Biology, Microbiology and Management of Enhanced Carbetamide Biodegradation

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Frontispiece

Above: A field of *Trifolium repens* c.v. haifa after treatment with carbetamide for the first time. Excellent grass weed control was achieved.

Below A field of *Trifolium repens* c.v. haifa after treatment for the third consecutive season with carbetamide. Treatment resulted in poor grass weed control which subsequently reduced yield by around 50 %. This thesis examines the cause of failure and management options which can be used to avoid failure.

ABSTRACT

The experiments described in this thesis establish that the herbicide carbetamide degrades rapidly upon repeated application to soil, when compared to a previously untreated soil. This rapid degradation results in poor performance of this herbicide under field conditions. Rapid degradation of carbetamide is associated with an increased population of carbetamide degrading soil micro-organisms. Using *Lolium rigidum* (annual ryegrass) as an indicator species, the efficacy of carbetamide was reduced by between 27 and 40 percent upon repeated application.

Rapid carbetamide degradation is inhibited by the broad-spectrum antibacterial chloramphenicol but not by the broad-spectrum antifungal cycloheximide, suggesting that bacteria are responsible for the rapid degradation. A bacterium was isolated from a soil exhibiting rapid degradation of carbetamide using carbetamide as a source of carbon. Based upon classical tests, FAME and 16s rRNA sequence analysis, the bacterium was assigned to the genus *Rhodococcus*. Strong circumstantial evidence indicates that the isolated bacterium contributes to carbetamide degradation in soil.

Studies suggested that the isolated *Rhodococcus* sp. degraded carbetamide via aniline and that this phenotype was plasmid encoded. Treatment of stationary phase cultures of the isolated bacterium with chlorpropham resulted in the accumulation of 3 chloroaniline within the

culture medium, indicating that the isolated bacterium degrades carbetamide via aniline. Accumulation of 3 chloroaniline occurred as the isolated bacterium was unable to use chlorinated compounds as growth substrates. The carbetamide degrading isolate harboured a number of large plasmids. Three spontaneous cured derivative strains, which were unable to degrade carbetamide, were selected after growth on rich media. All three cured derivatives had lost the same 185 kb plasmid. Ability to degrade carbetamide was not transferred through conjugation. Electroporation of a cured strain with plasmid extract from the wild-type bacterium resulted in transformant strains capable of carbetamide degradation. These strains had acquired the 185 kb plasmid lost through curing, providing additional evidence that the carbetamide degrading phenotype is plasmid encoded.

Enhanced carbetamide degradation declined after ceasing carbetamide application in the field, providing evidence that enhanced carbetamide degradation can be managed by applying carbetamide less frequently. Cross enhancement was evident to the structurally related herbicide propham but not to the other herbicides tested. For selective control of annual grasses in *Trifolium* seed crops, rotation between the herbicides carbetamide, propyzamide and EPTC is likely to be adopted. Rotation between these herbicides allows for *Trifolium* crops to be grown in successive seasons, whilst managing enhanced degradation of these herbicides and this practice is likely to increase the time taken to select for weeds resistant to carbetamide.

Abstract

Application of the methyl carbamate insecticide, carbaryl, increased the persistence of carbetamide in enhanced carbetamide degrading soils. Field application of carbetamide plus carbaryl yielded only slight improvements in weed control. Field application of carbaryl at the rates used in this study increased the cost of weed control by about 50 %. It is therefore considered unlikely that carbaryl would be used as an extender in the field due to economic considerations.

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DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made.

I give consent to this copy of my thesis, when deposited in the University Library, being available for photocopying and loan.

Signed: Date: 20/10/97

S.J.W. Hole

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ABBREVIATIONS

$^{14}CO_2$	radiolabelled CO_2 formed from degradation of ^{14}C ring labelled carbetamide
2,4-D	(2,4-dichlorophenoxyacetic) acid
carbaryl	1-napthyl methylcarbamate
carbetamide	(*R*)-(-)-1-(ethylcarbamoyl)ethyl phenylcarbamate
CFU	colony forming unit
chloramphenicol	D-threo-*N*-dichloroacetyl-1-*p*-nitrophenyl-2-amino-1,3-propanediol
chlorpropham	isopropyl 3-chlorophenylcarbamate
cycloheximide	4-[(2*R*)-2-[(1*S*,3*S*,5*S*)-(3,5-dimethyl-2-oxocyclohexyl)]-2-hydroxyethyl]piperidine-2,6-dione
DT_{50}	time taken for 50 % of the pesticide to degrade
ECD	electron capture detection
EPTC	S-ethyl dipropylcarbamothioate
FAME	fatty acid methyl ester
GC	gas chromatography
HPLC	high performance liquid chromatography
MPN	most probable number
PFGE	pulse field gel electrophoresis
propham	isopropyl phenylcarbamate
propyzamide	3,5-dichloro-*N*-(1-dimethyl-2-propynyl)benzamide
SBS buffer	sterile buffered saline (pH 7.0)
TBE	Tris-borate (89 mM), EDTA (2 mM) buffer (pH 8.3)
TE	Tris-HCl (10 mM) EDTA (1 mM) buffer (pH 8.0)

1. INTRODUCTION AND RATIONALE FOR THE STUDY

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Modern chemical herbicides are synthetic organic molecules which are applied to control or suppress weeds. Herbicides are an efficient method of controlling weeds and have contributed significantly to higher yields of food and fibre (Klingman and Ashton, 1982). An extremely useful characteristic of some modern herbicides is their "selectivity". This can be defined as the ability to control weeds whilst having minimal impact upon certain crop species. It is this "selectivity" which has resulted in the greatest improvement in crop yields. As herbicides have resulted in such vast improvements in agricultural production, rigorous examination of their failure is also important. This thesis is an investigation into the failure of the herbicide carbetamide to control grass weeds upon repeated application.

Carbetamide is a soil applied, grass selective herbicide which is used in Australia for control of annual grasses in *Trifolium* spp.(clover) seed crops. Weeds in *Trifolium* seed crops reduce yields, lower quality, increase disease and insect problems and cause premature stand loss, harvesting problems and can affect animals grazing them (Klingman and Ashton, 1982). Stands of perennial *Trifolium* spp. established for seed production are typically maintained for between three and five seasons, whilst annual *Trifolium* spp. grown for the same purpose are typically grown for two consecutive seasons. Australian *Trifolium* pasture seed producers use herbicides extensively for weed control. As stands are typically

maintained for two to five years, annual application of the same herbicides often occurs.

As carbetamide is expensive, other herbicides were initially used in preference for grass control in *Trifolium* seed crops. The most commonly used herbicides were from two chemical classes, the aryloxyphenoxypropionates and the cyclohexanediones. *L. rigidum* with resistance to these herbicide groups was first reported in 1982 (Heap and Knight, 1982). Due to frequent herbicide application in *Trifolium* seed crops, herbicide resistant *L. rigidum* is common in many fields used for pasture seed production in the south-east of South Australia (Hole, 1993). Limited alternative herbicides existed for selective grass control in *Trifolium* seed crops. Apart from carbetamide, other herbicide options were limited to either propyzamide, which was considered too expensive, and mixtures of less efficacious herbicides. Since grass weed control is required on an annual basis, many *Trifolium* seed producers applied carbetamide to the same field in consecutive seasons. Carbetamide initially provided excellent grass control with minimal crop damage. However, when applied during 1992, carbetamide failed to control a range of grass weeds in approximately 35 % of the fields to which it was applied (Andrew Glynn, Rhône-Poulenc, personal communication). The majority of fields in which carbetamide failed had been treated in at least the prior season with carbetamide. Enhanced carbetamide degradation was considered a possible cause of failure.

Enhanced degradation is not the only cause of soil applied pesticide failure. Other causes of failure could include poor application, herbicide resistance or unfavourable environmental conditions. Poor performance of carbetamide over several seasons, in many locations of varying soil types suggested that poor application was not a major cause of failure. There is varying "tolerance" to carbetamide between *L. rigidum* accessions (McAlister, 1992, Hole, 1993, McAlister *et al.*, 1995). However, when carbetamide is applied at the appropriate growth stage, as performed by McAlister (1992), effective control is achieved at rates much lower than the recommended rate.

As few alternative herbicides are available, the development of methods enabling enhanced carbetamide degradation to be managed will contribute significantly to grass weed control in *Trifolium* seed crops. Seed from these crops is sold both on the domestic and international markets. Also, the use of *Trifolium* based pastures increases livestock production and fixes nitrogen in the soil, which results in increased crop production in subsequent seasons (Henzell, 1981).

This thesis documents research examining enhanced carbetamide degradation upon repeated application. Research was conducted to determine: i) if enhanced carbetamide degradation was the cause of failure, ii) whether enhanced carbetamide degradation could be managed to provide more efficacious and reliable weed control and iii), the microbial basis for enhanced carbetamide degradation.

2. REVIEW OF THE LITERATURE

2.1 Introduction and Scope of this Review

Pesticides can be degraded more rapidly in soil upon repeated application than when initially applied. This has been termed "enhanced degradation" (Roeth, 1986) or "enhanced biodegradation" (Racke and Coats, 1990b). The latter term emphasises the involvement of living entities, soil micro-organisms, in this phenomenon. The first report of enhanced pesticide degradation upon repeated application was in 1949 for the herbicide 2,4-D (Audus, 1949). Since then, enhanced pesticide degradation upon repeated application (henceforth referred to as enhanced degradation) has been shown to occur with a wide range of structurally dissimilar pesticides (see Roeth (1986), Felsot (1989) and Racke and Coats (1990b) for reviews). Though reported for a wide range of pesticides, enhanced degradation of carbetamide has not been reported previously, nor have bacteria capable of utilising carbetamide as an energy source been studied in detail.

This thesis describes an investigation of whether the herbicide carbetamide is degraded more rapidly upon repeated application to South Australian soils. This literature review considers information relevant to the herbicide carbetamide and the phenomenon of enhanced pesticide degradation.

2.2 Carbetamide

Carbetamide is a phenyl carbamate herbicide which selectively controls grass weeds in broad-leaf crops. Phenyl carbamate herbicides were one of the first synthetic organic chemistries to be exploited commercially for weed control (Kaufman, 1974). Other phenyl carbamate herbicides include propham, chlorpropham, barban and phenmedipham. The structure and properties of these herbicides are shown in Figure 2.1. The primary mode of action of carbetamide and most phenyl carbamate herbicides is to inhibit or disrupt mitosis. Exceptions to this are phenmedipham and desmiedipham, which are herbicidally active by inhibiting photosynthesis (Ashton and Crafts, 1981).

2.2.1 Historical development

The first report of a phenyl carbamate compound affecting plant growth was in 1927 when it was noted that ethyl carbanilate inhibited root growth in some grass species (Fränkel, 1927). However, the use of phenyl carbamates as herbicides was not exploited until after the discovery in 1940 that propham selectively controls oats (*Avena* sp.) in charlock (Templeman and Sexton, 1945). Since the discovery of propham, numerous other phenyl carbamate compounds have been reported as having potent herbicidal activity (George *et al.*, 1954). The herbicidal properties of carbetamide were reported in 1967 (Desmoras *et al.*, 1967), although this herbicide was discovered in 1960 (Rhône-Poulenc, 1977).

Figure 2.1 Structures and chemical properties of various phenyl carbamate herbicides.

herbicide	structure	properties	
carbetamide	(benzene ring)—NH-C(=O)—O—CH(CH_3)-C(=O)—NH-C_2H_5	mol. wt.(g) m.p. water sol. vap. press. mode of action	236.3 119 C 3.5 g L^{-1} negligible mitotic inhibition
propham	(benzene ring)—NH-C(=O)—O—CH(CH_3)(CH_3)	mol. wt.(g) m.p. water sol. vap. press. mode of action	179.2 87-87.6 C 32-250 mg L^{-1} sublimes slowly mitotic inhibition
chlorpropham	(Cl-substituted benzene ring)—NH-C(=O)—O—CH(CH_3)(CH_3)	mol. wt(g). m.p. water sol. vap. press. mode of action	213.7 41.4 C 89 mg L^{-1} 13.3 mPa (25 C) mitotic inhibition
barban	(Cl-substituted benzene ring)—NH-C(=O)— O—CH_2-C≡C—CH_2-Cl	mol. wt.(g) m.p. water sol. vap. press. mode of action	258.1 75-76 C 11 mg L^{-1} 50 μPa (25 C) mitotic inhibition
phenmedipham	(CH_3-substituted benzene ring)—NH-C(=O)—O—(benzene ring)—NH-C(=O)—O—CH_3	mol. wt.(g) m.p. water sol. vap. press. mode of action	300.3 143-144 C 3 mg L^{-1} 13 pPa (25 C) photosynthetic inhibitor

2.2.2 Toxicological and physiochemical properties

Like most phenyl carbamate herbicides, carbetamide is a potent inhibitor of mitosis in grass plants (Desmoras *et al.*, 1967, Badr, 1983), although these compounds are almost non-toxic to mammals (Still and Herrett, 1976, Rhône-Poulenc, 1977). Carbetamide is relatively soluble in water (3.5 g L^{-1}) and has negligible vapour pressure at 20°C (Pedersen *et al.*, 1995). Carbetamide is stable under acidic conditions, but chemical hydrolysis occurs under alkaline conditions (Rhône-Poulenc, unpublished, cited by Cantier *et al.*, 1988).

2.2.3 Agronomic Uses

Carbetamide controls most grasses and some broadleaf weeds in a wide range of crops. Those grasses which are not controlled typically have deep root systems, thus avoiding herbicide uptake, or are perennial and sufficiently established to tolerate application. Crops to which carbetamide can be safely applied include clovers (*Trifolium* sp.), lucerne, winter rape, chicory, lentils, vines, lettuce, cabbage, oil seeds and winter beans (Brock, 1972, Soper and Hutchison, 1974, Haggar and Oswald, 1975, Rhône-Poulenc, 1977). In Australia, carbetamide controls economically important annual grass weeds in lupins, peas, oilseeds and pastures (Reeves and Lumb, 1974, Cuthbertson, 1976, Stephenson and Madin, 1984). Typically, carbetamide application rates in excess of

1,500 g a.i. ha^{-1} provided 60 - 80 % reduction in weed growth and significantly increase yields (Reeves and Lumb, 1974, Cuthbertson, 1976).

Although carbetamide suppresses some broadleaf species, the level of broad leaf weed control is markedly improved by application of carbetamide/dimefuron mixtures (Rhône-Poulenc, 1977). Typically this mixture is useful for weed control in beans, peas and Brassica crops (Badr, 1983, Davies *et al.*, 1990).

2.2.4 Behaviour of carbetamide in soil

Carbetamide exhibits similar behaviour to other phenyl carbamate and non-polar herbicides in soil (Harris and Sheets, 1965). Carbetamide is weakly adsorbed to soil (Pedersen *et al.*, 1995) and is readily degraded by soil micro-organisms (Rhône-Poulenc, 1977). Degradation is influenced by soil pH, moisture and temperature (Cantier *et al.*, 1988). Evidence that soil micro-organisms readily degrade carbetamide include the marked increase in degradation with increasing temperature (Rhône-Poulenc, 1977), production of radiolabelled CO_2 (Rouchaud *et al.*, 1988) and increased herbicide persistence in autoclaved soil (Cantier *et al.*, 1988).

As carbetamide is primarily root absorbed, placement of carbetamide within the soil is necessary for effective weed control. As carbetamide is only weakly adsorbed to soil particles, rainfall can be used to

move carbetamide into the soil. This allows for incorporation of carbetamide into soil, without the use of cultivation. As cultivation is not required, carbetamide may be applied to established or perennial plants. This method of incorporation is utilised when carbetamide is applied to *Trifolium* sp. stands in Australia. As leaching does occur, heavy rainfall may result in movement of carbetamide deeper into the soil profile (Rhône-Poulenc, 1977). However, as carbetamide is readily degraded by soil micro-organisms, it is unlikely to pollute ground water.

2.2.4.1 Degradation pathway and persistence in soil

In a study of the degradation of carbetamide, chlorpropham and propyzamide, Rouchaud *et al.* (1988) reported the production of aniline and 3-chloroaniline as metabolites from carbetamide and chlorpropham respectively. These metabolites were further metabolised at a rate sufficient such that they did not accumulate in the soil. The production of aniline from carbetamide suggests that initial cleavage of the carbetamide is likely to occur at the ester linkage - shown as microbial degradation in Figure 2.2. Rouchad *et al.* (1988) also reported that propyzamide degradation produced a ketone metabolite in soil. Therefore, it is likely that carbetamide and propyzamide are degraded by different pathways in soil.

Degradation of ^{14}C-labelled carbetamide under controlled conditions in a non-sterile alkaline soil demonstrated that significant amounts were converted to aniline and $^{14}CO_2$ (Cantier *et al.*, 1988). At 28°C

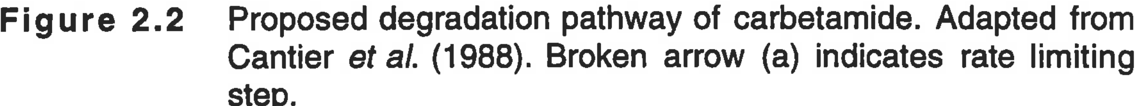
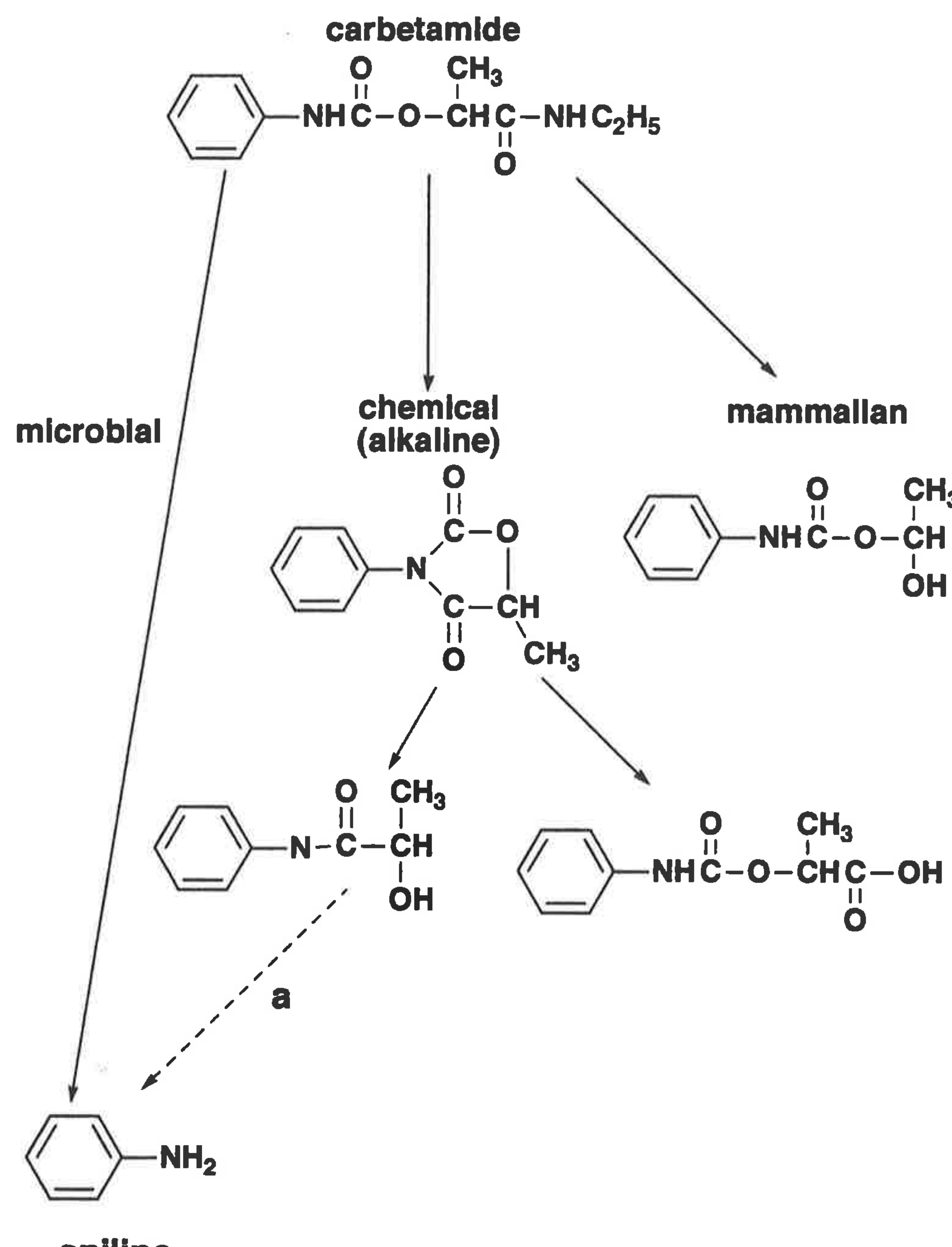


Figure 2.2 Proposed degradation pathway of carbetamide. Adapted from Cantier *et al.* (1988). Broken arrow (a) indicates rate limiting step.


carbetamide
microbial
chemical
(alkaline)
mammalian
a
aniline

the half life of carbetamide in non-sterile soil was 30 days. Not all possible carbetamide metabolites are observed in non-sterile soil (Cantier *et al.*, 1988). A possible explanation for this is that microbial degradation proceeds via hydrolytic cleavage of the carbamate linkage. This has been demonstrated for microbial degradation of propham and chlorpropham (Kaufman and Kearney, 1965, Kearney, 1965, Kearney and Kaufman, 1965).

Field studies of carbetamide efficacy under different climatic conditions suggest that carbetamide is degraded faster with increasing soil temperature (Rhône-Poulenc, 1977). This is supported by laboratory studies where degradation was faster at 37°C than at 28°C (Cantier *et al.*, 1988). The half life of carbetamide at these two temperature was 11.3 and 22.1 days, respectively. These results are similar to those observed with propham and chlorpropham, where degradation is considerably faster at 29°C than 15°C (Burschel and Freed, 1959). An important conclusion of Burschel and Freed (1959) is that their results with laboratory incubations were comparable with results obtained in the field.

As stated previously, carbetamide is weakly adsorbed to soil particles (Pedersen *et al.*, 1995). Propham is similar to carbetamide in this characteristic. Studies on the leaching of propham have demonstrated that this herbicide is leached in soil but tends to distribute within the soil profile (Freed, 1951). Leaching of carbetamide has also been noted (Rhône-Poulenc, 1977). Carbetamide leached beyond the first four or five centimetres is unlikely to control grasses. Also leaching of carbetamide

dilutes the concentration of carbetamide in the upper layers of the soil. When the soil dries the herbicide would be drawn toward the soil surface (Rhône-Poulenc, 1977). Based upon the results of Freed (1951), approximately 6 mm of saturated propham solution applied to a leaching column localised propham activity within the upper 5 cm of the column, which is the optimum depth suggested for carbetamide activity (Rhône-Poulenc, 1977).

2.2.5 Mode of Action in Plants

Once at the plant surface the selectivity and activity of all herbicides is directly influenced by herbicide i) absorption, ii) translocation, iii) metabolism and iv), activity at the site of action. Selectivity of carbetamide appears to be due primarily to activity at the site of action, the meristematic regions. Evidence for this is principally based upon the observed symptoms. Symptoms of treatment with carbetamide are indistinguishable to that of propham and chlorpropham, suggesting that these herbicides have similar mode of action.

2.2.5.1 Symptoms

Visual symptoms following treatment with carbetamide, propham or chlorpropham include cell hyperplasia (swelling) at the meristematic regions, darkening of leaves and greatly reduced growth (Allard *et al.*, 1946, Freed, 1953, Desmoras *et al.*, 1967, Badr, 1983). Leaves are visibly greener three days after treatment and continue to

become greener over the next eleven days (Allard *et al.*, 1946). This is the result of an increased leaf chlorophyll content (19 - 28 %) (Freed, 1953). During this period, little or no cell elongation occurs and no new leaves are produced (Allard *et al.*, 1946). Visible cell necrosis or browning begins two weeks after treatment and this begins at the leaf tips. Allard *et al.* (1946) reported that oat plants were completely dead 23 days after treatment with propham. Carbetamide induces symptoms similar to these in *Hordeum vulgare* and in the green alga *Chlorella vulgaris* (Bastide and Badon, 1988, Osman *et al.*, 1988). Of the species tested by Allard *et al.* (1946), oats and wheat were the most sensitive and rice was less sensitive. As these experiments were conducted in a glasshouse, it could be reasonably expected that these symptoms and plant death may proceed more slowly under field conditions, especially when temperatures are lower.

2.2.5.2 Absorption and translocation

Mass flow of water toward roots, in response to transpiration by plants, is thought to be responsible for the absorption of most soil applied herbicides (Sagar *et al.*, 1982, Moyer, 1987).

Although absorbed by both roots and shoots, it is likely that carbetamide must be absorbed by roots for optimum herbicidal activity. With propham and chlorpropham, the reasons for this are two-fold. Firstly, these herbicides are more readily absorbed by roots (Baldwin *et al.*, 1954). Secondly, once absorbed, translocation of propham and chlorpropham

occurs acropetally (Baldwin *et al.*, 1954). After absorption carbetamide translocation is similar in resistant and susceptible species (Bastide and Badon, 1987). As applications of carbetamide to soil are more effective than foliar applications, it is also likely that carbetamide is absorbed more readily by roots and is only translocated acropetally.

2.2.5.3 Metabolism

Unlike most other herbicides (Devine *et al.*, 1993), metabolism of carbetamide does not appear to play a major role in selectivity. Desmoras *et al.* (1967) reported that carbetamide accumulated in a tolerant species (*Medicago sp.*) but was rapidly degraded in a susceptible species (*Hordeum sp.*). However Bastide and Badon (1987) showed that carbetamide is metabolised faster in lucerne than in barley or lettuce. Such metabolism may reflect small differences in the susceptibility of plants (Bastide and Badon, 1987). However, carbetamide metabolism is almost certainly not the principal mechanism of selectivity.

2.2.5.4 Cytological effects

Disruption of microtubule arrangement appears to be the primary mode of action of carbetamide. Numerous reports demonstrate that carbetamide, propham and chlorpropham disrupt microtubule arrangement and as a consequence affect chromosome structure and movement during mitosis (Allard *et al.*, 1946, Mann and Storey, 1966, Jackson, 1969, Sanger

and Jackson, 1971, Palevitz and Helper, 1976, Badr, 1983, Holmsen and Hess, 1985). Additionally, these herbicides induce the formation of aberrant cell walls and interfere with cell elongation, processes which also require precise arrangement of microtubules. Symptoms of mitotic disruption include blocked metaphase, multinucleate cells, giant nuclei, altered spindle formation and an increased number of partly contracted chromosomes (Desmoras *et al.*, 1967, Jackson, 1969, Holmsen and Hess, 1985). Disruption of mitosis can occur with concentrations as low as 0.01 ppm. Increasing the concentration increases the speed and severity of observed effects. As the symptoms induced by all phenyl carbamates are similar, the biochemical mode of action is thought to be similar (Hess, 1987).

The biochemical basis for interaction between phenyl carbamates and microtubules has not been reported. However, it is presumed to be due to interference with the unknown mechanism by which plants co-ordinate microtubule arrangement (Hess, 1987, Lehnen *et al.*, 1990). This assumption is based upon observations of the cytological effects induced by these herbicides.

An alternative explanation for the herbicidal activity of phenyl carbamate herbicides has been proposed, and this explanation suggests that these herbicides regulate gene expression. Cartwright (1976) suggests that the action of phenyl carbamates at the molecular level inhibits metabolic changes or gene derepression (induction). Evidence cited for this includes: i) inhibition of α amylase production in barley endosperm, ii) inhibition of

cytokinin-induced cell proliferation in tobacco pith cultures by chlorpropham, iii) cell elongation is not inhibited (*Avena* spp.) in cells in which elongation has started (Cartwright, 1976). However, apart from the inhibitory effect on α amylase production, these results can be equally well explained by phenyl carbamates interfering with the mechanism that organises microtubules. It remains to be determined how phenyl carbamate herbicides physiologically and/or biochemically interact, and selectively interfere with mitosis.

2.2.5.5 Herbicide resistance

Herbicide resistance is where a weed population is less effectively controlled by a herbicide, due to prior selection with the same herbicide. Mechanisms of herbicide resistance include reduced adsorption, reduced translocation, increased metabolism and reduced activity at the active site (Hall *et al.*, 1994). Resistance mechanisms can be further grouped into either target site or non-target site mechanisms.

The only report of herbicide resistance to carbetamide is in the annual grass weed *L. rigidum.* McAlister *et al.* (1995) reported that root elongation of two populations (SLR 10 and SLR 31) was greater than the control population (SLR 2) in the presence of carbetamide. However unpublished data on the whole plant response of these two biotypes clearly demonstrates that: i) there is virtually no difference in the response of these populations and ii), excellent control is achieved at recommended field rates

(McAlister, 1992). In addition soil used for the entire plant assays was extremely high in organic matter (33 % peat moss). High levels of organic matter reduce carbetamide availability and hence activity in soil (Pedersen *et al.*, 1995). Therefore, although variability exists among *L. rigidum* populations in their response to carbetamide, good control is expected under field conditions.

2.3 Microbial Degradation and Enhanced Degradation of Pesticides

2.3.1 Introduction

The efficacy and activity of soil applied herbicides are strongly influenced by their persistence in soil. Microbial degradation is recognised as the major route by which most herbicides are degraded once they enter the soil (Kaufman and Kearney, 1976, Torstensson, 1980, Koskinen and Moorman, 1992). This not only applies to herbicides but also to a wide range of soil applied pesticides including insecticides, fungicides and nematicides. Therefore, for soil applied herbicides, a certain period of soil persistence is essential to their efficacy as herbicides. In contrast, rapid degradation of chemicals which contaminate the environment is advantageous (Felsot, 1989). Treatments which stimulate the soil microbial community tend to increase chemical degradation rates and conversely treatments which inhibit microbial activity tend to slow degradation (Skipper *et al.*, 1986).

Microbial degradation can pose an agronomic problem where micro-organisms can use a pesticide as a nutrient or energy source. In such cases, the application of a pesticide (or potentially any organic molecule) results in rapid degradation of a subsequent application. This phenomenon has been termed "enhanced degradation" (Roeth, 1986), "enhanced biodegradation upon repeated application" (Racke and Coats, 1990b), "accelerated biodegradation" (Kaufman *et al.*, 1986) and "growth linked

degradation" (Robertson and Alexander, 1994) and has been reported to occur with most classes of biodegradable soil applied pesticides. This phenomenon has also been observed to occur with chemicals which are not viewed as pesticides, for example acetylene (Terry and Leavitt, 1992).

Enhanced degradation of herbicides similar in structure to carbetamide has been demonstrated. Decreased efficacy of propham and chlorpropham upon repeated application infers that these herbicides are amenable to enhanced degradation (Gray and Joo, 1985). Enhanced degradation of propham and chlorpropham has been demonstrated (Chapman and Harris, 1990, Robertson and Alexander, 1994). Although these herbicides are similar in structure to carbetamide, a partially purified enzyme from a soil bacterium capable of hydrolysing propham and chlorpropham could not hydrolyse carbetamide (Marty and Vouges, 1987).

Where the cause of enhanced pesticide degradation has been examined, typically soil bacteria are the organisms associated with degradation (Lal and Saxena, 1982). Often pure cultures of microorganisms have been isolated capable of pesticide degradation. One exception is linuron where degradation was performed by a mixed microbial community (Roberts *et al.*, 1993). Degradation of chlorpropham, propham and phenmedipham has been shown with pure bacterial cultures (Clark and Wright, 1970, Vega *et al.*, 1985, Pohlenz *et al.*, 1992). Several studies have indicated that phenyl carbamate degrading phenotypes are typically plasmid encoded (Vega *et al.*, 1988, Gaubier *et al.*, 1992, Pohlenz *et al.*,

1992). Enhanced carbetamide biodegradation would suggest the presence of microorganisms which are well adapted to utilising this herbicide as a nutrient and/or energy source. Such microorganisms have not been described previously.

2.3.2 Terminology

To aid this discussion, the following terms are defined:

•"Pesticides" are commercial, chemical products which suppress, control or eradicate pests. This term embraces chemical products which control plants, insects, fungi and nematodes.

•"Metabolites" are products formed due to the degradation of a pesticide, or other compound, due to any degradative process.

•"Biodegradation" refers to the process whereby soil micro-organisms degrade a molecule to smaller and simpler metabolites. Usually the metabolites are less toxic, but not always. Complete biodegradation is the conversion to CO_2, H_2O and other simple molecules so that the initial structure is completely lost. This process is also termed mineralisation.

•"Enhanced Degradation" is evident by "an increase in the decomposition rate of a herbicide (*pesticide or chemical*) in a medium, usually soil, previously treated with a herbicide (*pesticide or chemical*), as

compared to its decomposition rate in an identical medium that was not previously treated (Roeth, 1986) - words in brackets added.

•"Cross Enhancement" is where the degradation rate of a chemical is increased due to prior application of a different chemical. Cross enhancement is usually observed where the two chemicals are similar in structure and implies that they are degraded by similar pathways.

2.3.3 Microbial degradation in soil

Once applied to a soil, all chemicals, including pesticides, are subject to an array of transport and transformation processes (Figure 2.3). These processes have been reviewed elsewhere (for example Kearney, 1977, Alexander, 1981, Shea, 1985, Walker, 1987, Nash, 1989, Koskinen and Moorman, 1992) and covered with reference to carbetamide earlier in this review (section 2.2.4). Whilst all of these processes can affect the performance of soil applied pesticides, microbial degradation is the most important. Microbial degradation is responsible for degradation of most soil applied pesticides (Torstensson, 1996). Also, microbial degradation, unlike most abiotic mechanisms, has the capacity to completely degrade compounds. Thus undesirable metabolites typically do not accumulate. Pesticide degradation has both advantages and disadvantages. Whilst degradation is necessary to prevent accumulation in the environment, if degradation occurs too rapidly the efficacy of soil applied pesticides can be eroded (Felsot, 1989).

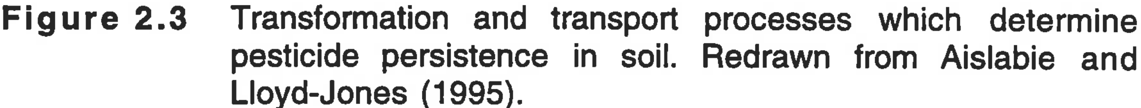
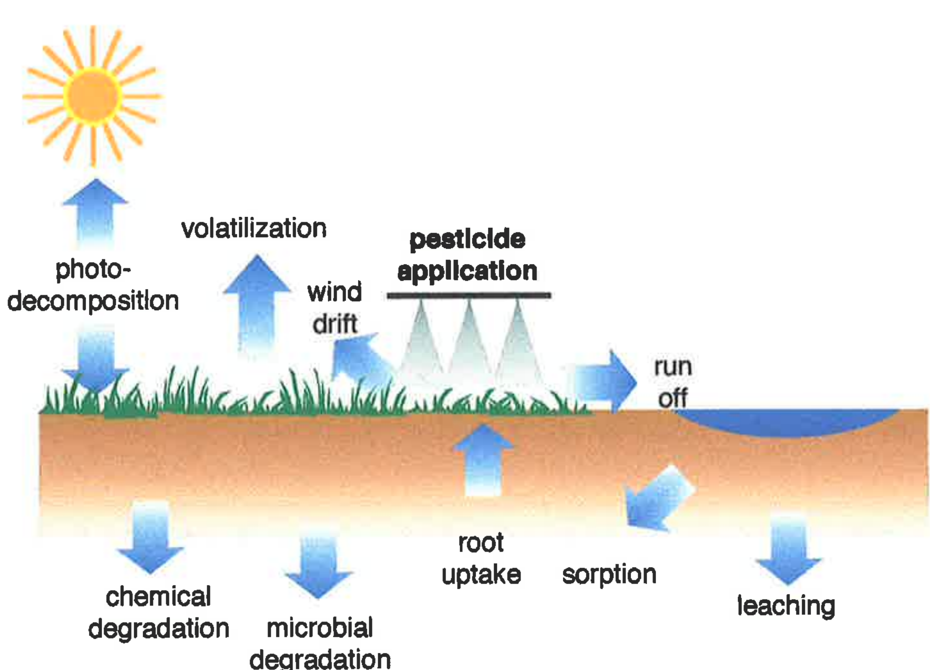


Figure 2.3 Transformation and transport processes which determine pesticide persistence in soil. Redrawn from Aislabie and Lloyd-Jones (1995).


volatilization
pesticide
application
photo-
decomposition
wind
drift
run
off
root
uptake
sorption
leaching
chemical
degradation
microbial
degradation

Virtually all commercially available pesticides are degraded to some extent by soil micro-organisms. If pesticides do not degrade, they accumulate in food chains causing significant environmental problems (Chapalamadugu and Chaudry, 1992). Some pesticides used in the past, such as the organochlorines, are very persistent in the environment. Public concern over the accumulation of such recalcitrant pesticides, leading to environmental contamination residues in food, highlighted a need for and stimulated the development of biodegradable pesticides (Felsot, 1989).

Soil micro-organisms degrade pesticides: i) utilising them as nutrient and/or energy sources, ii) removing them from their environment as they are toxic or iii) passively removing the compounds through co-metabolism using other energy/nutrient sources. This removal or degradation is impressive since these organisms often occupy less than 0.1 % of the soil by volume (Torstensson, 1980). Soil micro-organisms may use herbicides as sources of carbon, energy, nitrogen, phosphorus, or sulphur (Karns, 1990). Irrespective of whether the herbicide in the soil is toxic or is a source of energy and /or nutrients, the effect on the population of soil micro-organisms is that of simple natural selection (Karns, 1990).

Kaufman and Kearney (1976) described the degradation kinetics of herbicides as following one of two patterns chemical or biological degradation. The kinetics of these two types are shown in Figure 2.4. Chemical degradation is typically described as following a decay curve, also described as first order kinetics. However, biological degradation, whilst

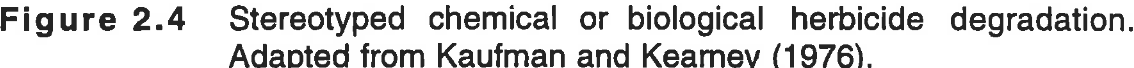
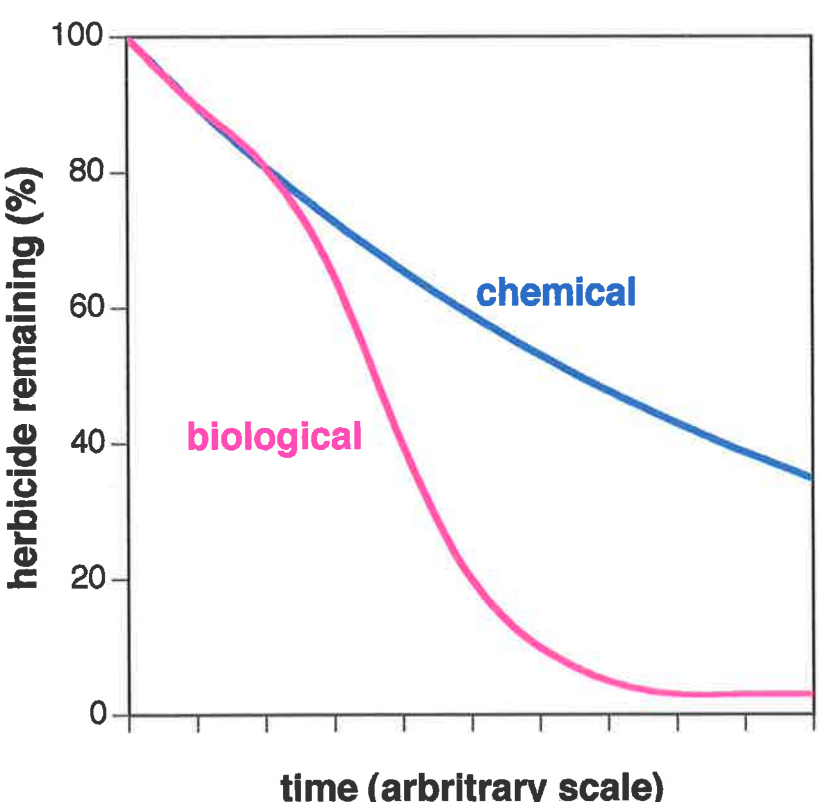


Figure 2.4 Stereotyped chemical or biological herbicide degradation. Adapted from Kaufman and Kearney (1976).


100
80
60
40
20
0
herbicide remaining (%)
chemical
biological
time (arbitrary scale)

initially following similar kinetics to that of chemical degradation, shows a period of more rapid degradation. This period of rapid degradation is assumed to be a result of degradation by an active or adapted soil micro-flora. Herbicides which exhibit biological degradation kinetics were considered by Kaufman and Kearney (1976) to be more prone to enhanced degradation.

Microbial degradation of herbicides is the result of transformations and cleavage reactions which are mediated by enzymes (Englert *et al.*, 1993). A vast array of enzymes are produced by soil micro-organisms. These enzymes vary immensely in their specificity to bind with and change compounds or fragments of compounds (Alexander, 1981). As there is great diversity amongst microbial enzymes, a wide range of compounds can be degraded within the soil (Table 2.1). A notable inclusion in Table 2.1 in terms of this review are carbamate structures. Some substitutions to chemicals, such as single or multiple halogenated aromatics, branched carbon chains and chlorine and sulfonate substitutions, are associated with increased soil persistence (Alexander, 1981, Shea, 1985). These groups are not naturally produced in soil. This may be because they are not transformed by enzymes or that transformation products may be toxic.

Table 2.1 Organic molecular fragments amenable to microbial transformation(s). From Englert *et al.* (1993).

alcohols	ketones
aldehydes	lactams
alicyclic aliphatics	lactones
aliphatics (saturated)	nitriles
aliphatics (unsaturated)	nitro compounds
amides	nitrosamines
aromatics (simple substituted)	organoarsenicals
aromatic heterocyclics	organomercurials
azides	organophosphorus compounds
carbamates	organosulfates
carboxylic acids	organotins
condensed Aromatics	oximes
dithiocarbamates	quaternary ammonium compounds
esters	sulfides
ethers	sulfonic acids
glycosides	thioamides
halides	thiol carbamates
heterocyclics	thiols
hydroxamic acids	ureas
hydroxyl amines	

2.3.4 Enhanced degradation upon repeated application

Enhanced degradation has been observed with a wide range of soil applied pesticides. Reports of this phenomenon have originated from many continents or regions including North America, Europe, Asia and the Middle East (Racke and Coats, 1990b) and it has been extensively reviewed - see Roeth, 1986, Felsot, 1989, Racke and Coats, 1990b. In Australia, research has been conducted examining enhanced degradation of the nematicide fenamiphos (Stirling *et al.*, 1992). However no reports of enhanced herbicide degradation affecting weed control in practical applications have been published.

The first herbicide reported to be susceptible to enhanced degradation was 2,4-dichlorophenoxyacetic acid (2,4-D) (Audus, 1949). Until the mid 1970's enhanced herbicide degradation received little attention as most herbicides amenable to enhanced degradation were foliar absorbed or unlikely to be applied repeatedly (Roeth, 1986). Since this time a number of agriculturally important soil applied herbicides have been reported as being susceptible to enhanced degradation (Table 2.2). Table 2.2 is not an exhaustive list but indicates the diversity of chemical structures amenable to enhanced degradation. Common characteristics of herbicides exhibiting enhanced degradation are: i) the herbicide is amenable to microbial degradation and ii) the herbicide is relatively non-persistent (Roeth, 1986).

Table 2.2 Herbicides amenable to enhanced degradation upon repeated application.

Herbicide	**Reference**
2,4-D	(Audus, 1949)
EPTC	(Obrigawitch *et al.*, 1982)
propham	(Gray and Joo, 1985)
chlorpropham	"
TCA	"
dalapon	"
amitrole	(Riepma, 1962)
vernolate	(Wilson, 1984)
butylate	(Tuxhorn *et al.*, 1986)
dicloprop	"
2,4,5-T	"
tri-allate	(Cotterill and Owen, 1989)
propyzamide	(Walker and Welch, 1991)
linuron	"
alachlor	"
napropamide	(Walker *et al.*, 1996)
simazine	(Saavedra and Pastor, 1996)
atrazine	(Pussemier *et al.*, 1997)

Enhanced degradation has also been reported for chemicals other than pesticides. An example is acetylene which is used as an industrial welding gas and in the study of denitrification in soil. By flooding a soil with

acetylene the enzyme N_2O reductase is inhibited. However, soils previously treated with acetylene can rapidly degrade this gas (Terry and Leavitt, 1992).

There is significant variability in the time taken for enhanced degradation to occur. Rapid degradation may be evident after only a single application (Roeth, 1986), though repeating application several times tends to amplify the effects of enhanced degradation. Two contrasting examples of this are 2,4-D and simazine. With 2,4-D, enhanced degradation may be evident after a single application (for example - Robertson and Alexander, 1994). Whilst with simazine, which has been used extensively at high rates since the 1950's, enhanced degradation was not confirmed until 1990 (Saavedra and Pastor, 1996). This is despite previous reports showing that simazine degradation was not affected or only affected slightly by prior application (Fryer and Kirkland, 1970, Robertson and Alexander, 1994). Simazine is biodegraded and micro-organisms capable of partial mineralisation were reported in 1965 (Kaufman *et al.*, 1965). Possible differences in the number of applications required to induce enhanced degradation may reflect the energy released during biodegradation or the time taken for genetic rearrangements or mutations to occur leading to novel catabolic capabilities.

Enhanced degradation may persist for several months to years after herbicide application has ceased (Roeth, 1986). Decline of enhanced degradation has important practical ramifications (see sections 2.3.9 and 5). The time taken for enhanced degradation to decline is most probably linked

to the time taken for the population of degrading organisms to decline or for these organisms to lose their capacity to degrade. Factors that are suggested as affecting the survival of degrading micro-organisms include: i) ability to produce spore-like structures, ii) continued availability of trace amounts of the herbicide, iii) availability of alternative substrates and iv), environmental conditions (Roeth, 1986). Persistence may be as short as one year, as with linuron (Walker and Welch, 1992) or may be still evident after several years as with 2,4-D (Smith and Aubin, 1994). Enhanced degradation of acetylene was still evident after 8 years (Terry and Leavitt, 1992).

The effect of enhanced degradation upon weed or pest control is variable between pesticides, fields and seasons. For example, attempts to correlate prior EPTC application and a range of soil characteristics including prior herbicide application could not predict weed control (Drost *et al.*, 1990). In a field in which enhanced degradation was confirmed, good weed control was observed (Drost *et al.*, 1990). Varying results may be partially explained by differing soil types, environmental conditions and weed emergence patterns.

Carbetamide is a soil active herbicide, the activity of which is strongly influenced by soil persistence (section 2.2.4). Kaufman and Kearney (1976) suggest that it is herbicides, such as carbetamide, which have little or no foliar activity that will be most affected by enhanced degradation.

2.3.5 Methods of studying enhanced degradation

Methods used in the study of enhanced degradation have reflected the available analytical techniques. Initially, degradation and enhanced degradation were examined using plant bioassays and ultra-violet absorption. These techniques are "non-specific and insensitive" (Smith and Lafond, 1989). More recently, gas and high pressure liquid chromatography techniques (GC/HPLC) have enabled accurate determination of microgram quantities of pesticides. Another widely used, alternative technique is monitoring the evolution of ^{14}C labelled CO_2 from appropriately labelled pesticides.

Results of laboratory based degradation studies cannot be directly extrapolated to the field. One of the greatest differences with herbicides applied to the soil surface, such as carbetamide, is that the concentration is not uniform down the soil profile. In a discussion on whether or not biphasic degradation (i.e. initially slow then rapid degradation) occurs, Smith and Lafond (1990) suggest that with 2,4-D, the effective soil concentration varies with depth and that this will affect the rate of degradation. As the rate of degradation may be altered in laboratory incubations, it is also evident that the effect of various treatments cannot be directly extrapolated to the field. Thus studies which examine options for the management of enhanced pesticide degradation use field based trials (see Drost *et al.*, 1990, Felsot and Tollefson, 1990, Harvey, 1990).

2.3.6 Effect of soil properties upon enhanced degradation

Numerous soil properties affect microbial degradation in soils (Aislabie and Lloyd-Jones, 1995) and hence affect enhanced degradation. These properties affect the diversity and activity of soil micro-organisms and the availability of the pesticide. However, once enhanced degradation is evident, soil temperature and moisture appear to have the greatest capacity to directly influence enhanced degradation.

2.3.6.1 Temperature

In general, increasing temperature within the range of 5 - 30°C, results in increased microbial activity and pesticide degradation. Under laboratory conditions it has been found that the optimum rate of degradation for most herbicides is around 20°C (Shea, 1985). As enhanced pesticide degradation is directly linked to microbial activity, increasing temperature tends to further decrease pesticide persistence in soils affected by this phenomenon.

Several reports have documented the effect of temperature upon enhanced pesticide degradation. Saavedra and Pastor (1996) reported a differential effect between soils depending on their prior simazine application and incubation temperature. Enhanced simazine degradation was clearly evident at incubation temperatures of 15°C and 25°C but not at

5°C. Similar results have been reported with the herbicide EPTC (Obrigawitch *et al.*, 1982), suggesting that at low temperatures enhanced degradation may be inhibited.

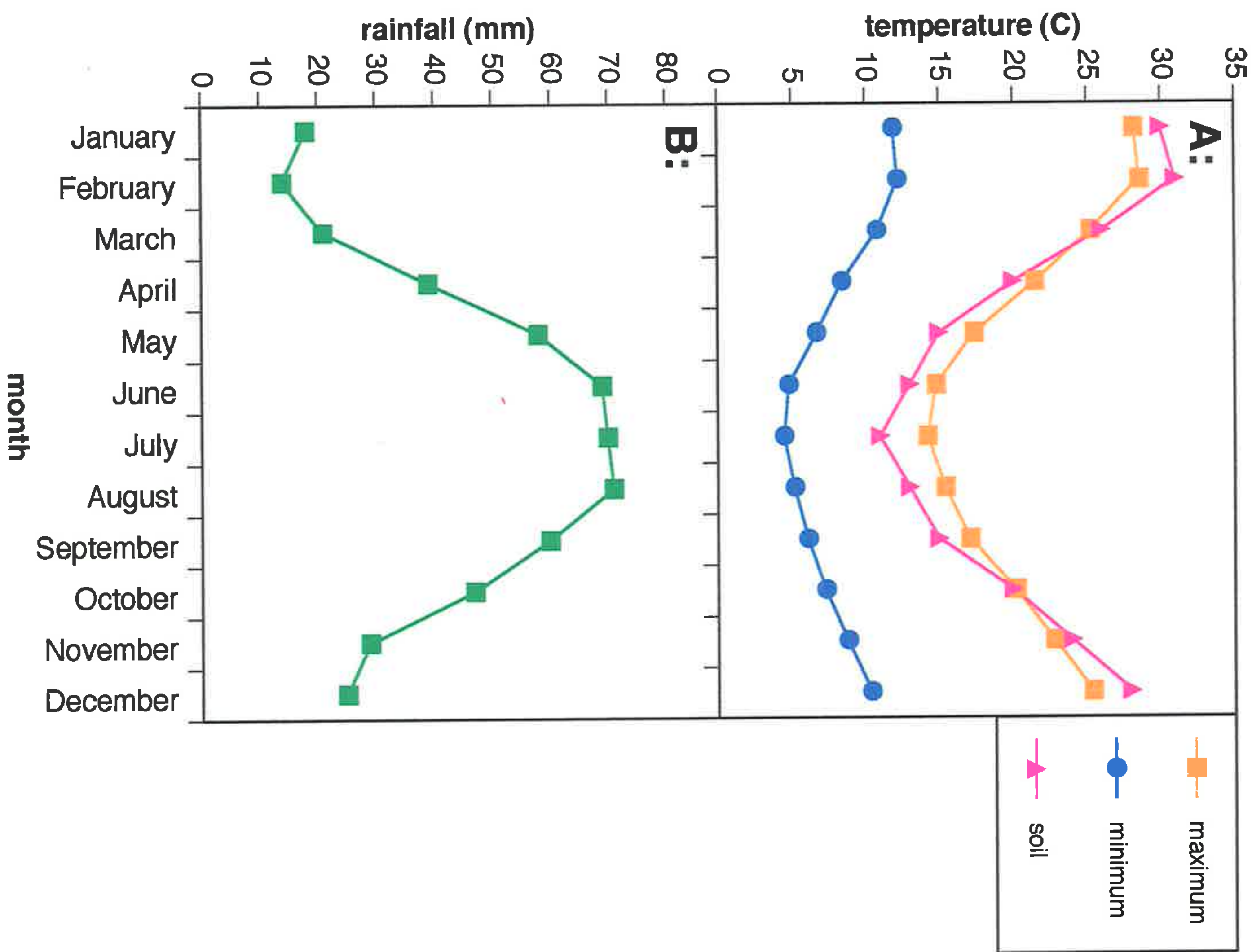
Low soil temperatures do not necessarily inhibit either the induction or expression of enhanced degradation. Enhanced degradation of the herbicide napropamide occurred at 5°C and could be induced by a prior treatment at 5°C (Walker *et al.*, 1996). This implies that in a cold environment, bacteria which are cold tolerant are more likely to be involved in enhanced degradation.

Under Australian conditions carbetamide is applied during June and July. During this period the average soil temperature is unlikely to fall below 10°C (Figure 2.5). Temperatures such as these are unlikely to inhibit enhanced pesticide degradation in soil.

2.3.6.2 Moisture content

As with temperature, soil moisture also affects several processes in the soil, both in the presence and absence of enhanced pesticide degradation. Typically, degradation is fastest when the soil moisture content is between 75 % and 100 % of field capacity, providing that no other essential elements are limiting degradation (Shea, 1985). Soil moisture affects the biomass and activity of soil micro-organisms and the availability of some pesticides in soil. However, enhanced pesticide

Figure 2.5 (A) Average monthly maximum and minimum air temperatures for Naracoorte and average soil temperature 10 cm below surface at 3 PM for Struan (near Naracoorte). (B) Average monthly rainfall recorded at Naracoorte. Source: Australian Bureau of Meteorology.



A:
B:
temperature (C)
0
5
10
15
20
25
30
35
rainfall (mm)
0
10
20
30
40
50
60
70
80
month
January
February
March
April
May
June
July
August
September
October
November
December
maximum
minimum
soil

degradation is typically only limited by extremely low or high soil moisture contents. For example, enhanced degradation of the herbicide EPTC was inhibited when soil moisture was 1.6 % or less (Obrigawitch *et al.*, 1982). Similar effects are observed when rainfall is absent under field conditions (Roeth *et al.*, 1990).

As with soil temperature, degradation or enhanced carbetamide degradation is unlikely to be limited by soil moisture when applied under normal conditions of use in Australia. In southern Australia, carbetamide is applied from June to August which is the period of highest monthly rainfall and low temperatures (Figure 2.5). Therefore soil moisture content is unlikely to preclude enhanced degradation from occurring in this region.

2.3.7 Cross-enhancement

In addition to enhanced degradation, it has been reported that other pesticides may be degraded faster due to prior application of a different herbicide. This is termed "cross enhancement" (Roeth, 1986). Cross enhancement has both practical and scientific implications. Practical implications relate to other herbicides which could be affected and the length of time enhancement will persist in a soil. Cross enhancement may be the result of degradation by the same pathway. If so, the spectrum of cross enhancement may provide insight into the degradation pathway.

Examples of cross enhancement include that between propham and chlorpropham, EPTC and vernolate, and 2,4-D and other phenoxy herbicides (Roeth, 1986). The degradation rate for the herbicides for which cross-enhancement is evident is usually slower than that for the herbicide which was originally applied (Roeth, 1986). Three explanations have been proposed to explain the slower degradation rate: i) the induction process benefits a number of soil micro-organisms and these organisms have varying catabolic efficiency for different herbicides; ii) the enzymes induced by the herbicide have the ability to degrade a range of herbicides with different rates for each herbicide; or iii) that the herbicides interact with different operons, thereby inducing different specific activities of the same or similar enzymes (Roeth, 1986).

Cross enhancement is more likely to occur for compounds which are similar in structure to the rapidly degraded chemical (Roeth, 1986). For example, enhanced soil degradation of the herbicide EPTC resulted in rapid butylate and vernolate degradation (Obrigawitch *et al.*, 1983). The initial step in biodegradation of phenyl carbamates is through cleavage of the ester linkage (Kaufman, 1967). This linkage is unique to phenyl carbamate compounds and enzymes which cleave this linkage typically do not degrade other compounds (Kearney and Kaufman, 1965). Thus for carbetamide, cross enhancement is most likely to other phenyl carbamate herbicides.

2.3.8 Effect on weed control

The persistence of soil applied herbicides is the single greatest determinant of their activity (Koskinen and Moorman, 1992), thus enhanced degradation is likely to impact upon weed control. However, even with detailed information on persistence it is often not possible to determine accurately the reduction in weed control (Racke, 1990). Under field conditions, Kaufman and Kearney (1976) suggest that the impact of enhanced degradation will depend upon: i) the herbicidal mode of action, ii) the cropping system, iii) the physical-chemical behaviour of the herbicide in soil, iv) weed density, v) the time of weed seed germination in comparison with the persistence of the herbicide and vi), environmental conditions. Early weed emergence can also reduce the impact of enhanced degradation (Drost *et al.*, 1990). Clearly, numerous factors combine to reduce weed control due to enhanced degradation.

Based upon the mode of action of carbetamide, it is likely that reduced persistence of carbetamide in soil would result in decreased herbicidal efficacy. In summary, carbetamide must be absorbed by roots in order to be translocated to the meristematic regions (section 2.2.5.2). Also carbetamide is slow acting, taking several weeks to kill weeds (section 2.2.5.1) and may be metabolised within plants (section 2.2.5.3). This suggests that susceptible plants need to absorb carbetamide over a period of time in order to be controlled. Therefore, significant reductions in soil persistence are likely to have a large impact upon weed control.

2.3.9 Management of enhanced degradation

Though enhanced pesticide degradation may persist for several years after the last application, partial reversion back to the natural state can provide significant improvements in weed control (Roeth, 1986). As only partial reversion is observed, the efficacy of pesticides which are applied early in the season, such as the insecticide carbofuran, may not be improved or remain unreliable (Felsot and Tollefson, 1990). Though enhanced degradation of phenyl carbamate herbicides has been documented (Gray and Joo, 1985), management of enhanced degradation for these herbicides has not been examined in detail. This probably reflects the fact that these herbicides are not used extensively or repeatedly. In Australia, carbetamide failure was primarily noted in fields which had been treated with the same herbicide in the previous season. As carbetamide had been available for approximately twenty years, this suggests that infrequent carbetamide application provides effective weed control. Management of enhanced pesticide degradation is further described in section 5.

2.3.10 Microbial involvement in enhanced degradation

Though often poorly documented, the role of an "adapted or conditioned" (Felsot, 1989) soil microbial population is usually implicated as the causal agent of enhanced degradation. Evidence for an elevated population of degrading micro-organisms as the causal agent of enhanced

degradation includes: i) increases in the Most Probable Number (MPN) or Colony Forming Units (CFU's) capable of degradation, ii) inhibition of enhanced degradation by chemicals which are specific microbial inhibitors (Skipper *et al.*, 1986), iii) transfer of enhanced degradation from one soil to another (Walker *et al.*, 1996) and iv), the isolation of microorganisms capable of pesticide mineralisation (Behki, 1994,). Whilst these methods have traditionally been used, each has limitations.

As soils are complex heterogeneous media, studying the involvement of micro-organisms in the process of enhanced degradation can be difficult and is often subject to several limitations. Not the least of these limitations is that many soil organisms, perhaps greater than 99 %, cannot be cultured using normal techniques (Wang and Wang, 1996). The use of molecular DNA/RNA techniques has overcome some of these limitations (Amann *et al.*, 1991, Steffan and Atlas, 1991).

Carbetamide and other phenyl carbamate herbicides are degraded via hydrolytic cleavage to yield aniline or a halogenated aniline. For chlorpropham, this initial transformation was suggested to be due to a single enzyme capable of hydrolysing the herbicide (Kearney and Kaufman, 1965), although it was unclear whether the hydrolysis was at the ester or amide linkage. Typically, the herbicide degrading enzymes of phenyl carbamate degrading bacteria are stable and inducible (Mateen *et al.*, 1994). Mateen *et al.* (1994) concluded that degradation of phenyl carbamate herbicides is catalysed by "broad-specificity" hydrolases and

oxygenases, although most esterases also exhibit "broad specificity" (Dixon and Webb, 1979).

2.3.10.1 Genetics of bacterial metabolism

Enhanced degradation is almost certainly due to elevated populations of soil micro-organisms capable of pesticide metabolism. In most cases the micro-organisms are soil bacteria (Head *et al.*, 1990). When a pesticide is initially applied to a soil, biodegradation is probably the result of a mixed population of organisms each of which partially degrade the compound. However, if one or more organisms obtain the ability to completely degrade the pesticide then this organism may have a selective advantage (Karns, 1990). Thus enhanced degradation may take time for the selection of a strain capable of complete biodegradation. Integration of a complete metabolic pathway into a single bacteria could be due to genetic changes which involve: i) plasmids, ii) transposable elements, iii) mutation or iv), other genetic modification. Where examined, plasmids are often associated with a pesticide degrading phenotype (Roeth, 1986), though the evolution of plasmid-borne catabolic pathways may involve the other mechanisms listed above.

Understanding the genetics of herbicide biodegradation provides a basis for understanding the development of enhanced degradation (Karns, 1990). Commonly the genes which encode for pesticide or an unusual catabolic function reside on a plasmid. Plasmids are

extrachromosomal pieces of DNA which enable bacteria to modify their biochemical activities (Kams, 1990).

Genes encoding for pesticide degradation in different bacteria often show a high degree of similarity. In discussing the similarity of 2,4-D degrading plasmids, Head *et al.* (1990) suggest that this is due to either i) independent evolution from common ancestral genes or ii), genes for degradation are transmitted through a microbial population via plasmids. It is likely that both postulated mechanisms occur as Head *et al.* (1990) cite examples of readily transmissible plasmids and gene homology from organisms isolated on different continents. More recent genetic examination of 2,4-D degrading bacteria suggests that both independent evolution and plasmids transfer between organisms has occurred (Vallaeys *et al.*, 1996). Similar observations of high similarity have been made with plasmid borne genes encoding *m*-toluate degradation (Assinder and Williams, 1990). The implication of this for carbetamide degradation is that the genes which encode degradation may show high similarity to genes previously described for degradation of esters and the metabolite aniline.

2.3.10.2 Influence of Pesticide Metabolites

Examination of pesticide metabolites provides insight into the likelihood of enhanced pesticide degradation. Features of metabolites which have been postulated to influence enhanced degradation include: i) nutrient value, ii) toxicity and iii) availability (Somasundaram and Coats, 1990).

Microorganisms are unlikely to gain energy or nutrients from initial degradation of a molecule. It is only when the metabolites are further broken down, such that they can be incorporated into central metabolic pathways, that the full value is realised. The importance of metabolite degradation is implied where pre-incubation with a herbicide metabolite induces enhanced degradation. Where soil is pre-treated with 2,4 dichlorophenol, a 2,4-D metabolite, 2,4-D is typically degraded at an enhanced rate (Somasundaram and Coats, 1990).

Hydrolysis of the ester linkage of phenyl carbamate herbicides yields aniline (or the corresponding halogenated compound). This compound is most likely converted to catechol and further degraded following either the *ortho* or *meta* cleavage pathways (Mateen *et al.*, 1994). Degradation of aniline and halogen substituted anilines has been extensively studied as they are the metabolites of many soil applied pesticides. These compounds can be degraded by both Gram positive and Gram negative bacteria (Kaminski *et al.*, 1983).

Studies of aniline degrading bacteria isolated from soil have indicated that degradation occurs via oxygenation to catechol (Aoki *et al.*, 1983a, Aoki *et al.*, 1983b). Products of oxygenation can be further degraded via either *ortho* or *meta* cleavage (Houghton and Shanley, 1994). Ultimately these products are “channelled” into the Krebs cycle, yielding energy. These pathways have been extensively studied in Pseudomonads (Houghton and Shanley, 1994) but are common to virtually all aromatic

degrading organisms. The ability to channel compounds through the Krebs cycle is seen as both an ability to efficiently degrade a wide variety of compounds (due to numerous insertion points) and to maintain metabolic dynamic equilibrium (Houghton and Shanley, 1994).

Halogenated aromatic compounds do not occur naturally and are degraded via modified pathways. Degradation of chlorpropham (Rouchaud *et al.*, 1988) yields 3-chloroaniline upon initial cleavage. Whilst non-halogenated aromatic compounds which can be converted to catechol and degraded via *ortho* or *meta* cleavage pathways, halogenated aromatics are typically degraded via a modified *ortho* cleavage pathway. Such pathways arise spontaneously after several months incubation of enrichment cultures (Reineke and Krackmuss, 1988) and are usually plasmid borne. Pathways are induced separately to conserve energy (Houghton and Shanley, 1994) and this may also prevent the formation of toxic intermediates.

Formation of aniline as an intermediate in the degradation of carbetamide is unlikely to be toxic to soil bacteria and this product is likely to be rapidly degraded by soil bacteria as a source of carbon and/or energy. Rapid degradation and low toxicity of aniline is expected due to the ubiquity of aromatic amines in the environment (Dagley, 1986). It is therefore the initial formation of aniline from carbetamide which is likely to involve enzyme(s) specialised for this reaction (Dagley, 1986). However as degradation of haloaromatic compounds requires specialised enzymes, the

ability to degrade these compounds may be limited depending upon prior exposure to these compounds.

2.3.11 Conclusion

Enhanced degradation of soil applied pesticides upon repeated application has been reported for numerous structurally diverse chemicals, including chemicals similar in structure to carbetamide. Reports of enhanced carbetamide degradation were not found. Enhanced degradation of propham and chlorpropham has been reported. These herbicides are similar in structure to carbetamide and the initial degradative steps are likely to be similar. Cross enhancement has been reported between propham and chlorpropham. As carbetamide is similar in structure to these herbicides and the degradation pathway is similar, it is most likely that cross enhancement would occur between these herbicides.

The occurrence of enhanced carbetamide degradation is likely to significantly reduce both pre- and post-emergent weed control. Carbetamide takes several weeks to kill plants and appears to require continual soil persistence during this period to control grasses effectively. Therefore enhanced degradation would result in short term suppression of established grass plants. Enhanced carbetamide degradation would reduce the period of soil activity such that weeds with extended or slow germination patterns would not be effectively controlled.

Karns (1990) suggests that taking into account the adaptive ability of soil micro-organisms, and the pressure of the environmental lobby, all soil applied pesticides will either become amenable to enhanced degradation or they will be banned through lack of biodegradability. As carbetamide is biodegradable, enhanced degradation is a likely cause of failure when used repeatedly.

Carbetamide has been applied on an annual basis for grass control in legume pasture seed fields in Australia. Initial applications of carbetamide are typically extremely efficacious, but after two to three years of continuous use efficacy declines substantially. Under such conditions *Trifolium* seed producers recorded yield losses in the order of 50 % (L. Badman, legume pasture seed producer, personal communication), though in some fields grass weed control was sufficiently poor that the crop was completely smothered. Poor or improper application were unlikely causes of failure. As discussed in the introduction, poor application over many fields in several seasons was considered unlikely and herbicide resistance within the grass weeds was not the cause of failure (McAlister, 1992, Hole, 1993). Therefore enhanced degradation of carbetamide was considered a possible cause.

Environmental conditions prevailing in the area where carbetamide failed are unlikely to inhibit the development or expression of enhanced pesticide degradation in soil. Rainfall and temperature data

presented in Figure 2.5 indicate that adequate soil moisture and moderate soil temperatures are prevalent when carbetamide is applied (July).

3. GENERAL METHODS AND MATERIALS

3.1 Fields

Experiments were conducted with soil collected from 5 fields with differing histories of carbetamide application. The soil characteristics and carbetamide application history of these fields are listed in Table 3.1. These fields were all suitable for the cultivation of *Trifolium* pasture seed crops.

Table 3.1 Carbetamide application history and soil physical and chemical properties of fields used in this study. Silt and clay measurements were made by soil hydrometer (Sheldrick and Wang, 1993), pH was measured by preparation of a 1:5 (w/v) soil slurry in 0.01 M $CaCl_2$ (Hendershot *et al.*, 1993).

field	carbetamide application history[1]	silt (%)	clay (%)	pH
A[2]	none	7	23	4.8
B[3]	1990-92	8	12	4.9
C[2]	none	7	4	4.6
D[4]	none	7	10	4.5
E(entire field)[4]	1989-92	6	8	5.8
E[II][5]	1989-92	6	8	5.8
E[III][5,6]	1989-93/94/95	6	8	5.8

notes:

1 - Years in which 2,100 g ha^{-1} carbetamide was applied to the field.
2 - Fields A and C had not been previously treated with any herbicides.
3 - Field B had been treated with a wide range of herbicides and insecticides. This field was adjacent to field A.
4 - Field D and E are adjacent.
5 - Soils E[II] and E[III] are plots (10 m x 2 m, 4 replicates) within field E.
6 - Plots were sampled prior to carbetamide application in 1994 and 1995 (sections 5.3.1 and 7.2.4).

3.2 Chemicals

Table 3.2 Formulation, source and detection parameters of pesticides used in this study. Further details of methods are in section 3.5. All compounds were analysed by HPLC except for propyzamide which was analysed by GC. Notation: T = technical, WP = wettable powder, EC = emulsifiable concentrate, FC = flowable concentrate, ECD = electron capture detection, - = not determined. Recovery values were calculated based upon soil samples taken 4 hours after herbicide treatment and compared to diluted herbicide stock solutions, or in the case of propyzamide comparison was made to a standard curve.

chemical	purity (%)	formulation	recovery from soil (%) field D	recovery from soil (%) field E	retention time (min)	wavelength (nm)	source
carbetamide	93.9	T	92	91	10.5	233	Rhône-Poulenc, France
"	70	WP	-	-	10.5	233	Rhône-Poulenc, Australia
carbaryl	50	FC	87	83	16.9	233	Rhône-Poulenc, Australia
"	99.9	T	-	-	16.9	233	Rhône-Poulenc, Australia
chlorpropham	98.5	T	-	-	14.9	233	Zeneca, UK
"	50	EC	87	91	14.9	233	Lane, Australia
diuron	80	T	-	-	12.2	254	Sigma, USA
"	50	FC	91	85	12.2	254	Agchem, Australia
EPTC	>95	T	-	-	16.4	220	ICI Crop Care, Australia
"	72	EC	84	86	16.4	220	ICI Crop Care, Australia
propham	99	T	-	-	12.9	233	Zeneca, UK
"	75	WP	93	91	12.9	233	Agchem, Australia
propyzamide	>95	T	-	-	10.3	ECD	Agchem, Australia
"	50	WP	93	93	10.3	ECD	Agchem, Australia
simazine	98	T	-	-	2.3	254	Ciba, Australia
"	50	FC	94	92	2.3	254	Agchem, Australia

3.3 Field Methods

3.3.1 Pesticide application

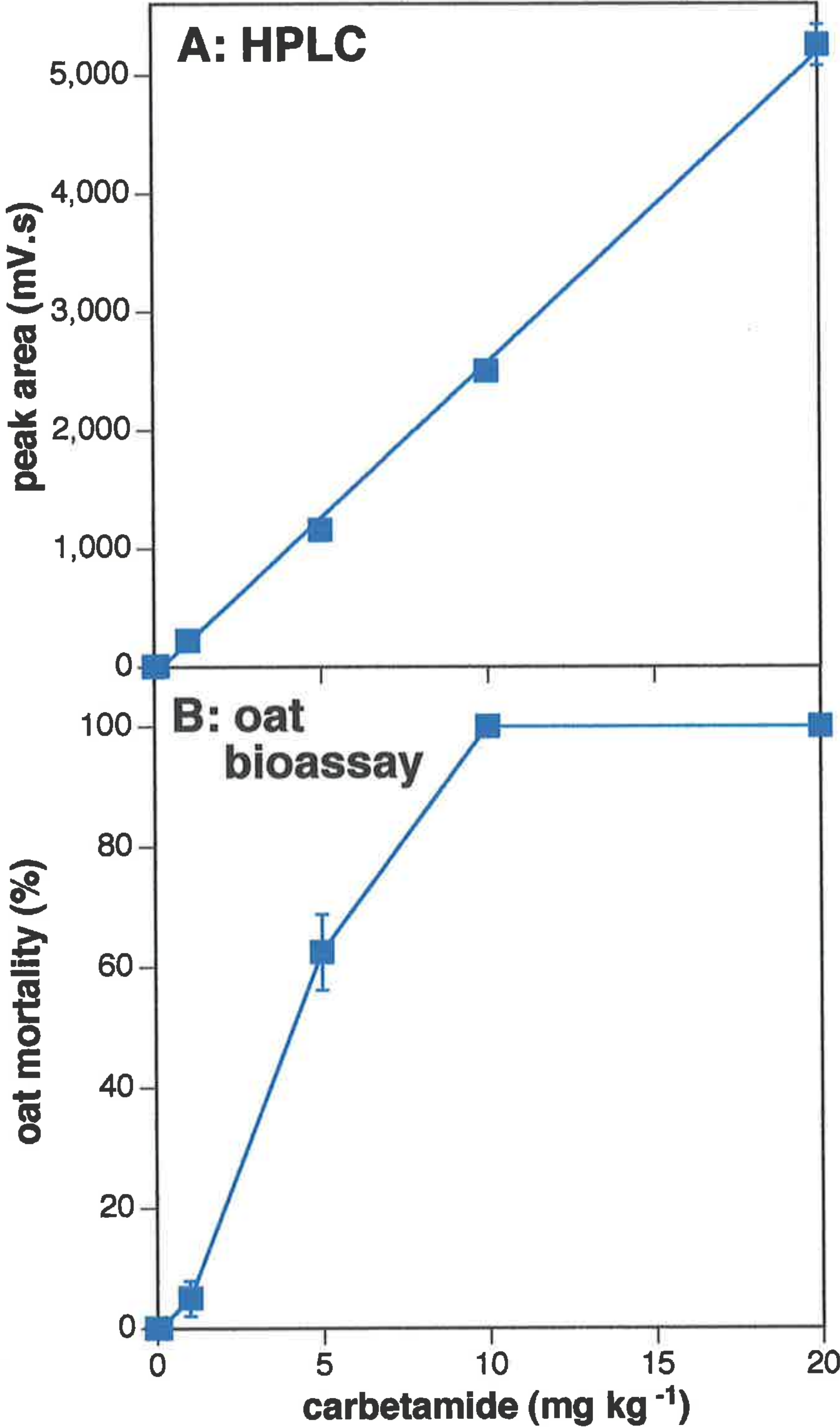
Field application of herbicides (and the insecticide carbaryl) were made using a propane pressurised (250 kPa) hand held sprayer of 2 m boom width fitted with flat fan nozzles (Hardi 4110-10). Sprayer output was 125 L ha^{-1} when moving at 1.0 m s^{-1} at a height of 40 cm above the plant canopy. Each plot was sprayed twice, giving a total water volume of 250 L ha^{-1}. Pesticides were applied as commercial formulations in all field experiments. Formulations and sources of pesticides are listed in Table 3.2.

3.4 Pesticide Degradation Methods

Carbetamide degradation kinetics were determined both chemically, using HPLC assay, and biologically, using an oat bioassay. These methods are described in sections 3.4.2, 3.4.3 and 3.5. Comparison of the results obtained from both methods in soil treated with a range of carbetamide concentrations is shown in Figure 3.1.

Pesticide degradation was examined in soil collected from the fields described in Table 3.1. Except where indicated in the text, all degradation experiments included four separate replicates. After collection from the field, moisture content was measured by drying a subsample at

Figure 3.1 Peak area of HPLC detector response (A) and oat mortality (B) in relation to soil carbetamide concentration. Vertical bars indicate standard errors of the mean. Each point is the mean of four replicates.


A: HPLC
peak area (mV.s)
5,000
4,000
3,000
2,000
1,000
0
B: oat bioassay
oat mortality (%)
100
80
60
40
20
0
0
5
10
15
20
carbetamide (mg kg -1)

120°C for 24 hours. From each sample the required number of duplicate samples were weighed into 300 mL plastic containers. The equivalent of 150 g of oven dry soil was placed into each container. Moisture content was adjusted by addition of double distilled water. For all experiments the moisture content was adjusted to between 15 and 20 % of oven dry weight. Containers of soil were fitted with clip-on lids to minimise evaporation and incubated at 15°C. During long term experiments, soil moisture content was periodically adjusted gravimetrically.

3.4.1 Pesticide addition

Pesticides were added to experimental units in 1 mL of water per container. Technical grade carbetamide was used for all experiments. All other pesticides were added as commercial formulations. After pesticide addition, soil in each container was emptied into a separate plastic bag and mixed thoroughly.

3.4.2 Extraction of pesticide residues from soil

Pesticide residues were extracted from 25 g soil subsamples by placing in a 250 mL polypropylene bottle with 25 mL acetonitrile and shaking on a rotary shaker for 1 hour. Samples were allowed to settle for 10 minutes, after which a subsample of the supernatant was filtered (0.2 µm nylon) and placed into an auto-sampler vial. For the herbicide propyzamide, residues were extracted in a similar method to the other herbicides except

for: i) the substitution of methanol for acetonitrile and ii) particulate matter was removed by centrifugation in a microfuge (12,566xg) for 5 minutes. Pesticide residues were analysed quantitatively using the chromatography methods detailed in section 3.5.

3.4.3 Oat bioassay for carbetamide analysis

The bioactivity of carbetamide residues in soil was evaluated using an oat bioassay similar to that previously described for detection of chlorpropham residues in soil (Eshel and Warren, 1967). After subsampling for residue analysis, 10 oat seeds (*Avena sativa* c.v. echidna) were sown into the remaining soil in each container. After a further seven days incubation at 15°C, seeds were recovered from the soil and those with coleoptiles failing to extend beyond the seed were classed as dead.

3.5 Pesticide Chromatography

Chromatography of all compounds, excluding propyzamide, was performed using an ICI HPLC system (LC 1100 pumps, LC1600 detector) equipped with a reverse phase C18 column (250 x 4.6 mm, Exsil 100/5 µm ODS). Samples (100 µL) were injected using a SGE LS3200 autosampler and carbetamide was eluted with a 10 - 100 % acetonitrile gradient over 20 minutes (0 min, 10 %, 3 min 32.5 %, 10 min, 34.3 %, 20 min 100 %). Propham, chlorpropham, EPTC, simazine and diuron were eluted with a 10 - 100 % concave acetonitrile gradient over 30 minutes. The

flow rate in all systems was 1.2 mL min^{-1}. Wavelengths, retention times and recoveries from soil are shown in Table 3.2. Appropriate wavelengths for detection were based upon UV spectra (DMS 100s, Varian) of technical grade compounds dissolved in acetonitrile. The UV absorption spectra of carbetamide is shown in Figure 3.2. Data was collected and analysed using DP800 (ICI) interface and software. For all pesticides, peak area was linearly correlated to concentration within the range 0 to 2 μg.

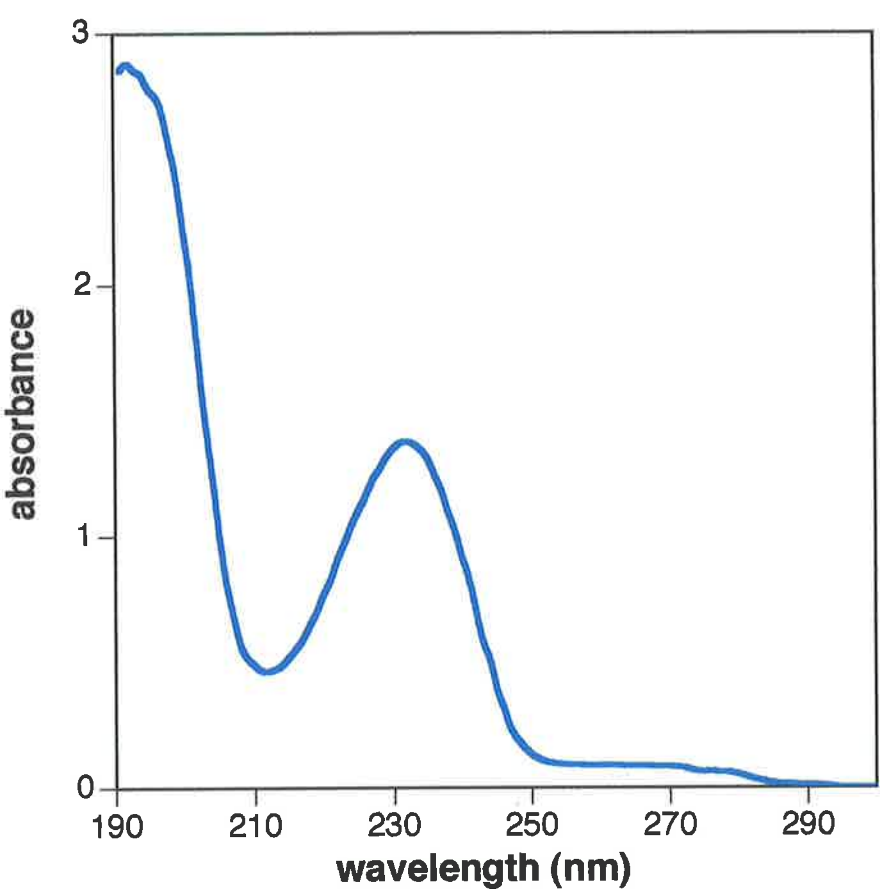
Soil extracts of propyzamide treated soil were separated by GC (Varian Star 340cx, DB5 column, injection volume 5 μL, injector 260°C, column 180°C, detector ECD 300°C) (Adler *et al.*, 1972). Data was collected and analysed using PC based software.

3.6 Bacterial Methods

3.6.1 Growth of organisms

The following Mineral Salts Medium (MSM) based upon that used by Kaufman and Kearney (1965) was used for the isolation of carbetamide degrading bacteria: K_2HPO_4 0.8 g L^{-1}, KH_2PO_4 0.2 g L^{-1}, $MgSO_4.7H_20$ 0.2 g L^{-1}, $CaSO_4$ 0.1 g L^{-1}, $(NH_4)_6Mo_7O_{24}.4H_20$ 0.001 g L^{-1}, and $(NH_4)_2SO_4$ 5.0 g L^{-1}. Media was autoclaved at 120°C for 20 minutes. For solid media, 15 g L^{-1} of agar (Difco, Bacto agar) was added prior to autoclaving. For active growth of pure isolates in liquid culture, 50 mg L^{-1} of yeast extract (Oxoid) was added. Carbetamide (200 mg L^{-1} for liquid media

Figure 3.2 Ultraviolet (UV) absorption spectrum of carbetamide (9.5×10^{-5} M, dissolved in acetonitrile).



3
2
1
0
absorbance
190
210
230
250
270
290
wavelength (nm)

and 500 mg L^{-1} for solid media) was added dissolved in methanol (50 % w/v) after autoclaving. For non-selective growth, cultures were grown in nutrient broth (Oxoid), or on nutrient agar (Oxoid). Cultures were established routinely in 50 mL of liquid medium in 250 mL Erlenmyer flasks. Except where mentioned in text, all cultures were incubated at 28°C on an orbital shaker (oscillating distance 2 cm, 80 rpm).

3.6.2 Pesticide degradation

Technical grade pesticides were used in all bacterial growth experiments. All pesticides were added dissolved in methanol, except for diuron, simazine and carbaryl which were dissolved in acetone. Source and purity of pesticides is shown in Table 3.2.

Degradation of carbetamide in 50 mL liquid cultures was monitored by removal of 1 mL subsamples which were centrifuged (microfuge, 12,566xg, 5 minutes) to remove bacteria. The supernatant was diluted 10 fold with acetonitrile and 800 µL was loaded into an autosampler vial. Quantitative HPLC was performed as described previously (section 3.5).

As propham and chlorpropham are relatively insoluble in water, degradation of these herbicides was monitored in 5 mL cultures (section 8.2.8). Entire cultures were destructively sampled at each time point by the addition of 15 mL acetonitrile to stop growth and dissolve remaining

herbicide. Quantitative HPLC was performed as described previously, the only modification being reduction of the injection volume to 50 μL.

3.6.3 Measurement of growth

Growth of cultures was monitored using the Miles and Misra drop plate technique (Vincent, 1970). Cultures were routinely sampled (100 μL) and 10 fold dilutions were performed in SBS buffer. Three replicate 20 μL drops were placed onto solid MSM (plus carbetamide) or nutrient media and incubated at 28°C. After two to four days the number of colonies was counted at the appropriate dilution.

Growth of organisms was estimated by monitoring the optical density (OD) of cultures using a spectrophotometer set at 600 nm (DMS 100s, Varian). This was initially performed in conjunction with drop plate counts to correlate OD_{600} with bacterial cell numbers.

3.6.4 Buffers

Serial dilutions and re-suspensions of cultures were performed in sterile buffered saline (SBS). This buffer was composed of NaCl 8.5 g L^{-1}, $NaH_2PO_4.H_2O$ 1.08 g L^{-1} and Na_2HPO_4 1.73 g L^{-1}.

3.6.5 Additional carbon sources

Sodium acetate, pyruvate and succinate, where supplied as additional carbon sources, were added at 5 mM, as filtered (0.2 µm) stock solutions in water.

3.6.6 Antibiotics

Antibiotics were added to autoclaved media after cooling to 50°C. Antibiotic stock solutions, rifampycin (10 µg mL^{-1} in methanol), streptomycin (10 mg mL^{-1} in water, filter sterilised 0.2 µm), gentamycin (10 mg mL^{-1} in water, filter sterilised 0.2 µm), naladixic acid (10 mg mL^{-1} in 0.1 M NaOH, filter sterilised 0.2 µm), chloramphenicol (34 mg mL^{-1} in methanol) and ampicillin (50 mg mL^{-1} in methanol), were stored at -20°C.

3.7 Measurement and detection of ^{14}C-labelled compounds

^{14}C labelled carbetamide (Rhône Poulenc) was dissolved in methanol and stored at 4°C. Measurement of total ^{14}C was performed using liquid scintillation counting. Scintillant (Ultima Gold XR, Canberra Packard) was added to samples and counting was performed using a Beckman scintillation counter (LS 5000TD). Corrections were made for efficiency and samples without ^{14}C were used to determine background levels. Detection of radiolabelled compounds after separation by HPLC was performed using a

YtSi solid cell flow through scintillation counter (FLO-ONE Beta, series A100, Radiomatic, Canberra Packard).

4. ENHANCED CARBETAMIDE DEGRADATION UPON REPEATED APPLICATION - CONFIRMATION AND CHARACTERISATION OF THE PHENOMENON

4.1 Introduction

In summarising enhanced insecticide degradation in soil, Racke and Coats (1990a) proposed three fundamental features which must be thoroughly investigated to confirm and characterise enhanced insecticide degradation. In applying these criteria to herbicides these are: i) increased rate of degradation upon repeated application when compared to an appropriate control soil, ii) examination of herbicide degradation kinetics and metabolite formation under controlled conditions and iii), to elucidate the microbial aspects of herbicide degradation, including isolation of adapted micro-organisms. Racke and Coats (1990a) suggested that enhanced degradation may or may not result in reduced pesticide efficacy. As this project was established to confirm the cause of poor grass weed control with carbetamide, demonstration of a significant reduction in weed control upon repeated application was an *"a priori"* objective.

The aim of research presented in this chapter was to determine whether repeated carbetamide application is a cause of carbetamide failure in the field. Specific characteristics to be quantified or examined were i) efficacy of weed control, ii) kinetics of carbetamide degradation and iii), the involvement of soil micro-organisms in this process.

4.1.1 An experimental system for monitoring the effects of enhanced degradation

Elucidating whether enhanced pesticide degradation is the cause of pest control failure may require extensive investigation. For initial confirmation, adjacent fields may be compared for herbicidal efficacy. An assumption of this method is that subtle differences in soil type, or prior application of other pesticides does not affect pesticide activity. Alternatively using a single soil, differing soil pesticide histories can be generated by differing pesticide application regimes. However for herbicides, this approach modifies the weeds present, either in type or density, thus the system needs to be manipulated such that the weed characteristics are the same. In the present study, both of these approaches were used. Where possible, conditions as close as practicable to commercial situations were simulated.

Grasses vary in their level of susceptibility to phenyl carbamate herbicides. Whilst most grass species are susceptible to carbetamide, some exhibit high levels of resistance (Rhône-Poulenc, 1977). Thus, inappropriate selection of a test species may affect results. At the commencement of this study, the weed species which were not effectively controlled by carbetamide included *L. rigidum.*, *Vulpia* sp. and *Phalaris minor.* All of these species are normally susceptible to carbetamide application. As *L. rigidum* seed was readily available, this species was used in this study.

Under Australian conditions, carbetamide is applied to the soil surface soon after weed emergence. Herbicide efficacy is dependent upon rainfall leaching the herbicide into the root zone from which it is absorbed. Variability in the timing of rainfall after treatment is an additional variable which may reduce the efficacy of carbetamide. Hence, although carbetamide is not volatile, if rainfall does not occur soon after application there is usually reduced carbetamide efficacy. To remove rainfall as a variable, an irrigation system was used to leach carbetamide into the soil, as described in section 6.2.3 (exceptions were the experiments described in sections 4.2.1 and 4.2.2). The irrigation system also allowed for application of carbetamide under low wind conditions, making herbicide application more precise.

For field experiments described in this thesis *L. rigidum* mortality after application of carbetamide was assessed by the presence of absence of new growth after spraying. After application carbetamide rapidly inhibits cell division and elongation, and results in treated leaves turning a visibly darker green (Desmoras *et al.*, 1967, Badr, 1983). As carbetamide is most likely translocated acropetally (section 2.2.5.2), leaf extension is continually inhibited while the amount of carbetamide absorbed from the soil remains high. Thus it can be assumed that if the soil carbetamide concentration declines significantly, the concentration within the plant would decline and plant growth may resume. Appropriate controls are required to demonstrate that any observed changes in efficacy are solely due to prior carbetamide application.

4.1.2 Chemical and bioassay detection of carbetamide in soil

After confirming that the observed decrease in efficacy was linked to repeated application, the next objective was to examine the kinetics of carbetamide degradation upon repeated application. An analytical assay and bioassay were developed for the quantification of carbetamide residues in soil. Quantification of carbetamide residues in soil was performed by reverse phase high performance liquid chromatography (HPLC) of soil solvent extracts using ultraviolet light absorption for detection. Spectroscopy (DMS 100s, Varian) of technical grade carbetamide (Table 3.2) confirmed that this compound has an absorption maximum at 233 nm (Figure 3.2) (Jendrzejczak *et al.*, 1992). Extraction of carbetamide residues from soil was achieved by shaking with acetonitrile for 1 hour. As carbetamide is not strongly bound to soil (Pedersen *et al.*, 1995), high recoveries were achieved with this procedure (Table 3.2).

Plant bioassays are a proven method for detecting herbicide residues in soil. Whilst chemical techniques can be faster and more accurate, bioassays are the only method which accurately reflect the biological availability of a herbicide in soil (Lavy and Santelmann, 1986). Bioassay methods still play a key role in the development of new herbicides (Schmidt, 1993). Several bioassay methods have been used to examine the persistence of propham and chlorpropham - for example (Crafts and Drever, 1960, Roberts and Wilson, 1962). Typically these methods are long term

assays running over several weeks, requiring large amounts of space. An alternative method, based upon inhibition of oat germination, was used in the present study. This allowed increased replication and a more rapid measurement of carbetamide concentration (see section 3.4.3).

4.1.3 Involvement of micro-organisms in enhanced degradation

The third aspect of enhanced carbetamide degradation examined was the association of soil micro-organism(s) responsible for this process. Enhanced pesticide degradation is most likely the result of "growth-linked" microbial degradation of a pesticide (Robertson and Alexander, 1994). Growth requires energy and this is obtained by the channelling of suitable substrates into the energy producing cycles. Under aerobic conditions this results in the production of CO_2. Thus, addition of appropriately ^{14}C labelled compounds to soil, when serving as a growth substrate, results in $^{14}CO_2$ evolution. $^{14}CO_2$ can be trapped in basic solutions (such as sodium hydroxide) and counted using liquid scintillation techniques, thus providing a simple way of measuring biodegradation (Skipper *et al.*, 1986). Due to the ease of use of this method, $^{14}CO_2$ evolution has also been extensively used to examine enhanced degradation of the herbicide EPTC - see various articles in Racke and Coats (1990b). The system employed for monitoring the evolution of $^{14}CO_2$ influences the total percentage evolved (Skipper *et al.*, 1986). Systems used are described as either "static" or "flow through". Typically, total $^{14}CO_2$ evolution is lower from

static systems than flow through systems, though Skipper *et al.* (1986) suggested that the static systems are probably more comparable to field values.

Enhanced degradation is a biological phenomenon. That is, rapid degradation is due to the presence of a high population of micro-organisms capable of degradation. As bacterial growth rates are rapid, it is possible to inoculate a control soil with a small amount of enhanced soil. Upon addition of the growth substrate, the population of bacteria rapidly increases and enhanced degradation becomes evident. This type of experiment has been used to demonstrate the potential to disperse degrading organisms from field to field (Walker and Welch, 1990) and to suggest contamination of control samples due to improper sample hygiene (Walker *et al.*, 1996) or spray drift.

Microbial inhibitors have been used extensively to implicate soil micro-organisms in many soil processes including degradation. These inhibitors can be described as either physical or chemical. Physical techniques include heat sterilisation and gamma irradiation. Chemical methods include compounds that selectively inhibit bacteria, fungi or protozoa. Though all of these treatments have limitations (Kaufman and Kearney, 1976), they are still a most effective means of elucidating the role of soil microorganisms in the degradation of herbicides and other compounds in soil.

Some chemical treatments are selective and can be used to determine the role a particular class of micro-organisms plays in degradation. For this technique to be valid, certain assumptions must prevail: i) all target organisms are susceptible to a particular inhibitor and ii) that an applied inhibitor does not affect other classes of organisms. In the present study, chloramphenicol and cycloheximide were used to inhibit bacteria and fungi respectively. Chloramphenicol inhibits the formation of proteins by ribosomes found only in bacteria, whilst cycloheximide inhibits eukaryote ribosomes (Brock *et al.*, 1994). As mentioned above an assumption in the use of microbial inhibitors is that all organisms of that class are susceptible. This is not strictly true as some groups within each class are typically tolerant or resistant. Roberts *et al.* (1993) exploited this natural variability in antibiotic resistance to characterise a mixed population of organisms which degraded the herbicide linuron.

4.1.4 Isolation of degrading organisms

The ability to isolate a pesticide degrading micro-organism from soil provides evidence that the isolated organism is specifically associated with enhanced degradation. However, the vast majority of soil micro-organisms, perhaps 99 %, are not culturable by normal techniques (Wang and Wang, 1996). Thus an isolated organism can only be considered as one of the organisms responsible. Secondly, it is highly likely that the organism(s) responsible for degradation would vary from field to field.

Despite these limitations, traditional culture isolation methods provide the easiest method to determine the degradation pathway.

The isolation of effective degrading organisms is commonly achieved by addition of degrading soil to a minimal (or mineral) salts medium containing the herbicide as the sole source of carbon. There are numerous "recipes" for such minimal salts media. More specific details of bacteria and fungal isolation procedures are given by Skipper *et al.* (1986). In the present study, a mineral salts media that had been previously used for the isolation of phenyl carbamate degrading bacteria was used (Kaufman and Kearney, 1965).

4.2 Methods

4.2.1 Treatment of naturally occurring *L. rigidum* infestations

The effectiveness of carbetamide in controlling natural infestations of the grass weed *L. rigidum* was tested in two adjacent fields. Field A had not been previously treated with carbetamide. Field B had been commercially treated with carbetamide annually (2,100 g a.i. ha^{-1}) for three consecutive seasons, with the most recent application 12 months before this experiment. The experimental design was a randomised complete block with three replicates in both fields A and B. Both sites were naturally infested with *L. rigidum*, though the plant density was different at each site (as shown in

Figure 4.1). Half of the plots at both sites were initially treated (section 3.3.1) with carbetamide (2,100 g ha^{-1}), the remaining plots were left unsprayed as controls. Treatments were assessed 28 days later to determine *L. rigidum* mortality. Assessment was made by counting the number of live plants in 3 randomly placed 0.1 m^2 quadrats within each plot. *L. rigidum* plants that had produced new leaves were classed as live.

4.2.2 Treatment of transplanted *L. rigidum* infestations

To assess the efficacy of a second carbetamide treatment to the plots described above (section 4.2.1), 120 *L. rigidum* seedlings were transplanted into each plot. Seeds of *L. rigidum* were germinated in dishes containing a 0.5 cm layer of 0.6 % agar. Dishes were maintained for 7 days in an incubator at 20°C with a 12 hour light period of 48 $\mu E\ m^{-2}s^{-1}$. Seedlings (one leaf stage) were sown into 10 subplots within each plot. Each subplot of 12 plants was transplanted to give an effective plant density of 400 plants m^{-2}.

When seedlings reached the 4 - 6 leaf stage, the number of plants which had become established was recorded and all plots were sprayed with carbetamide (2,100 g ha^{-1}). Two subplots of *L. rigidum* plants in each plot were covered to act as unsprayed controls so that other environmental influences on plant mortality could be excluded. Plant mortality was assessed by the presence or absence of new growth 28 days

after spraying. Treatments were compared and standard errors of the mean were generated by analysis of deviance assuming binomial error distribution using Genstat 5.0.

4.2.3 Influence of prior carbetamide application on the kinetics of carbetamide degradation - repeated application under laboratory conditions

The influence of prior carbetamide application on the kinetics of carbetamide degradation was examined under laboratory conditions. Four replicate 5 kg soil samples were collected to a depth of 10 cm from field B. From each sample, the equivalent of 150 g of oven dry soil was weighed into 10 disposable plastic containers. The moisture content of the soil in each container was raised to 15 % (w/w). Half the containers of soil from each replicate were treated with 1 mL of an aqueous carbetamide solution (3.0 g L^{-1} technical grade, Table 3.2) to give a final soil carbetamide concentration of 20 mg kg^{-1}. Remaining containers were treated with 1 mL of water. Soil in each container was then thoroughly mixed by emptying into a plastic bag which was then shaken for 60 s. Soil was returned to the same container, to which a clip-on lid was fitted to minimise evaporation. Containers of soil were then incubated at 15°C. After 56 days, all the soil samples were treated with 20 mg kg^{-1} carbetamide and mixed as described previously. Containers were removed at weekly intervals, mixed as described above and a 25 g subsample taken to determine residual

carbetamide by HPLC (sections 3.4.2, 3.5). Bioassays were performed on the remaining soil in each container (section 3.4.3).

4.2.4 Effect of a single field application of carbetamide on carbetamide degradation

The influence of a single prior carbetamide application upon subsequent carbetamide degradation was examined in a field with no prior history of carbetamide application (field C, Table 3.1). To exclude soil drift or other sources of soil contamination, a field 1 km from the nearest field treated with carbetamide was used. Eight soil samples were collected from field C in September 1995 by compositing three subsamples taken to 7 cm in depth from each of 4 plots (10 m x 2 m) which were treated with commercially formulated carbetamide (2,100 g a.i. ha^{-1}) in July 1994 and from four untreated control plots. Eight containers of soil were weighed from each soil sample and carbetamide added (20 mg kg^{-1}) (section 3.4.1). Sampling to determine carbetamide degradation kinetics occurred from 0 to 49 days after treatment. Quantification and biological activity of residual carbetamide was determined as described in the general methods section (section 3.4).

4.2.5 Radiolabelled $^{14}CO_2$ evolution from carbetamide treated soils

To indicate whether the enhanced carbetamide degradation evident in Figure 4.3 and Figure 4.4 was due to active degradation by soil

micro-organisms, evolution of $^{14}CO_2$ and degradation of carbetamide was monitored in soils with and without enhanced carbetamide degradation. The soils used were D (control) and E[III] (enhanced carbetamide degrading) (Table 3.1). Four replicate soil samples were collected from each site. Each soil sample comprised four composited subsamples collected from a separate plot. To increase uniformity, soil samples were passed through a 3.9 mm sieve and then weighed (150 g) into plastic containers (section 3.4). One container of soil was treated with carbetamide (20 mg kg^{-1}) and ^{14}C ring labelled carbetamide (8 x 10^{4} Bq kg^{-1}), mixed as described previously, divided into 25 g subsamples and placed into 250 mL high density polyethylene (HDPE, Nalgene) bottles with screw closures. Samples were incubated at 15°C. To trap CO_2, 2 mL of 0.5 M NaOH in a liquid scintillation vial was placed in each bottle. Traps were changed weekly in order not to exceed CO_2 absorption capacity. $^{14}CO_2$ was determined after addition of scintillant by liquid scintillation as described in section 3.7. At time points from 0 to 70 days, bottles were removed and frozen until analysis. Carbetamide was extracted from soil by addition of 30 mL methanol and shaking for 1 hour. Particulate matter was removed from the supernatant by passing through an 0.2 µm nitrocellulose filter (25 mm, Schleicher and Schuell). An 800 µL sample was placed into an autosampler vial and carbetamide detection was made via HPLC with UV detection (section 3.5). Results were averaged and expressed as the percentage remaining or evolved for carbetamide and $^{14}CO_2$ respectively.

4.2.6 Transfer of enhanced degrading ability between soils

Soil samples from fields previously treated and untreated with carbetamide (fields E[III] and D respectively, Table 3.1) were collected as described in section 3.4. These soils were mixed to give samples containing 0, 0.1, 1, 5, 10 and 100 % enhanced carbetamide degrading soil. Containers of soil were then treated with 20 mg kg^{-1} carbetamide, mixed and subsampled 0, 14 and 28 days after treatment to determine residual carbetamide (section 3.4).

4.2.7 Inhibition of enhanced degradation by microbial inhibitors

Containers of soil, with and without enhanced carbetamide degradation (collected from fields D and E respectively), were treated with either water, chloramphenicol (3 mL, 2.5 mg mL^{-1}, Sigma), or cycloheximide (3 mL, 2.5 mg mL^{-1}, Sigma), or were sterilised by autoclaving at 120°C for 3 hours. Two days after initial treatment, the soil samples were treated with carbetamide (20 mg kg^{-1}) and mixed (section 3.4.1). Containers of soil were subsampled at 0, 9 and 21 days after treatment to determine carbetamide remaining. This experiment was repeated and the results averaged.

4.2.8 Isolation of bacteria capable of degrading carbetamide

A process of enrichment and isolation, the fundamentals of which were described by Skipper *et al.* (1986), was used to isolate a carbetamide degrading bacterium. Enhanced carbetamide degrading soil from field E was treated with carbetamide (20 mg kg^{-1}) and incubated at 15°C for 7 days to stimulate carbetamide degrading organisms. Samples of this soil (0.1 g) were added to 50 mL of MSM (no yeast extract added, section 3.6) with carbetamide (1.0 g L^{-1}) and incubated at 28°C with shaking. After 5 days the media became turbid and 100 µL was transferred to a sterile flask containing carbetamide (200 mg L^{-1}) MSM media. After 4 further transfers, loopfuls of media were streaked onto solid MSM containing carbetamide (500 mg L^{-1}) and incubated at 28°C. After 5 days, actively growing colonies were transferred to solid carbetamide (500 mg L^{-1}) MSM media. As these colonies were very similar in appearance and growth rate, an individual isolate was selected for further characterisation.

4.2.9 Confirmation of carbetamide degradation by isolated bacterium

An individual colony of the isolated bacterium was transferred to MSM containing carbetamide (200 mg L^{-1}) and yeast extract (50 mg L^{-1}). After this culture reached stationary phase, 100 µL was used to inoculate flasks containing the same media (with and without carbetamide).

Measurements were made from 0 to 44 hours after inoculation. Carbetamide degradation was monitored by HPLC (section 3.6.2) and spectrophotometric analysis, and compared to uninoculated controls. For spectrophotometric analysis, absorbance of samples (as diluted for HPLC analysis, section 3.6.2) were measured in quartz cuvettes at 233 nm (DMS 100s, Varian). Growth of organisms was measured by dilution drop plates and absorption at 600 nm (section 3.6).

4.2.10 Gram stain, cell shape and biochemical tests

Gram staining was performed to determine whether the organism was Gram positive or negative and to observe cell shape via light microscopy (Norris and Swain, 1971). Colonies were taken from nutrient agar plates and mixed with a drop of water on a microscope slide. After smearing over the surface of the slide, the suspension was allowed to dry. After heat fixing, slides were treated with crystal violet followed by iodine, destained with 100 % ethanol and counterstained with safranin. Slides were viewed under X 100 and X 1000 magnification, photographs were taken under X 180 magnification. Cultures of *Streptococcus faecalis* and *Escherichia coli* were used as positive and negative controls respectively. Catalase activity was determined by adding a few drops of 3 % H_2O_2 to colonies grown on nutrient agar. Oxidase production was examined by smearing the isolated bacterium onto filter paper with Spot Test Oxidase reagent (Difco).

4.2.11 Biolog substrate utilisation

Substrate utilisation of 95 different carbon sources was performed using Biolog SFP microplates (attempts to use GP plates were unsuccessful as the tetrazolium dye was toxic to the bacterium). The bacterium was inoculated into the 96 wells of the microplate according to the manufacturer's directions. After incubation at 28°C for 72 hours the plates were scored visually. Results were analysed by Microlog PC based software (Release 3.50).

4.2.12 Fatty acid analysis - Midi Fame

Analysis of bacterial fatty acid composition can provide rapid identification of the organism (Trüper and Schleifer, 1992). Following extraction from micro-organisms, fatty acids were converted to their respective methyl esters and then separated by gas chromatography. Except for an extended incubation time on trypticase soy broth agar to increase cell material (48 h at 28°C), all other methods were performed as described in the MIDI FAME MIS Manual. Reagents were supplied and gas chromatography was performed by Dr. Bruce Hawke (CSIRO Division of Soils, Adelaide). Chromatograms were qualitatively and quantitatively analysed by Sherlock software (Version 1.06) interrogating the Trypticase Soy Broth Agar (TSBA) database (Revision 3.90).

4.2.13 16s rRNA sequence analysis

Ribosomal RNA (rRNA) molecules are ubiquitous and contain highly conserved and variable regions within their sequence (Gürtler and Stanisich, 1997). These characteristics make the rRNAs an ideal "universal macromolecular chronometer" (Solignac *et al.*, 1991) allowing for more accurate determination of phylogenetic relationships. To more accurately identify the carbetamide degrading bacterium, the 16S rRNA primary structure (nucleotide sequence, 16s rRNA) was determined by polymerase chain reaction (PCR) amplification and dideoxy sequencing. The theory and practice of these techniques is well described elsewhere (Arnheim and Erlich, 1992).

Chromosomal DNA was isolated from the bacterium using the initial steps for isolation of plasmid DNA from this strain. Steps prior to treatment with 3M NaOH were performed as described in section 8.2.9. Extracts were then purified using a Wizard Genomic DNA purfication kit (Promega cat # A1120) according to the manufacturer's instructions to remove RNA and protein. After washing twice with 70 % ethanol, DNA was resuspended in 100 µL of TE buffer.

PCR was initially performed using the fD1 (5' AGA GTT TGA TCC TGG CTC AG) and rD1 (5' AAG GAG GTG ATC CAG CC) primers designed to amplify DNA coding for the 16s rRNA from most eubacteria (Weisburg *et al.*, 1991). PCR reactions (50 µL) were performed in 200 µL

thin walled PCR tubes (Perkin Elmer). Optimised concentrations or volumes in each reaction were 1 µM of each primer (fD1, rD1), 3 mM $MgCl_2$ and 1 µL of template DNA solution. All other conditions were as specified in the Perkin Elmer Amplitaq Gold instructions (cat. # N808-0241). Thermal cycling was performed using an Idaho Technology thermo-cycler. After an initial denaturing step (95°C, 9 min), 35 cycles of annealing (50°C, 30 s), extension (72°C, 90 s) and denaturing (94°C, 30 s) were carried out, followed by a final extension step (72°C, 7 min). Amplified DNA (5 µL) was separated on a 1 % agarose gel (TBE, containing ethidium bromide) and compared to an appropriate DNA size marker (Lambda *Eco*R I *Hin*d III digest, Promega, cat. # G1731).

The PCR product described above was purified using affinity based chromatography according to the manufacturer's instructions (High Pure PCR Product Purification Kit, Boehringer Mannheim, cat. #17732668). Purified PCR product was diluted with water 5,000 or 50,000 fold and then re-amplified using the external primers of Weisburg *et al.* (1991) described above and the internal primers 704f (5' GTA GCG GTG AAA TGC GTA GA) and 765r (5' CTG TTT GCT CCC CAC GCT TTC) published by Damiani *et al.* (1997). Amplification and purification of PCR products was performed as described above. Products were then ligated with a PCR product cloning vector and transformed into *E. coli* (strain S17-1, supplied by Dr. N. McClure, Flinders University, Australia) using heat shock at 42°C according to the manufacturer's instructions (pGEM-T Vector System I,

Promega, cat. #A3600). White colonies containing recombinant plasmids were inoculated into 5 mL of nutrient broth containing 50 µg mL^{-1} ampicillin and incubated shaking overnight at 37°C.

Plasmid DNA was extracted from the *E. coli* transformants using a rapid plasmid isolation kit (Wizard Plus Minipreps, Promega, cat. #A7100). Subsamples (10 µL) of plasmid extracts were separated by agarose gel electrophoresis (1 % agarose, TBE buffer) to confirm the DNA insert was the appropriate size (results not presented). DNA concentration and purity was determined by measuring absorption at 260 and 280 nm using microcapillary tubes (Beckman 5 spectrophotometer). Samples were then diluted to 250 ng μL^{-1}. Sequencing reactions were performed using M13 or T7/SP6 primers and dye labelled dideoxy nucleotides. Forward and reverse sequences were determined for each insert. Sequencing reactions and electrophoresis were performed by the Nucleic Acid and Protein Chemistry Unit, University of Adelaide, using an Applied Biosystems 373A DNA Sequencing System.

For initial identification of the sequence, a 200 bp fragment was submitted for a "Blast" search (Altschul *et al.*, 1990). Results of this search indicated high similarity to the genus *Rhodococcus* sp. (results not shown). Partial sequences were aligned to a *Rhodococcus* strain (accession number X76704) using the program Seqed (vers. 1.0.3, Applied Biosystems). Based upon the alignment method, partial overlap of the sequences was indicated

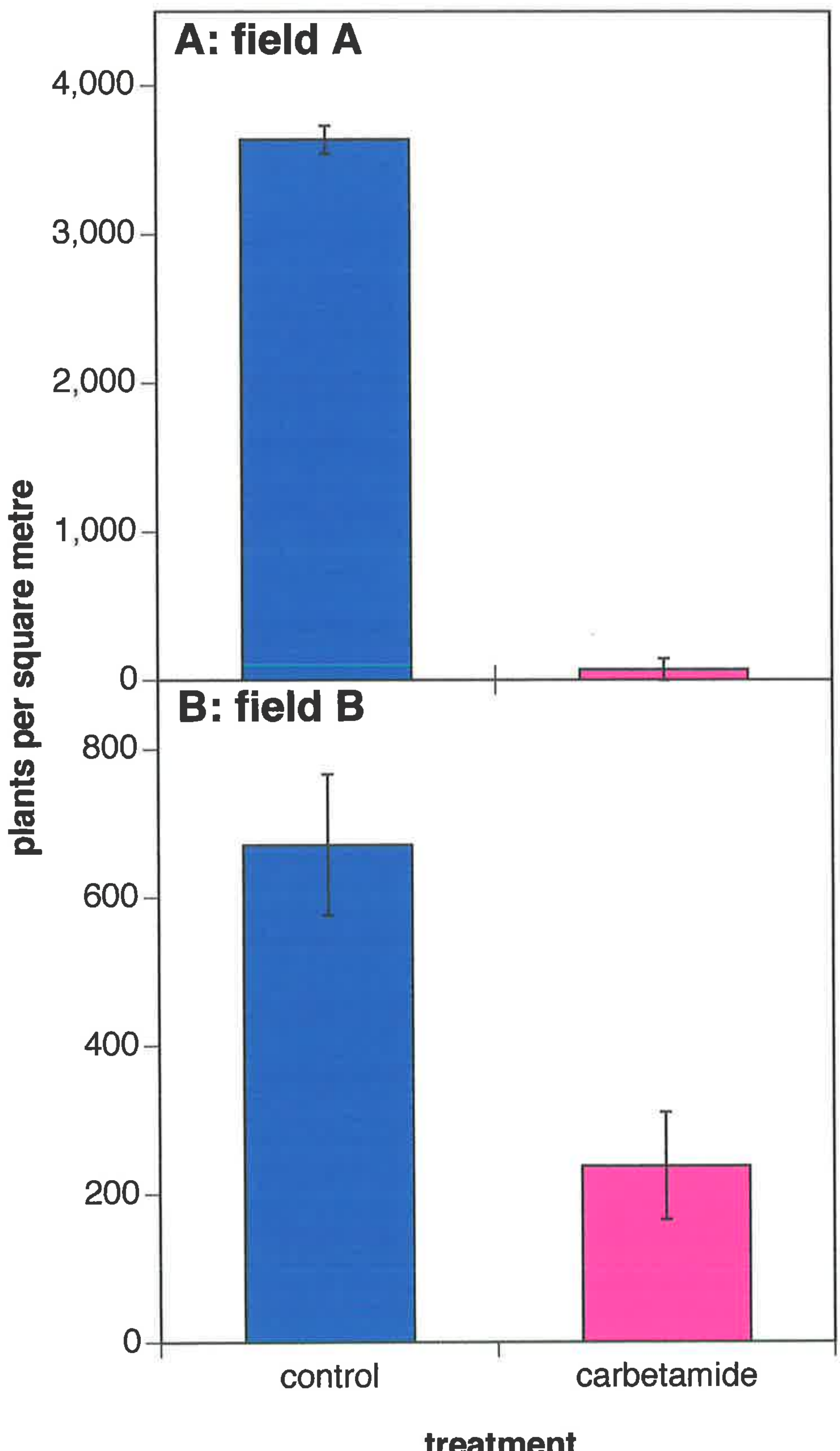
and a consensus sequence was calculated by Seqed. The consensus sequence (Appendix 1) was compared to type strain sequences of the mycolic acid containing actinomycetes, which encompasses the genera *Corynebacterium*, *Gordona*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, and *Tsukamurella* (Chun and Goodfellow, 1995). These sequences, as used by Chun and Goodfellow (1995), were accessed from the EMBL databases. Also comparison was made to two *E. coli* type strains for appropriate numbering. Alignment of these sequences with the sequence obtained in this study was performed by the computer program Pileup (Wisconsin Package, 1994). Similarity values were calculated manually and are shown in Table 4.1. Insertions, deletions and unknown nucleotides were excluded from the calculation. Comparison of the bacterium isolated in this study was evaluated with reference to published comparisons of the mycolic acid containing actinomycetes (Chun and Goodfellow, 1995, Pascual *et al.*, 1995, Rainey *et al.*, 1995).

4.3 Results

4.3.1 Treatment of naturally occurring *L. rigidum* infestations

Though the naturally occurring *L. rigidum* plants present in both fields A and B were severely affected by carbetamide application, there was a significant difference in carbetamide efficacy between these sites (Figure 4.1A and B). In the field not previously treated with carbetamide (A),

Figure 4.1 *L. rigidum* density (plants m^{-2}) in plots treated or untreated with carbetamide (2,100 g a.i. ha^{-1}) in a field previously untreated with any herbicide (A) and a field previously treated with carbetamide (B). Standard errors of the means are indicated by vertical bars.


A: field A
4,000
3,000
2,000
1,000
0
B: field B
800
600
400
200
0
plants per square metre
control
carbetamide
treatment

this herbicide controlled 98 % of the *L. rigidum* plants. In contrast, in field B only 64 % mortality occurred. As only field B had been previously treated with carbetamide, this result provided initial evidence that prior carbetamide application influences the efficacy of subsequent applications.

As the histories of these fields were not identical, it cannot be concluded definitively that prior carbetamide application was the sole cause of these differences. Therefore the remainder of the experiment was conducted to use the treated and untreated plots from this experiment to confirm further decreased carbetamide efficacy upon repeated application.

4.3.2 Treatment of transplanted *L. rigidum* infestations

The efficacy of carbetamide in controlling transplanted *L. rigidum* was significantly influenced in plots in both fields A and B by application of carbetamide earlier in the season. Figure 4.2A shows that carbetamide application resulted in 88 % mortality of *L. rigidum* seedlings when applied to plots within a field that had never been treated with carbetamide. However, at this same site, on plots pre-treated once only with carbetamide, the mortality of *L. rigidum* seedlings treated with carbetamide was only 51 %. Similar results were obtained in field B that had been treated in previous seasons with carbetamide. Figure 4.2B shows that application of carbetamide resulted in 89 % mortality of *L. rigidum* seedlings on control plots that received no carbetamide pre-treatment in the

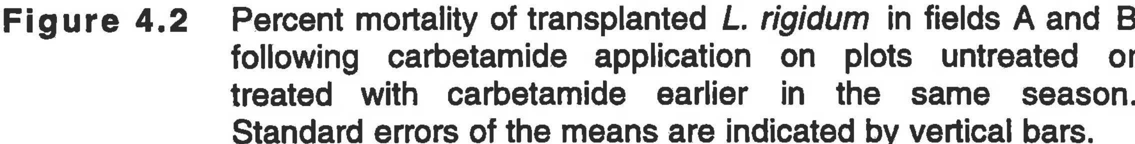
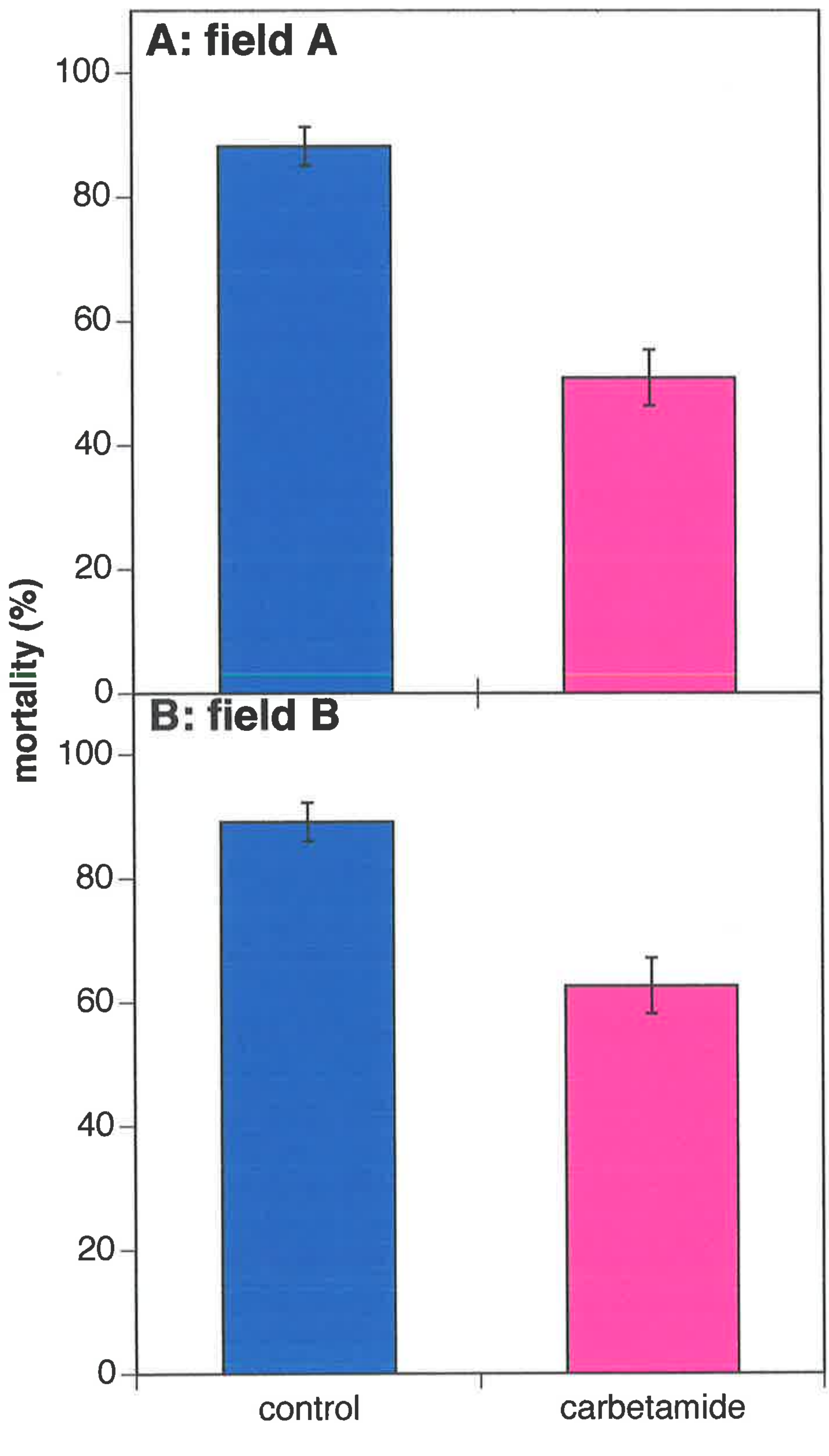


Figure 4.2 Percent mortality of transplanted *L. rigidum* in fields A and B following carbetamide application on plots untreated or treated with carbetamide earlier in the same season. Standard errors of the means are indicated by vertical bars.

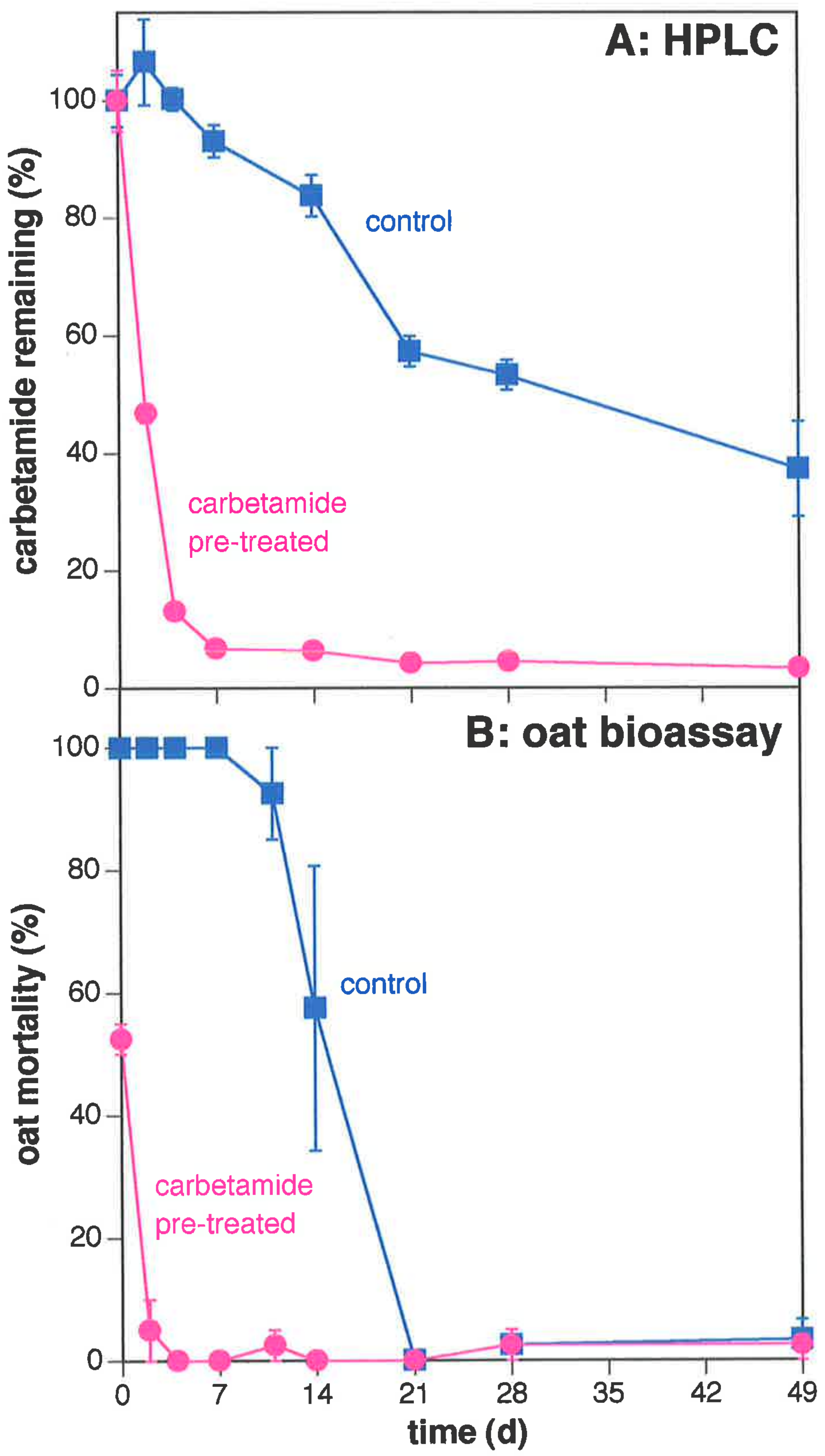

A: field A
B: field B
mortality (%)
100
80
60
40
20
0
control
carbetamide
soil pre-treatment

same season. Mortality of *L. rigidum* seedlings treated with carbetamide was only 63 % on plots that had been pre-treated with carbetamide earlier in the same season. Therefore, although carbetamide causes high mortality when first applied to a soil (Figure 4.1), there is a loss of herbicidal efficacy upon repeated application, even after only a single previous treatment (Figure 4.2A). At both sites, mortality of *L. rigidum* plants that were covered at the second time of carbetamide application, and thus not treated, was less than 4 %.

4.3.3 Influence of prior carbetamide application on the kinetics of carbetamide degradation - repeated application under laboratory conditions

The kinetics of carbetamide degradation in soil samples collected from field B and incubated under controlled conditions clearly demonstrated that carbetamide was degraded at a faster rate upon repeated application (Figure 4.3). After 7 days, only 8 % of the applied carbetamide remained in the soil pre-treated with carbetamide, whilst there was 78 % remaining in the control soil. The effect of rapid carbetamide degradation on weed control is reflected in the oat bioassay results (Figure 4.3B). In the control soil, oat mortality was 100 % when oat seeds were sown into soil for 0 - 7 days after carbetamide addition. However, in the carbetamide pre-treated soil, oat mortality was only 52.5 % when sown immediately after the addition of carbetamide and thereafter oat germination was not inhibited.

Figure 4.3 Kinetics of carbetamide degradation in soil as measured by (A) HPLC, (B) bioassay and influenced by pre-treatment with carbetamide or water (control) 8 weeks earlier in the laboratory. Standard errors of the means are indicated by vertical bars.


A: HPLC
carbetamide remaining (%)
100
80
60
40
20
0
control
carbetamide
pre-treated
B: oat bioassay
oat mortality (%)
100
80
60
40
20
0
control
carbetamide
pre-treated
0
7
14
21
28
35
42
49
time (d)

Therefore, by both direct chemical assay and a bioassay, there is a clear reduction in the persistence of carbetamide upon repeated application.

Over the period 0 - 14 days after carbetamide treatment, there is a good correlation between the chemical assay and the bioassay methods (Figure 4.3). However, from 14 days onwards whilst the chemical assay indicated relatively high amounts of carbetamide remaining, sufficient to theoretically inhibit germination (Figure 3.1), bioassay results indicated little effect of this remaining carbetamide. One possible explanation for this result is that physical mixing of the soil stimulated carbetamide degradation. Disparity between the chemical and biological assay was not observed in any other experiments.

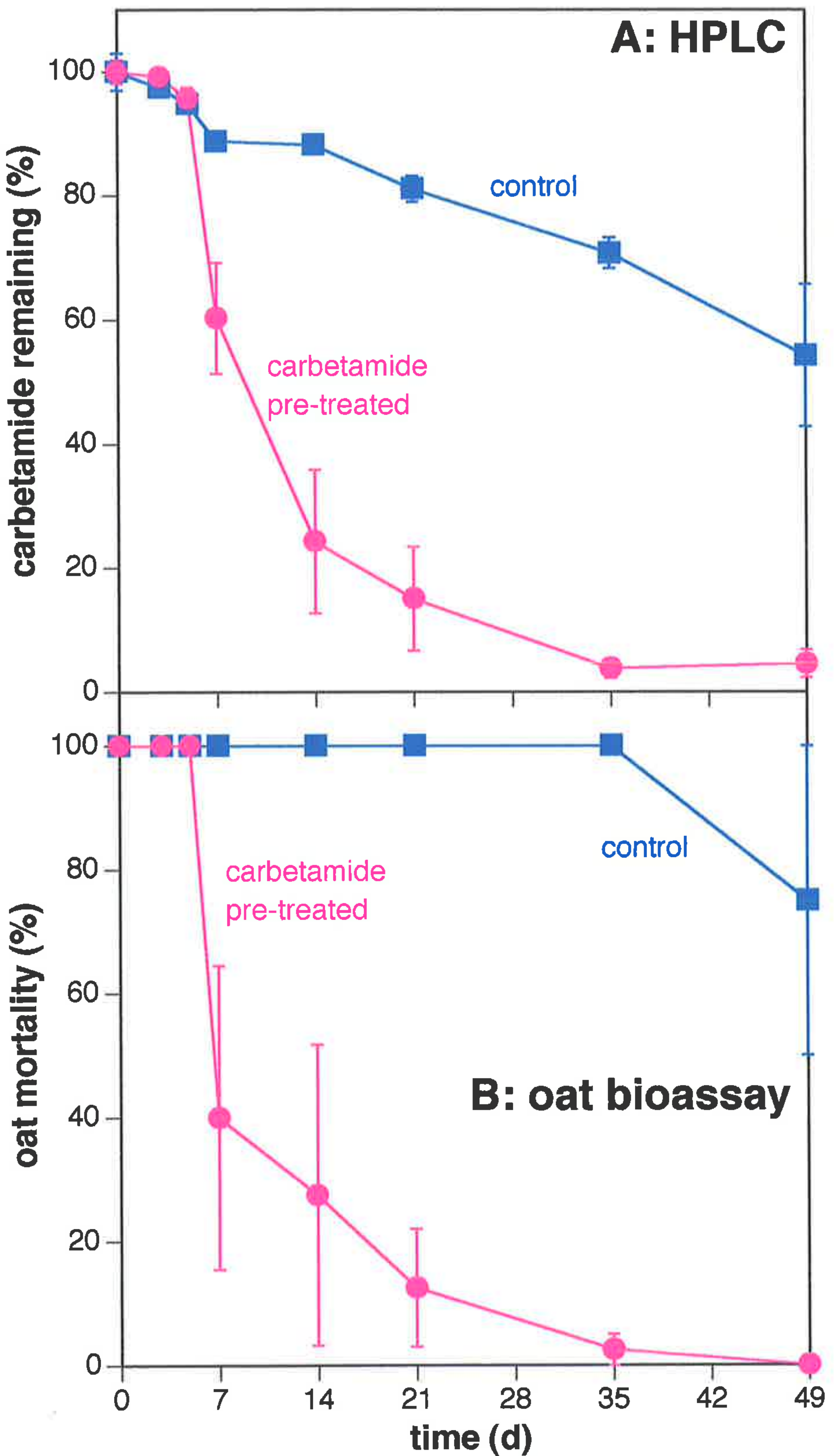
Results presented in Figure 4.3, suggest that poor carbetamide performance upon repeated application to fields A and B (Figure 4.1, Figure 4.2) is probably due to rapid carbetamide degradation. A potentially significant limitation of this study is that carbetamide treatments were made only 8 weeks apart, whereas under commercial conditions carbetamide is applied once annually. Enhanced degradation of linuron was reported to be undetectable after 57 weeks (Walker and Welch, 1992). Therefore the carbetamide application frequency is potentially an important determinant. This was addressed in the following experiment.

4.3.4 Effect of a single field application of carbetamide on carbetamide degradation

Figure 4.4 clearly demonstrates enhanced degradation of carbetamide in soil collected from plots within field C that had been treated only once with carbetamide 14 months earlier. Carbetamide had a DT_{50} of 54 days (extrapolation) in the untreated control soil which resulted in 100 % oat mortality when sown up to 35 days from carbetamide addition. However, in soil treated once in the field with carbetamide 14 months earlier, degradation of this herbicide was comparatively rapid, after a lag phase of 5d, with a DT_{50} of 9 days. In this soil, the mortality of oats was only 39 % when sown 7 days after herbicide addition.

This result (Figure 4.4) confirms that enhanced carbetamide degradation is the most likely cause of failure with this herbicide when used repeatedly on an annual basis. Enhanced rates of degradation were clearly evident in this experiment after a single application (Figure 4.4A). Bioassay results indicate that rapid degradation of carbetamide results in a shortened period of herbicidal activity (Figure 4.4B). Insufficient information exists to determine the effect of altered kinetics upon weed control, though such a large decrease in persistence is likely to significantly reduce weed control.

Figure 4.4 Kinetics of carbetamide degradation in soil as measured by (A) HPLC, (B) bioassay and influenced by prior treatment with carbetamide 14 months earlier in the field or previously untreated. Standard errors of the means are indicated by vertical bars.


A: HPLC
carbetamide remaining (%)
100
80
60
40
20
0
control
carbetamide
pre-treated
B: oat bioassay
oat mortality (%)
100
80
60
40
20
0
control
carbetamide
pre-treated
0
7
14
21
28
35
42
49
time (d)

4.3.5 Radiolabelled $^{14}CO_2$ evolution from carbetamide treated soils

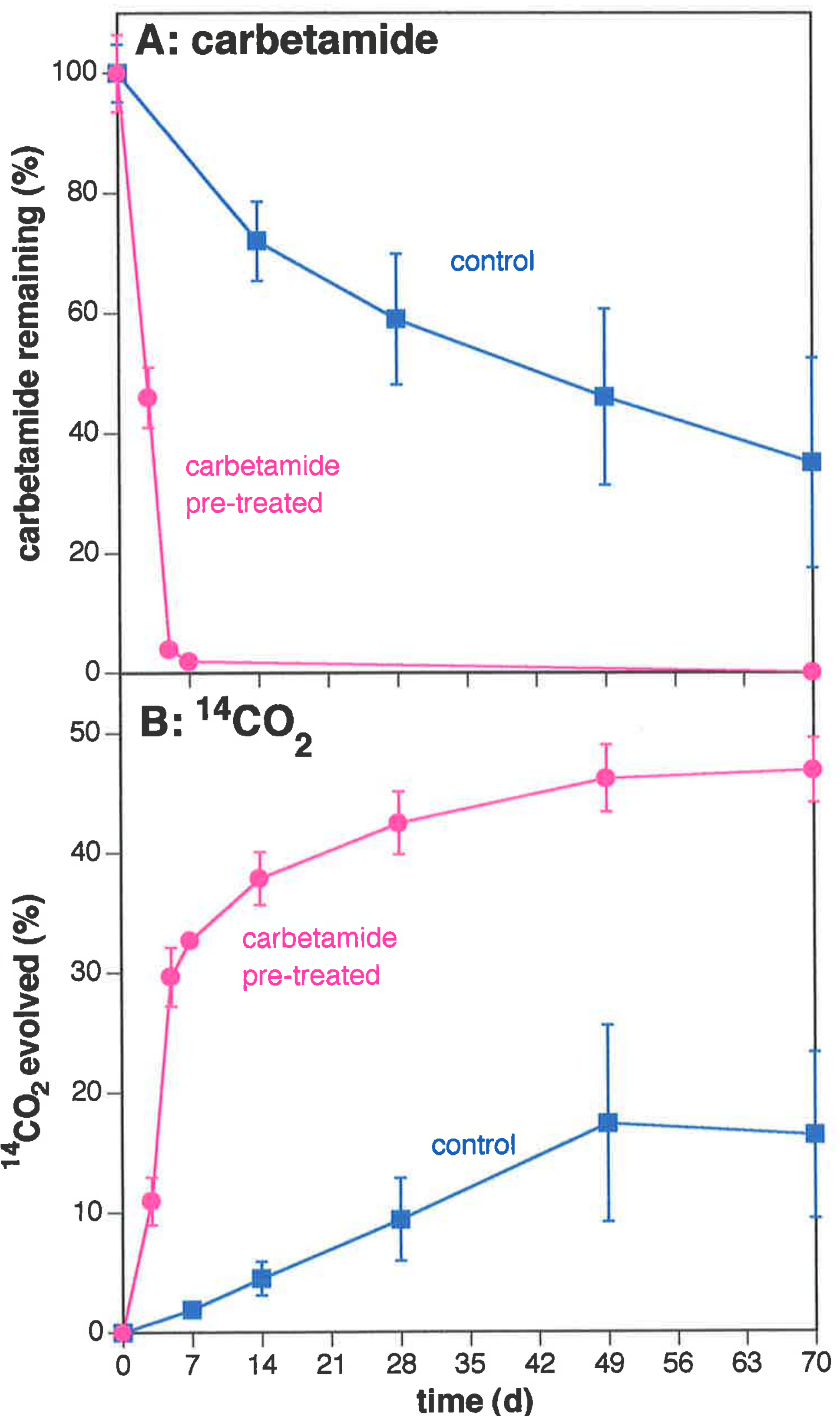
In soil that had been previously treated with carbetamide, enhanced carbetamide degradation was associated with radiolabelled carbetamide being converted to $^{14}CO_2$ at a faster rate than in the control soil (Figure 4.5). The DT_{50} of carbetamide (Figure 4.5A) in soil that had not been previously treated with carbetamide (field D) was 42.5 days, whereas in soil that had been treated annually with carbetamide for 7 consecutive seasons the DT_{50} was only 2.8 days.

After 49 days incubation in the soil that degraded carbetamide at an enhanced rate, 46.9 % of the applied carbetamide (as radioactivity) was evolved as $^{14}CO_2$. After the same period in the control soil only 16.4 % was evolved. These results are consistent with enhanced carbetamide degradation being due to elevated levels of soil micro-organisms which can use carbetamide as a source of carbon or energy. Also these results demonstrate that even in soil not previously treated with carbetamide this herbicide is at least partially biodegraded.

4.3.6 Transfer of enhanced degrading ability between soils

Enhanced carbetamide degradation was transferred to a control soil by addition of low percentages of enhanced carbetamide

Figure 4.5 Degradation of carbetamide in soil (A) and corresponding $^{14}CO_2$ evolution (B) from ^{14}C ring labelled carbetamide added to soil in carbetamide pre-treated and previously untreated soil. Carbetamide degradation was monitored by UV absorption. Standard errors of the means are indicated by vertical bars.


A: carbetamide
carbetamide remaining (%)
100
80
60
40
20
0
control
carbetamide
pre-treated
B: $^{14}CO_2$
$^{14}CO_2$ evolved (%)
50
40
30
20
10
0
carbetamide
pre-treated
control
0
7
14
21
28
35
42
49
56
63
70
time (d)

degrading soil. Figure 4.6 displays the mean percent carbetamide remaining 14 and 28 days after addition as influenced by the percentage of enhanced carbetamide degrading soil added to a soil previously untreated with carbetamide. In general, there is less carbetamide remaining with increasing percentage of enhanced degrading soil. The results are more pronounced after 28 days. Addition of as little as 1 % of enhanced carbetamide degrading soil significantly increased the rate of carbetamide degradation. Addition of 0.1 % enhanced carbetamide degrading soil did not increase the rate of carbetamide degradation.

4.3.7 Inhibition of enhanced degradation by microbial inhibitors

Carbetamide degradation kinetics in soil with and without enhanced carbetamide degradation was affected by treatment with anti-microbial agents and heat sterilisation (Figure 4.7). In soil which degraded carbetamide at an enhanced rate, there was only 9 % of the applied carbetamide remaining after 9 days. In this same soil, when pre-treated with chloramphenicol or heat sterilisation, there was 80 to 95 % of the applied carbetamide remaining. These levels were similar to that of the control soil after 9 days. Enhanced carbetamide degradation was not affected by pre-treatment with cycloheximide. Treatment effects, similar to those observed in the enhanced carbetamide degrading soil, were evident with the control soil 21 days after treatment (Figure 4.7B).

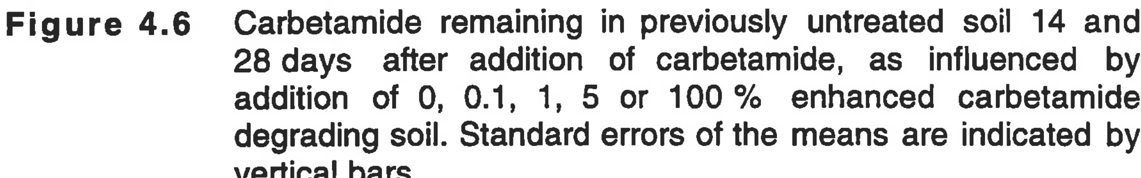


Figure 4.6 Carbetamide remaining in previously untreated soil 14 and 28 days after addition of carbetamide, as influenced by addition of 0, 0.1, 1, 5 or 100 % enhanced carbetamide degrading soil. Standard errors of the means are indicated by vertical bars.

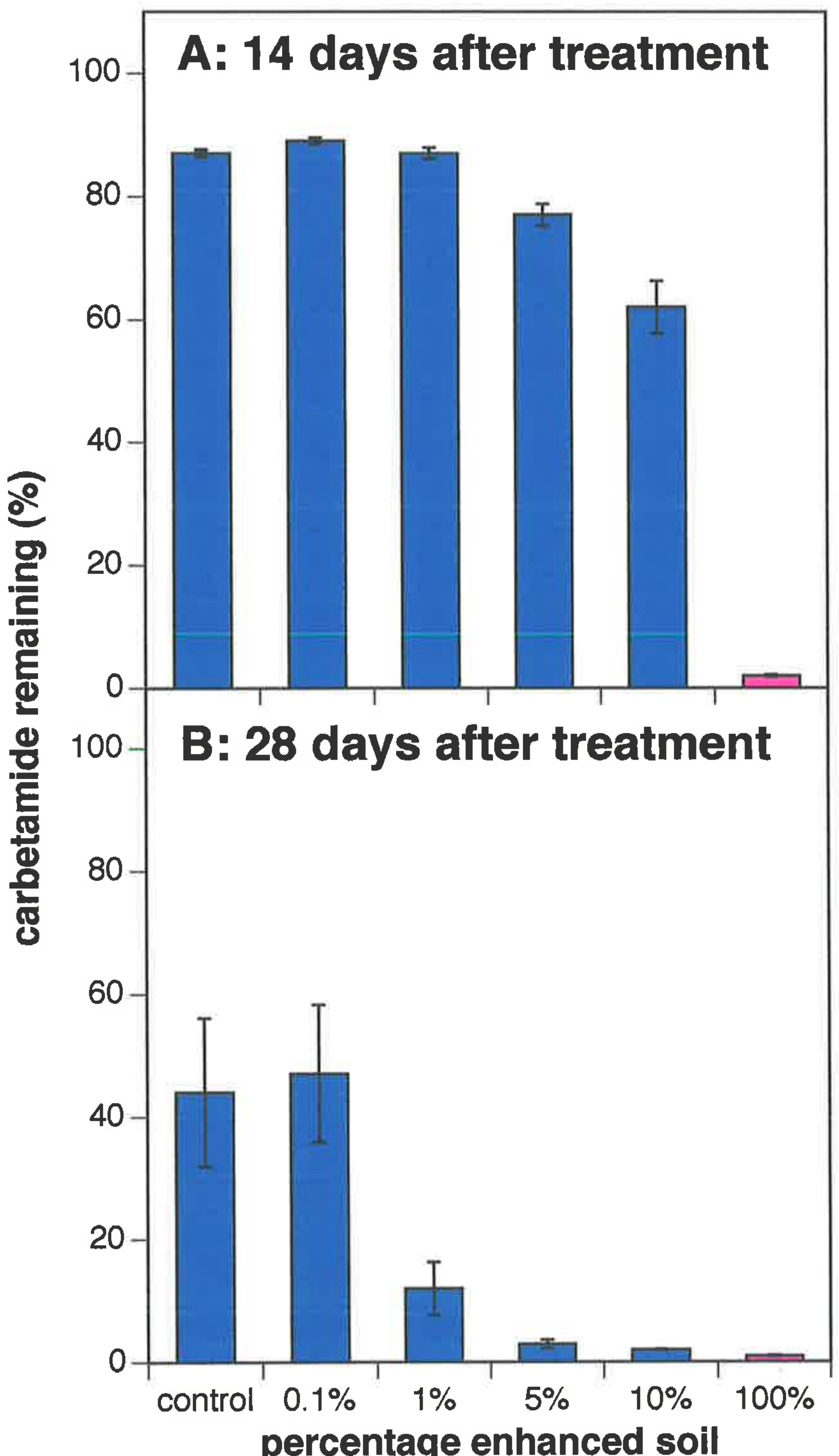
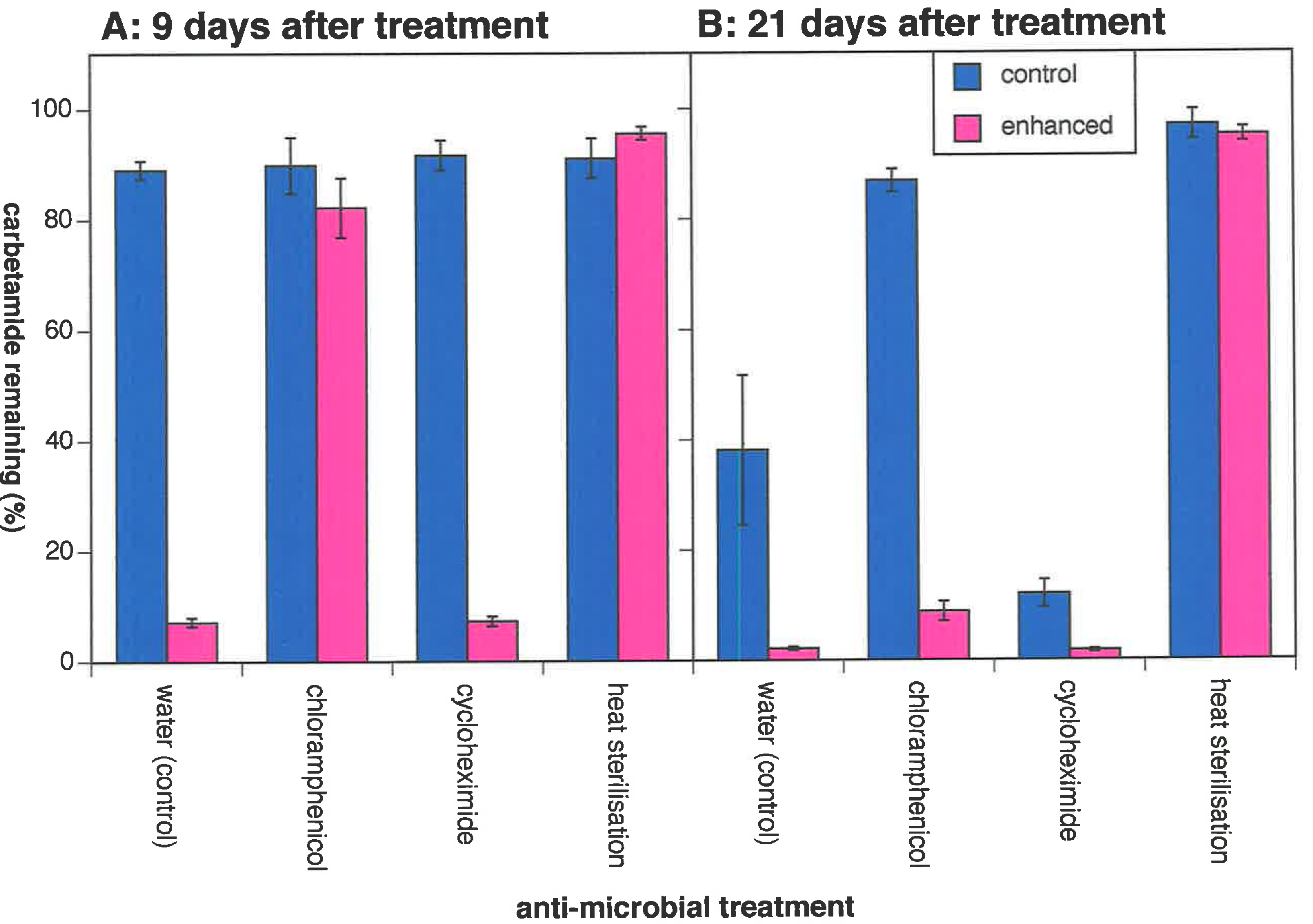

A: 14 days after treatment
B: 28 days after treatment
carbetamide remaining (%)
100
80
60
40
20
0
control
0.1%
1%
5%
10%
100%
percentage enhanced soil

Figure 4.7 Influence of the anti-microbial agents, chloramphenicol and cycloheximide, and heat sterilisation on degradation of carbetamide in enhanced carbetamide degrading and control soils. Samples were taken at 9 (A) and 21 (B) days after addition of carbetamide. Standard errors of the means are indicated by vertical bars.

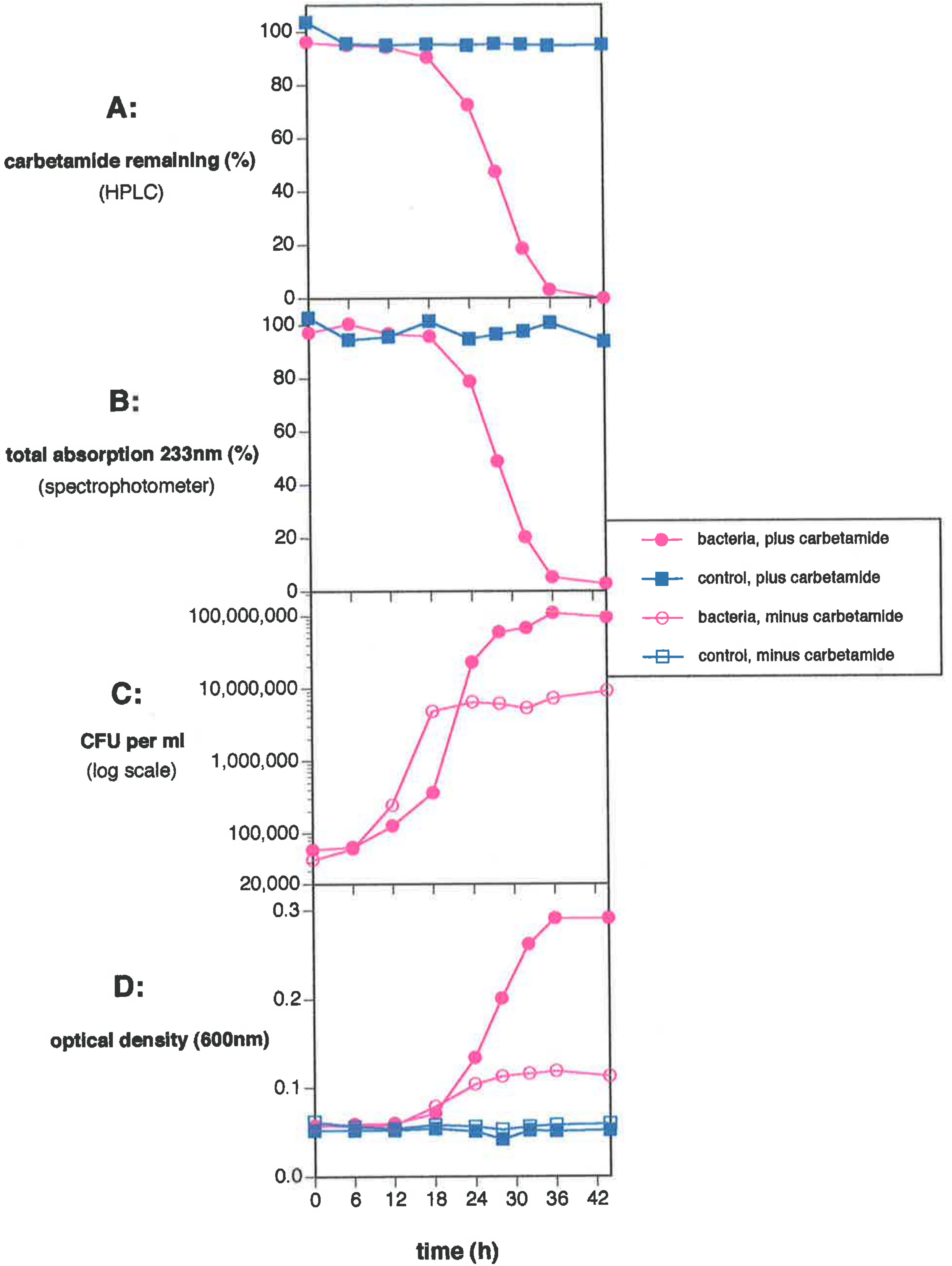

A: 9 days after treatment
B: 21 days after treatment
control
enhanced
carbetamide remaining (%)
100
80
60
40
20
0
water (control)
chloramphenicol
cycloheximide
heat sterilisation
water (control)
chloramphenicol
cycloheximide
heat sterilisation
anti-microbial treatment

In the unamended control soil, approximately 50 % of the applied carbetamide remained after 21 days. In this same soil, pre-treated with chloramphenicol, or heat sterilised, carbetamide degradation was inhibited, with 90 - 100 % of the applied carbetamide remaining. Treatment with cycloheximide resulted in 10 % of the applied carbetamide remaining in the control soil after 21 days, a lower amount than when treated with water. One possible explanation for this is that the low pH of this soil favours fungal growth (Klingman and Ashton, 1982). Therefore treatment with cycloheximide may have suppressed or killed fungi leading to enhanced bacterial activity and hence more rapid degradation. Whether cycloheximide increased the degradation of carbetamide in the enhanced soil was not determined as, apart from initial sampling, samples were collected after significant degradation had occurred in the enhanced soil.

4.3.8 Confirmation of carbetamide degradation by isolated bacterium

Degradation of carbetamide by the putative carbetamide degrading isolate was confirmed by examination of growth in liquid media. Growth of the isolated bacterium (Figure 4.8C, D) was associated with loss of carbetamide from the media (Figure 4.8A, B). The bacterial population in the flask increased from 6×10^4 CFU mL^{-1} to peak at 1.1×10^8 CFU mL^{-1} 36 hours after inoculation (Figure 4.8C). This was linked to an increase in the optical density (OD_{600}) of the culture (Figure 4.8D). However, growth ceased after 36 hours when all the carbetamide had been degraded

Figure 4.8 Degradation of carbetamide, as measured by HPLC (A) and spectrophotometer (B), during growth of the isolated bacterium as measured by colony forming units (CFU) per mL (C) and absorption of the cultures at 600 nm (D). Results are presented from single flasks.


A:
carbetamide remaining (%)
(HPLC)
100
80
60
40
20
0
B:
total absorption 233nm (%)
(spectrophotometer)
100
80
60
40
20
0
C:
CFU per ml
(log scale)
100,000,000
10,000,000
1,000,000
100,000
20,000
D:
optical density (600nm)
0.3
0.2
0.1
0.0
0
6
12
18
24
30
36
42
time (h)
bacteria, plus carbetamide
control, plus carbetamide
bacteria, minus carbetamide
control, minus carbetamide

(Figure 4.8A, C). The bacterial population grown in the absence of carbetamide was approximately one tenth of the population in the flasks to which carbetamide had been added (Figure 4.8C). These results, demonstrating a close association between growth of the isolate (Figure 4.8C, D) and carbetamide degradation (Figure 4.8A, B), confirm that the isolated bacterium was capable of using carbetamide as a primary source of carbon and energy.

Evidence for the possible degradative pathway used for deriving energy from carbetamide was not apparent during culture of the isolated carbetamide degrading organism (Figure 4.8). No metabolites were identified by HPLC (233 nm) in the culture medium at any stage during incubation. This result was confirmed by the correlation between the percentage of carbetamide remaining as determined by HPLC (Figure 4.8) and the relative absorption of the total culture at 233 nm as measured by spectrophotometer (Figure 4.8B). Likely metabolites included aniline (section 2.2.4.1) which absorbs light at 233 nm. These results suggest that once carbetamide metabolites are formed they are either rapidly degraded, or are removed from the culture medium.

4.3.9 Identification of the isolated carbetamide degrading organism

Preliminary examination of the isolated carbetamide degrading organism indicated that the isolate was Gram positive with rod shaped cells.

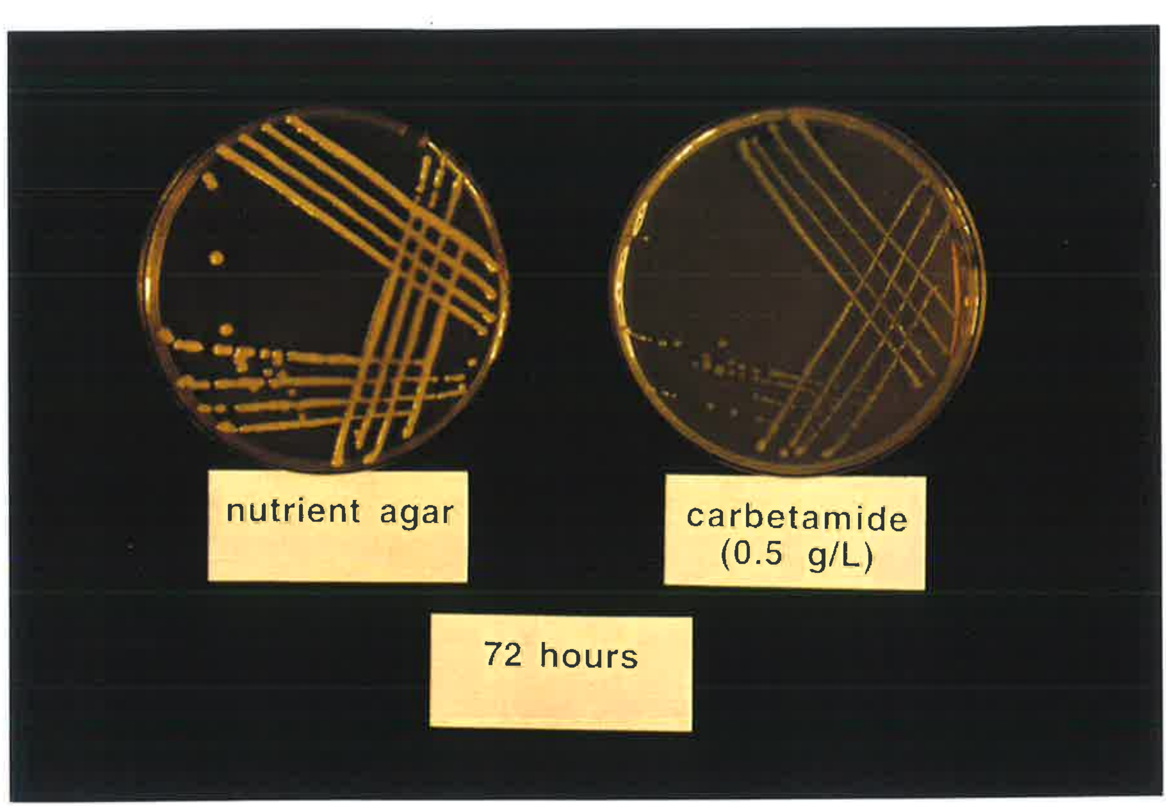
Cells of the isolated bacterium stained purple (Figure 4.9), indicative that this organism is Gram positive. For further confirmation of Gram stain, a small amount of cells was mixed with 10 µL of 3 % KOH on a microscope slide (Gregersen, 1978). This treatment did not alter cell viscosity (in contrast to cells of *E. coli*) confirming that the isolated bacterium was Gram positive. Gram stain slides were examined to determine cell shape and size. Cells were rod shaped, 0.5 to 0.75 µm in width by 1.0 to 1.5 µm in length. No grouping or clumping of cells was observed.

The range of substrates utilised by the isolated bacterium indicated, as determined using Biolog SFP plates, most closely matched that of *Rhodococcus fascians* with a probability of 0.507. This result is consistent with the classical tests (results described above) but was not very accurate due to the low probability obtained. Using a commercially available system for analysis (MIDI FAME Microbial Identification System), the fatty acid profile of the carbetamide degrading bacterium was determined. The closest match obtained was 0.561 with the organism *Rhodococcus erythropolis*. The genus from this method was the same as that obtained by examining substrate utilisation (described above). However, as the match was poor, further identification methods were pursued.

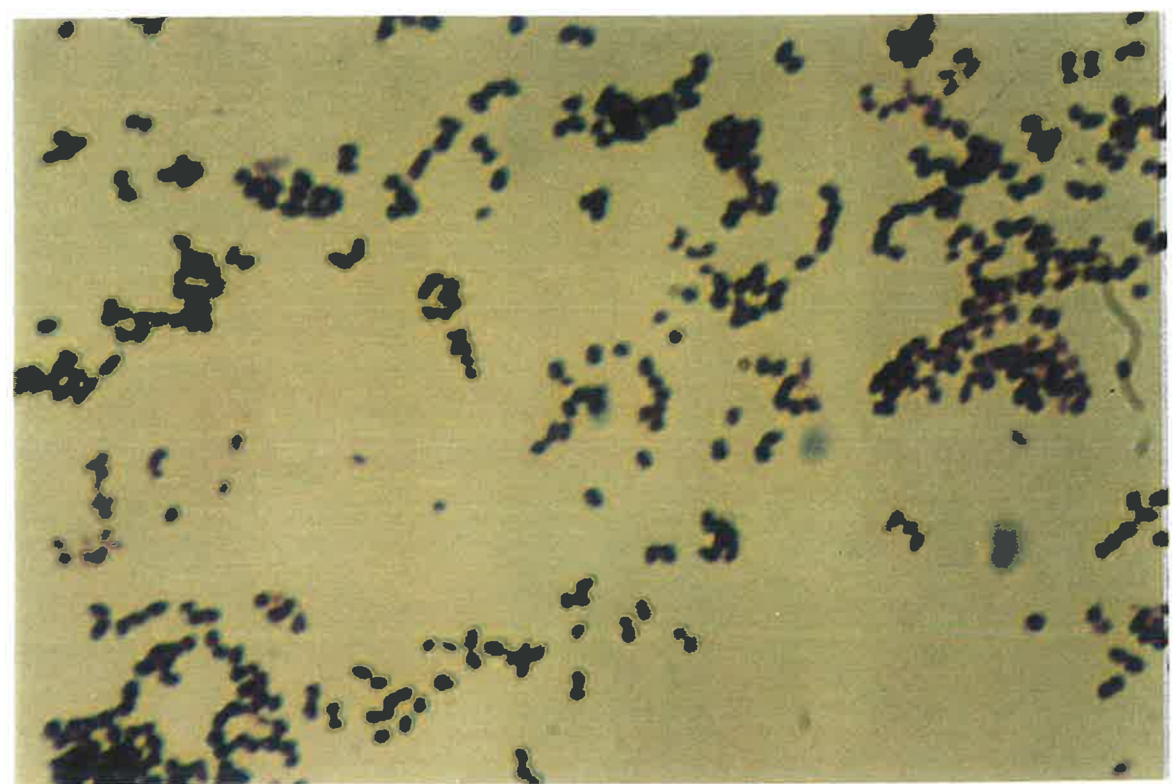
Using PCR primers described previously (Damiani *et al.*, 1997, Weisburg *et al.*, 1991), sufficient 16s rRNA sequence was obtained to identify the carbetamide degrading isolate more accurately. The sequence

Figure 4.9 Photographs of the isolated bacterium after 72 hours incubation on nutrient agar and carbetamide (500 mg L^{-1}) minimal salts medium (A), and as viewed after gram staining (B). Bar equals 5 μm.

A:



B: = 5µm



obtained comprised 1408 bases (appendix 1, excluding primers), spanning nearly the entire 16s gene. Based upon the similarity calculations (Table 4.1), the sequences used for comparison could be placed into four groups. Highest similarity was observed with the 16s rRNA sequence of *R. erythropolysis* (group I) (Figure 4.10). A second group (II) comprising the mycolic acid containing actinomycetes was evident with lower similarity. *A. globiformis* (group III), a non-mycolic acid containing bacterium (Chun and Goodfellow, 1995), had lower similarity than all of the mycolic acid containing strains (designated group III). Low similarity (77 %) was observed with *E. coli* (group IV).

16s rRNA sequence analysis of the bacterium isolated in this study supported and extended the results of substrate utilisation (Biolog) and cell wall fatty acid analysis (MIDI Fame). Where examined previously, representative strains of mycolic acid forming bacteria exhibit greater than 90 % similarity (Chun and Goodfellow, 1995). The strain examined in the present study exhibited between 91.3 and 96.3 % sequence similarity to mycolic acid forming bacteria, confirming the placement of the isolated bacterium within this complex. High similarity 96.3 % was observed to *R. erythropolis.* Visual examination of sequence alignments indicated that the majority of the observed mis-matches between the type sequence of *R erythropolis* occurred where sequence reliability was poor (refer Appendix 1, regions 300 - 400 and 1140 - 1200 base pairs). Indicating that, in this case, the true similarity value may have been underestimated.

Figure 4.10 Comparison of the similarity between the 16s rRNA sequence of the isolated carbetamide degrading bacterium and other mycolic acid forming bacteria, and the non-mycolic acid forming bacterium *Arthrobacter globiformis*.

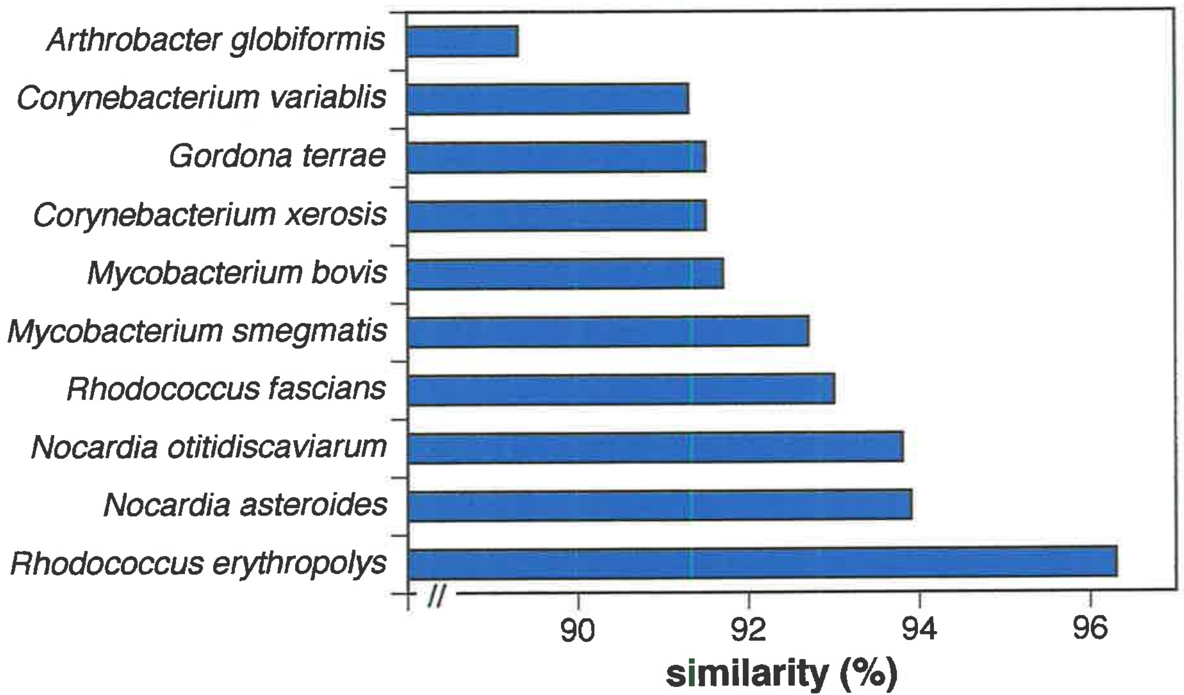

Arthrobacter globiformis
Corynebacterium variablis
Gordona terrae
Corynebacterium xerosis
Mycobacterium bovis
Mycobacterium smegmatis
Rhodococcus fascians
Nocardia otitidiscaviarum
Nocardia asteroides
Rhodococcus erythropolys
90
92
94
96
similarity (%)

Table 4.1 Similarity values of the bacterium isolated in this study and sequences of type strain mycolic acid containing actinomycetes. Similarity values between these organisms has been published elsewhere (Chun and Goodfellow, 1995).

organism	similarity	similarity group	Genbank accession number
Rhodococcus erythropolis	96.3	I	X53203
Nocardia asteroides	93.9	II	X57949
Nocardia otitidiscaviarum	93.8	II	M59056
Rhodococcus fascians	93.0	II	X53204
Mycobacterium smegmatis	92.7	II	X52922
Mycobacterium bovis	91.7	II	M20940
Corynebacterium xerosis	91.5	II	M59058
Gordona terrae	91.5	II	X53202
Corynebacterium variablis	91.3	II	X53185
Arthrobacter globiformis	89.3	III	M23411
Escherichia coli	76.7	IV	11775T
Escherichia coli	76.5	IV	25922

4.4 Discussion

The experiments reported in Figure 4.1 and Figure 4.2 demonstrate that upon repeated application the efficacy of carbetamide declines from above 80 % (mortality) to 50 and 68 % in fields A and B, respectively. Decreases in carbetamide efficacy as a herbicide were of this magnitude even after a single field application (Figure 4.2A). Laboratory incubations of soil from field B (plant responses shown in Figure 4.1B and Figure 4.2B) demonstrated that carbetamide degraded more rapidly upon

repeated application to soil (Figure 4.3A) and that this was associated with rapid loss of herbicidal activity (Figure 4.3B). These results strongly suggest, but are not definitive proof, that enhanced carbetamide degradation in the soil was the cause of carbetamide failure in this field.

Enhanced pesticide degradation results in reduced efficacy of many pesticides (Roeth, 1986, Felsot, 1989, Racke and Coats, 1990b). This phenomenon has been implicated as the cause of reduced herbicidal efficacy of propham and chlorpropham (Gray and Joo, 1985), herbicides similar in structure to carbetamide. Results of this study confirm that the efficacy of carbetamide is reduced upon repeated application (Figure 4.1, Figure 4.2). Reductions in weed control with carbetamide upon repeated application observed in the current study are similar to those reported with the herbicide EPTC and other herbicides upon repeated application (Gray and Joo, 1985, Roeth *et al.*, 1990, Skipper, 1990, Harvey, 1991). Reductions in EPTC efficacy of this level can reduce *Z. mays* yields by greater than 90 % (Harvey, 1990). Whilst the crops and environments are different, clearly if such reductions in the efficacy occur due to enhanced carbetamide degradation, crop yields would be significantly reduced.

A limitation of the initial study on carbetamide degradation kinetics (section 4.3.3) was that the challenge time between the first and second application was only 56 days. Previously it has been demonstrated that enhanced degradation of the herbicide linuron may be "lost" after as little as 57 weeks (Walker and Welch, 1992). Under Australian conditions,

carbetamide is applied on an annual basis for grass weed control. Thus it was possible that the effects shown in Figure 4.2 and Figure 4.3, though evident 8 to 16 weeks after treatment, may not be evident 12 months after application. However the results presented in Figure 4.1 suggested that the phenomenon did persist over seasons. To examine this further, the field based experiment described in section 4.3.4 was conducted.

Enhanced carbetamide degradation was evident 14 months after initial carbetamide treatment (Figure 4.4), confirming that enhanced carbetamide degradation is induced by even one prior application and enhancement persists from one season to another. A single prior carbetamide application reduced carbetamide DT_{50} from 54 to 9 days (Figure 4.4A). Such rapid degradation resulted in rapid loss of associated herbicidal bioactivity (Figure 4.4B). Therefore, enhanced carbetamide degradation is the likely cause of reduced field efficacy of this herbicide upon repeated application.

Evidence presented for the involvement of soil micro-organisms in enhanced carbetamide degradation included: i) increased conversion of ^{14}C-carbetamide to $^{14}CO_2$ in soil (Figure 4.5), ii) transfer of enhanced degradation to a control soil (Figure 4.6) and iii), inhibition of degradation by soil sterilisation (autoclaving) (Figure 4.7). Specific evidence for the involvement of bacteria is the inhibition of enhanced carbetamide degradation by the broad spectrum anti-bacterial chloramphenicol but not by the broad-spectrum antifungal cycloheximide (Figure 4.7). These methods

have been widely used to implicate soil microorganisms and specifically bacteria in pesticide degradation (Kaufman and Kearney, 1976, Walker and Welch, 1990).

Studies with microbial inhibitors provided circumstantial evidence that bacteria also play a key role in the degradation of carbetamide in soil not previously treated with carbetamide. Carbetamide degradation in the soil previously untreated with carbetamide was clearly inhibited by chloramphenicol (Figure 4.7B). Furthermore, as radiolabelled CO_2 was also evolved from this same control soil (Figure 4.5), it is likely that the biochemical pathways for degradation are already present in the soil. This is a possible explanation for enhanced degradation occurring after only a single carbetamide application (Figure 4.1 and Figure 4.4).

Transfer of a soil characteristic by addition of small quantities of an "active" soil has been used previously to indicate that an observed characteristic is a function of the soil biota (Wiseman *et al.*, 1996). Also, it has been suggested that as such low amounts of transfer are required, wind or cultivation movement may play a part in transferring the phenomena from one area to another (Walker and Welch, 1990).

A bacterium able to grow using carbetamide as a source of carbon was isolated from soil. Classical tests, substrate utilisation and analysis of cell wall fatty acid composition indicated that the isolated bacterium was a member of the mycolic acid forming group and a member of

the genus *Rhodococcus.* Typical characteristics of this genus are that they are aerobic, non-motile, slow growing bacteria commonly isolated form soil (Goodfellow, 1986, Goodfellow, 1992). Further work may identify the isolated bacterium more accurately, such work could include more accurate determination of the 16S rRNA sequence using primer sets which enable sequencing in both directions. A limitation of this approach is that for actinomycetes the 16s rRNA sequence does not necessarily provide the accuracy required to identity a member of this group (Embley, 1994).

Rhodococci capable of degrading a wide range of xenobiotics in soil have been isolated (Warhurst and Fewson, 1994). Herbicides degraded by *Rhodococci* in pure culture include the thiocarbamate herbicide EPTC (Behki, 1994) and the triazinone herbicide metatmitron (Parekh *et al.*, 1994). It is likely that more than one organism is responsible for enhanced carbetamide degradation. Evidence for this includes the diversity of organisms capable of degrading structurally related phenyl carbamate herbicides (Kaufman, 1977). *Rhodococcus* is a likely genus to be associated with enhanced degradation as members of this genus are typically environmentally robust, capable of surviving “starvation” treatment for several months (Warhurst and Fewson, 1994).

4.5 Conclusion

Research presented in this chapter confirmed *Trifolium* seed growers’ observations that repeated carbetamide applications were less

effective than initial applications. Reductions in weed control of the magnitude described here (Figure 4.1 and Figure 4.2) are likely to reduce crop yields significantly. Reduced carbetamide efficacy upon repeated application was linked to rapid or enhanced degradation kinetics, with a single carbetamide application sufficient to reduce the DT_{50} of a second carbetamide application from 54 to 9 days. Addition of radiolabelled carbetamide to enhanced carbetamide degrading soil resulted in the rapid evolution of radiolabelled $^{14}CO_2$, suggesting that carbetamide was being biologically degraded. Incorporation of the anti-bacterial chloramphenicol inhibited enhanced carbetamide degradation, suggesting that bacteria were the causal agents involved with degradation. A soil bacterium was isolated capable of using carbetamide as a source of carbon and energy.

It is likely that enhanced carbetamide degradation in the soils tested was the result of the activity of soil bacteria capable of catabolising carbetamide for metabolic gain. As a result carbetamide is degraded more rapidly upon repeated application to a soil. Rapid carbetamide degradation reduces the time in which grass weeds can absorb carbetamide from soil. Thus control of both established plants and germinating seeds is decreased. Decreases in efficacy are similar in magnitude to those reported for EPTC (Harvey, 1990). Therefore the practice of repeatedly applying carbetamide on an annual basis is the likely cause of reduced efficacy of this herbicide.

5. PERSISTENCE OF ENHANCED CARBETAMIDE DEGRADATION IN SOIL

5.1 Introduction

Carbetamide is an important herbicide in *Trifolium* seed production systems as it effectively controls many agronomically important annual grass weeds. Carbetamide effectively controls herbicide resistant biotypes of *L. rigidum* (McAlister, 1992), as well as *Hordeum* sp., *Vulpia* sp. and *Phalaris minor* (Hole, 1993), which are inadequately controlled with other herbicides. Thus, carbetamide plays a key role in the management of grass weeds in *Trifolium* seed crops. Research documented in the preceding chapter demonstrated that, upon repeated application, carbetamide is degraded rapidly in soil. This in turn reduces weed control. In this chapter, research is presented examining whether enhanced carbetamide degradation can be managed by applying this herbicide less frequently.

Enhanced soil degradation of the herbicide EPTC upon repeated application is a significant agronomic problem in the United States of America (Roeth *et al.*, 1990). However, enhanced EPTC degradation can be circumvented by using the following practices: i) herbicide and crop rotation, ii) chemical extenders to inhibit EPTC degradation, iii) row cultivation and iv) late season application of other herbicides (Harvey, 1990). With the exception of row cultivation, these techniques could be utilised in *Trifolium* seed production systems in Australia. During the present

study, insufficient time was available to conduct a long term field trial such as those documented by Harvey (1990). Therefore, the principles underlying the practices of herbicide rotation and chemical extenders were examined in relation to enhanced carbetamide degradation. The biological principles underlying herbicide and crop rotation are that: i) enhanced EPTC degradation declines over time and ii), other herbicides, which do not maintain enhanced EPTC degradation in soil, can be applied. Extenders function, as their name suggests, by extending the persistence of a pesticide (Roeth, 1986).

It is the combination of several techniques that allows enhanced EPTC degradation to be successfully managed (Drost *et al.*, 1990, Harvey, 1990). Therefore, all of the carbetamide degradation studies described in chapters 5, 6 and 7 were conducted with soil collected from two adjacent fields (D and E). This also allowed comparison with the carbetamide degrading soil bacterium, which was isolated from field E.

5.2 Persistence of enhanced carbetamide degradation in soil

Does enhanced carbetamide degradation, due to repeated application, decline over time once applications have stopped? The answer to this fundamental question will determine the long-term efficacy of carbetamide. Research with several herbicides subject to enhanced degradation has demonstrated that ceasing application typically allows soils

to return to their "natural state" (Roeth, 1986). Herbicides can often then be re-applied with greater likelihood of controlling weeds. For example, with the thiocarbamate herbicide EPTC (in a mixture with cyanazine), reducing application frequency from every year to every second or third year, increased *Panicum miliaceum* (wild proso millet) control from 46 percent to 88 and 91 %, respectively (Harvey, 1990).

The driving force influencing reversion of a soil back to its natural state is most likely to be competition between organisms. In the absence of selection pressure (herbicide application), micro-organisms must compete for other nutrient sources. If the herbicide degrading phenotype confers no selective advantage, or actually results in an increased metabolic burden, then either the phenotype may be lost, or other bacteria may simply out-compete the degrading organisms. As degrading phenotypes are frequently plasmid encoded (Roeth, 1986), plasmid loss is a common mechanism for loss of a phenotype (Stanisich, 1988, Karns, 1990). Under laboratory conditions, additional stresses, such as heat, tend to increase the rate or frequency of plasmid loss (Stanisich, 1988). In the field many factors, including heat, wet/dry cycling and predation, significantly influence communities of soil micro-organisms (Van Gestel *et al.*, 1992, England *et al.*, 1993, Van Gestel *et al.*, 1993, Nishiyama *et al.*, 1995). Roeth (1986), in quoting Koch (1981), suggests that loss of a herbicide degrading phenotype is fundamentally similar to the loss of antibiotic resistance in micro-organisms. With antibiotic resistance, loss of the

degrading phenotype is presumed to be proportional to the "burden" a mechanism places on a host.

5.3 Methods

5.3.1 Persistence of enhanced carbetamide degradation

A combined field and laboratory study was conducted over three seasons to monitor decline of enhanced carbetamide degradation after ceasing carbetamide application. Soil samples were collected in 1994, 1995 and 1996 from a field treated annually with carbetamide from 1989 to 1992 (E[II], Table 3.1). To enable comparison, carbetamide degradation kinetics were also examined in soil collected from an adjacent field (D) not previously treated with carbetamide, and soil from field E[III], treated annually with carbetamide from 1989 to 1995. In this latter soil, samples were collected prior to carbetamide application in 1994 and 1995. Soils E[II] and E[III] were maintained as subplots (10 m x 2 m, 4 replicates) within field E (Table 3.1). Soil sampling was performed by compositing 4 sub-samples taken to 7 cm depth from each plot. Similar sampling techniques were used for field D, with the same area sampled each year. Soil samples were treated with carbetamide (20 mg kg^{-1}). Carbetamide degradation kinetics were determined by HPLC and oat bioassay analysis of samples taken from 0 to 49 days after treatment (section 3.4).

Paraquat and diquat were used during the experimental period to control vegetation on the experimental areas. These herbicides are bound strongly to soil (Brian *et al.*, 1958) and are therefore less likely to interfere with soil microbial activity.

5.3.2 Most probable number of microorganisms capable of carbetamide degradation

Soils collected during 1996 for the previous experiment (section 5.3.1) were assayed to determine the Most Probable Number (MPN) of carbetamide degrading microorganisms per gram of soil. The method used was based upon the microtechnique of Rowe, Todd and Waide (1977). Soil dilutions were performed using a "Soil Extract Broth" (SEB). Soil (500 g) was diluted with H_2O to 1 L in a 3 L flask and mixed on a bench top shaker at room temperature for 1 hour. After autoclaving for 1 hour at 120°C, the flask was cooled to room temperature. The supernatant was removed (without filtering) and autoclaved again for 15 minutes at 120°C. After cooling, this broth was used for dilutions of soil. SEB (200 µL) was placed into all wells of the required number of 96 well microplates (Labsystems, 500 µL well volume). Soil samples to be assayed were passed through a 3.9 mm sieve to remove extraneous matter and increase homogeneity. For the initial dilution, 10 g of soil was added to 90 mL of SEB and mixed on a orbital shaker for 1 hour. After allowing particulate matter to settle for 60 seconds, 200 µL of this suspension was transferred into the first row of

wells in a 96 well microplate. Two-fold serial dilutions were performed with an octpette, using sterilised disposable tips for each dilution. Carbetamide and ^{14}C labelled carbetamide were then added in 70 µL of sterile water per well (final concentration 70 mg L^{-1} technical grade carbetamide and 4.2×10^5 Bq L^{-1} ^{14}C labelled carbetamide). After fitting lids, microplates were wrapped in foil to minimise evaporation and incubated at 15°C for 28 days.

To assess carbetamide degradation, a 50 µL subsample was removed from each well without disturbing the soil pellet. Radioactivity in subsamples was determined by liquid scintillation counting (section 3.7). Loss of 20 % or more of the applied radioactivity was assumed to indicate carbetamide degradation. Using a published MPN table (Rowe *et al.*, 1977), with adjustment for initial soil moisture content, MPN values for each soil sample were determined. These values were used to calculate the mean and standard error of the mean for the number of degraders in each soil treatment.

5.4 Results

5.4.1 Persistence of enhanced carbetamide degradation

Over the period 1994 to 1996, the capacity for enhanced carbetamide degradation declined in soil for which carbetamide application

Figure 5.1 Carbetamide degradation kinetics in three soils collected in 1994, 1995 and 1996, as measured by HPLC and oat bioassay. Soils were either untreated (A), treated with carbetamide from 1989 till 12 months before sampling (B), or treated with carbetamide from 1989 to 1992 (C). Vertical bars indicate standard errors of the mean.

A: previously untreated

B: treated annually from 1989 until 1 year before sampling

C: treated annually 1989 - 1992

sampling year

1994

1995

1996

carbetamide remaining (%)

oat mortality (%)

time (d, log scale)

ceased in 1992 (Figure 5.1C). However, even four years after the last carbetamide application, degradation in this soil was more rapid than in the previously untreated soil in any year (Figure 5.1A). Whilst in soil where annual carbetamide application was continued, carbetamide degraded rapidly in each year (Figure 5.1B). Bioassay results confirmed the trends observed (Figure 5.1).

Table 5.1 Carbetamide DT_{50} in days as influenced by prior carbetamide application, or time since last carbetamide application, determined in three successive years. DT_{50} values were determined by linear extrapolation between appropriate data points. Refer to Table 3.1 for further information on each field.

herbicide history year of sampling	previously untreated with carbetamide, field D	treated with carbetamide annually 1989 until 12 months before sampling, field E[III]	treated with carbetamide 1989-1992, field E[II]
1994	23.0	4.3	5.4
1995	43.6	4.2	9.6
1996	28.0	4.2	10.2

The time taken for 50 % of the applied carbetamide to degrade (DT_{50}) in the three soils in each year is presented in Table 5.1 Over the three year period there was variation in carbetamide DT_{50} in the untreated control soil, ranging from 23 to 43.6 days. Variations in many soil microbial activities are known to occur and can vary over much shorter time frames (Cook and Greaves, 1987). Therefore the results presented indicate that, due to variation in persistence, the efficacy of carbetamide is likely to vary between

seasons. In contrast, carbetamide degradation was extremely rapid in soil receiving annual carbetamide treatment up until 12 months prior to sampling. For this soil, over the three year study, the DT_{50} was 4.2 or 4.3 days, with around 70 % of the degradation occurring between 3 and 5 days after carbetamide treatment.

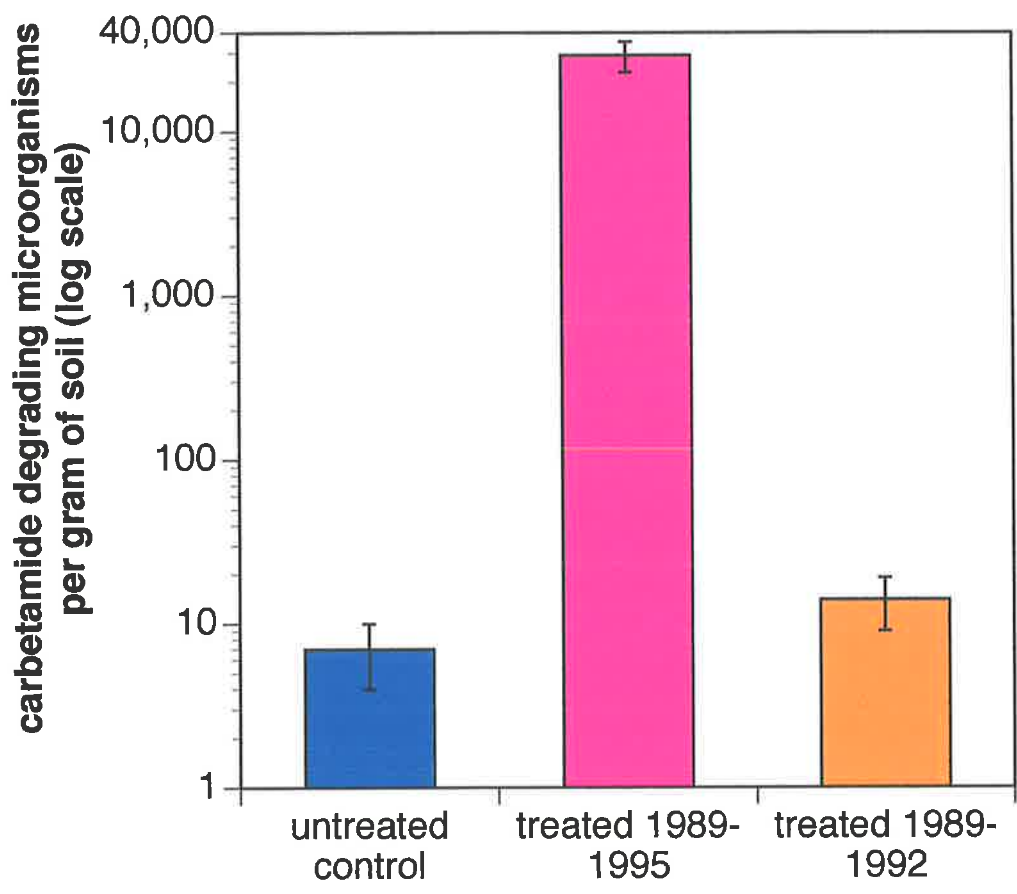
In contrast to the control soil (Figure 5.1A) and soil treated annually with carbetamide (Figure 5.1B), the carbetamide DT_{50} increased over time in soil where application of this herbicide stopped in 1992 (Figure 5.1C). Also, over the three year period the lag phase before rapid degradation occurred, increased from 3 to 5 days. The DT_{50} during 1994 was 5.4 days, a comparable value to soil treated in 1993 with carbetamide. This suggests that after carbetamide application in 1992, the kinetics of carbetamide degradation in both soils was similar. When sampled in the following two years, the DT_{50} was 9.6 and 10.2 days, demonstrating an increase in persistence if application of carbetamide ceases. Comparatively, carbetamide DT_{50} in this soil in 1996 was more than twice that in soil where annual carbetamide application continued (Figure 5.1B) but less than one third of the mean DT_{50} of the control soil (Figure 5.1A, 31.5 days, averaged over the three years). This result shows that in the absence of carbetamide application, the soil capacity for enhanced carbetamide degradation declines over time.

5.4.2 Most probable number of microorganisms capable of carbetamide degradation

Prior treatment with carbetamide and time without carbetamide application substantially influenced the most probable number of carbetamide degrading microorganisms in the soils sampled (Figure 5.2). In the control soil, previously untreated with carbetamide (field D), there were 7 carbetamide degrading microorganisms per gram of soil. Whilst in soil treated annually with carbetamide from 1989 to 1995, and sampled in 1996, there were 2.9×10^4 degrading organisms per gram of soil (Figure 5.2). In soil to which carbetamide application ceased in 1992, there were 14 carbetamide degrading microorganisms per gram of soil. This data confirms the experiments in sections 4.3.5, 4.3.6 and 4.3.7, that enhanced carbetamide degradation is linked to an elevated soil population of carbetamide degrading organisms.

Decline of the capacity for enhanced carbetamide degradation over time, as shown in Figure 5.1C, was correlated with a decline in the soil population of carbetamide degrading micro-organisms (Figure 5.2). Carbetamide degraded rapidly in soil sampled in 1994 which had been previously treated annually from 1989 to 1992. However, in this soil when sampled in 1996, carbetamide degradation was slower (Fig, 5.1C) and there were 14 carbetamide degrading microorganisms per gram of soil. This represents a population density more similar to the control soil (7 degrading microorganisms g^{-1}) than the soil to which carbetamide was applied

Figure 5.2 Most probable number of carbetamide degrading microorganisms per gram of soil collected during 1996 in: a previously untreated control soil (Field D), a soil treated annually with carbetamide from 1989 to 1995 (Field E[III]) and a soil treated annually with carbetamide from 1989 to 1992 (Field E [II]). Vertical bars indicate standard errors of the mean.


carbetamide degrading microorganisms per gram of soil (log scale)
40,000
10,000
1,000
100
10
1
untreated control
treated 1989-1995
treated 1989-1992
years of carbetamide treatment

annually from 1989 - 1995 (2.9×10^4 degrading microorganisms g^{-1}). This result provides further evidence that by ceasing carbetamide application a soil will tend to revert to its "natural state".

An assumption of this experiment is that continued annual carbetamide applications did not further enhance the population of carbetamide degrading organisms. Evidence for this is presented in Figure 5.1, where the degradation rate was approximately the same in the three consecutive years.

5.5 Discussion

In the absence of carbetamide application, the phenomenon of enhanced carbetamide degradation declined over time. However, only partial reversion back to the "natural" state was demonstrated (Figure 5.1C). A stable pattern of degradation appeared to be established in this soil, part way between the fully enhanced soil and the control soil (Table 5.1). Estimation of the population of carbetamide degrading microorganisms in soil collected in 1996 indicated a substantial decline in the most probable number of carbetamide degrading microorganisms in the absence of carbetamide application (Figure 5.2). Though there were relatively few carbetamide degrading microorganisms 4 years after the last carbetamide application, carbetamide still degraded rapidly (Figure 5.1). One possible explanation for this is that the degrading microorganisms multiply rapidly when carbetamide is re-applied. Alternatively, if carbetamide degradation is

plasmid encoded, application of the herbicide may stimulate plasmid transfer in the soil.

Persistence of enhanced pesticide degradation is also likely to be determined by bacterial survival. It has been proposed that soil micropores may provide physical protection from predation by protozoa and desiccation (Nishiyama *et al.*, 1995). Location of microorganisms within such pores could also potentially protect them from dispersal during dilution for a MPN assay.

Enhanced carbetamide degradation was found to be a persistent phenomenon, with enhancement still clearly evident 4 years after ceasing carbetamide application. For other pesticides and chemicals, there is considerable range in the reported persistence of enhanced degradation. Enhancement of linuron degradation is reported to last less than 57 weeks (Walker and Welch, 1992), whilst enhanced degradation of 2,4-D was still evident after 204 weeks (Smith and Aubin, 1994) and enhanced degradation of acetylene was still evident after 8 years (Terry and Leavitt, 1992). The results presented in Figure 5.1 demonstrate that enhanced carbetamide degradation is persistent in soil. The agronomic relevance of these results should be confirmed by conducting field based carbetamide degradation studies.

Enhanced carbetamide degradation, while persisting for at least four years, does decline slowly after ceasing carbetamide application

(Figure 5.1). This suggests that less frequent carbetamide application should provide improved weed control. Insufficient data correlating carbetamide persistence with efficacy is available to confirm this. A long term herbicide rotation trial is needed to determine the optimum frequency of carbetamide application. One possible experimental design has been used for enhanced EPTC degradation (Harvey, 1991). Such an experiment could also examination field based carbetamide degradation, thus enabling correlation between carbetamide persistence and weed control. Whilst a long term herbicide rotation trial is clearly needed, time constraints of the present study did not permit this to be undertaken.

6. SPECTRUM OF CROSS ENHANCEMENT AND CROSS DEGRADATION

6.1 Introduction

Cross enhancement is defined as the rapid degradation of a pesticide due to prior application of a different pesticide (Roeth, 1986). From a practical perspective, determining the spectrum of cross enhancement is important for determining appropriate herbicide rotations in both the short and long term. In the short term, or until enhanced degradation declines, applications of soil active herbicides to which there is cross enhancement are less likely to provide effective weed control. In the longer term, application of any herbicide to which there is cross enhancement will maintain or increase the population of degrading micro-organisms, increasing the time period over which the soil active herbicide is less effective. The latter phenomenon is independent of whether the herbicide is applied to the soil or foliage.

As enhanced degradation of phenyl carbamate herbicides has not been studied extensively, no studies of cross enhancement from phenyl carbamates have been published. However, the degradation of phenyl carbamate herbicides by bacteria isolated from soil has been studied. Kaufman and Blake (1973) reported that two soil isolates, *Pseudomonas striata* and an *Achromobacter* sp., were capable of degrading both propham and chlorpropham. Oat bioassay results suggested that these organisms

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could also degrade or partially detoxify CDEC, dicryl, propachlor, propanil, solan and swep (Kaufman and Blake, 1973). Only slight activity was recorded with the phenylurea herbicides diuron, DMU and fenuron. These results were obtained with isolates grown in pure culture with 0.1 g L^{-1} yeast extract (Kaufman and Blake, 1973), a good carbon source, therefore potentially different results may be observed in the field.

Typically, *Trifolium* seed crops are grown in the same field for 2 to 5 consecutive seasons (section 1). One economic reason for such lengthy monocultures is that, particularly with perennial *Trifolium* sp., yields from first year stands are typically half those of established stands. As demonstrated in the previous chapter, repeated applications of carbetamide do not provide effective weed control. Therefore, one objective of cross enhancement studies was to identify herbicides which could be used within *Trifolium* stands to control grass weeds. Such alternative herbicides include propham, chlorpropham, propyzamide and EPTC. Aryloxphenoxypropionate and cyclohexanedione herbicides were ignored as there is widespread resistance in *L. rigidum* to these herbicides (Hole, 1993). Secondly, cross enhancement to herbicides which are most commonly used in rotational crops was examined. The spectrum of herbicides applied to a field is a reflection of the crops grown, weeds present and herbicides available. All of these are strongly influenced by environmental and soil properties. Thus, for a given area, a restricted number of combinations of crops, weeds and herbicides are compatible. In areas where *Trifolium* production occurs the

most common rotational crops are perennial grasses such as *Phalaris*, *Festuca* and *Dactylus* spp. Where appropriate, diuron and simazine are applied for grass weed control in these crops.

EPTC is not currently registered in Australia for grass weed control in *Trifolium* sp. However, EPTC is recommended for use with *Trifolium* sp. in the United States of America (Klingman and Ashton, 1982). Also, as EPTC has not been extensively used in Australia, resistance to this herbicide is less likely. Small scale unreplicated field trials confirmed that EPTC effectively controlled annual grasses with minimal damage to annual *Trifolium* sp. (S. Hole and B. Badman - unpublished data). A problem with EPTC is that it requires immediate incorporation to reduce volatilisation (Gray and Weierick, 1965). Therefore, if used, EPTC should be applied in the first year when the stand is sown thus enabling soil incorporation using cultivation.

6.2 Methods

6.2.1 Spectrum of cross enhancement from carbetamide

To determine the spectrum of cross enhancement from carbetamide to other herbicides, soil samples collected (section 3.4) from fields D and E were treated (20 mg kg^{-1}) with carbetamide (technical grade) or formulated propham, chlorpropham, EPTC, propyzamide, diuron or

simazine (Table 3.2). After herbicide addition, the soil in each container was mixed and subsampled for analysis by HPLC or GC at 0, 9 and 14 days after treatment. Residual concentration of all herbicides, excluding propyzamide, were measured by HPLC. For the additional herbicides analysed by HPLC, separation was achieved with a 10 - 100 % concave acetonitrile gradient over 30 minutes (Table 3.2). Residual propyzamide was determined 0 and 14 days after treatment using GC of soil extracts. Soil samples treated with propyzamide were extracted with methanol (25 mL) and a subsample of the supernatant was centrifuged to remove particulate matter. Chromatography was performed as described in section 3.5.

6.2.2 Spectrum of growth by the isolated *Rhodococcus* sp. on other herbicides and carbaryl

The ability of the isolated carbetamide degrading bacterium to grow on a range of herbicides was examined. Subsamples (100 µL) of a late log phase culture of the isolated bacterium grown on carbetamide were used to inoculate 50 mL cultures of MSM media (with 50 mg L^{-1} yeast extract) containing 200 mg L^{-1} carbetamide, propham, chlorpropham, EPTC, propyzamide, diuron, simazine or carbaryl. Herbicides were added dissolved in methanol, except for diuron, simazine and carbaryl which were added dissolved in acetone. Flasks were incubated at 28°C. At time points from 0 to 72 hours after inoculation, 1 mL samples were removed from each

flask to determine changes in OD (section 3.6.3). Colony forming units per mL were determined according to section 3.6.3.

6.2.3 Efficacy of a range of herbicides in a field with enhanced carbetamide degradation

This experiment was conducted in field E during 1992. From 1988 to 1991 this field was devoted to *Trifolium resupinatum* (white clover) seed production and had been treated once every winter with 2,100 g ha^{-1} carbetamide. After this experiment had been conducted, enhanced carbetamide degradation was confirmed in this field (Figure 5.1). The site was densely infested with *Vulpia* sp. which had been grazed lightly prior to herbicide treatment. The trial was arranged as a randomised complete block design, with four replicates and each plot measured 10 m x 2 m. Carbetamide (2,100 g ha^{-1}), propham (3,750 g ha^{-1}), propyzamide (1,000 g ha^{-1}), diuron (1,500 g ha^{-1}) and simazine (2,000 g ha^{-1}) were applied using a propane pressurised hand held boom sprayer (section 3.3.1), with plots remaining untreated as controls. One day after spraying, simulated rainfall was applied to the trial area to leach the soil active herbicides into the soil. A total of 4,000 litres of water was applied evenly over the trial site using a petrol driven pump and a fanning nozzle. This irrigation equated to approximately 5.6 mm of rainfall.

Assessment was made 118 days after treatment by counting plants remaining in four randomly selected quadrats of 0.025 or 0.25 m^2

within each plot (depending on plant density). Data was used to generate the mean and standard error of the mean for each treatment.

6.2.4 Efficacy of rotation between carbetamide and propyzamide in the field

A randomised block experiment was conducted during 1994 in field B, which had been commercially treated with carbetamide once annually in the winters of 1990-92. Each of the four replicates consisted of nine 10 m by 2 m plots. Initial treatment was made to the nine plots within each block on July 10, three with carbetamide (2,100 g ha^{-1}), three with propyzamide (1,000 g ha^{-1}), and three remaining unsprayed. To assess a second herbicide application, 128 *L. rigidum* seedlings were transplanted into each plot. Plants within each plot were arranged into 8 groups of 16 plants (effective plant density 522 plants m^{-2}). When the *L. rigidum* plants were at the 3 to 4 leaf stage (Sept. 25), plots were treated with either carbetamide (2,100 g ha^{-1}) or propyzamide (1,000 g ha^{-1}), or remained unsprayed. These treatments were applied so as to generate the nine possible combinations of initial and final treatments. Plant mortality was assessed 28 days after the second treatment.

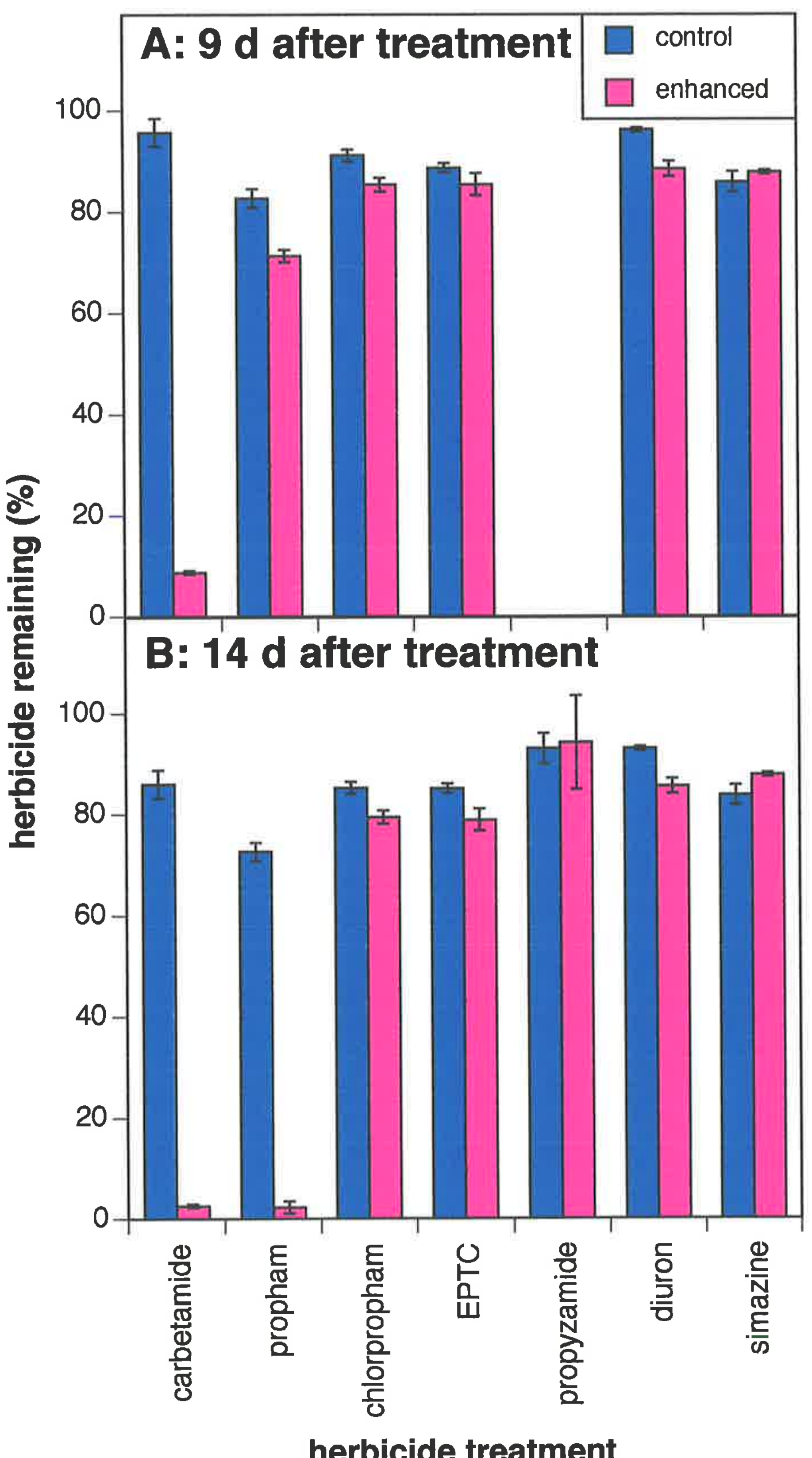
6.3 Results

6.3.1 Spectrum of cross enhancement from carbetamide

After 9 days incubation, enhanced carbetamide degradation was clearly evident but similar levels of all other herbicides remained (Figure 6.1A). However after 14 days, soil which degraded carbetamide at an enhanced rate, also degraded propham at an enhanced rate (Figure 6.1B), but none of the other herbicides tested. Fourteen days after the addition of carbetamide there was 85 % remaining in the control soil compared to 4 % remaining in the enhanced soil. Similarly, with propham there was 70 % remaining in the control soil but only 5 % remaining in the soil with enhanced carbetamide degradation (showing cross enhancement to propham). Thus, prior carbetamide application induces enhanced propham degradation but results after 9 days indicated that there was a lag period before propham degradation was observed.

After 14 days (Figure 6.1B), in both the control soil and the enhanced soil, there were relatively high and similar amounts of chlorpropham (79 - 85 %), EPTC (79 - 85 %), propyzamide (93 - 94 %), diuron (86 - 93 %) and simazine (84 - 88 %) remaining. Therefore, cross enhancement to these herbicides was not conferred by prior carbetamide application.

Figure 6.1 Degradation of a range of herbicides after 9 (A) and 14 days (B) in soil which degraded carbetamide at an enhanced rate and a soil previously untreated with carbetamide (control). Propyzamide treated soil samples were only analysed for 0 and 14 d. Vertical bars indicate standard errors of the mean.


A: 9 d after treatment
control
enhanced
B: 14 d after treatment
herbicide remaining (%)
0
20
40
60
80
100
carbetamide
propham
chlorpropham
EPTC
propyzamide
diuron
simazine
herbicide treatment

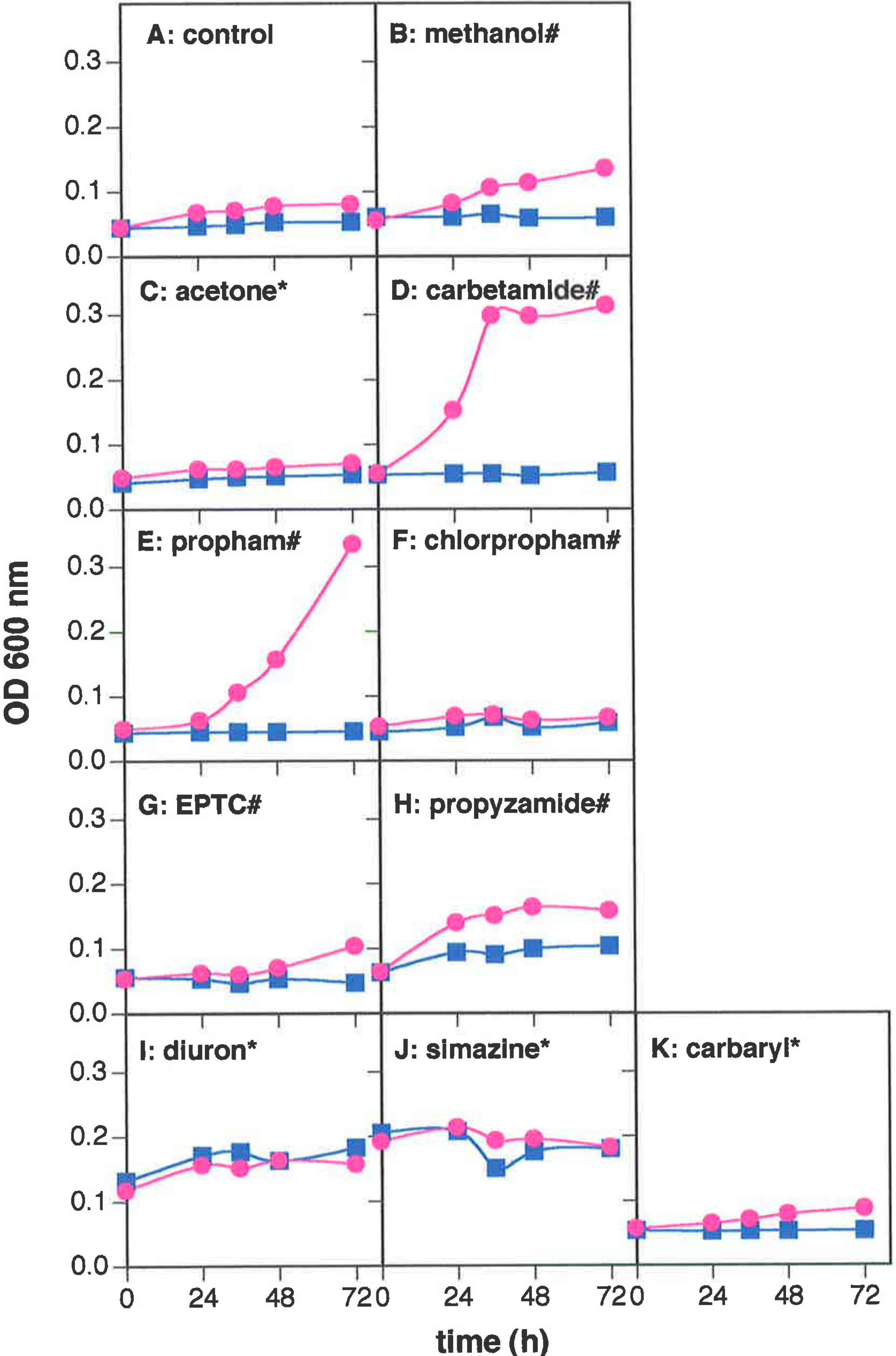
6.3.2 Spectrum of growth by the isolated *Rhodococcus* sp. on other herbicides

The soil bacterium isolated based upon its ability to degrade carbetamide (Figure 4.8) also demonstrated significant growth upon propham (Figure 6.2E), but not any of the other herbicides tested (Figure 6.2). Flasks containing carbetamide and the isolated bacterium were noticeably turbid 36 hours after inoculation (Figure 6.2D), with turbidity greater than the methanol control. This is indicative of bacterial growth and carbetamide degradation (Figure 4.8). Growth on propham, as indicated by increasing OD, was slower than on carbetamide (Figure 6.2D,E), though the final OD was similar to that of carbetamide. Growth on carbetamide and propham was confirmed by CFU measurement (Figure 6.3).

Growth comparable to that observed on carbetamide and propham was not evident with any of the other compounds tested. Flasks containing chlorpropham and simazine did not change in OD over the assay period. Flasks containing propyzamide and diuron increased in OD, even when not inoculated with the bacterium. Diuron, simazine and propyzamide have relatively low water solubilities and OD measurement was inappropriate for determining bacterial growth on these compounds.

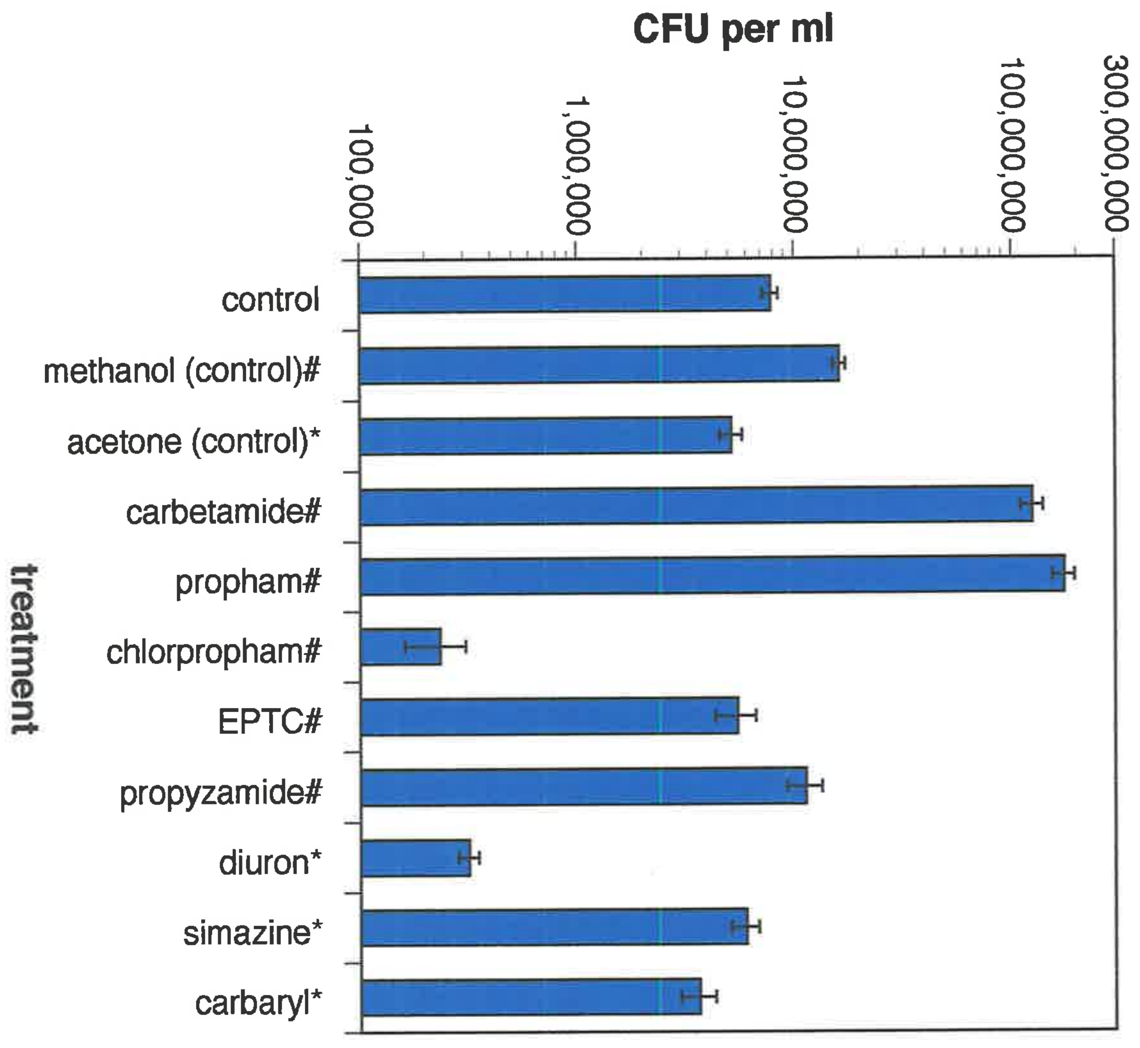
Examination of the CFU indicated that some herbicides were toxic (Figure 6.3). Compared to their appropriate controls (methanol or acetone), treatments of flasks with chlorpropham or diuron were inhibitory to

Figure 6.2 Growth of the isolated carbetamide degrading bacterium (●) with a range of herbicides compared to uninoculated controls (■). Treatments were added dissolved in either methanol (#) or acetone (*). Measurements are for a single flask, the experiment was repeated with similar results.



A: control
B: methanol#
C: acetone*
D: carbetamide#
E: propham#
F: chlorpropham#
G: EPTC#
H: propyzamide#
I: diuron*
J: simazine*
K: carbaryl*
0.0
0.1
0.2
0.3
OD 600 nm
0
24
48
72
time (h)

Figure 6.3 Colony forming units of the carbetamide degrading isolate after 72 hours incubation with a range of herbicides. Initial inoculum density was 6×10^4 CFU mL^{-1}. Treatments were added dissolved in either methanol (#) or acetone (*). Vertical bars indicate standard errors of the mean.


CFU per ml
100,000
1,000,000
10,000,000
100,000,000
300,000,000
treatment
control
methanol (control)#
acetone (control)*
carbetamide#
propham#
chlorpropham#
EPTC#
propyzamide#
diuron*
simazine*
carbaryl*

growth (Figure 6.3). Incubation with EPTC, propyzamide, simazine or carbaryl resulted in bacterial populations similar to their appropriate control, suggesting that these compounds did not affect growth of the bacterium. Further examination of the ability of the bacterium to degrade chlorpropham is reported in sections 8.3.3 and 8.3.5. The influence of carbaryl on carbetamide degradation in soil and by the isolated *Rhodococcus* sp. are examined in sections 7 and 8.3.5, respectively.

6.3.3 Efficacy of a range of herbicides in a field with enhanced carbetamide degradation

Carbetamide is registered for the control of *Vulpia* sp. in Australia. However, results presented in Table 6.1 and Figure 6.4 show that in this field previously treated with carbetamide, this herbicide no longer effectively controls *Vulpia* sp. Also, application of the structurally related herbicide propham provided unsatisfactory control. In contrast to carbetamide and propham, propyzamide, which like carbetamide requires root absorption and soil persistence, provided excellent control.

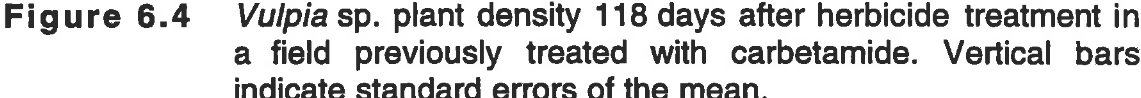


Figure 6.4 *Vulpia* sp. plant density 118 days after herbicide treatment in a field previously treated with carbetamide. Vertical bars indicate standard errors of the mean.

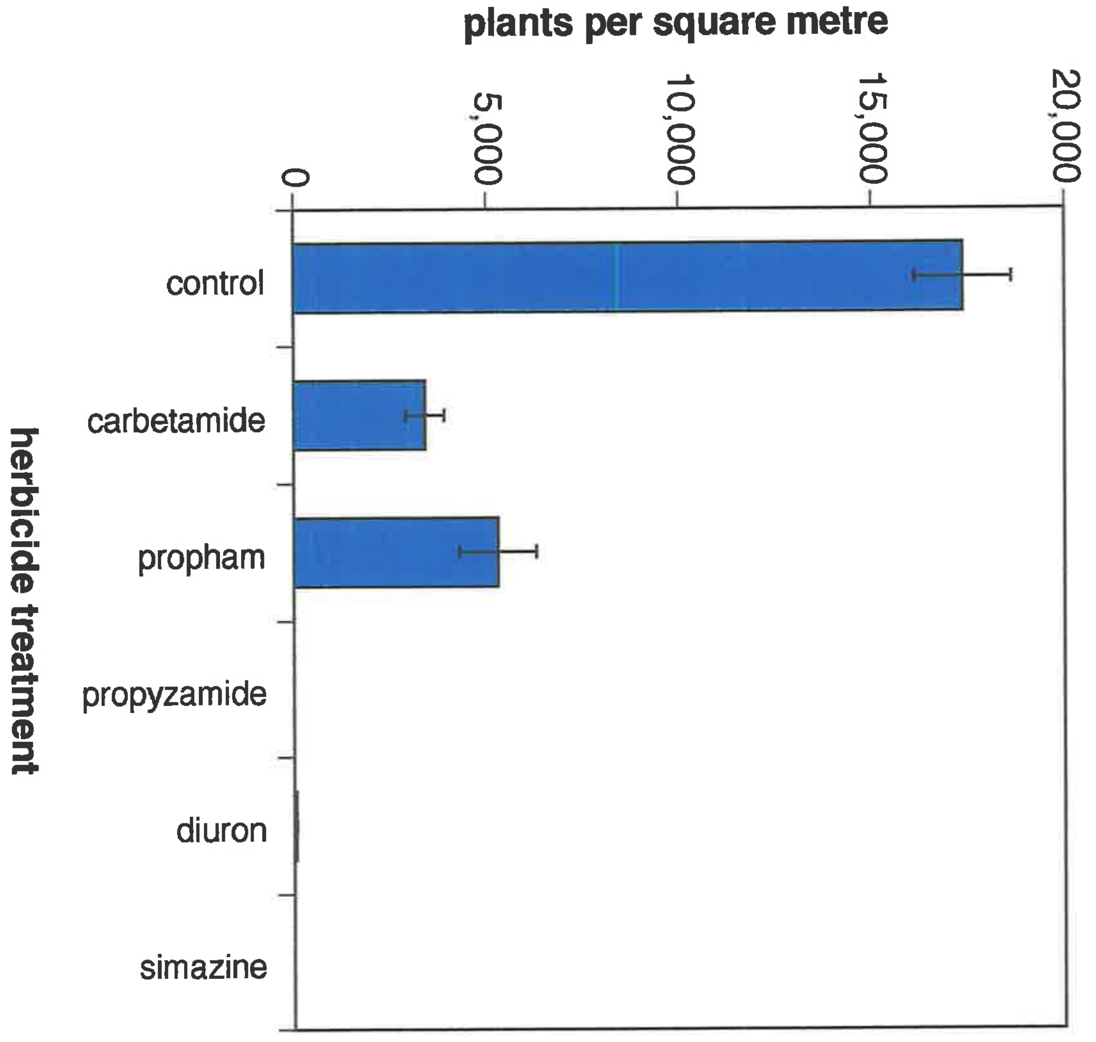

plants per square metre
0
5,000
10,000
15,000
20,000
herbicide treatment
control
carbetamide
propham
propyzamide
diuron
simazine

Table 6.1 Mean plant density of *Vulpia* sp. following herbicide treatment in a field (E) enhanced for carbetamide degradation due to prior carbetamide application. Values are also expressed as the percentage of control.

treatment	mean (plants per m^2)	SEM	% of control
control (unsprayed)	17376	1256	100
carbetamide	3419	501	20
propham	5307	995	31
propyzamide	0	0	0
diuron	59	13	<1
simazine	0	0	0

At the recommended field rate of carbetamide, 2,100 g a.i. ha^{-1}, there were 3,419 *Vulpia* plants m^{-2} in the plots sprayed with carbetamide as compared to 17,376 m^{-2} in the unsprayed control plots (20 % survival). Propham provided poor control of *Vulpia sp.*, with 31 % of the plants surviving herbicide application. The most effective treatments were propyzamide and simazine (Figure 6.4), which provided 100 % control of *Vulpia sp.* with no surviving plants evident 118 days after treatment. The herbicide diuron also provided extremely effective control.

These results demonstrate that in this field, rapid carbetamide degradation due to prior carbetamide application (Figure 5.1) is linked to poor field efficacy of this herbicide (Figure 6.4). Furthermore, results presented indicate that application of the herbicide propham, which is rapidly

degraded due to prior carbetamide application, also provides inadequate weed control. This result indicates that cross enhancement from carbetamide to other herbicides, can reduce the efficacy of these herbicides in the field. Comparison of carbetamide, propham and propyzamide only permits superficial comparison. A significant limitation of this study is absence of data confirming the efficacy of these herbicides if applied to fields previously untreated with carbetamide.

6.3.4 Efficacy of rotation between carbetamide and propyzamide in the field

The herbicides carbetamide and propyzamide are registered for control of grasses in *Trifolium* stands. As shown in this thesis, repeated carbetamide applications, on an annual basis, results in poor control of annual grasses (Figure 4.1, Figure 4.2, Figure 6.4), due to rapid degradation of carbetamide in soil (Figure 4.4, Figure 5.1). However, propyzamide remains effective, as the results presented in Figure 6.4 demonstrate that cross enhancement from carbetamide to propyzamide is not evident. This current experiment was conducted to examine whether: i) propyzamide fails to control weeds upon repeated application and ii) whether rotation between carbetamide and propyzamide would provide effective control.

Propyzamide, as with carbetamide, was less effective in controlling *L. rigidum* in plots that had been previously treated with the same

Figure 6.5 Influence of prior soil treatment with carbetamide or propyzamide, or remaining unsprayed (control), on mortality of *L. rigidum* 28 days after treatment with carbetamide (A) or propyzamide (B). Vertical bars indicate standard errors of the mean.

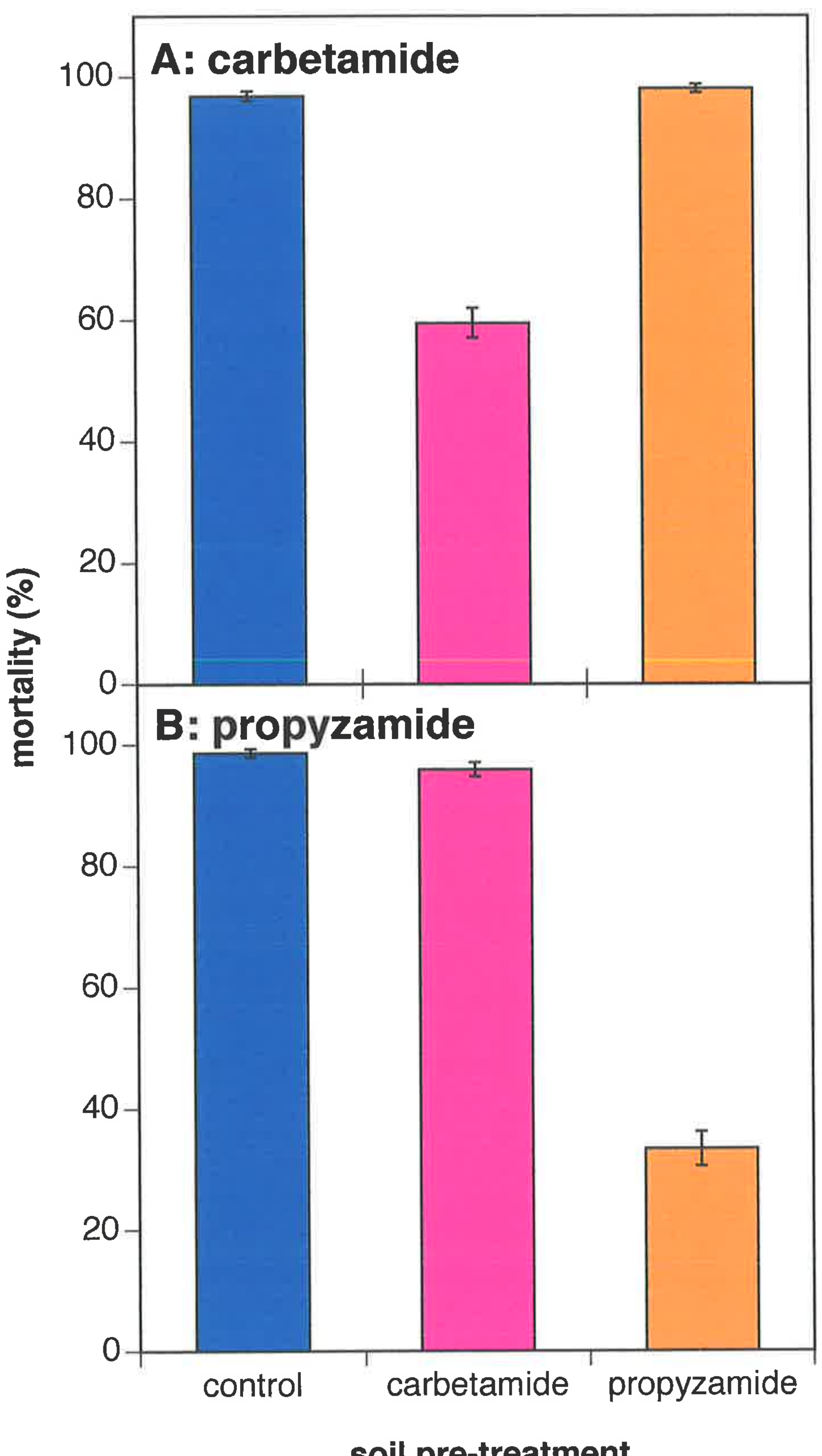
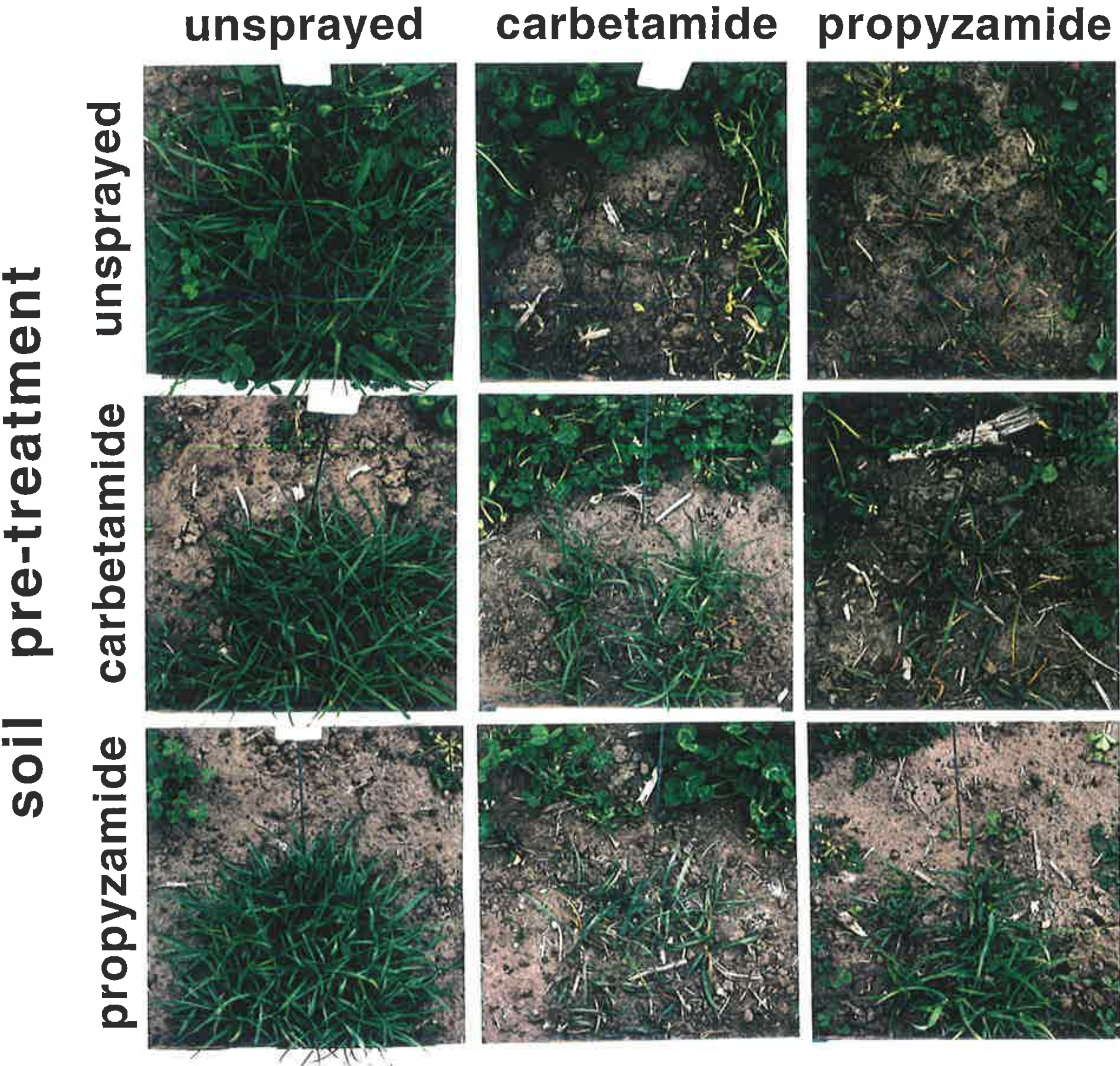

A: carbetamide
B: propyzamide
mortality (%)
0
20
40
60
80
100
control
carbetamide
propyzamide
soil pre-treatment

Figure 6.6 Photographs of plants 28 days after treatment with carbetamide or propyzamide, or remaining unsprayed, as influenced by soil pre-treatment with carbetamide, or propyzamide or initially unsprayed.


herbicide applied
to control *L. rigidum*
unsprayed
carbetamide
propyzamide
soil pre-treatment
unsprayed
carbetamide
propyzamide

herbicide (Figure 6.5). Results are presented photographically in Figure 6.6. Application of carbetamide to *L. rigidum* seedlings in previously untreated plots resulted in 97 % mortality (Figure 6.5A). However, on plots pre-treated earlier in the season with carbetamide, a second carbetamide treatment (77 days later) resulted in only 60 % mortality. *L. rigidum* plants, though visibly affected following treatment with carbetamide, survivors had produced new leaves (Figure 6.6). A similar result was found for propyzamide, a herbicide with a similar mode of action to carbetamide. Figure 6.5B shows that application of propyzamide to previously untreated plots resulted in 99 % mortality of *L. rigidum* seedlings. In plots pre-treated once with propyzamide, a second propyzamide treatment resulted in only 33 % mortality. Plants surviving had produced new foliage after application (Figure 6.6). Therefore, for both carbetamide (Figure 4.1, Figure 4.2 and Figure 6.5A) and propyzamide (Figure 6.5B), repeated application results in decreased efficacy.

This experiment examined whether pre-treatment with carbetamide reduced the subsequent efficacy of propyzamide, and *vice versa*. Rotation between carbetamide and propyzamide, in either order, resulted in high *L. rigidum* mortality (96-97 %) from the second herbicide treatment. Figure 6.5 shows that, although having similar modes of action, carbetamide (Figure 6.5A) and propyzamide (Figure 6.5B) acted independently. Thus, pre-treatment with one herbicide did not affect the subsequent efficacy of the other herbicide.

6.4 Discussion

In situations where soil has acquired the characteristic of enhanced carbetamide degradation, determining the spectrum of cross enhancement allows the identification of alternative soil active herbicides which will work effectively. In the longer term, application of herbicides to which there is cross enhancement is likely to increase the time before carbetamide can be re-applied and effectively control weeds. Cross enhancement was evident from carbetamide to propham, but not to chlorpropham, EPTC, propyzamide, diuron or simazine (Figure 6.1). This latter group of herbicides, (excluding chlorpropham, which was not tested) also provided more effective *Vulpia* sp. control than did propham in a field displaying enhanced carbetamide degradation (Figure 6.4). The carbetamide degrading *Rhodococcus sp.* isolated in this study is able to use propham as a source of carbon and energy (Figure 6.2E and Figure 6.3). This result with propham provides further evidence that the isolated bacterium is involved in carbetamide degradation in soil. This also indicates that application of propham is likely to maintain the soil population of carbetamide degrading micro-organisms, increasing the persistence of enhanced carbetamide degradation. This is not likely to be the case for chlorpropham, EPTC, propyzamide, diuron or simazine.

Rotation from carbetamide to propyzamide and vice versa maintained weed control at a level similar to when either herbicide was initially applied to soil (Figure 6.5 and Figure 6.6). However, if either

carbetamide or propyzamide was applied repeatedly, the efficacy of both herbicides was significantly less (Figure 6.5). This result indicates that propyzamide, like carbetamide, is amenable to enhanced degradation upon repeated application. Enhanced degradation of propyzamide has been previously reported (Walker and Welch, 1992). However, rotation between carbetamide and propyzamide, in either order, provided excellent control (Figure 6.5, Figure 6.6). As enhanced carbetamide and propyzamide degradation were mutually exclusive (Figure 6.5), this suggests that different soil micro-organisms are responsible for the degradation of each herbicide. As these herbicides are structurally different, the involvement of different organisms and/or different pathways is likely. Seed yields from *Trifolium* stands are typically higher in the second and third years after establishment, necessitating reliable annual grass control in successive seasons. Rotation between carbetamide and propyzamide enables reliable grass weed control for two successive seasons.

EPTC is a herbicide which has not been used extensively in Australia. Cross enhancement was not evident from carbetamide to EPTC (Figure 6.1). A limitation of EPTC is that physical incorporation into soil immediately after application is required to minimise volatilisation (Gray and Weierick, 1965). Therefore, if EPTC is to be used, it should be applied in the first year of a *Trifolium* stand when it can be incorporated. This would allow for carbetamide or propyzamide application in the second year, and the alternative herbicide to be applied in the third year. Therefore, registration of

EPTC for use in *Trifolium* seed crops would improve the spectrum of available herbicides and potentially improve yields and returns to producers.

Cross enhancement was evident from carbetamide to propham but not chlorpropham. Structurally these herbicides differ only by a chlorine substitution on the aromatic ring (Figure 2.1). Although cross enhancement between these two herbicides has not been examined in soil, bacteria capable of degrading both propham and chlorpropham have been reported (Kaufman and Kearney, 1965). One possible explanation for the absence of cross enhancement to chlorpropham in this study is that the carbetamide degraders in the soils used in this study cannot degrade halogen substituted aromatic compounds. This is examined in the next chapter. *Rhodococci* capable of degrading chlorinated aromatic structures have been isolated from soil treated with these compounds (Warhurst and Fewson, 1994). Isolates have included species from the same genus as the carbetamide degrading *Rhodococcus* isolated during the present study (Hale *et al.*, 1994). This suggests that given suitable selection pressure, cross enhancement between carbetamide and chlorpropham may occur. Therefore, repeated rotation between carbetamide and chlorpropham, or propham and chlorpropham may fail to provide adequate weed control in the field due to cross enhancement between these herbicides.

Cross enhancement was not evident to diuron or simazine (Figure 6.1). These herbicides are used extensively in crops grown in rotation with *Trifolium* seed crops. Application of these herbicides is therefore

unlikely to exacerbate or maintain enhanced carbetamide degradation. Growth of the isolated carbetamide degrading bacterium was inhibited by diuron but not affected by simazine (Figure 6.3). This suggests that if diuron was toxic to carbetamide degrading organisms in soil, application of diuron could potentially shorten the persistence of enhanced carbetamide degradation. This needs to be examined in the field, possibly as a part of the herbicide rotation trials recommended above.

7. EXTENDERS OF CARBETAMIDE PERSISTENCE

7.1 Introduction

Roeth (1986) described extenders as "feasible short-term aids for coping with enhancement in situations where the enhancing herbicide must be used". Extenders which increase persistence of EPTC and chlorpropham are well documented (Roeth, 1986). Fonophos and dietholate are effective extenders of EPTC persistence and hence increase weed control (Moorman *et al.*, 1992). However, repeated use of EPTC/dietholate mixtures may become ineffective in controlling weeds (Bean *et al.*, 1990, Harvey, 1991, Moorman *et al.*, 1992). Therefore extenders are best viewed as "short-term aids" (Roeth, 1986).

Several compounds have been shown to inhibit the degradation of phenyl carbamate herbicides in soil. For example, PCMC, phorate, diazinon and carbaryl can inhibit chlorpropham degradation (Kaufman, 1977). However these compounds have not been shown to slow carbetamide or enhanced degradation of any compound. In the present study, a range of compounds were tested for their ability to inhibit enhanced carbetamide degradation. Unlike screening for EPTC extenders, where over three thousand compounds were tested (Drost *et al.*, 1990), only a relatively small number of chemicals could be tested in the current study. The rate chosen was 10 mg kg^{-1}, giving a ratio to carbetamide of 1:2, a lower ratio than that used by Kaufman *et al.* (1970).

7.2 Methods

7.2.1 Screening for carbetamide extenders

A range of compounds were tested for their ability to inhibit enhanced carbetamide degradation. Many of these compounds had been previously shown to inhibit degradation of phenyl carbamate compounds (Kaufman *et al.*, 1970, Kaufman, 1977). Enhanced carbetamide degrading soil (field E) was treated with carbetamide (technical grade, 20 mg kg^{-1}) and one of the following: aldicarb (granules, 15 % a.i. kg^{-1}, Rhône-Poulenc), carbaryl (flowable concentrate, Table 3.2), chlorfenvinphos (emulsifiable concentrate, 500 g L^{-1} a.i., Shell), dazomet (granules, 98 % a.i. kg^{-1}, BASF), diazinon (emulsifiable concentrate, 800 g L^{-1}, Rhône-Poulenc), dietholate (emulsifiable concentrate, Zeneca), EPTC (emulsifiable concentrate, Table 3.2), malathion (emulsifiable concentrate, 500 g L^{-1}, Agchem) or phorate (granules, 10 % a.i. kg^{-1}, Agchem) added at 10 mg kg^{-1} soil. Granular products were added by weight. Soil from field E and D treated with carbetamide and additional water acted as positive and negative controls. Soil in each container was mixed and subsamples (25 g) were taken at 0 and 9 days. These were frozen until analysis by HPLC (section 3.4).

7.2.2 Influence of soil carbaryl concentration on carbetamide degradation

Different rates of carbaryl (5 - 40 mg kg^{-1}) were added to enhanced carbetamide degrading soil (field E, Table 3.1). Methods were otherwise identical to the previous experiment (section 7.2.1), with sampling at 0 and days after treatment.

7.2.3 Influence of time of carbaryl addition

Carbaryl (10 mg kg^{-1}) was added to enhanced carbetamide degrading soil (field E, Table 3.1) either at the same time as carbetamide or one week earlier. These treatments were compared to enhanced carbetamide degrading soil (field E) and the control soil (field D) treated with carbetamide only. Soil sub-samples (25 g) were taken at 0 and 14 days after addition of carbetamide. Remaining methods were as described in the previous experiment.

7.2.4 Effect of carbaryl in three soils with different carbetamide application histories

The effect of carbaryl (20 mg kg^{-1}) on the kinetics of carbetamide degradation (20 mg kg^{-1}) was evaluated in three soils with different histories of carbetamide application (fields D, E[II] and E[III]). Respectively, these fields were: i) previously untreated with carbetamide (D), ii) treated annually with carbetamide from 1989 until 1994 (E[III]) and iii),

treated with carbetamide from 1989 until 1992 (E[II]). Soils were collected during 1995. A modified 10 - 100 % acetonitrile gradient was used for the detection of both carbetamide and carbaryl at 233 nm (0 min, 10 %, 3 min, 32.5 %, 10 min 34.3 %, 12 min, 40.6 %, 16 min, 44.2 %, 24 min, 100 %). Methods were otherwise identical to those described in section 3.4. DT_{50} values for carbetamide and carbaryl were calculated by linear interpolation between data points.

7.2.5 Carbaryl as an extender in the field

The effectiveness of carbaryl as an extender in the field was examined using *L. rigidum* mortality as an indicator. The method for this experiment was essentially the same as for carbetamide/propyzamide rotation in the field (section 6.2.4).

Three plots (10 m x 2 m) in field B were initially treated with either carbetamide (2,100 g ha^{-1}), or a mixture of carbetamide (2,100 g a.i. ha^{-1}) and carbaryl (2,100 g ha^{-1}), or remained unsprayed. This was replicated four times as a randomised complete block design. Chemicals were applied using a hand held boom sprayer of 2 m boom width (section 3.3.1). Nine weeks later, 128 *Lolium rigidum* seedlings (1 leaf) were sown into each plot. When these plants reached the 3 - 4 leaf stage the plots were treated again with the same three treatments such that the nine possible treatment combinations were generated. After 28 days, the

presence or absence of new light green leaf growth was used as an indicator of plant survival or mortality respectively.

7.3 Results

7.3.1 Screening for carbetamide extenders

Carbaryl was the only compound which inhibited enhanced carbetamide degradation (Figure 7.1). Addition of 10 mg kg^{-1} carbaryl to the enhanced carbetamide degrading soil increased the percentage of carbetamide remaining (after 9 days) from 12 % to 42 %. In comparison, there was still 97 % remaining in the control soil. None of the other compounds tested inhibited enhanced carbetamide degradation, even though some of the compounds (e.g. diazinon and phorate) had been previously shown to inhibit chlorpropham degradation (Kaufman, 1977).

7.3.2 Influence of soil carbaryl concentration on carbetamide degradation

Increasing the soil carbaryl concentration resulted in increased levels of carbetamide remaining after 9 days (Figure 7.2). Each doubling of the carbaryl concentration from 5 to 10 to 20 mg kg^{-1} resulted in an approximate doubling of the amount of carbetamide remaining after 9 days. Carbaryl when added at 40 mg kg^{-1} resulted in a carbetamide level similar to that of the control soil after 9 days (90 % remaining). This result

Figure 7.1 Carbetamide remaining 9 days after addition to enhanced carbetamide degrading soil as influenced by addition of a range of compounds. Carbetamide remaining in soil previously untreated with carbetamide is presented for comparison. Vertical bars indicate standard errors of the mean.

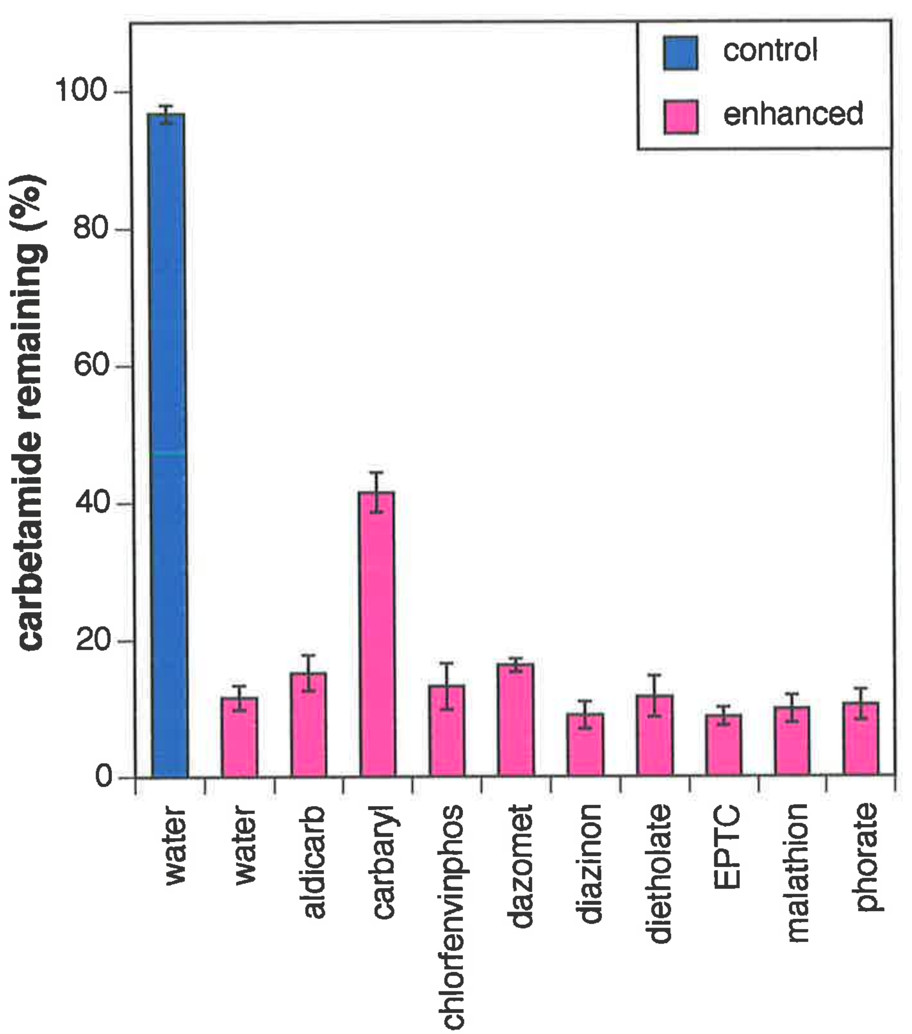
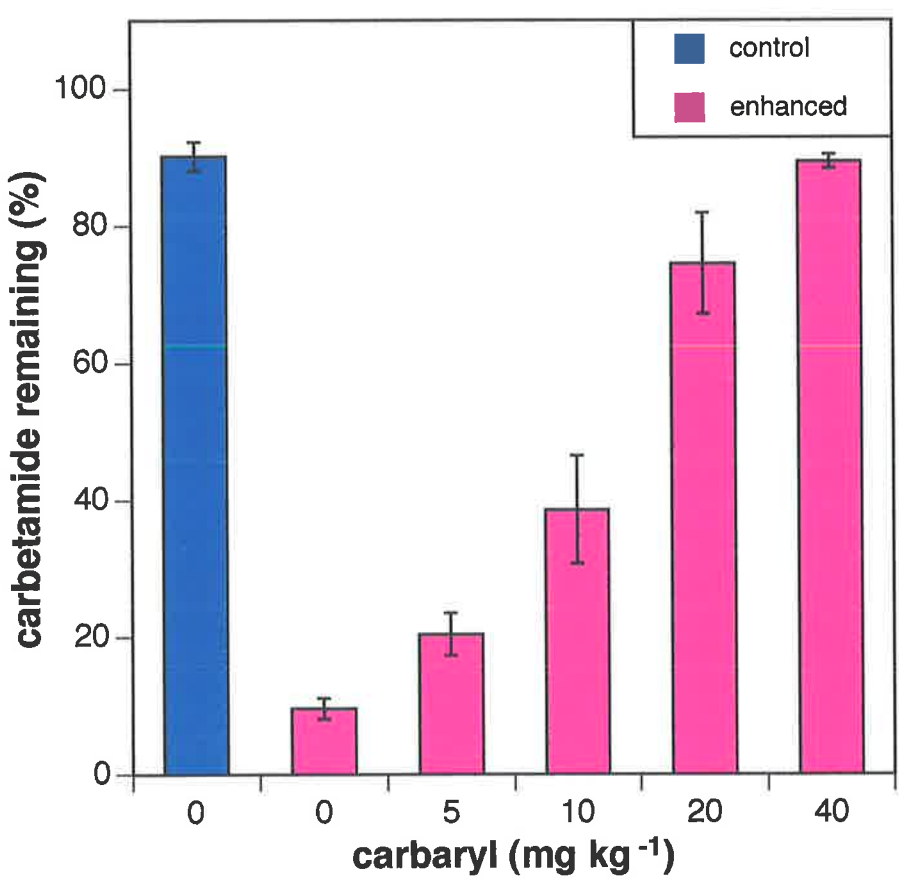

control
enhanced
carbetamide remaining (%)
100
80
60
40
20
0
water
water
aldicarb
carbaryl
chlorfenvinphos
dazomet
diazinon
dietholate
EPTC
malathion
phorate

Figure 7.2 Carbetamide remaining 9 days after addition as influenced by soil carbaryl concentration (0 - 40 mg kg^{-1}) in a soil enhanced for carbetamide degradation and compared to degradation in soil previously untreated with carbetamide (control). Vertical bars indicate standard errors of the mean.


control
enhanced
100
80
60
40
20
0
carbetamide remaining (%)
0
0
5
10
20
40
carbaryl (mg kg -1)

demonstrates that the inhibitory effect of carbaryl, in enhanced carbetamide degrading soil, is concentration dependant.

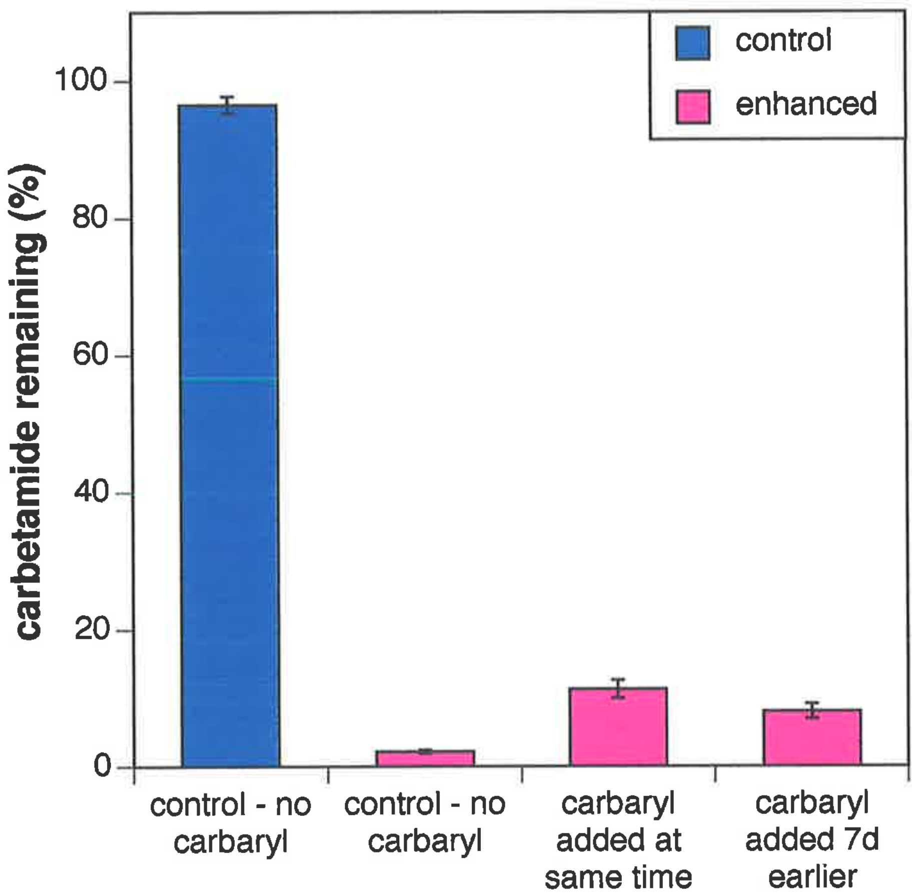
7.3.3 Influence of time of carbaryl addition

Timing of carbaryl addition was examined to determine if incubation of soil with carbaryl alone would reduce a soils ability to degrade carbetamide. Addition of carbaryl one week prior to the addition of carbetamide resulted in less effective inhibition of enhanced carbetamide degradation than if carbaryl was applied at the same time as the carbetamide (Figure 7.3). Therefore in this experimental system, it was most effective to add carbaryl concomitant with carbetamide.

7.3.4 Effect of carbaryl in three soils with different carbetamide application histories

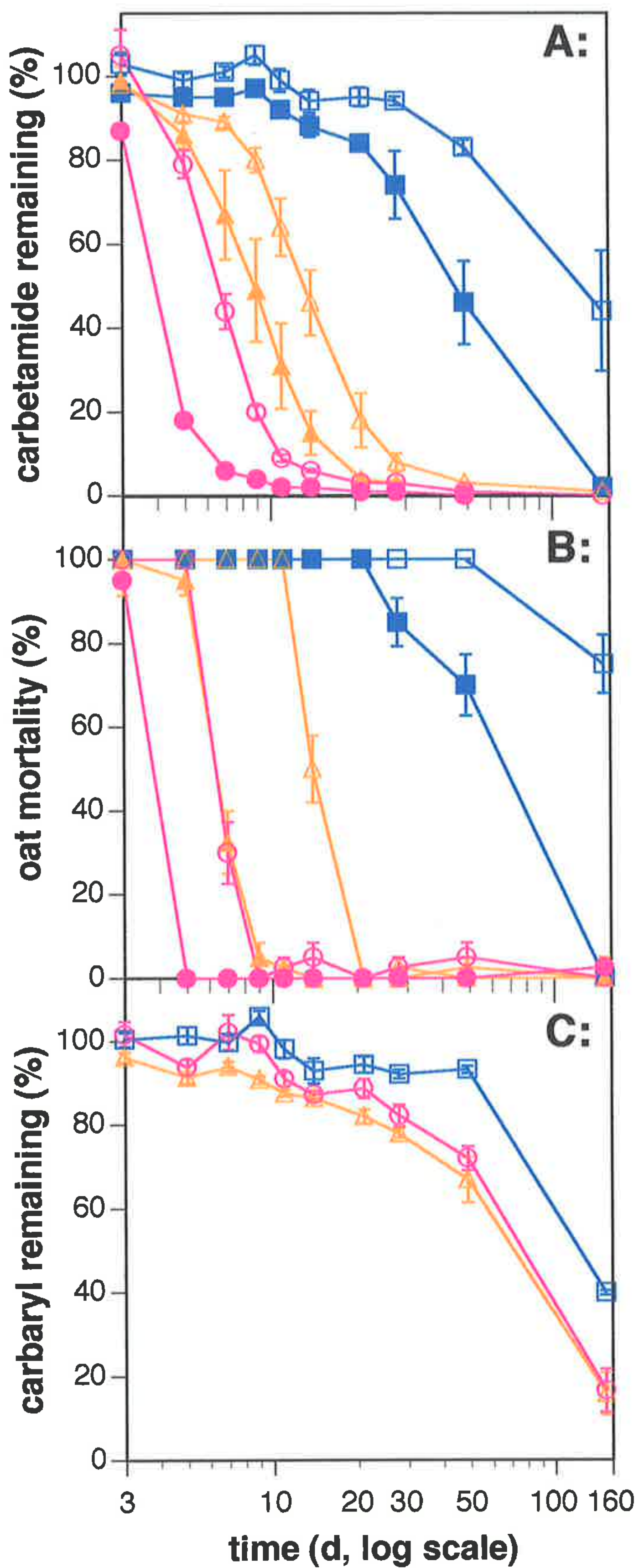
Carbaryl inhibited carbetamide degradation in all three soils tested (Figure 7.4A). Bioassay results (Figure 7.4B) closely reflected those obtained by chemical detection (HPLC), indicating that carbaryl did not influence the potency of carbetamide as a herbicide. Comparison of the effect of addition of carbaryl on carbetamide degradation kinetics are summarised in Table 7.1. By presentation of the results as DT_{50} ratios, it was evident that addition of carbaryl increased carbetamide persistence in the control soil (DT_{50} ratio 3.0) to a greater extent than in either soil treated previously with carbetamide (DT_{50} ratio 1.5 - 1.6). Extrapolation of this result

Figure 7.3 Carbetamide remaining 14 days after addition to a soil previously untreated with carbetamide, or an enhanced carbetamide degrading soil as influenced by addition of carbaryl (10 mg kg^{-1}) either at the same time as carbetamide, or 7 days prior to carbetamide addition. Vertical bars indicate standard errors of the mean.



control
enhanced
carbetamide remaining (%)
100
80
60
40
20
0
control - no carbaryl
control - no carbaryl
carbaryl added at same time
carbaryl added 7d earlier
time of carbaryl addition relative to carbetamide

Figure 7.4 Degradation of carbetamide in soil collected in 1995 as influenced by prior carbetamide application and/or addition of carbaryl in three soils. The soils were: previously untreated with carbetamide (■), treated with carbetamide annually from 1989 to 1994 (●), or treated with carbetamide annually from 1989 to 1992 (▲). Treatment with carbaryl is indicated by hollow symbols (□, O, Δ). Carbetamide and carbaryl degradation was measured by (A) HPLC and (B) oat bioassay, whilst carbaryl degradation (C) was measured by HPLC. Vertical bars indicate standard errors of the mean.


A:
B:
C:
carbetamide remaining (%)
oat mortality (%)
carbaryl remaining (%)
0
20
40
60
80
100
3
10
20
30
50
100
160
time (d, log scale)

to the field suggests that carbaryl may be more effective as a carbetamide extender in fields not previously treated with carbetamide. The results obtained with the oat bioassay, confirmed and closely reflected the chemical analysis (Figure 7.4A and B), suggesting that the insecticide carbaryl does not alter the potency of carbetamide toward germinating *Avena* seeds.

Carbaryl degradation was more rapid in the soils previously treated with carbetamide (Figure 7.4C). However, degradation was not sufficiently rapid in these soils to account for carbaryl being less effective as an inhibitor (Table 7.1). Possible explanations for the difference between the kinetics of carbaryl degradation in these soils could include differences in the soil physical and chemical properties, or due to prior crops pesticide history (carbaryl had not been applied previously to either field).

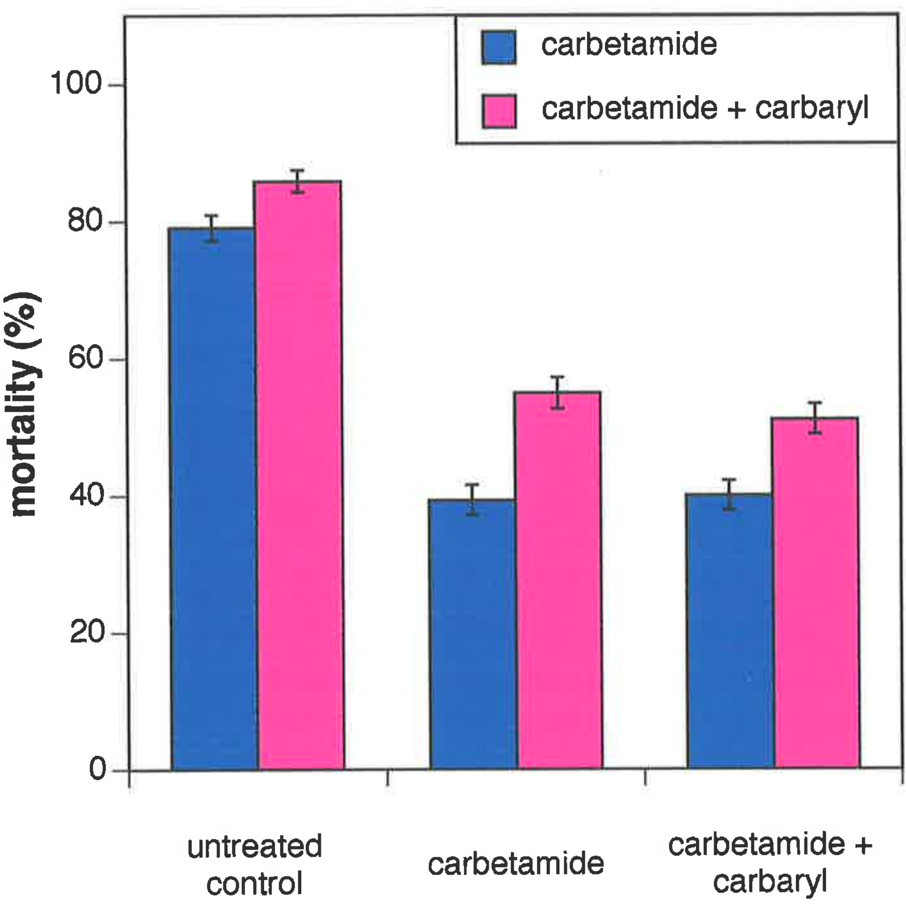
Table 7.1 Carbetamide DT_{50} in soil as influenced by carbetamide application history and addition of carbaryl. Soil samples were collected during 1995. Values were determined by linear interpolation between appropriate data points and are expressed in days.

field	years of carbetamide application	carbetamide alone	carbetamide plus carbaryl	DT_{50} ratio
D	none	46.0	136.2	3.0
E[II]	1989-92	8.9	13.3	1.5
E[III]	1989-94	4.1	6.7	1.6

7.3.5 Carbaryl as an extender in the field

Field applications of carbetamide plus carbaryl compared to carbetamide alone demonstrated that the mixture provided consistently greater *L. rigidum* control (Figure 7.5). Carbetamide application to plots which had not been treated earlier in the season resulted in 79 % mortality. Application of the carbetamide/carbaryl mixture provided 86 % control. Carbetamide application to plots treated earlier in the season with carbetamide (to induce enhanced carbetamide degradation) resulted in only 39 % mortality. Application of carbaryl in addition to carbetamide increased *L. rigidum* mortality to 55 %. Unlike the decrease in efficacy observed when carbetamide was applied to carbetamide pre-treated plots, no additional decrease in efficacy was observed when the carbetamide/carbaryl mixture

Figure 7.5 Influence of prior soil treatment with carbetamide or carbetamide plus carbaryl, or remaining unsprayed (control), on mortality of *L. rigidum* 28 days after treatment with carbetamide or carbetamide plus carbaryl. Vertical bars indicate standard errors of the mean.



carbetamide
carbetamide + carbaryl
100
80
60
40
20
0
mortality (%)
untreated control
carbetamide
carbetamide + carbaryl
soil pre-treatment

was applied to plots pre-treated earlier in the same season with the same mixture. Though application of carbaryl provided increased weed control, overall efficacy was still less than when carbetamide is applied to a soil for the first time (Figure 4.1).

L. rigidum plants which remained unsprayed at the time of second chemical treatment had 99 % survival (results not presented).

7.4 Discussion

Nine chemicals were examined for their ability to inhibit enhanced carbetamide degradation. Several of these compounds had been reported previously to inhibit phenyl carbamate degradation (Kaufman *et al.*, 1970). However, in this study, carbaryl was the only compound which inhibited enhanced carbetamide degradation (Figure 7.1). The use of granular formulations may have limited the availability of some of the compounds tested. However, diazinon was applied as an emulsified concentrate and displayed no effect. Dietholate, a commercial extender of EPTC persistence (Drost *et al.*, 1990), also had no observable effect on carbetamide persistence. Further work examined the potential of carbaryl as an extender in the field.

Carbaryl increased the persistence of carbetamide in soil, irrespective of whether the soil degraded carbetamide at an enhanced rate due to prior carbetamide application. Comparatively, the effect in a soil

previously untreated with carbetamide was greater than in soil previously treated with carbetamide (Table 7.1), with increases in the DT_{50} of 3.0- and 1.5-fold respectively. Cross enhancement from carbetamide to carbaryl was not the cause of this observed variation in effect (Figure 7.4C). The soil half life of carbaryl varied from 83 to 132 days. The reason for such variation was not examined. Laboratory persistence studies identified that increasing soil carbaryl concentration (Figure 7.2) and applying carbaryl at the same time as carbetamide increased the persistence of carbetamide (Figure 7.3). Under field conditions, application of carbetamide plus carbaryl increased *L. rigidum* mortality by 6.8 % to 15.6 % (Figure 7.5).

The field (Figure 7.5) and laboratory based studies (Figure 7.4) identified carbaryl as a moderately good extender of carbetamide persistence. However, in order for carbaryl to be used commercially as an extender, cost effectiveness must be demonstrated. Based upon current prices, carbaryl application at the rate used in the field assay costs approximately 50 % of the cost of carbetamide. Unless substantially improved efficacy from using carbaryl as an extender can be obtained, it is unlikely that carbaryl will be used commercially as an extender.

As with carbetamide, enhanced degradation of carbaryl can occur upon repeated application (Rajagopal *et al.*, 1983). Furthermore, bacterial cultures capable of carbaryl metabolism have been isolated from soil (Rajagopal *et al.*, 1984, Chapalamadugu and Chaudry, 1991). The

impact of enhanced carbaryl degradation was not evaluated in these experiments but could result in the decreased efficacy of a carbetamide/carbaryl mixture. This has also been demonstrated with repeated applications of the EPTC extender dietholate (Bean *et al.*, 1990, Moorman *et al.*, 1992).

8. STUDIES WITH THE CARBETAMIDE DEGRADING *RHODOCOCCUS* SP. ISOLATED FROM SOIL

8.1 Introduction

Since the development of synthetic pesticides, research has been conducted to determine the fate and effects of these compounds in the environment. Aspects which have been examined include the formation and toxicity of metabolites and the effects on soil micro-flora. Enhanced degradation of soil applied pesticides demonstrates the adaptive ability of the soil micro-flora to degrade xenobiotics. This phenomenon has led to the isolation of soil micro-organisms capable of degrading pesticides with diverse chemical structures. Felsot (1989) suggested that these organisms, or their degradative pathways, have the potential to assist in the remediation of concentrated pesticide wastes. Alternatively, herbicide degrading genes sourced from degrading organisms can be used in the development of herbicide tolerant crops (Oxtoby and Hughes, 1990). Such practical applications, and a greater understanding of enhanced pesticide degradation (Felsot, 1989), may be obtained by examining the biochemistry and genetics of pesticide degradation.

The purpose of research presented in this chapter was to examine further the microbiology, biochemistry and genetics of a carbetamide degrading bacterium isolated from soil which showed enhanced degradation of this herbicide (section 4.2.8). Specifically,

experiments were conducted to determine the catabolic route of degradation and the genetic basis of carbetamide degradation.

8.1.1 Biochemical Analysis

Biochemical studies of catabolism aim to describe the process as a series of catalytic transformations which lead to the formation of central metabolites (Houghton and Shanley, 1994). The production of central metabolites usually involves several steps. If any particular reaction in the pathway is limiting then metabolites can accumulate. For example, degradation of the herbicide metamitron in liquid culture by an isolated *Rhodococcus* sp. is associated with the production of an unidentified degradation product (Parekh *et al.*, 1994). The production of metabolites can also be observed in soil. In the current study, carbetamide metabolites were not evident by the analysis techniques used in either soil (section 3.4), or in pure culture of the isolated bacterium (section 4.2.8). This is likely to be due to rapid metabolism of metabolites once formed. The close correlation between carbetamide degradation and $^{14}CO_2$ evolution shown earlier (Figure 4.5), suggests that rapid degradation of carbetamide metabolites does occur. Also, evolution of $^{14}CO_2$ from soil treated with ring labelled ^{14}C-carbetamide suggests that the degrading organisms possess aromatic ring cleavage enzymes.

The degradation of aromatic compounds has been extensively studied (Aoki *et al.*, 1983a, Aoki *et al.*, 1983b, Kaminski *et al.*, 1983,

Harayama and Timmis, 1989). Non-halogenated aromatic compounds are ubiquitous in nature and are readily degraded. Methyl and halogenated ring compounds are present in the environment as degradation products of pesticides and by-products of manufacturing (Smith, 1994) and are more resilient to degradation (Harayama and Timmis, 1989). Aniline, an amine substituted aromatic compound, has been reported as a degradation product of carbetamide (Rouchaud *et al.*, 1988). If formed, it is likely that aniline would then be converted to catechol, as this later compound is viewed as a key intermediate in the degradation of aromatic compounds by bacteria (Harayama and Timmis, 1989, Fuchs *et al.*, 1994, Houghton and Shanley, 1994).

Chlorpropham is a compound likely to assist in elucidating the pathway of carbetamide degradation. Although able to degrade and grow upon propham, the isolated *Rhodococcus* sp. was unable to utilise chlorpropham as a growth substrate (Figure 8.4). The only structural difference between propham and chlorpropham is a chlorine substitution on the phenyl ring. Such substitutions are more resilient to degradation, suggesting that degradation products of chlorpropham are more likely to accumulate. The accumulation of dead-end metabolites from chlorpropham may provide useful information on the metabolic pathway for degradation of propham and carbetamide by the isolated bacterium.

8.1.2 Genetic Analysis

Plasmids are extra chromosomal DNA elements which can code for a variety of functions including catabolism of compounds such as pesticides (Sayler *et al.*, 1990). Other functions or properties which can be plasmid encoded include antibiotic resistance, pathogenicity, conjugation and symbiosis (Stanisich, 1988). As not all catabolic pathways are encoded on plasmids (Harayama and Timmis, 1989, Sayler *et al.*, 1990) determining the genetic location of catabolic genes is an important first step in genetic analysis. Stanisich (1988) summarised the experimental approaches which can be used to determine whether a phenotype is plasmid encoded. These include: i) spontaneous loss or curing, ii) transfer via conjugation or transduction, or transformation, and iii) examination of the DNA. Ideally, a combination of these methods should be used to confirm that a phenotype is plasmid encoded.

Spontaneous loss or curing is loss of a phenotype in the absence of appropriate selection (Stanisich, 1988). If the phenotype is plasmid encoded, the basis of such loss is assumed to be due to an error in plasmid replication or segregation into dividing cells (Stanisich, 1988). Frequency of curing can often be increased by a range of chemical and physical treatments, protoplast formation and incompatibility (reviewed by Stanisich, 1988). Effective curing agents are typically determined by trial and error.

Plasmid transfer is inferred when a phenotype is transferred from one bacterium to another. Conjugation, transduction and transformation are processes by which a plasmid may be transferred. Conjugation is a function encoded on many plasmids which allows genetic recombination to occur between cells, which can result in plasmid transfer. Transduction is the transfer of DNA between cells via a bacteriophage and transformation is the uptake of a plasmid directly from the surrounding medium (reviewed by Stanisich, 1988). All of these methods require a recipient strain. Suitable recipients can either be closely related species, as is presumed to occur in nature (Sayler *et al.*, 1990), or cured strains selected with an appropriate marker, for example antibiotic resistance (Tam *et al.*, 1987).

The aim of the genetic studies presented in this chapter were to determine whether the carbetamide degrading phenotype was plasmid encoded.

8.2 Methods

8.2.1 Degradation of ^{14}C ring labelled carbetamide by the isolated bacterium

Three 50 mL flasks containing 20 mL of MSM media with 50 mg L^{-1} yeast extract, 200 mg L^{-1} carbetamide and 4.2 x 10^5 Bq L^{-1} of ^{14}C ring labelled carbetamide were inoculated with 100 µL of the carbetamide degrading bacterium grown to late log phase on carbetamide

(200 mg L^{-1}) MSM media (section 3.6). Evolved $^{14}CO_2$ was trapped within sterile 2 mL microfuge tubes (with lids removed) which contained 1 mL of 0.5 M NaOH. Microfuge tubes were supported inside 5 mL glass vials, to prevent contamination from the medium. Flasks were sealed with laboratory film to minimise gas exchange. Three uninoculated flasks and a flask without ^{14}C, served as degradation and background ^{14}C controls respectively.

Sampling of flasks occurred from 0 to 36 hours after inoculation. At each time point 100 µL of media was removed to determine total ^{14}C remaining in the culture medium, 2 mL of media was removed to determine the percentage carbetamide remaining and ^{14}C incorporated into the bacteria, and CO_2 traps were changed. The 2 mL samples were centrifuged for 5 minutes in a microfuge (12,566xg) to pellet the bacteria. The supernatant was transferred to a 5 mL glass vial to which 500 µL of methanol was added to prevent bacterial growth. Bacterial pellets were resuspended in 500 µL of water and then centrifuged as described above. The supernatant was added to the initial supernatant and the bacterial pellet was resuspended in scintillation fluid. Degradation of carbetamide was monitored in the bacteria-free supernatant using reverse phase HPLC with a solid cell ^{14}C detector (section 3.7). The supernatant (1 mL) was injected into the HPLC system and separation was performed using the gradient described in section 3.5. Subsamples (100 µL) of the bacteria-free supernatant were counted using liquid scintillation to determine HPLC ^{14}C detector efficiency. Corrections were also made for the impurities present in

the ^{14}C labelled carbetamide. ^{14}C measurements were performed as described in the general methods (section 3.7).

8.2.2 Degradation of carbetamide and growth of the bacterium in the presence of alternative carbon sources

Carbetamide degradation and growth of the bacterium was monitored in flasks containing 50 mL of MSM media, with and without 50 mg L^{-1} yeast extract, and pyruvate, acetate or succinate as additional carbon sources. Pyruvate (Ajax), acetate (Ajax) and succinate (BDH) were added as filter sterilised stock solutions of the appropriate sodium salt to give a final concentration of 5 mM. Flasks were inoculated with 100 µL of the carbetamide degrading bacterium grown to late log phase in MSM media containing 200 mg L^{-1} carbetamide and 50 mg L^{-1} yeast extract. Sampling was performed from 0 to 67 hours after inoculation. Subsamples were removed to determine carbetamide degradation and growth of the bacterium. These were determined by HPLC and absorption at 600 nm, respectively, by the methods described in sections 3.5 and 3.6.3.

8.2.3 Metabolite production from chlorpropham

In order to increase the likelihood of detecting metabolites formed from chlorpropham, experiments were conducted with carbetamide cultures grown to stationary phase in carbetamide MSM media

(section 3.6.1). Cultures of the isolated bacterium (5 mL) grown to stationary phase on carbetamide (500 mg L^{-1}), were treated with 200 mg L^{-1} chlorpropham. After incubation at 28°C for 24 hours on a rotary shaker, 15 mL of acetonitrile was added to each culture. Subsamples (1.5 mL) were removed from each culture, centrifuged for 5 minutes in a microfuge (12,566xg) and 800 µL was loaded into autosampler vials. Samples (50 µL) were chromatographed by reverse phase HPLC using a linear acetonitrile:water gradient (10 - 100 %) over 30 minutes at a flow rate of 1.2 mL min^{-1} with the detection at 233 nm. Standards of chlorpropham (technical grade, Table 3.2) and 3-chloroaniline (Sigma) were used to identify compounds formed.

8.2.4 Growth on aniline and phenol, and chlorine or methyl substituted analogues of these compounds

Experiments were conducted to determine the ability of the isolated bacterium to utilise aniline and phenol, and chloro- or methyl-substituted analogues, as growth substrates. Whilst the isolated bacterium could utilise propham as a carbon source, a halogenated analogue was clearly toxic (Figure 6.3). Results presented in Figure 8.3 demonstrate that the bacteria was capable of degrading chlorpropham to 3-chloroaniline. To examine whether 3-chloroaniline and similar compounds could be used as growth substrates, flasks containing 50 mL of MSM media (plus 50 mg L^{-1}

yeast extract) were treated with aniline, phenol, their 3 or 4-chloro substituents or 3-methylaniline at 200 mg L^{-1}. Flasks were inoculated with 100 μL of the carbetamide degrading bacterium grown to late log phase and then incubated at 28°C. Flasks were subsampled from 0 to 72 hours after inoculation to determine growth by optical density (section 3.6.3). Drop plate counts (section 3.6.3) were performed 72 hours after inoculation to determine the colony forming units per mL.

8.2.5 Effect of inhibitory compounds on growth of the isolated bacterium and carbetamide degradation

The effect of chlorpropham, 3-chloroaniline, 3-chlorophenol and carbaryl on growth and carbetamide degradation by the isolated bacterium was examined. Flasks containing 50 mL of MSM media (section 3.6.1) plus carbetamide (200 mg L^{-1}) and varying concentrations of chlorpropham, 3-chloroaniline, 3-chlorophenol or carbaryl (0.1 - 100 mg L^{-1}) were inoculated with 100 μL of a culture of the carbetamide degrading bacterium grown to late log phase in carbetamide MSM media. Growth and carbetamide degradation was measured at various time up to 96 hours after inoculation by optical density and HPLC (section 3.5).

8.2.6 Selection of cured strains

The following method was used to isolate the cured strains denoted CI and CII. Stationary phase cultures of the carbetamide degrading bacterium, grown in carbetamide MSM media, were used to inoculate (100 µL) flasks containing 50 mL of nutrient broth. After reaching late log phase (18 - 24 h at 28°C), these cultures were used to inoculate (100 µL) flasks containing the same medium. After reaching late log phase, dilutions (10 fold) of this culture were made and aliquots (100 µL) were spread onto nutrient agar. Colonies arising from individual cells were then plated onto nutrient agar and then replica plated onto carbetamide (500 mg L^{-1}) MSM agar. Cured strains exhibited poor growth on this media (Figure 8.7). In each case only one cured strain was identified per 500 colonies tested.

A further cured strain (denoted CIII) was isolated, the only modification to the method above involved incubation of the nutrient broth cultures at 33°C instead of 28°C. This increased temperature did not appear to affect curing rate. Acridine orange (5 mg mL^{-1}), SDS (50 mg mL^{-1}) and mitomycin C (100 µg L^{-1}) were used in an attempt to increase the curing rate. However, the bacteria were sensitive to these compounds and no cured strains were produced.

8.2.7 Confirmation that cured strains were derived from the wild type strain

To confirm that the cured strain CI was indeed derived from the original isolate, analysis of its 16S rRNA coding region and substrate utilisation profile were performed. A 1,500 bp region of the 16s rRNA coding region from the wild type (X) and cured strain (CI) was PCR amplified and digested with either *Hpa* II or *Rsa* I (Promega) according to the manufacturer's instructions (section 4.2.13). Restriction fragments were then separated by agarose gel electrophoresis (2.0 % agarose, 1X TBE), stained with ethidium bromide and visualised under UV light. Substrate utilisation comparison was made using Biolog® SFP plates according to the manufacturer's directions (section 4.2.11).

8.2.8 Confirmation of loss of phenotype in cured strains

Cultures of the wild type (X) and three cured strains (CI, CII, CIII) grown to stationary phase in 5 mL of nutrient broth (with shaking at 28°C) were used as inoculum (100 µL) for 5 mL of MSM (section 3.6) to which either carbetamide, propham, chlorpropham or aniline was added (200 mg L^{-1}, in methanol). Cultures were incubated at 28°C on a bench-top shaker at 120 rpm for 48 hours. Acetonitrile (15 mL) was then added to dilute and dissolve compounds remaining in each vial. The amount of each compound remaining for each culture was determined by HPLC and

expressed as the percentage remaining in control cultures (no bacteria) after 48 hours (minimal losses occurred in uninoculated control flasks). Results were calculated upon the basis of three replicates.

8.2.9 Plasmid Extraction from carbetamide degrading and cured strains

Plasmid DNA was isolated using a large scale preparative method (Schreiner *et al.*, 1991). Late log phase cultures (50 mL) of bacteria grown in nutrient broth (Oxoid) were centrifuged at 11,951xg for 10 minutes (Sorvall SS34 rotor). The bacterial pellet was resuspended in 6.7 % sucrose (50 mM Tris-HCl, 1 mM EDTA, pH 8.0) to which 2.0 mL of lysozyme (40 mg mL^{-1}, 10 mM Tris-HCl, 50 mM EDTA, pH 8.0) was added. After 15 minutes incubation at 37°C, 970 µL of 250 mM EDTA (50 mM Tris-HCl, pH 8.0) was added and tubes were incubated a further 15 minutes at 37°C. Lysis was achieved by the addition of 600 µL SDS (20 % w/v, 50 mM Tris-HCl, 20 mM EDTA, pH 8.0) and gentle rocking at room temperature for 30 minutes. Chromosomal DNA and membranes were denatured by the addition of 560 µL of freshly prepared NaOH (3 M) and mixed by gentle rocking for 10 minutes. The pH of the solution was reduced by addition of 1 mL of Tris-HCl (2.0 M, pH 7.0) and then gentle rocking for 5 minutes. Chromosomal DNA and membranes were precipitated by the addition of 2.1 mL SDS (20 % w/v, 50 mM Tris-HCl, 20 mM EDTA, pH 8.0) and 4.2 mL of ice cold NaCl (5.0 M) and incubation at 4°C

overnight. After a clearing spin (4°C, 48,000xg, 90 min), the supernatant was transferred to a clean centrifuge tube and plasmid DNA precipitated by addition of 1 volume cold isopropanol (-20°C) and incubation at -20°C for 30 minutes. DNA was pelleted by centrifugation (4°C, 10,000xg, 20 min) and, after discarding the supernatant, was resuspended in 5 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) assisted by gentle rocking for 30 minutes. DNA was again precipitated by addition of 1 volume cold isopropanol (-20°C), incubation at -20°C and centrifugation (as described above). Finally DNA was resuspended in 0.5 to 1.5 mL of TE buffer.

8.2.10 Electrophoresis

Initially, plasmid extracts were separated using submerged agarose gel electrophoresis, however separation and visualisation of plasmids was inconsistent when extracts were not digested. Electrophoresis of plasmid extracts digested with the restriction enzymes *Hin*d III and *Bam* HI demonstrated the loss of several large DNA fragments in preparations from the cured strain CI (results not shown). Uncut plasmid DNA from the wild-type strain and the three cured derivatives was then separated using Pulse Field Gel Electrophoresis (PFGE, CHEF DNRIII, BioRad). Plasmid extracts (25 - 40 µL) were loaded onto an agarose gel (150 mL, 1 %) and separated with a field strength of 6 V cm^{-1} and pulse times linearly ramped from 4 to 20 seconds over 14 hours. TBE (0.5 X) was used as the electrophoresis buffer which was maintained at 14°C. Concatemers of lambda cI857 Sam7 DNA (48.5 kb, BioRad) was used as a

size marker. After separation, DNA was stained with ethidum bromide (0.5 μg L^{-1}, 30 min), destained in water (1 - 2 h) and then visualised under UV light.

To determine the approximate size of plasmids harboured in strains examined this study, plasmids of known size from *Agrobacterium radiobacter* (strain K84) were used (Clare *et al.*, 1990). This was performed as the linear DNA fragments which were used for markers in this study have different mobility to circular plasmid DNA (Picardeau and Vincent, 1997). Plasmids preparations of *A. radiobacter* (strain K84) containing plasmids of 47.7 kb, 173 kb and approximately 300-400 kb (Farrand *et al.*, 1985, Clare *et al.* 1990, Donner *et al.*, 1993) were supplied by C. Dandie (Flinders University). After separation by PFGE, mobility was measured and the data was best described by a linear relationship (as was the linear DNA marker). This relationship was used to estimate the size of plasmids in the isolated carbetamide degrading *Rhodococcus* sp.

8.2.11 Selection of antibiotic resistant strains of bacteria

In order to perform matings and electroporation, antibiotic resistant strains of the wild type (X) and cured type (CI) were selected. Each strain was grown to stationary phase in nutrient broth (Oxoid) and 100 μL of each resulting culture was spread onto nutrient agar (Oxoid) containing

streptomycin (1, 10 and 50 μg mL^{-1}), rifampycin (1, 10 and 50 μg mL^{-1}), naladixic acid (2.5, 25 and 250 μg mL^{-1}), chloramphenicol (2.5, 25 and 250 μg mL^{-1}) or gentamycin (1, 10, 50 μg mL^{-1}). Dilution drop plate counts (section 3.6.3) were also performed to determine CFU inoculated onto each plate. Plates were incubated at 28°C for 48 h and scored for the number of colonies present on each plate. Actively growing colonies on antibiotic plates were streaked onto nutrient agar and solid MSM with carbetamide 500 mg L^{-1} with the range of antibiotics to confirm resistant strains and determine cross resistance and compatibility with carbetamide degradation.

8.2.12 Conjugation between carbetamide degrading and cured strains

Matings were attempted between a streptomycin resistant wild type strain and rifampycin resistant cured strain. Cultures (5 mL, nutrient broth) of these strains were grown for 24 h at 28°C. Subsamples (1.5 mL) were centrifuged for 5 minutes in a microfuge (12,566xg), the supernatant discarded and the pellet resuspended in 300 μL of sterile nutrient broth (Oxoid). Aliquots (50 μL) of each strain were added to sterile filters (25 mm, 0.2 μm nylon, Whatman) on nutrient agar (Oxoid) plates, with aliquots of each strain also added to separate filters to act as controls. Plates containing filter papers were then incubated at 28°C for 48 hours. Cells on the filter papers were resuspended in 2 mL of sterile saline buffer (section 3.6.4)

and serial dilutions (10-fold) were performed in sterile saline buffer. Aliquots (100 µL) of appropriate dilutions were placed onto nutrient agar or carbetamide (500 mg L^{-1}) MSM agar, with antibiotics as necessary.

8.2.13 Plasmid transformation into cured strain by electroporation

Cells were prepared for electroporation based upon the method of Shao *et al.* (1995). The cured strain CI, with resistance to the antibiotic rifampycin, was grown in 5 mL nutrient broth (Oxoid) to stationary phase and then 1 mL was used to inoculate 200 mL of the same media. Cells were harvested after reaching an OD_{600} of 0.8 by centrifugation (4,068xg, 15 minutes, 4°C, Sorvall GSA rotor). After discarding the supernatant, the cells were washed in 100 mL of ice-cold sterile water, centrifuged as described above and then washed twice in 100 mL of ice cold 10 % glycerol. After a final centrifugation, cells were resuspended in 4 mL of 10 % glycerol (50-fold concentration of cells). Aliquots of cells (200 µL) were dispensed into microcentrifuge tubes and either placed on ice, for immediate use, or were stored at -70°C.

For electroporation, 40 µL of prepared cells were combined with 10 µL of total plasmid extract from the wild-type carbetamide degrading isolate (X) (section 8.2.9) or TE buffer (control) in a microcentrifuge tube. Half (25 µL) of this mixture was suspended between the electrodes of an

electrocuvette which was then placed on ice for 10 minutes. The electric pulse was supplied by a BRL Technologies Cell Porator with a voltage booster (BRL). Settings were capacitance 330 μF, resistance of 4 Ω and 300 mV. After electroporation, the electrocuvette was placed on ice for 10 minutes. Cells were then diluted with 0.6 mL of nutrient broth and incubated at 28°C for 3 hours. Aliquots of cells (100 μL) were plated onto solid MSM media with carbetamide 500 mg L^{-1} (no rifampycin) and incubated at 28°C for 4 days. Total viable cells were determined by serial dilution and drop plate counts (section 3.6.3). Transformed cell lines were streaked onto nutrient agar containing 10 μg mL^{-1} rifampycin and solid carbetamide (500 mg L^{-1}) MSM media and growth was compared to appropriate cell lines. Plasmid extracts of electroporated strains were performed and analysed as described previously (section 8.2.9), the only difference in method was that PFGE pulse time were ramped from 7 to 20 seconds (instead of 4 to 20 seconds).

8.3 Results

8.3.1 Degradation of ^{14}C ring labelled carbetamide by the isolated bacterium

The carbetamide degrading bacterium isolated in this study was capable of mineralising a significant portion of the phenyl ring of carbetamide to CO_2. ^{14}C ring labelled carbetamide was undetectable

36 hours after inoculation with the isolated bacterium (Figure 8.1), with 24 % of the ^{14}C incorporated into the bacterial mass and 24 % converted into $^{14}CO_2$. A significant proportion of ^{14}C (32.8 %) was converted into compound(s) which could not be identified in the culture medium by HPLC (no ^{14}C detector response). After 36 hours 19 % of the added ^{14}C was lost from the system. This may have been lost as $^{14}CO_2$, or may represent inefficiency ^{14}C detection (for example for ^{14}C incorporated into bacterial cells). No appreciable loss of ^{14}C or carbetamide degradation was detected in uninoculated control flasks (results not presented). These results established that the isolated *Rhodococcus* sp. mineralised a significant proportion of the carbon within the phenyl ring of carbetamide to $^{14}CO_2$, indicating that the isolated bacterium possesses ring cleavage enzymes.

Results obtained with the isolated bacterium (Figure 8.1) were similar to those in soil (Figure 4.5). During the period of active carbetamide degradation (0 to 5 days from addition) in enhanced carbetamide degrading soil, 30 % of the applied radioactivity was evolved as $^{14}CO_2$, a comparable proportion to that evolved *in vitro* by the isolated bacterium (24 %).

8.3.2 Degradation of carbetamide and growth of the bacterium in the presence of alternative carbon sources

The patterns of carbetamide degradation and growth of the bacterium in the presence of alternative carbon sources were markedly

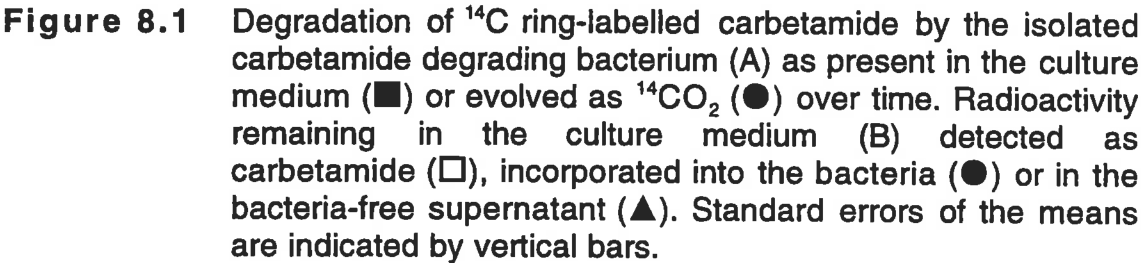
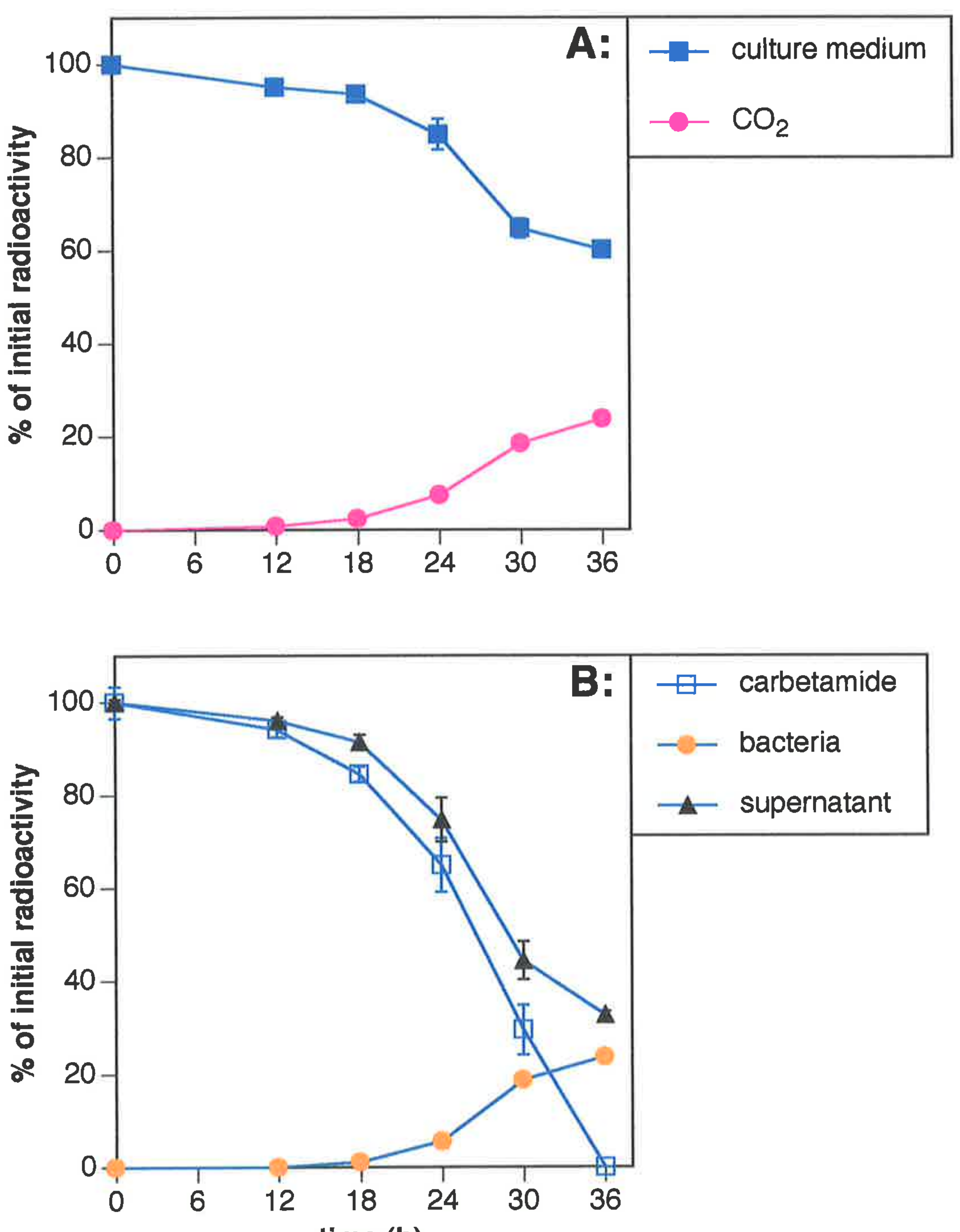


Figure 8.1 Degradation of ^{14}C ring-labelled carbetamide by the isolated carbetamide degrading bacterium (A) as present in the culture medium (■) or evolved as $^{14}CO_2$ (●) over time. Radioactivity remaining in the culture medium (B) detected as carbetamide (□), incorporated into the bacteria (●) or in the bacteria-free supernatant (▲). Standard errors of the means are indicated by vertical bars.

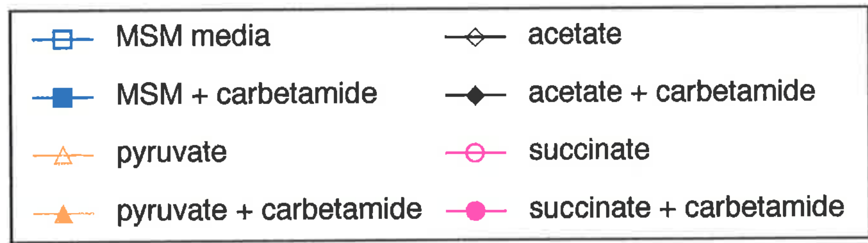

A:
culture medium
CO_2
% of initial radioactivity
100
80
60
40
20
0
0
6
12
18
24
30
36
B:
carbetamide
bacteria
supernatant
% of initial radioactivity
100
80
60
40
20
0
0
6
12
18
24
30
36
time (h)

different depending upon whether yeast extract was present in the media (Figure 8.2). In flasks without additional pyruvate, acetate or succinate, addition of yeast extract increased the rate of carbetamide degradation and the growth of degrading organisms. In the absence of yeast extract (Figure 8.2A), addition of pyruvate had no distinguishable effect on either carbetamide degradation or growth of the bacterium. Addition of acetate to similar flasks slowed carbetamide degradation and growth of the bacterium. With addition of succinate, carbetamide degradation was further slowed and no detectable growth of the bacterium occurred using the method employed.

In contrast to the results described above, addition of pyruvate, acetate and succinate to flasks to which 50 mg L^{-1} yeast extract had been added (Figure 8.2B) stimulated growth but had only a slight effect on carbetamide degradation. In these flasks, carbetamide degradation was approximately as rapid as in flasks not amended with pyruvate, acetate and succinate. In comparison to growth on carbetamide, growth on pyruvate alone was slower but growth on acetate or succinate alone was slightly faster. However, growth on pyruvate, whilst slower than on carbetamide, was greater after 66 hours than on carbetamide. Addition of both carbetamide and the additional carbon sources resulted in proportionally increased total growth but had little effect on growth rate.

No evidence of catabolic repression of carbetamide degradation was observed in the presence of alternative carbon sources. Catabolic repression is where an alternative carbon source, such as

Figure 8.2 Degradation of carbetamide and growth of the isolated bacterium as influenced by additional carbon sources (pyruvate, acetate or succinate) and addition of 50 mg L^{-1} yeast extract. Values are shown for one flask, the experiment was repeated and similar results were obtained.


A: without yeast extract
B: yeast extract added
OD (600 nm, log scale)
1.00
0.80
0.60
0.40
0.20
0.10
0.04
carbetamide remaining (%)
100
80
60
40
20
0
0
12
24
36
48
60
time (h)
MSM media
MSM + carbetamide
pyruvate
pyruvate + carbetamide
acetate
acetate + carbetamide
succinate
succinate + carbetamide

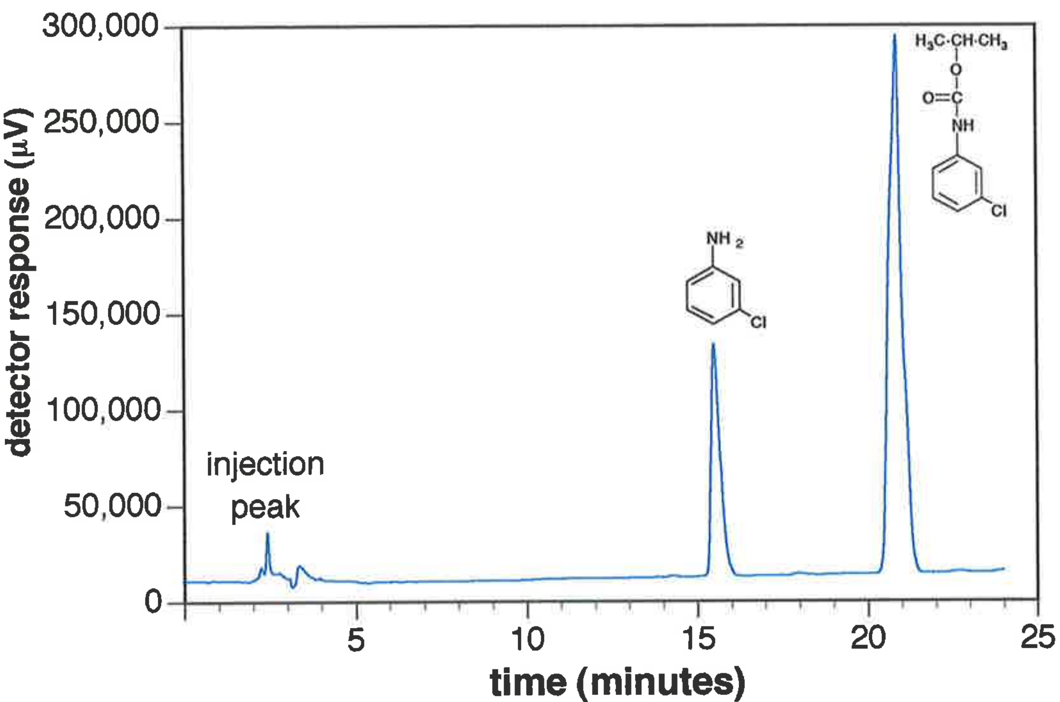
pyruvate, acetate or succinate is metabolised in preference to the compound of interest (i.e. carbetamide). When yeast extract was present in the media, addition of succinate, acetate or pyruvate did not slow carbetamide degradation. Whilst in the absence of yeast extract, addition of acetate or succinate slowed carbetamide degradation but also inhibited growth.

Results presented in Figure 8.2 indicate that in the presence of alternative carbon sources, degradation of carbetamide is not inhibited by preferential use of alternative growth substrates. Addition of low concentration of yeast extract had a dramatic influence on growth of the isolated bacterium and carbetamide degradation (Figure 8.2A vs. B, note log scale). Carbon sources were added during this experiment but had little effect on growth (Figure 8.2A). Therefore, yeast extract may be a source of otherwise limiting micronutrients and/or essential amino acids.

8.3.3 Metabolite production from chlorpropham

The isolated *Rhodococcus* sp. is unable to utilise chlorpropham as a growth substrate (Figure 6.2, Figure 6.3). Chromatography of cultures grown to late log phase on carbetamide and then treated with chlorpropham demonstrated the production of a metabolite which eluted at 15.5 minutes (Figure 8.3). This metabolite coeluted with 3-chloroaniline. This result suggests that the isolated bacterium transformed chlorpropham to form 3-chloroaniline and implies that carbetamide is degraded to aniline.

Figure 8.3 HPLC chromatogram of chlorpropham and metabolite formed after incubation with stationary phase cultures of the isolated carbetamide degrading bacterium. Structures shown indicate standards (chlorpropham and 3-chloroaniline) with identical retention times.



300,000
250,000
200,000
150,000
100,000
50,000
0
detector response (µV)
injection
peak
NH 2
Cl
H3C-CH-CH3
O
O=C
NH
Cl
5
10
15
20
25
time (minutes)

The formation of aniline as a metabolite of carbetamide in soil has been reported previously (Rouchaud *et al.*, 1988). In the current study, aniline was not detected as a metabolite either after soil incubation or with the isolated bacterium *in vitro*. However, cultures of the isolated bacterium grown to stationary phase on carbetamide and then treated with chlorpropham, converted the chlorpropham to 3-chloroaniline, suggesting that carbetamide, as with many other phenyl carbamate herbicides (Kearney and Kaufman, 1965, Kaufman, 1967, Marty *et al.*, 1986, Chapalamadugu and Chaudry, 1992, Pohlenz *et al.*, 1992), is initially degraded at the ester linkage by the isolated *Rhodococcus* sp.

8.3.4 Growth on aniline and phenol, and chlorine or methyl substituted analogues of these compounds

The carbetamide degrading *Rhodococcus* sp., isolated in this study (section 4.2.8), was also able to grow on both aniline and phenol, though a lag phase was observed before growth occurred on phenol (Figure 8.4). The observed lag phase may represent the time taken to induce the appropriate enzymes for phenol degradation, or may be due to toxicity of the phenol (Straube *et al.*, 1990). In contrast, growth was not observed on these same compounds when substituted with chlorine or a methyl group (Figure 8.4). Poor growth, less than the methanol control, was observed with 3-chlorophenol, 3-chloroaniline, 4-chlorophenol, 4-chloroaniline and 3-methylaniline. Drop-plate counts confirmed that the

Figure 8.4 Growth of the carbetamide degrading bacterium on aniline and phenol, and a range of chlorinated/methylated analogues as measured by absorption at 600nm.

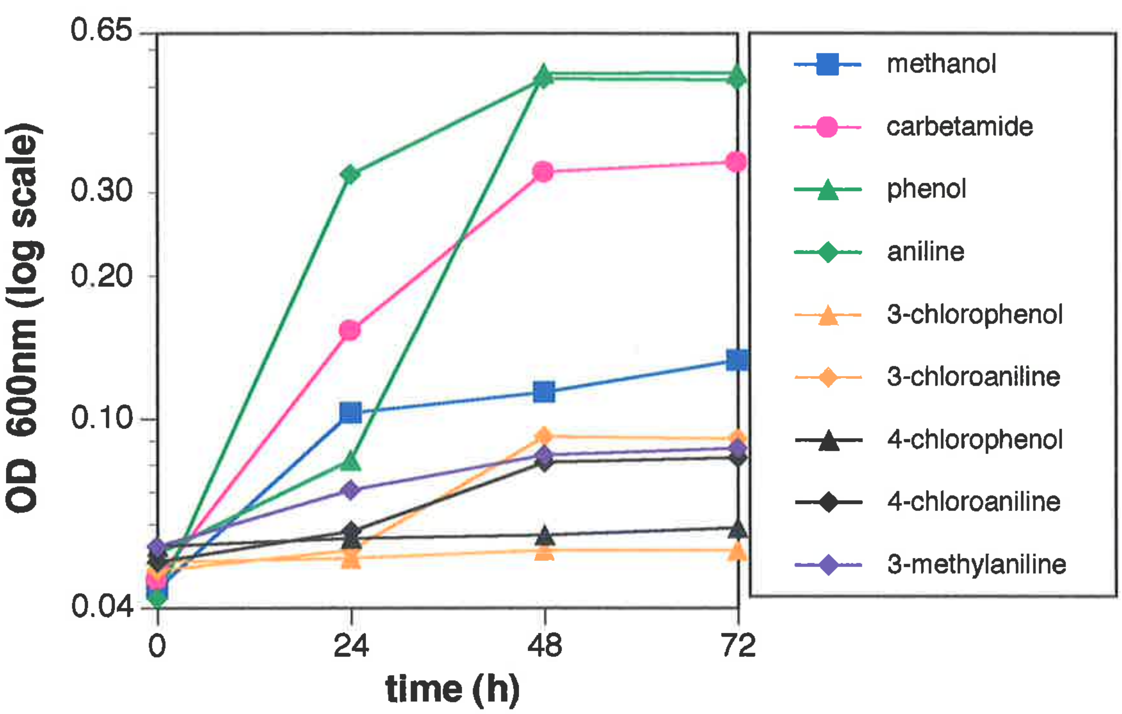
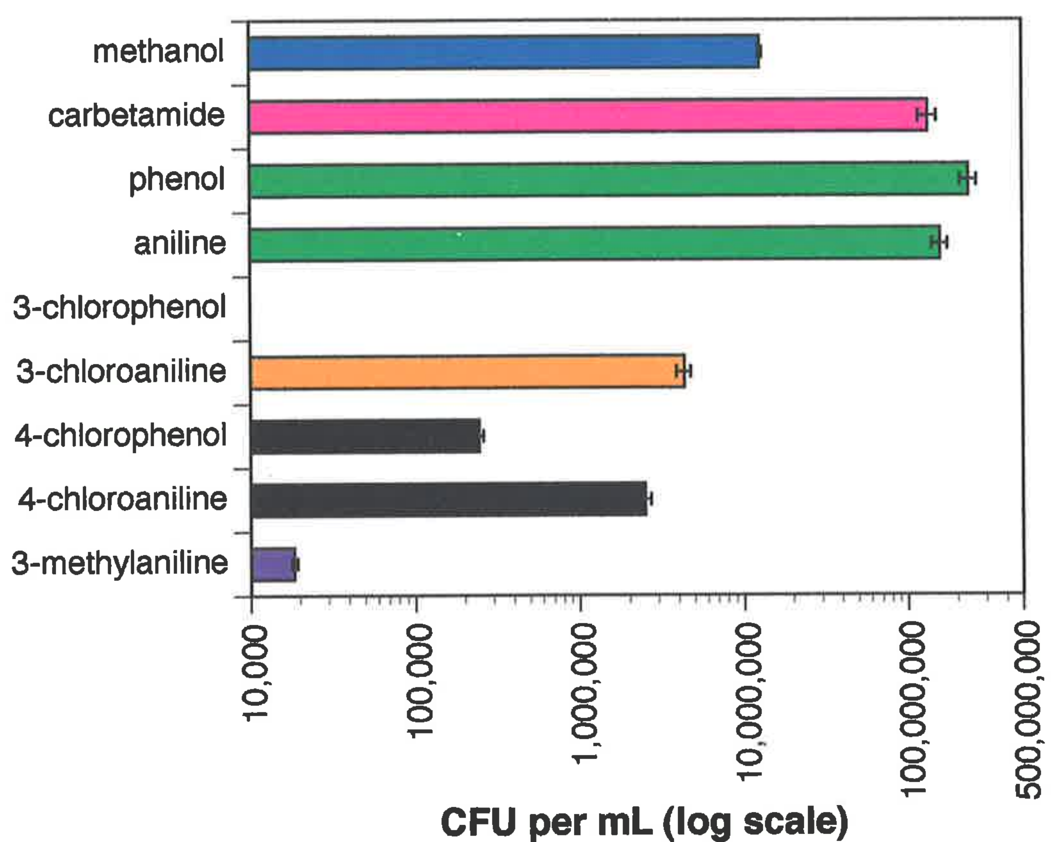

OD 600nm (log scale)
0.65
0.30
0.20
0.10
0.04
0
24
48
72
time (h)
methanol
carbetamide
phenol
aniline
3-chlorophenol
3-chloroaniline
4-chlorophenol
4-chloroaniline
3-methylaniline

Figure 8.5 Colony forming units (CFU) capable of growth on nutrient agar 72 hours after inoculation of flasks containing aniline and phenol, and their chlorinated/methylated derivatives. Horizontal bars indicate standard errors of the mean.


methanol
carbetamide
phenol
aniline
3-chlorophenol
3-chloroaniline
4-chlorophenol
4-chloroaniline
3-methylaniline
10,000
100,000
1,000,000
10,000,000
100,000,000
500,000,000
CFU per mL (log scale)

chlorinated compounds exhibited varying toxicity to the isolated bacterium (Figure 8.5). The most toxic compound was 3-chlorophenol, with no viable colonies observed at any dilution (Figure 8.5). Results indicate that although the *Rhodococcus* isolate is capable of initially degrading chlorpropham to 3-chloroaniline (Figure 8.3), the isolated bacterium appears unable to usefully degrade this metabolite (Figure 8.4, Figure 8.5). The apparent toxicity the substituted aromatic compounds tested is possibly due to formation of lethal and inhibitory metabolites (Bartels *et al.*, 1984).

8.3.5 Effect of inhibitory compounds on growth of the isolated bacterium and carbetamide degradation

Inhibition of both growth and carbetamide degradation was evident with addition of 3-chloroaniline, 3-chlorophenol, chlorpropham and carbaryl, though a unique concentration-dependent result was observed with each compound (Figure 8.6). Based upon the lowest concentration required to completely inhibit growth and degradation, inhibition was greatest with carbaryl > 3-chlorophenol > chlorpropham > 3-chloroaniline. At the highest concentration used (100 mg L^{-1}), all the compounds, except 3-chloroaniline, completely inhibited growth and little carbetamide degradation occurred. No metabolites were evident on HPLC chromatograms as a result of treatment with any of the inhibitory compounds.

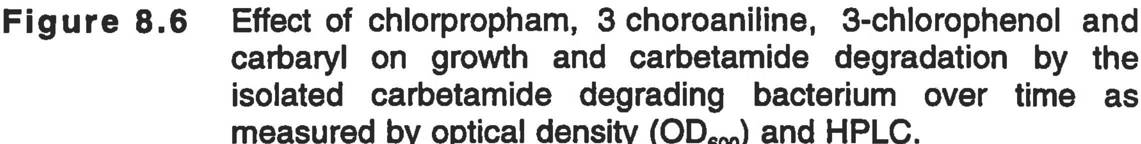
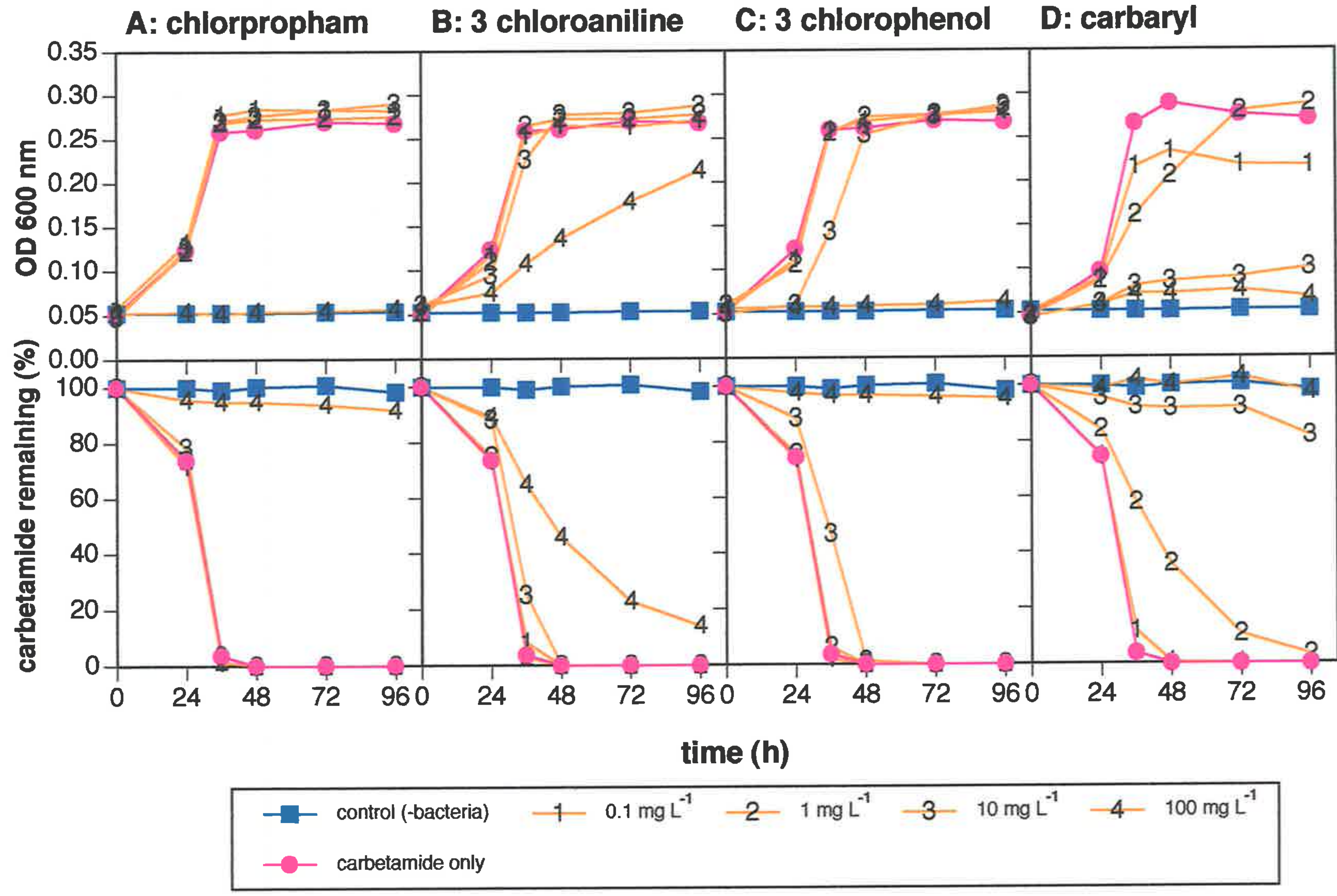


Figure 8.6 Effect of chlorpropham, 3 choroaniline, 3-chlorophenol and carbaryl on growth and carbetamide degradation by the isolated carbetamide degrading bacterium over time as measured by optical density (OD_{600}) and HPLC.



A: chlorpropham
B: 3 chloroaniline
C: 3 chlorophenol
D: carbaryl
OD 600 nm
carbetamide remaining (%)
time (h)
control (-bacteria)
0.1 mg L-1
1 mg L-1
10 mg L-1
100 mg L-1
carbetamide only

8.3.6 Selection of cured strains

Initial evidence that the carbetamide degrading phenotype was plasmid-borne was achieved by the selection of cured strains. Curing was performed by culture of the wild type carbetamide degrading bacterium in nutrient broth, a medium which did not directly select for the carbetamide degrading phenotype. Cured strains exhibited poor growth on this media (Figure 8.7). Three cured strains were independently selected. In each case only one cured strain was identified per 500 colonies tested. The rate of curing was not affected by increasing temperature from 28 to 33°C. The bacterium was very sensitive to acridine orange and mitomycin C and no cured strains were produced after treatment with these compounds.

8.3.7 Confirmation that cured strains were derived from the wild type strain

The cured derivative strain CI was shown to be indistinguishable from the wild-type carbetamide degrading isolate. Restriction digests of amplified 16S rRNA were identical for both the wild type bacterium (X) and the cured strain (CI) (Figure 8.8). The range of substrates used by both the carbetamide degrading and cured strain were identical (as indicated by Biolog® SFP plates, results not presented). These results confirmed that the cured strain CI was derived from the wild type strain.

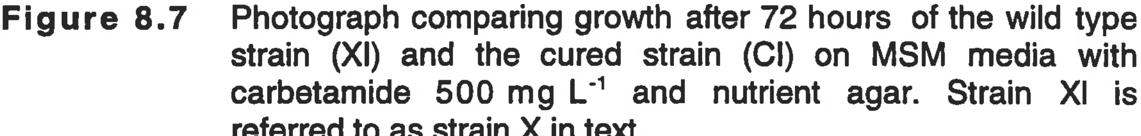


Figure 8.7 Photograph comparing growth after 72 hours of the wild type strain (XI) and the cured strain (CI) on MSM media with carbetamide 500 mg L^{-1} and nutrient agar. Strain XI is referred to as strain X in text.

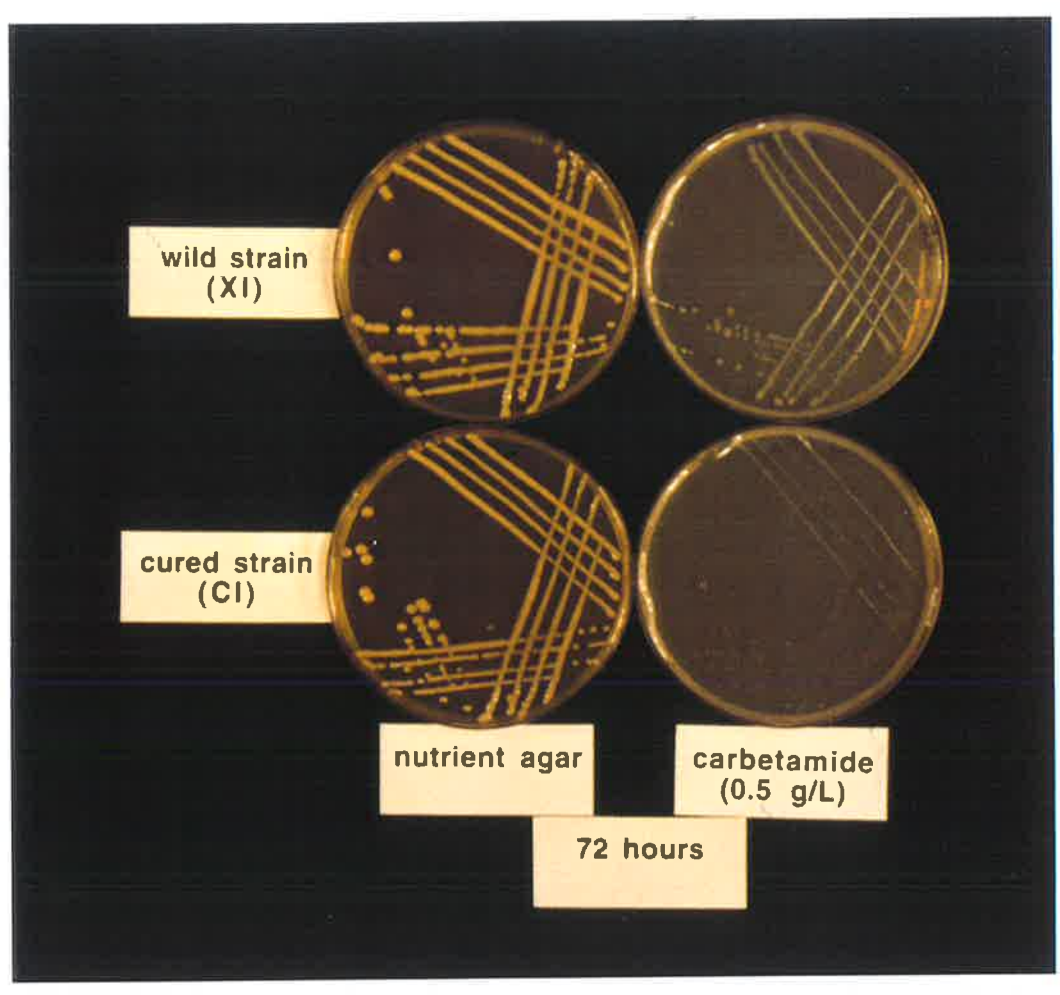
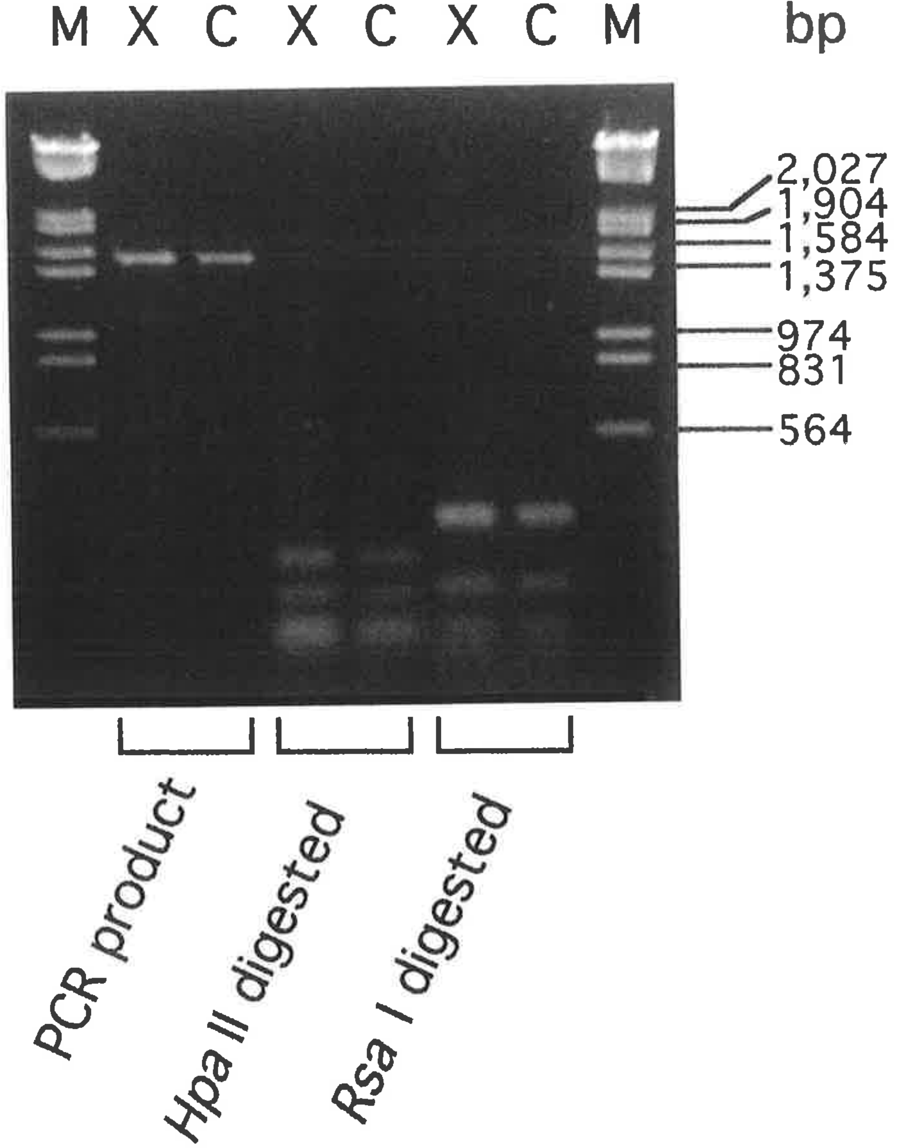

wild strain
(XI)
cured strain
(CI)
nutrient agar
carbetamide
(0.5 g/L)
72 hours

Figure 8.8 Restriction digests (*Hpa* II and *Rsa* I) of 16s rRNA amplified by PCR from the carbetamide degrading strain (X) and a spontaneous cured strain (C). Marker (M) is lambda DNA digested with *Eco*R I and *Hin*d III

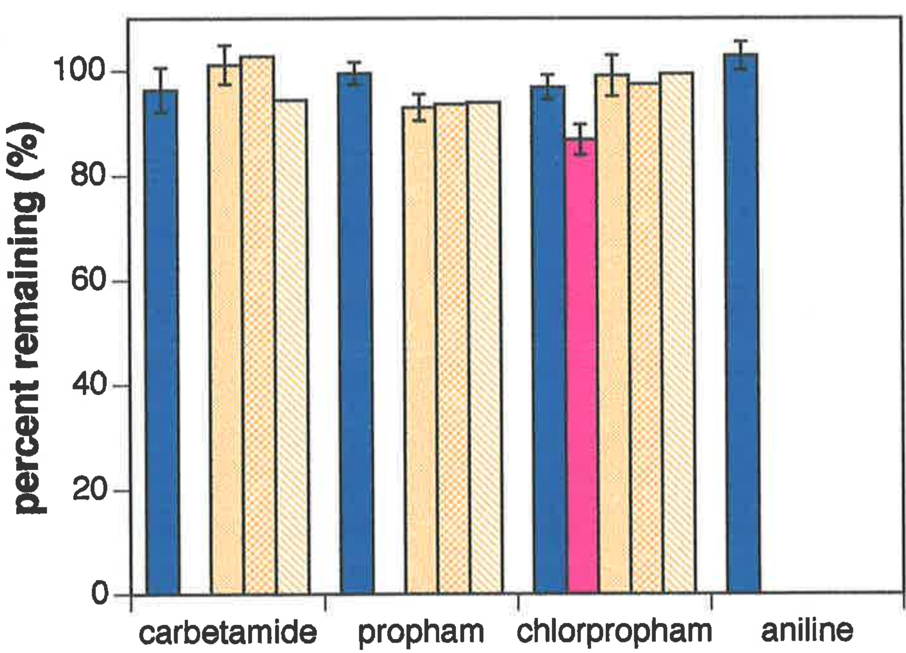

M X C X C X C M
bp
2,027
1,904
1,584
1,375
974
831
564
PCR product
Hpa II digested
Rsa I digested

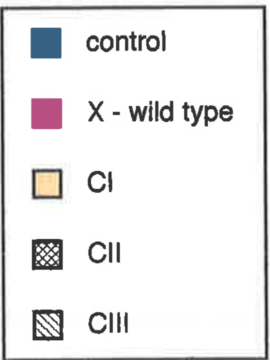
8.3.8 Confirmation of loss of phenotype in cured strains

The cured strains were unable to degrade carbetamide, propham or chlorpropham but retained the ability to degrade aniline (Figure 8.9). 3-chloroaniline was detected in cultures of the wild type bacterium when treated with chlorpropham. Though the cured strains were unable to degrade propham, growth was clearly evident in these cultures. Growth was probably due to carry over of nutrients from the stock cultures. Though growth of the cured strains was clearly evident in cultures containing propham, no growth was evident in cultures containing chlorpropham. This visual result suggests that chlorpropham is also toxic, through another mechanism apparently not associated with carbetamide degradation.

Experiments in this thesis have linked carbetamide degradation with propham degradation (Figure 6.2, Figure 6.4). By the selection of cured strains incapable of degrading carbetamide, loss of the ability to degrade propham (Figure 8.9) and transform chlorpropham was also observed. This indicates that changes or losses which have resulted in the absence of the carbetamide degrading phenotype, are also probably involved in propham degradation and chlorpropham transformation.

Figure 8.9 Carbetamide, propham, chlorpropham or aniline remaining in cultures 24 h after inoculation with the carbetamide degrading isolate and three independently isolated cured derivatives. Values are average of three replicates. Vertical bars indicate standard errors of the mean.

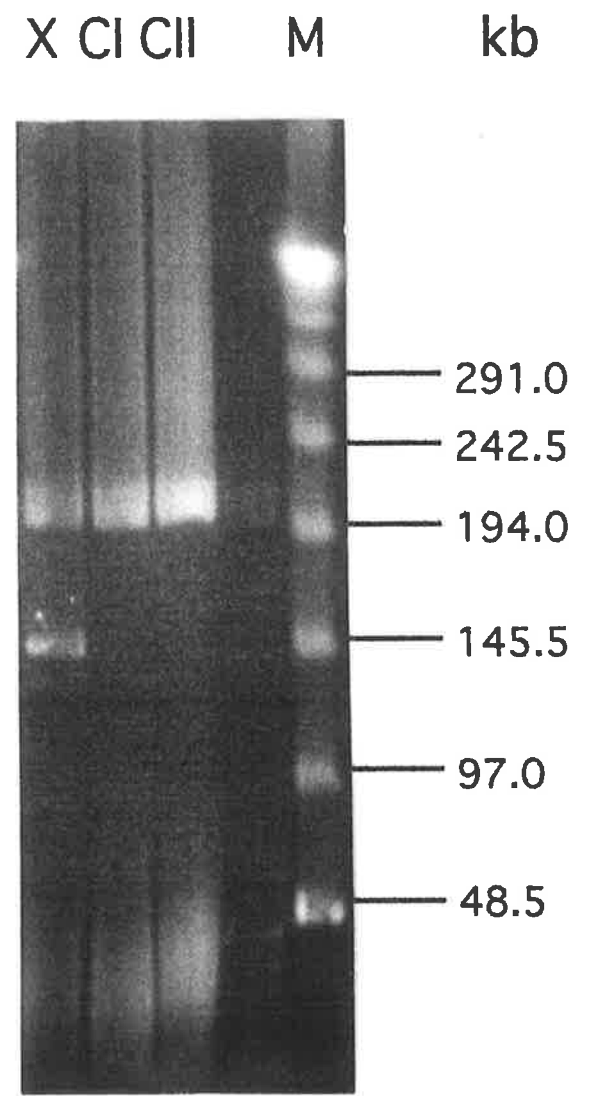

percent remaining (%)
100
80
60
40
20
0
carbetamide
propham
chlorpropham
aniline


control
X - wild type
CI
CII
CIII

8.3.9 Plasmid analysis of carbetamide degrading and cured strains

Isolation of plasmid DNA from the carbetamide degrading bacterium and the three cured derivatives proved successful using the large scale method of Schreiner *et al.* (1991). Separation of uncut plasmid extract of the wild type carbetamide degrading isolate (X) and cured derivative (CI), using submerged gel electrophoresis for long periods (2-3 days), resulted in inconsistent visualisation of bands corresponding to large plasmids. Separation of the same extracts digested with restriction enzymes (*Hin*d III and *Bam* HI) revealed several plasmid DNA fragments common to both strains (results not presented). However, there were several fragments absent from the cured strain (CI), indicating loss of DNA. Hence pulse field gel electrophoresis (PFGE) was used to separate the extracts. PFGE of plasmid extracts from the wild type carbetamide degrading isolate indicated the presence of four large plasmids (Figure 8.10). Little separation of the three larger plasmids occurred and in Figure 8.10 they appear as a single diffuse band. In contrast to the wild type strain, all three independently isolated cured strains which could no longer degrade carbetamide (Figure 8.8) only contained the larger three plasmids present in the wild type strain (results not shown for cured strain CIII, Figure 8.10). Mobility of all plasmids was dependent upon electrophoresis conditions (compare Figure 8.10 and Figure 8.12), demonstrating that the plasmid DNA was circular (Picardeau and Vincent, 1997).

Figure 8.10 Plasmid DNA of the carbetamide degrading wild type bacterium (X) and two cured strains (CI, CII) as separated by pulse field gel electrophoresis. Size marker (M) is concatemers of lambda cI857 Sam7 DNA (48.5 kb).



X CI CII M kb
291.0
242.5
194.0
145.5
97.0
48.5

Using plasmids isolated from *A. radiobacter* (strain K84) as size markers (results not presented), the plasmid lost through curing was estimated to be approximately 185 kb. The three larger cryptic plasmids, present in both the wild type carbetamide degrading isolated and the three cured derivatives, were estimated to be 240 to 250 kb approximately.

Loss of the carbetamide degrading phenotype through curing was associated with the loss of the same 185 kb plasmid in three independently cured derivatives (Figure 8.10). These results provide initial evidence that the carbetamide degrading phenotype is encoded on the 185 kb plasmid (Stanisich, 1988).

8.3.10 Selection of antibiotic resistant strains of bacteria

Strains of both the wild type bacterium (X) and the cured strain (CI) were selected with resistance to rifampycin and streptomycin at 10 and 50 μg mL^{-1} respectively. No colonies were evident on agar containing naladixic acid, chloramphenicol or gentamycin. Colonies with resistance to streptomycin occurred at a greater frequency than for rifampycin. Streptomycin resistant colonies arose at a frequency of one per 1.32 x 10^6 CFU, whereas for rifampycin colonies arose at a frequency of on per 3.5 x 10^7 CFU. Resistance to rifampycin or streptomycin did not result in resistance to streptomycin or rifampycin, respectively, or any of the other antibiotics. MSM plates containing 500 mg L^{-1} carbetamide and

10 $\mu g\ mL^{-1}$ rifampycin effectively selected between the rifampycin resistant and susceptible carbetamide degrading strains. Media containing carbetamide and streptomycin did not effectively select against streptomycin sensitive strains. Hence in conjugation attempts, a rifampycin resistant cured strain was used as the recipient.

8.3.11 Conjugation between carbetamide degrading and cured strains

Transfer of the carbetamide degrading phenotype was not observed to occur through conjugation. No colonies arose on plates containing carbetamide (500 mg L^{-1}) and rifampycin (10 $\mu g\ mL^{-1}$) when inoculated with cells from the filter inoculated with the donor and recipient cells. There were on average 15×10^7 recipient and 1.65×10^6 donor cells on these plates. As there were fewer donor cells, this experiment was repeated using the antibiotic sensitive wild type strain and the rifampycin resistant cured strain. Again, no colonies of transconjugant cells were evident on carbetamide-rifampycin plates and the ratio of donor to recipient cells was similar. These results indicate that either the rate of conjugation was very low, that the appropriate plasmid was not transferred during conjugation, or that conjugation did not occur.

8.3.12 Plasmid transformation into cured strain by electroporation

Electroporation enabled the transformation of the cured derivative with wild-type plasmid DNA. Approximately 100 colonies were evident on plates that contained carbetamide and were inoculated with cells that were electroporated in the presence of the plasmid extract from the wild-type carbetamide degrading *Rhodococcus* (Figure 8.11). No colonies were evident on plates inoculated with cells that were either not electroporated or electroporated with TE buffer. Electroporation resulted in approximately 67 % cell mortality. Electroporated strains grew equally well on carbetamide media as the wild-type carbetamide degrading strain (X) and were clearly resistant to the antibiotic rifampycin. As transformed strains were rifampycin resistant the origin of these strains was confirmed. All transformed strains degraded carbetamide. Similar results were observed when the cured strain without antibiotic resistance was used as the recipient strain.

Plasmid analysis of the transformed strains indicated the presence of a large plasmid (Figure 8.12), the same size as the 185 kb plasmid lost through curing (Figure 8.10). All electroporated strains still harboured the other larger plasmids of approximately 240-250 kb. This result provided further evidence that the carbetamide degrading phenotype was encoded on the plasmid lost through curing.

Figure 8.11 Photograph of MSM media containing 200 mg L^{-1} carbetamide and inoculated with 100 µl of the cured strain (CI) which had been treated with either plasmid DNA from the wild type bacterium and/or subjected to electroporation.

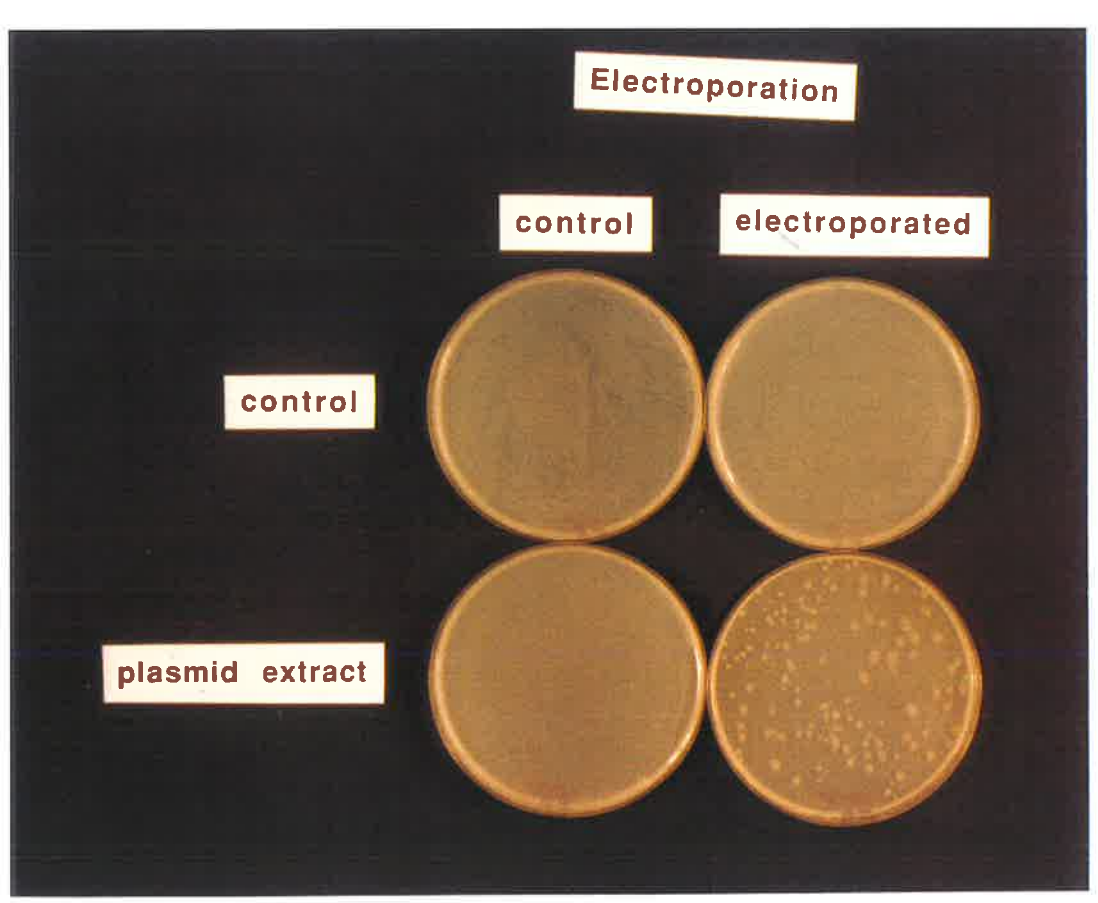
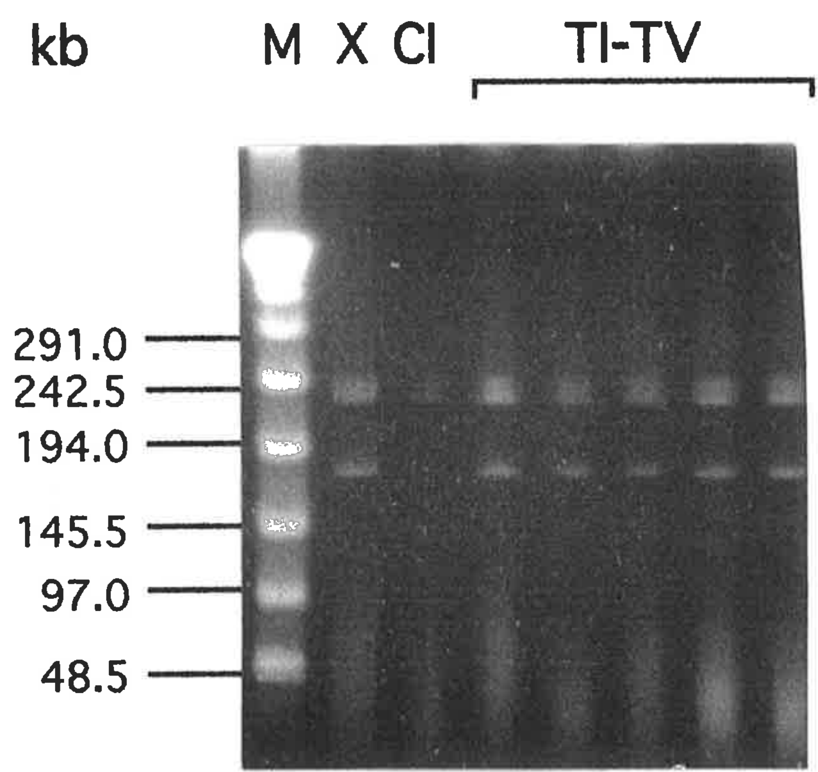

Electroporation
control
electroporated
control
plasmid extract

Figure 8.12 Plasmid DNA of the carbetamide degrading wild type bacterium (X), the cured strain (CI) and five strains transformed by electroporation (TI-TV) as separated by pulse field gel electrophoresis. Size marker (M) is concatemers of lambda cI857 Sam7 DNA (48.5 kb).


kb
M
X
CI
TI-TV
291.0
242.5
194.0
145.5
97.0
48.5

8.4 Discussion

Research presented in chapters 4 and 6 described a *Rhodococcus* sp. capable of utilising carbetamide as a source of carbon and energy. Studies presented in this chapter suggest that this bacterium initially degrades carbetamide to form aniline and that this phenotype is plasmid encoded. Formation of 3-chloroaniline by stationary phase cultures of the bacterium treated with chlorpropham (Figure 8.3) suggests that initial degradation step occurs adjacent to the amide bond. Accumulation of 3-chloroaniline in the media occurs because the bacterium cannot use this compound as a growth substrate (Figure 8.4). Rapid growth of the bacterium in the presence of aniline (Figure 8.4), which is degraded by the isolated bacterium (Figure 8.8), also suggests that aniline is a metabolite formed during degradation of carbetamide by the bacterium isolated in this study. Though aniline is a likely initial metabolite, the bacterium further mineralises the phenyl ring as significant amounts are converted to $^{14}CO_2$ (24 %) and incorporated into the bacteria (24 %) (Figure 8.1). The fate of the remainder of the carbetamide molecule (non phenyl portion) was not determined. However, irrespective of the initial degradation step, the remainder of the molecule has several moieties which are anticipated to be readily degraded by soil micro-organisms. One method for determining the fate of the remainder of this molecule would be to use carbetamide radiolabelled on the alkyl portions.

Substantial evidence was obtained suggesting that the initial step in carbetamide degradation by the isolated *Rhodococcus* sp. is plasmid encoded. Cured strains of the bacterium, unable to degrade carbetamide, arose spontaneously when grown in non-selective media (Figure 8.7). Plasmid analysis of these cured strains indicated the loss of a large (185 kb) plasmid (Figure 8.10). Though not transferred by conjugation, electroporation allowed for the production of transformed strains and provided further evidence that the carbetamide degrading phenotype is plasmid encoded. Electroporation of a cured strain with plasmid extract of the wild type carbetamide degrading bacterium resulted in transformant strains which had regained the ability to degrade carbetamide (Figure 8.11). Plasmid analysis of transformant strains demonstrated the presence of the 185 kb plasmid lost through curing (Figure 8.12), providing further evidence that the carbetamide degrading phenotype is plasmid encoded.

Numerous studies have demonstrated that plasmids can encode pesticide degradation, or the initial steps required to channel products into central metabolic pathways (Karns, 1990). Such plasmids can vary in size from a few to several hundred kb. For example, Gaubier *et al.* (1992) sequenced a 2 kb plasmid which encoded for chlorpropham degradation, whilst degradation of the herbicide EPTC by a *Rhodococcus* was encoded on a 77 kb plasmid (Tam *et al.*, 1987). In the present study, a large plasmid of approximately 185 kb appears to encode carbetamide degradation (Figure 8.10, Figure 8.12). Cured strains, although unable to

degrade carbetamide, still degraded aniline (Figure 8.8), demonstrating that the ability to degrade aniline is either encoded on another plasmid or the chromosome. However, this does not exclude the possibility that aniline degradation is also encoded on the plasmid lost through curing as multiple pathways for catabolism of aromatic substrates may reside in a single bacterial strain (Hughes *et al.*, 1984, Kitayama *et al.*, 1996). Also, it is likely that other functions or genes are encoded on the plasmid which encodes carbetamide degradation. As only small sections of DNA appear to be required for encoding phenyl carbamate degradation in *Pseudomonas cepacia* (Gaubier *et al.*, 1992), substantial portions of the plasmid linked to carbetamide degradation may encode other functions.

It has been proposed that the ability of degradative plasmids to be transferred by conjugation, both within and between species, may be responsible for rapid spread of pesticide degradative ability (Clarke, 1984, Head *et al.*, 1990). However, not all plasmids are transferred by conjugation (Broda, 1979). In the present study the plasmid which appears to encode for carbetamide degradation was not transferred by conjugation (section 8.3.11). This suggests that either the plasmid is non-conjugative or transfer occurred at rates below those detectable with the test method used.

Further examination of carbetamide as a source of carbon and energy was gained by inclusion of pyruvate, acetate and succinate in the culture medium (Figure 8.2). The influence of these additional carbon sources on bacterial growth and carbetamide degradation was dependant

upon whether yeast extract (50 mg L^{-1}) was added to the medium. When yeast extract was present in the medium, the addition of the carbon sources increased the total growth observed but had little effect on carbetamide degradation (Figure 8.2). When compared to growth on carbetamide, growth on pyruvate alone was slower, whilst growth on acetate and succinate was more rapid. In the absence of yeast extract, growth on all substrates including carbetamide was much less. However, in contrast to cultures containing yeast extract, addition of acetate or succinate to flasks containing carbetamide repressed growth and slowed carbetamide degradation (Figure 8.2). Addition of pyruvate had little effect on growth or carbetamide degradation. The biochemical basis for the observed effect with yeast extract is unknown. Yeast extract has been shown to also increase growth of a bacterium capable of chlorpropham degradation (Vega *et al.*, 1985). Results presented here in cultures without yeast extract demonstrate that under nutrient limiting conditions, such as might be expected in soil, the isolated bacterium can actively degrade carbetamide.

Evolution of $^{14}CO_2$ (Figure 8.1) from flasks and soil (Figure 4.5) treated with radiolabelled carbetamide indicated that degradation of the phenyl ring must occur. The ability of the isolated bacterium to use aniline and phenol, and their chlorinated or methyl analogues as growth substrates was examined in liquid culture (Figure 8.4, Figure 8.5). The isolated bacterium readily degraded aniline and phenol (after a lag phase) but could not degrade any of the chlorinated or methylated analogues. Drop plate

counts of the cultures indicated that the substituted compounds were toxic. This suggests that the carbetamide degrading bacterium was unable to effectively degrade chlorpropham and use this compound as a carbon source (Figure 6.2) as the 3-chloroaniline formed is toxic (Figure 6.3).

Inclusion of chlorpropham, 3-chloroaniline, 3-chlorophenol or carbaryl in addition to carbetamide slowed growth and inhibited carbetamide degradation (Figure 8.6). For all these compounds, inhibition was concentration dependent (Figure 8.6). Though these compounds were inhibitory, no metabolites were observed to accumulate in the culture media when these compounds were present. It is noteworthy that at an equivalent concentration, chlorpropham exhibited greater inhibition than 3-chloroaniline. This latter compound was shown to be a metabolite of chlorpropham (Figure 8.3). Possible explanations for the observed result include that chlorpropham is also inhibitory due to another mechanism or that 3-chloroaniline is not readily taken up by the bacterium.

On a concentration basis, carbaryl was more effective in inhibiting growth and carbetamide degradation than chlorpropham, 3-chloroaniline and 3-chlorophenol. In contrast to these latter three compounds (Figure 6.3, Figure 8.5), carbaryl was apparently non-toxic to the isolated bacterium (Figure 6.3). One possible explanation for this is that carbaryl inhibits an enzyme involved in carbetamide degradation, as has been described previously in the degradation of propham (Kaufman *et al.*, 1970). Evidence supporting this includes the fact that the bacterium isolated

in this study apparently degrades carbetamide via aniline, the same intermediate has been reported in the degradation of propham (Kaufman, 1967).

The wild type carbetamide degrading strain and the three cured strains all contained three large plasmids of similar size (approximately 240 to 250 kb). No phenotype was attributed to these larger plasmids, hence their description as cryptic. Large plasmids in Mycobacteria have proved to be stable (Picardeau and Vincent, 1997). Further work could be undertaken to determine phenotypes associated with the other large plasmids.

9. CONCLUSIONS AND IMPLICATIONS

9.1 Enhanced Carbetamide Degradation

This project was conducted to determine the cause of carbetamide failure when this herbicide is applied repeatedly to the same field. Carbetamide, when applied during 1992, failed to adequately control annual grass weeds in many fields in the south-east of South Australia. The majority of fields where carbetamide failure was observed had been treated in at least the prior season with carbetamide. A field experiment demonstrated that, upon repeated application, the efficacy of carbetamide in controlling *L. rigidum* was approximately halved (Figure 4.2). This carbetamide failure was due to rapid degradation of this herbicide (Figure 4.3) by soil micro-organisms (Figure 4.7). Rapid degradation and reduced efficacy were observed even after a single prior carbetamide application (Figure 4.4, Figure 4.2), demonstrating that this phenomenon can develop rapidly in soil. As reported with enhanced degradation of EPTC (Drost *et al.*, 1990), varying environmental conditions are the probable cause of variations between replicate portions of experiments conducted in three successive seasons (Figure 4.2, Figure 6.5, Figure 7.5). Although variable, the observed reductions in carbetamide efficacy due to enhanced degradation are likely to lead to dramatically reduced weed control and therefore crop yields. Enhanced degradation has been reported as a cause of failure for many soil applied pesticides (Roeth, 1986, Felsot, 1989, Racke

and Coats, 1990b). This study establishes that this phenomenon is also a cause of carbetamide failure.

Inhibition of enhanced carbetamide degradation by the broad-spectrum antibiotic chloramphenicol (Figure 4.7) provided evidence that bacteria were the cause of rapid carbetamide degradation. Subsequently, a *Rhodococcus* sp. was isolated from soil capable of utilising carbetamide as a source of carbon and energy (Figure 4.8). Circumstantial evidence, based upon similar results in soil and pure culture of the isolated bacterium indicated that the isolate may be responsible for degradation in soil. Evidence for this included both cross enhancement/degradation to propham (Figure 6.1, Figure 6.2 and Figure 6.3), inhibition by carbaryl (Figure 7.4, Figure 8.6) and susceptibility to chloramphenicol (Figure 4.7, section 8.3.10). *Rhodococci* have often been associated with soil degradation of organic pollutants (Warhurst and Fewson, 1994). Hence it is likely that the isolated bacterium is at least partially responsible for carbetamide degradation in the field from which it was isolated. In particular the capacity of *Rhodococci* to tolerate extreme environmental conditions (Warhurst and Fewson, 1994) and location within the aerobic zone in soil (Ensign, 1992), makes them likely candidates for involvement in enhanced pesticide degradation. However, it is possible that bacteria from other genera may also be capable of carbetamide degradation both within this field and in other fields. A range of organisms, both Gram positive and negative, have previously been isolated capable of phenyl carbamate degradation

(Kaufman and Kearney, 1965). In examination of soils enhanced for 2,4-D degradation, many organisms have been isolated, even when isolated from the same area (Soulas, 1993, Ka *et al.*, 1994b). Although the *Rhodococcus* strain was the only carbetamide degrading bacterium isolated in this study, this may be the result of the isolation and culture methods used which will undoubtedly select organisms with certain characteristics, for example rapid growth. *Rhodococci* are well adapted for growth in the laboratory under aerobic conditions (Finnerty, 1992).

Formation of 3-chloroaniline from chlorpropham indicated that microorganisms degrade carbetamide via aniline (Figure 8.3). Further supporting evidence includes rapid growth on aniline (Figure 8.4) and earlier reports that aniline is a carbetamide metabolite in soil (Rouchaud *et al.*, 1988). Preliminary genetic studies indicated that the gene(s) encoding the conversion of carbetamide to aniline are located on a large plasmid (approximately 185 kb) (Figure 8.10, Figure 8.12) Although bacteria described previously have been capable of degrading phenyl carbamate compounds (Clark and Wright, 1970, Marty *et al.*, 1986), where tested they were unable to degrade carbetamide (Marty and Vouges, 1987). However in this study, although only carbetamide, propham and chlorpropham were tested, the bacterium isolated was capable of degrading or transforming these molecules (Figure 8.9).

Cycloisomerisation of carbetamide does not appear to occur in the degradation of carbetamide by soil microorganisms. Formation of cyclic

degradation products in sterile soil has been reported for many phenyl carbamate herbicides including carbetamide, chlorbufam, desmedipham and phenmedipham (Sabadie *et al.*, 1985, Sabadie and Coste, 1986a, Sabadie and Coste, 1986b, Tam *et al.*, 1987). In the present study, degradation of chlorpropham to 3-chloroaniline implies that carbetamide is degraded through aniline (section 8.3.3). This initial step is the same as reported for microbial degradation of several other phenyl carbamate compounds (Kearney and Kaufman, 1965, Kaufman, 1967, Chapalamadugu and Chaudry, 1992, Gaubier *et al.*, 1992, Pohlenz *et al.*, 1992, Mateen *et al.*, 1994). Although this does not preclude the formation of a cyclic intermediate, it does appears unlikely. Cell free and purified enzyme extracts of phenyl carbamate degrading organisms have converted these compounds directly to the corresponding aniline (Kearney and Kaufman, 1965, Marty *et al.*, 1986, Marty and Vouges, 1987, Pohlenz *et al.*, 1992). Furthermore, a gene encoding for a single hydrolase enzyme which catalyses the degradation of phenmedipham (Pohlenz *et al.*, 1992), is functional even after transformation into *Nicotiana tabacum* (tobacco) (Streber *et al.*, 1994). Therefore, it appears that in non-sterile soil, carbetamide is degraded through aniline without prior cycloisomerisation, as enzymes which catalyse the formation of such compounds do not appear to form these products.

Rhodococci are Gram positive bacteria noted for their ubiquity in the environment, capacity for degrading a wide range of compounds and

tolerance of extreme environmental conditions (Warhurst and Fewson, 1994). Thus *Rhodococci* are organisms likely to be associated with enhanced degradation. Herbicides which *Rhodococci* have been shown capable of degrading include glufosinate, EPTC, simazine, atrazine and metamitron (Tebbe and Reber, 1988, Behki *et al.*, 1993, Parekh *et al.*, 1994, Ankumah *et al.*, 1995). The *Rhodococcus* sp, isolated as a part of this thesis is capable of degrading the herbicide carbetamide (Figure 4.8). Enhanced carbetamide degradation was still clearly evident four years after ceasing carbetamide application in the field (Figure 5.1). Persistence in the environment is a noted feature of the genus *Rhodococci* (Warhurst and Fewson, 1994), with enhanced degradation of acetylene still clearly evident four to eight years after the last treatment (Terry and Leavitt, 1992).

9.2 Management of Enhanced Carbetamide Degradation

Research presented in chapters 5, 6 and 7 examined whether methods employed for management of enhanced pesticide degradation in other agricultural systems are effective in managing enhanced carbetamide degradation. Also, where possible, attempts were made to understand the biological basis for the effectiveness, or lack of effectiveness, and limitations of treatments. The three areas examined were: i) persistence of enhanced carbetamide degradation, ii) cross enhancement from carbetamide to other herbicides and iii), the use of chemical extenders to increase carbetamide persistence.

Less frequent carbetamide application, rotation to herbicides which are dissimilar in structure to carbetamide and use of carbaryl as a carbetamide extender are all potential management options which could be used to manage enhanced carbetamide degradation. Importantly, these management practices are complimentary and use of all three techniques, where practical, is likely to improve effective grass control with carbetamide.

Other factors are also likely to determine the efficacy of carbetamide. These include weed germination characteristics, weed density and timing of herbicide application. Optimisation and appropriate management of these is likely to increase the efficacy of carbetamide.

Research presented in this thesis examined whether less frequent carbetamide application and chemical extenders could be used to manage enhanced carbetamide degradation. Results indicated that enhanced carbetamide degradation capacity declined with time (Figure 5.1) due to decreases in the soil microbial population capable of carbetamide degradation (Figure 5.2). This suggests that less frequent carbetamide application is likely to result in improved weed control. Results indicated that this could be achieved practically by herbicide rotation (Figure 6.4, Figure 6.5). The methyl carbamate insecticide carbaryl partially inhibited enhanced carbetamide degradation (Figure 7.4). Due to the high cost of carbaryl and only moderate increase in efficacy (Figure 7.5), it is unlikely that carbaryl would be used commercially to extend the persistence of carbetamide in soil.

The capacity for soil to exhibit degradation of carbetamide at an enhanced rate, as a result of prior carbetamide application declined in the absence of carbetamide application (Figure 5.1). This suggests that less frequent application of carbetamide will provide more efficacious grass control. A most probable number (MPN) assay further confirmed decline of enhanced carbetamide degradation (Figure 5.2). Four years after ceasing carbetamide application the most probable number of carbetamide degrading microorganisms had declined to a level similar to that of the control soil (Figure 5.2). Only 14 degrading microorganisms per gram of soil were evident 4 years after ceasing carbetamide application, a relatively low population (Fournier *et al.*, 1993). However carbetamide still degraded relatively rapidly in this soil (Figure 5.1). Further field-based research should be conducted to determine the appropriate frequency of carbetamide application for reliable and efficacious weed control. Throughout this project *Trifolium* seed producers communicated that application of carbetamide in every third or fourth season provided excellent grass control.

Propyzamide and EPTC were identified in this study as key herbicides which, in rotation with carbetamide, may allow for effective grass weeds control in *Trifolium* seed crops. Propyzamide controls a similar spectrum of weeds to carbetamide but is also less efficacious upon repeated application (Figure 6.5). This is most likely due to enhanced propyzamide degradation (Walker and Welch, 1991). However, propyzamide is structurally dissimilar to carbetamide and, based upon cytological evidence, appears to

have a different herbicidal mode of action to carbetamide (Vaughn and Vaughn, 1987, Vaughn and Lehnen, 1991). As propyzamide is structurally dissimilar, degradation most likely occurs via a different pathway (Rouchaud *et al.*, 1988) and cross enhancement from carbetamide to propyzamide, and vice versa, is therefore unlikely. This was evident when this rotation was tested in the field (Figure 6.5). Therefore these herbicides could be used sequentially on an annual basis to provide effective weed control.

Though EPTC is not registered in Australia for use in *Trifolium* crops, it is registered for this purpose in the United States of America. As EPTC requires physical incorporation into soil, application of this herbicide is limited to the year when the stand is established. As with propyzamide, EPTC is different in both chemical structure and mode of action to carbetamide (Vaughn and Lehnen, 1991). Thus cross enhancement between these carbetamide and EPTC herbicides is unlikely. By using EPTC, carbetamide and propyzamide in rotation it should be possible to control grasses in three successive seasons. *Trifolium* seed producers consider this particularly important with perennial *Trifolium* sp. as yields are typically low in the first year. After 2 to 3 years of continuous *Trifolium* sp. cropping, fields are normally sown to either cereals (wheat or barley) or grass pasture seed (*Phalaris, Dactylus* or *Festuca* spp.). Carbetamide, EPTC and propyzamide are not applied in these crops, hence it should be a

number of seasons between each application of carbetamide, EPTC or propyzamide.

9.3 Herbicide Resistance

Herbicide resistance in key weed species is a significant agronomic problem in *Trifolium* seed production and selection of carbetamide resistant *L. rigidum*, or other weed species, could render carbetamide ineffective. Variability in the response of *L. rigidum* to carbetamide has been demonstrated (McAlister *et al.*, 1995). However, carbetamide still controls all biotypes examined at recommended rates (McAlister, 1992).

The time taken for selection of resistant individuals is based upon the frequency, efficacy and duration of selection and the frequency of resistance genes (Maxwell and Mortimer, 1994). Research documented in this thesis suggests that due to enhanced carbetamide degradation, carbetamide is likely to be applied less frequently in the future. Carbetamide, as with other herbicides which disrupt mitosis as their primary mode of action (Smeda and Vaughn, 1994), is often less efficacious than desired. Throughout this study, the maximum recommended field rate of carbetamide was used, but often only 80 - 90% *L. rigidum* mortality was observed (Figure 4.2, Figure 4.7 and Figure 7.5). Currently there are no weed biotypes with resistance to carbetamide by a mechanism which renders the herbicide ineffective under field conditions (McAlister, 1992). As there are no

weeds which have been selected with resistance to carbetamide, the frequency of resistance genes is also unknown. Based upon: i) reduced application frequency and low efficacy, when compared to other herbicides, the time taken for selection of carbetamide resistant weeds will probably be longer than for herbicides to which there is widespread resistance in this area.

9.4 Molecular Aspects of Carbetamide Degradation

In this study, strong evidence was presented that the ability of an isolated soil bacterium to degrade carbetamide is plasmid encoded (section 8). Though genetic methods for analysis of Gram positive bacteria have lagged behind those developed for Gram negative, significant advances have been made recently (Warhurst and Fewson, 1994). Use of a *Rhodococcus* cloning vector may allow for further genetic analysis and such vectors have recently been described (Shao *et al.*, 1995). After ligation of endonuclease restriction digested plasmid extract into a suitable vector, this recombinant product could be transformed into a recipient cured strain by electroporation (section 8.3.13). This may allow further confirmation that the phenotype is plasmid encoded and cloning and sequencing of the carbetamide degrading gene.

Further genetic analysis may allow molecular DNA methods to be used for soil analysis. The use of PCR and/or gene probing has allowed for more in depth examination of bacterial degradation of 2,4-D

(Holben *et al.*, 1992, Neilson *et al.*, 1992, Ka *et al.*, 1994a, Ka *et al.*, 1994b, Ka *et al.*, 1994c). In the present study a MPN method was used, which are subject to several assumptions (Cochran, 1950). Analysis of soil DNA could either target the carbetamide degradative genes, or the 16s rRNA sequence of the organism isolated in this study (section 4.3.9). By targeting a carbetamide degradation gene, detection would be possible even in other, possibly non-culturable, organisms (Head *et al.*, 1990). However, this technique would not allow for identification of the organism(s) responsible. Alternatively, molecular DNA techniques could be used to determine the contribution of the bacterium isolated in this study to carbetamide degradation in soil. Probing soil bacterial DNA extracts with the 16s DNA sequence of the *Rhodococcus* sp. isolated in this study (section 4.3.9), may allow for correlation of degrading activity with populations of similar *Rhodococci* (Holben *et al.*, 1988, Amann and Ludwig, 1994). Although this method would only permit correlation as the 16s rRNA sequence is not directly linked to carbetamide degradation.

Another potential method for analysis of soil microbial populations is PCR amplification of a 16s rRNA fragment (<500 bp) combined with denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.*, 1993). Such fragments, though virtually identical in length, can be separated by this method. Individual bands may then be excised from the gel, cloned into an appropriate vector and sequenced. Thus allowing identification of bacteria present, including bacteria unculturable by normal

techniques (Rölleke *et al.*, 1996). By comparing soils which differ in their carbetamide application history it may be possible to determine not only bacteria which benefit from carbetamide application (increased band intensity) but those to which carbetamide is toxic or inhibitory (decreased band intensity). However, a limitation of this method is that it would not indicate whether a particular bacterium actually degrades carbetamide.

Through the application of molecular DNA methods it may be possible to gain a more intimate understanding of enhanced pesticide degradation by microorganisms in soil. It has been proposed that this may allow for development of appropriate strategies for management of enhanced pesticide degradation (Head *et al.*, 1990). Alternatively, examination of the catabolic route of degradation may allow for development of specific microbial inhibitors.

9.5 Wider Implications of the Study

A potentially large market exists for carbetamide in Australia, in both broad-acre crops and intensive horticulture. To date, cheaper and more efficacious herbicides have been available for annual grass control in these crops. As with *Trifolium* seed production, herbicide resistance may drive producers of other crops to use herbicides with alternative modes of action, such as that of carbetamide. Failure to take account of results such as those presented in this thesis could also lead to failure due to enhanced degradation.

It is likely that other pesticides are affected by enhanced degradation upon repeated application in Australia. For example, as a part of this study reduced efficacy of the herbicide propyzamide upon repeated application was demonstrated (Figure 6.5). This was probably due to enhanced degradation (Walker and Welch, 1991). In Australia propyzamide is registered for use in turf and lettuces, uses where repeated annual applications are made either within one season or on an annual basis. Therefore a review of current recommendations for propyzamide is required. Further research needs to be conducted to determine other pesticides which are affected by enhanced degradation.

9.6 Concluding Remarks

Research presented in this thesis demonstrates that enhanced degradation is the cause of carbetamide failure upon repeated application. Research also indicated that enhanced carbetamide degradation can be managed by less frequent carbetamide application. Less frequent carbetamide application can be achieved by rotation with a range of other herbicides. Therefore carbetamide is likely to continue playing a key role in the control of annual grasses in *Trifolium* seed crops. Other key herbicides which could be used in rotation with carbetamide for annual grass control are propyzamide and EPTC.

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Appendix 1: 16s rRNA sequence

Consensus 16s rRNA sequence of the bacterium isolated in this study. Letters shown in lower case are those a consensus sequence was calculated by the program Sequed (Applied Biosystems).

```
AGAGTTTGAT CCTGGCTCAG GACGAACGCT GGCGGCTGCT TAACACATGC 50
AAGTCGAGCG GTAAGGCCTT TCGGGGTACA CGAGCGGCGA ACGGGTGAGT 100
AACACGTGGG TGATCTGCCC TGCACTTCGG GATAAGCCTG GGAAACTGGG 150
TCTAATACCG GATATGACCT CCTATCGCAT GGTGGGTGGT GGAAAGATTT 200
ATCGGTGCAG GATGGGCCCG CGGCCTATCA GCTTGTTGGT GGGGTAATGG 250
CCTACCAAGG CGACGACGsG kwkmnsrmCT GAGAGGTGAC CGvCCACACT 300
GGAnTGArAC ACGGCCCAGA yCTACGGAGG CAGCAnTGGG AATATGCACA 350
wkGGssrArm kmTGATGCAG CGACGCCGCG TGAGGGATGA CGGCTTCGGG 400
TTGTAAACyC TTCAGCAGGG ACGAAGCGCA AGTGACGGTA CCTGCAGAAG 450
AAGCACCGGC TAACTACGTG CCAGCAGCCG CGGTAATACG TAGGGTGCAA 500
GCGTTGTCCG GAATTACTGG GCGTAAAGAG TTCGTAGGCG GTTTGTCGCG 550
TCGTTTGTGA AAAnCAGCAG CTCAACTGCT GGCTTGCAGG CGATACGGGC 600
AGACTTGAGT ACTGCAGGGG AGACTGGAAT TCCTGGTGTA GCGGTGAAAT 650
GCGyAGATAT CAGGAGGAAC ACCGGTGGCG AAGGCGGGTC TCTGGGCAGT 700
AACTGACGCT GAGGAACGAA AGCGTGGGTA GCGAACAGGA TTAGATACCC 750
TGGTAGTCCA CGCCGTAAAC GGTGGGCGCT AGGTGTGGGT TCCTTCCACG 800
GAATCCGTGC CGTAGCTAAC GCATTAAGCG CCCCGCCTGG GGAGTACGGC 850
CGCAAGCTAA AACTCAAAGG AATTGACGGG GGCCCGCACA AGCGCGGAGC 900
ATGTGGATTA ATTCGATGCA ACGCGAAGAA CCTTACCTGG GTTTGACATA 950
TACCGGAAAG CTGCAGAGAT GTGGCCCCCT TGTGGTCGGT ATACAGGTGG 1000
TGCATGGCTG TCGTCAGCTC TGTCGTGAGA TGTTGGGTTA AGTCCCGCAA 1050
CGAGCGCAAC CCTATCTATG TTGCAGCACG TTATGTGGAG TCyTAGAArw 1100
CTGCGGsGGG TCAACTCGGA GAAGTGGGGA CGACGTCAAG TCATCATGCC 1150
CTTATGTCCA GGGCTTCACA CATGCTACAA TGGCCAGTAC AGAGGGCTGC 1200
GAGACCGTGA GGTGGAGCGA ATCCCTTAAA GCTGGTCTCA GTTCGGATCG 1250
GGGTCTGCAA CTCGACCCCG TGAAGTCGGA GTCGnTAGTA ATCGCAGATC 1300
AGCAACGCTG CGGTGAATAC GTTCCCGGGC CTTGTACACA CCGCCCGTCA 1350
CGTCATGAAA GTCGGTAACA CCCGAAGCCG GTGGCTTAAC CCCTTGTGGG 1400
AGGGAGCCGT CGAAGGTGGG ATCGGCGATT GGGACGAAGT CGTAACAAGG 1450
TAGCCGTACC GGAAGGTGCG GCTGGATCAC CTCCTT
```

Erratum

page/line	
i/15	..and 16S rRNA..
10/14	..indistinguishable from those of propham..
13/20	Cartwright (1976) suggested..
16/19	..may result in..
27/5	..(Smith and Lafond, 1990).
35/13	..a single bacterium..
35/21	..encode for pesticide degradation..
38/7	..aromatic compounds can be..
45/18	..rotary shaker (approx. 80 rpm)..
47/9	..DB5 column (10 m),..
49/7	..nutrient agar..
58/2	..a particular group of..
59/8	.. such minimal salts medium.
68/1	16S rRNA sequence analysis
68/8&21	16S rRNA
79/16	These results suggested..
80/21	16S rRNA
81/2, 4, 12	16S rRNA
81/5	*R. erythropolis*..
Fig. 4.10	..the 16S rRNA.., *Rhodococcus erythropolis*
86/7	16S rRNA
86/9&11	Rhodococci..
86/12	..triazinone herbicide metamitron..
103/16	..grown on carbetamide minimal salts agar..
116/15	However these compounds have not been shown to affect carbetamide degradation in soil.
118/6	.. at 0 and 9 days after treatment.
129/8	..as this latter compound..
134/20	..the bacterium..
135/18	.. at various times..
136/9	..replica plated (48 prong stainless steel replicator)..
136/11	.. on this medium..
137/5	..16S rRNA..
139/23	..DNA (48.5 kb, BioRad) were used..
142/9	..of the same medium.
143/11	..MSM medium. Growth was compared..
143/13	.. strains were analysed as..
143/14	..pulse time was..
144/7	..represent inefficient ^{14}C..
148/7	..toxicity of the..
Fig. 8.8	..of 16S rRNA amplified..
152/5	..be approximately 240..
155/7	..that the initial degradation..
155/9	..in the medium occurs..
165/10	..of the genus *Rhodococcus*..
170/2	..Based upon reduced application..
171/3	..which is subject..
171/4, 11, 15 & 18	..16S rRNA
171/14	..of similar *Rhodococci*..
174/10	..of chemicals of..
178/3	pp. 11-48.
183/2	D.A. Hopwood
184/17	..Mitosis and Cell Plate..
186/5	..Acylanilide, Azide,..
190/22	..upon 16S rRNA Gene..
192/19	Reineke, W. and Knackmuss, H.-J.
193/20	..Coding for 16S rRNA..
195/12	..improved *Escherichia coli-Rhodococcus*..
197/7	Soper, D. and Hutchison, A.S. (1974)
197/26	..Carbaniliate and Acylanilides.
202/1&2	16S rRNA sequence..