Microbial degradation of the herbicide mecoprop by a rhizosphere community

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Declaration

I declare that this thesis is a report of my own original work undertaken during the years 1980-1983 at Warwick University and U.W.I.S.T. To the best of my knowledge it has not been previously reported by any other person.

SUMMARY

Rhizosphere microorganisms were grown in batch culture with mecoprop, a selective herbicide, as the sole carbon and energy source. Growth was detected only if the mecoprop concentration was 2.08 mM or less and lag phases of up to 37 days were required before growth commenced. After consecutive subculturing the length of the lag phase decreased to less than 24 hours. Degradation of the herbicide was followed by monitoring inorganic chloride ions released from the mecoprop structure, culture absorbance increases and the disappearance of the maximum ultra-violet absorption peak of mecoprop. The culture enriched to degrade 2.08 mM was able to utilize mecoprop concentrations up to 66.7 mM, but prolonged lag phases were recorded.

Analysis of the mecoprop-degrading culture revealed that it contained two Pseudomonads, an Alcaligenes species, Acinetobacter calcoaceticus and a Flavobacterium species. None of the pure cultures was able to utilize mecoprop but some combinations of two or more organisms degraded the herbicide stressing the importance of interacting communities for the degradation of xenobiotic compounds. The two Pseudomonads made up 83.5% of the community, the remaining organisms were minor components. A specific relationship existed between the Pseudomonads based on the provision of a growth factor. The community was a stable association as subculturing for over 300 days failed to reduce the complexity.

Growth studies using mixed carbon sources demonstrated that the two Pseudomonads and Alcaligenes species were able to utilize mecoprop when succinate was present as a co-substrate. Pronounced two-phase growth curves were produced with mecoprop degradation occurring after initial growth on succinate.

A biodegradation pathway for mecoprop was proposed based upon degradation studies of the structurally related herbicides 2,4-D, MCPA and dichlorprop by the community and the oxidation of these herbicides by mecoprop-grown cells.

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CHAPTER ONE

INTRODUCTION

1.1 THE RHIZOSPHERE

1.1.1 Definition of the Rhizosphere

The term 'the rhizosphere' refers to the zone of soil influenced by the proximity of a plant's root system (Hiltner, 1904). Conditions exist within this region which stimulate intense microbial activity, producing microbiological gradients, with the highest numbers closest to the plant roots. The rhizosphere is difficult to define as there are no sharp dividing lines between it and the surrounding soil.

The rhizosphere effect can be detected in plant root seedlings only a few hours old, and has been noted during various stages of plant growth. As senescence approaches the rhizosphere ceases to be distinguishable from the adjacent soil.

1.1.2 Quantitative Aspects of the Rhizosphere

Dilution plate counting permits a numerical expression of the rhizosphere effect; that is, the ratio of the number of microorganisms per unit weight of rhizosphere soil, denoted R, to the number in a unit weight of soil further away from the roots, denoted S. The effect is greatest for bacteria with R:S values of between 5 to 20, but sometimes as high as 100 (Rovira & McDougall, 1967). The expression of an R:S value for fungi and actinomycetes is more difficult due to their filamentous nature. However, both fungi and actinomycetes are not as prolifically stimulated as bacteria.

1.1.3 Microbiology of the Rhizosphere

Since the first description of the rhizosphere in 1904, much work has shown that a preferential stimulation of certain groups of microorganisms existed, illustrating both quantitative and qualitative effects. Lochhead (1940) compared the relative incidence of certain bacterial types in rhizospheres of different plants with controls using soil further away from

the roots. In all plants studied the selective enhancement of Gram-negative rod-shaped bacteria was noted, but Gram-positive, spore forming, short rods were less numerous in the rhizosphere than in the control soil. Clark (1940) showed that Pseudomonads predominate over organisms such as Bacillus and Arthrobacter.

Further work by Lochhead and Rouatt (1955) demonstrated the presence in the rhizosphere of organisms with certain nutritional requirements, particularly amino acid requirements. The incidence of this group of organisms decreased with an increase in distance away from the roots.

Pseudomonads represent the most numerous group of bacteria in the rhizosphere of plants. The occurrence of fluorescent Pseudomonads in rhizosphere soil was demonstrated to be greater than in the soil microflora generally. Vancura (1980) postulated that the predominance of this group was due to the ability of Pseudomonads to grow rapidly, thus making them good competitors for growth substances.

1.1.4 Factors affecting the Rhizosphere

The rhizosphere is influenced by a complex set of factors which included sloughed off root cells, organic materials, solute gradients caused by root absorption, pH fluctuations, varying oxygen and carbon dioxide concentrations and microbial interactions. Vrany et al. (1962) considered that the main factor influencing the rhizosphere was the plant itself.

The presence of readily utilizable compounds is considered to be the main cause of microbial stimulation in the rhizosphere. The origin, nature and nomenclature of the organic materials is shown in Table 1.1, but in order to reduce the complexity, the organic materials will be collectively referred to as root exudates in this Introduction.

1.1.5 Composition of root exudates

Quantitative analyses of exudates are difficult due to the very low concentration of compounds exuded, which are too low to be determined by

TABLE 1.1 Sources of carbon material in the rhizosphere.

(after Rovira et al. 1979).

1. Exudates: Compounds of low molecular weight which leak from all cells into either the intracellular junctions, or directly through the epidermal cell walls into the soil. The release of these compounds is not metabolically mediated.

2. Secretions: Compounds of low molecular weight as well as high molecular weight mucilages which are released as a result of metabolic processes.

3. Plant Mucilages: Four sources of plant mucilages which contribute to the organic materials in the rhizosphere are:

   (a) mucilage originating in the root cap and secreted by Golgi,

   (b) hydrolysates of the polysaccharide of the primary cell wall between the epidermal cells and sloughed root cap cells,

   (c) mucilage secreted by the epidermal cells which still only have primary walls. This includes mucilages secreted by root hairs, and

   (d) mucilage produced by bacterial degradation of the outer multilamellate primary cell walls of old, dead epidermal cells.

4. Mucigel: The gelatinous material at the surface of roots grown in normal non-sterile soils. It includes natural and modified plant mucilages, bacterial cells and their metabolic products (such as capsules, glycocalyx materials and slimes) as well as colloidal material and organic matter from the soil. Whereas root mucilages are

entirely of plant origin and microbially produced mucilages can only be studied and characterized in axenic culture, mucigel is a product of the entire root-soil-microbial complex with its own distinctive morphological and biochemical properties. The mucigel is important in maintaining contact between the root and the soil as the root shrinks during daytime water stress so permitting continuous uptake of nutrients and water.

5. Lysates: These are compounds released from autolysis of older epidermal cells when the plasmalemma fails. With further time the walls of these epidermal cells are digested by microorganisms and the cells become heavily colonized releasing the products of microbial activity into the rhizosphere.

techniques available and to the lack of knowledge of the volume of soil into which the exudate flowed. However, numerous studies have revealed the following compounds in exudates: sugars, amino acids, organic acids, enzymes, vitamins, growth substances, growth inhibitors and attractants. An indication of the range of compounds detected in wheat root exudates is given in Table 1.2. Rovira and McDougall (1967) considered that it was of little value to assess such composite tables without realizing that comparisons between plants are only valid if conducted under identical environmental conditions and at similar stages of plant growth.

Sugars. At least 10 sugars have been identified in a wide range of plants, with glucose and fructose being the most abundant.

Amino acids. These are the most studied group of compounds, their importance can be appreciated as some rhizosphere inhabitants require amino acids for growth (Section 1.1.3).

Vitamins. Rovira and Harris (1961) found biotin at low levels in the exudates from lucerne, field pea, tomato, phalaris plants and six clover species. Pantothenate and niacin were also found in most exudates, whereas thiamine was detected only occasionally. Despite low levels of vitamins, they may be sufficient to meet the requirements of some rhizosphere community members.

Organic acids. These are important in the exudate as they are readily utilizable growth substrates for rhizosphere populations. Ten different organic acids have been detected in wheat plants (Table 1.2).

1.1.6 Factors affecting exudation

The analysis of exudate composition has revealed that it is variable depending upon the particular plant studied. Other factors affecting exudation include; the age of the plant, presence of microorganisms, light and temperature, foliar sprays and soil moisture levels.

Plant species. Smith (1976) studied the inorganic and organic components of root exudates of three mature trees and found much variation in the exudation

TABLE 1.2 **Compounds Reported in Wheat Root Exudates**

(after Rovira, 1965).

Sugars	Amino Acids	Organic Acids	Nucleotides Flavonones	Enzymes
Glucose	Leucine	Oxalic	Adenine	Invertase
Fructose	Isoleucine	Malic	Guanine	Amylase
Maltose	Valine	Acetic	Flavonone	Protease
Galactose	γ-amino butyric acid	Propionic		
Ribose	Glutamine	Butyric		
Xylose	α-Alanine	Valeric		
Rhamnose	β-Alanine	Citric		
Arabinose	Asparagine	Succinic		
Raffinose	Serine	Fumaric		
Oligo-saccharides	Glutamic	Glycolic		
	Aspartic acid			
	Glycine			
	Phenylalanine			
	Threonine			
	Tyrosine			
	Lysine			
	Proline			
	Methionine			
	Cystathionine			

content of the different species. Fagus grandiofoliar released the largest amount of amino acids and organic acids per hectare while Betula alleghaniensis exuded the largest amount of sugars.

Age of the plant. More amino acids and sugars were exuded with increased plant age of both pea and oat plants (Rovira, 1956a).

Light and temperature. Rovira (1959) demonstrated that different environmental conditions affect exudation. By altering the intensity of light exposed to clover plants, the quantitative exudation of amino acids changed. Rovira also noted that increasing temperatures increased amino acid exudation, especially for asparagine, by tomato and clover plants.

Presence of microorganisms. It is difficult to study root exudation in natural field or greenhouse conditions as microbial activity influences exudation. Root systems must be sterile, as any substances detected in unsterile systems could be attributed to microbial synthesis rather than solely by exudation. Moreover, microorganisms:

a) affect permeability of root cells and lyse them, so releasing compounds,

b) utilize exudates and make intermediates available, and

c) alter nutrient availability of the plant

Studies by Martin (1977) using labelled organic compounds showed the loss of (^{14}C)-carbon from roots was influenced by the presence of microorganisms, and that absence of bacteria reduced plant growth.

By comparing quantities of carbohydrates released in the presence and absence of microorganisms, Lynch and Barber (1977) suggested that the organisms around the root enhanced substrate release. Yields of organisms were greater than could be supported by carbohydrate concentrations exuded under sterile conditions.

Soil moisture. There was increased release of amino acids when plants were allowed to wilt and then were rewetted (Katznelson et al. 1954). This factor

is important in field conditions where wilting followed by rain must occur. The release of amino acids would cause prolific growth by rhizosphere inhabitants that have amino acid requirements.

Foliar sprays. Wheat plants sprayed with growth regulators and antibiotics exuded greater quantities of amino acids than untreated plants (Vrany et al. 1962). Foliar applications of 2,4-D (25 ppm) at different time intervals onto sorghum and sunhemp enhanced the numbers of amino acids of both plants and also increased concentrations of amino acids in sorghum (Balasubramanian & Rangaswami, 1973).

1.1.7 Sites of exudation. Pearson and Parkinson (1961) used the detection of ninhydrin-positive substances, that is principally amino acids, to locate sites of exudation from broad bean seeds. As the new root emerged ninhydrin-positive substances were detected over the whole root, however, as the root grew it became apparent that a specific region, just behind the root tip, was responsible for the production (Fig. 1.1).

Between 1965 and 1973 McDougall and Rovira undertook a series of experiments using pulse labelling of photosynthates with (^{14}C)-carbon dioxide. By scanning the roots the sites of exudation were detected as the zone of elongation of roots.

1.1.8 Microbiological studies using artificial rhizosphere

The need to use model systems to increase knowledge of the rhizosphere environment has widely been appreciated (Timonin, 1941; Rovira, 1965). The complexity of the rhizosphere must be reduced in models. One of the first workers to try to create an artificial rhizosphere was Timonin (1941). He placed sacks containing growth substances into soil. The 'exudates' diffused through the sack producing rhizosphere conditions on the soil that was attached to the sack. Timonin demonstrated that the density of microbial population in the artificial rhizosphere was greater than in the control soil.

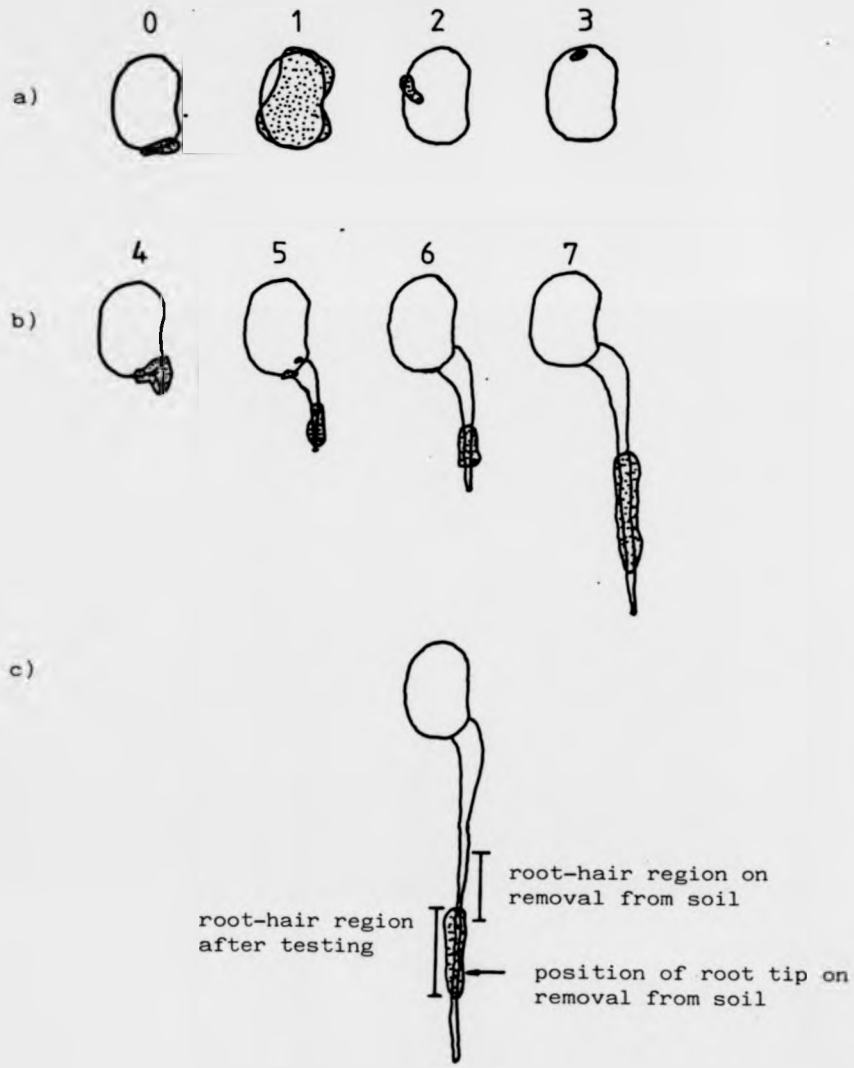
Fig. 1.1 The detection of ninhydrin-positive substances to locate sites of exudation (after Pearson & Parkinson, 1961). The shading represents the areas of detection of the substances.

a. Ninhydrin colour patterns obtained from germinating seeds.

b. Ninhydrin patterns from germinating seeds and their roots.

c. Patterns showing that the detection of substances was not due to damage of root hairs on removal of the roots from soil.

days after planting



Rovira (1956b) created an artificial rhizosphere by the daily addition of root exudates to air dried soil. Rovira considered that a rhizosphere had been successfully established as similar conditions were obtained to those in the natural rhizosphere. But the author did concede that many rhizosphere conditions, such as carbon dioxide accumulation, mineral uptake and the physical nature of root surfaces cannot be simulated by this method.

Chan et al. (1963) realized the need for rhizosphere studies with mixed cultures and investigated the growth of five bacterial species on root extracts of different plants from various stages of growth. The organisms used were Agrobacterium radiobacter, Arthrobacter citreus, Azotobacter chroococcum, Bacillus cereus and a Pseudomonas species. The root extracts were prepared from two, four and eight week old oat, wheat and soybean plants. Roots were washed, air-dried, soaked in agitated water for 2-3h, filtered, centrifuged then sterilized by filtering through millipore membranes. The results showed that the Pseudomonas species (Gram-negative) predominated over Arthrobacter citreus (Gram-positive, coccoid rods) in mixed culture as found in the general rhizosphere microflora (Section 1.1.3). Using soil extracts the Pseudomonas species was not as good a competitor as Arthrobacter citreus. The age of the plant was of more importance than the species; for example, the root extracts from all four week old plants gave the same results, that is, the Pseudomonas species produced maximum growth but the Bacillus cereus and Azotobacter chroococcum were poor competitors. This study assumed that prepared root extracts produced an equivalent effect to root excretions.

Kunc and Macura (1966) copied the continuous enrichment of soil by exudates by adding synthetic exudates containing mixtures of organic acids, sugars and amino acids to soils. The analysis of the microbial populations which resulted showed an increase in the numbers of microorganisms in the vicinity of the exudate source. The nutritional requirements of bacteria were as found in the rhizosphere, that is, requiring amino acids.

Using two concentrations of synthetic exudates Abdel-Nasser and Moawad (1975) found that the numbers of the heterotrophic microflora increased after exudate additions. The numbers were correspondingly higher for the higher exudate concentration.

1.1.9 The Importance of the Rhizosphere

The rhizosphere microflora can be beneficial to plant growth. The microflora can mineralize organic forms of nitrogen, phosphorus and sulphur which would normally be unattainable to plant roots. Inorganic ions so generated can sustain plant growth. By breaking down compounds the rhizosphere microorganisms are contributing to soil fertility and nutrient cycling. The rhizosphere microflora produce compounds that are toxic to soil-borne plant pathogens.

All nutrients reaching the plant from the soil must pass through the rhizosphere, so any change, either biological or chemical, made by rhizosphere microorganisms could affect the plant.

Rhizosphere inhabitants can synthesize plant growth regulators (Jackson et al. 1964).

1.1.10 Pesticides in the Rhizosphere

The presence of several pesticides in the rhizosphere alters exudation patterns (Section 1.1.6) as well as some metabolic processes.

Rao and Sharma (1978) studied the effect of the additions of several fungicides to cauliflower plants. The bacteria, fungi, actinomycetes and free living nitrogen fixing bacteria were all increased numerically when compared to control soils further away from the roots. Activity of amylase, invertase and cellulase enzymes were also correspondingly higher. There was a significant correlation between the fungal populations and enzyme activity. The authors interpretation included that the rhizosphere fungi could have contributed to enzyme levels, so increasing biodegradative opportunities.

Hsu and Bartha (1979) used two radiolabelled organophosphate

insecticides, diazinon and parathion. After addition of the labelled compounds the evolution of (^{14}C)-carbon dioxide of bush bean rhizosphere and control soil was quantified. The presence of rhizosphere microorganisms was necessary for insecticide degradation. The authors concluded that the process of cometabolism in mineralization (Section 1.5.3) was possibly responsible for degradation. The root exudates in the rhizosphere provide many easily utilizable carbon sources that aid the transformation of xenobiotic compounds such as organophosphate insecticides.

1.2 CHLORINATED PHENOXYALKANOIC HERBICIDES

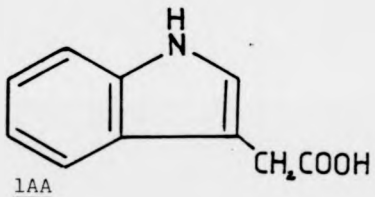
1.2.1 Development of selective herbicides

To feed a growing population the efficiency of agricultural techniques must be maximized. One method that has greatly increased productivity is the control of the growth of unwanted plants or weeds. The definition of a weed is subjective, Fletcher and Kirkwood (1982) described them as 'plants growing in the wrong places'. Weeds compete with crops for light, water, nutrients and space, so are harmful to crops as they substantially reduce yields.

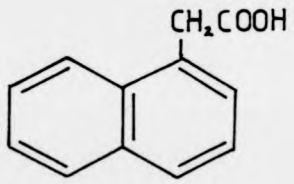
Earlier usage of herbicides (pre-1940) was confined to broad ranged inorganic compounds that tended to accumulate in soils and crops, pointing to the need to develop compounds that would selectively kill weeds in cereal crops.

In the 1920's substances that controlled plant growth were identified. One of the first of these hormones was called indole-acetic acid (IAA, Fig. 1.2). Work in the early 1940's at Imperial Chemical Industries' Research Station at Jeallots Hill investigated the role of hormones in plant growth and sprayed a plant growth regulator α-naphthylacetic acid (NAA) onto oats and charlock (a *Brassica* species). The *Brassica* species was killed, but the cereal was left unharmed. Similar results were obtained when NAA was sprayed onto other weeds. The workers then examined compounds which were similar in both structure and growth regulatory properties to NAA, and tried

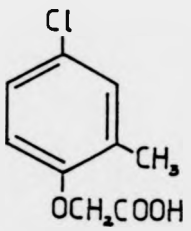
Fig. 1.2 The chemical structures of some natural and synthetic auxins (after Fletcher & Kirkwood, 1982).



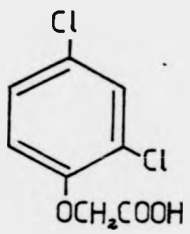
IAA



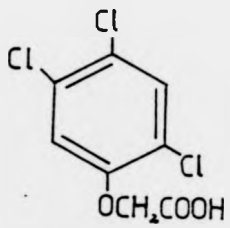
NAA



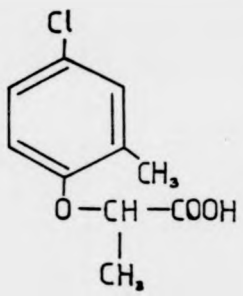
MCPA



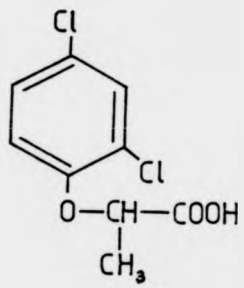
2,4-D



2,4,5-T



mecoprop



dichlorprop

4-chloro-2-methyl phenoxyacetic acid (MCPA, Fig. 1.2) which was active at low concentrations.

Workers at the Rothamsted Experimental Station (1945) (cited by Fletcher & Kirkwood, 1982) also realised the potential of synthetic plant growth hormones as weed killers. They investigated 2,4-dichlorophenoxyacetic acid (2,4-D, Fig. 1.2) for herbicidal properties. By the late 1940's both MCPA and 2,4-D were widely used in both Britain and the USA as they were cheap to produce, virtually non-poisonous to animals, active at low concentrations and translocated to all parts of the plant (Fletcher & Kirkwood, 1982).

A third phenoxy herbicide was introduced, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T, Fig. 1.2) as it was more effective than 2,4-D as a brushkiller. In 1956 the Boots Pure Drug Company applied to use 2-(4-chloro 2-methyl phenoxy) propionic acid (mecoprop, Fig. 1.2) as it was more effective against chickweeds and cleaver than 2,4-D and MCPA.

1.2.2 Uptake and mode of action of phenoxy herbicides

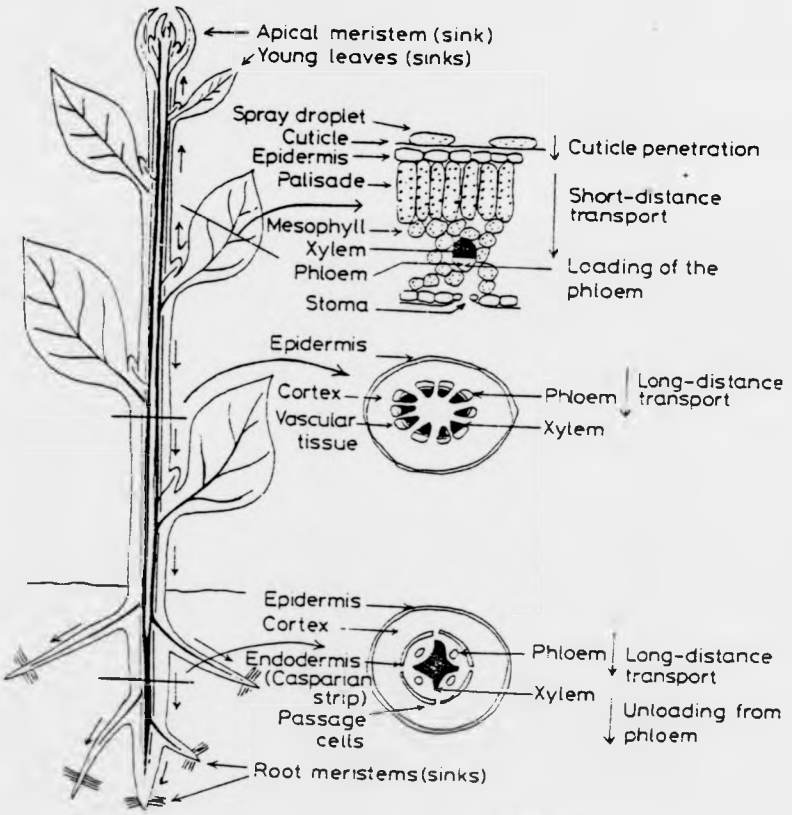
The herbicide becomes active when it is taken up into the plant and the concentrations that reach the plant depend upon the leaf structure. The leaf cuticle acts as a barrier and the rate of uptake is dependent upon the cuticle thickness which is species dependent. The leaf stomata may act as a specialised entry site (Fig. 1.3).

From Figure 1.3 it can be noted that the foliage applied herbicides, which include mecoprop, reach the areas of the root meristem and leave the plant via the root systems to the surrounding soil.

When the phenoxy herbicides reach the active sites in the weeds proliferation of tissues and chaotic growth results caused by interference of the RNAase enzyme giving synthesis of RNA and protein. The cell proliferation and swelling of leaves and stems disrupts the transport systems in the plants and starvation in roots occurs, eventually leading to senescence.

Phenoxy herbicides also inhibit and uncouple oxidative phosphorylation.

Fig. 1.3 Sites of entry and loss of foliage applied herbicides and their transport around the plant (after Fletcher & Kirkwood, 1982).


Apical meristem (sink)
Young leaves (sinks)
Spray droplet
Cuticle
Epidermis
Palisade
Mesophyll
Xylem
Phloem
Stoma
Cuticle penetration
Short-distance transport
Loading of the phloem
Epidermis
Cortex
Vascular tissue
Phloem
Xylem
Long-distance transport
Epidermis
Cortex
Endodermis (Casparian strip)
Passage cells
Phloem
Xylem
Long-distance transport
Unloading from phloem
Root meristems (sinks)

1.2.3 Biodegradation of phenoxy herbicides

Degradation of the phenoxy herbicides in soil is important otherwise repeated applications of persistent herbicides would cause accumulation in soils (Alexander, 1961a). As the herbicides are organic molecules they may be utilized by soil microorganisms as sources of carbon or energy. Loos (1975) suggested that the ability of soil populations to degrade phenoxy herbicides was the result of enzyme systems which catalyzed the breakdown of compounds structurally related to the herbicides but lacked specificity, which resulted in the breakdown of the phenoxy compounds.

Audus (1950) reported the first isolation of a soil bacterium, '*Bacterium globiforme*', that could degrade 2,4-D. Further studies by Audus (1951) used soil perfusion techniques to examine 2,4-D, MCPA and 2,4,5-T degradation. Three distinct phases were described:

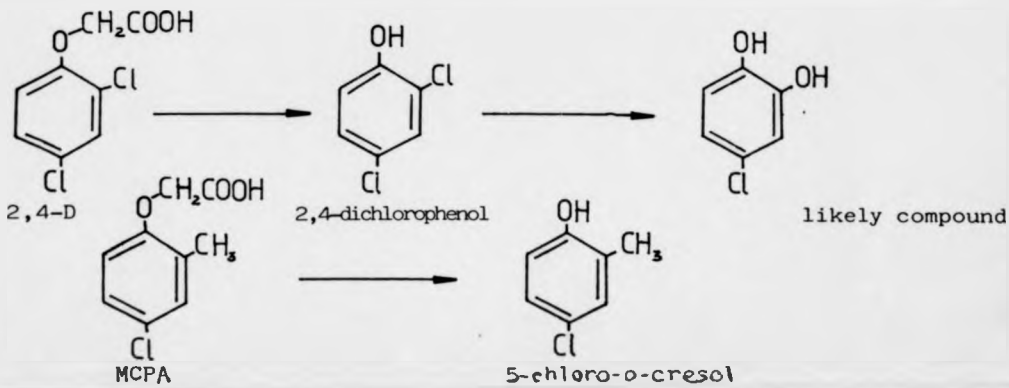
a) absorption of the three herbicides onto soil colloids,

b) lag phases of varying lengths during which no degradation occurred, and

c) rapid degradation followed.

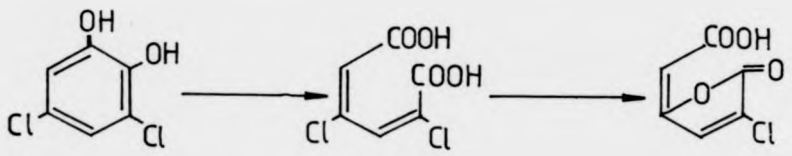
Enriched soil populations that resulted were capable of rapid degradation of further herbicide additions. Preliminary evidence that 2,4-dichlorophenol was an intermediate of 2,4-D breakdown was reported.

Attempts to confirm the identity of the metabolites of the first stages of degradation of 2,4-D and MCPA were undertaken (Steenson & Walker, 1957) using manometric techniques. The authors postulated that the first step in degradation of both herbicides involved the oxidation of the acetic acid side chain, to give the corresponding phenol, and that 4-chlorocatechol was a likely intermediate in 2,4-D metabolism.



Loos et al. (1967a,b) used an Arthrobacter species to investigate formation of metabolites during 2,4-D degradation. By comparing infra red spectra and gas chromatograms of metabolites with authentic samples they demonstrated that 2,4 dichlorophenol was the first intermediate produced, but that the second metabolite was 2,4-dichloroanisole. Bollag et al. (1968a) used the same organism to show 2,4-dichlorophenol was converted to 3,5-dichlorocatechol.

In further studies Bollag et al. (1968b) investigated the fate of the catechol compound produced by the Arthrobacter species. The catechol as metabolized and the intermediate produced still contained the two halogens. In a subsequent reaction one of the chlorines was released. Using mass spectrometry they established that the compound generated from 3,5-dichlorocatechol had a probable formula of $C_6H_3Cl\ O_4$ and considered that α-chloro-γ-carboxy-methylene-Δ^{α}-butenolide would have the correct structure. The proposed pathway was:



3,5 dichlorocatechol

α-chloro-γ-carboxy methylene-Δ^{α}-butenolide

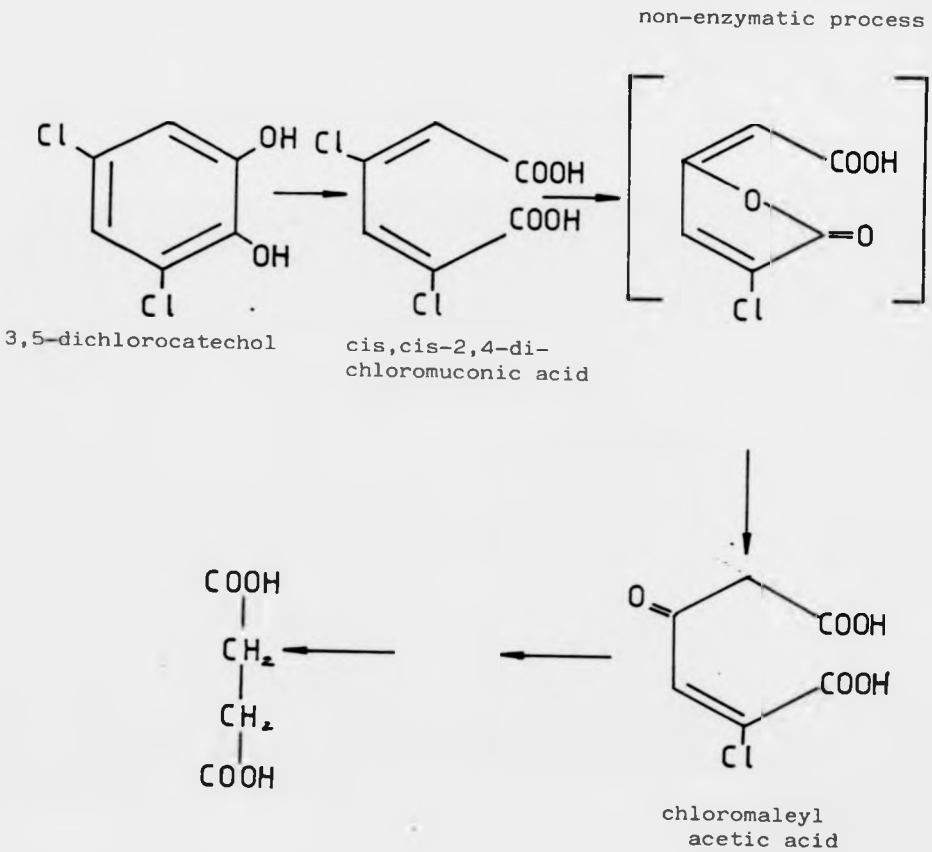
This suggested that β-chloromuconate was the intermediate between 3,5-dichlorocatechol and α-chloro-γ-carboxy-methylene-Δ^{α}-butenolide.

The final products of the reaction sequence for Arthrobacter species were identified by Tiedje et al. (1969) using mass spectra and infra red spectrometry. Enzymes from the bacterium grown on 2,4-D were used, 2,4 dichloromuconic acid was metabolized to yield a product identified as chloromaleyl acetic acid, which in turn yielded succinic acid (Fig. 1.4).

Fig. 1.4 Pathway of chlorocatechol degradation by enzymes from an *Arthrobacter* species (after Tiedje *et al*. 1969).

FIG. 1.4

Pathway of chlorocatechol degradation by enzymes from Arthrobacter sp. From Tiedje et al., (1969).



Gaunt and Evans (1971a) presented evidence of the metabolic pathway of MCPA degradation by a Pseudomonas species. They identified intermediates, using chromatography, as 5-chloro-o-cresol and γ-chloro-α-methylmuconate. The authors considered the latter compound could only arise from the ortho cleavage of 5-chloro-3-methylcatechol although this compound was not detected.

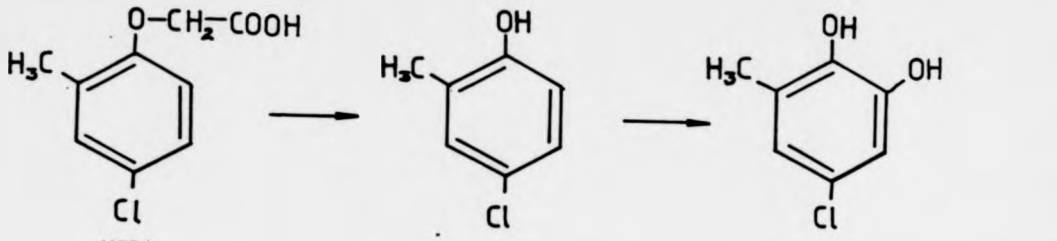
γ-carboxy-methylene-α-methyl-$\Delta^{\alpha\beta}$-butenolide was also identified and its percursor was postulated as cis-cis-γ-chloro-α-methylmuconate. A metabolic pathway was suggested (Fig. 1.5).

Cell-free systems of Pseudomonas species NCIB 9340 were used to resolve three enzyme systems which catalyzed several of the sequences in MCPA degradation (Gaunt & Evans, 1971b). The ring-fission, lactonizing and delactonizing enzymes were separated and their requirements were elucidated.

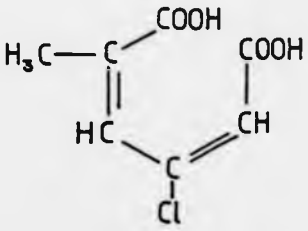
Despite the enormous amount of data generated during the years of 1950 to the 1970's on 2,4-D and MCPA degradation little, if any, research had shown degradation of 2,4,5-T or mecoprop. Studies have pointed to the effect of molecular structure on microbial breakdown (Alexander & Aleem, 1961). Simple differences of molecular structure between 2,4-D and 2,4,5-T namely a third chloride ion at the meta position in relation to the phenoxy group, causes the aromatic nucleus to remain intact for longer. No microorganisms capable of utilizing 2,4,5-T as the sole source of carbon and energy were reported until recently. A mixed culture of organisms capable of 2,4,5-T degradation arose by a technique termed 'plasmid-assisted molecular breeding' (Kellog et al. 1981). From the mixed culture a pure culture, Pseudomonas cepacia AC1100 was isolated (Kilbane et al. 1982) that was capable of growth on 2,4,5-T as the sole carbon and energy source. The loss of the carbon source was followed in laboratory cultures using spectrophotometric analysis and gas chromatography.

Further work undertaken by Chakrabarty's group examined the capacity of the pure culture to degrade 2,4,5-T in contaminated soils rather than laboratory cultures (Chatterjee et al. 1982). Concentrations up to 1,000

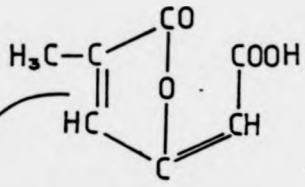
Fig. 1.5 Proposed pathway for the metabolism of MCPA by Pseudomonas sp. 9340 (after Gaunt & Evans, 1971a).



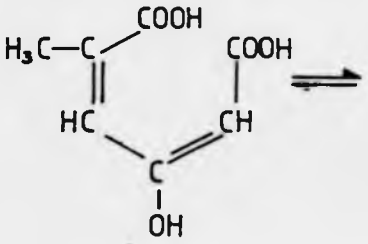
MCPA — 5-chlor-o-cresol — 5-chloro-3-methylcatechol



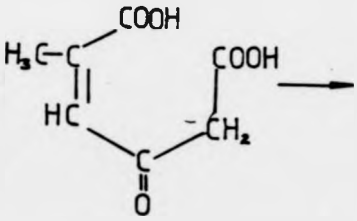
γ-chloro-α-methylmuconic acid



γ carboxymethylene-α-methyl-$\Delta^{\alpha\beta}$-butenolide



γ-hydroxy-α-methylmuconic acid



β-methylmaleyl acetic acid

split before entering a terminal respiratory cycle

ppm 2,4,5-T in soils were effectively removed by the organism. Degradation in soils only occurred in the presence of Pseudomonas cepacia AC1100. Optimal conditions of moisture and temperature were established. Heavier contamination, up to 20,000 ppm 2,4,5-T per gram of soil was removed so effectively by AC1100 that the soil was able to support plant growth (Kilbane et al. 1983). AC1100 was able to completely dehalogenate 2,4,5-trichlorophenol, 2,3,4,6-tetrachlorophenol and pentachlorophenol and many brominated and fluorinated aromatic compounds. Evidence was presented which showed that 2,4,5-trichlorophenol was an intermediate in 2,4,5-T degradation (Karns et al. 1983a).

The mode of regulation of 2,4,5-T metabolism by strain AC1100 was investigated (Karns et al. 1983b). AC1100 was grown on different carbon sources and the response to several chlorinated compounds was monitored. Dechlorination of 2,4,5-trichlorophenol (2,4,5-TCP) and pentachlorophenol (PCP) occurred when the organism was grown on 2,4,5-TCP and 2,4,5-T, but not when the strain was grown on lactate, glucose or succinate. Karns et al presented evidence which demonstrated that constitutive enzymes controlled the conversion of 2,4,5-T to 2,4,5-TCP, but inducible enzymes controlled the degradation of 2,4,5-TCP to further, as yet unidentified, metabolites.

An 'agent' that emulsified 2,4,5-T into a stable colloid suspension, was produced by AC1100 at the stationary phase of growth (Banerjee et al. 1983). More of the emulsifying agent was produced by 2,4,5-T-grown cells than by glucose-grown cells. Optimal activity of the agent was between pH 7.0 and 9.0. Investigations into the nature of the agent showed several enzymes, including DNase and RNase had no effect on activity. Prolonged heating at 100^{o}C reduced the emulsifying activity, whereas solvent extraction using a lipid-extracted mixture removed activity completely. The authors suggested that the agent played a role in transporting 2,4,5-T into cells.

Compared to 2,4-D and MCPA, little is known of the persistence or

degradation of mecoprop by soil organisms. Kilpi (1980) used soil samples which had been agriculturally exposed to mecoprop for three years in an attempt to isolate herbicide degrading bacteria. However, these attempts were unsuccessful. A mixed culture growing on dichlorprop and benzoic acid could grow on mecoprop and dichlorprop, but only when benzoic acid was present as a co-substrate. This ability was lost on extensive subculturing. Kilpi suggested that the difficulty in obtaining mecoprop-degrading bacteria could have been due to growth culture techniques which encouraged rapidly growing bacteria in preference to slower growing bacteria (Section 1.5.1).

Samples of three Saskatchewan soils were used to compare the rates of degradation of mecoprop. Half lives of between seven and nine days were reported depending upon soil type (Smith & Hayden, 1981).

1.2.4 Degradation of carbon-chloride bonds

Chlorinated aromatic compounds are known to lose their halogenated substituents upon degradation. However the enzymes that catalyze the breaking of the aromatic carbon and halogen bond have not been extensively studied. The enzymes responsible for breaking the carbon-halogen bond of aliphatic compounds have been more widely investigated.

Slater's group have examined the activities of dehalogenase enzymes using a range of chlorinated aliphatic compounds. Hardman and Slater (1981a) isolated 16 soil bacterial strains capable of degrading 2-monochloropropionic acid or monochloroacetic acid. The bacteria were divided into five groups based on their reponse to four straight chain chlorinated compounds. Gel electrophoresis revealed that there were four different dehalogenase enzymes. Further studies by Hardman and Slater (1981b) used one of the bacterial strains previously isolated, denoted E4. Two dehalogenase enzymes were detected when E4 was grown in continuous culture with either monochloroacetate or 2-monochloropropionate as the growth-limiting substrate. The activity of dehalogenase I to several chlorinated alkanoics was described. A third

dehalogenase was detected under certain growth conditions.

Slater et al. (1979) compared the enzyme activities of two strains of Pseudomonas putida P3 and S3. The former had greater enzyme activities than the latter, and its dehalogenase activity was induced by chlorinated compounds that did not support growth. S3 was unable to grow on several halogenated aromatics.

1.3 MIXED MICROBIAL COMMUNITIES

1.3.1 Traditional methods: pure culture techniques

Pure culture techniques have been most beneficial and their importance in aiding our understanding of environmental biodegradation is recognised (Bull, 1980). The isolation and growth of a pure culture from soil samples growing on a compound as the sole source of carbon and energy has been considered to be evidence of the organisms role in degradation.

However, Harder (1981) considered there may be some disadvantages of focusing attention on pure culture techniques. Compounds could be reported to be persistent because the correct culture conditions were not selected.

Several studies by Slater and Bull (Slater, 1978; Bull, 1980; Slater, 1981; Slater & Bull, 1982) have reminded the reader of the heterogeneous nature of soil and the great diversity of growth substances, natural habitats and populations that occur within it. The authors have referred to the diversity of organisms found in several extreme environments (such as extremes of pH or temperature). From this they concluded that there are probably only a few, if any, environments where organisms grow in isolation, pointing to the greater relevance of studying mixed communities of organisms in preference to pure cultures. Pure culture techniques have been emphasized since Koch's work in 1881, and have been preferentially taught.

1.3.2 The advantages of using mixed culture techniques for studying biodegradation of xenobiotic compounds

Xenobiotic compounds are defined as being environmentally foreign and include many synthetic chemicals that are released into natural environments as pesticides (Table 1.3). Microorganisms which have the potential to degrade many naturally-occurring compounds may not have the genetic potential to degrade new chemical structures. The greater biodegradative capabilities of mixed microbial communities may increase opportunities to breakdown some xenobiotic compounds (Bull, 1980).

Slater (1978) considered a situation which could arise, where individual community members could not degrade a specific carbon source, but together the mixed community generated the necessary catabolic capacity. Conversely, a mixed community may be better adapted to utilize complex carbon compounds than single species (Slater & Godwin, 1980). Beam and Perry (1974) were unsuccessful in attempting to isolate organisms in pure culture which could degrade cycloparaffins, however mixed cultures using a co-substrate, hexadecane, were capable of utilizing cyclohexane.

Similar results were obtained by Bollag and Liu (1971) and Ou and Sikka (1977). In the former study, sevin, a carbamate insecticide was used as the growth substrate. A mixed community comprising of three microorganisms, one fungus and two bacterial species, was more effective in transforming the insecticide than the individual community members. The latter study used silvex ((2,4,5-trichlorophenoxy)propionic acid) and yeast extract as a co-substrate. A mixed culture of a Pseudomonas species and an Achromobacter species degraded silvex, but the two single cultures were not capable of degradation.

Senior et al (1976) described the structure of a seven-membered microbial community that utilized Dalapon, (2,2-dichloropropionic acid). The community could be divided into three primary utilizers and four secondary

TABLE 1.3 **Origins of pollutants in the environment**

(after Higgins & Burns, 1975).

A. NATURALLY OCCURRING	B. TRANSFORMED AND CONCENTRATED	C. SYNTHESIZED
Oxides of nitrogen	Sewage	Pesticides
Nitrate	Fertilizers	Surfactants
Nitrite	Acid waste	Radionuclides
Asbestos	Fuel combustion products	Synthetic polymers
Heavy metals	Pesticides	Petrochemicals
Radionuclides	Surfactants	
Hydrocarbons and their derivatives	Hydrocarbons	
	Petrochemicals	
	Heavy metals	
	Radionuclides	

utilizers. After 3,000h of continuous growth in a chemostat, a fourth primary utilizer, P3, was detected. It was thought that P3 arose from a mutation from one of the secondary utilizers.

The degradation of synthetic industrial sewage mixtures were studied using combinations of either two or three organisms (Schmidt et al. 1983). The mixture included methanol, ethanol, acetone, isopropanol, phenol and either 4-chlorophenol or mixtures of 2-, 3- and 4-chlorophenol. Initially, Alcaligenes species strain A7 and Pseudomonas extorquens were introduced to a fermenter containing the 4-chlorophenol mixture. Degradation of the carbon sources was not achieved and the culture washed out. The study was repeated but a third organism, Pseudomonas species strain B13, was introduced. Full degradation of 4-chlorophenol was achieved. Similar results were obtained when the isomeric chlorophenols were included in the growth media. Alcaligenes species A7 and Pseudomonas extorquens washed out, but when strain B13 was present a stable community established with full degradation of the carbon source.

1.3.3 Validity of mixed community studies

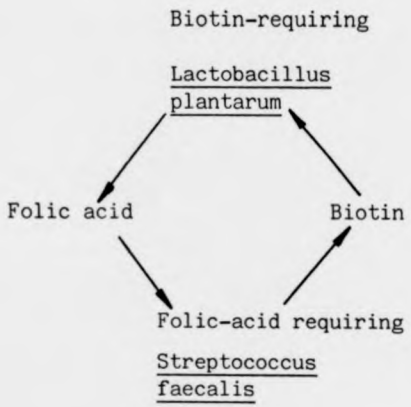
It can be argued that mixed culture biodegradation studies no more reflect natural environments than pure culture studies. Slater (1981) answered an hypothetical question by considering that the potential of such communities must exist in a particular sample if it is possible to isolate a stable community from that sample.

1.3.4 Interactions between community constituents

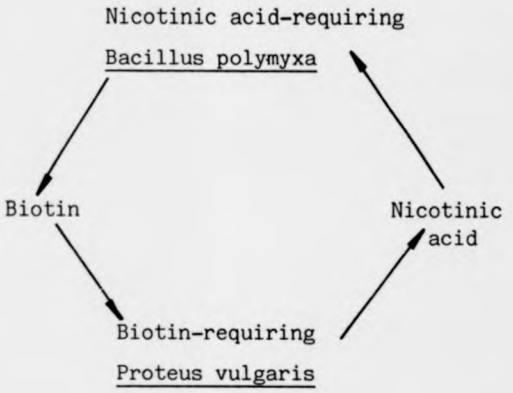
Several studies have reported the mechanisms of interactions between community members as being mutually beneficial or beneficial to one organism based on specific carbon sources. For example, one member of the community, A, was unable to synthesize a necessary growth factor. This was grown in the presence of a second organism, B, which synthesized and excreted the necessary growth factor for organism A (Slater, 1978). Further examples of interactions

Fig. 1.6 Examples of two-membered associations of microorganisms based upon the provision of specific growth compounds (after Slater, 1978).

Microbial community involving
Lactobacillus plantarum and
Streptococcus faecalis



Microbial community involving
Bacillus polymyxa and
Proteus vulgaris



between two-membered communities are illustrated in Figure 1.6.

Interactions between community constituents can be based on parameters other than the carbon source(s), such as the removal of inhibitory compounds, combined metabolic capabilities, alterations of growth constants or electron transfer (Slater, 1978).

It is difficult to determine if such community interactions play an important role in natural systems. In a recent review Slater (1978) suggested that they could be important in the study of microorganisms in relation to their environment.

1.4 PLASMIDS

1.4.1 Introduction to Plasmids

Plasmids are extrachromosomal DNA molecules which can exist in an autonomous state. They are capable of replication independently from the chromosomal DNA and may pass on copies to both daughter cells upon host cell proliferation. The genetic information carried on plasmids is not essential for its hosts survival, but enhances it in the presence of certain substances (Williams, 1978). Their occurrence throughout soil microorganisms is widespread (Reanney et al. 1982).

Novick (1980) described plasmids as subcellular organisms as 'any nucleic acid system that controls its own replication should therefore be regarded as an organism'. Plasmids can exist in cells in a variety of numbers of copies, between 1-50 and in different sizes from 1-200 Megadaltons.

Plasmids confer specific biological functions upon the host cell, for example, resistance to one or more antibiotics, heavy metal resistance or the ability to degrade a particular carbon source.

Plasmids that are able to transfer themselves by cell to cell contact from host to donor cell are called conjugative and the process is conjugation. Many plasmids over 20 Megadaltons have the necessary genes for conjugation, but some plasmids of this size, together with smaller plasmids, are not capable of this process and are termed non-conjugative.

The role of the plasmid genes can be examined by chemically removing

them then looking at the phenotypic responses in the cured cells. The chemicals, such as acridine dyes, mitomycin C, ethidium bromide or rifampicin are mutagens, but other environmental or nutritional conditions can bring about loss of the plasmid. Once the extrachromosomal element is lost it can only be introduced back into the cell by another conjugative plasmid.

1.4.2 Degradative plasmids

Degradative plasmids have gene sequences that code for catabolic enzymes for certain compounds. Some plasmids contain codes for the degradation of many naturally occurring compounds including camphor, salicylate, naphthalene, octane, xylene and toluene (Fig. 1.7).

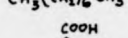
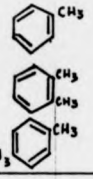
The CAM plasmid does not have the complete set of genes for camphor degradation. Instead camphor is degraded to isobutyrate and recipient cells, that have chromosomal borne genes for isobutyrate degraded it further. The recipient genes are not induced by camphor (Chakrabarty, 1976).

The SAL plasmid codes for the biodegradation of salicylate (Fig. 1.7). Unlike CAM it specifies the complete mineralization of the organic molecule.

The NAH plasmid specifies the degradation of both salicylate and naphthalene as the former is a metabolite of naphthalene, however the converse is not true (Fig. 1.7).

The XYL and TOL plasmids are frequently grouped together as they can carry out the same functions in the host cell. The TOL plasmid specifies the degradative pathway of *para* or *meta* toluate or xylenes. The XYL plasmids host cell can degrade xylene, *p*-*m*-toluenates are intermediates in xylene degradation so they can also be degraded. The plasmids do differ as TOL is a conjugative plasmid and XYL is not (Chakrabarty, 1976). Reineke and Knackmuss

Fig. 1.7 Degradative plasmids in Pseudomonas (after Williams, 1978).

Plasmid	Molecular weight ($\times 10^{-6}$)	Primary growth substrate		Plasmid coded pathway
CAM	160	camphor		camphor → acetate + isobutyrate
OCT	27 *	octane		octane → octanoate
SAL	51	salicylate		salicylate
NAH	49	naphthalene		naphthalene → salicylate → catechol → acetaldehyde + pyruvate
TOL+	60 to 170	toluene m-xylene p-xylene		toluene → benzoate → catechol → acetaldehyde + pyruvate

* 27×10^6 is MW for OCT fragment of the complete OCT plasmid, whose intact MW is $60\text{-}70 \times 10^6$.

+Molecular weight range for a number of different isofunctional plasmids

(1979) transferred the TOL plasmid from Pseudomonas putida mt-2 (WR101) to Pseudomonas species B13 (WR1). The latter could degrade 3-chlorobenzoate and 4-chlorophenol. The strains produced were capable of degrading novel compounds including 4-chloro- and 3,5-dichlorobenzoate.

The OCT plasmid codes for the degradation of several straight chain alkanes, such as octane and decane.

1.4.3 The role of plasmids in phenoxy herbicide degradation

Section 1.4.2 described the occurrence of plasmids coding for degradation of naturally occurring compounds. Plasmids have also been indicated to play a role in the degradation of two of the phenoxy herbicides, (Pemberton & Fisher, 1977; Fisher et al. 1978). The first report of the existence of pesticide degrading plasmids was by Pemberton and Fisher (1977). A strain of Alcaligenes paradoxus JMP 116, containing a plasmid, was capable of growth on 2,4-D as the sole carbon and energy source. The plasmid was cured using mitocycin C and the cured isolate was not capable of degrading the herbicide.

Further studies using JMP 116 (Fisher et al. 1978) demonstrated that the plasmid, pJP1, encoded for both 2,4-D and MCPA degradation. When the plasmid was cured the host cell could not degrade 2,4-D but 2,4-dichlorophenol, the first intermediate in 2,4-D degradation (Section 1.2.3), was degraded. The authors concluded that the plasmid specified the conversion of 2,4-D to 2,4-dichlorophenol. The chromosomal material of the host cell contained the genes for 2,4-dichlorophenol degradation. pJP1 was a conjugative plasmid.

Don and Pemberton (1981) showed that the herbicide 2,4,5-T was not degraded by any of six plasmid bearing strains. The authors concluded that as the six plasmids from different species of Alcaligenes were physically and genetically alike they could have a common ancestry.

The environmental importance of pesticide degrading plasmids cannot be over emphasized. If the potential exists in natural soil populations to

degrade one or more synthetic pesticides and this property can be distributed throughout the population by conujugation, then this removal can halt the build-up of pollutants in the environment (Fisher et al. 1978; Pemberton et al. 1979; Pemberton, 1979).

1.5 ENRICHMENT TECHNIQUES

1.5.1 Batch system cultures

Several methods are available both for studying microbial growth and enrichment of biodegrading organisms. The traditional method involves the use of batch cultures as they are effective and simple to analyze (Slater, 1979). Batch grown organisms are alternatively described as closed cultures as a reflection of growth conditions; that is, there is no input or output of materials after culture inoculation. All growth nutrients are initially present in excess, permitting growth at the maximum specific growth rate (μ_{max}) of the organisms. However, exponential growth is only maintained for a short time before nutrient depletion or the build-up of toxic by-products halts growth, showing that the process is self-limiting.

Closed cultures, therefore, lack continuity, and the most successful organism(s) are those with the highest μ_{max} values.

Bull (1980) considered this to be a disadvantage if isolating microbial communities as their selection would be based upon their μ_{max} values. The intricate reactions of microbial community growth are difficult to control in closed systems (Harder et al. 1977).

1.5.2 Continuous-flow cultures

Continuous cultures are characterized by a balance between input of growth nutrients and an output of organisms, unused and spent materials. Alternatively, they are called open cultures. There are several types of continuous-flow systems:

a) chemostats, which are described as having the dilution rate externally controlled by the flow rate;

b) turbidostats, where the culture absorbance readings electrically control the flow rate, so producing internal control and allowing growth at the highest specific growth rate (Bull, 1980), and

c) multi-stage chemostats that have more than one growth chamber, each at a different dilution rate and providing continuous inoculation of the successive chambers (Bull, 1980).

The growth rate in open cultures is limited by one nutrient, so exponential growth is maintained. Theoretical mathematical equations describing open cultures have been adequately described elsewhere (Harder et al. 1977; Slater, 1979) and will not be repeated in this Introduction. However, details from the equations have shown growth is self-regulating to produce steady state conditions, allowing growth to proceed at a range of growth rates up to μ_{max}.

There are few, if indeed any, natural environments where conditions prevail to give the maximum specific growth rates of organisms. Growth at submaximal levels, as permitted by open culture systems, may simulate natural conditions (Slater, 1979).

1.5.3 The study of pesticide metabolism using continuous culture techniques

Several recent reports discussed the advantages of continuous culture techniques for analyzing possible environmental degradation of pesticides (Bull, 1980; Harder, 1981). It is surprising to note that very few such studies have been undertaken.

A widely used organophosphorus insecticide was used as the sole source of carbon and energy for continuous culture studies. Parathion (O,O-dimethyl O-p-nitrophenyl phosphorothionate) was added as the growth limiting substrate to a two stage chemostat (Daughton & Hsieh, 1977). After two years of continuous cultivation two bacterial species were isolated that degraded parathion synergistically. A strain of Pseudomonas stutzeri hydrolyzed the

the insecticide to p-nitrophenol which was in turn utilized by Pseudomonas aeruginosa. Intermediates of p-nitrophenol degradation were utilized by Pseudomonas stutzeri. This was the first report of parathion degradation by a defined microbial community. Daughton and Hsieh considered the continuous culture techniques allowed the use of low substrate concentrations favourable to enrichment conditions. Batch cultures may contain parathion at toxic concentrations.

A second report of pesticide studies in chemostats was undertaken by Senior et al. (1976). Dalapon, a herbicide used for controlling monocotyledons, was used as the sole carbon and energy source. A microbial community capable of degrading the herbicide was isolated from the chemostat (see Section 1.3.2).

1.5.4 The rhizosphere as a chemostat

Few studies have used chemostat growth conditions as a model for microbial growth in the rhizosphere. Lynch (1982) stated that the continuous additions of root exudates to the rhizosphere were similar in principle to a continuous-flow culture, but he was uncertain of microbial removal from the area. This led Lynch to postulate that a fed-batch culture may provide a more precise rhizosphere analogy than continuous culture.

Studies undertaken at Colorado State University provided a series of reports using chemostat techniques. The interactions of a constructed microbial community were studied growing on a mixture of glucose, amino acid and vitamin supplements (Anderson et al. 1978; Cole et al. 1978; Coleman et al. 1978). The validity of this study was rightly questioned by Lynch (1982); firstly, because an isolated community would have been more representative than a constructed one; secondly, there was an incorrect emphasis on glucose additions rather than other commonly found exudate components.

1.6 AIMS OF THE STUDY

Many studies have demonstrated that interacting communities isolated from soils are capable of degrading some xenobiotic compounds, but little is known of the existence of such systems in the root region. The aim of this study was the isolation of microbial communities from the root environment capable of degrading mecoprop - a widely used herbicide. An enrichment programme included batch and chemostat techniques for the isolation of degrading organisms. The resulting microbial community was characterized in terms of microbial species and their population sizes. Attempts were made to elucidate interactions occurring between the community constituents.

CHAPTER TWO

MATERIALS AND METHODS

2.1 GROWTH MEDIA

2.1.1 Minimal medium

Medium of the following composition was used for growth of organisms in both batch and chemostat cultures; (gl^{-1} glass distilled water) $MgSO_4.7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.5; KH_2PO_4, 0.5; K_2HPO_4, 1.5; trace elements, 10 ml.

The trace element solution contained (gl^{-1} glass distilled water) NaEDTA, 12.0; NaOH, 2.0; $MgSO_4.7H_2O$, 1.0; $ZnSO_4.7H_2O$, 0.4; Na_2SO_4, 10.0; $Na_2MoO_4.2H_2O$, 0.1; $Fe_3SO_4.7H_2O$, 2.0.

The minimal medium was sterilized by autoclaving at 15 lbs in^{-2} for 15 min. The pH of the medium was 7.0 but was altered before autoclaving by suitable additions of concentrated H_2SO_4 or NaOH when required.

2.1.2 Addition of carbon sources

Unless otherwise stated all carbon sources were autoclaved at 10 lbs in^{-2} for 10 min and added aseptically to the defined growth medium to give a final concentration of 0.25g carbon l^{-1} (gCl^{-1}).

2.1.3 Nutrient Agar, Malt Extract Agar and Nutrient Broth

All were made according to the manufacturers specifications and were sterilized by autoclaving.

28g of nutrient agar was suspended in 1.0 litre of glass distilled water and stirred until fully dissolved. It was not necessary to boil the mixture to dissolve the nutrient agar. The agar was sterilized by autoclaving at 121°C for 15 min.

Malt extract agar (50g) was dissolved in 1.0 litre of glass distilled water and sterilized by autoclaving at 115°C for 10 min.

13.0g of nutrient broth was mixed into 1.0 litre of glass distilled water. The broth was sterilized by autoclaving at 121°C for 15 min.

2.1.4 King's B medium

King's B medium (King et al. 1954) contained (gl^{-1} distilled water) glycerol, 10.0 ; K_2HPO_4, 1.5 ; Protease peptone, 20.0 ; $MgSO_4.7H_2O$, 1.5 ; Bacteriological agar, 20.0 . All of the ingredients were dissolved by stirring prior to autoclaving at 10 lbs in^{-2} for 20 min.

2.1.5 Minimal medium agar

The ingredients of minimal medium (Section 2.1.1) were mixed thoroughly with $20gl^{-1}$ bacteriological agar, then autoclaved at 121°C for 15 min.

2.1.6 Mecoprop agar

Minimal medium agar (Section 2.1.5) was supplemented after autoclaving with $0.25gCl^{-1}$ mecoprop as the sole carbon and energy source.

2.2 PREPARATION OF RHIZOSPHERE SAMPLES

2.2.1 Growth of wheat plants

Five wheat seeds (cultivar Maris Dove) were sown in each of a series of pots containing Begbrooke North soil (Table 2.1). When the seeds had germinated one plant per pot was selected and grown in the original pot in a greenhouse. The greenhouse temperature was controlled to give a maximum day temperature of 15°C and a minimum night temperature of 10°C. Illumination, operated by a time switch, provided at 14h light to 10h dark regime. In the early stages of growth the plants were watered with approximately 50 ml additions when the top soil was dry. Watering was increased to 100 ml once the plant's three to four leaved stage had been reached.

TABLE 2.1 **Analysis of Begbrooke north soil**
Grid Reference: SP. 476137

Source: a field at the Weed Research Organisation, Begbrooke, Oxford

Contents:

Available phosphate	21.8 ppm
Organic carbon	2.1% (w/w)
Total nitrogen	0.14% (w/w)
Ammonium-nitrogen	0.8 ppm
Nitrate-nitrogen	15.5 ppm
Cation Exchange Capacity	21.09 milliequivalents $100g^{-1}$
pH in H_2O	5.9
pH in $CaCl_2$	5.4

Mechanical analysis

Composition	Percentage
Clay	15
Silt	13
Coarse sand	46
Fine sand	26

2.2.2 Rhizosphere washings

When the plants were up to 27 days old they were carefully removed from the pots and all loosely adhering soil was gently shaken off. The remaining roots and associated soil were added to 25 ml of minimal growth medium in a sterile bottle. After hand shaking the bottle for 60 seconds the root washings were used as the inoculations for herbicide degradation studies.

2.3 GROWTH AND MAINTENANCE OF MICROORGANISMS

2.3.1 Batch culture growth

Either 250 ml or 2 litre conical flasks were used to adequately aerate 100 ml or 400 ml respectively of defined growth medium (Section 2.1.1). After inoculation the cultures were incubated in an orbital shaker at 25°C $\pm$ 0.5°C at 140 revolutions min^{-1}. Aliquots were removed aseptically for sampling and growth was monitored as described in Section 2.4.

2.3.2 Chemostat culture growth

Quickfit glass chemostats with a working volume of 0.8 litre were used. The chemostat lids had five ports which allowed the input and output of materials and organisms. Fresh media was supplied from 20 litre Pyrex glass aspirators through a Watson Marlow peristaltic pump (Falmouth, Cornwall) by silicon tubing. It was possible to change the rate of media flow into the chemostat by altering the peristaltic pump as required.

The cultures were aerated with sterile air supplied at a rate of 800 ml min^{-1}. An outlet port allowed silicon tubing to carry waste, under pressure, from the chemostat to a second 20 litre glass aspirator that served as a waste pot. A T-shaped junction in the waste tubing led into a sterile bijou bottle, and was used as a sample port. The flow from the chemostat into the waste pot was temporarily halted by an

appropriately placed metal clip. This diverted the flow into the bijou bottle to collect the sample.

The chemostat contents were kept at 25°C by the circulation of heated water in a cold finger introduced through the vessel lid. The water was thermostatically controlled at the correct temperature by a Churchill heating unit (Churchill Instruments, Uxbridge, Middlesex). The chemostat was inoculated by transferring organisms from a bijou bottle attached by silicon tubing to one of the lid ports into the growth vessel.

Cultures were kept homogeneous by a magnetic follower controlled by a Gallenkamp magnetic stirrer. The lid was fastened to the vessel by a metal clip and sealed with vacuum grease. The chemostat was sterilized at 15 lb in^{-2} for 30 min prior to growth experiments.

2.3.3 Maintenance of microorganisms

Isolates from the mecoprop degradation studies were maintained on nutrient agar plates and subcultured regularly. Stock cultures were stored in sterile glycerol (30% v/v) at -18°C.

2.3.4 Specific growth rate

The specific growth rate (μ) of a culture was calculated using the Method of Least Squares as an unbiased estimation of the observed data.

2.3.5 Culture doubling time

The culture doubling time (t_d) was calculated from the specific growth rate value (Section 2.3.4) using the following equation:

$$t_d = \frac{0.693}{\mu}$$

2.4 ANALYTICAL PROCEDURE FOR BATCH AND CHEMOSTAT SAMPLES

Chemostat samples (Section 2.3.2) and batch culture samples (Section 2.3.1) were monitored for culture absorbance, chloride ion release and viable counts ml^{-1}.

2.4.1 Culture absorbance determination

The culture absorbance of batch and chemostat samples was monitored at 600 nm using a Corning colorimeter model 252.

2.4.2 Determination of free chloride ions

Samples were tested for the presence of free chloride ions in the growth medium (indicating dechlorination of the aromatic compounds). A Marius Chlor-O-counter (F.T. Scientific, Glos., U.K.) titrated free chloride ions against silver ions generated colormetrically. The time taken for the titration to be completed was proportional to the chloride ion concentration as given in a numerical readout. For example, a readout of 100 counts at range 10 was equivalent to 1.0 µmoles chloride ml^{-1}.

Batch and chemostat samples (1.0 ml) were added to 25 ml of base solution (l^{-1} glass distilled water, glacial acetic acid, 100 ml; concentrated nitric acid, 8.0 ml; 0.5M NaCl, 1.0 ml) and 1.0 ml of thymol-gelatin solution (gl^{-1} glass distilled water, white powder gelatin, 6.0; thymol, 0.1; thymol blue pH indicator, 0.1). The thymol-gelatin solution was stored at 4°C. A maximum of 15-20 samples could be assayed per solution mixture.

2.4.3 Determination of viable counts

Samples were serially diluted to 10^{-6} using 0.1M-phosphate buffer pH 7.0. Aliquots (0.1 ml) were spread plated onto nutrient agar, malt extract agar (Section 2.1.3) and King's B agar (Section 2.1.4). The plates were incubated at 25°C for two to three days. Colonies were counted and recorded as the number of colony forming units ml^{-1} (c.f.u).

2.4.4 Identification of isolated organisms

After repeated streaking of isolates onto three types of solid medium, that is, nutrient agar, malt extract agar and King's B medium, pure cultures were obtained. Identification of the isolates was undertaken in conjunction with Torry Research Station, Aberdeen, on the basis of biochemical tests such as catalase, oxidase, urease, citrate, indole production, nitrate to nitrite conversion and ONPG tests. Morphological tests included Gram staining, spore formation, shape and formation of clusters of organisms.

2.5 SPECTROPHOTOMETRIC ANALYSIS

2.5.1 Spectrophotometric analysis of mecoprop

A spectrophotometric scan from 240 to 340 nm was undertaken with defined growth medium (Section 2.1.1) containing mecoprop at a final concentration of $0.25gCl^{-1}$ (2.08 mM). A Pye-Unicam SP1700 spectrophotometer allowed the detection of the maximum absorbance peak of mecoprop at 279 nm. The light path was 1.0 cm and the cuvette size was 3.0 ml. The carbon source referred to as mecoprop was used as a technical formulation called Compitox Extra and contained mixtures of sodium and potassium salts of mecoprop (Section 2.9).

2.5.2 Culture supernatant absorbance readings

A mecoprop degrading community was grown in minimal medium containing the herbicide. Samples (10 ml) were removed at various time intervals during growth and centrifuged at 10,000 rev min^{-1} for 10min. The absorbance of the supernatant was read at 279 nm to monitor the disappearance of the maximum absorbance peak of mecoprop during community growth.

This procedure was repeated using pure mecoprop acid (Section 2.9) to compare the disappearance at 279 nm of the pure acid with the technical formulation.

2.6 ESTIMATION OF ENZYME ACTIVITY

2.6.1 Whole cell dehalogenase assay

Organisms (400 ml) from late exponential growth phase in minimal medium (Section 2.1.1) and mecoprop were harvested at 10,000 rev min^{-1} for 10 min at 4°C. The pellet was washed with 0.02M-phosphate buffer pH 7.9 centrifuged as described above and resuspended in 5 ml of 0.02M-phosphate buffer pH 7.9.

The assay reaction mixture, maintained at 30°C contained 4 ml of 0.02M-phosphate buffer pH 7.9, 0.1 ml 0.05M NaCl, 0.2 ml distilled water and 1.0 ml of resuspended cells. The assay was commenced by the addition of 0.2 ml 10% (v/v) mecoprop. At various time intervals over a 35-45 min period, 1.0 ml samples were removed from the reaction mixture and the free chloride ions were determined (Section 2.4.2).

2.6.2 Preparation of cell-free extracts for dehalogenase assays

The cultures were harvested and washed as previously described (Section 2.6.1). The cell walls were disrupted by passage through a cooled French pressure cell (American Inst. Co. Ltd., Maryland, U.S.A) at 83 MPa. To minimize temperature fluctuation in the pressure cell the cultures were packed in ice. Any cell debris was separated from the disrupted cell contents by centrifugation at 18,000 rev min^{-1} for 45 min. The cell free supernatant was assayed for dehalogenase activity as previously described (Section 2.6.1).

2.6.3 Effect of altering substrate concentration and buffer pH on dehalogenase activity

The dehalogenase assay procedure was repeated but with several modifications to investigate their influence on enzyme activity, for example, the buffer in the assay was replaced in separate experiments by:

a) Tris-sulphate buffer pH 7.0,

b) Tris-sulphate buffer pH 7.9,

c) 0.02M-phosphate buffer pH 7.0, and

d) 0.02M-phosphate buffer pH 7.9

The effect of varying the carbon source concentration in the assay reaction mixture was investigated using;

a) 1% (v/v) mecoprop, and

b) 5% (v/v) mecoprop

2.6.4 Determination of protocatechuic acid

The production of protocatechuate as an intermediate in mecoprop degradation was followed by the method of Arnow (1937), that is, 1.0 ml aliquots were aseptically removed from the growth medium and added to test tubes containing 1.0 ml of 0.5M HCl and 1.0 ml of nitrite-molybdate reagent (gl^{-1} distilled water, sodium nitrite, 100; sodium molybdate, 100). A yellow colour developed but was replaced by a red colour after addition of 1M NaOH. Sufficient distilled water was added to give a final volume of 12 ml and the absorbance was measured at 540 nm, using a Pye-Unicam SP1700 spectophotometer.

2.7 MEASUREMENT OF OXYGEN UPTAKE

Measurement of oxygen uptake was undertaken using an oxygen electrode (Rank Brothers, Bottisham, Cambridge, England), connected to a chart recorder.

2.7.1 Calibration of the oxygen electrode

Air saturated distilled water containing 240 nmoles oxygen ml^{-1} was added to the oxygen chamber and allowed the calibration of 100% oxygen saturation on the chart recorder. After the 100% calibration sodium dithionite crystals (0.1g) were added to the air saturated water in the oxygen chamber. The sodium dithionite absorbed any oxygen present, so the chart recorder was calibrated to zero oxygen saturation.

The oxygen chamber was thoroughly cleaned after the calibration

procedure to ensure that all sodium dithionite was removed before cell respiration was measured.

2.7.2 Measurement of cell suspension respiration

Batch culture techniques (Section 2.3.1) were used to grow either pure culture isolates or microbial communities on different carbon sources until the late exponential growth phase was reached. Organisms were harvested at 10,000 rev min^{-1}, washed and resuspended in 0.1M phosphate buffer pH 7.0 and aerated for 2h. Samples (3 ml) of a known concentration of cells were added to the oxygen chamber taking care to exclude air bubbles which would influence the oxygen concentration in the chamber. The basal rate of oxygen uptake was measured over a 10 min period. Different carbon sources at 0.25gCl^{-1} such as mecoprop, 2,4-D, MCPA, catechol, dichlorprop, glucose or succinate were injected (0.1ml) into the oxygen chamber. The oxygen consumption rate was measured for each carbon source.

The oxygen uptake rates for different carbon sources were compared to the basal rate of oxygen consumption. Relative rates of oxidation of carbon sources by the cell suspensions were compared by dividing the endogenous rate into the oxygen uptake rate.

2.8 PLASMID DNA

2.8.1 Gel Preparation

Agarose (0.7% w/v) was dissolved in 150 ml Tris-borate buffer (gl^{-1} glass distilled water, Tris, 10.8; NaEDTA, 0.93; boric acid, 5.5) by gentle boiling. The agarose was cooled slightly before being poured onto clean glass slides. Side props were attached to the glass slide using bulldog clips. Care was taken not to allow bubbles to form in the agarose. A comb was gently placed onto the agarose to form wells for lysate loading. When the gel solidified the clips, props and comb were removed prior to gel usage.

The methods of plasmid visualisation of Wheatcroft and Williams (1981) and Birnboim and Doly (1979) were used.

2.8.2 Sample preparation for the Wheatcroft and Williams method

The organisms were grown for 48h in minimal medium (Section 2.1.1) and 0.25gCl^{-1} mecoprop. The rapid plasmid screen of Wheatcroft and Williams (1981), with some procedural modifications by P.C. Gowland, was as follows: 1.0 ml aliquots of the mecoprop-degrading culture were spun down in a microcentrifuge for 2 min. After the supernatant was discarded the pellets were dried and resuspended in 100 µl of solution A (Tris, 50 mM; EDTA, 50 mM; xylene cyanol FF, 0.1 mg ml^{-1}; antifoam 5% v/v, solution A was stored at 4°C) by whirlimixing for 1 min. Reagent B (saturated solution of sodium dodecyl sulphate in 1M NaOH) was added (25 µl) and the tubes were hand shaken by inverting the tubes repeatedly for 1 min. After whirlimixing for 1 min the lysate (20 µl) was loaded into separate gel wells (Section 2.8.1). The gel was run at 60 mA for 90 min then stained with 1 µg ml^{-1} ethidium bromide solution for 30 min. The gel was rinsed carefully with distilled water, visualized under U.V. light and photographed with Polaroid type 665 film using a Polaroid CU5 Land camera (Polaroid Corp., U.S.A) fitted with two U.V. filters and one orange filter.

2.8.3 The Birnboim and Doly method of plasmid visualization

The organisms were grown in minimal medium containing 0.25gCl^{-1} mecoprop as previously described (Section 2.8.2). Aliquots (1.0 ml) were centrifuged in Eppendorf tubes, the pellet was resuspended in 100 µl lysis solution (Tris-HCl pH 8.0, 25 mM; EDTA pH 8.0, 10 mM; glucose, 50 mM; fresh lysozyme, 2 mg ml^{-1}) and stored at 0°C for 30 min. After addition of 200 µl of alkaline SDS (1% w/v sodium dodecyl sulphate in 0.2M NaOH) the Eppendorf tubes were kept at 0°C for 5 min. 150 µl of 3M sodium acetate pH 4.8 was added to the Eppendorf tubes. After

storage at 0°C for 60 min a coarse heavy precipitate formed. The tubes were microcentrifuged for 5 min at room temperature. Care was taken to remove 400 µl of supernatant without disturbing the precipitate. Cold ethanol (1.0 ml) was added to the supernatant and the tubes were stored at -70°C for 30 min.

After microcentrifuging for 5 min at 4°C the supernatant was carefully removed before the pellet was resuspended in Tris buffer. Lysate was loaded onto the agarose gel (Section 2.8.1) for electrophoretic analysis.

The gel was stained, visualized and photographed as previously described (Section 2.8.2).

2.9 CHEMICALS

All chemicals were of the highest purity which was commercially available. The mecoprop used throughout unless otherwise stated was a technical formulation, called Compitox Extra, kindly donated by May and Baker (Ongar, Essex, U.K). The preparation contained 600g per litre of mixtures of the sodium and potassium salts of mecoprop. Further information on the mixture was not available from the company. Pure mecoprop acid and dichlorprop were kindly synthesized by Trevor Byast (Weed Research Organisation, Oxford), 2,4-D, 2,4,5-T and MCPA were all obtained as free acids from Fluka (Bucks, U.K).

Glucose, succinate, catechol and 3-chlorobenzoate were supplied by Sigma (Poole, Dorset, U.K). 2-chloro- and 4-chlorobenzoate were purchased from Fluka (Bucks, U.K).

Nutrient agar was purchased from London Analytical and Bacteriological Media Ltd (London), malt extract agar in nutrient broth and bacteriologial agar from Oxoid (Basingstoke, Hants). The minimal medium ingredients were all available from Fisons (Loughborough, Leics) and BDH (Poole, Dorset).

Ethidium bromide, agarose Type II, sodium dodecyl sulphate were all supplied by Sigma (Poole, Dorset), xylene cyanol FF by BDH (Poole, Dorset) and Dow Corning antifoam by Hopkin and Williams (Chadwell Heath, Essex).

CHAPTER THREE

ISOLATION OF A MICROBIAL COMMUNITY CAPABLE OF GROWTH ON MECOPROP

3.1 BATCH AND CHEMOSTAT ENRICHMENT CULTIVATION OF RHIZOSPHERE MICROORGANISMS

Batch enrichment. In an attempt to establish if mecoprop was stable in liquid culture, that is, to exclude the possibility that inorganic chloride ions were released from the mecoprop molecule by non-microbial sources, three conical flasks were prepared containing 100 ml of defined growth medium (Section 2.1.1) and mecoprop at concentrations of 0.5, 0.25 and 0.1g carbon l^{-1} (gCl^{-1}) or 4.17 mM, 2.08 mM and 0.83 mM respectively. The flasks were incubated at 25°C in an orbital shaker for 28 days (Section 2.3.1). Aliquots (1 ml) were removed at various time intervals and subsequent determinations revealed that no chloride ions (Section 2.4.2) were released in any of the three conical flasks. As mecoprop was shown to be stable in liquid culture any chloride ions detected after the addition of microorganisms was attributed to microbial activity.

Wheat plants were grown under controlled conditions for 12 days (Section 2.2.1). The root washings (Section 2.2.2) were used to inoculate three batch enrichment cultures containing mecoprop at concentrations of 0.5, 0.25 and $0.1gCl^{-1}$. Over a 50 day period there was no chloride ion release in the batch containing $0.5gCl^{-1}$ mecoprop (Fig. 3.1). However, in the other two batch cultures lag phases of 37-50 days for the culture containing $0.1gCl^{-1}$ and 30-37 days for the $0.25gCl^{-1}$ culture were noted before growth commenced (Fig. 3.1). In both cases the theoretical maximum chloride ion release was attained; that is 0.83 µmoles ml^{-1} and 2.08 µmoles ml^{-1} for 0.1 and $0.25gCl^{-1}$ mecoprop respectively.

The three batch cultures were subcultured every 14 days. After four consecutive subcultures more detailed studies of the cultures were undertaken

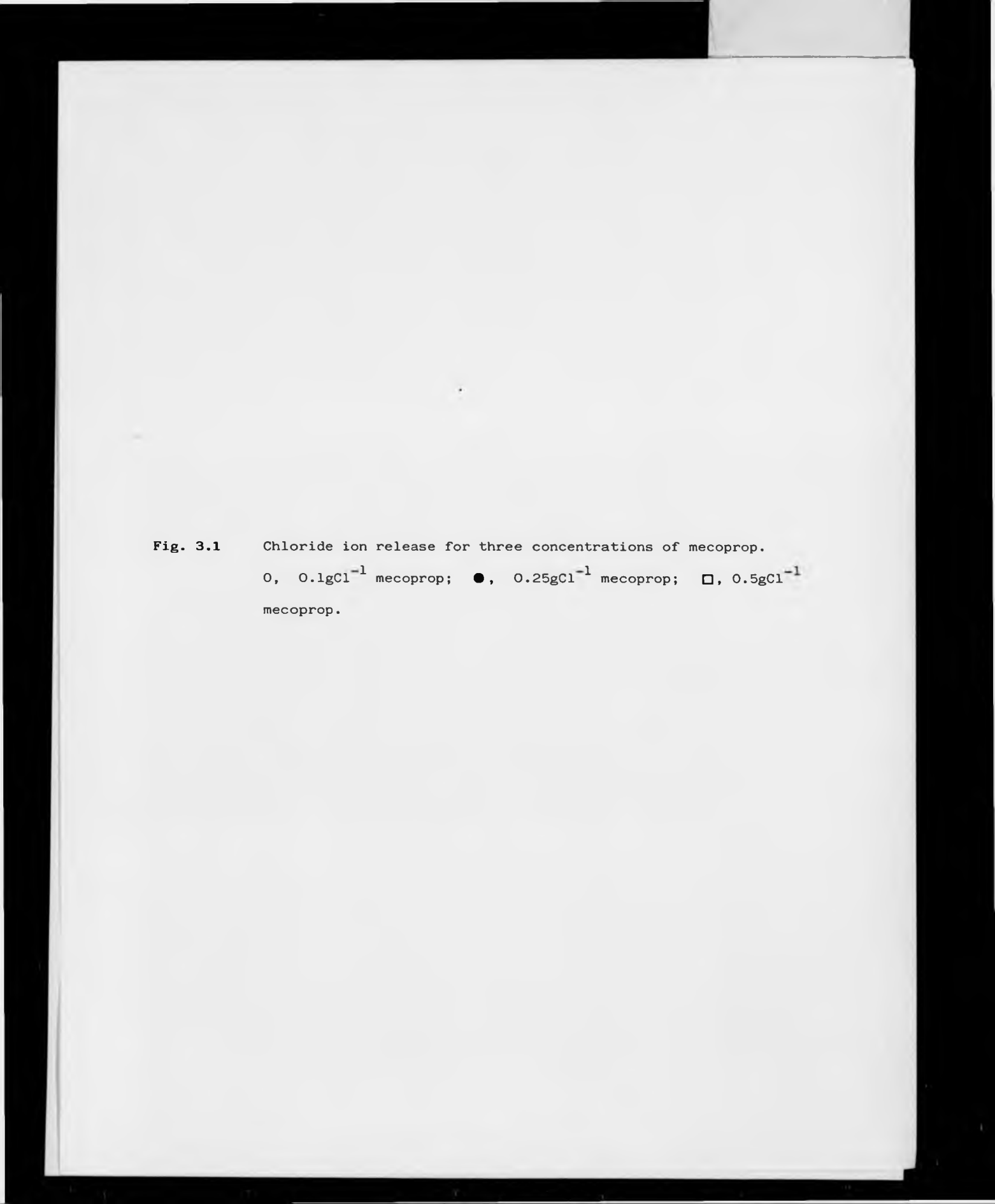
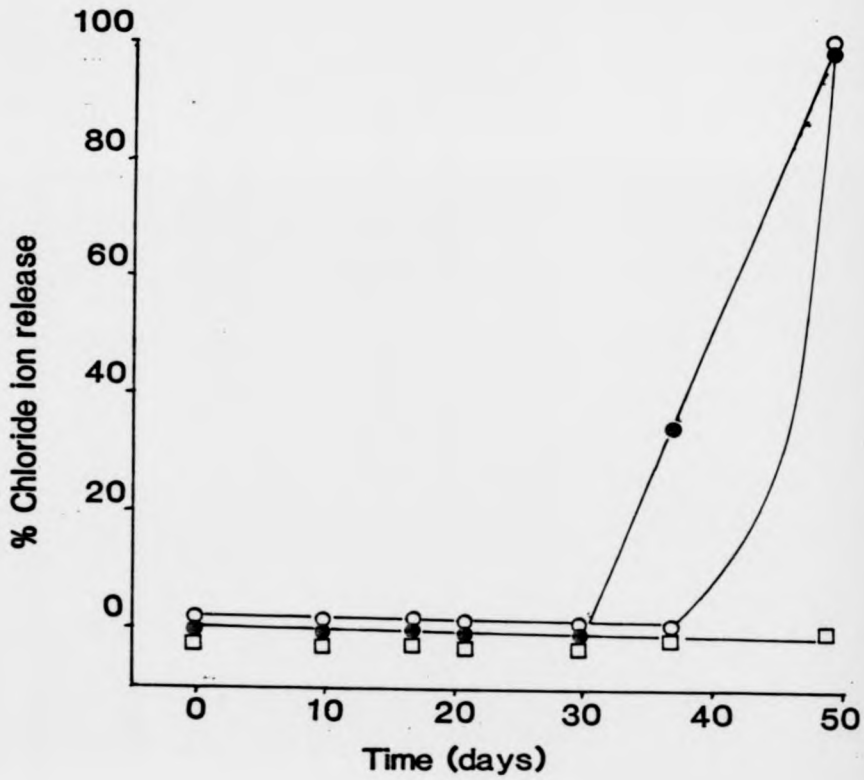


Fig. 3.1 Chloride ion release for three concentrations of mecoprop. ○, 0.1gCl^{-1} mecoprop; ●, 0.25gCl^{-1} mecoprop; □, 0.5gCl^{-1} mecoprop.


100
80
60
40
20
0
% Chloride ion release
0
10
20
30
40
50
Time (days)

to confirm that the chloride ion release indicated growth on the herbicide. The culture absorbance (Section 2.4.1), pH, and chloride ion release (Section 2.4.2) were monitored during growth (Table 3.1). After a lag phase of between two and four days both culture absorbance and chloride ion release increases were noted in the culture containing $0.25gCl^{-1}$ mecoprop. By the sixth day the chloride ion release reached the theoretical maximum, that is 2.08 µmoles ml^{-1} of chloride ions. There was little change in the culture pH throughout the growth cycle (Table 3.1). The $0.5gCl^{-1}$ culture did not release chloride ions and no increase in culture absorbance was detected after 14 days incubation (Table 3.1).

The inorganic chloride ion release reached 100%, that is 0.83 µmoles ml^{-1} within 13 days for the $0.1gCl^{-1}$ culture. However, there was little increase in the culture absorbance with a maximum of 0.025. Again, the pH did not change substantially during the incubation period.

In an attempt to investigate the contents of the three cultures, all were spread plated separately onto mecoprop agar (Section 2.1.6). After four days incubation at $25^{o}C$ very small colonies (less than 0.5 mm in diameter) were detected making isolation based upon morphological descriptions difficult. However, five bacteria were isolated based on slight differences in colony colour and texture.

The five bacterial isolates were inoculated separately into two series of batch enrichment cultures: one containing $0.25gCl^{-1}$ mecoprop and supplemented with 0.05% (w/v) yeast extract and the other containing $0.25gCl^{-1}$ mecoprop as the sole carbon and energy source. The pure cultures grew quickly in the presence of the yeast extract, giving culture absorbance readings of between 0.18 and 0.22 in 24h, but with little chloride ion release. There was no growth in the pure culture batch systems which contained mecoprop as the sole carbon source.

As it appeared that the cultures only utilized the yeast extract and not

TABLE 3.1 DETAILED STUDIES OF THE ABILITY OF THE THREE CONCENTRATIONS OF MECOPROP TO SUPPORT GROWTH

Time (days)	0.5gCl^{-1} mecoprop			0.25gCl^{-1} mecoprop			0.1gCl^{-1} mecoprop		
	Cl^- release (µmoles ml^{-1})	Absorbance (E_{600})	pH	Cl^- release (µ moles ml^{-1})	Absorbance (E_{600})	pH	Cl^- release (µmoles ml^{-1})	Absorbance (E_{600})	pH
0	0	0.025	6.98	0	0.02	7.00	0	0.015	7.00
1	0	0.025	7.04	0	0.02	7.00	0	0.015	7.02
2	0	0.025	7.03	0	0.02	6.98	0	0.015	7.02
4	0	0.025	n.d.	0.66	0.035	6.88	n.d.	n.d.	n.d.
5	0	0.025	n.d.	1.14	0.065	6.86	n.d.	n.d.	n.d.
6	0	0.025	7.00	2.12	0.18	6.86	0.66	0.025	6.92
10	0	0.025	7.01				0.79	0.025	6.92
13	0	0.025	7.14				0.94	0.025	6.95
14	0	0.025	7.10						

n.d. = not done

mecoprop, the percentage of yeast extract was decreased to 0.005% (w/v) and the batch enrichment cultivation of the five bacterial isolates were repeated. It was hoped that the lower concentration of the easily utilized carbon and energy source (yeast extract) would increase the biomass of the pure cultures sufficiently to allow the cultures to then utilize the second carbon source upon the exhaustion of the yeast extract. However, this concentration of yeast extract was too low to sustain growth of the five isolates as neither chloride ion release nor culture absorbance increases were noted after 120h incubation. Therefore, none of the purified organisms was able to utilize mecoprop as the sole carbon and energy source in liquid culture.

The five organisms picked off the mecoprop agar plates were spread separately onto agar plates without carbon source additions (Section 2.1.5). Colonies were detected after four days incubation at 25°C indicating that colony growth was supported by some other energy source, possibly a constituent of the agar, and not by mecoprop. It was concluded that no organism existed in any of the three batch cultures, that is, the 0.5, 0.25 or $0.1gCl^{-1}$ cultures, that was capable of degrading mecoprop in pure culture.

After four months of batch enrichment cultivation at 25°C, rhizosphere microorganisms were still unable to utilize mecoprop in the $0.5gCl^{-1}$ culture. Studies with this concentration were terminated and the two remaining cultures (that is the 0.25 and $0.1gCl^{-1}$ cultures) were more extensively studied.

The $0.25gCl^{-1}$ and $0.1gCl^{-1}$ mecoprop cultures were subcultured whenever the chloride ion release was 100%, that is, 2.08 µmoles ml^{-1} for $0.25gCl^{-1}$ and 0.83 µmoles ml^{-1} for $0.1gCl^{-1}$ mecoprop.

To investigate if the $0.25gCl^{-1}$ culture was able to degrade other concentrations of mecoprop, the culture was added to two batch cultures containing minimal medium (Section 2.1.1) and mecoprop at 0.5 or $0.1gCl^{-1}$. The chloride ion release (Section 2.4.2), culture absorbance (Section 2.4.2) and pH were recorded over a 12 day incubation period (Fig. 3.2a & b). Growth

Fig. 3.2a The growth of the 0.25gCl^{-1} mecoprop-degrading culture on 0.1gCl^{-1} mecoprop. O, culture absorbance; ●, chloride ion release; □, pH.

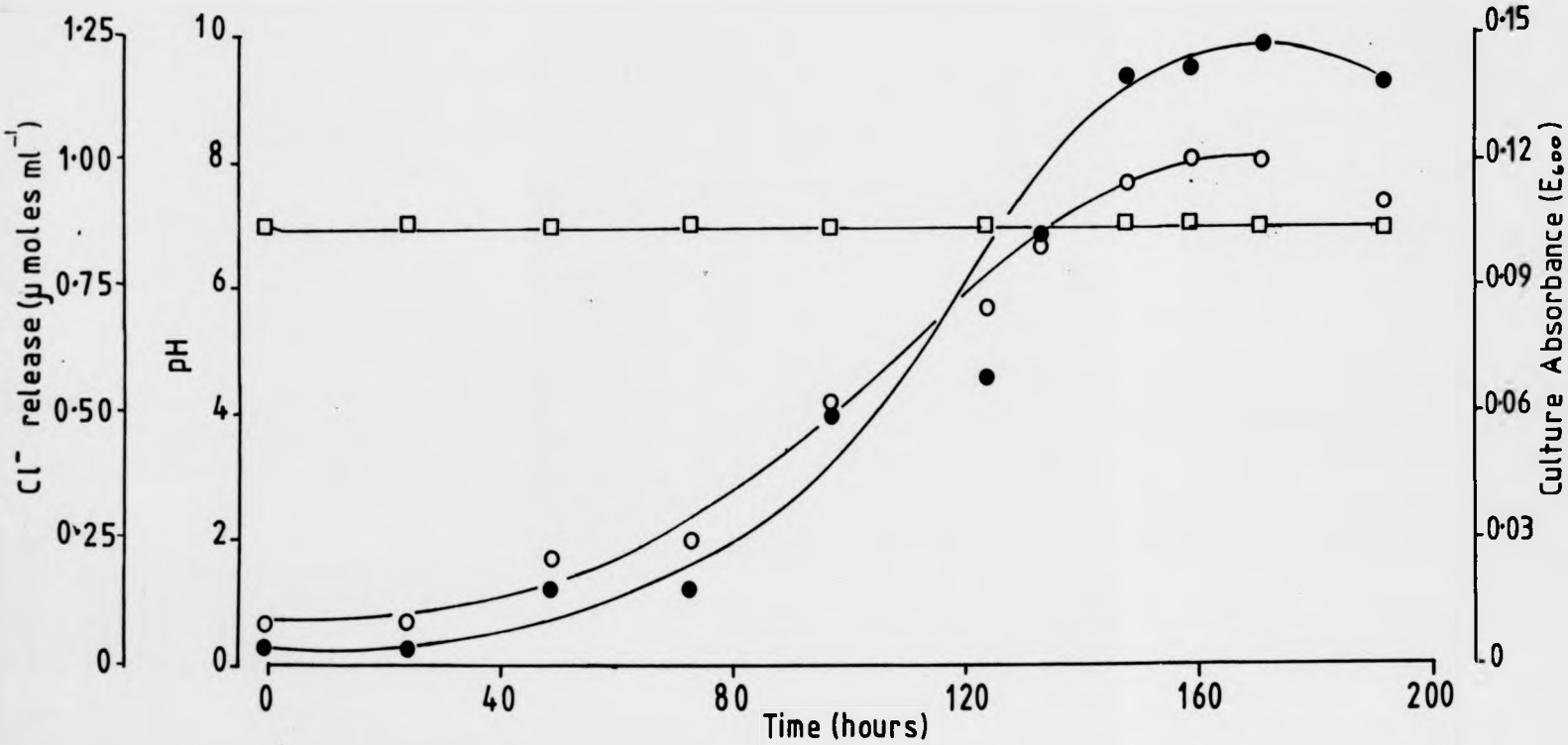
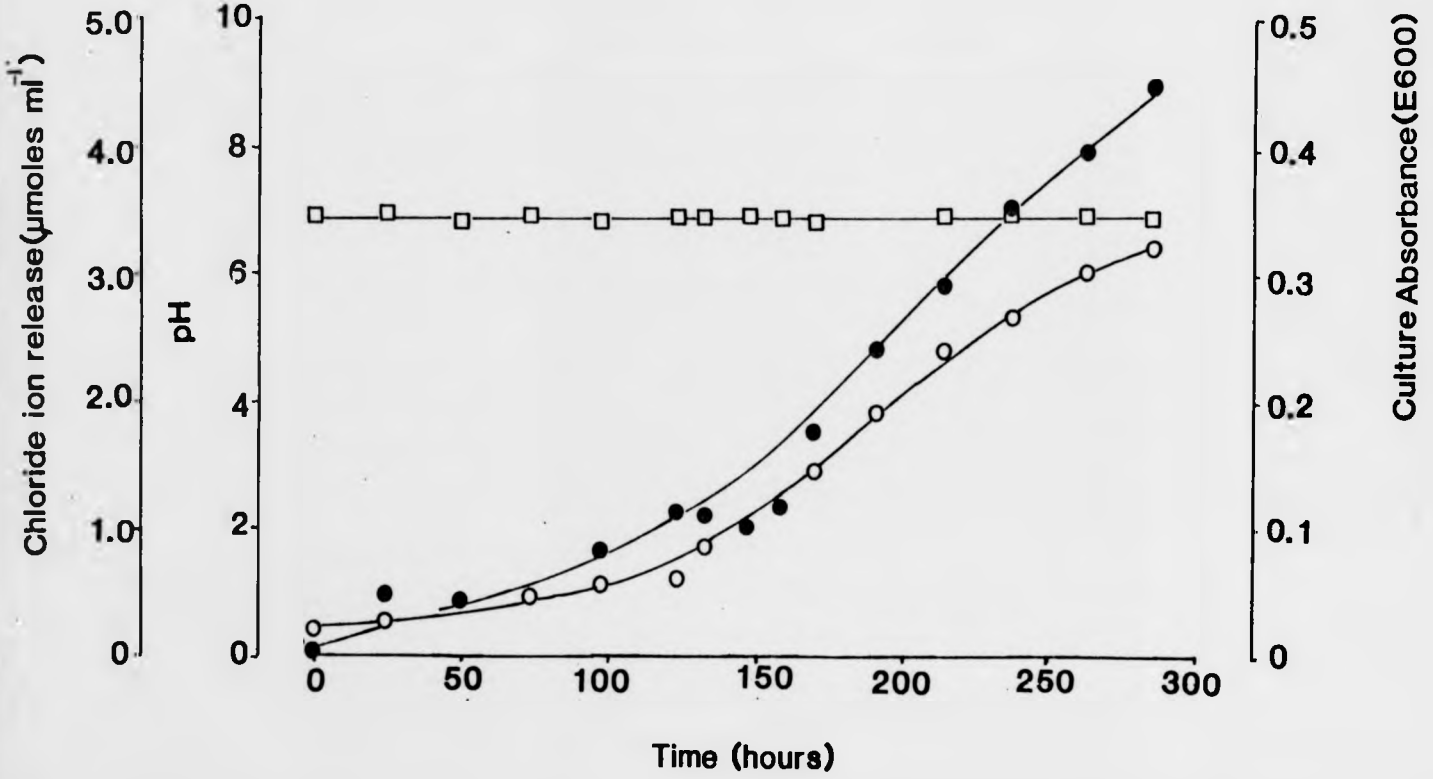

1·25
1·00
0·75
0·50
0·25
0
Cl⁻ release (μ moles ml⁻¹)
10
8
6
4
2
0
pH
0·15
0·12
0·09
0·06
0·03
0
Culture Absorbance (E₆₀₀)
0
40
80
120
160
200
Time (hours)

Fig. 3.2b The growth of the $0.25gCl^{-1}$ mecoprop-degrading culture on $0.5gCl^{-1}$ mecoprop. O, culture absorbance; ●, chloride ion release; □, pH.


Chloride ion release(μmoles ml⁻¹)
5.0
4.0
3.0
2.0
1.0
0
pH
10
8
6
4
2
0
0
50
100
150
200
250
300
Time (hours)
0.5
0.4
0.3
0.2
0.1
0
Culture Absorbance(E600)

was noted at $0.5gCl^{-1}$ for the first time: both chloride ion release and culture absorbance increased after a lag phase of three to four days (Fig. 3.2b). In the batch enrichment culture containing $0.1gCl^{-1}$ mecoprop a similar three to four day lag phase was detected. Growth ceased after six to seven days at the same time as all the available chloride ions were released (0.83 µmoles ml^{-1}).

This procedure was repeated for the $0.1gCl^{-1}$ culture to investigate if it had a similar ability to the $0.25gCl^{-1}$ culture, that is, the ability to degrade other concentrations of mecoprop other than the concentration at which enrichment was established. Again, this culture was capable of degrading $0.5gCl^{-1}$ of the herbicide.

Consecutive subculturing of the $0.25gCl^{-1}$ culture showed that the lag phase rapidly decreased. The initial lag phase duration was 30-37 days (Fig. 3.1); after two further subcultures it was five days; after seven subcultures the length of the lag phase was reduced to four days; after 12 subcultures the lag phase was less than 48h. For all further experimental work it was decided to concentrate on this culture. Aliquots of the $0.25gCl^{-1}$ culture were spread plated onto nutrient agar, malt extract agar (Section 2.1.3) and King's B agar (Section 2.1.4). After 48h incubation at 25°C the colonies were picked off the plates and purified by repeated streaking onto the three different agars. Analysis of the purified organisms revealed that the $0.25gCl^{-1}$ culture consisted of five different bacterial isolates. Identification of the organisms (Section 2.4.4) was undertaken in conjunction with the Torry Research Station, Aberdeen (Section 4.1). None of the purified organisms was capable of growing on $0.25gCl^{-1}$ mecoprop as the sole carbon and energy source after 14 days incubation, however some combinations of two, three or four different organisms were capable of fully degrading the herbicide (Section 4.2).

Detailed analysis of several growth parameters were monitored during one

growth cycle of the $0.25gCl^{-1}$ culture (Fig. 3.3). The theoretical maximum chloride release (2.08 µmoles ml^{-1}) was attained in less than 60h and this coincided with the cessation of growth as determined by the culture absorbance. A plot of the increase in absorbance against the increase in chloride ion release showed a linear relationship existed between these two parameters (Fig. 3.4). The specific growth rate (Section 2.3.4) of the $0.25gCl^{-1}$ culture was calculated as $0.087h^{-1}$. The $0.25gCl^{-1}$ culture was subcultured whenever the chloride ion release indicated total mecoprop degradation. After approximately 40 subcultures the length of the lag phase was less than 24h. No further reductions in the length of the lag phase were detected although subculturing was undertaken for a period of approximately 330 days. None of the community constituents was lost from the culture.

Chemostat cultures. Chemostat cultures of rhizosphere microorganisms (Section 2.3.2) were undertaken in conjunction with batch enrichment cultures. Wheat plants were grown under controlled conditions (Section 2.2.1) for 14 days and the root washings (Section 2.2.2) were used to inoculate the chemostat. Initially the carbon source concentration was $0.5gCl^{-1}$ mecoprop and the dilution rate was set at $0.005h^{-1}$. The chemostat was left for 48h before the pump was switched on to allow the rhizosphere organisms to proliferate. Sampling (Section 2.3.2) was undertaken at regular intervals over 86 days for chloride ion release (Section 2.4.2) and viable organism count (Section 2.4.3) analysis.

The viable counts were initially between 3×10^7 and 5×10^7 viable organisms ml^{-1} (Table 3.2). Chloride ions released from the mecoprop molecules were first detected between the 18th and 25th day after inoculation at 11.75% of the theoretical maximum release, that is 0.49 µmoles ml^{-1}. This increased to 47.00% of the maximum release, 1.96 µmoles ml^{-1} by the 53rd day. Aliquots (0.1 ml) were spread plated onto nutrient agar, malt extract agar (Section 2.1.3) and King's B agar (Section 2.1.4) to investigate the species diversity in the

Fig. 3.3 Detailed growth curve of the enriched 0.25gCl^{-1} degrading culture. O, viable counts ml^{-1}; ●, chloride ion release; □, culture absorbance.

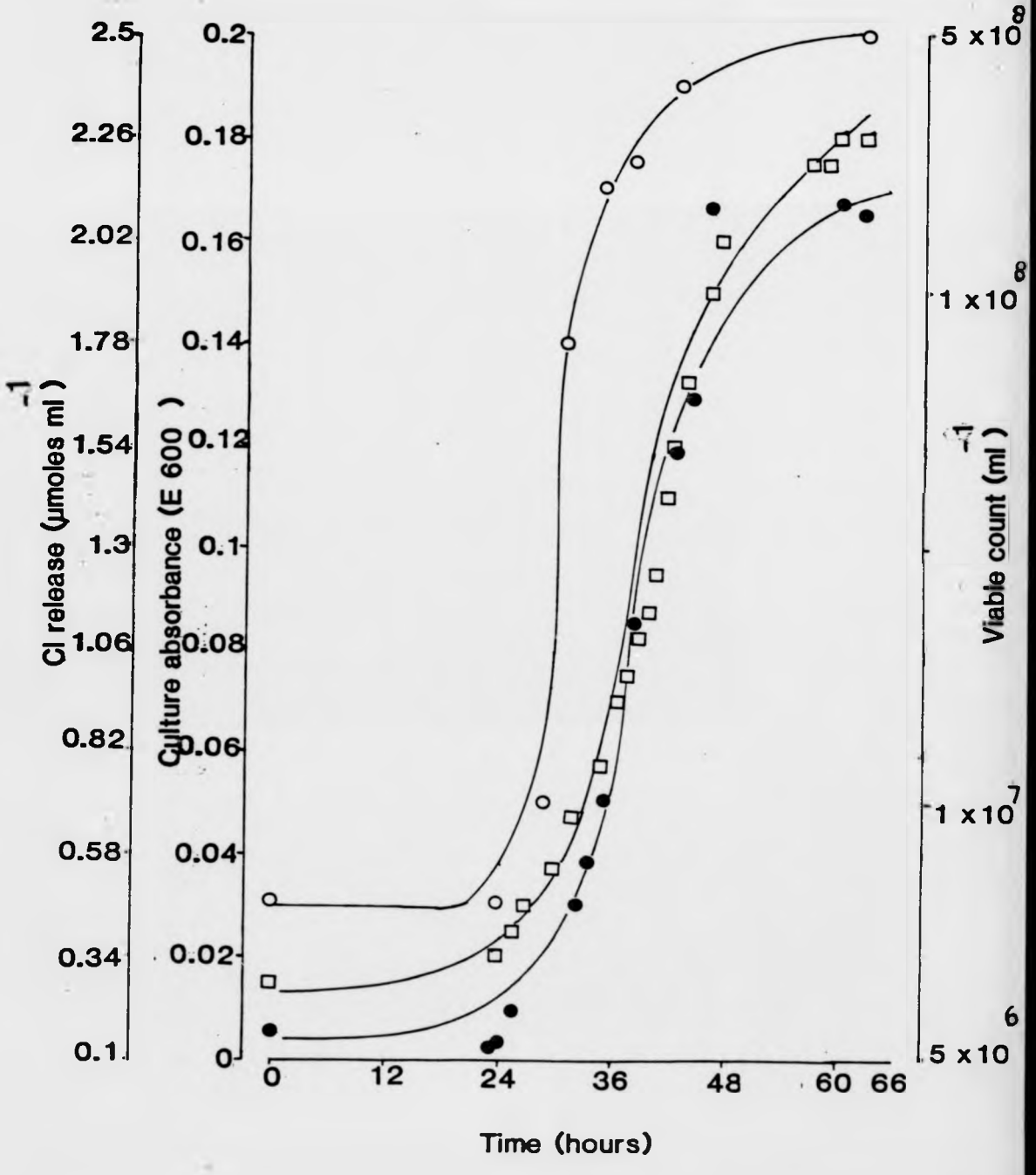
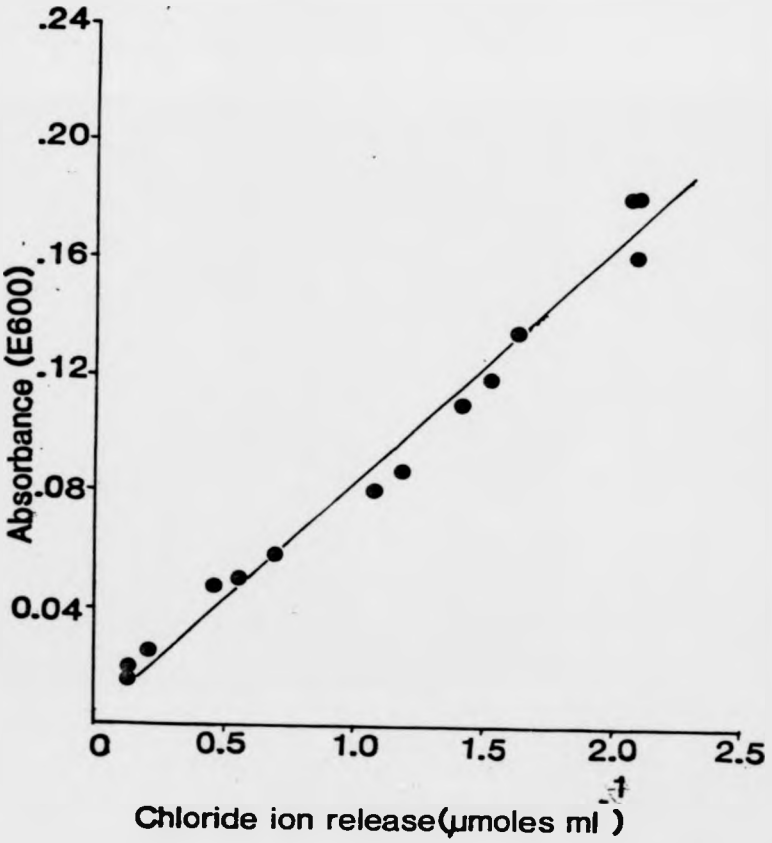

2.5
2.26
2.02
1.78
1.54
1.3
1.06
0.82
0.58
0.34
0.1
Cl release (µmoles ml⁻¹)
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Culture absorbance (E 600)
5 x 10⁸
1 x 10⁸
1 x 10⁷
5 x 10⁶
Viable count (ml⁻¹)
0
12
24
36
48
60
66
Time (hours)

Fig. 3.4 The plot of culture absorbance against chloride ion release for the enriched $0.25gCl^{-1}$ degrading culture.


.24
.20
.16
.12
.08
0.04
Absorbance (E600)
0
0.5
1.0
1.5
2.0
2.5
Chloride ion release(μmoles ml⁻¹)

chemostat culture. The isolates were compared by cross-plating techniques, that is, each organism was streaked separately onto nutrient agar, malt extract agar and King's B agar to compare colony morphology. The same three bacterial isolates were consistently isolated at different sample times:

Organism A was a Gram-negative bacterium which produced off-yellow pigments on King's B agar.

Organism B was a Gram-negative bacterium which produced smooth cream-coloured colonies on King's B agar.

Organism C was a Gram-negative fluorescent Pseudomonas species.

Two other organisms were occasionally isolated, but were numerically fewer than organisms A, B or C. The organisms were described as:

Organism D, a Gram-negative bacterium that produced yellow concentric rings on King's B agar.

Organism E, a Gram-negative bacterium that produced pale yellow coloured colonies on nutrient agar.

Batch culture techniques suggested that $0.5gCl^{-1}$ mecoprop was too high a concentration to detect the enrichment of herbicide degradation, so the carbon source supplied to the chemostat concentration was lowered to $0.25gCl^{-1}$. The percentage chloride ion release then increased (Table 3.2). Samples from the chemostat culture were used to inoculate a series of batch cultures containing minimal medium (Section 2.1.1) and mecoprop at 0.5, 0.25 and $0.1gCl^{-1}$. The highest concentration could not support growth, whereas cultures growing on $0.25gCl^{-1}$ and $0.1gCl^{-1}$ released all of the available chloride ions.

Chemostat culture samples were spread plated on nutrient agar, malt extract agar and King's B agar. Analysis of the colonies after 48h incubation revealed that the five organisms previously described were all still present after 3,360h continuous growth. Investigation of the ability

TABLE 3.2 CHEMOSTAT CULTIVATION OF RHIZOSPHERE MICROORGANISMS

Initial Mecoprop concentration 0.5 gCl^{-1}

TIME (days)	CHLORIDE ION RELEASE (μ moles ml^{-1})	VIABLE COUNT (ml^{-1})
0	0	3×10^7
4	0	5×10^7
11	0	5×10^7
18	0	2×10^6
25	0.49	2×10^6
32	0.87	2×10^7
39	1.48	1×10^7
46	1.82	2×10^6
53	1.96	1×10^7
86	Mecoprop concentration reduced to $0.25 gCl^{-1}$	
92	1.90	1×10^7
95	2.35	4×10^7
104	1.53	8×10^7
108	1.42	n.d.
113	1.88	n.d.
120	2.27	2×10^7
140	2.31	4×10^7

n.d. = not done

of the purified organisms to grow in batch culture on $0.25gCl^{-1}$ mecoprop, showed that none of the cultures was capable of utilizing the herbicide.

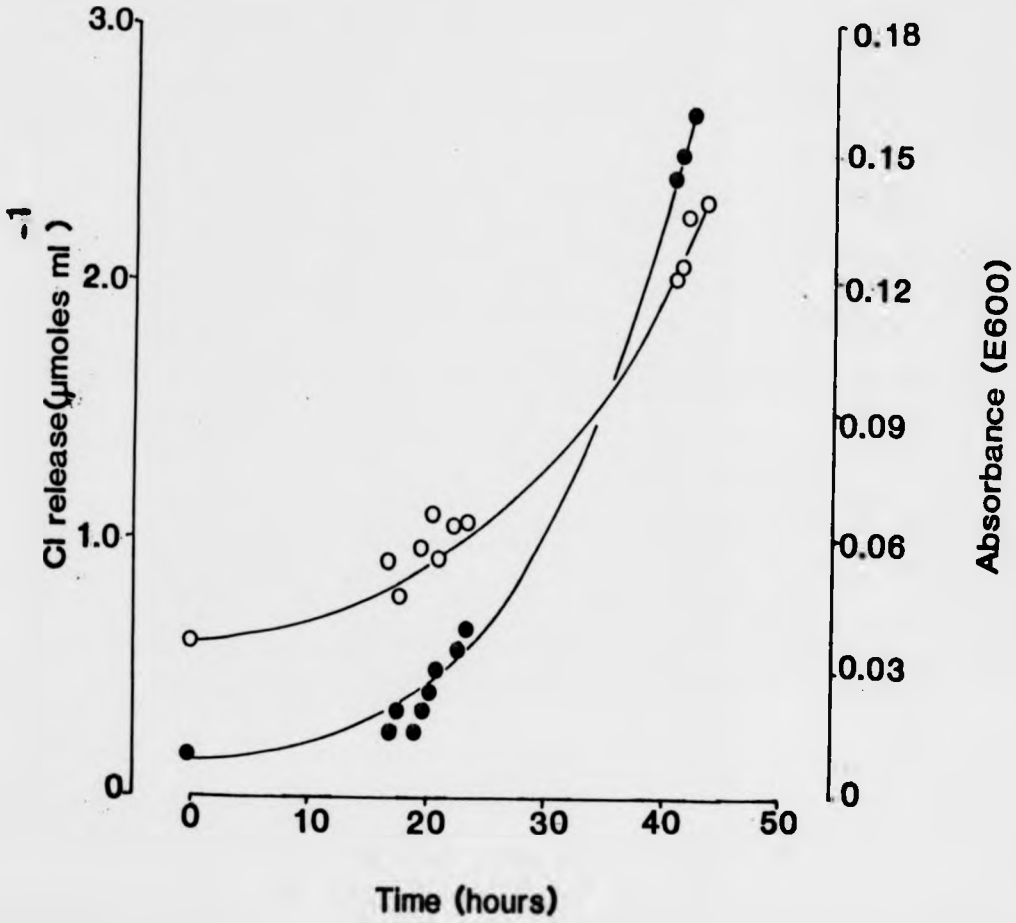
The microbial community enriched to degrade mecoprop by batch procedures was used as an inoculation for chemostat growth to investigate if chemostat cultures could lose any loosely associated organisms. Initially the dilution rate was $0.024h^{-1}$ and the mecoprop concentration was $0.25gCl^{-1}$. After the chemostat had been in operation for 23 days samples (Section 2.3.2) were removed and inoculated into batch cultures containing $0.25gCl^{-1}$ mecoprop. A lag phase of approximately 20h was noted before increases in chloride ion release and culture absorbance were detected (Fig. 3.5). The maximum population phase was reached at the same time as the chloride ion release was completed.

The chemostat dilution rate was gradually increased over a 5,000h period from $0.024h^{-1}$ to $0.086h^{-1}$. The chloride ion release remained at 100% of the theoretical maximum (2.08 µmoles ml^{-1}) and the culture absorbance at 0.18–0.2, a value that batch cultures achieved when the herbicide was totally degraded (Fig. 3.3). Chemostat cultures were spread plated onto nutrient agar, malt extract agar (Section 2.1.3) and King's B agar (Section 2.1.4) to allow analysis of the bacterial species composition. None of the community constituents was lost from the culture during the 5,000h of chemostat operation.

3.2 THE INFLUENCE OF pH, CARBON SOURCE CONCENTRATION AND CHEMOSTAT DILUTION RATE ON MECOPROP DEGRADATION

pH. A series of 250 ml conical flasks containing 100 ml minimal medium and $0.25gCl^{-1}$ mecoprop had a range of pH values from 2.5 to 11.5 (Section 2.1.1). The community enriched by batch culture techniques at $0.25gCl^{-1}$ from the 24h lag phase enrichment regime was used to inoculate the batch cultures. Chloride ion release (Section 2.4.2) was determined at specific intervals over a seven day incubation period (Fig. 3.6). No chloride ion release was detected

Fig. 3.5 Growth curves of chemostat samples on $0.25gCl^{-1}$ mecoprop.
O, chloride ion release; ●, culture absorbance.


3.0
2.0
1.0
0
Cl release(μmoles ml⁻¹)
0.18
0.15
0.12
0.09
0.06
0.03
0
Absorbance (E600)
0
10
20
30
40
50
Time (hours)

at pH values between 2.5 and 4.5, with a slight increase to 19.5% (0.41 µmoles ml^{-1}) of the theoretical maximum at pH 5.0. At pH values of 6.0 to 10.5 the community utilized all of the supplied mecoprop as the chloride ion release reached 100% (2.08 µmoles ml^{-1}). In the batch cultures at pH values above 10.5 a sharp decrease in chloride ion release was recorded (Fig. 3.6). Examination of the percentage species composition at several different pH values showed a preferential stimulation of certain community constituents at some alkaline values (Section 4.3).

Mecoprop concentration. The effect of supplying higher concentrations of mecoprop to the $0.25gCl^{-1}$ culture was studied. Initially, final concentrations of between 0.5 and $1.0gCl^{-1}$ mecoprop were used in batch cultures, but the culture quickly degraded all of the available carbon sources. Higher concentrations, ranging up to $8.0gCl^{-1}$ were used.

The length of the lag phase before community growth commenced increased with increased mecoprop concentration, from 18h at $1.0gCl^{-1}$ to 528h at $8.0gCl^{-1}$. The percentage of mecoprop degraded after the cessation of growth, calculated from the percentage of chloride ions released, indicated that this decreased with increased carbon source concentration (Table 3.3). The specific growth rates were calculated for the culture growing on different mecoprop concentrations (Section 2.3.4). The growth rate remained in the region of 0.08 to $0.09h^{-1}$, values close to that reported for the culture growing on $0.25gCl^{-1}$ mecoprop (Section 3.1). However, at concentrations higher than $4.0gCl^{-1}$ the specific growth rate dramatically declined (Table 3.3). pH values were recorded after growth ceased to investigate whether the significant release of chloride ions from high mecoprop concentrations caused acidic conditions that may inhibit growth. However this hypothesis was rejected as all pH values were recorded at between 6.7 and 7.32.

Fig. 3.6 pH profile of the microbial degradation of mecoprop.

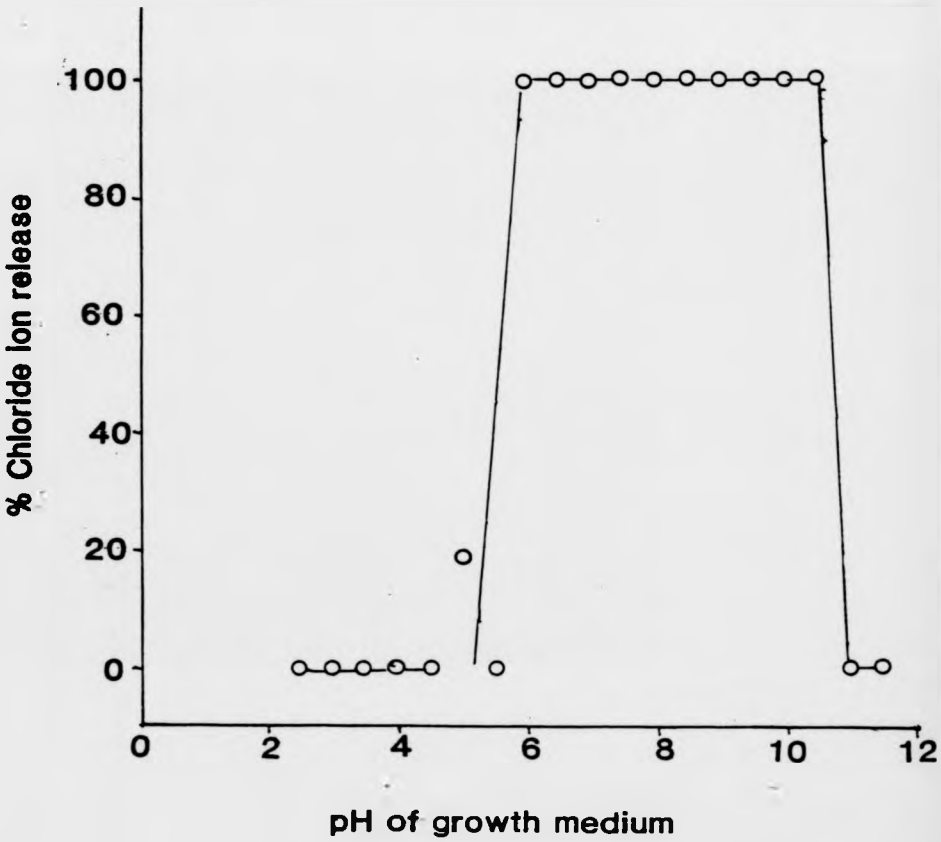

% Chloride ion release
100
80
60
40
20
0
0
2
4
6
8
10
12
pH of growth medium

TABLE 3.3 THE GROWTH CHARACTERISTICS OF THE MECOPROP UTILIZING COMMUNITY ON DIFFERENT MECOPROP CONCENTRATIONS

Mecoprop concentration gCl^{-1}	mM	Length of lag phase (hours)	Specific growth rate (h^{-1})	% mecoprop utilized after growth ceased
1.0	8.33	18.0	0.09	67
2.0	16.7	31.0	0.08	58
3.0	25.0	47.0	0.06	51
4.0	33.3	61.0	0.08	32
5.0	41.67	144.0	0.02	39
6.0	50.0	240.0	0.02	30
7.0	58.3	288.0	0.02	27
8.0	66.7	528.0	0.01	14

Chemostat dilution rate. The effect of increasing the chemostat dilution rate and therefore increasing the maximum specific growth rate, was investigated. A stable population of the $0.25gCl^{-1}$ community grown continuously for 1,500h using chemostat techniques was used as an inoculation for the study. Whenever the chemostat dilution rate was increased a period of 14 days elapsed before the chemostat was sampled (Section 2.3.2). Chloride ion release (Section 2.4.2), culture absorbance (Section 2.4.1) and viable counts ml^{-1} (Section 2.4.3) were determined for each dilution rate.

Both the viable counts ml^{-1} and the culture absorbance decreased as the dilution rate was increased from $0.085h^{-1}$ to $0.12h^{-1}$ (Fig. 3.7). The chloride ion release remained at the theoretical maximum (2.08 µmoles ml^{-1}) until the highest dilution rate, when it decreased to 1.75 µmoles ml^{-1} or 84.13% of the theoretical maximum.

3.3 SPECTROPHOTOMETRIC ANALYSIS OF THE MECOPROP-DEGRADING CULTURE

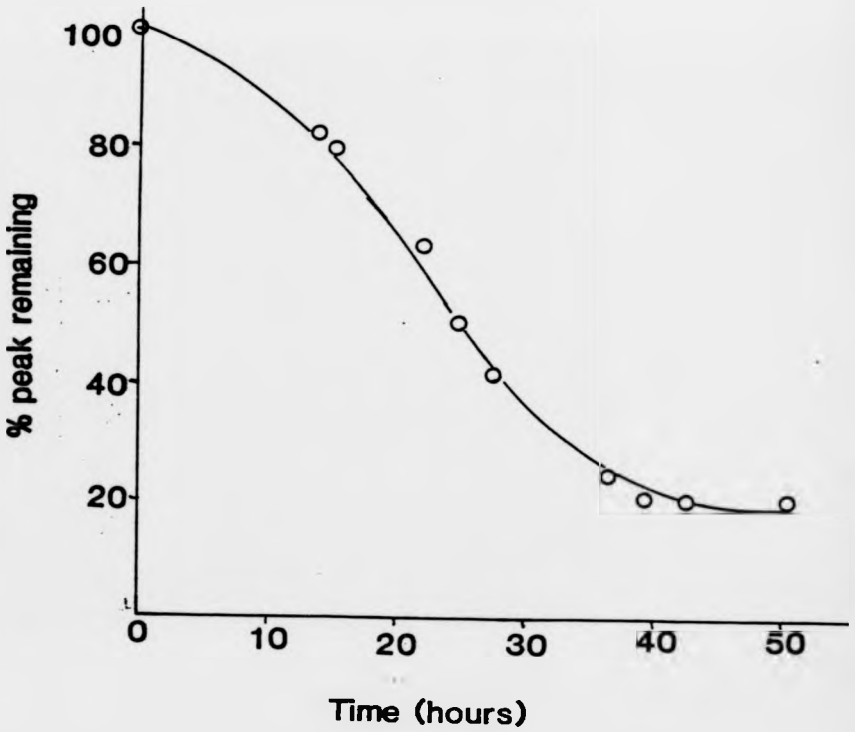
An ultraviolet scan of $0.25gCl^{-1}$ mecoprop in minimal medium (Section 2.1.1) over the range 240 to 340 nm revealed that the maximum absorbance peak was at 279 nm (Section 2.5.1). Several other chlorinated phenoxy compounds such as MCPA, 4-(2-methyl-4-chlorophenoxy) butyrate and 2-(4-chlorophenoxy) propionate also have maximum absorbance peaks at 279 nm so it is not specific for mecoprop (Alexander & Aleem, 1961).

The absorbance at 279 nm was monitored during one growth cycle of the $0.25gCl^{-1}$ culture to investigate herbicide degradation (Section 2.5.2). The disappearance of the absorption peak at 279 nm denoted the utilization of mecoprop by the community. The absorbance at 279 nm was measured before culture inoculation and this value was assigned 100%, subsequent measurements were expressed as a percentage of this value. The absorbance reading at 279 nm after a 50h incubation period was 20% of the original value (Fig. 3.8). As the mecoprop molecules were responsible for the absorption of light at this wavelength, the disappearance of the absorption peak illustrated the

Fig. 3.7. The effect of the chemostat dilution rate on several parameters. O, chloride ion release; ●, viable count ml^{-1}; □, culture absorbance.

% Chloride release
0
20
40
60
80
100
Absorbance
0
.04
.08
.12
.16
.2
.24
Dilution rate (h-1)
.08
.09
0.1
.11
.12
.13
Viable counts (ml-1) X 10 7
14
20
24
28
32
36
40

Fig. 3.8 The disappearance of the 279 nm peak for the mecoprop formulation during community growth.


100
80
60
40
20
% peak remaining
0
10
20
30
40
50
Time (hours)

disappearance of the mecoprop structure or its transformation to another compound. A plot of chloride ions released against absorption peak height at various sample times during community growth revealed a linear relationship between the two variables (Fig. 3..9),.

The possibility existed that the 20% of the absorption peak which remained after community growth had ceased could have been attributed to some constituent of the technical formulation of mecoprop (Section 2.9) interfering with the absorbance. Therefore, the procedure was repeated using pure mecoprop acid (Section 2.9).

Initially the degradation of pure mecoprop acid by the community was more rapid than that of the technical formulation showing a 35% reduction in absorbance peak height after 15h (Fig. 3.10). The total disappearance of the peak at 279 nm was 60% of the pre-inoculation value when the culture growth ceased, however the chloride ion release was 100% (2.08 µmoles ml^{-1}). Again, a plot of chloride ion release against the decrease in peak height during community growth was linear (Fig. 3.11).

3.4 DEHALOGENASE STUDIES

The release of inorganic chloride ions into the growth medium by the $0.25gCl^{-1}$ culture growing on mecoprop indicated the existence of dehalogenating enzymes. Estimation of the activity of dehalogenating enzymes was undertaken using the procedure described in Section 2.6.1.

Whole cell assays (Section 2.6.1) were undertaken after the $0.25gCl^{-1}$ culture was grown on minimal medium and mecoprop for 55h. The assay period was extended from between 35 and 45 mins to 24h but dehalogenating activity was not detected as determined by chloride ion release. The possibility existed that the assay conditions were not favourable to detect activity, so the procedure was modified. The buffer used in the assay mixture, that is 0.02M-phosphate buffer pH 7.9, was replaced in separate experiments by Tris buffer and 0.1M-phosphate buffer at different pH values (Section 2.6.3) and

Fig. 3.9 The disappearance of the 279 nm peak against chloride ion release for the mecoprop formulation.

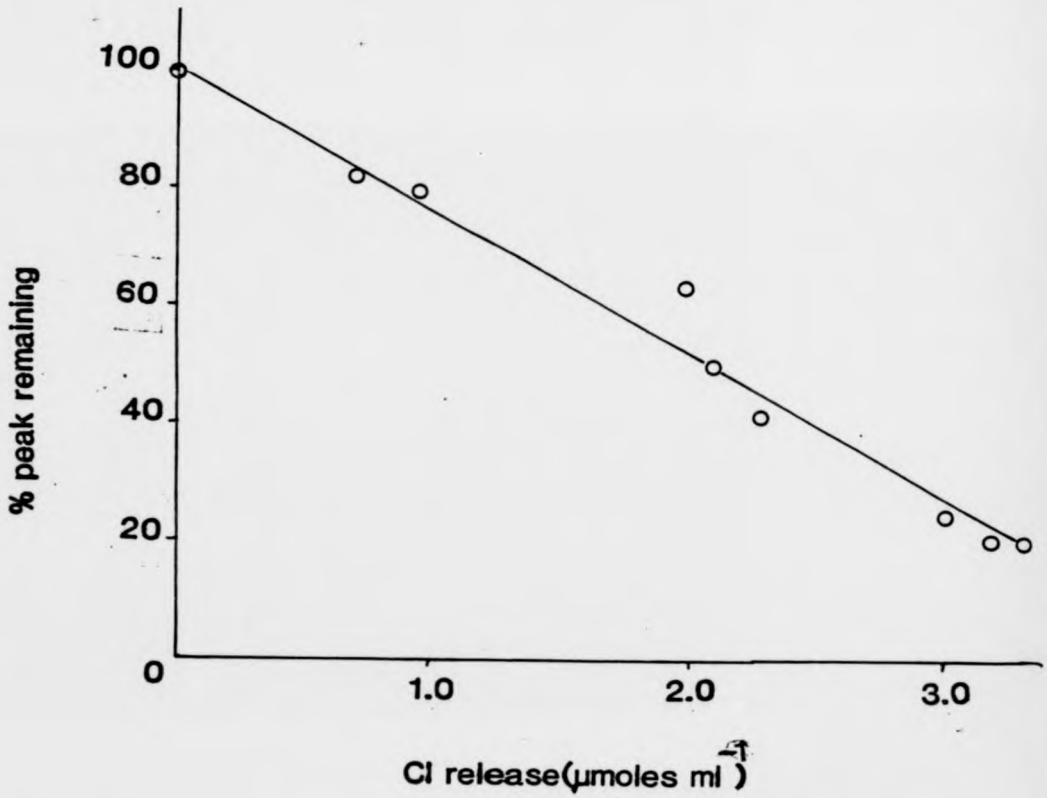

100
80
60
40
20
0
% peak remaining
1.0
2.0
3.0
Cl release(μmoles ml⁻¹)

Fig. 3.10 The disappearance of the 279 nm peak for pure mecoprop acid during community growth.

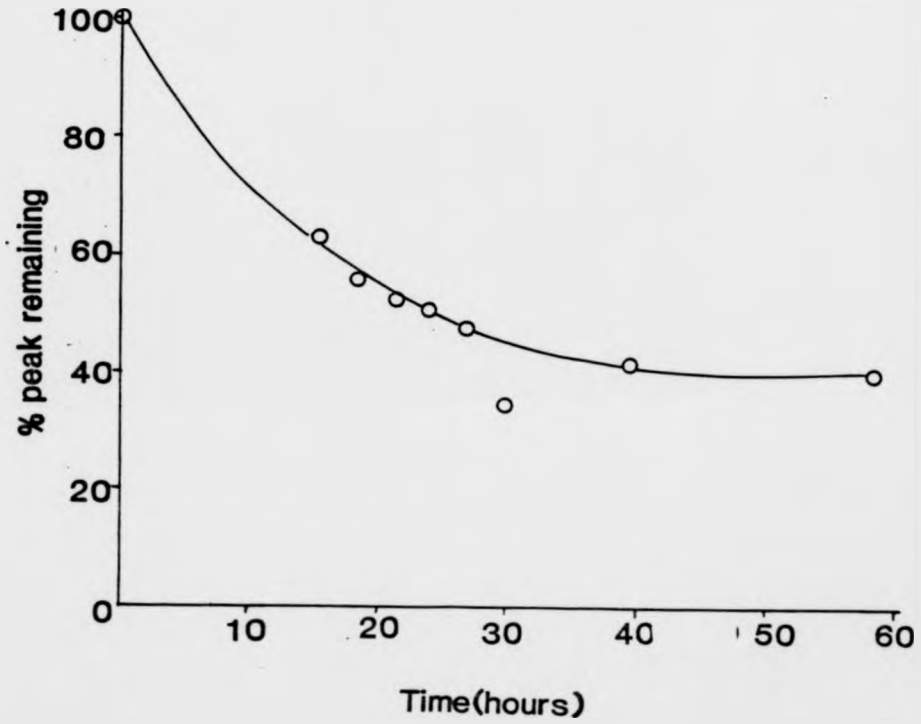
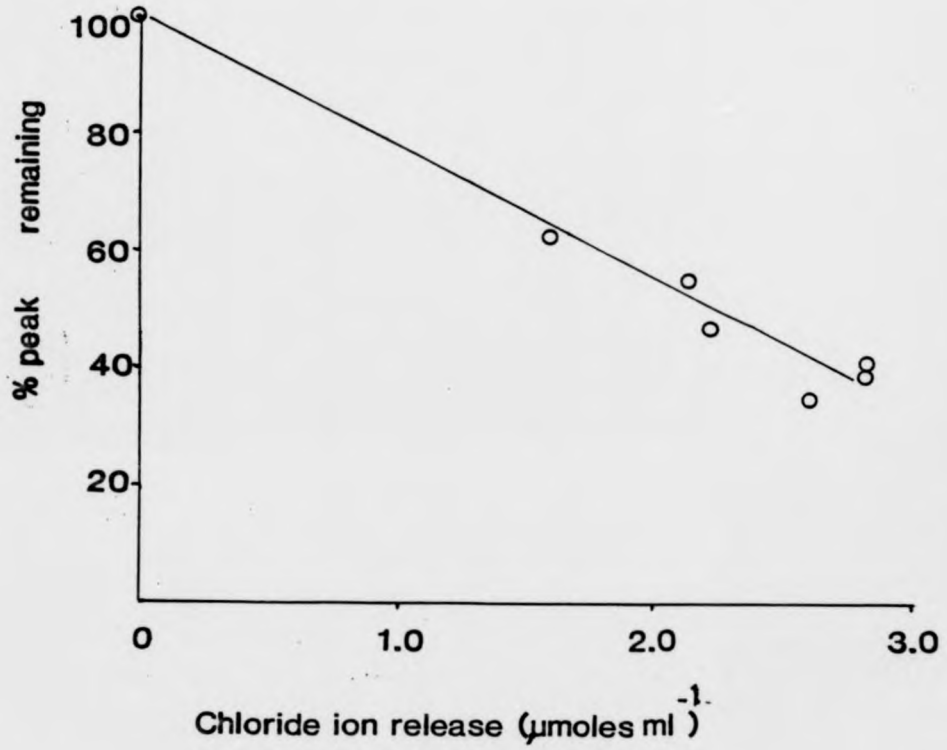

100
80
60
40
20
0
% peak remaining
10
20
30
40
50
60
Time(hours)

Fig. 3.11 The disappearance of the 279 nm peak against chloride ion release for pure mecoprop acid.


100
80
60
40
20
% peak remaining
0
1.0
2.0
3.0
Chloride ion release (μmoles ml -1)

the whole cell assay procedure was repeated. However, no dehalogenating activity was detected after 24h. The same result was obtained when the assay was repeated using lower concentrations of mecoprop in the assay mixture (Section 2.6.3).

As the experimental procedure failed to demonstrate the presence of any dehalogenating enzymes further modifications were introduced. Cell-free extracts were used as well as whole cells as the enzymes may have been bound to cellular components. Samples (10 ml) were removed from batch enrichment flasks at several stages in the assay procedure; that is, after harvesting and after cell washing, and added to reaction mixtures containing:

a) defined growth medium (Section 2.1.1),

b) 0.1M-phosphate buffer, pH 7.0,

c) 0.1M-phosphate buffer, pH 7.9,

d) Tris-sulphate buffer, pH 7.0,

e) Tris-sulphate buffer, pH 7.9.

Unfortunately, with all of the whole cell assays, growth occurred so that culture absorbance increases were detected in the assay mixture as well as chloride ion release. No chloride ion release and therefore no dehalogenating enzymes were detected using cell-free extracts.

3.5 DISCUSSION

Carbon source concentrations of $0.25gCl^{-1}$ and $0.1gCl^{-1}$ mecoprop were able to support the growth of rhizosphere organisms after lag phases of 30-37 days and 37-50 days respectively. Lag phases have been reported in herbicide degradation by soil organisms and feasible explanations for the duration of lag phases have been offered.

Audus (1951) undertook much of the pioneering work on microbial degradation of phenoxy herbicides using soil perfusion techniques. Herbicide degradation by soil microorganisms was monitored using a biological assay based upon comparisons of inhibition of root growth of cress seedlings

(Lepidium sativum). Root inhibition by known concentrations of the herbicides allowed a plot of standard curves and quantitative analysis. Lag phases of 13.7 ± 2.8 days for 2,4-D and 50-80 days for MCPA were reported before herbicide degradation occurred (Audus, 1952), however fresh herbicide additions were rapidly degraded. Audus termed this an enriched soil and suggested that the lag phase before growth commenced represented the time required for the soil to enrich in herbicide degraders. The specific method of enrichment was not known but two possible explanations were offered:

a) the lag phase represented the time required for a few strains in a soil population that had the ability to degrade the herbicides to proliferate to a sufficient number to make herbicide degradation detectable, or

b) the time required for the adaptation of enzyme systems in the organisms that lack specificity (Section 1.2.3).

More recent publications drew similar conclusions for possible lag phase explanations (Loos, 1975; McCall et al. 1981; Duah-Yentumi & Kuwatsuka, 1982; Spain & Van Veld, 1983). These included the time for soil population to:

a) synthesize inducible enzymes,

b) adapt non-specific enzyme systems, or

c) for mutations to occur.

Torstensson (1980) argued that inducible enzymes were less likely to be involved as it was difficult to explain how adaptation remained after the herbicide exposure was terminated, thus making mutation or enzyme adaptation more probable explanations.

During the lag phases before $0.25gCl^{-1}$ and $0.1gCl^{-1}$ mecoprop degradation commenced an adaptation of rhizosphere organisms by strain selection, mutation or enzyme adaptation or a combination of all three processes occurred to produce an enriched population of herbicide degraders. After approximately 40

subcultures the length of the lag phase was reduced from 30-37 days to less than 24h for the $0.25gCl^{-1}$ culture. Fresh herbicide additions to the community produced rapid mecoprop degradation, demonstrating adaptation to the herbicide. Spain and Van Veld (1983) defined adaptation to a xenobiotic compound as 'the change in the microbial community that increases the rate of transformation of a test compound as a result of a prior exposure to the test compound'.

The enriched microbial community obtained at $0.25gCl^{-1}$ mecoprop was able to grow in batch cultures containing up to $8.0gCl^{-1}$. A reduction in growth was noted with an increase in mecoprop concentration (Table 3.3). The influence of concentration on herbicide degradation was studied by Hurle (1973), who observed that an increase in 2,4-D concentration caused an increased duration of the lag phase. Moreover, there was a linear relationship between herbicide concentration and the lag phase duration. Hurle's interpretation of his results included a toxicity theory which implied that the applied herbicide would kill off some organisms, thus providing a growth substrate for any soil organisms that were capable of degrading the herbicide. The degraders utilized the dead organisms and so increased numerically. Higher concentrations of applied herbicide killed off a greater proportion of the soil organisms and produced more substrate for the survivors and less competition for the growth substrate. The lag phase, therefore, terminated at the point of exhaustion of the easily metabolized substrate, that is the dead organisms. In summary, the longer the lag phase the more toxic the herbicide is to the soil organisms.

Kilpi (1980) used a mixed culture of organisms enriched on vanillic acid and MCPA and exposed the culture to a range of different MCPA concentrations from 0.04 to 0.2% (w/v) and 0.05 to 0.2% (w/v) 2,4-D. Growth, monitored by chloride ion release and increase in culture turbidity, was less prolific at higher concentrations of MCPA and 2,4-D. Increased concentration

decreased the amount of 2,4-D degraded.

In all of the batch cultures containing different mecoprop concentrations growth ceased before 100% degradation occurred. The total percentage of herbicide degraded by the $0.25gCl^{-1}$ culture decreased with increased concentrations (Table 3.3). The possibility existed that a build-up of toxic waste products in the batch cultures or a depletion of one or more of the basal salts in the growth medium halted growth.

Prolonged incubation of primary enrichment batch cultures containing $0.5gCl^{-1}$ mecoprop were not successful in producing degradative organisms. This was confirmed by chemostat studies. The higher mecoprop concentration, $0.5gCl^{-1}$, appeared to be toxic in some manner for the rhizosphere organisms. For instance, the higher concentration may have poisoned sites of enzyme action, or conversely been impermeable to the cell membranes. Possible explanations for this phenomenon must explain the observation that both of the communities enriched at lower mecoprop concentrations, that is the $0.25gCl^{-1}$ and $0.1gCl^{-1}$ cultures, degraded $0.5gCl^{-1}$ mecoprop and the $0.25gCl^{-1}$ culture degraded concentrations up to $8.0gCl^{-1}$.

The site of action of mecoprop degradation in the rhizosphere organisms may have been inactivated when initially exposed to higher concentrations ($0.5gCl^{-1}$). Initial exposure to lower concentrations (for example $0.25gCl^{-1}$), that are not toxic to action sites, followed by an increase in concentration may have permitted mutations at these sites which enabled them to utilize higher herbicide concentrations. Conversely after long exposures to lower concentrations the permeability of cell membranes may have been modified to allow the passage of higher concentrations into the cells.

The disappearance of the maximum ultraviolet absorption peak during microbial growth has frequently been used to follow herbicide degradation by soil organisms (Alexander & Aleem, 1961; Boethling & Alexander, 1979; Kilpi, 1980). The decrease in the size of the 279 nm peak demonstrated the

disappearance of the primary mecoprop structure during growth. The linear relationship between the chloride ions released by the 0.25gCl^{-1} culture and the disappearance of the peak suggested that the 279 nm peak represented the chlorinated compound that disappeared upon dechlorination.

Several recent studies have utilized the dehalogenase assay described in Section 2.6.1to examine aspects of microbial dehalogenation. The assay was used in conjunction with gel electrophoresis techniques by Hardman and Slater (1981a & b), and different dehalogenase enzymes were separated and classified according to their activities on a range of substrates. Slater et al. (1979) used the assay to compare dehalogenating activity in two Pseudomonas putida strains. In all cases the compounds studied were chlorinated aliphatic compounds and although dehalogenating reactions of haloaromatics do occur the specific enzymes responsible for dehalogenation have not been investigated (Horowitz et al. 1983; Suflita et al 1983). In this study dehalogenase enzymes were not detected despite much adaptation of the procedure. The possibility must exist that the enzymes remained tightly bound to some cellular constituent. However, later work with the 0.25gCl^{-1} culture (Section 6.1) demonstrated that temperatures above 25°C were not conducive to growth. Although several parameters in the assay procedure, such as buffer content and pH as well as carbon source concentration, were altered to ensure suitability for the dehalogenating reaction, the effect of the temperature of the assay mixture was not investigated.

In conclusion, several recent reviews have stressed that microbial communities are useful to observe degradation of xenobiotics or recalcitrant compounds (Bull, 1980; Harder, 1981; Slater & Bull, 1982). Mecoprop was considered to be a recalcitrant compound as the propionic acid sidechain is linked in the α position to the phenoxy group (Alexander & Aleem, 1961; Kilpi, 1980). This study is the first report of mecoprop degradation by soil organisms growing in laboratory cultures. In this instance no one organism

was capable of degrading the herbicide, but combinations of two or more organisms were required to generate the necessary catabolic capability (Section 4.2). Previous work that failed to demonstrate mecoprop degradation (Kilpi, 1980; Smith & Hayden, 1981) could have placed an undue emphasis on pure culture techniques or chosen inappropriate initial concentrations or enrichment times.

CHAPTER FOUR

IDENTIFICATION OF THE MECOPROP-DEGRADING COMMUNITY CONSTITUENTS

4.1 MORPHOLOGICAL AND BIOCHEMICAL TESTS

Aliquots from the enriched mecoprop community were spread plated onto nutrient agar, malt extract agar, (Section 2.1.3) and King's B agar (Section 2.1.4). After incubation at 25°C for two to three days a loopful of each colony type was picked off and repeatedly streaked onto fresh plates. This allowed the comparison of pure cultures and assisted the determination of the number of community constituents in the mecoprop-degrading culture.

The same five bacteria were isolated over a four week period and were taken to represent the stable community. The cultures were very closely associated and made separation difficult. Identification of the isolates, numbered one to five, was undertaken in conjunction with the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen.

Morphological Tests. All of the five community constituents were non-fermentative, non-spore forming Gram-negative rods, except HL4 which was Gram-variable.

Organism HL1. Microscopic observation revealed large clusters of short rods. Colonies were smooth, round, regular, opaque and grew to 1mm in diameter in two days.

Organism HL2. Colonies were an off-yellow colour on nutrient agar, grew to 2mm in diameter in two days and were low convex, round, regular, entire and smooth. The organism had no flagella and was non-motile. Microscopic observation showed singular or pairs of rods.

Organism HL3. A small proportion of cells were motile, possessing peritrichous flagella. Colonies were individual rod shaped bacteria,

and grew to 0.5 mm in diameter in two days and were round, regular, low convex, smooth and opaque.

Organism HL4. Colonies were off-white, low convex, smooth, regular, round, opaque and grew 1.5 mm in diameter in two days. The short twitching rods were predominantly Gram-negative, but some of the rods were Gram-variable. The organism did not possess flagella.

Organism HL5. The colonies produced were raised cream-yellow with closely packed individual rods, 1.0 mm in diameter after two days incubation and were smooth, opaque, round and regular.

Biochemical Tests. The results of the biochemical tests of the five mecoprop community constituents are summarized in Table 4.1.

All of the isolates were catalase and oxidase positive, except isolate number HL4. None of the isolates could convert NO_3^- to NO_2^- except isolate HL5; isolate HL1 could convert NO_3^- to N_2. Varying responses were produced by the isolates to ONPG tests antibiotic sensitivity, urease and Simmon's citrate tests. Isolates HL2 and HL5 had growth factor requirements of L-methionine and flexirubin respectively. The identification based upon the biochemical and morphological tests are described in Table 4.2.

Species Composition. The microbial community was grown in defined growth medium (Section 2.1.1) and 0.25gCl^{-1} mecoprop. Aliquots (0.1 ml) were aseptically removed from the batch culture at the stationary growth phase and spread plated onto nutrient agar (Section 2.1.3). After incubation at 25°C for two to three days the colonies were divided into the different bacterial groupings and counted to give the percentage composition of each member of the microbial community. Pseudomonas species HL1 and Pseudomonas maltophilia made up the majority of the community, together comprising approximately 83.5% of the community (Table 4.3). The Acinetobacter calcoaceticus and the Flavobacterium species

TABLE 4.1 Biochemical Tests of the Five Bacterial Isolates
(Incubation at 30°C)

Courtesy of Torry Research Station, Aberdeen

	Isolate HL1	Isolate HL2	Isolate HL3	Isolate HL4	Isolate HL5
Catalase	+	+	+	+	+
Oxidase	+	+ -25°C	+	-	+
O-F glucose	-alkaline	-alkaline	-	-alkaline	weak oxidative acid
Pyocyanine	-	-	-	-	-
Fluorescence	-	-	-	-	-
L-Arganine CSU	not determined	-	-	-	-
Betanine CSU	-	-	-	-	-
Glucose CSU	-	-	-	-	doubtful result
Lactate CSU	+	with L-methionine at 10 µg ml^{-1} +	+	+	-
Acetate CSU	+	-	+	+	-
Penicillin G	-	-	-	not determined	-
Streptomycin	-	+	-	+	±
Chloramphenicol	-	+	+	+	+
Tetracycline	+	-	+	+	±
Novobiocin	-	-	(+)	±	+
Polymyxin B	+	+	(+)	+	-
O/129	-	-	-	-	(+)
Levan	-	weak brown diff.pigment -	-	-	-
Growth factor requirement	-	satisfied with L-methionine +	-	-	Flexirubin +
Maltose	-	O	-	not	not
O-F open	(alk)	(weak acid)	(alk)	determined	determined
Gas glucose	-	-	-	-	-
Acid glucose	-	-	-	-	-
ONPG	-	+	-	-	+
Arg. Møller	-	-	-	-	-
Arg. Thornley	+	(+)	+	-	-
Lys. Møller	-	+	-	-	-
Orn. Møller	-	-	-	-	-
NO_3^- to NO_2^-	-	-	-	-	+

continued....

NO_3^- to N_2	+	–	–	–	–
DNA ase	–	(+)	–	–	not determined
Gel Stab 20°C	–	+	not determined	not determined	+
Gel plate	+	+	–	–	+
Casein	–	brown pigment slightly diffusable +	–	–	+yellow
Starch	–	–	–	–	+ under growth
Lecith egg	–	–	–	–	–
Lipase egg	–	+	–	–	–
NH_3	–	+	–	–	+
Indole	–	–	–	–	(+)
H_2S	–	–	–	–	–
Tween 80	–	+	–	+	+ yellow
Urease	–	–	–	+	not determined
Simmon's citrate	+	–	+	–	–

TABLE 4.2 **Identification of the mecoprop-degrading community constituents**

Courtesy of Torry Research Station, Aberdeen

Isolate HL1	The genus could not be identified with a high degree of confidence, but was considered likely to be a non-motile strain of *Pseudomonas* species.
Isolate HL2	*Pseudomonas maltophilia* .
Isolate HL3	*Alcaligenes* species .
Isolate HL4	*Acinetobacter calcoaceticus* .
Isolate HL5	*Flavobacterium* species as defined by Holmes & Owen (1979). Their amended definition restricts *Flavobacterium* species allied to section I of Bergey's Manual.

HL5 were only minor components of the community totalling 4.2%. Alcaligenes species HL1 represented 12.33% of the mecoprop-degrading community (Table 4.3).

4.2 RECOMBINATIONS OF THE COMMUNITY CONSTITUENTS

Reconstituted Community

The mecoprop-degrading community was demonstrated to contain five bacterial isolates. An investigation to regenerate the community from its constituent members to restore degradative capabilities was undertaken. The five bacterial isolates of the $0.25gCl^{-1}$ community were grown separately in nutrient broth to provide a good inoculation size for reconstitution experiments. After overnight growth 1.0 ml of each isolate was inoculated with 100 ml defined growth medium (Section 2.1.1) containing $0.25gCl^{-1}$ mecoprop. After 48h incubation at 25°C prolific growth was noted with increases in culture absorbance from 0.1 to 0.33. The theoretical maximum chloride ion release was obtained, that is 2.08 µmoles chloride ions ml^{-1}. Thus, the mecoprop-degrading community could be reconstituted from its original components.

In an attempt to determine if any of the microorganisms were required in order to generate the necessary catabolic potential to degrade mecoprop various combinations of two, three and four mixed cultures were established. This involved ten different combinations of two organisms, ten different combinations of three organisms and five different combinations of four organisms.

Pure culture studies. All of the five community constituents were grown overnight in nutrient broth and then inoculated separately into 100 ml defined growth medium (Section 2.1.1) containing $0.25gCl^{-1}$ mecoprop as the sole carbon and energy source. After a 13 day incubation period at 25°C none of the purified organisms were capable of degrading the herbicide.

TABLE 4.3 **Percentage composition of the five community constituents**

The percentage varied depending on the age of the culture, but typically resulted in the following composition:

Organism	Percentage Composition*
Pseudomonas species HL1	58.89
Pseudomonas maltophilia	24.66
Alcaligenes species HL3	12.33
Acinetobacter calcoaceticus	2.74
Flavobacterium species HL5	1.37

*(two decimal places)

Two-Membered Studies. The culture absorbance and chloride ion release were measured after inoculation, then at 24h intervals for six days for the ten possible combinations of two community constituents. Six different combinations of two community members were able to fully degrade the supplied mecoprop (Table 4. 4). An interesting finding was that Pseudomonas maltophilia which had a growth factor requirement of L-methionine (Table 4.1) was not able to degrade mecoprop in any of the two-membered combinations except when Pseudomonas species HL1 was present. Pseudomonas species HL1 was able to fully degrade mecoprop in all of the two-membered mixtures. Alcaligenes species HL3 degraded mecoprop in all of the two-membered experiments except when combined with Pseudomonas maltophilia (Table 4.4).

Three-Membered Studies. All of the ten possible combinations of three community members were capable of growth on mecoprop as the sole carbon and energy source. Culture absorbance increases from between 0.06 and 0.075 to between 0.22 and 0.27 were noted after approximately 100h incubation. The maximum chloride ion release (2.08 µmoles chloride ions ml^{-1}) was recorded for each combination.

Four-Membered Studies. Again, mecoprop was utilized as the sole carbon and energy source in all of the five different combinations of four community members. Culture absorbance increases and chloride ion release was noted in all of the four-membered mixtures.

4.3 THE INFLUENCE OF pH AND CHEMOSTAT FLOW RATE ON SPECIES COMPOSITION

pH. The pH of the defined growth medium was altered with suitable additions of NaOH and H_2SO_4 to give a range of values between 2.5 and 11.5 (Section 2.1.1). It was established that microbial community growth occurred in growth medium containing $0.25gCl^{-1}$ mecoprop at pH values between 6.0 and 10.5 (Section 3.2). In several of the batch enrichment cultures a distinct yellow colouration was observed during

TABLE 4.4 **Combinations of two community constituents grown on mecoprop as the sole carbon and energy source**

	Pseudomonas maltophilia	Alcaligenes species HL3	*Acinetobacter calcoaceticus*	Flavobacterium species HL5
Pseudomonas species HL1	+	+	+	+
Pseudomonas maltophilia		-	-	-
Alcaligenes species HL3			+	+
Acinetobacter calcoaceticus				-

+ denotes increased culture absorbance and chloride ion release

- denotes no increases in culture absorbance or chloride ion release

microbial community growth. In an attempt to investigate specific effects of alkaline conditions on the community constituents the percentage species composition was elucidated for several pH values. The percentage of the Pseudomonas species HL1 decreased slightly with an increase in pH values from approximately 59% to 55.5% (Table 4.5). The yellow colouration noted in the growth medium at pH values between 7.0 and 9.0 was probably due to increased levels of the two organisms, Pseudomonas maltophilia and Flavobacterium species HL5, which produced yellow pigments (Section 4.1).

Chemostat dilution rate. The effect of increasing the dilution rate on the species composition of the mecoprop-degrading community was examined (see also Section 3.2). The flow rate of growth medium into the chemostat was altered by regulation of the Watson-Marlow flow-inducer (Section 2.3.2). After altering the dilution rate the chemostat culture was left for a 14 day period before the chemostat was sampled to allow steady state conditions to establish.

An increase in dilution rate, and therefore an increase in specific growth rate, from 0.085 to $0.12h^{-1}$ produced little change in the level of the Pseudomonas species HL1 which remained at between 70% to 72.5% of the community (Table 4.6). The levels of Alcaligenes species HL3 and Pseudomonas maltophilia both changed markedly in their percentage composition in the mecoprop community, but no pattern emerged with an increase in dilution rate (Table 4.6). Both the Acinetobacter calcoaceticus and Flavobacterium species HL5 remained as minor components in the community, comprising of less than 10% of the community.

4.4 PLASMID DNA STUDIES

Studies by Pemberton and his co-workers between 1977 and 1981 demonstrated the role of extrachromosomal elements in the degradation of

TABLE 4.5 THE PERCENTAGE SPECIES COMPOSITION OF THE MECOPROP COMMUNITY AT DIFFERENT pH VALUES

(one decimal place)

Organism	Percentage species composition			
	pH 7.0	pH 8.0	pH 8.5	pH 9.0
Pseudomonas species HL1	58.9	58.8	53.8	55.8
Pseudomonas maltophilia	24.7	25.5	28.2	28.8
Acinetobacter calcoaceticus	2.7	2.0	2.6	1.9
Alcaligenes species HL3	12.3	9.8	10.3	7.8
Flavobacterium species HL5	1.4	3.9	5.1	5.8

TABLE 4.6 THE PERCENTAGE SPECIES COMPOSITION OF THE MECOPROP COMMUNITY AT A RANGE OF CHEMOSTAT DILUTION RATES (one decimal place)

Organism	Dilution rate (h^{-1})			
	0.085	0.095	0.105	0.12
Pseudomonas species HL1	70.0	72.6	72.5	72.0
Pseudomonas maltophilia	1.8	6.6	3.10	13.3
Alcaligenes species HL3	25.4	13.1	15.0	7.3
Acinetobacter calcoaceticus	2.5	6.1	6.3	5.3
Flavobacterium species HL5	0.3	1.6	3.1	1.3

two of the phenoxy herbicides, 2,4-D and MCPA (Section 1.4.3). The possibility that plasmids were involved in mecoprop degradation by the microbial community was investigated using a plasmid DNA search.

The initial screening of the $0.25gCl^{-1}$ mecoprop-degrading community for plasmid DNA was undertaken using the Wheatcroft and Williams method (1981) with modifications as described in Section 2.8.2. The community was grown in defined growth medium (Section 2.1.1) containing $0.25gCl^{-1}$ mecoprop for 48h before the cells were centrifuged and lyzed (Section 2.8.2). The agarose gel was prepared (Section 2.8.1) and the lyzed cells were loaded onto the gel at concentrations of 20 µl. Initially concentrations of cells up to 0.75 ml were used for plasmid preparations but these concentrations were found to be too high to allow plasmid visualization, so the concentration was lowered to between 0.1 to 0.4 ml of culture.

Unpublished observations by D. Godwin-Thomas, J.R. Beeching and J.H.Slater have suggested that old cultures, two to three days after the stationary growth phase had been reached, were often more successful to use for visualizing plasmid DNA than exponentially growing cultures. A possible explanation was that older cultures may lyse better than growing cultures so making extractions of nucleic acids easier. In an attempt to compare the plasmid DNA visualized by the Wheatcroft and Williams method, cultures of different ages were employed. The microbial community was grown in defined medium containing $0.25gCl^{-1}$ mecoprop to various stages of growth, which included:

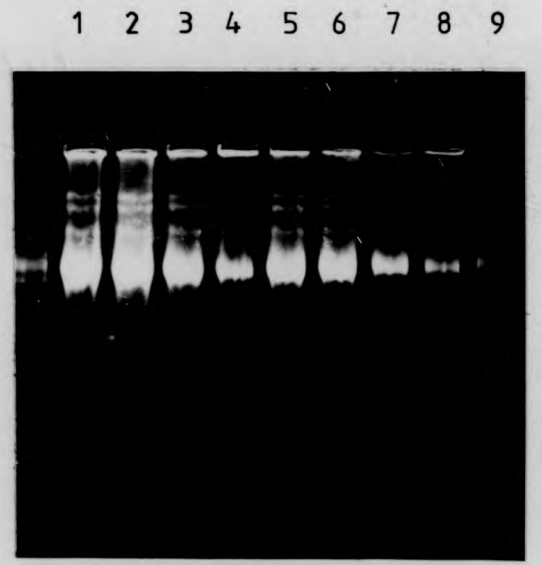
a) exponential growth phase,

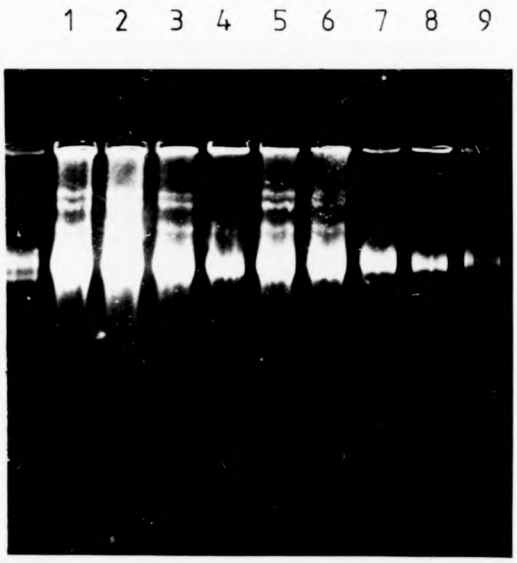
b) the stationary growth phase, and

c) an old culture, two to three days after the stationary growth had been reached.

The initial plasmid DNA search used a rapid screen (Section 2.8.2)

Fig. 4.1 A Wheatcroft & Williams screen of plasmid visualization of the mecoprop-degrading community at various stages of growth. The wells were loaded from left to right as follows: 1, 0.4 ml old culture, 2-3 days after the stationary phase; 2, 0.2 ml of old culture; 3, 0.1 ml of old culture; 4, 0.4 ml culture from the stationary growth phase; 5, 0.2 ml of culture from the stationary growth phase; 6, 0.1 ml of culture from the stationary growth phase; 7, 0.4 ml of culture from the exponential growth phase; 8, 0.2 ml of exponential growth phase culture; 9, 0.1 ml of exponential growth phase culture.


1 2 3 4 5 6 7 8 9


1 2 3 4 5 6 7 8 9

and the gel was run for 2h at 60 milliamps. After staining, plasmid bands were visualized using ultraviolet light. Plasmids were observed in all three stages of microbial community growth, but the bands were better defined in the old culture. The experimental procedure was repeated but a slower screen was used at 15 milliamps for 15h. Three plasmid bands were observed in the community, again the better defined and clearest bands were from the oldest culture (Fig. 4.1).

The Birnboim and Doly method (1979) of plasmid visualization was employed to investigate the possibility of producing better defined plasmid bands (Section 2.8.3). The advantage of this method was that the chromosomal DNA was eliminated during the procedure leaving only the plasmid DNA. A Birnboim and Doly screen of the mecoprop-degrading culture at both stationary growth phase and an old culture, two to three days after the stationary phase showed two plasmid bands when run at 50 milliamps for 26h.

Cultures containing plasmids of known molecular weight were included in a Birnboim and Doly plasmid screen together with the mecoprop-degrading culture to give an indication of the size of the community plasmids. The organisms used were R68-45 (32 Megadaltons), TP120 (32 Megadaltons) and RA1 (86 Megadaltons). But despite repeated attempts it was not possible to size the unknown plasmids as favourable operating conditions for the community plasmids and the marker plasmids differed so it was not possible to achieve suitable conditions for the plasmid bands to be visualized simultaneously. However, there were indications that the distance travelled by the community plasmids was appreciably less than that travelled by the smaller plasmids of R68-45 and TP120 suggesting community plasmids in the area of 150-200 Megadaltons (Fig. 4.2).

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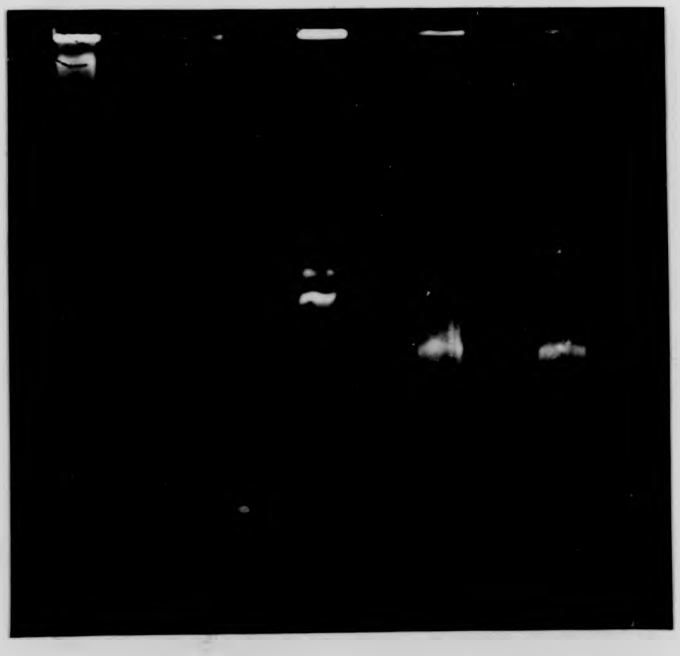
Fig. 4.2 A Birnboim & Doly screen of plasmid visualization for the mecoprop-degrading community and several marker plasmids. The wells were loaded from left to right as follows: 1, RA1; 2, TP120; 3, R68-45; 4, The mecoprop community from the stationary growth phase; 5, The mecoprop community in the exponential growth phase.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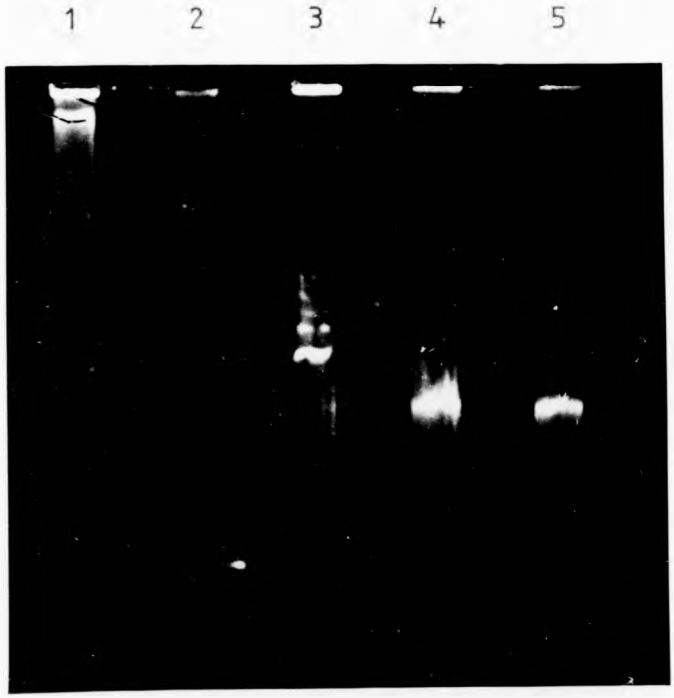
1
2
3
4
5

A brief investigation of the plasmids in the pure cultures of the mecoprop community constituents was undertaken. As none of the pure cultures could utilize mecoprop as the sole carbon and energy source (Section 4.2) they were grown overnight on nutrient broth. The cultures were prepared for plasmid visualization using the Wheatcroft and Williams method (1981) (Section 2.8.2). Plasmid bands were observed in Pseudomonas species HL1 and Flavobacterium species HL5, but none were detected in Pseudomonas maltophilia, Alcaligenes species HL3 or Acinetobacter calcoaceticus.

4.5 DISCUSSION

Between 1940 and 1950, Lochhead and his associates undertook much of the foundation work on the qualitative analysis of the rhizosphere inhabitants (Section 1.1.3). The earliest investigation (Lochhead, 1940) compared the relative incidence of certain groups of microorganisms in the rhizosphere with soil further away from the roots. A selective enhancement of Gram-negative, short, rod-shaped bacteria, such as Pseudomonas species, was reported with few incidence of Gram-positive, coccoid-shaped organisms such as Bacillus species, than in the surrounding soil (Clark, 1940; West & Lochhead, 1940). Classification of rhizosphere organisms into nutritional groups showed that the microflora had more complex nutritional requirements than the control organisms from soil further away from the plant root system (West & Lochhead, 1940).

A more detailed study of nutritional requirements (Wallace & Lochhead, 1950) revealed a high incidence of amino acid requiring bacteria, more specifically a requirement for the sulphur containing amino acids, particularly methionine. The authors concluded that methionine may have been required by the organisms not just as a readily available form of nitrogen or sulphur, but that the mobile methyl group

in methionine may have been utilized to synthesize an essential metabolite.

The identification of all of the mecoprop community constituents as Gram-negative short rods was as predominantly found in the rhizosphere by Lochhead and his co-workers. Moreover, the two organisms that were numerically dominant in the mecoprop-degrading community, that is Pseudomonas species HL1 and Pseudomonas maltophilia (Table 4.3), was in agreement with numerous reports showing Pseudomonads being the dominant organism in the rhizosphere (Clark, 1940; Alexander, 1961b; Vancura, 1980; Kleeberger et al. 1983). The mecoprop-degrading community can, therefore, be described as a typical group of rhizosphere inhabitants.

Pseudomonas maltophilia had a growth requirement of L-methionine, again a characteristic of rhizosphere microorganisms.

None of the purified community constituents were capable of degrading mecoprop as the sole carbon and energy source, yet combinations of three or more organisms (and in some instances combinations of two organisms), generated the necessary catabolic capability to degrade the herbicide. The community may, therefore, have been isolated as it was better adapted to degrade the herbicide, which was previously considered to be recalcitrant (Section 3.5), than the individual organisms (Slater & Godwin, 1980). Studies of microbial communities are well documented and investigation of the interactions between the participating organisms frequently pointed to an interdependence of organisms based upon essential growth compounds. That is, two populations grew together when one population required a specific compound which the second population provided (Slater, 1978).

Nurmikko (1954) provided an example of a two membered association based upon provision of specific compounds (cited by Slater, 1978, Fig.

1.6). Populations of Lactobacillus plantarum and Streptococcus faecalis were able to grow together as the former excreted folic acid that was required by Streptococcus faecalis which in turn excreted biotin required by Lactobacillus plantarum. A similar relationship was described by Yeoh et al. (1968). This involved Proteus vulgaris and Bacillus polymyxa. The two populations had growth requirements of biotin and nicotinic acid respectively which the organisms synthesized and excreted for each other (cited by Slater, 1978, Fig. 1.6).

This study showed that six different combinations of two community constituents had the capacity of degrading mecoprop (Section 4.2). More specifically, all of the combinations that included Pseudomonas species HL1 were able to utilize mecoprop (Table 4.4). Pseudomonas maltophilia could only degrade mecoprop when grown in the presence of the Pseudomonas species HL1. The possibility must exist that the degradation of mecoprop by the two membered association of Pseudomonas maltophilia and Pseudomonas species HL1 was the result of a specific relationship between the organisms. The Pseudomonas species HL1 may have provided the growth factor requirement (methionine) for Pseudomonas maltophilia. However, Pseudomonas species HL1 was not capable of utilizing mecoprop in pure culture (Section 4.2) so the Pseudomonas maltophilia must in turn have contributed a necessary 'factor' which allowed mecoprop degradation to occur in the presence of both organisms. As Pseudomonas species HL1 did not have a particular growth requirement its inability to degrade mecoprop in pure culture was not easily attributed to any one cause.

Stirling et al. (1976) isolated a mixed culture of a Nocardia species and a Pseudomonas species that was capable of growth on cyclohexane. Both organisms were required before degradation occurred because the Nocardia species was an auxotroph with growth factor

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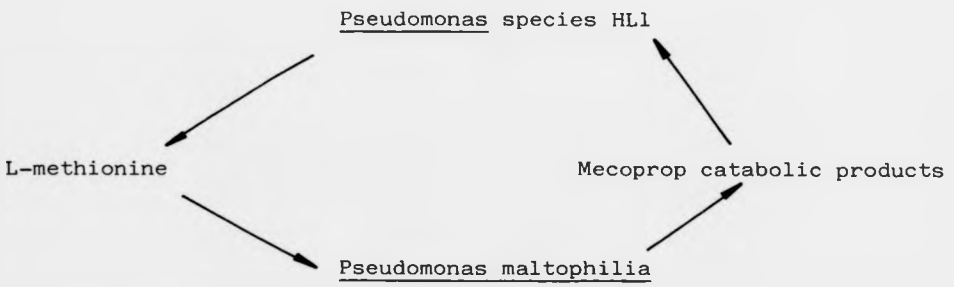
requirements that the Pseudomonas species supplied. The role of the Pseudomonas species in the mixed culture association was not as well understood, but it was considered to grow on products of cyclohexane degradation by the Nocardia species.

In the two-membered association comprising of Pseudomonas species HL1 and Pseudomonas maltophilia the 'factor' that the latter provided allowed the Pseudomonas species HL1 to grow in the culture and mecoprop degradation occurred. A feasible explanation was that the Pseudomonas maltophilia was able to commence some of the early stages of mecoprop degradation which then permitted Pseudomonas species HL1 to degrade the catabolic products. Therefore, a possible relationship of the two organisms could be summarized as:



Numerous combinations of organisms were also capable of degrading mecoprop (Section 4.2), in particular combinations of three and four community constituents as well as the total community of five organisms.

The importance of plasmids and their transfer between components of microbial communities to increase metabolic versatility to xenobiotic compounds has been discussed (Chatterjee et al. 1981; Slater & Bull, 1982). The existence of two or three plasmids in the mecoprop-degrading community was established using both the Wheatcroft and Williams (1981) and Birnboim & Doly (1979) methods of plasmid visualization (Section

4.4). The role of degradative plasmids may be examined by curing the pure culture plasmids then investigating the response of the cured strains to the particular compound (Section 1.4.1). However, none of the community constituents was capable of degrading mecoprop as the sole carbon and energy source, making it more difficult to assess the role, if any, of the plasmids in mecoprop degradation. Fisher et al. (1978) described plasmid pJP1 which encoded for the conversion of 2,4-D to 2,4-dichlorophenol, but not for the complete degradative pathway. The presence of plasmids contribute genetic information to the microbial community and may similarly assist in degradation by encoding for some of the catabolic enzymes in the mecoprop degradative pathway.

In conclusion, it has been acknowledged (Slater, 1978; Slater & Bull, 1982) that microbial interactions are frequently intricate making analysis difficult. Elucidation of all of the mecoprop interactions was not possible, and in common with several recent reports (Ou & Sikka, 1977; Kim & Rehm, 1982) it must be concluded that the nature of the interactions are not yet fully understood.

Many different combinations of organisms capable of degrading mecoprop existed within a stable microbial community. Although the relationships were not fully understood they were not mutually exclusive as none of the organisms were lost from the community despite 5,000h continuous growth.

CHAPTER FIVE

GROWTH CHARACTERISTICS OF THE MECOPROP-DEGRADING COMMUNITY

Organisms enriched for the ability to degrade one of the chlorinated phenoxy herbicides have been demonstrated to degrade other phenoxy herbicides (Audus, 1951; Kirkland & Fryer, 1972). Audus demonstrated that soils enriched with 2,4-D degrading organisms could also degrade MCPA, and soils enriched with MCPA degrading organisms could similarly degrade 2,4-D. Kirkland and Fryer (1972) used soils regularly sprayed with MCPA over a four year period. Soils enriched with the ability to degrade MCPA could also rapidly utilize 2-methyl 4-chlorophenoxy butanoic acid (MCPB). However, no such enrichment of ability to degrade two other phenoxy herbicides, mecoprop or dichlorprop was demonstrated.

The rhizosphere microbial community enriched to degrade mecoprop (Section 3.1) was similarly exposed to a range of phenoxy herbicides including MCPA, 2,4-D, 2,4,5-T and dichlorprop to investigate if the enriched community could utilize the structurally related compounds.

5.1 GROWTH OF THE MECOPROP-DEGRADING COMMUNITY ON 2,4-D, 2,4,5-T AND MCPA

The enriched mecoprop-degrading community (Section 3.1) was used as an inoculation for a series of batch growth systems containing minimal medium (Section 2.1.1) and $0.25 gCl^{-1}$ of either 2,4,5-T ($0.67 gl^{-1}$; 2.60 mM), MCPA ($0.46 gl^{-1}$; 2.31 mM) or 2,4-D ($0.58 gl^{-1}$; 2.60 mM). The batches were incubated at 25°C, culture absorbance (Section 2.4.1) and chloride ion release (Section 2.4.2) were monitored over a 20 day period. The mecoprop-degrading community was capable of utilizing MCPA and 2,4-D as the sole sources of carbon and energy, but it was not capable of utilizing 2,4,5-T.

Growth commenced between the fourth and fifth day after inoculation in the batch enrichment culture containing 2,4-D. Within 264h the culture

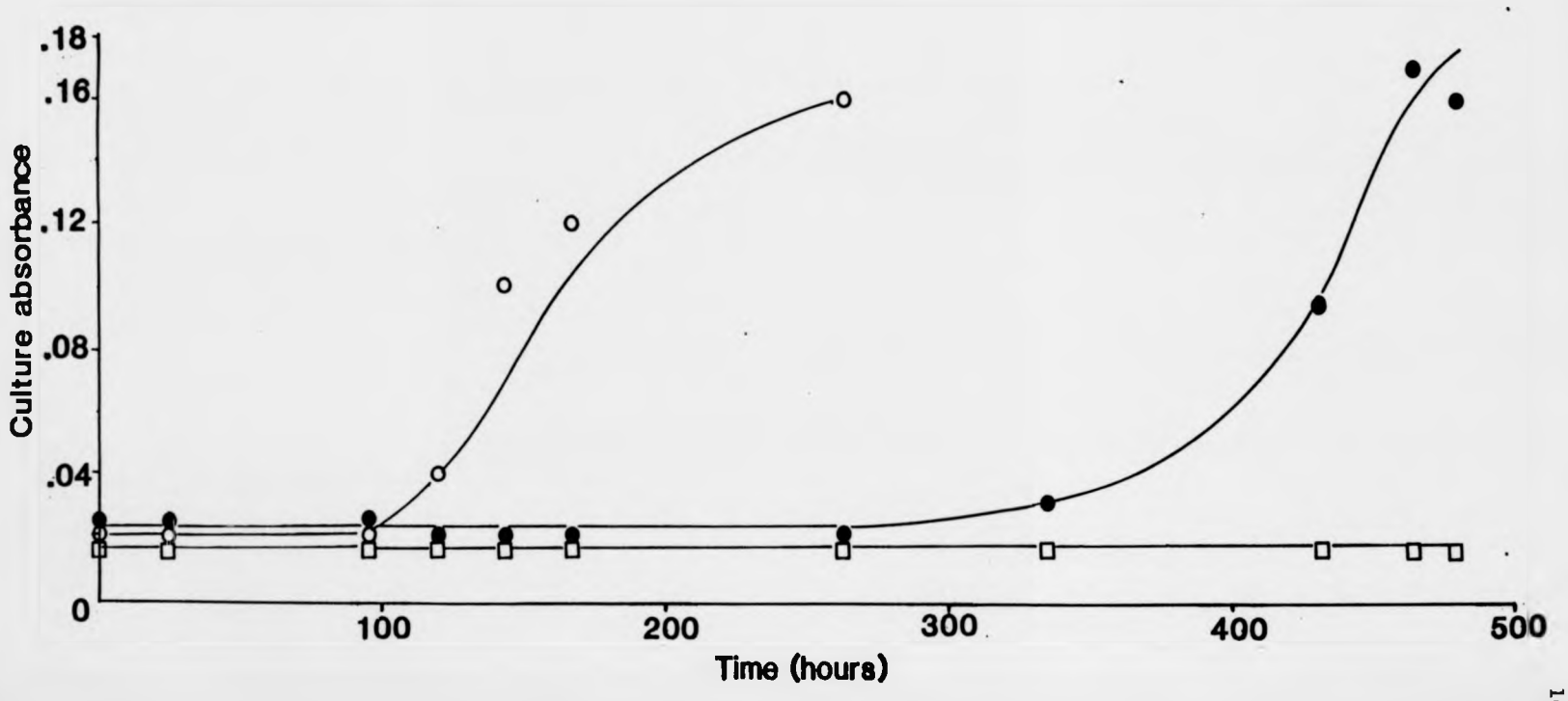
absorbance had increased from 0.02 to 0.16 and the chloride ion release from 0.77 to 5.89 µmoles ml^{-1} (98.46% of the theoretical maximum) (Fig. 5.1). The batch enrichment culture containing MCPA had a longer lag phase than the 2,4-D culture, with growth commencing between the 11th and 14th day. The culture absorbance increased from 0.02 to 0.17 and the chloride ion release from 0.60 to 2.20 µmoles ml^{-1} (69.56% of the theoretical maximum release) (Fig. 5.1).

Despite a prolonged incubation period of approximately 60 days, no increases in either culture absorbance or chloride ion release were detected in the 2,4,5-T batch culture (Fig. 5.1). It was concluded that the herbicide was not capable of supporting the growth of the mecoprop-degrading community.

In an attempt to enrich for 2,4,5-T degradation by the community, mixed substrates of 2,4,5-T and mecoprop were used as carbon sources. It was anticipated that the organisms would first degrade the more easily utilized carbon source, that is mecoprop, thereby increasing the cell biomass. With the increased biomass and increased catabolic potential a greater possibility of 2,4,5-T degradation existed.

Both 2,4,5-T and mecoprop were added to minimal medium to give a final concentration of $0.1gCl^{-1}$ of each herbicide. After incubation at 25°C for 60 days, increases in both chloride ion release and culture absorbance were recorded. However the increases only corresponded to mecoprop degradation as values of culture absorbance (0.2 - 0.25) and chloride ion release (2.0 µmoles ml^{-1}) were similar to values previously recorded for mecoprop degradation (Section 3.1). As chloride ion release and culture absorbance would have been higher if 2,4,5-T had also been degraded, it was concluded that the community did not enrich in 2,4,5-T degraders after 60 days. This was confirmed when a sample of the 2,4,5-T and mecoprop batch culture was subcultured into a batch system containing

Fig. 5.1 The growth of the mecoprop-degrading culture on 2,4-D, MCPA and 2,4,5-T. O, 2,4-D; ●, MCPA; □, 2,4,5-T.


.18
.16
.12
.08
.04
0
Culture absorbance
100
200
300
400
500
Time (hours)

$0.1gCl^{-1}$ 2,4,5-T as the sole carbon and energy source. No growth was detected after 16 days incubation.

To establish the maximum concentration of 2,4-D that could be degraded, the mecoprop community was inoculated into a series of 250 ml conical flasks containing 100 ml minimal medium (Section 2.1.1) with different concentrations of 2,4-D. The batch cultures were incubated at 25°C for up to 17 days and the chloride ion release (Section 2.4.2) culture absorbance (Section 2.4.1), and pH were monitored at suitable time intervals. Batch cultures containing more than $0.5gCl^{-1}$ 2,4-D were not capable of supporting growth of the mecoprop community. However, growth commenced after a lag phase of five days in the batch culture containing $0.5gCl^{-1}$ 2,4-D. The chloride ion release increased from 0.08 to 3.72 µmoles ml^{-1} or 35.0% of the theoretical maximum release (Fig. 5.2) and the culture absorbance from 0.02 to 0.1 within nine days.

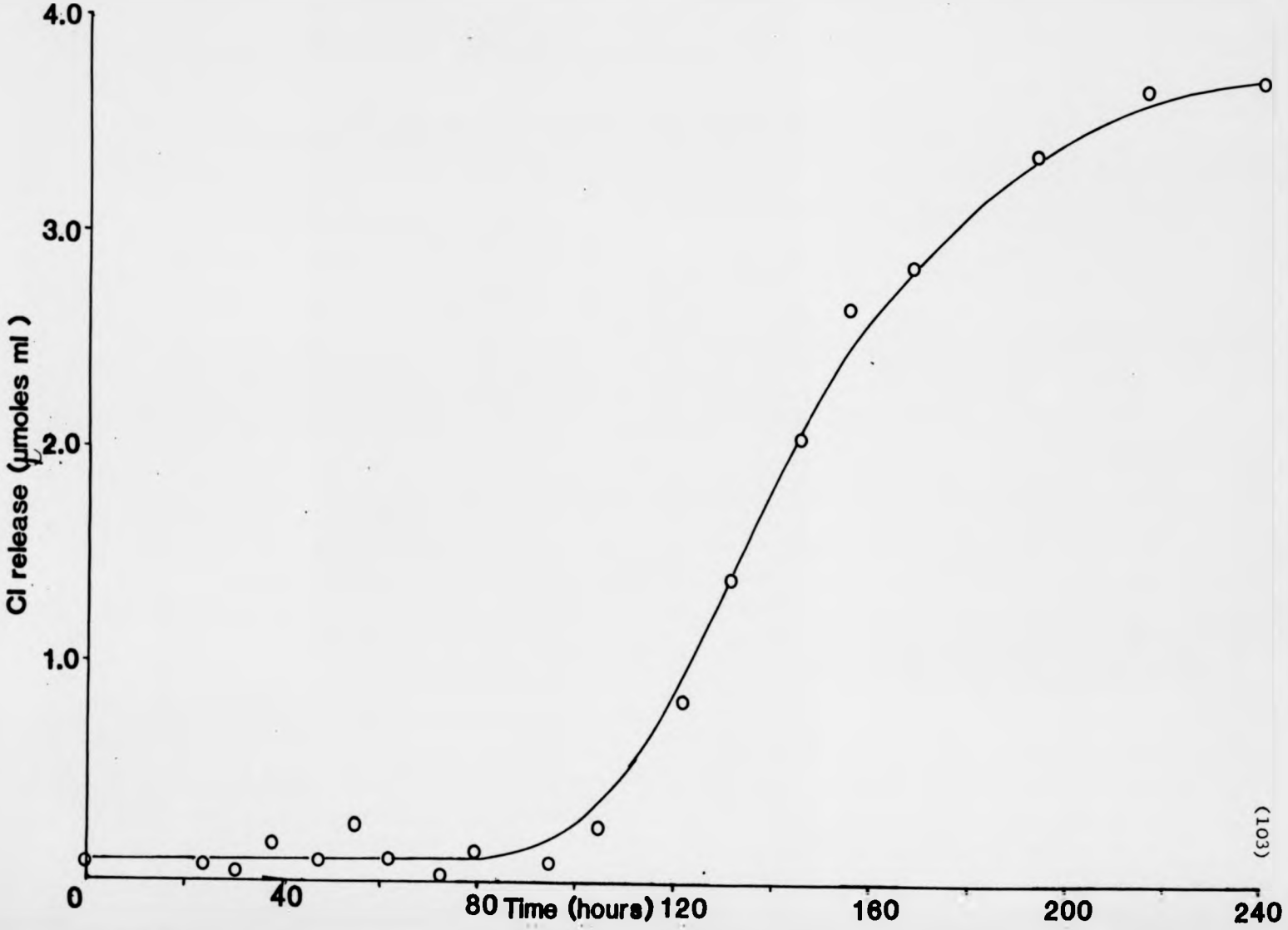
5.2 ENRICHMENT OF AN MCPA-DEGRADING COMMUNITY

Rhizosphere populations that had successfully enriched to degrade mecoprop were also able to degrade 2,4-D and MCPA. As a useful comparison it was decided to attempt enrichment of organisms using another phenoxy herbicide then to investigate if the new enriched population could similarly degrade mecoprop.

Wheat plants were grown under controlled conditions for 15 days (Section 2.2.1). The root washings (Section 2.2.2) were used to inoculate a series of 250 ml conical flasks containing 100 ml minimal medium (Section 2.1.1) and MCPA at concentrations of either $0.1gCl^{-1}$ ($0.187gl^{-1}$; 0.93 mM) or $0.5gCl^{-1}$ ($0.93gl^{-1}$; 4.63 mM). Chloride ion release was detected in the $0.1gCl^{-1}$ MCPA batch enrichment culture 21 days after inoculation. The 0.1gCl-l batch culture as subcultured whenever the chloride ion release reached the theoretical maximum, that is 0.93 µmoles ml^{-1}.

The higher concentration of MCPA, $0.5gCl^{-1}$ was not capable of

Fig. 5.2 The growth of the mecoprop community on $0.5gCl^{-1}$ 2,4-D.


4.0
3.0
2.0
1.0
Cl release (μmoles ml)
-1
0
40
80
Time (hours)
120
160
200
240

supporting growth of rhizosphere organisms despite a three month incubation period. Investigations of mecoprop enrichment also illustrated that rhizosphere organisms were not able to degrade mecoprop at the same carbon concentrations (Section 3.1).

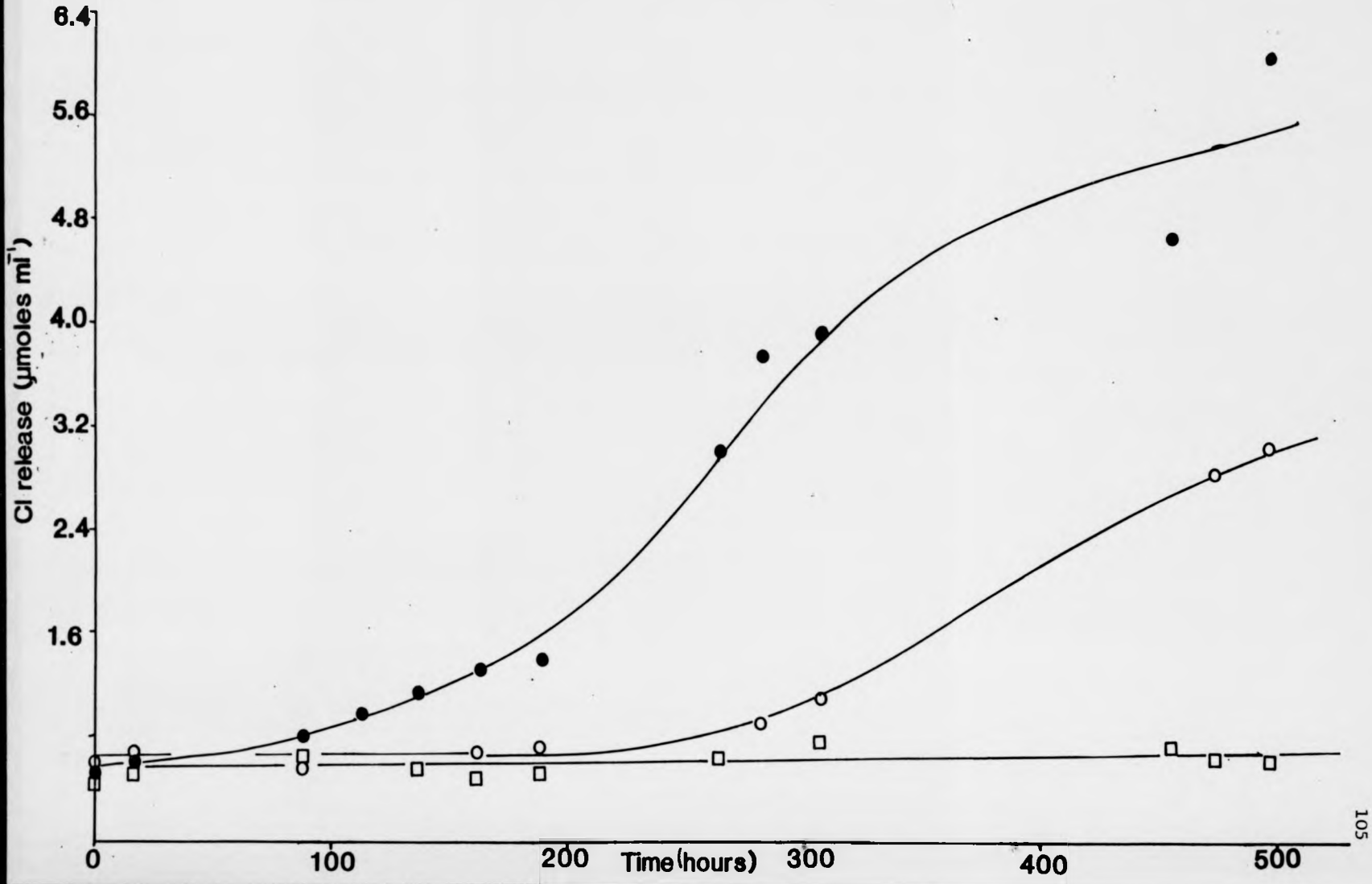
MCPA cross perfusion technique. Once an enriched population of MCPA degraders had been established cross perfusion techniques were undertaken. Cross perfusion, or cross adaptation, involved the growth of one herbicide-degrading population on other related compounds to investigate if the population was enriched to degrade other structurally similar herbicides (Kirkland & Fryer, 1972). Therefore, the rhizosphere organisms enriched to degrade MCPA were inoculated into a series of 250 ml conical flasks containing 100 ml of defined growth medium (Section 2.1.1) and $0.25gCl^{-1}$ of either mecoprop, 2,4-D or 2,4,5-T as the sole carbon and energy source.

A lag phase of 113h was recorded before chloride ion release commenced in the batch enrichment culture containing 2,4-D (Fig. 5.3). Chloride ion release increased from 0.80 to 6.04 µmoles ml^{-1} producing total chloride ion release of 5.24 µmoles ml^{-1} a value equal to the theoretical maximum for the supplied 2,4-D (5.20 µmoles ml^{-1}). However, the culture absorbance remained at 0.02 throughout the incubation period.

The MCPA enriched culture required a longer lag phase of 307h before mecoprop dehalogenation commenced (Fig. 5.3). The total chloride ions released from mecoprop after 500h equalled the theoretical maximum release for the supplied mecoprop, that is 2.08 µmoles ml^{-1}. Again, after an incubation period of 500h, no change in the culture absorbance was detected.

Neither chloride ion release nor culture absorbance increases were detected by the MCPA enriched culture after a 500h incubation period with 2,4,5-T as the sole carbon and energy source (Fig. 5.3). The batch culture

Fig. 5.3 An enriched MCPA-degrading community growing on mecoprop, 2,4-D and 2,4,5-T. O, mecoprop; ●, 2,4-D; □, 2,4,5-T.


6.4
5.6
4.8
4.0
3.2
2.4
1.6
Cl release (μmoles ml⁻¹)
0
100
200
Time (hours)
300
400
500

was incubated for a further 60 days but no growth was observed.

Details of the MCPA culture. After four consecutive subcultures a brief investigation of the constituents of the $0.1gCl^{-1}$ MCPA-degrading culture was undertaken. Cultures were spread plated onto nutrient agar, malt extract agar (Section 2.1.3) and King's B agar (Section 2.1.4). After two to three days incubation at 25°C colonies were picked off the plates and loopfuls were repeatedly streaked onto fresh plates until purified organisms were obtained. Six different bacterial isolates and one fungus were isolated from the MCPA culture. Identification was not undertaken but all of the bacterial isolates were shown to be Gram-negative.

The community constituents were grown separately in nutrient broth (Section 2.1.3) overnight at 25°C and then inoculated (5% v/v) into separate batch cultures containing $0.1gCl^{-1}$ MCPA to investigate pure culture degradation. None of the organisms were able to utilize MCPA as the sole carbon and energy source after 300h incubation as determined by monitoring chloride ion release and culture absorbance.

The enriched batch culture of MCPA degraders was transferred to a one litre fermenter (Section 2.3.2) and was grown continuously at a dilution rate of $0.024h^{-1}$ for 2,300h. Chemostat cultures failed to reduce the complexity of the MCPA community and no pure culture degraders emerged.

No further investigations of the MCPA culture were undertaken, and all further work concentrated on the mecoprop-degrading community.

5.3 GROWTH OF THE MECOPROP COMMUNITY ON A VARIETY OF AROMATIC COMPOUNDS

An investigation of the ability of the mecoprop enriched community to degrade a wide variety of aromatic compounds was undertaken.

Dichlorprop, (2-(2,4-dichlorophenoxy) propionic acid) a chlorinated phenoxy herbicide structurally related to mecoporop, was used as a growth substrate for the mecoprop-degrading community. A 5% (v/v) inoculation was

added to 100 ml minimal medium (Section 2.1.1) containing $0.25gCl^{-1}$ dichlorprop ($0.48gl^{-1}$; 2.31 mM). Growth commenced 18h after inoculation (Fig. 5.4), the chloride ion release increased from 0.31 to 3.79 µmoles ml^{-1} in 72h, corresponding to 75.32% dehalogenation of the theoretical maximum chloride ion release (4.62 µmoles ml^{-1}). The culture absorbance increased from 0.03 to 0.18 between the 16th and 72nd hour after inoculation (Fig. 5.4). The specific growth rate (Section 2.3.4) was $0.080h^{-1}$.

Growth of the mecoprop community on several chlorinated benzoic acids was investigated. It was necessary to generate the sodium salts of 2-, 3- and 4-chlorobenzoic acids by additions of sodium hydroxide as the free acids were only sparingly soluble. Each of the chlorobenzoic acids were autoclaved separately at 115°C for 10 mins. The chloride ion release (Section 2.4.2) was assayed to check that none of the carbon sources had broken down during autoclaving. Each chlorobenzoic acid was added separately to minimal medium (Section 2.1.1) to give a final concentration of $0.25gCl^{-1}$ ($0.47gl^{-1}$). The batch enrichment flasks were inoculated (5% v/v) with the mecoprop-degrading community and incubated at 25°C. None of the chlorinated benzoic acids were capable to supporting growth of the mecoprop community after 20 days incubation. In all cases the chloride ion release remained at between 0.80 and 1.0 µmoles chloride ions ml^{-1}.

Phenoxyacetic acid, a derivative of the chlorinated phenoxy herbicides, occurs naturally in soils. It was added to defined growth medium (Section 2.1.1) as the sole carbon and energy source to give a final concentration of $0.25gCl^{-1}$ ($0.118gl^{-1}$). A lag phase of 47h was noted before growth commenced (Fig. 5.5). The culture absorbance then increased from 0.04 to 0.14 within 53h. The specific growth rate (Section 2.3.4) was calculated as $0.026h^{-1}$.

4-chlorophenylacetic acid was added to 100 ml minimal medium as the

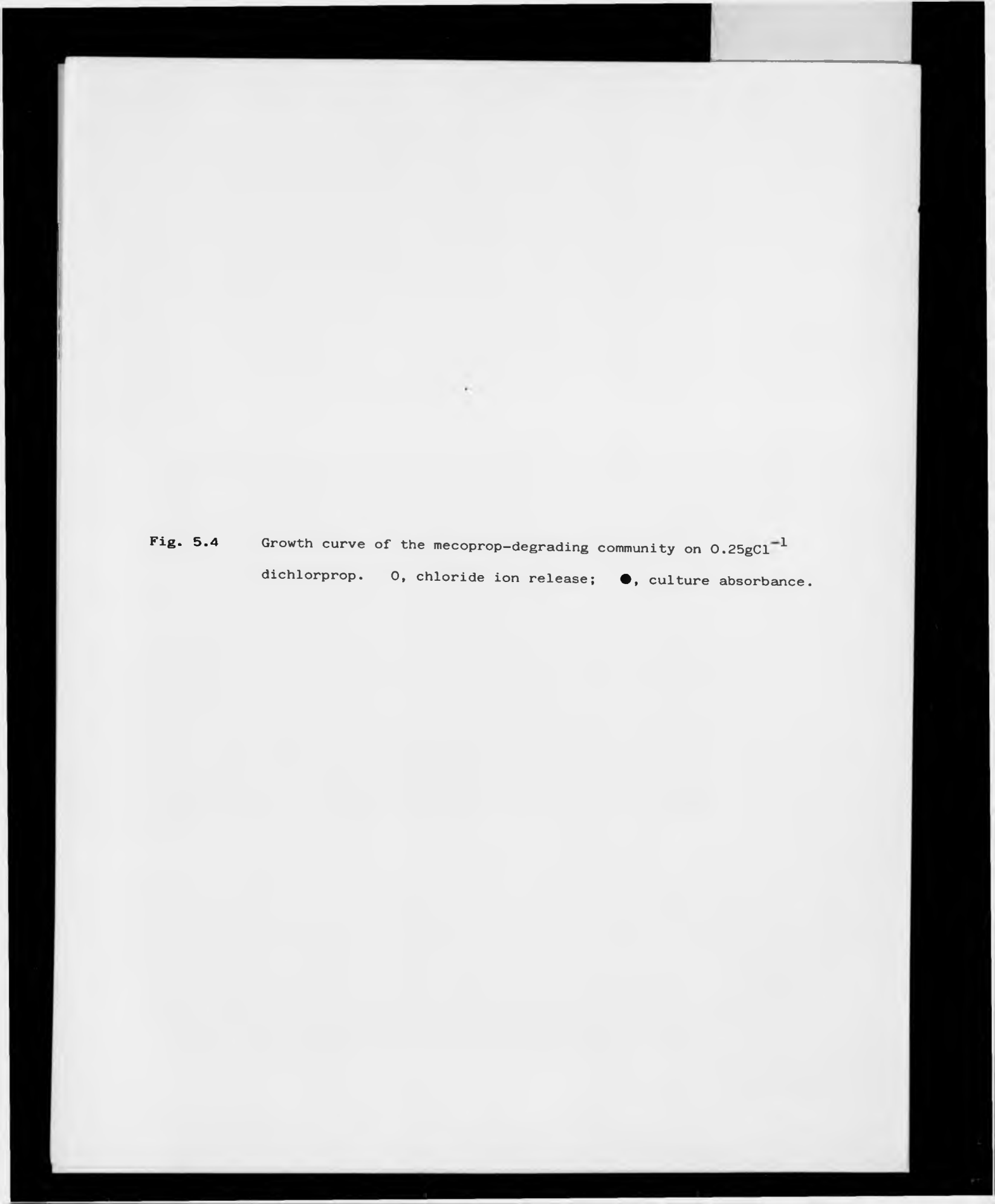


Fig. 5.4 Growth curve of the mecoprop-degrading community on 0.25gCl^{-1} dichlorprop. O, chloride ion release; ●, culture absorbance.

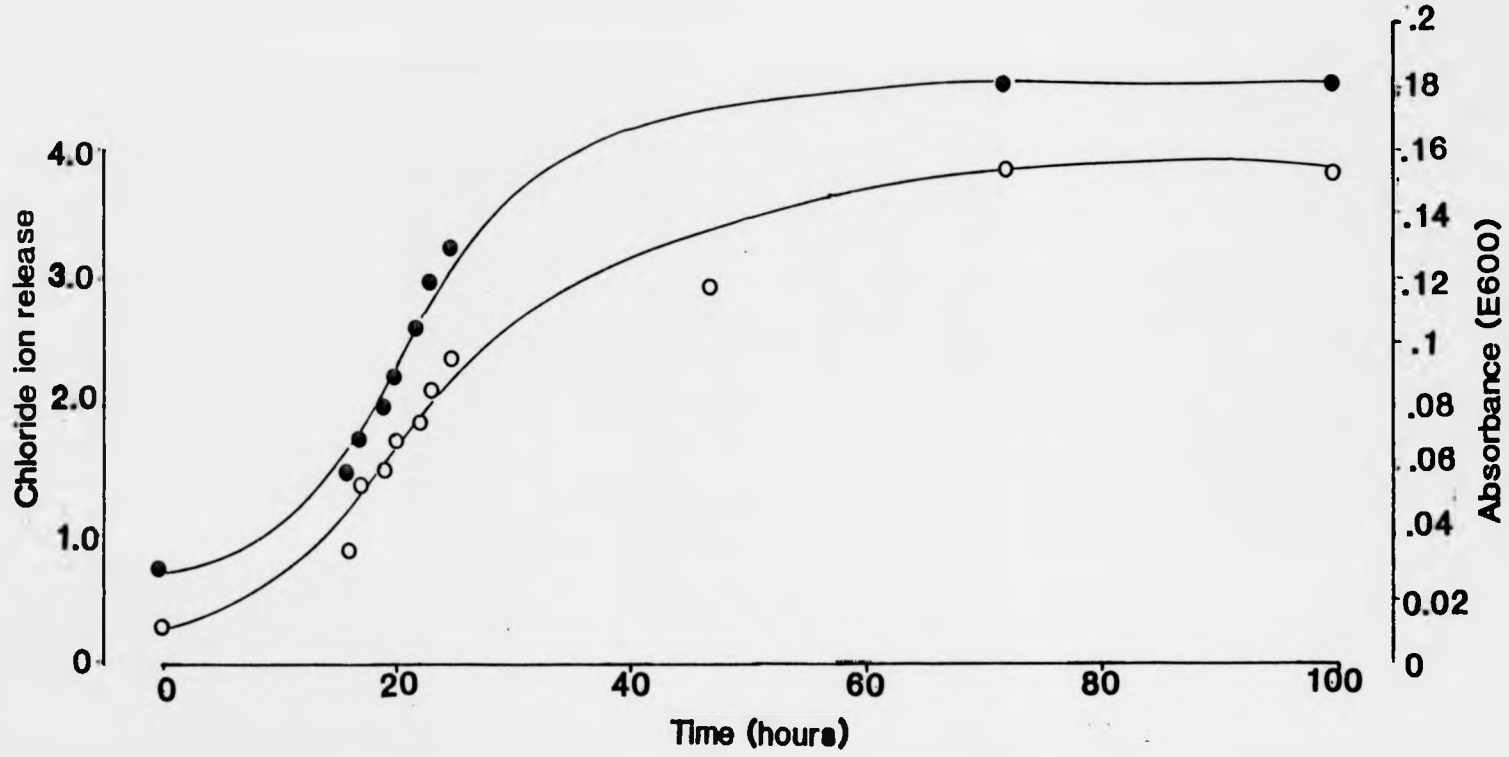
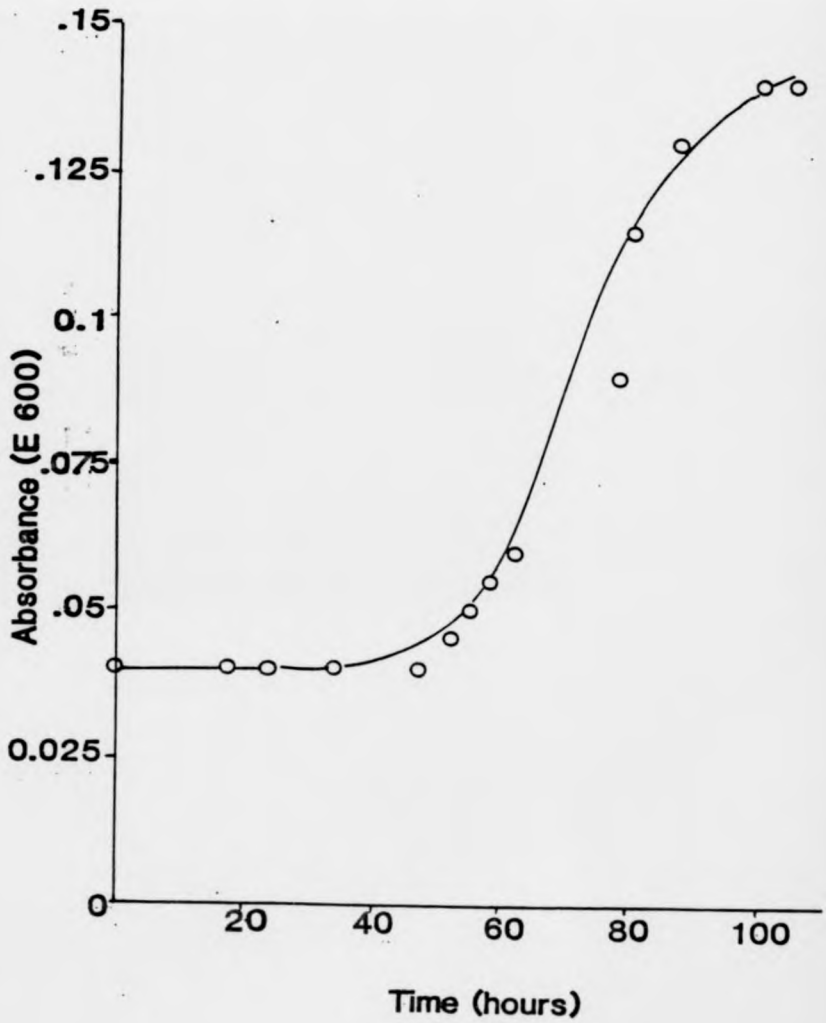

Chloride ion release
4.0
3.0
2.0
1.0
0
0
20
40
60
80
100
Time (hours)
.2
.18
.16
.14
.12
.1
.08
.06
.04
0.02
0
Absorbance (E600)

Fig. 5.5 Growth of the mecoprop-degrading community on phenoxyacetic acid.


.15
.125
0.1
.075
.05
0.025
0
Absorbance (E 600)
20
40
60
80
100
Time (hours)

sole source of carbon and energy to give a final concentration of $0.25gCl^{-1}$ ($0.44gl^{-1}$). After mecoprop community inoculation the batch enrichment culture was incubated for 22 days at 25°C. No increases in either culture absorbance or chloride ion release were noted during the incubation period, so it was concluded that 4-chlorophenylacetic acid was not capable of supporting growth of the mecoprop enriched community.

Catechol and catechol-like compounds occur as intermediates in the biodegradative pathways of two of the chlorinated phenoxy herbicides, 2,4-D and MCPA (Bollag et al. 1968a & b; Tiedje et al. 1969). Evidence of catechol degradation by the mecoprop community may illustrate its occurrence in the mecoprop biodegradative pathway. After 30h incubation at 25°C the absorbance of the batch enrichment culture increased from 0.05 to 0.28 when $0.25gCl^{-1}$ catechol ($0.38gl^{-1}$; 3.47 mM) was the sole carbon and energy source for the community. As catechol was rapidly degraded by the community an assay procedure was used to determine if any dihydroxy aromatic compounds were formed as intermediates in mecoprop degradation. The microbial community was grown in minimal medium (Section 2.1.1) containing $0.25gCl^{-1}$ mecoprop using batch culture growth (Section 2.3.1). Aliquots (1.0 ml) were aseptically removed from the batch culture after inoculation, at the mid-exponential growth phase and at the stationary phase. The samples were prepared as previously described (Section 2.6.4) and the absorbance was measured at 540 nm. However, no protocatechuic acid or protocatechuate-like compounds were detected in the samples studied.

5.4 GROWTH OF THE COMMUNITY CONSTITUENTS ON SEVERAL STRUCTURALLY RELATED HERBICIDES

The mecoprop-degrading community has been demonstrated to utilize MCPA, 2.4-D (Section 5.1) and dichlorprop (Section 5.3) but not 2,4,5-T (Section 5.1). The five bacterial isolates of the mecoprop community (Section 4.1) were similarly exposed to four of the phenoxy herbicides, MCPA, 2,4-D, 2,4,5-T and dichloroprop to investigate if any organism(s) was

capable of degrading these structurally related herbicides. It has already been established that none of the purified community constituents were able to utilise mecoprop as the sole carbon and energy source (Section 4.2).

The five pure cultures were grown overnight in nutrient broth (Section 2.1.3), a growth media that contains many easily utilized carbon compounds to produce rapid increases in cell biomass. After overnight growth at 25°C each of five the pure cultures were inoculated (1% v/v) separately into five 250 ml conical flasks containing 100 ml of defined growth medium (Section 2.1.1) and $0.25gCl^{-1}$ of 2,4-D. This procedure was repeated for four other herbicides: MCPA, dichloroprop, 2,4,5-T and phenoxyacetic acid. Thus 25 batch enrichment cultures, containing all the binary combinations of pure culture and herbicide, were produced. The batch enrichment cultures were incubated at 25°C (Section 2.3.1) for up to seven days.

Dichlorprop. After six days incubation with dichlorprop as the sole carbon and energy source, no increases in culture absorbance (Section 2.4.1) or chloride ion release (Section 2.4.2) were detected in any of the five batch cultures containing the mecoprop community constituents. It was concluded that dichlorprop was not capable of supporting the growth of any of the community constituents (Table 5.1).

2,4,5-T. No growth was detected by any of the five pure cultures after a seven day incubation period when 2,4,5-T was supplied as the sole carbon and energy source (Table 5.1).

MCPA. After four days incubation with MCPA as the sole carbon and energy source, none of the purified organisms were capable of degrading the herbicide; that is, no chloride ion increases or culture absorbance increases were noted (Table 5.1).

TABLE 5.1 **Growth of the mecoprop community constituents on a variety of structurally related herbicides**

Herbicides	*Pseudomonas* species HL1	*Pseudomonas maltophilia*	*Alcaligenes* species HL3	*Acinetobacter calcoaceticus*	*Flavobacterium* species HL5
2,4 D	+	+	-	-	-
MCPA	-	-	-	-	-
2,4,5-T	-	-	-	-	-
Dichlorprop	-	-	-	-	-
Phenoxyacetic acid	-	-	-	-	-

+ indicates chloride ion release and culture absorbance increase

- indicates no growth

Phenoxyacetic acid. Phenoxyacetic acid was not degraded by any of the five organisms after four days incubation (Table 5.1).

2,4-D. Both the Pseudomonas species HL1 and Pseudomonas maltophilia were able to degrade 2,4-D as the sole carbon and energy source after inoculation from overnight growth on nutrient broth (Table 5.1). Over a 72h incubation period the culture absorbance increased from 0.02 to 0.23 in the Pseudomonas maltophilia batch culture. The chloride ion release increased from 2.83 to 7.06 μmoles Cl^- ml^{-1} that is 81.35% of the theoretical maximum release (5.20 μmoles ml^{-1}) over the same time period.

In the batch enrichment culture containing the Pseudomonas species HL1 the culture absorbance increased from 0.03 to 0.2 and the chloride ion release from 2.10 to 6.38 μmoles Cl^- ml^{-1}, that is 82.31% of the theoretical maximum release in 72h incubation. More detailed growth curves of the two Pseudomonads were attempted with 2,4-D as the sole carbon and energy source and to allow the calculation of the specific growth rates. The experiment was repeated on numerous occasions but all attempts to produce growth curves failed. On several occasions the Pseudomonas maltophilia failed to grow at all. The growth of the Pseudomonas species HL1 on 2,4-D was not consistent: taking between four and nine days to fully degrade the supplied herbicide on separate occasions.

None of the three remaining community constituents, that is the Alcaligenes species HL3, the Flavobacterium species HL5 or the Acinetobacter calcoaceticus were capable of degrading 2,4-D in pure culture batch systems. After an incubation period of seven days no increases in culture absorbance or chloride ion release were noted for any of the three batch cultures (Table 5.1).

5.5 DISCUSSION

The rhizosphere community enriched to degrade mecoprop was also capable of degrading several structurally related herbicides, including 2,4-D, MCPA and dichlorprop. One of the earliest studies of 2,4-D degradation by soil organisms was undertaken by Audus (1951). In an attempt to elucidate the 2,4-D breakdown mechanisms, or more specifically the initial attack on the herbicide molecule, Audus studied the growth responses of an enriched soil to analogues of 2,4-D. The 2,4-D enriched soil was exposed to MCPA and the time taken for complete degradation was compared to soils not previously exposed to either 2,4-D or MCPA. A second enrichment was undertaken using MCPA and the resulting soil was exposed to 2,4-D. Audus noted that the time taken for MCPA to be degraded by the 2,4-D enriched soil was less than for the organisms not previously exposed to MCPA. However, the 2,4-D enriched organisms were not able to degrade MCPA at a comparable rate to organisms enriched on MCPA.

Audus reported similar results when the MCPA enriched organisms were grown on 2,4-D, that is, 2,4-D degradation occurred, but at a slower rate than the 2,4-D enriched organisms. He was uncertain if one species of bacteria was responsible for 2,4-D and MCPA degradation, if one enzyme system was responsible or each molecule required its own enzyme system.

Kirkland and Fryer (1972) undertook cross adaptation studies using MCPA enriched soils. The soil was exposed to MCPB, dichlorprop and mecoprop and bioassay procedures using either sorghum or cotton to follow herbicide degradation. Although MCPA enriched soils could rapidly degrade MCPB they could not utilize mecoprop or dichlorprop at a faster rate than control soils not previously exposed to any phenoxy herbicides.

Other studies have also shown a similar cross adaptation of organisms to the phenoxy herbicide group (Steenson & Walker, 1956; MacRae & Alexander, 1965). Loos (1975) offered a possible explanation for this

phenomenon. He considered that the potential to degrade the phenoxy herbicides could be attributed to non-specific enzymes that metabolize structurally related compounds, that is, the enzyme systems are sufficiently similar to allow the same enzymes to degrade either 2,4-D or MCPA. Although the 2,4-D and MCPA biodegradation pathways have been fully elucidated, little is known of the mecoprop biodegradation pathway (Lappin et al. 1984). However, as the mecoprop community was also capable of degrading 2,4-D and MCPA it can be suggested that the enzyme systems are sufficiently similar to degrade the three herbicides.

A microbial community enriched in MCPA degrading organisms was not capable of degrading 2,4-D or mecoprop (Section 5.2) as the sole carbon and energy source as the culture absorbance did not increase in either cultures. However, the MCPA enriched community could dehalogenate both 2,4-D and mecoprop molecules (Section 5.2). The mecoprop structure differs from that of MCPA by an extra methyl group in the phenoxy sidechain (Fig. 1.2). Possibly the biodegradation of mecoprop initially involved the cleavage of this methyl group to produce MCPA, with degradation then proceeding via the MCPA pathway (Section 1.2.3). This hypothesis was supported by the evidence that the mecoprop enriched community was able to degrade MCPA as it contained all of the necessary catabolic enzymes. But the MCPA enriched community could not degrade mecoprop as it did not contain the initial enzyme to cleave the extra methyl group. However, the MCPA community could dehalogenate the mecoprop molecule so it did contain some of the catabolic potential of the mecoprop pathway.

The herbicide 2,4,5-T, a component of the defoliant Agent Orange, has long been considered to be a recalcitrant compound (Alexander, 1961a). The structural difference of 2,4-D and 2,4,5-T, that is, an extra chlorine substitution on the aromatic ring at the five carbon position of the latter herbicide (Fig. 1.2), produce vast differences in microbial attack of the

two herbicides. Numerous reports have confirmed the inability of 2,4,5-T to support microbial growth (Alexander, 196la; MacRae & Alexander, 1965).

In the last three years a series of publications by Chakrabarty and his co-workers at the University of Illinois, Chicago, have reported evidence of 2,4,5-T degradation as the sole carbon and energy source (Kellog et al. 1981). A mixed microbial culture was established using 'plasmid assisted molecular breeding' techniques (Section 1.2.3). From this mixed culture a pure culture of Pseudomonas cepacia AC1100 emerged with the capability of 2,4,5-T degradation (Kilbane et al. 1982). Pseudomonas cepacia was capable of degrading 2,4,5-T in soils containing 1,000 µg 2,4,5-T per gram of soil (Chatterjee et al. 1982). The organism detoxified more heavily contaminated soils, up to 20,000 µg 2,4,5-T per gram of soil so effectively that the soil was able to support plant growth (Kilbane et al. 1983). An examination of the substrate specificity revealed a broad range of substituted phenols were dehalogenated by Pseudomonas cepacia AC1100. Karns et al. (1983a) were uncertain whether to attribute this to one enzyme system with a wide substrate specificity or several separate dehalogenation mechanisms. 2-4,5-trichlorophenol was considered to be an intermediate in 2,4,5-T degradation by AC1100.

To date this is the only report of pure culture 2,4,5-T degradation. Other researchers have stressed that cometabolism is necessary for 2,4,5-T degradation by soil organisms (Rosenberg & Alexander, 1980). Therefore, it can be argued that no natural organism has yet been isolated that can degrade 2,4,5-T. Pseudomonas cepacia AC1100 is a laboratory constructed organism, not a natural isolate. Although AC1100 is useful as it illustrated that 2,4,5-T degradation was a possibility, it does not illustrate that natural soil populations may acquire degradative capacity by exposure to the herbicide.

Neither the mecoprop enriched community (Section 5.1) nor the MCPA enriched community (Section 5.2) was able to utilize 2,4,5-T after prolonged incubation periods. However, both of the enriched communities did degrade the structurally related herbicides 2,4-D (or more precisely, the MCPA community dehalogenated the 2,4-D). It was apparent that the cross adaptation phenomena did not extend to 2,4,5-T. This showed the inherent stability of the covalent carbon-chloride bond at the five position to the phenoxy moiety.

MCPA is widely used agriculturally for the control of some broad-leaved weeds in cereal crops. Following its introduction in the 1940's there have been numerous reports of its biodegradation by soil micro-organisms (Audus, 1951; Steenson & Walker, 1956, 1957; Loos et al. 1967a) including the elucidation of the degradative pathway (Section 1.2.3). A rhizosphere community of six bacterial isolates and one fungus was able to utilize MCPA as the sole carbon and energy source (Section 5.). Investigations of the MCPA community revealed that it had several features in common with the mecoprop-degrading community, which included:

(a) all of the bacterial isolates were Gram-negative, a characteristic of rhizosphere microbial inhabitants (Section 1.1.3),

(b) none of the purified community constituents could utilize the herbicide on which they were enriched, that is either MCPA or mecoprop respectively,

(c) the complexity of the two communities was not reduced despite long-term chemostat cultivation, and

(d) no mecoprop or MCPA degradation was noted when the initial carbon source concentration was $0.5 gCl^{-1}$.

It seems probable that, as with mecoprop, low initial concentrations of MCPA were required before enrichment of degrading organisms occurred,

(Lappin et al. 1983).

Few degradation studies have been undertaken using the herbicide dichlorprop despite its wide field applications (Kirkland & Fryer, 1972; Kilpi, 1980). The chemical structure of dichlorprop is analogous to mecoprop and 2,4-D, that is, there are two chlorine atoms substituted on the aromatic ring, and a three carbon sidechain to the phenoxy group.

The microbial community enriched to degrade mecoprop could also degrade dichlorprop. A lag phase of 18h was noted before growth commenced, suggesting that prolonged adaptation periods were not required before degradation occurred (Fig 5.4). It is possible that similar enzyme systems may degrade both mecoprop and dichlorprop because of their structural similarity.

The microbial degradation of catechol via the ortho and meta pathways is well documented (Feist & Hegeman, 1969; Franklin et al. 1981; Higgins & Burns, 1975) and the enzyme systems involving both catechol-1,2-dioxygenase and catechol-2,3-dioxygenase are understood. The substrate specificities of the catechol dioxygenases were investigated under different growth conditions (Kilpi et al. 1983). A Pseudomonas species HV3 was used as it could degrade MCPA, 2,4,-D, 3- and 4-chlorobenzoic acids as well as several unchlorinated aromatic compounds. Kilpi et al. (1983) reported that both catechol-2,3-dioxygenase and 1,2-dioxygenase were induced by benzoic, 4-chlorobenzoic and salicyclic acid, illustrating that the strain HV3 was able to use both the ortho and meta pathways. However, only catechol-1,2-dioxygenase was present when HV3 grew on MCPA, 2,4-D or 3-chlorobenzoic acids.

The mecoprop-degrading community was able to rapidly utilize catechol (Section 5.3). Although the mecoprop degradation pathway has yet to be elucidated, the possibility exists that a catechol-like compound occurs as an intermediate in the pathway. Assay procedures undertaken to determine

the presence of dihydroxy aromatic compounds (Section 5.3) failed to detect any such compounds in the samples studied. However, only a few samples were taken throughout the community's growth cycle on mecoprop, and if dihydroxy compounds were formed their appearance as intermediates would be brief before conversion to another intermediate. The assay procedures were probably not undertaken at the precise time that the dihydroxy aromatic compound was present in the growth medium.

Chlorocatechols were detected as intermediates in the degradation of chlorobenzene (Reineke & Knackmuss, 1984), 3-chloro, 4-chloro and 3,5-dichlorobenzoate metabolism (Hartman et al. 1979). Pseudomonas species WR912 was obtained from chemostat cultures growing on a succession of chlorinated carbon sources (Hartman et al. 1979). A degradation pathway for several chlorobenzoates was proposed based upon enzyme activities. Anaerobic dehalogenation of halobenzoates have recently been studied. High performance liquid chromatography allowed the sequencing of intermediates (Horowitz et al. 1983). Michaelis-Menten equations were used to predict accumulation of intermediates in chlorobenzoate degradation (Sulflita et al. 1983). However, despite reports suggesting that chlorobenzoate degradative capabilities do exist in some soil and sediment organisms, the mecoprop-utilizing rhizosphere community was not able to degrade 2-chloro, 3-chloro, or 4-chlorobenzoates.

None of the purified community constituents was able to utilize MCPA, 2,4,5-T or dichlorprop (Section 5.4). As mecoprop degradation required two or more community constituents to generate the necessary catabolic capacity (Section 4.2), combinations of organisms may have been successful at degrading some of the structurally related herbicides.

Both Pseudomonas maltophilia and Pseudomonas species HL1 were capable of utilizing 2,4-D as the sole carbon and energy source, although growth was erratic and reproducible growth responses were not observed.

Pseudomonas maltophilia had a growth factor requirement for L-methionine (Section 4.1). Overnight growth on nutrient broth prior to inoculation into 2,4-D media should have satisfied the amino acid requirement and enabled Pseudomonas maltophilia to utilize the herbicide as the sole carbon and energy source. An explanation for the failure of Pseudomonas maltophilia to grow on 2,4-D was that any residual growth factors required by the organism in the nutrient broth decreased with an increase in incubation period, so making growth less likely.

In conclusion, it is a common agricultural practice to apply combinations of phenoxy herbicides to maximize the efficiency of weed killing (Section 7), so several different phenoxy herbicides may be present in the soil at any one instant. Therefore, the importance of microbial cross adaptation to these herbicides can be appreciated.

CHAPTER SIX

OXYGEN CONSUMPTION AND MIXED SUBSTRATE UTILIZATION BY THE MECOPROP-DEGRADING COMMUNITY

Two of the mecoprop community constituents, Pseudomonas maltophilia and Pseudomonas species HL1, were able to degrade 2,4 - D, but growth on the other phenoxy herbicides was not observed (Section 5.4). In order to calculate and compare specific growth rates and culture doubling times, the community members were grown on a variety of carbon sources at different temperatures, then in the presence of mecoprop with a co-substrate.

The rates of oxygen consumption by the mecoprop community and several community constituents were calculated and compared.

6.1 GROWTH OF THE COMMUNITY CONSTITUENTS ON A RANGE OF CARBON SOURCES AT 25°C AND 30°C

Experiments undertaken in conjunction with the Torry Research Station, Aberdeen, indicated that the community constituents were unable to grow on glucose at 30°C (Table 4.1). Although a temperature profile for mecoprop degradation was not carried out, it was observed that when temperatures exceeded 25°C in an uncooled incubator that higher temperatures were not conducive to growth. Several growth studies using easily metabolized carbon sources were repeated at the lower temperature of 25°C.

In general, heterotrophs are able to metabolize glucose via the Embden-Meyerhof pathway to pyruvate with the subsequent production of ATP (Stanier et al. 1977). Glucose metabolism by the community constituents was followed after overnight growth in nutrient broth (Section 2.1.4). Minimal medium (Section 2.1.1) was supplemented with $0.25 g Cl^{-1}$ glucose and the batch cultures were incubated at 25°C. The

culture absorbance (Section 2.4.1) was monitored at suitable time intervals.

In all cases growth was completed within 500 mins. Lag phases before growth commenced were of various duration, that is, 190-315 mins for Pseudomonas species HL1 (Fig. 6.1a), Alcaligenes species HL3 (Fig. 6.1b) and Flavobacterium species HL5 (Fig. 6.1c). The growth responses of Pseudomonas maltophilia and Acinetobacter calcoaceticus were less prolific (Fig. 6.2a & b), that is, the culture absorbance increased little from 0.055 to 0.08 for Pseudomonas maltophilia and from 0.07 to 0.11 in the Acinetobacter calcoaceticus batch culture.

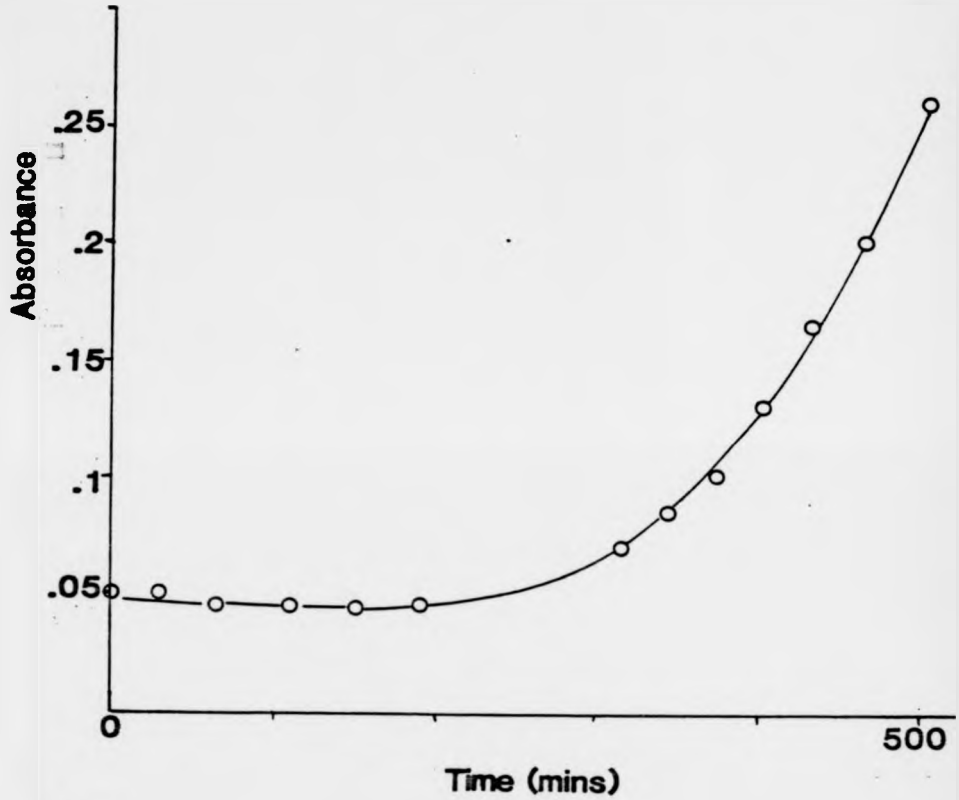
Succinate, a four carbon acid , can feed into the tricarboxylic acid cycle to generate energy (Stanier et al. 1977). Four of the mecoprop community members were able to utilize succinate as the sole carbon and energy source at 25°C. Culture absorbance increases from between 0.055 - 0.085 to 0.21 - 0.285 were recorded for the four community constituents within a 500 min incubation period (Fig. 6.3 and 6.4). The specific growth rates (Section 2.3.4) and culture doubling times (Section 2.3.5) were calculated and compared to the values of the same organisms growing on glucose (Table 6.1). The highest growth rate on glucose was for Pseudomonas species HL1 and the lowest was Flavobacterium species HL5, whereas for succinate the highest growth rate was Alcaligenes species HL3, the lowest being for Pseudomonas maltophilia.

The culture doubling times varied greatly depending on the organism and carbon source. For example, Pseudomonas maltophilia and Pseudomonas species HL1 required longer to double in population size when grown on succinate than on glucose. The doubling time on glucose was more than 5h for both Flavobacterium species and Acinetobacter calcoaceticus, but only 1.61h for Pseudomonas species HL1.

Fig. 6.1a Growth of Pseudomonas species HL1 on glucose.

Fig. 6.1b Growth of Alcaligenes species HL3 on glucose.

a)


Absorbance
.25
.2
.15
.1
.05
0
500
Time (mins)

b)

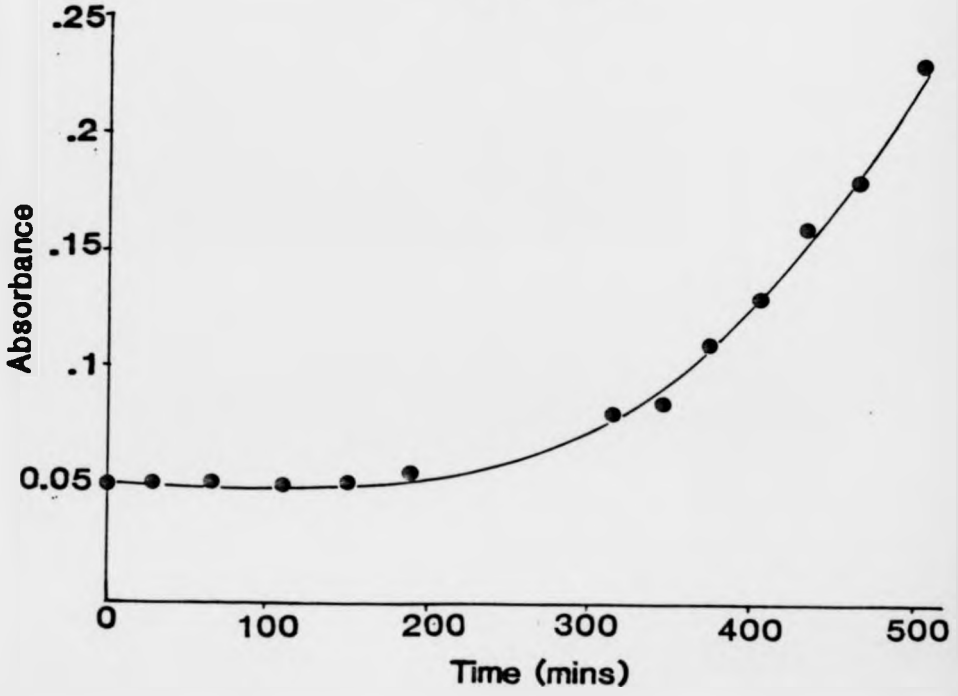

Absorbance
.25
.2
.15
.1
0.05
0
100
200
300
400
500
Time (mins)

Fig. 6.1c Growth of Flavobacterium species HL5 on glucose.

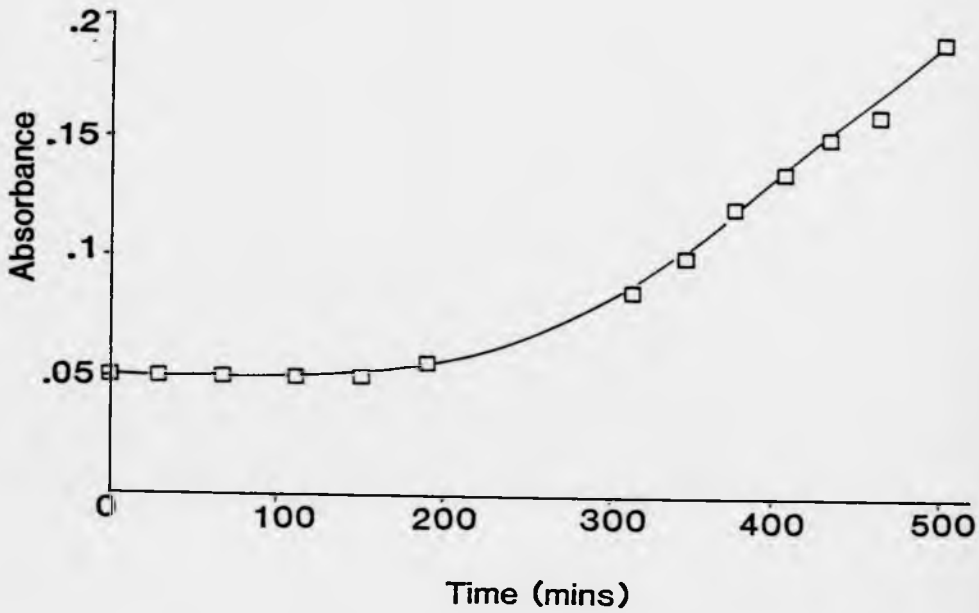
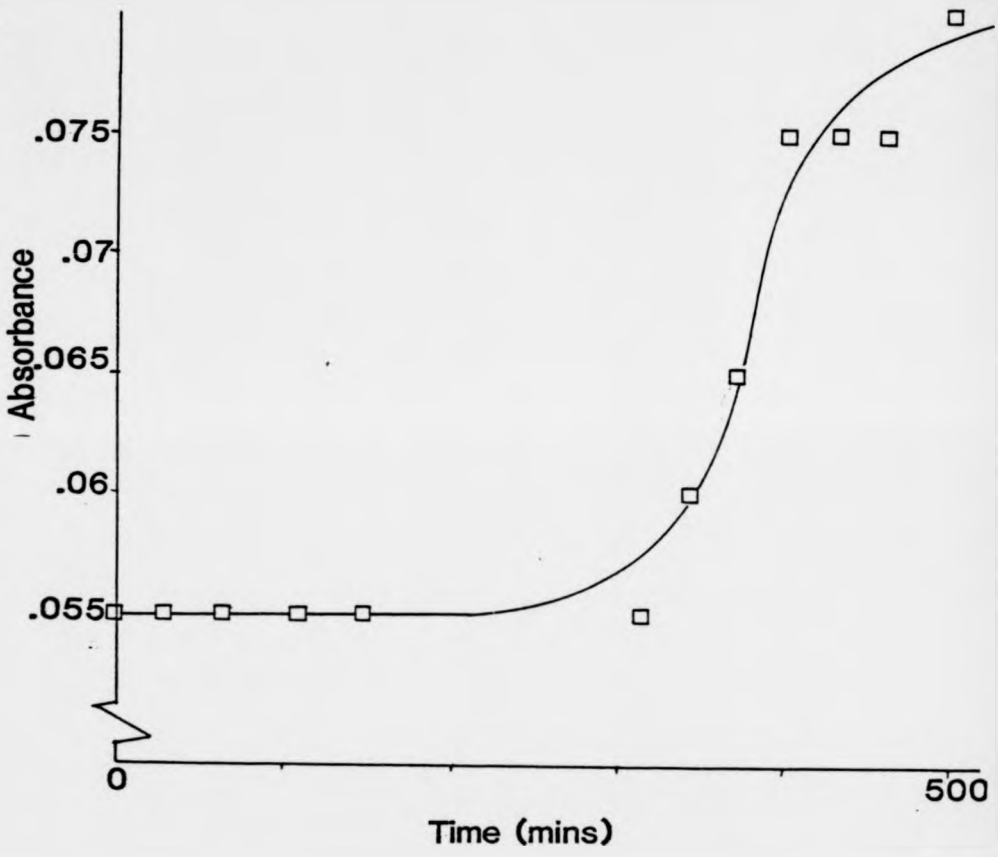

.2
.15
.1
.05
Absorbance
0
100
200
300
400
500
Time (mins)

Fig. 6.2a Growth of Pseudomonas maltophilia on glucose.

Fig. 6.2b Growth of Acinetobacter calcoaceticus on glucose.

a)


.075
.07
.065
.06
.055
Absorbance
0
500
Time (mins)

b)

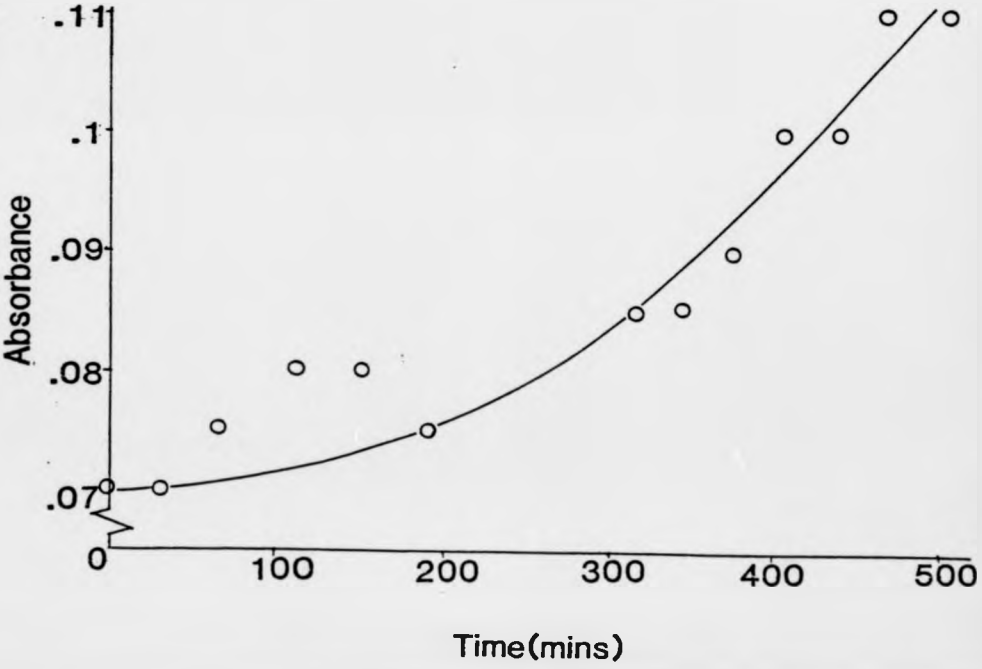
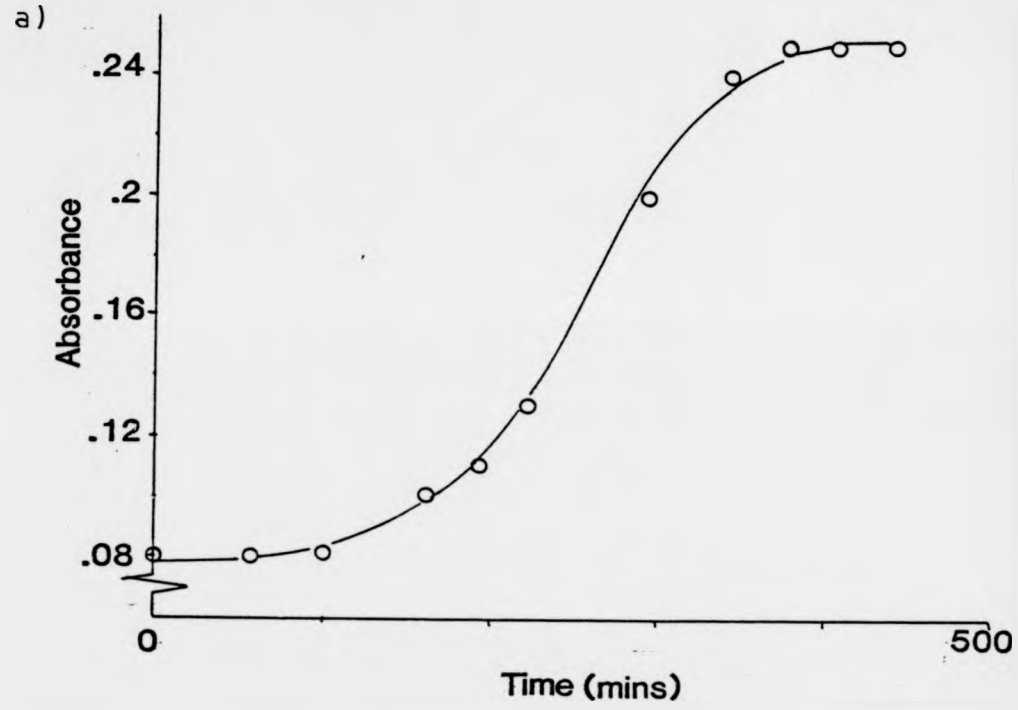

.11
.1
.09
.08
.07
Absorbance
0
100
200
300
400
500
Time(mins)

Fig. 6.3a Growth of Pseudomonas species HL1 on succinate.

Fig. 6.3b Growth of Pseudomonas maltophilia on succinate.


a)
.24
.2
.16
.12
.08
Absorbance
0
500
Time (mins)

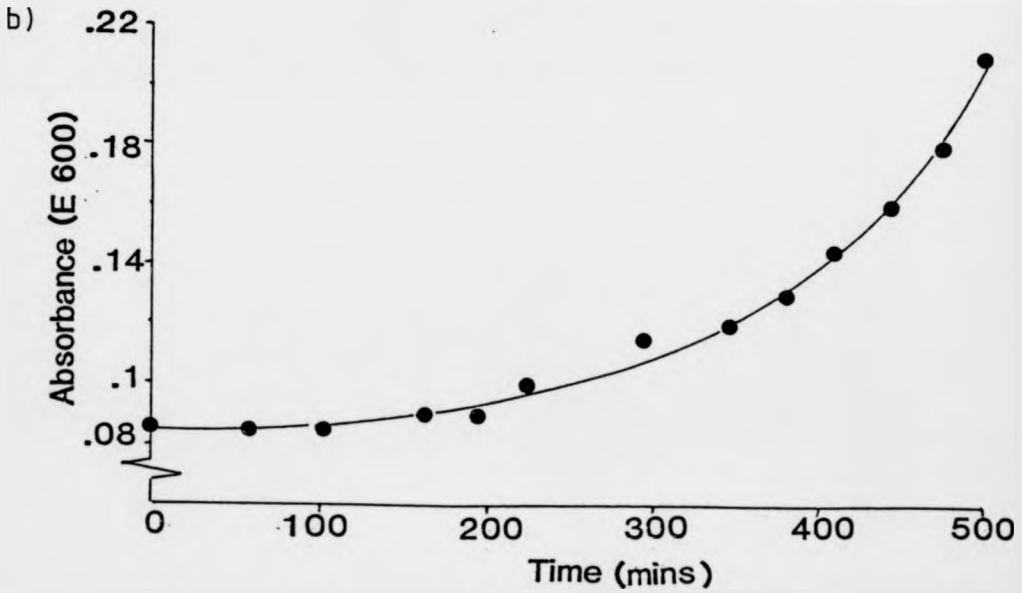
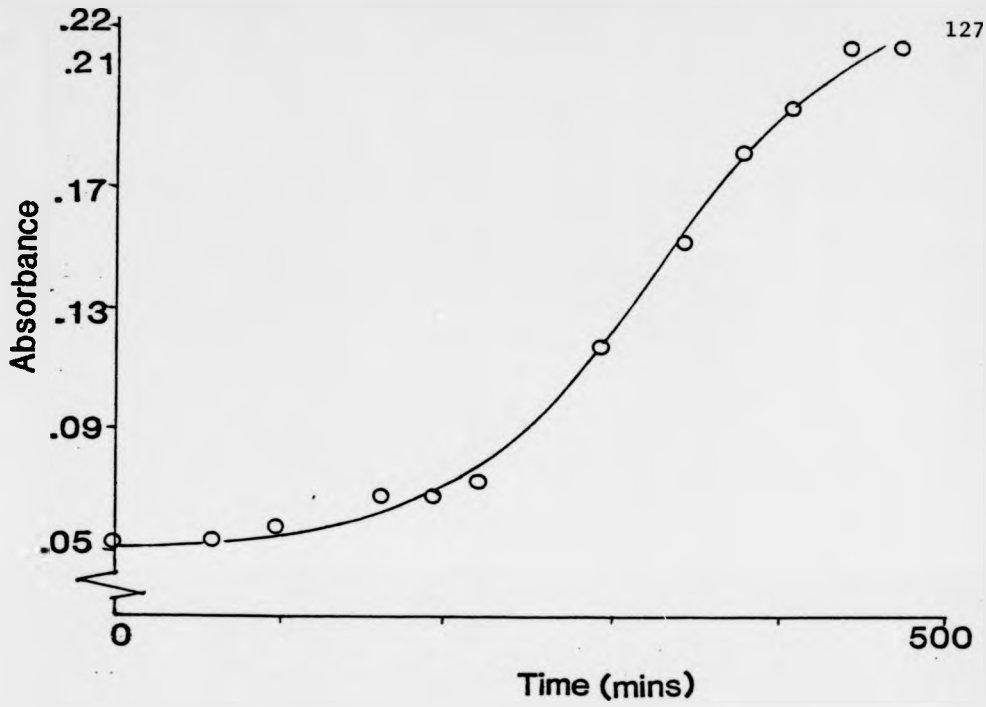

b)
.22
.18
.14
.1
.08
Absorbance (E 600)
0
100
200
300
400
500
Time (mins)

Fig. 6.4a Growth of Alcaligenes species HL3 on succinate.

Fig. 6.4b Growth of Acinetobacter calcoaceticus on succinate.

a)


.22
.21
.17
.13
.09
.05
Absorbance
0
500
Time (mins)

b)

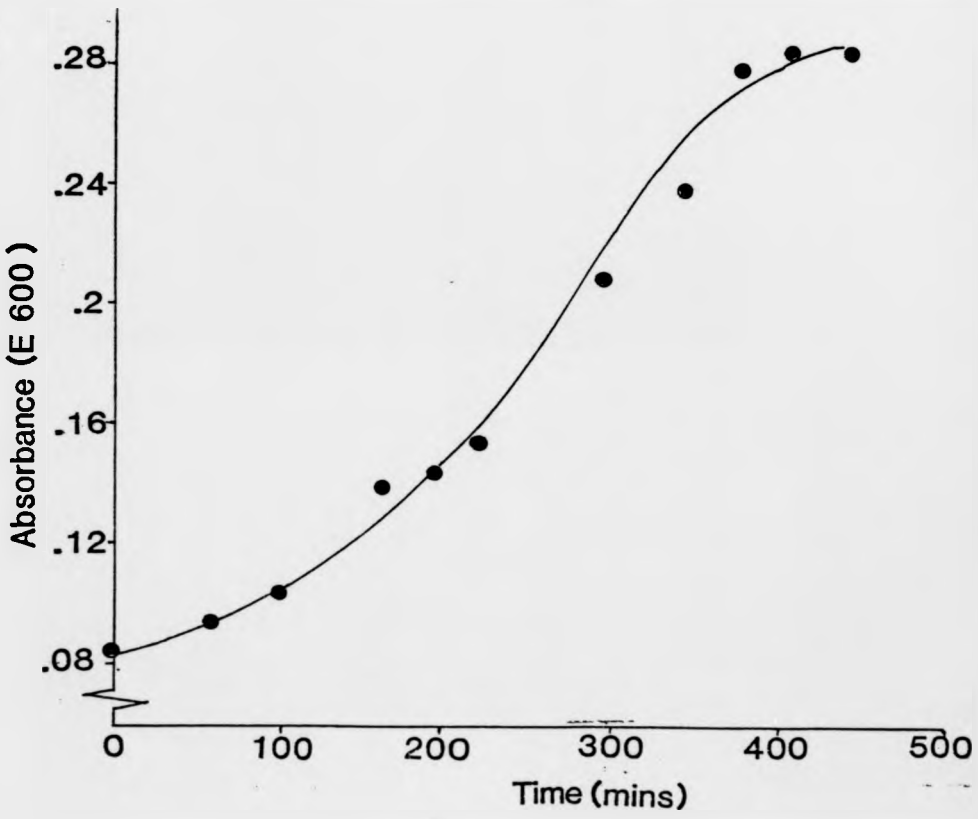

.28
.24
.2
.16
.12
.08
Absorbance (E 600)
0
100
200
300
400
500
Time (mins)

TABLE 6.1 SPECIFIC GROWTH RATES (h^{-1}) AND CULTURE DOUBLING TIMES (h) FOR THE COMMUNITY CONSTITUENTS GROWING ON GLUCOSE AND SUCCINATE

Carbon source	*Pseudomonas* species HL1		*Pseudomonas maltophilia*		*Alcaligenes* species HL3		*Acinetobacter calcoaceticus*		*Flavobacterium* species	
	μ	t_d	μ	t_d	μ	t_d	μ	t_d	μ	t_d
Glucose	0.43	1.61	0.32	2.19	0.28	2.48	0.14	5.02	0.13	5.17
Succinate	0.33	2.10	0.14	4.85	0.34	2.03	0.21	3.33	n.d.	

- not done

Carbon source profile. The growth characteristics of the mecoprop community members were examined using a variety of carbon sources. The presence or absence of growth was monitored by following changes in the culture absorbance (Section 2.4.1). Isolates were grown overnight in nutrient broth prior to inoculation (2% v/v) into minimal medium containing $0.25gCl^{-1}$ of either benzoate, acetate or lactate. The culture absorbance was measured at inoculation and again after three days of incubation at 25°C.

The community members were not able to grow on all of the different carbon sources. For example, sodium acetate and lactate were only utilized by two or three isolates at 25°C (Table 6.2). Alternatively, benzoic acid was utilized as the sole carbon and energy source by all five bacterial isolates at 25°C (Table 6.2).

Comparison of growth responses to the same carbon source at the different incubation temperatures of 30°C and 25°C demonstrated that growth was temperature related (Table 6.2). The Pseudomonas species was able to grow on both acetate and lactate at both temperatures, but Pseudomonas maltophilia was not able to utilize either carbon source unless L-methionine was present (Section 4.1). Acetate was able to support growth of Alcaligenes species at 25°C and 30°C but the organism was not able to degrade lactate at the lower temperature (Table 6.2). Acinetobacter calcoaceticus degraded lactate at both temperatures, however acetate was only utilized at 30°C. Finally, Flavobacterium species HL5 grew on lactate at 25°C but not 30°C, acetate could not support growth at either temperature.

TABLE 6.2 GROWTH CHARACTERISTICS OF THE COMMUNITY CONSTITUENTS ON SEVERAL CARBON SOURCES AT 25°C AND 30°C

Carbon source	*Pseudomonas* species HL1		*Pseudomonas maltophilia*		*Alcaligenes* species HL3		*Acinetobacter calcoaceticus*		*Flavobacterium* species HL5	
	25°	30°	25°	30°	25°	30°	25°	30°	25°	30°
Benzoate	+	n.d.	+	nd	+	n.d.	+	n.d.	+	nd
Acetate	+	+	-	-	+	+	-	+	-	-
Lactate	+	+	-	+ in presence of L-methionine	-	+	+	+	+	-

+; denotes increases in culture absorbance after three days incubation

-; denotes no increase in culture absorbance after three days incubation

n.d.; not done

6.2 MIXED SUBSTRATE UTILIZATION BY THE MECOPROP DEGRADING COMMUNITY AND COMMUNITY CONSTITUENTS

Mecoprop community. The growth response of the mecoprop-degrading community to a mixture of carbon sources was investigated using succinate and mecoprop as mixed substrates. After growth on $0.25gCl^{-1}$ mecoprop in minimal medium (Section 2.1.1), the community was inoculated into 100 ml minimal medium with $0.25gCl^{-1}$ of both mecoprop and succinate. Incubation was at 25°C, culture absorbance (Section 2.4.1) and chloride ion release (Section 2.4.2) were monitored at suitable time intervals.

The results suggested that the mecoprop was degraded after succinate had first supported growth, but detailed information was not available, so the time intervals between readings were decreased. A distinct growth pattern was observed, comprising of two phases of community growth. In the first phase the culture absorbance increased from 0.035 to 0.22 in 23h (Fig. 6.5). No chloride ion release was detected during this period, demonstrating that the unchlorinated carbon source, that is succinate supported initial growth. After a lag phase of approximately 5h when no change in culture absorbance was noted, a second growth phase commenced. Increases in culture absorbance from 0.22 to 0.36 and chloride ion release from zero to 1.61 µmoles ml^{-1} were then recorded. This implied that mecoprop was degraded after growth on succinate was complete (Fig. 6.5).

The study was repeated using sodium acetate and mecoprop as co-substrates for the mecoprop-degrading community. Again, a two phase growth pattern emerged when culture absorbance increased from 0.065 to 0.2 in 24h, but with no subsequent chloride ion release (Fig. 6.6). A lag phase of 6-7h followed before a second growth phase was detected when chloride ions were released and the culture absorbance increased

Fig. 6.5 Mixed substrate utilization by the mecoprop-degrading community growing on succinate and mecoprop. O, culture absorbance; ●, chloride ion release.

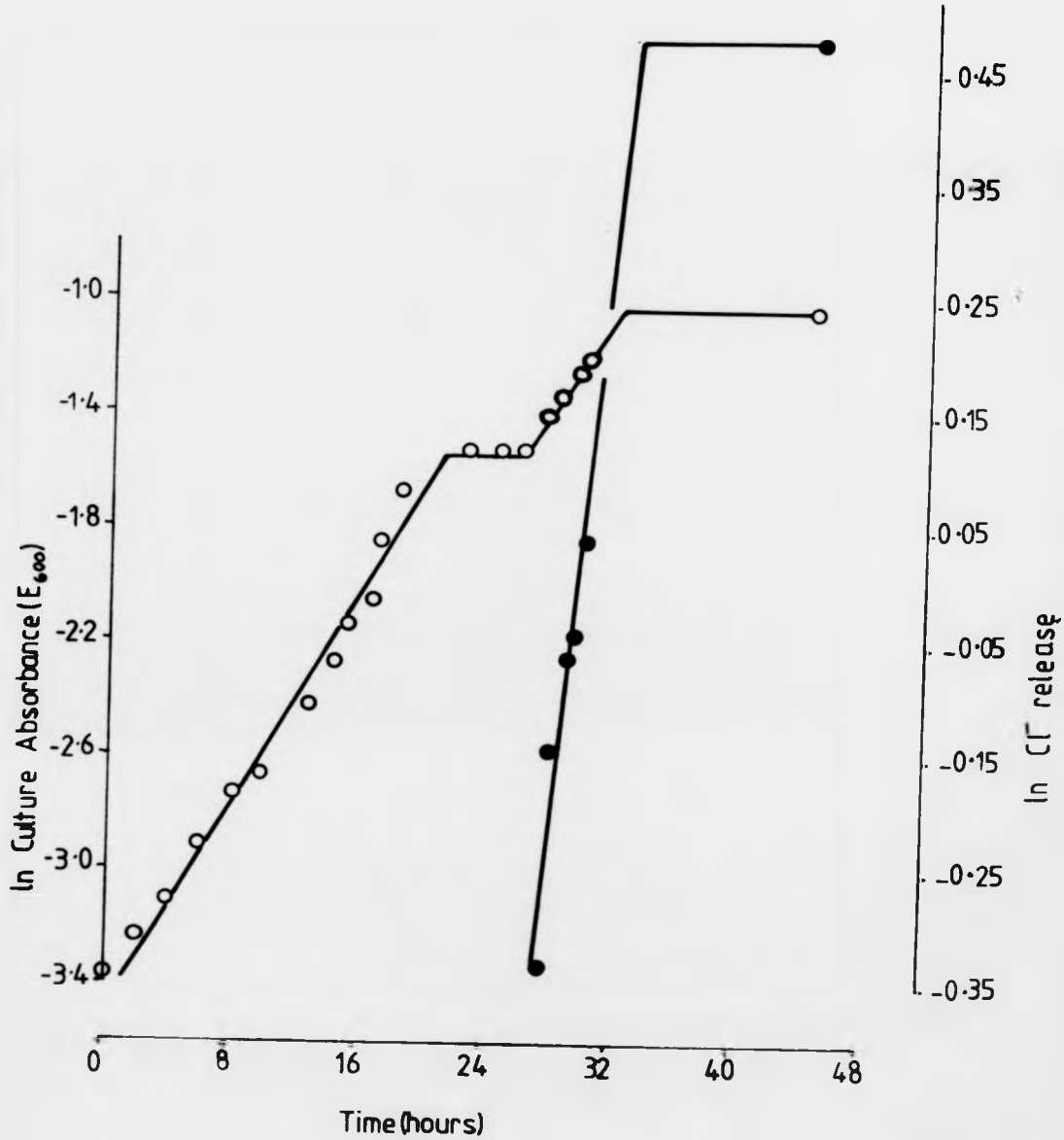
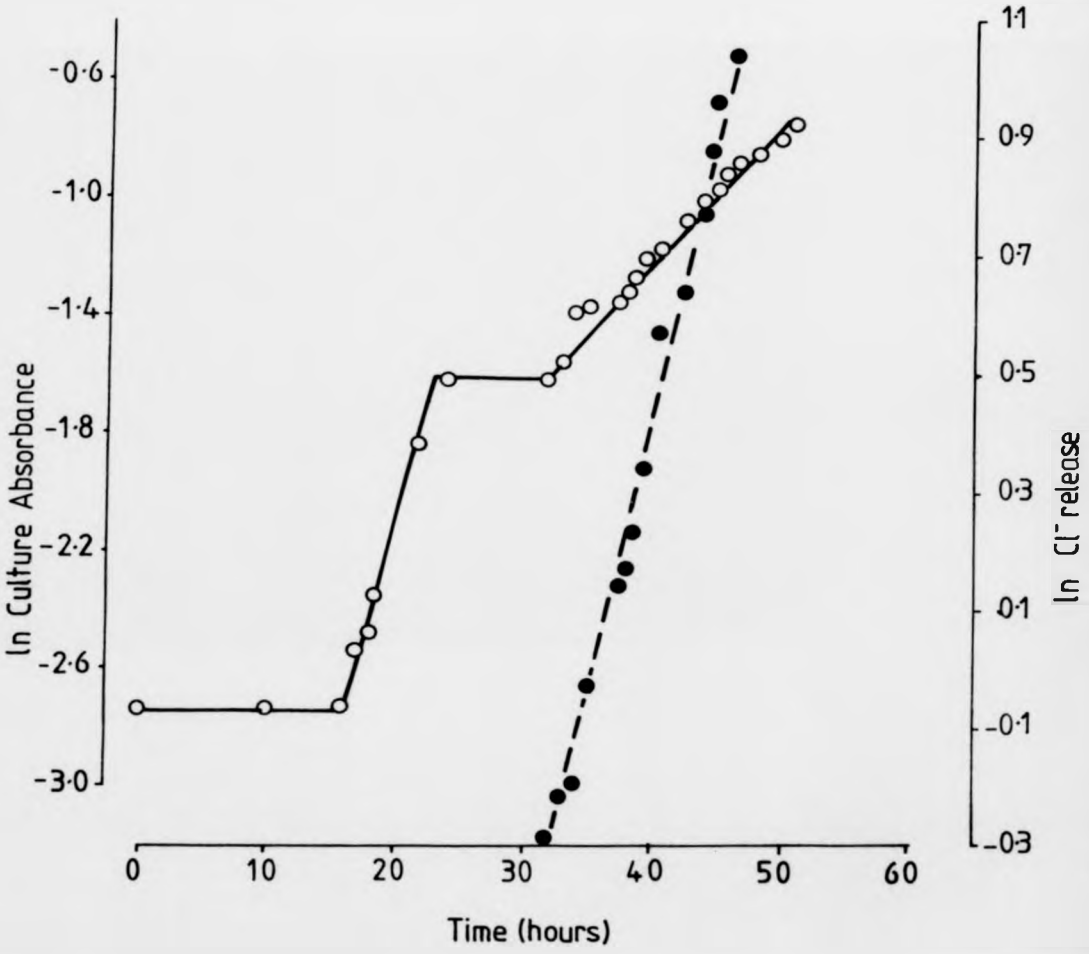

-1·0
-1·4
-1·8
-2·2
-2·6
-3·0
-3·4
ln Culture Absorbance (E_{600})
0·45
0·35
0·25
0·15
0·05
-0·05
-0·15
-0·25
-0·35
ln Cl⁻ release
0
8
16
24
32
40
48
Time (hours)

Fig. 6.6 Mixed substrate utilization by the mecoprop-degrading community growing on sodium acetate and mecoprop. O, culture absorbance; ●, chloride ion release.


-0·6
-1·0
-1·4
-1·8
-2·2
-2·6
-3·0
ln Culture Absorbance
1·1
0·9
0·7
0·5
0·3
0·1
-0·1
-0·3
ln Cl⁻ release
0
10
20
30
40
50
60
Time (hours)

from 0.2 to 0.5. This indicated that mecoprop degradation occurred in the second growth phase after initial community growth on sodium acetate.

The specific growth rates and culture doubling times for the two growth phases of the mecoprop community on mecoprop and succinate and mecoprop and sodium acetate were calculated (Table 6.3).

Community constituents. None of the mecoprop-degrading community members were able to degrade mecoprop as the sole carbon and energy source (Section 4.2). However, the responses of the constituents to mecoprop as a co-substrate in liquid culture were not known. To examine the ability of the five members to degrade mecoprop in the presence of a chemically simpler and more readily utilizable substrate, investigations using succinate or benzoate and mecoprop as a second carbon source were undertaken.

The five bacterial isolates were grown overnight in nutrient broth prior to inoculation (1% v/v) into minimal medium supplemented with $0.25gCl^{-1}$ of both succinate and mecoprop. Culture absorbance (Section 2.4.1) and chloride ion release (Section 2.4.2) were assayed over an 85h incubation period. Background chloride ion content was high due to transfer from nutrient broth.

In the batch enrichment culture containing Flavobacterium species the culture absorbance increased from 0.04 to 0.19 in 21h, but no other increases in culture absorbance or chloride ion release were detected despite a further 40h incubation (Fig, 6.7a). Three of the community constituents, that is, Pseudomonas maltophilia, Pseudomonas species and Alcaligenes species, were able to degrade mecoprop after first growing on succinate. Growth by Pseudomonas maltophilia on succinate was prolific, the culture absorbance increased from 0.03 to 0.2 after 15h growth, with no corresponding increases in chloride ion release (Fig. 6.7b). After a lag period of 48h a second growth phase commenced when

TABLE 6.3 SPECIFIC GROWTH RATE AND CULTURE DOUBLING TIMES FOR THE TWO GROWTH PHASES OF THE MECOPROP COMMUNITY GROWING ON MECOPROP AND SUCCINATE OR MECOPROP AND SODIUM ACETATE

Growth Phase	Specific Growth Rate (h^{-1})	Culture Doubling Time (h)
Succinate Phase	0.09	7.45
Mecoprop Phase	0.08	8.88
Sodium acetate Phase	0.14	4.91
Mecoprop Phase	0.04	15.75

Fig. 6.7a Growth of *Flavobacterium* species HL5 on succinate and mecoprop. O, culture absorbance; ●, chloride ion release.

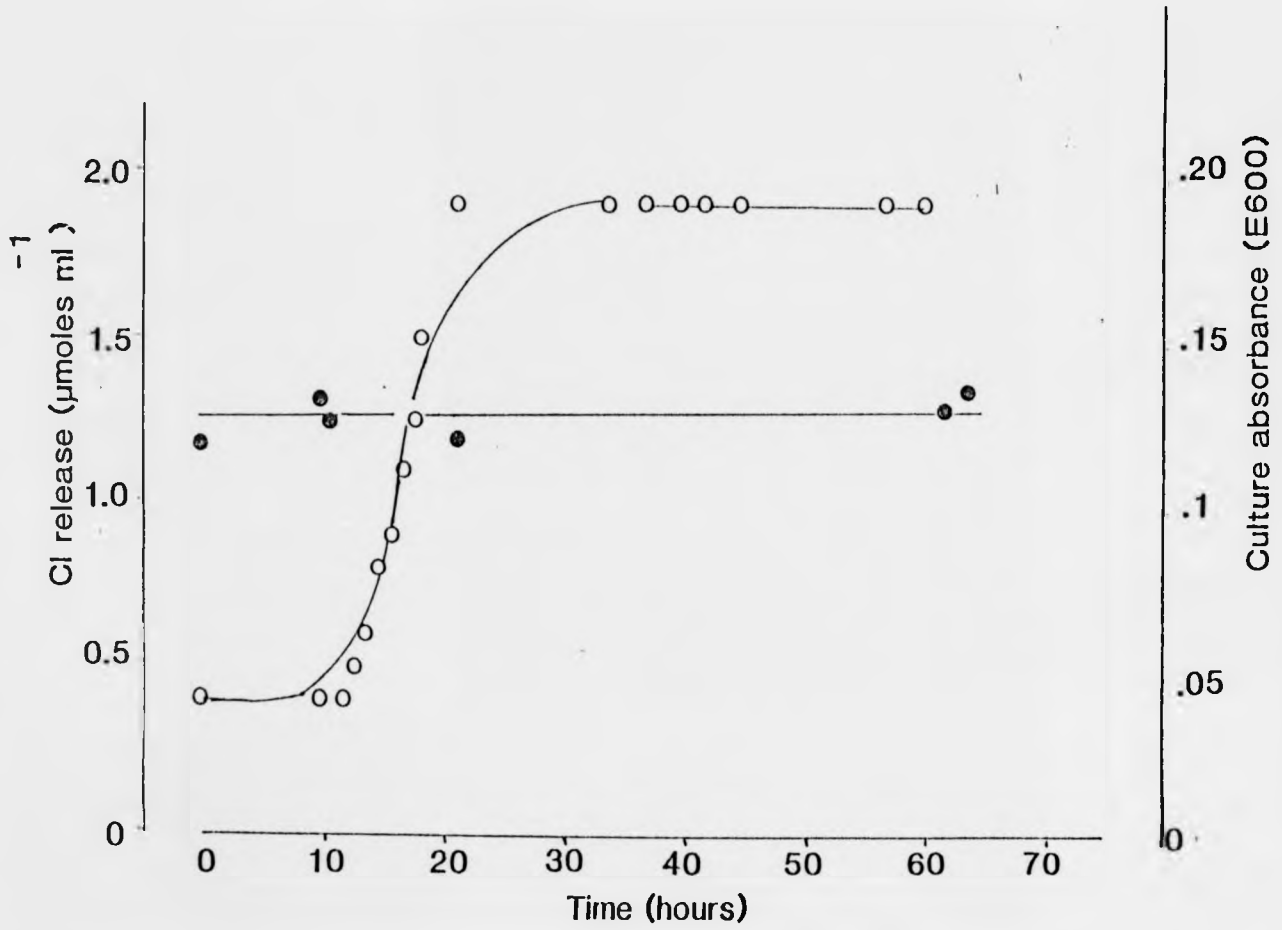
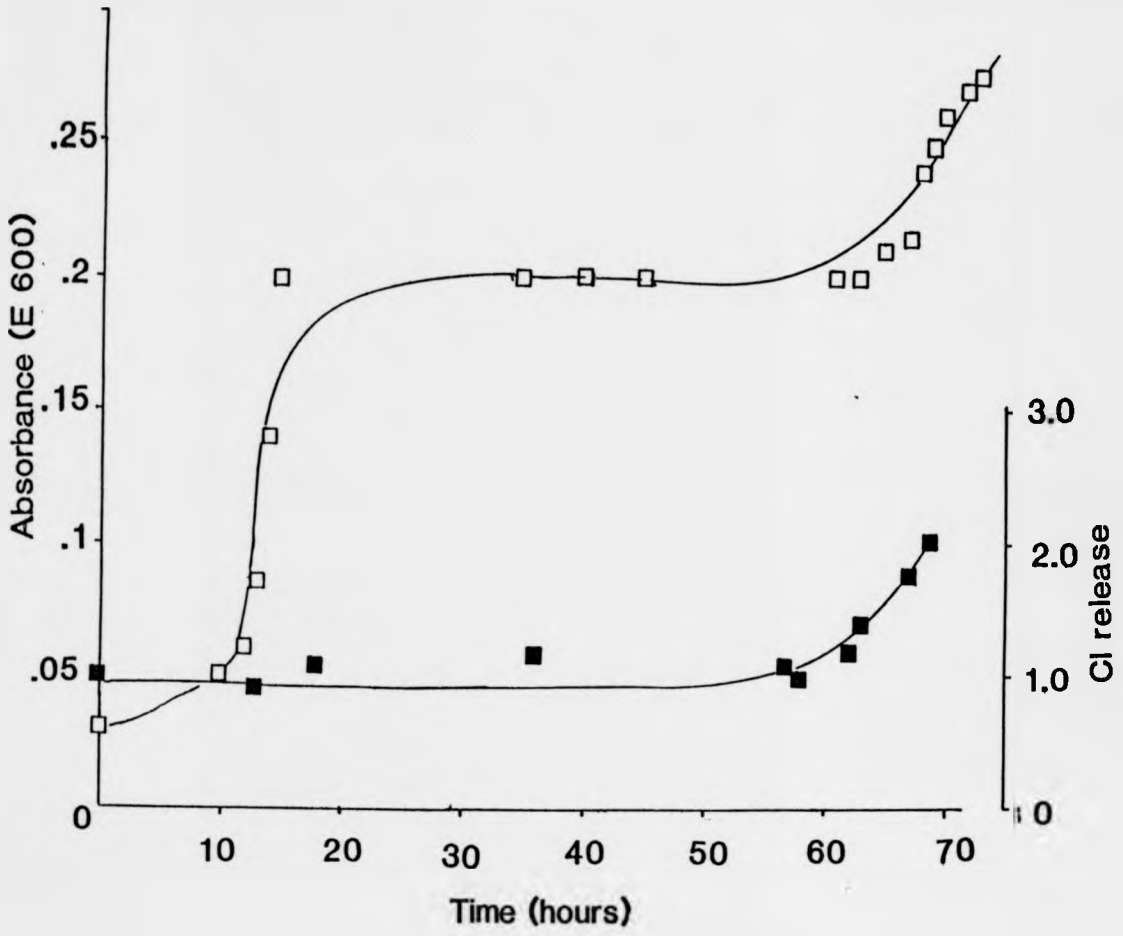

Cl release (µmoles ml -1)
2.0
1.5
1.0
0.5
0
0
10
20
30
40
50
60
70
Time (hours)
.20
.15
.1
.05
0
Culture absorbance (E600)

Fig. 6.7b Growth of Pseudomonas maltophilia on succinate and mecoprop.

□, culture absorbance; ■, chloride ion release.


Absorbance (E 600)
.25
.2
.15
.1
.05
0
10
20
30
40
50
60
70
Time (hours)
3.0
2.0
1.0
0
Cl release

the culture absorbance increased from 0.2 to 0.26 and small chloride ion releases were detected from 1.36 to 2.02 µmoles ml^{-1}.

The two growth phases were more pronounced in Pseudomonas species HL1 (Fig. 6.8a). The first growth phase of Pseudomonas species HL1 involved culture absorbance increases from 0.04 to 0.17 in 16h without corresponding chloride ion release, which indicated that the unchlorinated carbon source, that is succinate, was utilized first. After a lag phase of between 30-40h, mecoprop degradation occurred denoted by chloride ion release (1.77 µmoles ml^{-1}) and culture absorbance increases (Fig. 6.8a). Alcaligenes species produced a similar two phase growth response to the mixed substrates with mecoprop degradation following succinate degradation (Fig. 6.8b).

The specific growth rates and culture doubling times for the two growth phases of Pseudomonas maltophilia, Pseudomonas species and Alcaligenes species were calculated and compared (Table 6.4). Values for the specific growth rates and culture doubling times for Pseudomonas species and Pseudomonas maltophilia were substantially lower for growth on mecoprop than on succinate (Table 6.4) showing that growth proceeded at a much slower rate on mecoprop. Data for Acinetobacter calcoaceticus on succinate and mecoprop was not available as initial growth on nutrient broth was not successful.

The experimental procedure for mixed substrate studies with succinate and mecoprop was repeated using benzoate and mecoprop as carbon sources for the community constituents.

Detailed studies to produce growth curves were not undertaken, so growth phases were not pronounced. In the batch enrichment culture containing Pseudomonas species HL1 the culture absorbance increased from 0.05 to 0.56 in 90h. Between 26 and 46h incubation chloride ion release commenced and the total production reached 1.10 µmoles ml^{-1}. Acinetobacter

Fig. 6.8a Growth of Pseudomonas species HL1 on succinate and mecoprop. O, culture absorbance; ●, chloride ion release.

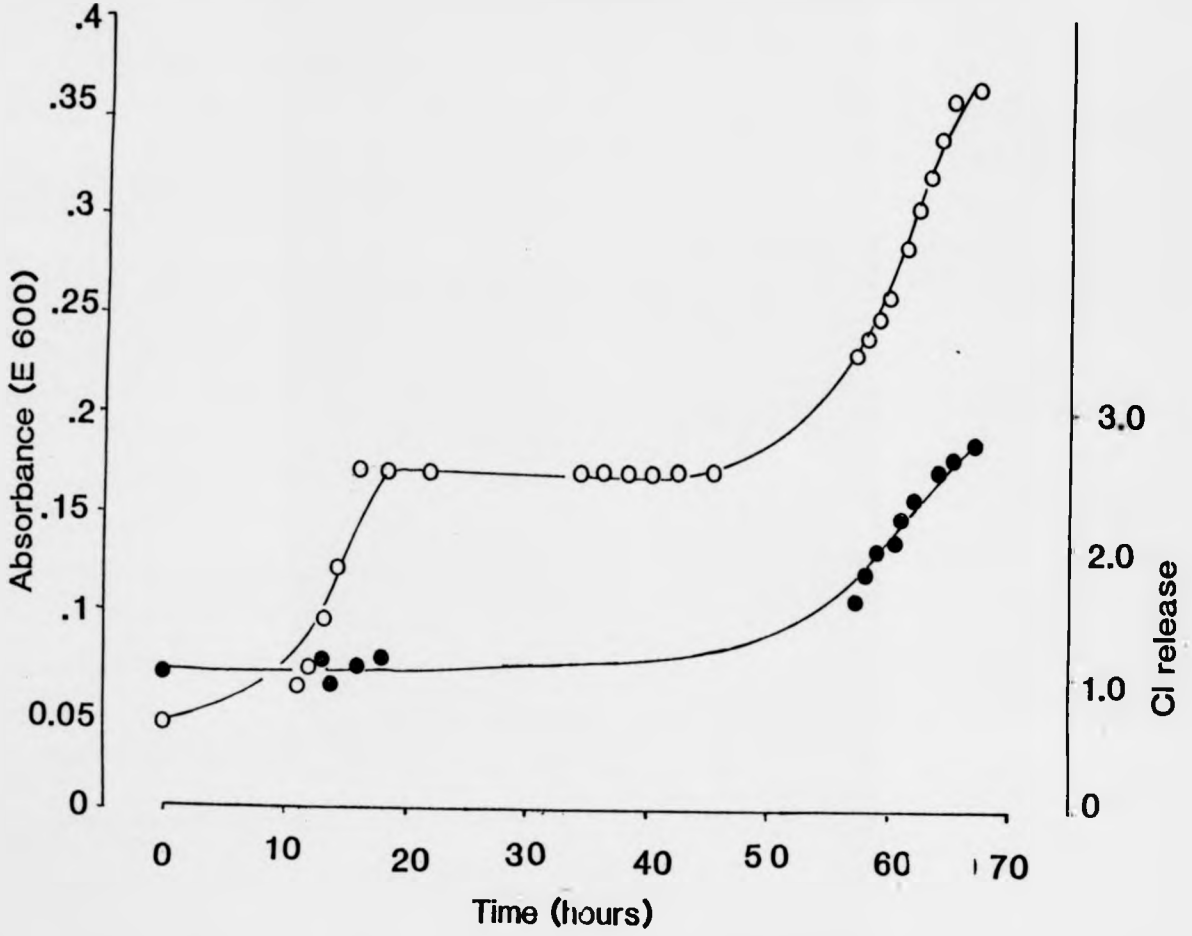

.4
.35
.3
.25
.2
.15
.1
0.05
0
Absorbance (E 600)
3.0
2.0
1.0
0
Cl release
0
10
20
30
40
50
60
70
Time (hours)

Fig. 6.8b Growth of *Alcaligenes* species HL3 on succinate and mecoprop. □, culture absorbance; ■, chloride ion release.

Absorbance
0
0.1
.2
.3
.4
Time (hours)
0
10
20
30
40
50
60
70
Chloride ion release (μmoles ml⁻¹)
1.0
2.0
3.0

TABLE 6.4 SPECIFIC GROWTH RATES AND CULTURE DOUBLING TIMES FOR THE TWO GROWTH PHASES OF THREE COMMUNITY CONSTITUENTS GROWING ON SUCCINATE AND MECOPROP.

Growth Phase	Pseudomonas species		Pseudomonas maltophilia		Alcaligenes species	
	$\mu(h^{-1})$	$t_d(h)$	$\mu(h^{-1})$	$t_d(h)$	$\mu(h^{-1})$	$t_d(h)$
Succinate growth phase	0.22	3.15	0.11	6.30	0.04	19.80
Mecoprop growth phase	0.06	12.16	0.04	19.80	0.08	8.77

calcoaceticus produced culture absorbance increases from 0.06 to 0.4 in 90h. After 50h, chloride ion release was detected and increased gradually over the next 40h, producing 1.60 µmoles ml^{-1}.

In both Pseudomonas maltophilia and Alcaligenes species HL3 batch cultures the culture absorbance increased from 0.04-0.05 to between 0.47 and 0.55. The values are higher than those previously reported for the growth of the two organisms on 0.25gCl^{-1} benzoate alone. However, chloride ion release was not detected in the batch flasks containing either organism.

6.3 MECOPROP COMMUNITY OXYGEN CONSUMPTION USING VARIOUS CARBON SOURCES

The microbial community was grown in a two - litre conical flask containing 400 ml minimal medium (Section 2.1.1) and 0.25gCl^{-1} mecoprop. After 35h growth the chloride ion release (Section 2.4.2) indicated that mecoprop degradation was complete. The cells were harvested (Section 2.7.2) resuspended in 0.1M-phosphate buffer pH 7.0 (Section 2.7.2). The oxygen electrode was prepared and calibrated while the culture was aerated (Section 2.7.2).

The oxygen consumption basal rate was recorded prior to injection of each carbon source. The oxygen required to metabolize each different carbon source was noted before the oxygen electrode was emptied and cleaned. The experimental procedure was repeated using 2,4 - D mecoprop, MCPA, catechol and dichlorprop, and the oxygen consumption by the microbial community was calculated. The basal rate of consumption represented the oxygen uptake required for the basic metabolic functions, such as cell maintenance and ionic transport. The oxygen uptake in the presence of a carbon source indicated that oxygen-requiring biological reactions, such as carbon metabolism, occurred. The rate of increase in oxygen consumption was determined by dividing the oxygen consumed in the presence of each carbon source by the basal rate (Table 6.5).

TABLE 6.5 OXYGEN CONSUMPTION OF THE MECOPROP-DEGRADING COMMUNITY

Endogenous oxygen uptake ≡ 0.265 µmoles O_2 1 x 10^9 cells^{-1} h^{-1}

Carbon source ($0.25gCl^{-1}$)	Oxygen uptake (µmoles O_2 $1x10^9$ cells^{-1} h^{-1})	Stimulated Increase (calculated by dividing the O_2 uptake by the endogenous uptake rate)
Mecoprop	0.696	2.63
2,4-D	0.561	2.12
MCPA	0.417	1.57
Catechol	1.809	6.83
Dichlorprop	0.991	3.74

The mecoprop-degrading community produced increased rates of oxygen consumption when compared to the basal rate, for all of the carbon sources investigated (Table 6.5). Although the community was grown on mecoprop it could still utilize the structurally related compounds 2,4 - D, MCPA and dichlorprop (Section 5.1 & 5.3) as demonstrated by the oxygen uptake by the community. The rate of oxygen consumption, which is related to the rate of carbon source utilization by the community, differed for the various phenoxy compounds. The microbial community consumed oxygen when two of the phenoxy herbicides, 2,4 - D and MCPA, were injected separately into the oxygen chamber, but the rate of uptake was less than the rate for mecoprop, whereas the rate of oxygen consumption for dichlorprop was higher (Table 6.5).

Catechol, a dihydroxy substituted benzene ring commonly encountered as an intermediate in phenoxy herbicide degradation (Section 1.2.3) had the highest rate of oxygen uptake, indicating its rapid utilization by the community (Table 6.5).

6.4 OXYGEN CONSUMPTION OF SEVERAL MECOPROP COMMUNITY MEMBERS

The pure cultures were grown on minimal medium (Section 2.1.1) and $0.25gCl^{-1}$ succinate and incubated at 25°C until the culture absorbance (Section 2.4.1) measured between 0.15 to 0.2. The cultures were centrifuged, resuspended in 0.1M -phosphate buffer and aerated as previously described (Section 6.3). The oxygen electrode was assembled and calibrated (Section 2.7.1). A variety of carbon sources were investigated to allow the determination of the rate of oxygen uptake by the community members.

After the calculation of the basal consumption rate for Pseudomonas maltophilia succinate, catechol, glucose and mecoprop were introduced separately to the oxygen chamber. When compared to the basal rate increased oxygen consumption was only recorded in the presence of

succinate (Table 6.6). The oxygen uptake for the other carbon sources studied remained at the same level as the basal rate, that is, these carbon sources were not utilized by the succinate-grown Pseudomonas maltophilia.

Increased rates of oxygen uptake were recorded by Alcaligenes species HL3 in the presence of succinate, demonstrating that succinate was utilized by the organism.. No such increased rates were noted in the presence of either glucose or mecoprop (Table 6.6).

The oxygen uptake by Flavobacterium species by mecoprop equalled the basal rate, suggesting that mecoprop was not utilized by this succinate-grown organism. However, succinate was rapidly utilized by Flavobacterium species as reflected in the oxygen uptake (Table 6.6).

The oxygen uptake of succinate was the only carbon source studied for Pseudomonas species. The rate of oxygen consumption in the presence of succinate showed that it was rapidly utilized by this organism (Table 6.6).

A comparison of the rates of increase of consumption for the community constituents studied demonstrated that Flavobacterium species HL5 and Alcaligenes species HL3 utilized succinate more rapidly than Pseudomonas species or Pseudomonas maltophilia (Table 6.6).

6.5 DISCUSSION

The mecoprop-degrading community produced a two-phase growth curve when grown on either (a) succinate and mecoprop; or, (b) sodium acetate and mecoprop. For both growth curves the first growth phase involved culture absorbance increases without corresponding chloride ion release. This demonstrated that despite enrichment on mecoprop, the community utilized the more easily metabolized substrates first. After a short lag phase of 5h for succinate and mecoprop growth curves and between 6-7h for sodium acetate and mecoprop, a second growth phase commenced.

TABLE 6.6 OXYGEN UTILIZATION BY THE MECOPROP COMMUNITY CONSTITUENTS

a. Alcaligenes species HL3

Endogenous rate: 0.209 µmoles O_2 $1x10^9$ $cells^{-1}h^{-1}$

Carbon source	Oxygen uptake µmoles O_2 $1x^9$ $cells^{-1}$ h^{-1}	Stimulated Increase
Succinate	2.77	13.25
Mecoprop	0.209	0
Glucose	0.209	0

b. Flavobacterium species HL5

Endogenous rate: 0.104 µmoles O_2 $1x10^9$ $cells^{-1}h^{-1}$

Carbon source	Oxygen uptake µmoles O_2 $1x10^9$ $cells^{-1}$ h^{-1}	Stimulated Increase
Succinate	2.07	19.90
Mecoprop	0.104	0

c. Pseudomonas species HL1

Endogenous rate: 0.104 µmoles O_2 $1x10^9$ $cells^{-1}h^{-1}$

Carbon source	Oxygen uptake µmoles O_2 $1x10^9$ $cells^{-1}$ h^{-1}	Stimulated Increase
Succinate	0.591	5.68

d. Pseudomonas maltophilia

Endogenous rate: 0.346 µmoles O_2 $1x10^9$ $cells^{-1}h^{-1}$

Carbon source	Oxygen uptake µmoles O_2 $1x10^9$ $cells^{-1}$ h^{-1}	Stimulated Increase
Succinate	0.833	2.41
Catechol	0.346	0
Mecoprop	0.346	0
Glucose	0.346	0

Mecoprop degradation, denoted by simultaneous chloride ion release and culture absorbance increases, was detected in both batch cultures (Fig. 6.5 & 6.6).

An important finding was that in both diauxic growth curves the length of the lag phase before mecoprop degradation commenced was appreciably less than the lag phase duration for community growth on mecoprop as the sole carbon and energy source. Lag phases of approximately 24h were common for the enriched community (Section 3.1). Possible explanations for the shorter lag phases in diauxic growth curves included:

(a) an increase in the cell biomass as a result of initial growth on either succinate or sodium acetate enabled a more rapid attack of mecoprop; or

(b) non-specific enzymes responsible for succinate and sodium acetate degradation were able to catalyze a biochemical attack on the mecoprop molecule.

Degradation of the three-carbon sidechain of mecoprop (Fig 1.4), that is the methyl group or the $-CH_2COOH$ group, or both, during succinate/sodium acetate growth would not involve chloride ion release. After the exhaustion of the easily metabolized substrate the lag phase may be shorter as less time was required for the induction of enzymes to degrade the chemically modified structure of mecoprop. The likelihood of sodium acetate or succinate degrading enzymes catalyzing phenoxy sidechain loss is feasible as degradation of both carbon sources is via β-oxidation pathways involving the loss of two carbon units such as $-CH_2COOH$ (Stanier et al. 1977).

Although the mecoprop community constituents were not able to degrade mecoprop as the sole carbon and energy source (Section 4.2) several of the community constituents produced two-phase growth curves

on succinate and mecoprop (Section 6.2). Pseudomonas species, Pseudomonas maltophilia and Alcaligenes species all produced pronounced diauxic growth curves (Fig. 6.7 & Fig. 6.8), with chloride ion release detected in the second growth phase only. The lag phase duration between the two growth phases, that is, after succinate degradation and prior to mecoprop degradation, for all of the three cultures were longer than for the mixed community growing on the same two carbon sources. It is interesting to note that both Pseudomonas species HL1 and Pseudomonas maltophilia could utilize mecoprop after initial 'priming' on succinate as these organisms play a major role in the mixed culture, together comprising over 83.5% of the community (Table 4.3).

It was apparent that adaptation of the pure cultures to enable them to degrade mecoprop had not occurred as samples taken from the three batch cultures when in the second growth phase were transferred to growth medium with mecoprop as the sole carbon and energy source, and no degradation was detected. As the presence of succinate was, therefore, required for the three pure cultures to degrade mecoprop, succinate played some role in herbicide degradation. Mecoprop degradation by the three cultures may have been the result of succinate-grown cells attacking sidechain components of mecoprop, so altering the chemical structure to produce a more easily metabolizable substrate, as described above. The community constituents may however, already possess all of the necessary enzymes required for mecoprop degradation but the large molecule may be structurally unsuitable to enter the cells. Succinate-induced enzymes that catalyzed sidechain degradation would change the chemical structure of the molecules to allow them to pass into the cells for degradation.

The only report to date of mixed substrate utilization involving phenoxy herbicides was undertaken by Kilpi (1980). Initial attempts to

isolate mecoprop degrading organisms from soil agriculturally exposed to mecoprop were not successful. Kilpi tried enrichment of the soil organisms using various combinations of aromatic carbon sources and herbicides which included:

a) vanillic acid and MCPA,

b) vanillic acid and dichlorprop,

c) benzoic acid and MCPA, and

d) benzoic acid and dichlorprop.

The degradation of the herbicides was followed by monitoring the disappearance of their characteristic ultraviolet spectra. Organisms enriched on benzoate and dichlorprop were inoculated into media that contained mecoprop and benzoate. The more easily utilized substrate, that is benzoate, was degraded first before mecoprop degradation occurred. Organisms enriched in laboratory culture on MCPA were not able to utilize mecoprop even with benzoate as a co-substrate. Kilpi did not offer any explanations of why mixed substrate utilization of mecoprop was observed. However he suggested that an incorrect enrichment strategy had prevented the detection of mecoprop degradation as the sole carbon and energy source.

The specific growth rates and culture doubling times (Section 2.3.4 & 2.3.5) for the mecoprop community growing on mixed carbon sources were calculated (Table 6.3). In both batch cultures growth proceeded at a faster rate when the more easily metabolized compound was utilized, with culture doubling times of 4.91h for the acetate growth phase and 15.75h for the mecoprop growth phase (Table 6.3). The two phases were not so pronounced when co-substrates of succinate and mecoprop were degraded. The culture doubling times were 7.45h and 8.88h for the succinate and mecoprop growth phases respectively (Table 6.3). A

comparison of the specific growth rate of the mixed community growing on mecoprop as the sole carbon and energy source, that is $0.087h^{-1}$ (Section 3.1) and a culture doubling time of 7.96h (Section 2.3.5) demonstrated growth proceeded at a faster rate than when degraded in the presence of co-substrates (Table 6.3). However, growth by the mixed community on succinate and mecoprop proceeded at a faster rate than Pseudomonas species, Pseudomonas maltophilia or Alcaligenes species when grown on the same mixed substrates (Table 6.3 & 6.4).

The specific growth rates and culture doubling times of the pure cultures on mixed substrates were calculated for the two separate growth phases. For Pseudomonas species HL1 and Pseudomonas maltophilia the specific growth rate and culture doubling times demonstrated slower growth on mecoprop than on succinate (Table 6.4) however with Alcaligenes species HL3 growth proceeded at a faster rate on mecoprop than on succinate. The calculated values for the three pure cultures in the mecoprop growth phase demonstrated slower growth responses than when the same cultures were grown on glucose or succinate as the sole carbon and energy source (Table 6.1).

Finally, the specific growth rates and culture doubling time for Pseudomonas species, Pseudomonas maltophilia and Alcaligenes species growing on (a) succinate as the sole carbon and energy source (Table 6.1) and (b) succinate as a co-substrate (Table 6.4) were compared. For all three organisms the specific growth rates showed slower growth when mecoprop was present as a co-substrate, and the culture doubling times were longer. Apparently the presence of a second substrate, that is mecoprop, slowed down the rate of pure culture succinate utilization.

The pure cultures produced different growth responses to benzoate and mecoprop co-substrates. Both Pseudomonas species HL1 and Acinetobacter calcoaceticus released chloride ions from the supplied mecoprop, but neither equalled the theoretical maximum release (1.10 μmoles ml^{-1} or 52.88% and 1.60 μmoles ml^{-1} or 76.92% respectively). Neither Pseudomonas

maltophilia nor Alcaligenes species released chloride ions, although the culture absorbance was higher than that recorded for the cultures growing on benzoate as the sole carbon and energy source. Some aspect of incomplete mecoprop degradation must have occurred in order to support culture absorbance increases, but without subsequent chloride ion release or incomplete release.

Oxidation rates. The oxidation rates of various carbon sources was shown to be dependent upon the compound on which the organisms were grown. The oxidation rates of three different phenoxy herbicides, 2,4 - D, MCPA and dichlorprop by the microbial community grown on mecoprop were studied. All three herbicides have previously been demonstrated to be degraded by the community (Section 5.1 & 5.3). The findings were similar to those of Reineke and Knackmuss (1984) who reported that chloro-benzene-grown cells produced different responses to a variety of halogen-substituted benzene compounds. For example, the oxidation rate for benzene and chlorobenzene were similar but bromobenzene was oxidized at a slower rate whereas fluorobenzene was not oxidized at all.

The mecoprop-grown cells oxidized mecoprop and dichlorprop, which both contain a three-carbon sidechain, at faster rates than either 2,4 - D or MCPA, which both contain a two-carbon sidechain. The oxidation of the three structurally related herbicides by the mecoprop-grown cells suggested that the enzymes responsible for their oxidation were present in the mecoprop-grown cells and did not require induction. Therefore, the same enzyme systems may have oxidized all of the four herbicides investigated, pointing to common biodegradative pathways (Section 7). Also, the rapid oxidation rate of catechol by mecoprop-grown cells was an indication of its role as an intermediate in mecoprop degradation.

Conversely, the succinate-grown community constituents were not able to oxidize any other carbon sources except the compound on which they were grown (Table 6.6). Glucose was utilized as the sole carbon and energy source by all of the community constituents (Fig. 6.1) yet Alcaligenes species and Pseudomonas maltophilia could not oxidize glucose. Glucose-oxidizing enzymes were, therefore, not present in the succinate-grown cells. The degradation of glucose by the mecoprop community constituents must have been the result of induced rather than constitutive enzyme systems.

In conclusion, agriculturally applied mecoprop reaching the rhizosphere by translocation may be degraded by either microbial communities or pure cultures in the presence of co-substrates. The enzyme systems for mecoprop degradation are also responsible for 2,4 -D, MCPA and dichlorprop oxidation.

CHAPTER SEVEN

DISCUSSION

Since the introduction of the chlorinated phenoxy alkanoic compounds as selective herbicides in the 1940's (Section 1.2.1) their use in agriculture has greatly increased. By 1981 the total U.K. sales of the phenoxy derivatives of acetic, propionic and butyric acids was worth £53 million (anon, 1981).

The phenoxy herbicides are usually commercially available in combinations to maximize weed clearance. The listings in "Approved Products for Farmers and Growers" (Ministry of Agriculture, Fisheries & Food, 1983) have demonstrated that mecoprop is widely available in combinations with 2,4-D, MCPA and dichlorprop as well as with many other non-phenoxy pesticides (Table 7.1) to clear chickweed and cleavers in cereals and fruit crops. Nevertheless there has not been a build-up of mecoprop and dichlorprop in the environment. Long-term commercial field studies undertaken at the Weed Research Organisation, Oxford, have shown that when mecoprop was applied to soils it rapidly disappeared, but no mecoprop-degrading organisms were isolated (M.P. Greaves, personal communication). Few degradative studies of mecoprop have been published despite its widespread agricultural applications.

This study is the first report of the isolation of mecoprop-degrading organisms and illustrates the importance of both the initial enrichment strategy and of interacting microbial communities for the degradation of xenobiotic compounds. It was apparent that long term exposure to concentrations of 2.08 mM or less was necessary to enrich mecoprop-degrading organisms (Section 3.1). As no one organism was able to degrade mecoprop any study that emphasized pure culture techniques to demonstrate degradation would certainly have failed to isolate this

TABLE 7.1 PREPARATIONS OF PHENOXY HERBICIDES COMMERCIALLY AVAILABLE

A
The numbers of phenoxy preparation mixtures with other non-phenoxy pesticides

	Mecoprop	Dichlorprop	2,4-D	2,4,5-T	MCPA
The number of non-phenoxy herbicide preparations available containing the denoted phenoxy compounds	10	6	4	2	10

B
Commercial preparations containing combinations of phenoxy herbicides

	Mecoprop	Dichlorprop	2,4-D	2,4,5-T	MCPA
Mecoprop		+	+		+
Dichlorprop			+		+
2,4-D				+	+

+ denotes preparations available containing the two herbicides

community (Lappin et al. 1983).

This study has also demonstrated that the cross-adaptation capabilities by microorganisms extends to mecoprop and dichlorprop as well as 2,4-D and MCPA (Section 5.1 & 5.3). The soil microflora has, therefore, the ability to degrade several phenoxy herbicides, so reducing opportunities of environmental contamination. This demonstrates the significance of microbial cross-adaptation in field conditions.

The mecoprop-degrading community was able to utilize 2,4-D and MCPA as the sole sources of carbon and energy. Two of the community constituents were able to degrade 2,4-D in pure culture (Section 5.4). The enzyme systems responsible for the degradation of the three herbicides, that is, 2,4-D, MCPA and mecoprop, were considered to be sufficiently similar to support the proposal of a mecoprop biodegradative pathway. The proposal is based upon consideration of the cross-adaptation experiments (Section 5) and a knowledge of the degradative pathways of 2,4-D and MCPA (Section 1.2.3 and Fig. 1.3 & 1.4).

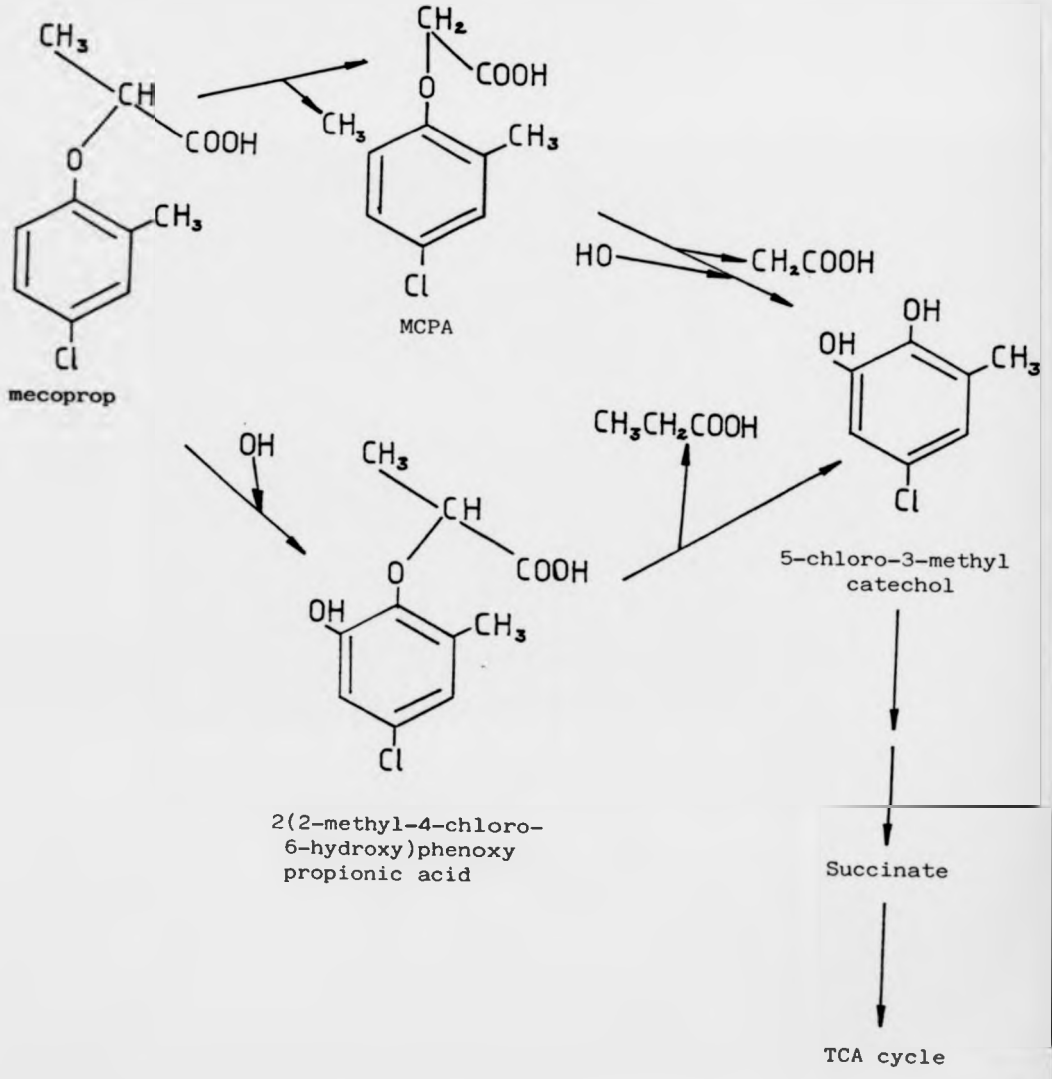
Two initial degradative steps are suggested:

a) the cleavage and removal of the $-CH_3$ group in the propionic side chain to produce MCPA. The pathway would then proceed via the MCPA degradative route (Fig. 7.1), and

b) the hydroxylation at the carbon six position on the aromatic ring (in relation to the phenoxy group) prior to the removal of the three carbon propionic sidechain (Fig. 7.1).

Both of the proposed initial steps produce a dihydroxy compound (5-chloro-3-methyl catechol). The 2,4-D and MCPA pathways show that

Fig. 7.1 Proposed pathway for the degradation of mecoprop.


mecoprop
MCPA
5-chloro-3-methyl
catechol
2(2-methyl-4-chloro-
6-hydroxy)phenoxy
propionic acid
Succinate
TCA cycle

catechol-like compounds are further degraded by ring cleavage to muconic acids and eventually feed into the TCA cycle; mecoprop is possibly also degraded via this route.

The biodegradative pathway of mecoprop may be confirmed by high-performance liquid chromatography techniques using a comparison of authentic standards with the hypothetical intermediates. Knowledge of the pathway derived from the studies of growth responses of the mecoprop community constituents to intermediates would greatly assist the elucidation of any existing community interactions.

As a consequence of traditional microbiological techniques employing pure culture studies, many compounds are reported as recalcitrant when combinations of two or more organisms may together break down the molecule. Natural environments are heterogeneous, containing a variety of substrates, growth conditions, and usually more than one organism, making pure culture approaches less representative than mixed culture studies (Slater, 1978; Slater, 1981). Despite the obvious complexities, an understanding of interactions would assist descriptions of microbial communities (Bull & Slater, 1982).

Harder (1981) argued that microbial communities play an important role in the degradation of xenobiotic compounds. Assemblages of organisms may be better adapted to attack environmentally foreign compounds than pure cultures (Slater & Bull, 1978). Reports of microbial communities acting synergistically to degrade xenobiotic compounds have included the degradation of sevin, an insecticide (Bollag & Liu, 1971), ring cycloalkanes (Beam & Perry, 1974), parathion (Daughton & Hsieh, 1977), silvex (Ou & Sikka, 1977) and mixtures of alkanes that is, C_{13} - C_{18} hydrocarbons (Kim & Rehm, 1982).

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Although the importance of microbial communities for degradation studies is acknowledged, the majority of reviews discussing community interaction studies have been principally restricted to summarizing two-membered associations (Slater & Bull, 1978). This is the simplest possible association but has allowed initial classification methods to be suggested. Classification based upon the influence of two populations of organisms, A and B, produced a maximum of six possible interactions from matrix studies (Slater & Bull, 1978; Bull & Slater, 1982). The organisms may have a beneficial or a detrimental influence on the other, or may produce no response (Table 7.2). The six basic effects are described as neutralism, commensalism, mutualism, parasitism/predation, amensalism and competition (for definitions, see Table 7.2).

Classification systems of this type are useful as they allow generalizations to be made, but are severely limited to assist investigations of more complex communities of three or more organisms (Bull & Slater, 1982). An example of a complex microbial community is the seven membered Dalapon community (Senior et al. 1976). The community comprised of three primary utilizers and four secondary utilizers as the preferred stable entity. Continuous cultivation for 18,000h failed to reduce the complexity, although the three primary utilizers were all competing for the same growth limiting substrate. Apart from suggesting that unknown nutritional and physical interactions occurred, the authors acknowledged that the specific relationships in the community were not understood (Slater & Bull, 1978).

A second classification system has now been introduced which can be used to describe more complex community structures (Slater, 1981). Microbial communities may be placed into seven separate classes, although some overlap would occur when interactions are more fully

TABLE 7.2 MATRIX OF INTERACTIONS OF TWO MICROBIAL POPULATIONS A & B (after Slater & Bull, 1978).

		The effect on the growth of organism A by the activity of organism B.		
		+	0	-
The effect on the growth of organism B by the activity of organism A.	+	++	+0	+-
	0	0+	00	0-
	-	-+	-0	--

Neutralism: (0,0) Lack of interaction between the two organisms

Mutualism: (+,+) reciprocal benefit

Commensalism: (+0 or 0+) one organism benefits, the other does not derive benefit or harm from the association

Amensalism: (-0, 0-) one organism is restricted by the presence of the other organism, the latter is unaffected

Parasitism/Predation: (+-, -+) the feeding of one population on another organism

Competition: (- -) both populations mutually restricted by each other, probably based on a common growth factor

elucidated (Table 7.3). The majority of microbial communities reported fit into either Class 1 (communities based upon specific nutrient requirements between constituent members) or Class 4 (communities based upon a combined metabolic capability) (Slater, 1981).

The mecoprop-degrading community was a stable association of organisms presumably as a result of a series of mutually beneficial interactions. Subculturing and growth for a total period of over 16,000h in batch culture and continuous chemostat cultivation for 5,000h (Section 3.1), failed to reduce the community structure although simpler combinations of just two organisms, for example, Pseudomonas maltophilia and Pseudomonas species HL1 would have degraded mecoprop (Section 4.2).

An investigation of the initial classification of the mecoprop community was whether it was a 'loose' or a 'tight' association of organisms. Slater (1978) described loose associations as having no necessity or compulsion in the relationship, whereas tight associations were defined as having a common need to remain as communities. It was apparent that the mecoprop-degrading community was tightly associated because:

a) none of the community members were able to degrade the herbicide as the sole carbon and energy source (Section 4.2) so a combined metabolic attack was necessary, and

b) as a reflection of a tight physical association, the community constituents were difficult to separate.

The community is unusual in that no obvious divisions into primary and secondary utilizers can be made, unlike the Dalapon community (Senior et al. 1976), so the interactions are more difficult to assess.

The principle association in the mecoprop-degrading community

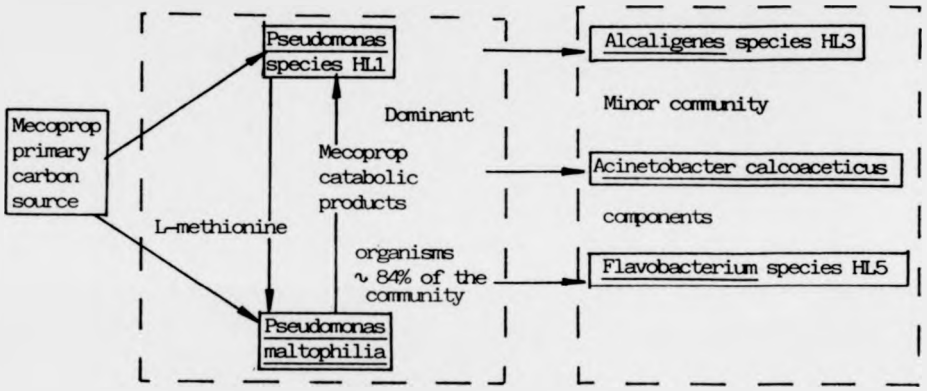
undoubtedly exists between Pseudomonas species HL1 and Pseudomonas maltophilia, first because of their association based upon the provision and requirement of L-methionine (Section 4); and secondly because the two organisms have a dominant influence on the community by making up approximately 84% of the community. In conjunction with this dominant two-membered association are the three remaining community members, Acinetobacter calcoaceticus, Alcaligenes species HL3 and Flavobacterium species HL5, comprising approximately 16% of the community and making some unspecified contribution to the community's stability (Fig. 7.2A).

By using another method of categorization the mecoprop community can be described as a Class 1 community (Table 7.3) due to the provision of a growth factor requirement, that is, L-methionine for Pseudomonas maltophilia or as a Class 4 community because mecoprop degradation was the result of a combined metabolic attack as none of the pure cultures could degrade the herbicide as the sole carbon and energy source. More importantly, more than one type of interaction may occur in a mixed community and there are numerous possible interactions which may occur in such a five-membered association.

A simple study of the mecoprop community interactions when grown on another phenoxy herbicide, 2,4-D, revealed that the two dominant organisms in the mecoprop community were again of importance as the two primary 2,4-D utilizers, that is, Pseudomonas maltophilia and Pseudomonas species HL1. The three remaining organisms, Alcaligenes species HL3, Flavobacterium species HL5 and Acinetobacter calcoaceticus were all secondary utilizers of 2,4-D (Fig. 7.2B).

Fig. 7.2 Community interactions with two phenoxy herbicides

A. MECOPROP-DEGRADING COMMUNITY



B. MICROBIAL COMMUNITY DEGRADING 2,4-D

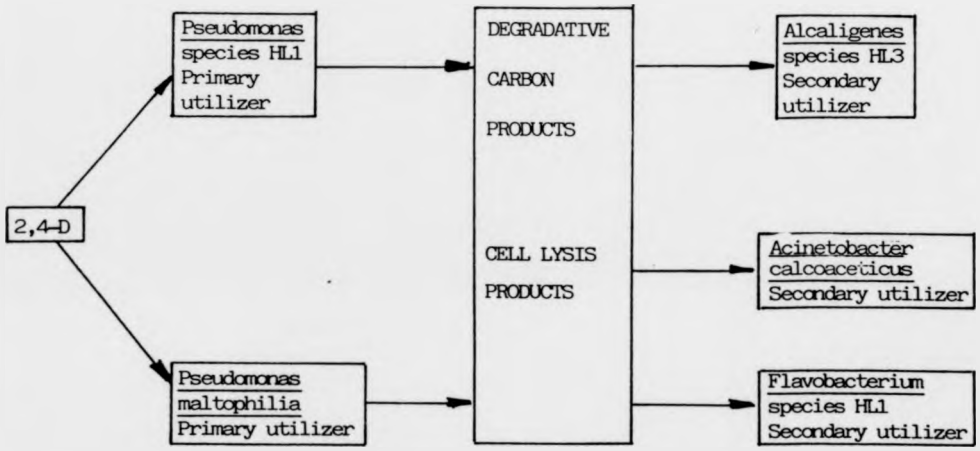


TABLE 7.3 CLASSES OF MICROBIAL COMMUNITIES (after Slater, 1981)

Class 1:	Structure based on provision of specific nutrients by different members of the community.
Class 2:	Structure based on the alleviation of growth inhibition, including removal of metabolites which are inhibitory to the producer species including Class 6.
Class 3:	Structure and stability due to interactions which may result in the modification of individual population growth parameters resulting in a more competitive or efficient community.
Class 4:	Structure due to the effect of a concerted, combined metabolic capacity, not expressed by the individual populations acting alone.
Class 5:	Structure due to a cometabolic stage.
Class 6:	Structure due to the transfer of hydrogen ions.
Class 7:	Structure is the result of the presence of more than one primary substrate utilizer - in many cases the nature of the interactions are unknown.

The microbial community capable of degrading mecoprop was enriched from rhizosphere organisms using batch culture techniques. Batch culture techniques are not considered to be the most appropriate method for the isolation of microbial communities. Slater (1978) argued that the sampling of batch culture growth onto a solidified enrichment medium would physically separate any interacting organisms, so destroying their association. An example cited was of a two-membered community where organism A was unable to degrade a specific carbon source but provided an essential growth factor for organism B, which could degrade the carbon source and consequently provided A with degradative products. Slater (1978) suggested that such an association would not be selected using solidified enrichment medium. A modification of batch culture sampling using a non-selective growth medium, followed by examination of pure culture degradation using liquid cultivation allows organisms such as A and B to be isolated. This disagrees with Slater's argument against batch cultivation for microbial community isolation. Because chemostat cultivation permits communities to be selected on parameters other than their specific growth rates, this technique is preferred for isolating microbial communities.

Parkes (1982) stated that chemostat cultures produce different enrichment conditions to batch cultures. Yet when the mecoprop-degrading community, which was established using batch culture techniques, was transferred to a chemostat and grown continuously for 5,000h no change in the community structure was detected. Therefore, the significant differences in batch and chemostat growth, including the build-up of toxic materials and μ_{max} growth rates in batch growth had no influence on this community otherwise fluctuations or changes would have

occurred during chemostat growth. Conversely the mecoprop-degrading community may have been such a stable arrangement that chemostat cultivation could not disrupt it.

Rhizosphere microflora possess diverse metabolic capabilities (Section 1.1). There are a few reports of degradation of xenobiotic compounds in the rhizosphere. Hsu and Bartha (1979) investigated the degradation of two insecticides, diazinon and parathion, by comparing rhizosphere soils with control soils. The authors concluded that the rhizosphere was a favourable habitat as synergistically acting organisms were responsible for a more rapid mineralization of both insecticides than in the control soil.

Microbial stimulation in the rhizosphere is due to the presence of exudates from a variety of different sources (Rovira et al. 1983), and include organic acids and sugars (Table 1.2). The mixed substrate studies undertaken with the mecoprop-degrading community and community constituents provided a crude analogy to the substrate rich rhizosphere environment. As mecoprop is a translocated herbicide it reaches the rhizosphere environment where many other carbon sources are also present so producing situations where mecoprop and easily utilizable substrates are available for microbial degradation.

This study has demonstrated that two possible mechanisms exist for mecoprop degradation in the rhizosphere by:

a) microbial communities consisting of typical rhizosphere inhabitants (Section 4) such as the five-membered association isolated in this study, and

b) individual rhizosphere organisms growing on combinations of root exudates, for example, easily degradable sugars or

organic acids and mecoprop (Section 6).

It is difficult to postulate an extrapolation of laboratory based experiments to field conditions to establish if mecoprop degradation occurs via one or both of these routes.

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It is difficult to postulate an extrapolation of laboratory based experiments to field conditions to establish if mecoprop degradation occurs via one or both of these routes.

REFERENCES

Abdel-Nasser, M. & Moawad, H. (1975). Changes in numbers of microorganisms during decomposition of root exudates in soil. Zbl. Bakt. Abt. 11, Bd. 130: 738-744.

Alexander, M. (1961a). Transformation of hydrocarbons and pesticides. In Introduction to soil microbiology, p.227-244. John Wiley & Sons Inc. New York.

Alexander, M. (1961b). Microbiology of the rhizosphere. In Introduction to soil microbiology, p.442-459. John Wiley & Sons Inc. New York.

Alexander, M. & Aleem, M.I.H. (1961). Effect of chemical structure on microbial decomposition of aromatic herbicides. Journal of Agricultural and Food Chemistry. 9: 44-47.

Anderson, R.V., Elliot, E.T., McClellan, J.F., Coleman, D.C., Cole, C.V. & Hunt, H.W. (1978). Trophic interactions in soil as they affect energy and nutrient dynamics. III. Biotic interactions of bacteria, amoebae, and nematodes. Microbial Ecology. 4: 361-371.

Anonymous, (1981). The Business Monitor, Government Statistics Office.

Arnow,L.E. (1937). Colorimetric determination of the components of 3,4-dihroxy phenylalanine-tyrosine mixture. Journal of Biological Chemistry 118: 531-538.

Audus, L.J. (1950). Biological detoxification of 2,4-dichlorophenoxy-acetic acid in soils: Isolation of an effective organism. Nature 166: 356.

Audus, L.J. (1951). The biological detoxification of hormone herbicides in soil. Plant and Soil. 3: 170-192.

Audus, L.J. (1952). The decomposition of 2:4-dichlorophenoxyacetic acid and 2-methyl-4-chlorophenoxyacetic acid in the soil. Journal of the Science of Food and Agriculture. 3: 268-274.

Balasubramanian, A. & Rangaswami, G. (1973). Influence of foliar application of chemicals on the root exudations and rhizosphere

microflora of Sorghum vulgare and Crotolaria juncea. Folia Microbiol. 18: 492-498.

Banerjee, S., Duttagupta, S. & Chakrabarty, A.M. (1983). Production of emulsifying agent during growth of Pseudomonas cepacia with 2,4,5-trichlorophenoxyacetic acid. Arch. Microbiol. 135: 110-114.

Beam, H.W. & Perry, J.J. (1974). Microbial degradation of cycloparaffinic hydrocarbons via co-metabolism and commensalism. J. gen. Microbiol. 82: 163-169.

Birnboim, H.C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic acids Research 1: 1513-1523.

Boethling, R.S. & Alexander, M. (1979). Effect of concentration of organic chemicals on their biodegradation by natural microbial communities. Applied and Environmental Microbiology 37: 1211-1216.

Bollag, J-M., Helling, C.S. & Alexander, M. (1968a). 2,4-D metabolism. Enzymatic hydroxylation of chlorinated phenols. Journal of Agricultural & Food Chemistry 16: 826-828.

Bollag, J-M, Briggs, G.G., Dawson, J.E. & Alexander, M. (1968b). 2,4-D Metabolism. Enzymatic degradation of chlorocatechols. Journal of Agricultural and Food Chemistry 16: 829-833.

Bollag, J-M. & Liu, S-Y. (1971). Degradation of Sevin by soil microorganisms. Soil Biology and Biochemistry 3: 337-345.

Bull, A.T. (1980). Biodegradation: some attitudes and strategies of microorganisms and microbiologists. In, Contemporary Microbial Ecology, p.107-136. Ellwood, D.C., Hedger, J.N., Latham, M.J., Lynch, J.M. & Slater, J.H. (eds.) Academic Press, London.

Bull, A.T. & Slater, J.H. (1982). Microbial interactions and community structures. In, Microbial Interactions and Communities, Vol. 1, p.13-44. Bull, A.T. & Slater, J.H. (eds.). Academic Press, London.

Chakrabarty, A.M. (1976). Plasmids in Pseudomonas. Annual Review of Genetics, 10: 7-30.

Chan, E.C.S., Katznelson, H. & Rouatt, J.W. (1963). The influence of soil and root extracts on the associative growth of selected soil bacteria. Canadian Journal of Microbiology 9: 187-197.

Chatterjee, D.K., Kellog, S.J., Watkins, D.R. & Chakrabarty, A.M. (1981). Plasmids in the biodegradation of chlorinated aromatic compounds. In Molecular Biology, Pathogenicity and Ecology of Bacterial Plasmids, p519-528. Levy, S.B, Clowes, R.C. & Koenig, E.L. (eds). Plenum Press, New York & London.

Chatterjee, D.K., Kilbane, J.J. & Chakrabarty, A.M. (1982). Biodegradation of 2,4,5-Trichlorophenoxyacetic acid in soil by a pure culture of Pseudomonas cepacia. Applied and Environmental Microbiology 44: 514-516.

Clark, F.E. (1940). Notes on types of bacteria associated with plant roots. Trans. Kansas Acad. Science 43: 75-84.

Cole, CV., Elliott, E.T., Hunt, H.W. & Coleman, D.C. (1978). Trophic interactions in soils as they affect energy and nutrient dynamics. V. Phosphorus transformations. Microbial Ecology 4: 381-387.

Coleman, D.C., Anderson, R.V., Cole, C.V., Elliott, E.T., Woods, L. & Campion, M.K. (1978). Trophic interactions in soils as they affect energy and nutrient dynamics. IV. Flows of metabolic and biomass carbon. Microbial Ecology 4: 373-380.

Daughton, C.G. & Hsieh, D.P. (1977). Parathion utilization by bacterial symbionts in a chemostat. Applied and Environmental Microbiology. 34: 175-184.

Don, R.H. & Pemberton, J.M. (1981). Properties of six pesticide degradation plasmids isolated from Alcaligenes paradoxus and Alcaligenes eutrophus. Journal of Bacteriology 145: 681-686.

Duah-Yentumi, S. & Kuwatsuka, S. (1982). Microbial degradation of Benthiocarb, MCPA and 2,4-D herbicides in perfused soils amended with organic matter and chemical fertilizers. Soil Sci. Plant Nutrition 28: 19-26.

Feist, C.F. & Hegeman, G.D. (1969). Phenol and benzoate metabolism by Pseudomonas putida: Regulation of tangential pathways. Journal of Bacteriology 100: 869-877.

Fisher, P.R., Appleton, J. & Pemberton, J.M. (1978). Isolation and characterization of the pesticide-degrading plasmid pJP1 from Alcaligenes paradoxus. Journal of Bacteriology 135: 798-804.

Fletcher, W.W & Kirkwood, R.C. (1982). Herbicides and Plant Growth Regulators. Granada, London.

Franklin, F.C.H., Bagdasarian, M. & Timmis, K.N. (1981). Manipulation of degradation genes of soil bacteria. In Microbial Degradation of Xenobiotic and Recalcitrant Compounds, p109-130. Leisinger, T., Cook, A.M., Hutter, R. & Nuesch, J. (eds). Academic Press, London.

Gaunt, J.K. & Evans, W.C. (1971a). Metabolism of 4-chloro-2-methyl-phenoxyacetate by a soil Pseudomonad. Preliminary evidence for the metabolic pathway. Biochem. Journal 122: 519-526.

Gaunt, J.K. & Evans, W.C. (1971b). Metabolism of 4-chloro-2-methyl-phenoxyacetate by a soil Pseudomonad. Ring-fission, lactonizing and delactonizing enzymes. Biochem. Journal 122: 533-542.

Harder, W., Kuenen, J.G & Matin, A. (1977). A review. Microbial selection in continuous culture. Journal of Applied Bacteriology. 43: 1-24.

Harder, W. (1981). Enrichment and characterization of degrading organisms. In Microbial Degradation of Xenobiotic and Recalcitrant compounds, p77-96. Leisinger, T, Cook, A.M., Hutter,

R. & Nuesch, J. Academic Press, London.

Hardman, D.J. & Slater, J.H. (1981a). Dehalogenases in soil bacteria. Journal of General Microbiology 123: 117-128

Hardman, D.J. & Slater, J.H. (1981b). The dehalogenase complement of a soil Pseudomonad grown in closed and open cultures on haloalkanoic acids. Journal of General Microbiology 127: 399-405.

Hartman, J., Reineke, W. & Knackmuss, H-J. (1979). Metabolism of 3-chloro-, 4-chloro-, and 3,5-dichlorobenzoate by a Pseudomonad. Applied and Environmental Microbiology 37: 421-428.

Higgins, I.J. & Burns, R.G. (1975). The Chemistry and Microbiology of Pollution. Academic Press, London.

Hiltner, L. (1904). Uber neuere Erfahrungen und Probleme auf dem Gebiete der Bodenbakteriologie unter besonderer Berucksichtigung der Grundungung und Brache. Arbeiten der Deutschen Landwirtschafts Gesellschaft 98: 59-78.

Holmes, B. & Owen, R.J. (1979). Proposal that Flavobacterium breve be substituted as the type species of the genus in place of Flavobacterium aquatile and amended description of the genus Flavobacterium: status of the named species of Flavobacterium. Request for an opinion. Int. J. Syst. Bact. 29: 416-426.

Horowitz, A., Sulflita, J.M., Tiedje, J.M. (1983). Reductive dehalogenation of halobenzoates by anaerobic lake sediment microorganisms. Applied and Environmental Microbiology 45: 1459-1465.

Hsu, T-S. & Bartha, R. (1979). Accelerated mineralization of two organophosphate insecticides in the rhizosphere. Applied and Environmental Microbiology 37: 36-41.

Hurle, K. (1973). Lag-phase Studien mit DNOC und 2,4-D im Boden. In Proc. Eur. Weed Res. Counc. Symp. Herbicides-soil, p151-162.

Jackson, R.M., Brown, M.E. & Burlingham, S.K. (1964). Similar effects on tomato plants of Azotobacter inoculation and application of gibberellins. Nature 203: 851-852.

Karns, J.S., Kilbane, J.J., Duttagupta, S. & Chakrabarty, A.M. (1983a). Metabolism of halophenols by 2,4,5-Trichlorophenoxyacetic acid-degrading Pseudomonas cepacia. Applied and Environmental Microbiology 46: 1176-1181.

Karns, J.S., Duttagupta, S. & Chakrabarty, A.M. (1983b). Regulation of 2,4,5-Trichlorophenoxyacetic acid and chlorophenol metabolism in Pseudomonas cepacia AC1100. Applied and Environmental Microbiology 46: 1182-1186.

Katznelson, H., Rouatt, J. & Payne, T.M.B. (1954). The liberation of amino acids by plant roots in relation to desiccation. Nature 174: 1110-1111.

Kellog, S.T., Chatterjee, D.K. & Chakrabarty, A.M. (1981). Plasmid-assisted molecular breeding: New techniques for enhanced biodegradation of persistent toxic chemicals. Science 214: 1133-1135.

Kilbane, J.J., Chatterjee, D.K., Karns, J.S., Kellog, S.t & Chakrabarty, A.M. (1982). Biodegradation of 2,4,5-Trichlorophenoxyacetic acid by a pure culture of Pseudomonas cepacia. Applied and Environmental Microbiology 44: 72-78.

Kilbane, J.J., Chatterjee, D.K. & Chakrabarty, A.M. (1983). Detoxification of 2,4,5-Trichlorophenoxyacetic acid from contaminated soil by Pseudomonas cepacia. Applied and Environmental Microbiology 45: 1697-1700.

Kilpi, S. (1980). Degradation of some phenoxy acid herbicides by mixed cultures of bacteria isolated from soil treated with 2-(2-methyl-4-chloro) phenoxypropionic acid. Microbial Ecology 6: 261-270.

Kilpi, S., Backstrom, V. & Korhola, M. (1983). Degradation of catechol, methylcatechols and chlorocatechols by Pseudomonas sp. HV3. FEMS

Microbiology Letters 18: 1-5.

Kim, Y.B. & Rehm, H.J. (1982). Studies on a mixed culture of Candida parapsilosis and different Bacilli on alkane. I. Characterization of the mixed culture systems. European Journal of Applied Microbiology and Biotechnology 14: 105-111.

King, O.E., Ward, M.K. & Raney, D.E. (1954). Two simple media for the demonstration of pyocyanin and fluorescin. Journal of Laboratory and Clinical Medicine 44: 301-307.

Kirkland, K. & Fryer, J.D. (1972). Degradation of several herbicides in a soil previously treated with MCPA. Weed Research 12: 90-95.

Kleeberger, A., Castorph, H. & Klingmuller, W. (1983). The rhizosphere microflora of wheat and barley with special reference to gram-negative bacteria. Archives of Microbiology 136: 306-311.

Kunc, F. & Macura, J. (1966). Decomposition of root exudates in soil. Folia Microbiologica 11: 239-247.

Lappin, H.M., Greaves, M.P. & Slater, J.H. (1983). Growth of rhizosphere populations on the herbicide mecoprop. Society for General Microbiology Quarterly 10: M16.

Lappin, H.M., Greaves, M.P. & Slater, J.H. (1984). Degradation of the herbicide mecoprop 2-(2-methyl 4-chlorophenoxy)-propionic acid by a synergistic microbial community. Applied and Environmental Microbiology (in press).

Lochhead, A.G. (1940). Qualitative studies of soil microorganisms. III. Influence of plant growth on the character of the bacterial flora. Canadian Journal of Research 18: 42-53.

Lochhead, A.G. & Rouatt, J.W. (1955). The "Rhizosphere Effect" on the nutritional groups of soil bacteria. Soil Science Society of America Proceedings 19: 48-49.

Loos, M.A., Roberts, R.N. & Alexander, M. (1967a). Phenols as intermediates in the decomposition of phenoxyacetates by an Arthrobacter species. Canadian Journal of Microbiology 13: 679-690.

Loos, M.A., Roberts, R.N. & Alexander, M. (1967b). Formation of 2,4-dichlorophenol and 2,4-dichloroanisole from 2,4-dichlorophenoxyacetate by Arthrobacter sp. Canadian Journal of Microbiology 13: 691-699.

Loos, M.A. (1975). Phenoxyalkanoic acids. In Herbicides, their Chemistry, Degradation and mode of action, Vol. 1, p1-128. Kearney, P.C. & Kaufman, D.D. (eds). Marcel Dekker, New York.

Lynch, J.M. & Barber, D.A. (1977). Microbial growth in the rhizosphere. Soil Biology and Biochemistry 9: 305-308.

Lynch, J.M. (1982). The rhizosphere. In Methods in Microbial Ecology, p395-411. Burns, R.G. & Slater, J.H. (eds). Blackwell Scientific Publications, Oxford.

McCall, P.J. Vrona, S.A & Kelley, S.S. (1981). Fate of uniformly carbon-14 ring labelled 2,4,5-Trichlorophenoxyacetic acid and 2,4-dichlorophenoxyacetic acid. Journal of Agricultural and Food Chemistry 29: 100-107.

MacRae, I.C. & Alexander, M. (1965). Microbial degradation of selected herbicides in soil. Journal of Agricultural and Food Chemistry 13: 72-76.

Martin, J.K. (1977). Factors influencing the loss of organic carbon from wheat roots. Soil Biology and Biochemistry 9: 1-7.

Ministry of Agriculture, Fisheries and Food (1983). Approved Products for Farmers and Growers. Her Majesty's Stationery Office.

Novick, R.P. (1980). Plasmids. Scientific American 243: 77-90.

Nurmikko, V. (1954). Symbiosis experiments concerning the production and biosynthesis of certain amino acids and vitamins in association of lactic acid bacteria. Annals Academiae Scientianim Fenniniae 54: 7-58.

Ou, L.T. & Sikka, H.C. (1977). Extensive degradation of Silvex by

Agricultural and Food Chemistry 25: 1336-1339.

Parkes, R.J. (1982). Methods for enriching, isolating and analysing microbial communities in laboratory systems. In Microbial Interactions and Communities Vol.1, p45-103. Bull, A.T. & Slater, J.H. (eds). Academic Press London.

Pearson, R. & Parkinson, D. (1961). The sites of excretion of ninhydrin positive substances by broad bean seedlings. Plant and Soil 13: 391-396.

Pemberton, J.M. & Fisher, P.R. (1977). 2,4-D plasmids and persistence. Nature 268: 732-733.

Pemberton, J.M. (1979). Pesticide degrading plasmids: a biological answer to environmental pollution by phenoxyherbicides. Ambio 8: 202-205.

Pemberton, J.M., Corney, B. & Don, R.H. (1979). Evolution and spread of pesticide degrading ability among soil microorganisms. In Plasmids of Medical, Environmental and Commercial Importance, p287-299. Timmis, K.N. & Puhler, A. (eds). Elsevier North Holland Biomedical Press.

Rao, A.V. & Sharma, R.L. (1978). Influence of chemicals on the microflora and enzyme activities in the cauliflower rhizosphere. Acta Botanica Indica 6: 71-74.

Reanney, D.C., Roberts, W.P. & Kelly, W.J. (1982). Genetic interactions among microbial communities. In Microbial Interactions and Communities Vol.1, p287-322. Bull, A.T. & Slater, J.H. (eds). Academic Press London.

Reineke, W. & Knackmuss, H-J (1979). Construction of haloaromatics utilising bacteria. Nature 277: 385-386.

Reineke, W. & Knackmuss, H-J (1984). Microbial metabolism of haloaromatics: isolation and properties of a chlorobenzene-degrading bacterium. Applied and Environmental Microbiology 47:

Agricultural and Food Chemistry 25: 1336-1339.

Parkes, R.J. (1982). Methods for enriching, isolating and analysing microbial communities in laboratory systems. In Microbial Interactions and Communities Vol.1, p45-103. Bull, A.T. & Slater, J.H. (eds). Academic Press London.

Pearson, R. & Parkinson, D. (1961). The sites of excretion of ninhydrin positive substances by broad bean seedlings. Plant and Soil 13: 391-396.

Pemberton, J.M. & Fisher, P.R. (1977). 2,4-D plasmids and persistence. Nature 268: 732-733.

Pemberton, J.M. (1979). Pesticide degrading plasmids: a biological answer to environmental pollution by phenoxyherbicides. Ambio 8: 202-205.

Pemberton, J.M., Corney, B. & Don, R.H. (1979). Evolution and spread of pesticide degrading ability among soil microorganisms. In Plasmids of Medical, Environmental and Commercial Importance, p287-299. Timmis, K.N. & Puhler, A. (eds). Elsevier North Holland Biomedical Press.

Rao, A.V. & Sharma, R.L. (1978). Influence of chemicals on the microflora and enzyme activities in the cauliflower rhizosphere. Acta Botanica Indica 6: 71-74.

Reanney, D.C., Roberts, W.P. & Kelly, W.J. (1982). Genetic interactions among microbial communities. In Microbial Interactions and Communities Vol.1, p287-322. Bull, A.T. & Slater, J.H. (eds). Academic Press London.

Reineke, W. & Knackmuss, H-J (1979). Construction of haloaromatics utilising bacteria. Nature 277: 385-386.

Reineke, W. & Knackmuss, H-J (1984). Microbial metabolism of haloaromatics: isolation and properties of a chlorobenzene-degrading bacterium. Applied and Environmental Microbiology 47:

395-402.

Rosenberg, A. & Alexander, M. (1980). Microbial metabolism of 2,4,5-Trichlorophenoxyacetic acid in soil, soil suspensions, and axenic culture. Journal of Agricultural and Food Chemistry 28: 297-302.

Rovira, A.D. (1956a). Plant root excretions in relation to the rhizosphere effect I. The nature of root exudate from oats and peas. Plant and Soil 7: 178-194.

Rovira, A.D. (1956b). Plant root excretions in relation to the rhizosphere effect. III. The effect of root exudate on the numbers and activity of microorganisms in soil. Plant and Soil 7: 209-217.

Rovira, A.D. (1959). Root excretions in relation to the rhizosphere effect. IV. Influence of plant species, age of plant, light, temperature and calcium nutrient on exudation. Plant and Soil 11: 53-64.

Rovira, A.D. & Harris, J.R. (1961). Plant root excretions in relation to the rhizosphere effect. Plant and Soil 14: 199-214.

Rovira, A.D. (1965). Plant root exudates and their influence upon soil microorganisms. In Ecology of Soil-borne Plant Pathogens, p170-186. Baker, K.F. & Snyder, W.C. (eds). Murray, London.

Rovira, A.D. & McDougall, M. (1967). Microbiological and biochemical aspects of the rhizosphere. In Soil Biochemistry, Vol.1, p417-463. McLaren, A.D. & Peterson, G.H. (eds). Arnold and Dekker.

Rovira, A.D., Foster, R.C. & Martin, J.K. (1979). Notes on terminology: origin, nature and nomenclature of the organic materials in the rhizosphere. In The Soil Root Interface, p1-4. Harley, J.H. & Russel, R.S. (eds). Academic Press, London.

Rovira, A.D., Bowen, G.D. & Foster, R.C. (1983). The significance of rhizosphere microflora and mycorrhizas in plant nutrient. In Inorganic Plant Nutrition, p61-93. Lauchli, A. & Bieleski, R.L. (eds). Springer-Verlag, Berlin.

Schmidt, E., Hellwig, M. & Knackmuss, H-J. (1983). Degradation of chlorophenols by a defined mixed microbial community. Applied and Environmental Microbiology 46: 1038-1044.

Senior, E., Bull, A.T. & Slater, J.H. (1976). Enzyme evolution in a microbial community growing on the herbicide Dalapon. Nature 263: 476-479.

Slater, J.H. (1978). The role of microbial communities in the natural environment. In The Oil Industry and Microbial Ecosystems, p137-154. Chater, K.W.A. & Somerville, H.J. (eds). Heyden & Son Ltd, London.

Slater, J.H. & Bull, A.T. (1978). Interactions between microbial populations. In Companion to Microbiology p181-207. Bull, A.T. & Meadow, P.M. (eds). Longman Group, London.

Slater, J.H. (1979). Microbial populations and community dynamics. In Microbial Ecology, a Conceptual Approach, p45-63. Lynch, J.M. & Poole, N.J. (eds). Blackwell Scientific Publications, Oxford.

Slater, J.H., Lovatt, D., Weightman, A.J., Senior, E. & Bull, A.T. (1979). The growth of Pseudomonas putida on chlorinated aliphatic acids and its dehalogenase activity. Journal of General Microbiology 114: 125-136.

Slater, J.H. & Godwin, D. (1980). Microbial adaptation and selection. In Contemporary Microbial Ecology, p137-160. Ellwood, D.C., Hedger, J.N., Latham, M.J., Lynch, J.M. & Slater, J.H. (eds). Academic Press, London.

Slater, J.H. (1981). Mixed cultures and microbial communities. In Mixed Culture Fermentations, pl-24. Bushel, M.E. & Slater, J.H. Academic Press, London.

Slater, J.H. & Bull, A.T. (1982). Environmental microbiology: biodegradation. Phil. Trans. R. Soc. London. 297: 575-597.

Smith, W.H. (1976). Character and significance of forest tree root exudates. Ecology 57: 324-331.

Smith, A.E. & Hayden, B.J. (1981). Relative persistence of MCPA, MCPB and mecoprop in Saskatchewan soils, and the identification of MCPA in MCPB-treated soils. Weed Research 21: 179-183.

Spain, J.C. & Van Veld, P.A. (1983). Adaptation of natural microbial communities to degradation of xenobiotic compounds: Effect of concentration, exposure, time, inoculum, and chemical structure. Applied and Environmental Microbiology 45: 428-435.

Stanier, R.Y., Adelberg, E.A. & Ingraham, J.L. (1977). General Microbiology - 4th edition. Macmillan Press, London.

Steenson, T.I. & Walker, N. (1956). Observations on the bacterial oxidation of chlorophenoxyacetic acids. Plant and Soil 8: 17-32.

Steenson, T.I. & Walker, N. (1957). The pathway of breakdown of 2:4-Dichloro- and 4-chloro-2-methyl-phenoxyacetic acid by bacteria. Journal of General Microbiology 16: 146-155.

Stirling, L.A., Watkinson, R.J. & Higgins, I.J. (1976). The microbial utilization of cyclohexane. Proceedings of the Society for General Microbiology 4: 28.

Suflita, J.M., Robinson, J.A. & Tiedje, J.M. (1983). Kinetics of microbial dehalogenation of haloaromatic substrates in methanogenic environments. Applied and Environmental Microbiology 45: 1466-1473.

Tiedje, J.M., Duxbury, J.M., Alexander, M. & Dawson, J.E. (1969). 2,4-D Metabolism: Pathway of degradation of chlorocatechols by Arthrobacter sp. Journal of Agricultural and Food Chemistry 17: 1021-1026.

Timonin, M.I. (1941). The interactions of higher plants and soil microorganisms: III. Effect of by-products of plant growth on activity of fungi and actinomycetes. Soil Science 52: 395-413.

Torstensson, L. (1980). Role of microorganisms in decomposition. In Interactions between herbicides and the soil, p159-178. Hance, R.J. (ed). Academic Press, London.

Vancura, V. (1980). Fluorescent Pseudomonads in the rhizosphere of plants and their relation to root exudates. Folia Microbiologica 25: 168-173.

Vrany, J., Vancura, V. & Macura, J. (1962). The effect of foliar applications of some readily metabolized substrates, growth regulators and antibiotics on rhizosphere microflora. Folia Microbiologica 7: 61-70.

Wallace, R.H. & Lochhead, A.G. (1950). Qualitative studies of soil microorganisms. IX. Amino acid requirements of rhizosphere bacteria. Canadian Journal of Research 28: 1-6.

West, P.M. & Lochhead, A.G. (1940). Qualitative studies of soil microorganisms. IV. The rhizosphere in relation to the nutritive requirements of soil bacteria. Canadian Journal of Research 18: 129-135.

Wheatcroft, R. & Williams, P.A. (1981). A rapid method for the study of both stable and unstable plasmids in Pseudomonas. Journal of General Microbiology 124: 433-437.

Williams, P.A. (1978). The biology of plasmids. In Companion to Microbiology, p77-108. Bull, A.T. & Meadow, P.M. (eds). Longman Group, London.

Yeoh, H.T., Bungay, H.R. & Krieg, N.R. (1968). A microbial interaction involving a combined mutualism and inhibition. Canadian Journal of Microbiology 14: 491-492.