analysis by gas chromatography it must be extracted from aqueous samples into a suitable solvent. The following extraction method (Baker, 1975) was routinely used:

A well-shaken 100 ml aqueous sample was transferred to a 250 ml bottle fitted with a ground glass stopper, to which 25 ml of xylene (Analar grade) containing 0,1% (v/v) Sumithion (internal standard) was added. The bottle was stoppered and shaken vigorously for 60 min. The ground glass stopper was replaced with a cork and the bottle was centrifuged at 2000 rev. min^{-1} 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 the gas chromatograph

The instrument used was a Hewlett Packard 5830 A gas chromatograph (Fig. A1) fitted with a H.P. 18850 A gas chromatograph terminal and a H.P. 7671 A automatic sampler. The following 'optimum' operating conditions were determined by Ettre, 1973 (cited by Baker, 1975 as a 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% (QFL + OV17) on gas chrom Q (QFL = 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 24 h with a flow of 30 ml min^{-1} N_2 to remove any excess solvent or volatile materials from the stationary phase.

Sample injection. Samples of amitraz in xylene to be determined were transferred to special automatic sampler vials of 2 ml capacity and sealed with septum caps. These were loaded into the sampler tray. When in operation the automatic sampler would withdraw the required sample volume and inject it into the chromatograph using a 10 µl Hamilton syringe. The temperature of the

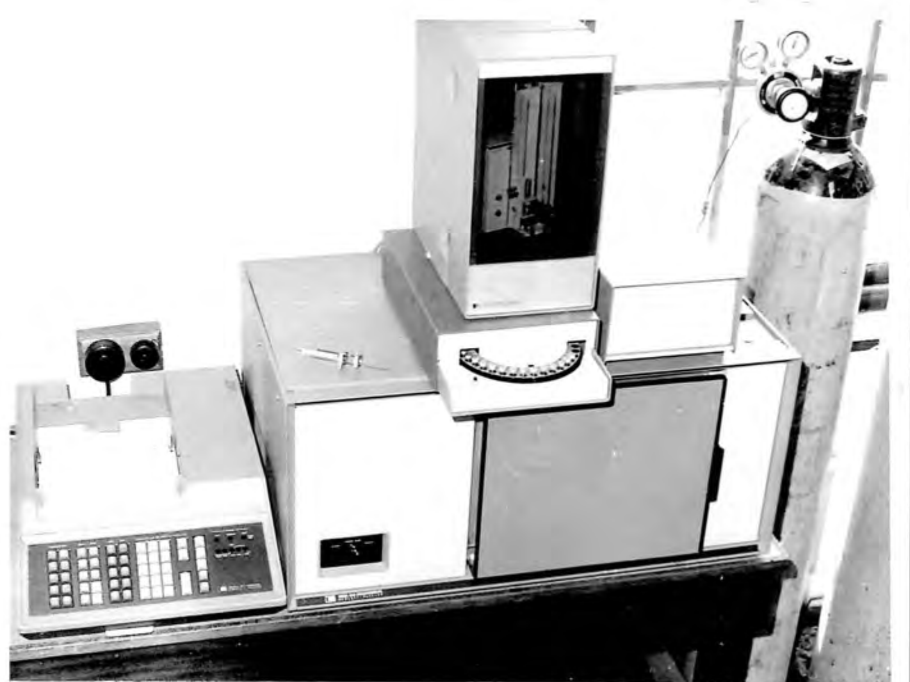


Fig. A1. Hewlett Packard 5830 A gas chromatograph used for the analysis of amitraz.

injector port was 250°C.

Sample detection. The gas chromatograph was equipped with flame ionization detectors. The temperature of the detector manifold was 250°C. During a run the gas chromatograph terminal traced the chromatogram on heat-sensitive paper and printed peak retention times near each peak apex.

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 hydrogen 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}.

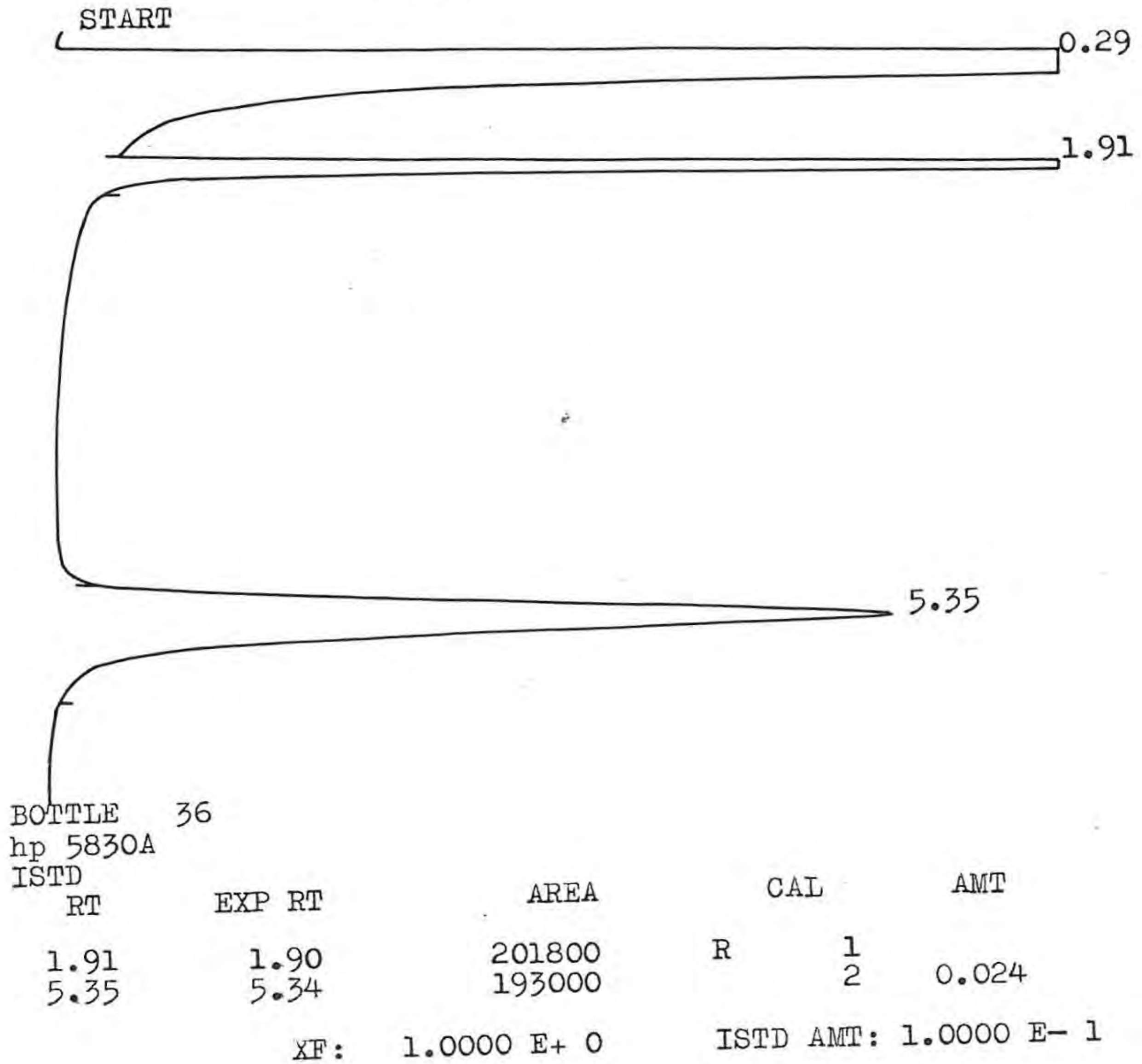
In each gas line a filter dryer was placed between the cylinder regulator and the instrument. These filters removed any water and organic contaminants.

A.3 Analysis of gas chromatograms

A typical gas chromatogram and report is given in Fig. A2. The Hewlett Packard 18850 A gas chromatograph terminal houses a multi-function digital processor. The

TEMP1 230 230
TIME1 25.0
INJ TEMP 250 250
FID TEMP 250 250
OVEN MAX 240

CHT SPD 1.00
ATTN 2 7
FID SGNL +B
SLP SENS 0.00
AREA REJ 1
FLOW A 30
FLOW B 42
OPTN 21
10.0 STOP



BOTTLE 36
hp 5830A
ISTD

RT	EXP RT	AREA	CAL		AMT
1.91	1.90	201800	R	1	
5.35	5.34	193000		2	0.024

XF: 1.0000 E+ 0   ISTD AMT: 1.0000 E- 1

Fig. A2. A typical list of operating conditions, gas chromatogram and report.

processor establishes temperature controls of the gas chromatograph as well as analysing integration data by basing it's calculations on the method entered before a run is started. At the end of each run it automatically prints the associated report. Quantitative analysis of amitraz was determined by the internal standard method. Sumithion at a fixed concentration of 0,1% (v/v) was used as the internal standard, and added to each sample. The concentration or amount of the sumithion peak in the resulting chromatogram was therefore known, and the others could be determined by simple proportion.

Thus:

if the peak due to amitraz = Y

and the peak due to sumithion = IS

XF = dilution factor

$$\text{absolute amount of } Y = \frac{\text{area } Y \times \text{response } Y}{\text{area } IS \times \text{response } IS} \times \text{amount } IS \times XF$$

In practice, a standard solution of 0,0250% (w/v) technical amitraz in xylene + 0,1% sumithion was made and injected at the beginning of each set of analyses, and the method calibrated against this solution.

APPENDIX B

IXODICIDES MENTIONED IN THE TEXT AND THEIR CHEMICAL DESIGNATIONS

Common or trade name	Chemical designation
Aldicarb	2-methyl-2-(methylthio)-O-[(methyl-amino) carbonyl]oxime
Amitraz	1,5-di-(2,4-dimethylphenyl)-3-methyl-1,3,5 - triazapenta-1,4-diene
Amitrole	3-amino-1,2,4 - triazole
Bifenox	5-(2,4-dichlorophenoxy)-2-nitro-benzoic acid
Diazinon	O,O-Diethyl-O-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothioate
DDT	2,2-Bis(p-chlorophenyl)-1,1-trichloroethane
Ethion	tetraethyl S,S'-methylene bis (phosphorothiolothionate)
Ioxynil	3,5-diiodo-4-hydroxybenzonitrile
Lindane (γ-BHC)	γ-1,2,3,4,5,6-hexachlorocyclohexane
Oxadiazon	2-tert-butyl-4-(2,4-dichloro-5-isopropoxyphenyl-Δ^2-1,3,4-oxadiazolin-5-one
Phosphalone	O,O-diethyl-5-(6-chloro-2-oxobenzoxazolin-3-yl) methyl phosphorodithioate
Sumithion	O,O-Dimethyl O-(3-methyl-4-nitrophenyl) phosphorothrionate

REFERENCES

Allcock, E.R., and D.R. Woods. 1977. Bacterial degradation products of the ixodicide amitraz. J. Appl. Bacteriol. (In press).

Ambrosi, D., and C.S. Helling. 1977. Leaching of Oxadiazon and Phosphalone in soils. J. Agric. Food Chem. 25: 215-217.

Baker, J.A.F., R.J. Taylor, and G.D. Stanford. 1973. A new triazapenta-diene compound active against cattle ticks of major importance in South Africa. Proc. 7th Br. Insectic. Fungic. Conf. pp. 291-300.

Baker, P.B. 1975. Bacterial Degradation of the Acaricide Amitraz. M.Sc. thesis, Rodes University.

Baker, P.B., and D.R. Woods. 1977. Co-metabolism of the Ixodicide Amitraz. J. Appl. Bacteriol. 42: 187-196.

Beard, W.E., and W.D. Guenzi. 1976. Separation of soil atmospheric gases by gas chromatography with parallel columns. Soil Sci. Soc. Am. J. 40: 319-321.

Blok, J. 1975. Methods for measurement of biodegradability of chemical compounds. Int. Biodeterior. Bull. 11: 78-84.

Bollag, J.M. 1974. Microbial transformations of pesticides. Advan. Appl. Microbiol. 18: 75-130.

Boots. 1973. BTS 27 419. Methods of analysis (for sales and registration purposes). Boots report code D/104. The Boots Company Ltd., Nottingham, England.

Bransome, E.D., ed. 1970. The current status of liquid scintillation counting. Grune and Stratton, Inc. New York.

Buchanan, R.E., and N.E. Gibbons, eds. 1974. Bergey's manual of determinative bacteriology, 8th ed. Williams & Wilkins Co., Baltimore, Md.

Campacci, E.F., P.B. New, and Y.T. Tchan. 1977. Isolation of amitrole-degrading bacteria. Nature 266: 164-165.

Cowan, S.T., and K.J. Steel. 1966. Manual for the Identification of Medical Bacteria. Cambridge University Press, England.

Dixon, M., and E.D. Webb. 1958. Enzymes. Longmans, Green and Co., London. pp. 49.

Eastin, E.F. 1976. Separation of Bifenox and related compounds by thin-layer chromatography. J. J. Chromatog. 124: 422-423.

Eichenlaub, R., and U. Winkler. 1974. Purification and mode of action of two bacteriocins produced by Serratia Marcesens HY. J. Gen. Microbiol. 83: 83-94.

Focht, D.D., and M. Alexander. 1971. Aerobic co-metabolism of DDT analogues by Hydrogenomonas sp. J. Agric. Food Chem. 19: 20-22.

Golterman, H.L. 1969. Methods for chemical analysis of fresh waters. Blackwell Scientific Pubns. Oxford and Edinburgh.

Harrison, I.R., A. Kozlik, J.F. McCarthy, B.H. Palmer, S.B. Wakerly, T.I. Watkins, and D.M. Weighton. 1972. 1,5-di-(2,4-dimethylphenyl)-3-methyl-1,3,5-triaza-penta-1,4-diene, a new acaricide active against strains of mites resistant to organophosphorous and bridged diphenyl compounds. Pestic. Sci. 3: 679-680.

Harrison, I.R., B.H. Palmer, and E.C. Wilmshurst. 1973. Chemical control of cattle ticks — resistance problems. Pestic. Sci. 4: 531-542.

Horvath, R.S. 1970. Co-metabolism of methyl- and chloro-substituted catechols by an Achromobacter species possessing a new meta-cleaving oxygenase. Biochem. J. 19: 871-876.

Horvath, R.S. 1972. Microbial co-metabolism and the degradation of organic compounds in nature. Bacteriological reviews. 36: 146-155.

Hsu, J.C., and N.D. Camper. 1976. Isolation of ioxynil degraders from soil enrichment cultures. Can. J. Microbiol. 22: 537-543.

Nelson, L.M., and H.G. Hedrick. 1976. Influence of an experimental herbicide on soil nitrogen-fixing bacteria and other microorganisms. Soil Sci. 122: 206-215.

Palmer, B.H., J.F. McCarthy, A. Kozlik, and I.R. Harrison. 1971. A new chemical group of cattle acaricides. Proc. 3rd Int. Congr. Acarol. pp. 687-691.

Phares, E.F. 1971. Methods in enzymology. Colowick, S.P., and N.D. Kaplan, eds. 22: 441-476. Academic press, New York.

Richey, F.A., W.J. Bartley, and K.P. Sheets. 1977. Laboratory studies on the degradation of (the pesticide) Aldicarb in soils. J. Agric. Food Chem. 25: 47-51.

Seki, H. 1976. Method for estimating the decomposition of hexadecane in the marine environment. J. Appl. Environ. Microbiol. 31: 439-441.

Skerman, V.B.D. 1967. A guide to the identification of the genera of bacteria, 2nd ed. Williams & Wilkins Co., Baltimore.

Sparacino, C.M., and J.W. Hines. 1976. High-performance liquid chromatography of carbamate pesticides. J. Chromatogr. Sci. 14: 549-556.

Takimoto, Yoshiyuki, Jun-ichi Ohnishi, and Miyamoto. 1976. The sensitivity and selectivity of a potassium-bromide thermionic detector (PTD) of a gas chromatograph for the microanalysis of organophosphorous compounds. J. Pestic. Sci. 1: 193-199.

Weather Bureau Reports 19 (1954) & 29 (1960) taken from Climate of South Africa, published by the Weather Bureau, Dept. of Transport.

Wharton, R.H., and W.J. Roulston. 1970. Resistance of ticks to chemicals. Ann. Rev. Entomol. 15: 381-404.

Willis, R.C., R.G. Morris, C. Cirakaglu, G.D. Schellenberg, N.H. Gerber, and C.E. Furlong. 1974. Preparation of the periplasmic binding proteins from Salmonella typhimurium and Escherichia coli. Archives of Biochemistry and Biophysics. 161: 64-75.