BIODEGRADATION OF PENTACHLOROPHENOL

MARTHA FLYNN
1999

A. ADDITIONAL EXPLANATORY NOTES

PAGE	NOTES
9 para 3	Pentachlorophenol is a persistent chemical in the environment because of the absence of microorganisms in the natural environment able to degrade PCP at the concentrations at which it is present.
57 Fig 2.13	Compounds XII – XVIII should not to be listed in the legend because they are not presented in the diagram.
76 last para	The negative results of the BBL Oxiferm® tube for ET01 were inconclusive and therefore the identification of ET01 by Yu (Pers. Comm., 1997) as *Bradyrhizobium* sp. was adopted.
96 first para	The increase in lag period does not accurately reflect the proportional reduction of PCP-degrading ET01 cell numbers for all combinations tested. For example at 150mg/l initial PCP concentration for the three combinations should theoretically produce a lag period three times that of ET01 in pure culture. The lag period was closer to twice that for ET01 in pure culture. This is one example of the three cultures in combination achieving a better result than was expected or seen with ET01 with only one of the other isolates (lag period would have had to be well below twice that for ET01 in pure culture). For initial PCP concentration of 50mg/l for the three isolates, the lag period was almost four times that of ET01 in pure culture at the same initial PCP concentration. The full inoculation of ET01 cells was not tested in combination with ET02 and ET03.
97 Fig 4.13	The following legend applies. PCP concentration mg/l (o); Ln Cell numbers (•).
101	Viable counts were repeatedly attempted and were initially the main aim of the project however due to technical difficulties were never deemed scientifically acceptable. Because ET02 and ET03 contributed minimally to the biomass (as measured by OD) only ET01 was considered for Ki calculations.
111 line 3	This statement reconfirms that ET02 was unlikely to be *Sphingomonas* spp. and the isolate was tentatively classified as *Pseudomonas putida* (Hussein *et al.*, 1996). Some *Pseudomonas* spp. were reclassified as *Sphingomonas* spp. by Nohynek *et al.*,.(1995) and Haggblom *et al.*, (1995).
125 para 2	There were no phenotypic tests of ET01 undertaken. The level of 16s-rRNA sequence similarity between *Bradyrhizobium* and ET01 was "very high" (Yu, Pers. Comm., 1997).

B. ERRATA LISTING

PAGE	LINE	CURRENTLY READS	SHOULD READ
10	8	H=ggblom	Häggblom
18	13	Yu and Shepherd, 1997	Yu and Shepherd, 1997
21	26	KF1^{T}	KF1
59	18	16sRNA	16S rRNA
72	2	"incubation temperature"	"incubation temperatures"
125	4	(Yu, 1998)	(Yu, 1997)

C. BIBLIOGRAPHY

PAGE

143 Tchan, Y-T and P. B. New. 1984. Genus I. *Azotobacter* Beijerinck 1901, 567^{AL}, in Bergey's Manual of Systematic Bacteriology. Editor in Chief - Holt, J.G.. Williams and Wilkins. Baltimore London. Volume 1, Pages 220 – 229.

145 World Health Organization (WHO). 1989. Pentachlorophenol Health and Safety Guide No. 19 0259 – 7268. WHO, Geneva, Switzerland.

146 Yu, P-L and J. Shepherd. 1997. Pentachlorophenol in New Zealand: A Biological Treatment Option. Australasian Biotechnology, **7**: 340–344.

BIODEGRADATION OF PENTACHLOROPHENOL

A THESIS PRESENTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF TECHNOLOGY IN ENVIRONMENTAL ENGINEERING AT MASSEY UNIVERSITY, PALMERSTON NORTH, NEW ZEALAND.

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ABSTRACT

Three isolates previously isolated from pentachlorophenol (PCP) contaminated soil as a consortium were tested for their ability to remove PCP from a minimal mineral salts medium with and without vitamin supplementation. Only one of the isolates, designated *Bradyrhizobium* sp. strain ET01, could utilise PCP as a sole source of carbon and energy. The other two isolates designated *Pseudomonas putida* strain ET02 and formerly *Pseudomonas aureofaciens* strain ET03 could grow in the presence of PCP but could not utilise it as a sole source of carbon and energy. The effects of various initial PCP concentrations and vitamin supplementation on the kinetics of PCP removal and the cell numbers for ET01 and culture combinations was tested.

An increasing initial PCP concentration affected the PCP removal rate, the lag period, the cell yield, cell numbers and specific growth rate. PCP removal by ET01 ceased at a concentration of 175mg/l. The PCP removal rate increased for ET01 in pure culture through the course of the experiments. The rate of removal at 150mg/l initial PCP concentration improved from 1.48mg/l/hr to 1.85mg/l/hr. The rate of removal at 120mg/l initial PCP concentration improved from 1.38mg/l/hr to a maximum of 2.10mg/l/hr. The shortest lag period was 4 hours for ET01 in pure culture on 20mg/l initial PCP concentration. The lag period for ET01 in pure culture was 0.30 of the initial PCP concentration. The size of the inoculum of ET01 had an effect on the lag period and the rate of PCP removal. Cell yield was extremely low for ET01 and the culture combinations at all initial PCP concentrations tested. Measurable PCP removal was observed when the cell density of ET01 reached approximately $1x10^7$ cells/ml. The final number of cells for ET01 for initial PCP concentrations over the range of 20mg/l to 150mg/l was approximately $5.5x10^7$ cell per ml (0.09mg/l). The highest specific growth rate for ET01, $0.06hr^{-1}$, occurred in media containing yeast extract and at an initial PCP concentration of 40mg/l.

Continuous subculturing under the selective pressure of PCP as the sole carbon and energy source in media containing yeast extract led to an increased PCP removal rate and a decreased lag period for ET01. There was a slight increased rate effect of combining ET01 and ET02, but generally ET01 in pure culture removed PCP at a higher rate than any of the culture combinations.

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TABLE OF CONTENTS

		Page
ABSTRACT		i
ACKNOWLEDGEMENTS		ii
TABLE OF CONTENTS		iii
LIST OF FIGURES		viii
LIST OF TABLES		xvii
1. Introduction		**1**
2. Literature Review		**3**
2.1	**Pentachlorophenol**	**3**
	2.1.1 Toxicity of Pentachlorophenol	3
	2.1.2 Production of Pentachlorophenol	5
	2.1.3 PCP Contamination in New Zealand	7
2.2	**Bioremediation of PCP Contaminated Sites**	**9**
	2.2.1 Biological Treatment Technologies	10
	2.2.1.1 Solid Phase Treatment	11
	2.2.1.2 *In Situ* Bioremediation	11
	2.2.1.3 Slurry-Phase Biotreatment	12
	2.2.2 Bioremediation Case Studies	13
	2.2.3 Phytoremediation and Anaerobic Bioremediation	15
	2.2.4 Feasibility of Bioremediation	17

		Page
2.3	**PCP Degrading Microorganisms**	**20**
	2.3.1 Pseudomonadaceae	20
	2.3.2 Sphingomonadaceae	21
	2.3.3 Actinomycetes	23
	2.3.4 Other Aerobic PCP Degrading Strains	26
	2.3.5 Anaerobic PCP Degraders	27
	2.3.6 PCP Degrading Fungi	28
	2.3.7 PCP Degrading Microalgae	29
2.4	**Growth Kinetics**	**30**
	2.4.1 Parameters Affecting Growth	31
	2.4.1.1 Initial PCP Concentration	31
	2.4.1.2 Inoculum	32
	2.4.1.3 Carbon Supplementation	34
	2.4.1.4 Temperature	35
	2.4.1.5 pH	36
	2.4.1.5 Plasmids	36
	2.4.2 Growth Rates	37
2.5	**Biochemistry of PCP Degradation**	**42**
	2.5.1 Aerobic Degradation of PCP	43
	2.5.1.1 The *Sphingomonas* spp. Pathway	44
	2.5.1.2 The *Mycobacterium* spp. Pathway	48
	2.5.2 Anaerobic Degradation of PCP	50
	2.5.3 Composite PCP Degradation Pathways	53
	2.5.3.1 *O*-Methylation of PCP	56
	2.5.3.2 Photodegradation of PCP	56
3.	**Materials and Methods**	**59**
3.1	Cultures	59
3.2	Growth and Maintenance of Cultures	60

		Page
	3.2.1 Inoculum	60
	3.2.2 Culture Growth	61
3.3	HPLC Analysis	62
3.4	Chloride Analysis	63
3.5	Epifluorescence Staining	64
	3.5.1 Acridine Orange Staining	65
	3.5.1.1 Reagents	65
	3.5.1.2 Staining Procedure and Cell Counts	66
	3.5.2 Cell number conversion	68
	3.5.3 CTC Staining	68
	3.5.3.1 Reagents	68
	3.5.3.2 Staining Procedure	70
	3.5.3.3 Staining Parameters Investigated	71
	3.5.4 Sequential CTC/AO Staining	72
3.6	Vitamin Experiment	72
3.7	Rate Determination	74
3.8	Calculation of Specific Growth Rate	74
3.9	Calculation of Inhibition Constant	74
3.10	Calculation of 95% Confidence Intervals	75

4. Results **76**

4.1	Isolate Growth and Characteristics	76
4.2	Initial PCP Removal	77
	4.2.1 Lag Period for Initial PCP Removal	81
4.3	PCP Removal and Chloride Release	82
	4.3.1 Theoretical and Actual Chloride Release	87
	4.3.2 Lag Period	89
4.4	Synergism Experiments	90
	4.4.1 Lag Period for Culture Combinations	94
4.5	PCP Removal and Organism Growth	96

		Page
4.6	Vitamin Experiment	102
	4.6.1 Lag Period for Vitamin Experiment	107
5.	**Discussion**	**109**
5.1	Introduction	109
5.2	Isolates	110
5.3	PCP Removal	111
5.4	Chloride Release	114
5.5	Lag Period	115
5.6	Cell Density and Yield	117
5.7	Specific Growth Rate	119
5.8	Culture Combinations	121
5.9	Vitamins	123
6.	**Conclusions**	**125**
7.	**Literature Cited**	**129**
A8.	**Appendix**	**148**
A8.1	Fluorescence Microscopy Staining Techniques	148
	A8.1.1 Direct Epifluorescence Microscopy	148
	A8.1.2 Fluorescent Dyes	151
	A8.1.2.1 Total Bacterial Counts	151
	A8.1.2.2 Metabolically active bacterial counts	153
A8.2	CTC Validation Experiment	156
	A8.2.1 CTC Staining	156
A8.3	Minerals and Vitamins	157
	A8.3.1 Minerals	157
	A8.3.2 Vitamins	158

		Page
A8.4	Initial PCP Removal Regression Equations	161
A8.5	PCP Removal and Chloride Release	163
A8.6	PCP Removal Regression Equations	167
A8.7	Chloride Release Regression Equations	168
A8.8	Theoretical versus Actual Chloride Release	169
A8.9	Culture Combination PCP Removal	172
A8.10	Culture Combination PCP Removal Regression Equations	173
A8.11	PCP Removal and Organism Growth	174
A8.12	Specific Growth Rates for ET01 and Culture Combinations	180
A8.13	Yield for ET01 and Culture Combinations	181
A8.14	Yield Regression Equations	187
A8.15	Composite Table of PCP Removal, Chloride Release, Yield and Specific Growth Rates	188
A8.16	Vitamin Experiment Regression Equations	189

LIST OF FIGURES

Figure number		Page
2.1	The effect of initial PCP concentration on lag time for *Arthrobacter* sp. strain NC in batch culture (Stanlake and Finn, 1982).	31
2.2	Cell yield (dry weight) produced at various PCP concentrations for *Arthrobacter* sp. strain NC in batch culture (Stanlake and Finn, 1982).	33
2.3	Effect of PCP concentration on specific growth rate (Gu and Korus, 1995).	38
2.4	Specific growth rate of *Arthrobacter* sp. strain NC (Stanlake and Finn, 1982).	39
2.5	The relationship between initial PCP concentration and specific growth rate calculated by using the Haldane modification of the Monod equation. Values of the parameters used for the calculation were; $\mu_{max} = 0.074h^{-1}$, $K_s = 0.060$mg/l and $K_i = 1.375$mg/l (Klecka and Maier, 1985).	40
2.6	Dehalogenation mechanisms involved in the degradation of PCP (McAllister *et al.*, 1996).	43
2.7	Summary of the aerobic *Sphingomonas* spp. pathway with steps from VI on derived from *Azotobacter* and *Streptococcus*.	45
2.8	Degradation of tetrachloro-*p*-hydroquinone (TeCHQ) to trichlorohydroquinone (TCHQ) by tetrachloro-*p*-hydroquinone reductive halogenase via the *S*-chloromethyl glutathione conjugate.	46

Figure number Page

2.9 Reaction sequence of 2-chloromaleylacetate catalysed by maleylacetate reductase producing 3-oxoadipate (Kaschabek and Reineke, 1992; Vollmer *et al.*, 1993). 48

2.10 Summary of the *Mycobacterium* spp. aerobic PCP degradation pathway. 50

2.11 Summary of the anaerobic pathway of PCP degradation by pure and mixed cultures. 52

2.12 A composite diagram illustrating the links between the anaerobic and aerobic PCP degradation pathways. 55

2.13 The products of photodegradation of PCP (Kaufman, 1978; McAllister *et al.*, 1996). 57

4.1 PCP removal by ET01 at three initial PCP concentrations (a) with 25mg/l yeast extract; (b) without 25mg/l yeast extract. 78

4.2 PCP removal by ET01 at varying initial PCP concentrations (a) with 25mg/l yeast extract; (b) without 25mg/l yeast extract. 79

4.3 PCP removal rates by ET01 at various initial PCP concentrations with and without 25mg/l yeast extract. 80

4.4 Lag period versus initial PCP concentrations for ET01. 82

4.5 Removal of PCP by ET01 at an initial concentration of 20mg/l and concomitant chloride release in media (a) with 25mg/l yeast extract; (b) without 25mg/l yeast extract. 83

Figure number Page

4.6 PCP removal rates for ET01 at various initial PCP concentrations with and without 25mg/l yeast extract. 86

4.7 Chloride release rates for ET01 at various initial PCP concentrations with and without 25mg/l yeast extract. 87

4.8 Theoretical and actual chloride release for ET01 at an approximate initial PCP concentration of 20mg/l (a) with 25mg/l yeast extract; (b) without 25mg/l yeast extract. 88

4.9 Lag period versus PCP concentration for ET01. 90

4.10 PCP removal from 50mg/l initial PCP concentration by culture combinations as indicated (see legend) in a minimal mineral salts (MMS) medium with 25mg/l yeast extract. 92

4.11 PCP removal rates for the culture combinations at three initial PCP concentrations as indicated (see legend) in a minimal mineral salts (MMS) medium with 25mg/l yeast extract. 94

4.12 The effect of initial PCP concentration on the lag period for ET01 and culture combinations as indicated (see legend) in a minimal mineral salts (MMS) medium with 25mg/l yeast extract. 96

4.13 Increase in ET01 cell numbers at an initial PCP concentration of 20mg/l. 97

4.14 Yield for ET01 in pure culture and culture combinations (see legend) in a minimal mineral salts (MMS) medium with 25mg/l yeast extract. 99

Figure number Page

4.15 Specific growth rates for ET01 in pure culture and culture combinations (see legend) in a minimal mineral salts (MMS) medium with 25mg/l yeast extract. 100

4.16 Removal of 50mg/l PCP by ET01 in pure culture with different vitamin supplementation (see legend). 102

4.17 Removal of 50mg/l PCP by ET01 and ET02 with different vitamin supplementation (see legend). 103

4.18 Removal of 50mg/l PCP by ET01 and ET03 with different vitamin supplementation (see legend). 103

4.19 Removal of 50mg/l PCP by ET01, ET02 and ET03 with different vitamin supplementation (see legend). 104

A8.1 The structure of acridine orange (Kodak, 1996). 151

A8.2 The structure of DAPI (Haughland, 1996). 152

A8.3 CTC reduced to the water-insoluble fluorescence CTC-formazan (Haughland, 1996). 153

A8.4 Removal of PCP at an initial concentration of 40mg/l and concomitant chloride release (a) with 25mg/l yeast extract; (b) without 25mg/l yeast extract. Theoretical chloride release from 40mg/l PCP is 26.8mg/l. 163

A8.5 Removal of PCP at an initial concentration of 50mg/l and concomitant chloride release (a) with 25mg/l yeast extract; (b) without 25mg/l yeast extract. Theoretical chloride release from 50mg/l PCP is 33.5mg/l. 163

Figure number		Page
A8.6	Removal of PCP at an initial concentration of 60mg/l and concomitant chloride release (a) with 25mg/l yeast extract; (b) without 25mg/l yeast extract. Theoretical chloride release from 60mg/l PCP is 40.2mg/l.	164
A8.7	Removal of PCP at an initial concentration of 80mg/l and concomitant chloride release (a) with 25mg/l yeast extract; (b) without 25mg/l yeast extract. Theoretical chloride release from 80mg/l PCP is 53.6mg/l.	164
A8.8	Removal of PCP and concomitant release of chloride at an initial concentration of 100mg/l PCP. Theoretical chloride release from 100mg/l PCP is 67.7mg/l.	165
A8.9	Removal of PCP and concomitant release of chloride at an initial concentration of 120mg/l PCP. Theoretical chloride release from 120mg/l PCP is 80.4mg/l.	165
A8.10	Removal of PCP and concomitant release of chloride at an initial concentration of 150mg/l PCP. Theoretical chloride release from 150mg/l PCP is 100mg/l.	165
A8.11	Removal of PCP and concomitant release of chloride at an initial concentration of 175mg/l PCP. Theoretical chloride release from 175mg/l is 116.7mg/l.	166
A8.12	Theoretical and actual chloride release for PCP at an initial concentration of 40mg/l (a) with 25mg/l yeast extract; (b) without 25mg/l yeast extract.	169

Figure number Page

A8.13 Theoretical and actual chloride release for PCP at an initial concentration of 50mg/l (a) with 25mg/l yeast extract; (b) without 25mg/l yeast extract. 169

A8.14 Theoretical and actual chloride release for PCP at an initial concentration of 60mg/l (a) with 25mg/l yeast extract; (b) without 25mg/l yeast extract. 170

A8.15 Theoretical and actual chloride release for PCP at an initial concentration of 80mg/l (a) with 25mg/l yeast extract; (b) without 25mg/l yeast extract. 170

A8.16 Theoretical and actual chloride release for PCP at an initial concentration of 100mg/l with 25mg/l yeast extract. 170

A8.17 Theoretical and actual chloride release for PCP at an initial concentration of 120mg/l with 25mg/l yeast extract. 171

A8.18 Theoretical and actual chloride release for PCP at an initial concentration of 150mg/l with 25mg/l yeast extract. 171

A8.19 PCP removal from 100mg/l initial PCP concentration by three bacterial cultures in pure culture and in combinations as indicated. 172

A8.20 PCP removal from 150mg/l initial PCP concentration by ET01 and culture combinations as indicated. 172

A8.21 Increase in ET01 cell numbers as PCP is depleted from an initial PCP concentration of 40mg/l. 174

Figure number Page

A8.22 Increase in ET01 cell numbers as PCP is depleted from an initial PCP concentration of 60mg/l. 174

A8.23 Increase in ET01 cell numbers as PCP is depleted from an initial PCP concentration of 80mg/l. 175

A8.24 Increase in ET01 cell numbers as PCP is depleted from an initial PCP concentration of 50mg/l. 175

A8.25 Increase in ET01 and ET02 cell numbers as PCP is depleted from an initial PCP concentration of 50mg/l. 176

A8.26 Increase in ET01 and ET03 cell numbers as PCP is depleted from an initial PCP concentration of 50mg/l. 176

A8.27 Increase in ET01, ET02 and ET03 cell numbers as PCP is depleted from an initial PCP concentration of 50mg/l. 176

A8.28 Increase in ET01 cell numbers as PCP is depleted from an initial PCP concentration of 100mg/l. 177

A8.29 Increase in ET01 and ET02 cell numbers as PCP is depleted from an initial PCP concentration of 100mg/l. 177

A8.30 Increase in ET01 and ET03 cell numbers as PCP is depleted from an initial PCP concentration of 100mg/l. 177

A8.31 Increase in ET01, ET02 and ET03 cell numbers as PCP is depleted from an initial PCP concentration of 100mg/l. 178

A8.32 Increase in ET01 cell numbers as PCP is depleted from an initial PCP concentration of 150mg/l. 178

Figure number Page

A8.33 Increase in ET01 and ET02 cell numbers as PCP is depleted from an initial PCP concentration of 150mg/l. 178

A8.34 Increase in ET01 and ET03 cell numbers as PCP is depleted from an initial PCP concentration of 150mg/l. 179

A8.35 Increase in ET01, ET02 and ET03 cell numbers as PCP is depleted from an initial PCP concentration of 150mg/l. 179

A8.36 Biomass produced for PCP removed for ET01 at 20mg/l initial PCP concentration. 181

A8.37 Biomass produced for PCP removed for ET01 at 40mg/l initial PCP concentration. 181

A8.38 Biomass produced for PCP removed for ET01 at 60mg/l initial PCP concentration. 182

A8.39 Biomass produced for PCP removed for ET01 at 80mg/l initial PCP concentration. 182

A8.40 Biomass produced for PCP removed for ET01 at 50mg/l initial PCP concentration. 182

A8.41 Biomass produced for PCP removed for ET01 and ET02 in combination at 50mg/l initial PCP concentration. 183

A8.42 Biomass produced for PCP removed for ET01 and ET03 in combination at 50mg/l initial PCP concentration. 183

A8.43 Biomass produced for PCP removed for ET01, ET02 and ET03 in combination at 50mg/l initial PCP concentration. 183

Figure number Page

A8.44 Biomass produced for PCP removed for ET01 at 100mg/l initial PCP concentration. 184

A8.45 Biomass produced for PCP removed for ET01 and ET02 in combination at 100mg/l initial PCP concentration. 184

A8.46 Biomass produced for PCP removed for ET01 and ET03 in combination at 100mg/l initial PCP concentration. 184

A8.47 Biomass produced for PCP removed for ET01, ET02 and ET03 in combination at 100mg/l initial PCP concentration. 185

A8.48 Biomass produced for PCP removed for ET01 at 150mg/l initial PCP concentration. 185

A8.49 Biomass produced for PCP removed for ET01 and ET02 in combination at 150mg/l initial PCP concentration. 185

A8.50 Biomass produced for PCP removed for ET01 and ET03 in combination at 150mg/l initial PCP concentration. 186

A8.51 Biomass produced for PCP removed for ET01, ET02 and ET03 in combination at 150mg/l initial PCP concentration. 186

LIST OF TABLES

Table Number		Page
2.1	The physical properties of PCP and NaPCP (WHO, 1989).	5
2.2	Degradation rates of PCP by pure cultures of bacteria.	37
3.1	Summary of staining procedure information as recommended by Kepner and Pratt (1994).	65
4.1	Line drawing of each isolates' cellular morphology and cell size.	76
4.2	Biochemical characteristics of ET01, ET02 and ET03.	77
4.3	PCP removal rates at three initial PCP concentrations with and without 25mg/l yeast extract.	78
4.4	PCP removal rates at various initial PCP concentrations.	79
4.5	Lag periods for a range of initial PCP concentrations for ET01 with yeast extract.	81
4.6	PCP removal and chloride release rates for ET01 at various initial PCP concentrations with and without 25mg/l yeast extract.	85
4.7	Lag periods for an range of initial PCP concentrations for ET01.	89
4.8	PCP removal rates for culture combinations at three initial PCP concentrations in a minimal mineral salts (MMS) medium with 25mg/l yeast extract.	93
4.9	Lag periods for ET01 and culture combinations at three initial PCP concentrations in a minimal mineral salts (MMS) medium with 25mg/l yeast extract.	95

Table Number		Page
4.10	Yields and specific growth rates for ET01 and culture combinations in MMS broth containing 25mg/l yeast extract.	98
4.11	K_i values for ET01 and the culture combinations.	101
4.12	PCP removal rates for culture combinations with different vitamin supplementation (MMS medium containing an initial PCP concentration of 50mg/l).	105
4.13	Lag periods for culture combinations with different vitamin supplementation (MMS medium containing an initial PCP concentration of 50mg/l).	107
A8.1	Regression equations for PCP removal at various initial PCP concentrations with and without 25mg/l added yeast extract.	161
A8.2	Regression equations for PCP removal at various initial PCP concentrations with and without 25mg/l added yeast extract.	162
A8.3	Regression equations for PCP removal by culture combinations at 50, 100 and 150mg/l PCP.	167
A8.4	Regression equations for chloride release at various initial PCP concentrations with and without 25mg/l added yeast extract.	168
A8.5	Regression equations for PCP removal by culture combinations at 50, 100 and 150mg/l PCP.	173
A8.6	Regression equations for cell growth, ln (cell numbers), of ET01 at various PCP concentrations and culture combinations at 50, 100 and 150mg/l PCP.	180

Table Number		Page
A8.7	Biomass yield for ET01 and culture combinations.	187
A8.8	PCP removal rate, chloride release rate, yield and specific growth rate for ET01 in pure culture with yeast extract.	188
A8.9	Regression equations for the culture combinations with different vitamin supplementation.	189

1. INTRODUCTION

Pentachlorophenol (PCP) is a highly toxic and persistent organochlorine compound. Pentachlorophenol and its sodium salt, sodium pentachlorophenate (NaPCP), have been utilised in New Zealand for their fungicidal and bactericidal properties since the 1950's (Bingham, 1992). PCP and its derivatives have a relatively low production cost compared to other biocides. In addition they offer the advantages of oil solubility, water solubility (in the sodium salt form), persistence and fairly widespread microbiological activity. This has meant that historically these materials have been applied not only for wood preservation and protection but also across a spectrum of manufactured materials and situations where antimicrobial activity is required (Bingham, 1992).

Freshly sawn green softwood timber which is susceptible to attack by sapstain and decay-causing fungi and must be protected by chemical treatment. Timber treatment with PCP has included both surface treatment to provide short-term protection, as an antisapstain, and less frequently, pressure treatment to afford long-term protection of the timber (Nadebaum *et al.*, 1992). As a result the soil of a large number of timber treatment sites around New Zealand are contaminated by PCP and its polychlorinated dioxin (PCDD) and dibenzofuran (PCDF) impurities (Bingham, 1992; Jackman, 1992; Szabo, 1993).

The timber industry was made aware of the significance of the environmental and health problems associated with NaPCP in the period preceding 1988, so that by the middle of that year the industry had moved substantially to adopt alternative antisapstain treatments. On 31 December 1991 the Pesticide Board de-registered all sodium pentachlorophenate timber treatment products (Bingham, 1992).

Pentachlorophenol poses a serious environmental risk because it is acutely toxic and is relatively resistant to chemical and biological degradation (Knackmuss and Hellwig, 1978). It is important that PCP is removed from the environment as it affects reproduction, impairs the immune system and poses carcinogenic, foetotoxic and teratogenic risks (Jackman, 1992) and is easily absorbed through the skin (WHO, 1989).

Because PCP has been present in the environment since early this century micro-organisms present in the contaminated water and soils have adapted to growth in the presence of PCP and micro-organisms have been isolated that can utilise PCP as a growth substrate (Watanabe, 1973; Stanlake and Finn, 1982; Shelton and Tiedje, 1984; Saber and Crawford, 1985; Utkin *et al.*, 1994; Bouchard *et al.*, 1996; Hussein *et al.*, 1996). Micro-organisms which are able to degrade pentachlorophenol (PCP) can still be killed or experience growth inhibition if they are exposed to a sufficiently high PCP concentrations as it is an uncoupler of oxidative phosphorylation (Klecka and Maier, 1985; Stanlake and Finn, 1982; Gonzalez and Hu, 1995; Miethling and Karlson, 1996).

The method of choice to remove PCP from the environment is by biological remediation or bioremediation. Bioremediation combines biology and technology and the resulting biotechnology has applications to environmental problems (Fetzner and Lingens, 1994). Bioremediation utilises the ability of certain micro-organisms to degrade organic compounds to simpler molecules and ultimately to carbon dioxide and water. This project contributes to the characterisation of PCP utilising micro-organisms and the biodegradation technology of this recalcitrant compound.

The following literature review will examine aspects of bioremediation of PCP contaminated sites, the various micro-organisms that can utilise PCP and the kinetic parameters of PCP degradation. The biochemistry of PCP utilisation by aerobic and anaerobic micro-organisms will also be presented.

2. LITERATURE REVIEW

2.1 PENTACHLOROPHENOL (PCP) IN NEW ZEALAND

New Zealand produces approximately 2 million cubic metres of sawn timber per annum (Jackman, 1992). Traditionally pine was used in the pulp and paper industry, but as chemical timber preservation techniques developed and mass production became possible, pine became a widely used construction material in New Zealand and an important export product. Thus the development of timber preservation techniques enabled construction material to have an extended life time. Over the past thirty to forty years various synthetic chemicals have been used to stop fungal and bacterial attack of freshly sawn timber, to prevent sapstain and for the permanent preservation of timber from decay caused by attacks from fungi, bacteria, mites and insects. The anti-sapstain biocides pentachlorophenol (PCP) and sodium pentachlorophenol (NaPCP) are two of the most widely used industrial timber preservatives (Jackman, 1992).

2.1.1 TOXICITY OF PCP

Pentachlorophenol is a highly toxic, persistent organochlorine chemical used widely as a timber preservative and pesticide over the past 40 years in New Zealand (Jackman, 1992). Pentachlorophenol and its sodium salt (NaPCP) are among the most widely distributed and versatile biocides used in the world and because of their efficiency, broad spectrum and low cost, they have been utilised as algicides, bactericides, fungicides, herbicides, insecticides, and molluscicides with a variety of applications in the industrial, agricultural and domestic fields (WHO, 1989).

The first time attention was drawn to the toxicity of PCP was in 1906 by Bechold and Ehrlich, but not until 1938 were experiments conducted in the USA that extended into the 1940s able to show that PCP was toxic to humans and was readily absorbed through the skin. Associated health and neurological disorders were described by the WHO as early as 1953 (Jackman, 1992). Thus, the toxicity and lethality, towards humans, of PCP has been known for more than 50 years (Jackman, 1992).

PCP is an inhibitor of oxidative phosphorylation and is toxic to a variety of organisms (Mileski *et al.*, 1988). Pentachlorophenol is acutely toxic to fish and a concentration of 0.6mg/l is lethal for most species (Edgehill and Finn, 1983). Adverse effects on aquatic organisms have been observed at PCP concentrations >34μg/l (Gifford *et al.*, 1994). PCP has been shown to bioaccumulate (i.e. an increase in body concentration relative to the environment) in aquatic biota (Gifford *et al.*, 1994). As PCP was formulated as an antimicrobial agent, it's recalcitrance in the environment and resistance to microbial attack is no surprise. The high degree of chlorination and toxicity of PCP make biodegradation a slow process. Many organisms, however, have been isolated from PCP contaminated soils, and some degrade and mineralise PCP, albeit slowly.

The main occupational health problems reported among PCP users in New Zealand have tended to be dermatitis related. However, PCP and its contaminants have been linked to types of cancer (Jackman, 1992). The USEPA has determined that PCP poses carcinogenic, foetotoxic and teratogenic risks (Jackman, 1992). Of particular concern is the growing body of evidence that shows that organochlorines affect reproduction, impair the immune system and cause cancer (Jackman, 1992). The acute health effects of exposure to high concentrations of technical grade PCP are generally the result of the biological action of the PCP molecule itself. In contrast to this the chronic effects and the effects of long-term exposure to technical grade PCP are most probably related largely to the biological action of the polychlorodibenzo-*p*-dioxins (PCDDs) and polychlorodibenzo-*p*-furans (PCDFs) (WHO, 1989). Pentachlorophenol is absorbed through the intact skin and the respiratory and gastrointestinal tracts and is distributed in tissues (WHO, 1989). Pentachlorophenol does not remain in the human body as long as many other organochlorines and is mostly removed in the urine with a half-life of 30 hours (Morton, 1997). Median urinary PCP concentrations of approximately 11 mg/l are typical for workers in contact with PCP, compared with urinary concentrations of approximately 0.01 mg/l for the general population (WHO, 1989). Exposure of the general population to low doses of PCP is common. Pentachlorophenol has been found in air, food, water, and consumer products (WHO, 1989).

The exact duration and concentration of exposure to PCP needed to cause adverse effects in humans are not well known, though 0.09mg/l in air will cause irritation of eyes and nose and 14mg/l is immediately dangerous to life and health in humans. Its current virtually safe dose (VSD) level is set at 0.006 pg/kg body weight per day (WHO, 1989). In 1977, the US National Academy of Sciences set the Acceptable Daily Intake (ADI) at 3µg/kg body weight per day (WHO, 1989). In New Zealand, the National Task Group has adopted a level of 10 pg/kg body weight, a standard which allows the highest level of contamination and requires the lowest standard for clean-up (Jackman, 1992).

2.1.2 PRODUCTION OF PCP

Pentachlorophenol consists of colourless to light brown flakes or crystals, with a characteristic phenolic odour (WHO, 1989). It is practically insoluble in water and decomposes on heating in the presence of water, forming hydrochloric acid. It is non-corrosive and non-flammable in an unmixed state, however dissolved in oil, PCP causes deterioration of rubber. The sodium salt of PCP, sodium pentachlorophenate (NaPCP) is marketed as a tan powder, pellets, or briquettes with a phenolic odour. NaPCP is readily soluble in water. It decomposes on heating forming toxic fumes, chlorides and sodium oxide. The physical properties of PCP and NaPCP are noted in Table 2.1.

Table 2.1 The physical properties of PCP and NaPCP (WHO, 1989) (ND = not determined).

	PCP	NaPCP
Molecular formula	C_6Cl_5OH	C_6Cl_5ONa
Boiling point	310°C (decomposition)	decomposition
Melting point	191°C	decomposition
Relative density (H_2O = 1)	2.0	2.0
(air = 1)	9.2	ND
Vapour pressure (20 °C)	2 mPa	ND
Relative molecular mass	266.3	288.3
Octanol/ water part. coeff. (pH 6.5)	3.56	ND
Solubility in water	2 g/l (20°C, pH 7)	330 g/l (25°C)
Solubility in organic solvents (25 °C) acetone	500 g/l	350 g/l
benzene	150 g/l	insoluble
ethanol	1200 g/l	650 g/l
methanol	1800 g/l	250 g/l

Pentachlorophenol and its derivatives have a relatively low production cost compared to other biocides. In addition they offer the advantages of oil solubility, water solubility (in the sodium salt form), persistence and a wide spectrum of anti-microbiological activity. This has meant that historically these materials have been applied not only for wood preservation and protection but also across a range of manufactured materials and applications where antimicrobial activity is required (Bingham, 1992).

Pentachlorophenol is produced by reacting chlorine gas with phenol in the presence of catalysts such as anhydrous aluminium chloride or ferrous chloride. It is also possible to manufacture it via the alkaline hydrolysis of hexachlorobenzene (McAllister *et al.*, 1996). During the manufacture of PCP "a large number" of toxic impurities such as polychlorodibenzo-dioxins and furans (dioxins and furans) are also produced. They represent some of the most toxic substances known. Dioxins and furans comprise a family of over 200 individual compounds and are highly persistent in the environment and have been shown to accumulate in mammalian tissue (Bingham, 1992). The extent of contamination of pentachlorophenol by dioxins and furans impurities during it's production is closely related to the care exercised during manufacture. Over chlorination of the product can be prevented by minimising the temperature of the reactor system at it's various chlorination stages reducing the impurities (Bingham, 1992).

Pentachlorophenol has been imported in to New Zealand and distributed by a number of companies since its introduction in the early 1950's. Pentachlorophenol has not at any time been manufactured in New Zealand, however a number of companies have been involved in the production of sodium pentachlorophenate by addition of base to pentachlorophenol raw material. Where contamination of the pentachlorophenol starting material is significant, a sludge waste may be generated during the pentachlorophenate production process which is enriched in PCDDs and PCDFs. A sample from this waste stream was analysed and contained a concentration of 56mg/l toxic equivalents (Jackman, 1992). No data exists on the quantities of this waste which have been produced (Jackman, 1992).

Up to 200 tonnes of PCP were used per annum in New Zealand over a period of approximately 40 years and approximately 5000 tonnes are believed to have been used in total. In Finland, the estimated total use of PCP since the 1930's was 25, 000 tonnes (McAllister *et al.*, 1996). Spills, leaks and dumping have resulted in PCP entering the soil and groundwater around the treatment plants in New Zealand (Szabo, 1993). It has been estimated that about 70% of all sawmills in New Zealand have used PCP (Szabo, 1993).

2.1.3 PCP CONTAMINATION IN NEW ZEALAND

Prior to 1970 NaPCP was the only prophylactic wood preservative, insecticide and herbicide used commercially in New Zealand (Gonzalez and Hu, 1995). From the early 1970s concerns were raised about potential wide scale sawmill site contamination with PCP, dioxins and furans, and their possible effects on the environment and human health in New Zealand (Gifford *et al.*, 1994). Antisapstain researchers embarked on trials to secure alternative replacements (Bingham, 1992). The timber industry was made aware of the significance of the environmental and health problems associated with PCP and NaPCP in the period preceding 1988. By the middle of 1988, when PCP was voluntarily withdrawn from use by the timber industry (Jackman, 1992), there had already been a substantial move to adopt alternative antisapstain treatments.

A major problem only recognised recently is that PCP can form dioxins in the environment (Jackman, 1992). Over the last few years, researchers have come to suspect that chlorinated dioxins and furans can be formed from chlorophenols under ambient conditions as well as at the high temperatures normally associated with their creation in industrial settings. It was discovered that enzymes could catalyse these reactions *in vitro*. A labeling study has indicated that PCP reacts to form highly chlorinated dioxins and furans in sewage sludge (Jackman, 1992).

In 1990 the New Zealand Government appointed the New Zealand National Task Group (NTG) to investigate site contamination from the use of timber treatment chemicals (Jackman, 1992) and on 31 December 1991 the Pesticide Board de-registered all sodium

pentachlorophenate timber treatment products (Bingham, 1992). In August 1992 the NTG reported that there are more than 600 contaminated timber treatment sites and PCP dump sites in New Zealand. Of the 600 plus known sites contaminated with PCP and its derivatives, only the Waipa sawmill and Lake Rotorua have been studied by the New Zealand National Task Group to date (Bingham, 1992).

As well as contaminated timber treatment sites throughout the country there are an unknown number of dump sites containing contaminated waste. There is significant contamination of the wider environment by PCP, dioxins and furans and other constituents of the PCP formulations through leaching and movement into groundwater and accumulation in food chains (Bingham, 1992; Jackman, 1992; Nadebaum *et al.*, 1992; Szabo, 1993). Where large areas of subsurface soils are polluted by PCP, there are limited options for clean up. Studies have shown that biological treatment using microbial biodegradation is a possible means to deal with this type of waste (Radehaus and Schmidt, 1992).

Pentachlorophenol has not apparently been used in New Zealand as a dressing for seeds, as a preservative in the leather or tanning industries, as a biocide in drilling rigs, as an antimicrobial in wallpaper adhesives or as a protection agent in the wool manufacturing industry, as it has in other countries (Bingham, 1992). PCP has been applied to soil surfaces in flooded paddy fields in the USA and infiltrates into the soil with percolating water (Murthy *et al.*, 1979). It has also been used as a soil and timber sterilant in mushroom culture (Bingham, 1992).

2.2 BIOREMEDIATION OF PCP CONTAMINATED SITES

Bioremediation is a biologically-based treatment process used to degrade industrial based organic compounds and waste materials into simpler molecules and ultimately to carbon dioxide, water and inorganic salts (Hayes, 1996). Bioremediation combines biology and technology and the resulting biotechnology has applications to environmental problems (Fetzner and Lingens, 1994). Bioremediation methods are extremely advantageous in that they ultimately mineralise a contaminant and involve little, if any, movement of the contaminated material. Biological treatment of hazardous wastes has been practiced successfully for many years, and the bioremediation industry has experienced sustained rapid growth over the past decade. The costs of bioremediation can vary from very cheap, as in land tilling and composting, to very expensive in the case of controlled leaching followed by bioreactor treatment of leachate.

Halophenols pose a serious environmental risk because of their acute toxicity and relative persistence to chemical and biological degradation (Knackmuss and Hellwig, 1978). It is apparent from the studies on contaminated wood-preserving sites that polychlorinated phenols and dioxins are persistent in soil and groundwater. A variety of analytical methods have become available for analytical detection and measurement of PCP and its related compounds (Crosby, 1981), this means contamination can be measured and treatment appropriate to the contamination implemented.

Pentachlorophenol is a persistent chemical in the environment because of the absence of micro-organisms with the capability to degrade it. Factors such as temperature, pH, salinity and water activity, oxygen availability, permeability of the surface for air and water and availability of nutrients can affect bioremediation. The concentration of PCP may be either too high, proving toxic for micro-organisms, or too low because of incorporation into humic substances or absorption to soil particles thereby failing to induce the degradative enzymes. Biological remediation aims to optimise the environmental conditions so that indigenous or inoculated organisms can degrade the contaminant at the maximum possible rate (Fetzner and Lingens, 1994).

Chlorinated phenolic compounds may be removed from the environment by complete or partial biodegradation or by biotransformation. Highly halogenated aromatic compounds such as PCP are particularly resistant to biodegradation (McCarthy *et al.*, 1996). Biodegradation can be considered complete only when the carbon skeleton of PCP is converted into intermediary metabolites and the chloride atoms are returned to the mineral state (Reineke and Knackmuss, 1988). Biotransformation reactions, where the carbon skeleton of the substrate remains unaltered, are alternative biodegradation mechanisms (H≅ggblom *et al.*, 1988). Many biotransformation products resist aerobic microbial attack and have a high potential for bioaccumulation. There may, however, be compounds present in the degrading environment that contribute to the success of PCP biodegradation, by adsorbing the PCP and it's degradation products, thereby mitigating their toxic effects. The rate of PCP degradation is more rapid in soils with a high organic matter content (Crosby, 1981).

Successful bioremediation of PCP is a function of three independent but interrelated factors; contaminant, microorganisms and environment (Aislabie *et al.*, 1997). The concentration and distribution of PCP, the physiological and genetic makeup of the microbial population and the environmental framework in which they are situated control the success of bioremediation (Aislabie *et al.*, 1997). These three factors are combined in different ways to maximise the effectiveness of bioremediation for waste treatment.

2.2.1 BIOLOGICAL TREATMENT TECHNOLOGIES

Biological treatment technologies for hazardous-waste management involve the development of systems that use biological catalysts to detoxify, degrade, or accumulate environmental pollutants (Fetzner and Lingens, 1994). Aislabie *et al.* (1997) have characterised biological treatment technologies for contaminated soils and groundwater into three main categories: 1) solid-phase biotreatment (landfarming); 2) *in situ* biotreatment; 3) slurry-phase biotreatment (bioreactors). Laboratory evaluation of the chemical, physical and biological characteristics of representative soils and waters from the site for monitoring concentrations of contaminants, nutrients, microbial activity and

other parameters critical to implementation of biological remediation is necessary (Compeau *et al.*, 1991). Biotechnological approaches which have been applied successfully so far involve the development of bioreactors to deal with specific contaminants and bioaugmentation, the addition of 'outside' microorganisms to sites with the ability to degrade the target contaminant to cope with contaminated landfill sites, industrial sites, and groundwater (Barnhart and Hyzy, 1993; Fetzner and Lingens, 1994).

2.2.1.1 SOLID PHASE TREATMENT

Solid phase treatment involves the excavation of contaminated soil or treatment lagoon sludge and mounding it either in an enclosure or on top of a liner (Litchfield, 1991). Landfarming, composting and engineered soil cells also come under the heading of solid phase treatment (Aislabie *et al.*, 1997). The merits of solid phase treatment methods are their cost efficiency and low cost, and they can be done on-site. Land farming is a solid phase treatment where contaminated surface soil is treated on site using conventional agricultural practices such as tilling, irrigation, and fertilisation. Composting is a thermophilic treatment process combining contaminated soil with a bulking agent to stimulate degradation. The static piles, or windrows, are not usually aerated or tilled but are turned (mixed) at regular intervals but they may be artificially ventilated. Engineered "soil cells" are a hybrid of landfarming and composting. The "cells" are constructed as aerated compost piles to control physical losses of the pollutant by leaching and volatilisation (Aislabie *et al.*, 1997).

2.2.1.2 *IN SITU* BIOREMEDIATION

The primary concept of *in situ* bioremediation (ISB), or biostimulation, is that indigenous bacteria in groundwater and subsurface soils can be induced to grow and degrade the contaminant by augmenting some environmental factors that are limiting growth and degradation. Biostimulation uses only the microbes already present in the contaminated site (Barnhart and Hyzy, 1993) and is a bioremediation method where there is an acceleration of what would eventually happen as a result of the indigenous

microbial population. Indigenous organisms may be removed from contaminated sites and returned as inocula, after enrichment and selection of adapted organisms, to the contaminated site. These microorganisms are assumed to have adapted to the contaminants and to their physical/chemical environment and these adapted organisms will degrade the organic contaminants. However, degradation may be retarded when a nutrient reaches a limiting concentration. *In situ* bioremediation is a natural, ongoing process which is simply the stimulation of the indigenous microflora by the external addition of the limiting nutrients, most often oxygen, to facilitate biodegradation (Litchfield, 1991). Bioventing and biosparging are two other *in situ* bioremediation technologies (Aislabie *et al.*, 1997). The former is the process of supplying oxygen to indigenous soil microbes in the unsaturated zone to stimulate the biodegradation of organic contaminants (Aislabie *et al.*, 1997). The latter is the technology of introducing air (or other gases) beneath the water table to promote localised site remediation. Organic constituents are removed by a combination of physical air stripping as well as oxygen-enhanced *in situ* degradation (Aislabie *et al.*, 1997). Vapourisation is a problem and the hazardous material(s) needs to be trapped in scrubbers. These treatment processes are the most cost efficient, relatively passive and treat soil and water simultaneously, though there are extended treatment times and monitoring difficulties.

2.2.1.3 SLURRY-PHASE BIOTREATMENT

Bioreactors may be utilized to deal with chemical contamination in either the liquid or solid phase or as a slurry. Bioreactors use suspended microbial growth or growth on an fixed solid support (Litchfield, 1991). The inoculum can come from the contaminated materials, an activated sludge treatment plant, pure cultures, or genetically engineered micro-organisms. The addition of bacterial cultures to a contaminated medium is called bioaugmentation and is frequently used in bioreactors and *ex situ* systems. The use of genetically engineered organisms in the bioreactor is another option, however as there are concerns about the construction and the environmental release of genetically engineered micro-organisms, this is still at the laboratory stage in most countries (Fetzner and Lingens, 1994). Bioaugmentation is the addition of "outsider"

microorganisms known to be efficient degraders of the target contaminant (Barnhart and Hyzy, 1993).

These above three main types of bioremediation technology are often applied in combination to suit the waste type, concentration and circumstance.

2.2.2 BIOREMEDIATION CASE STUDIES

Wood treating operations conducted at sites all over the world over a period of 60 years have resulted in soil and groundwater contamination with PCP. Solid phase biological treatment was selected as the technology of choice for the remediation of 30, 000 tonnes of wood treatment site soils from Minnesota. The soil contained PCP at concentrations of up to 3,000mg/l and was to be reduced to 150mg/l (Compeau *et al.*, 1991). A 3.25 hectare Land Treatment Unit (LTU) was designed and constructed to include all necessary provisions for watering systems and containment of excess water, security systems and equipment utilisation. The remediation was conducted as an aerobic process and based on previous experience and treatability study data. Concentrations of PCP up to approximately 1000mg/l were considered appropriate to treat in the soils present at this site (Compeau *et al.*, 1991). Evaluations of microbial numbers and activity clearly demonstrated that the indigenous microorganisms in these soils were capable of PCP degradation, although a PCP-degrading inoculum was prepared in case microbial activity was diminished to unacceptable levels during treatment operations. Remediation required 13 weeks, although the calculation from the laboratory treatability work predicted a remediation time of 10-11 weeks in duration. Calculations and evaluation of the buffering capacity of the soil indicated that no application of alkaline material would be required during the bioremediation. A baseline sampling of the LTU soils was conducted and a starting concentration of 410mg/l of PCP was detected. In the case of PCP degradation in soil, the rate of degradation was dependent on PCP concentration. Within the range of PCP concentrations encountered in these soils, the rate of degradation increased as the PCP concentration increased to an upper limit of 1000mg/l (Compeau *et al.*, 1991).

Landfarming employing fungal cultures has been used to remediate soil contaminated with PCP at concentrations ranging from 25 to 5000 mg/kg (Glaser and Lamar, 1995). Excavated soil was mechanically sieved through a 2.5cm screen, mixed, and placed on plots, 21m by 30m, to a depth of 25cm. The plots were inoculated with *Phanerochaete chrysosporium*, and wood chips were added as a nutrient substrate to sustain growth of the fungi. The plots were regularly tilled and irrigated. A 69% PCP reduction was achieved for initial PCP concentration of 1000mg/kg treated with *Ph. chrysosporium* and an 89% PCP reduction for initial PCP concentration of 600-700mg/kg was achieved with *Phanerochaete sordida* (Glaser and Lamar, 1995). Further studies with *Ph. sordida* were conducted with soil contaminated with up to 5200mg/kg PCP. Contaminated soil was diluted with clean soil to yield a PCP concentration of 100mg/kg. Soils beds, 21m by 30m, were inoculated with 10% (w/w) *Ph. sordida* and a 64% PCP reduction was achieved (Glaser and Lamar, 1995).

Composting is another solid phase treatment that has been used for bioremediation of PCP contaminated soils. Composting is a thermophilic treatment process combining contaminated soil with a bulking agent e.g., bark and ash, to stimulate degradation. In a large scale experiment on bacterial degradation of chlorophenols, using *Rhodococcus chlorophenolicus*, $100m^3$ of soil contaminated with up to 500mg/kg dry weight of chlorophenols from around dipping basins of sawmills was excavated for composting (Valo and Salkinoja-Salonen, 1986). There was no forced aeration of the windrows but they were irrigated weekly. Conditions for the activity of chlorophenol-degrading microbes were created in the field and these lead to 80% removal of chlorophenols within 4 months (Valo and Salkinoja-Salonen, 1986). Addition of chlorophenol-degrading bacteria to sterilised soil sped up degradation in the laboratory. Bark chips promoted chlorophenol degradation by protecting chlorophenol degraders against the toxicity of PCP, providing organic matter, maintaining the temperature and promoting aeration in the compost. The bark chips were also suggested to serve as a source of chlorophenol-degrading organisms (Valo and Salkinoja-Salonen, 1986). Over 90% of PCP in soil containing 7-8% organic matter was degraded compared with no degradation in soil with 0.04% organic matter (Valo and Salkinoja-Salonen, 1986).

Bioremediation of PCP contaminated soil by soil washing and the feasibility of a bioremediation programme using a slurry phase bioreactor was tested by bench scale biotreatability studies. The studies were performed to develop a microbial culture and biodegradative process which could treat PCP at higher concentrations than previously reported (Litchfield, 1991). Treatability studies were performed on soil samples for a contaminated site with PCP concentrations of between 2mg/kg and 9000mg/kg, to determine the efficacy of a slurry phase bioremediation process for these soils. The first two weeks of the study showed little if any indigenous microbial degradative potential in any of the various slurries tested. On day 13 each of the slurries received an inoculum of the PCP consortium to yield approximately 1 x 10^8 cells/ml of slurry. Inoculation with the PCP degrading consortium resulted in the degradation of PCP to below detectable levels (<1.0mg/l) as measured by HPLC (Litchfield, 1991). Data from laboratory treatability studies were used to design and implement the full-scale remediation process. The process involves soil washing to remove PCP from the soils followed by bioremediation of the pregnant wash solution in a slurry-phase bioreactor. The resulting wash solution was slurry treated on-site in slurry phase bioreactors which had been inoculated with the PCP degrading consortium. Bench scale studies performed before and during remediation supported the use of inoculation in enhancing the degradation of PCP in slurry reactors (Litchfield, 1991).

2.2.3 PHYTOREMEDIATION AND ANAEROBIC BIOREMEDIATION

PCP not only contaminates the soil around wood treatment plants but also surface water and wastewater from these sites is also contaminated and depending on the hydrogeology of the site PCP can leach into groundwater. Many different approaches have been explored for the removal of PCP from ground and waste-water. These treatment methods (Tikoo *et al.*, 1997) can be broadly classified as physical (adsorption, foam flotation), chemical (oxidation, photodecomposition, ultrasound) and biological (activated sludge process, anaerobic digestion, immobilised bacteria, cometabolism). Photolysis was deemed the fastest known PCP degradation mechanism in nature and could lead to total mineralisation of PCP to CO_2 and Cl^- (Valo *et al.*, 1985). Nonetheless, the process of photolysis is slow and the contamination can persist for

decades. The physical and chemical methods for treatment of waters contaminated with PCP are expensive to implement and are not always efficient enough for complete removal of the contaminant (Tikoo *et al.*, 1997). Above ground bioreactors (AGB) have since been used for groundwater bioremediation of PCP and have been applied successfully. During a single passage through a bioreactor, 70% of the PCP in contaminated surface water was removed, and greater than 99% of the PCP in groundwater was removed over a period of four weeks (Litchfield, 1991).

Combined biotechnologies with chemical or physical treatment (Compeau *et al.*, 1991), phytoremediation, i.e. the use of green plants and their associated microbial communities, (Anderson *et al.*, 1993; Aislabie *et al.*, 1997) and anaerobic treatment processes (Fetzner and Lingens, 1994) are other bioremediation techniques that are not commonly used.

Plant roots, in conjunction with their associated microbial communities, offer a potentially important treatment strategy for *in situ* bioremediation of chemically contaminated soils. This technique of bioremediation is known as phytoremediation (Anderson *et al.*, 1993). Under a variety of environmental conditions, vegetation has been shown to enhance microbial degradation rates of organic chemical residues in soils (Anderson *et al.*, 1993). These findings are important because vegetation may provide a low-cost alternative or supplement to expensive, capital-intensive technologies for soil cleanup. Investigations of the fundamental mechanisms whereby rhizosphere microbial degradation occurs would provide insights for applications of the plant-microbe system for *in situ* remediation. The rhizosphere is a zone of increased microbial activity and biomass at the root-soil interface that is under the influence of the plant root. This zone is distinguished from bulk soil by this root influence. The overall effect of the plant-microbe interaction is an increase in microbial biomass by an order of magnitude or more compared with that of microbial populations in bulk soils. The common theme to the literature on microbial degradation of herbicides in the rhizosphere is the possibility that rhizosphere microbial communities are involved in protecting the plant from chemical injury. Microbial consortia, rather than individual microbial species, are likely to be involved in the degradation of numerous toxicants in the rhizosphere. There is a

potential role of vegetation in facilitating microbial degradation for *in situ* bioremediation of surface soils contaminated with hazardous organic compounds (Anderson *et al.*, 1993).

Anaerobic bacteria are not used for bioremediation as often as aerobic bacteria (Aislabie *et al.*, 1997) and studies on the anaerobic treatment of PCP, combining anaerobic sewage sludge and bioaugmentation, resulted in the accumulation of less highly chlorinated phenols as opposed to complete mineralisation of PCP (Fetzner and Lingens, 1994). PCP was, however, able to be removed by anaerobic fixed film and upflow anaerobic sludge blanket reactors, as well as in an anaerobic semicontinuous-stirred tank reactor (Fetzner and Lingens, 1994). The fate of [^{14}C]PCP in flooded soil, i.e. anaerobic conditions was investigated and different mechanisms of PCP transformation were found (Fetzner and Lingens, 1994). A major part of the radioactivity was incorporated into insoluble macromolecules, i.e., bound to humin and humic acid (Fetzner and Lingens, 1994).

2.2.4 FEASIBILITY OF BIOREMEDIATION

Bioremediation is appropriate whenever it is certain that no toxic by-products will be formed either biologically or abiologically, that there are microorganisms which can degrade the contaminants, and that it is economical (Litchfield, 1991). To be certain that more toxic by-products are not formed, it is helpful to know the metabolic pathways that have been described based on studies of pure cultures and single compounds. Another factor to consider in determining whether to use bioremediation is the availability of the waste and it's concentration. The laboratory studies must involve testing the mixture of contaminants at the *in situ* concentrations. The economics of the various bioremediation options must be evaluated. Important factors include: time to develop the necessary data base, length of time for the remediation, costs of construction, location of the contamination in the soils or in soils and groundwater, the hydrogeology of the site, location of the contamination in relation to site boundaries and surface structures, and space for the treatment system (Litchfield, 1991).

Literature on bioremediation of PCP contaminated sites in New Zealand is sparse despite there being over 600 contaminated sites around the country (Jackman, 1992). Bioremediation is being considered for clean-up of contaminated soils and liquids from timber treatment sites in New Zealand (Aislabie *et al.*, 1997). Biotreatment of landfill leachate by *in situ* seeding with microbes has also been considered. The potential for the development of a biological treatment process for the organochlorine pollutant such PCP is being recognized in the light of the recent advances of environmental biotechnology and the applications of bioremediation in the treatment of a wide range of organic pollutants. Because such technology requires minimal energy input, only minor requirements of chemicals in the form of nutrients and operates at ambient temperature, the costs for these systems are much lower than those for other methods used for clean-up of environmental contaminants. Therefore, biotreatment of organochlorine wastes such as PCP should be the first technology considered, not the last (Yu and Shepherd, 1997).

A bioprocess for the successful remediation of PCP contaminated soils and liquids will further develop by integrating sound microbiological and biochemical research with good materials and process engineering. Only by continuing efforts in which microbiology, chemistry and engineering are integrated in the early stages of research can there be efficient transfer of new technology to the field of hazardous waste remediation (Fetzner and Lingens, 1994).

The forest and timber industry appears to be the culprit for the majority of the environmental chemical pollution in New Zealand. Much of the contamination is the result of poor management of hazardous chemicals in manufacturing, spillage, leakage or illegal disposal (Szabo, 1993). Pollution by chlorinated phenols, heavy metals, pesticides, polyaromatic hydrocarbons, petroleum products and industrial waste occurs in up to 10, 000 sites in New Zealand (Szabo, 1993; Aislabie *et al.*, 1997). Contaminants are not evenly distributed within the environment and may be dissolved in the groundwater, adsorbed into soil or distributed as free products or non-aqueous phase liquid. Soil at the industrial sites is often contaminated and sewerage and storm-water drainage systems act as a collection system transporting the pollutants to streams

and groundwater. The application of bioremediation to these contaminated sites involves the manipulation of environmental parameters to enhance microbial growth and hence degradation. Innovative bioremediation treatment facilities can be designed to suit the circumstances of the pollution problem. The presence of the required microbial population is not enough to ensure adequate bioremediation and environmental conditions *in situ* must be optimised to permit microbial growth and/or activity. The chemical structure of a contaminant, it's concentration and distribution, and the physiological and genetic makeup of the microbial population, all of which are situated in an environmental framework, control the success of bioremediation. Selection of microorganisms with the appropriate metabolic potential to degrade specific contaminants is possible because of their short generation time and rapid evolution. There are no limits to the potential of microorganisms to degrade pollutants. Remediation progress must be monitored to ensure that full mineralisation of the substrate is occurring. Direct and indirect methods may be used to demonstrate that contaminant removal can be attributed to microbial activity. Loss of substrate, activity of the microbes responsible and their assessment are important in understanding and improving on the bioremediation technology being used. To make bioremediation a more effective technology, further research is needed to gain a better understanding of the basic mechanisms underlying enhancement and inhibition of microbial degradation of high concentration of toxic compounds (Radehaus and Schmidt, 1992).

2.3 PCP DEGRADING MICROORGANISMS

Pentachlorophenol degrading microorganisms have been enriched by various investigators (Watanabe, 1973; Stanlake and Finn, 1982; Apajalahti and Salkinoja-Salonen, 1984; Shelton and Tiedje, 1984; Saber and Crawford, 1985; Radehaus and Schmidt, 1992; Utkin *et al.*, 1994; Bouchard *et al.*, 1996; Hussein *et al.*, 1996) from PCP contaminated sites and the wider environment. There is a variety of bacteria and fungi which are able to grow on and degrade this toxic compound, some microorganisms are able to live in the presence of high concentrations (<200mg/l) of PCP (Stanlake and Finn, 1982; Topp *et al.*, 1988; Radehaus and Schmidt, 1992; Rutgers *et al.*, 1993) even though they do not utilise it for growth. PCP degrading consortia are often developed thus allowing various microorganisms to participate in the metabolism of different intermediates along the degradation pathway. The ability to utilise PCP as the sole source of carbon and energy is found among both basidiomycete fungi and bacteria. Microorganisms able to degrade PCP have been characterised since the mid sixties. Axenic bacterial cultures able to degrade PCP include *Flavobacterium* spp., *Rhodococcus* spp., *Arthrobacter* spp., *Pseudomonas* spp., *Sphingomonas* spp., and *Mycobacterium* spp., and fungal cultures, *Phanerochaete* spp. and *Trametes* spp. are also able to degrade PCP (McAllister *et al.*, 1996). A number of the above bacteria have been recently reclassified as either *Sphingomonas chlorophenolicus* or *Mycobacterium chlorophenolicus* (Nohynek *et al.*, 1995; Häggblom *et al.*, 1995). In New Zealand, a PCP-degrading bacterium has been isolated from PCP-polluted soil (Hussein *et al.*, 1996) and has been tentatively identified as a *Bradyrhizobium* sp. strain ET01 (Yu, 1997).

2.3.1 PSEUDOMONADACEAE

Chu and Kirsch (1972) isolated an organism they designated bacterium KC-3 from a continuous-flow enrichment culture and which metabolised PCP as a sole source of organic carbon and energy at a concentration of 200mg/l,. The morphological and physiological characteristics of KC-3 suggested a relationship to the saprophytic coryneform bacteria.

Watanabe (1973) isolated bacteria from soil capable of degrading PCP at 40mg/l. These isolates were considered to be species of *Pseudomonas* or a closely related genus of the family Pseudomonadaceae (Watanabe, 1973). Many microorganisms that degrade PCP have been designated pseudomonads. The Pseudomonadaceae comprise one of the most complex groups of Gram-negative bacteria, with phenotypic similarities to many other genera (Palleroni, 1984). The type genus, *Pseudomonas*, is characterised by straight or curved Gram-negative rods which are strictly aerobic and motile by polar flagella (Palleroni, 1984). Many aromatic compounds may be used for growth by *Pseudomonas* spp. and the pathways for degradation of a number of aromatic compounds have been extensively analyzed (Palleroni, 1984).

Pseudomonas sp. B13 was isolated from sewage on 3-chlorobenzoate media (Knackmuss and Hellwig, 1978) and completely degraded many chloroaromatic compounds (Reineke and Knackmuss, 1988; Kaschabek and Reineke, 1992, 1993). It totally metabolised chlorophenol and dichlorophenol when grown in continuous culture (Knackmuss and Hellwig, 1978). The enzymology of the degradative route of chlorocatechols, key intermediates in the degradation of chlorophenols, by *Pseudomonas* sp. B13 has been investigated (Kaschabek and Reineke, 1993). Karns *et al.* (1983) described the ability of *Pseudomonas cepacia* AC1100 to partially and completely dehalogenate a wide range of halophenols including PCP.

2.3.2 SPHINGOMONADACEAE

The type species of the genus *Sphingomonas* is *Sphingomonas paucimobilis* and has masqueraded as a *Pseudomonas* sp. for many years (White *et al.*, 1996). *Sphingomonas* species have an aerobic, heterotrophic, soil-based life-style similar to that of members of the genus *Pseudomonas* with which they were confused for much of their history (White *et al.*, 1996). Several pseudomonads have been reclassified in the genus *Sphingomonas*. *Pseudomonas saccharophila* strains KF1^{T}, KF3, and NKF1 have been reclassified as *Sphingomonas subarctica* sp. nov., on the basis of chemotaxic, genetic and physiological methods and by electron microscopy studies (Noyhnek *et al.*, 1996;

Balkwill *et al.*, 1997). *Pseudomonas* spp. RA2 and SR3 have been reclassified as a single new species *Sphingomonas chlorophenolica* sp. nov. (Noyhnek *et al.*, 1995).

The genus *Sphingomonas* was described by Yabuuchi *et al.* in 1990 and in 1993 was amended by Takeuchi *et al.* (Balkwill *et al.*, 1997). Organisms in this genus are Gram-negative, non-spore forming rods that have a single polar flagellum when they are motile. They have yellow pigmentation, are obligately aerobic and produce catalase. The G+C contents of the genomic DNAs range from 61.6 to 67.8 mol% (Balkwill *et al.*, 1997). The genus *Sphingomonas* appears to be ubiquitous in soil, water, and sediments. *Sphingomonas* isolates from these environments have broad catabolic capabilities and, therefore, have high potential for bioremediation and waste treatment processes. PCP is one of the various contaminants that can be degraded by various *Sphingomonas* species (Karlson *et al.*, 1995).

Pentachlorophenol degraders other than *Pseudomonas* have also been reclassified as *Sphingomonas* spp. for example *Sphingomonas chlorophenolica* sp. nov. ATCC 39723 (formerly *Flavobacterium* sp. strain ATCC 39723), *S. chlorophenolica* sp. nov. ATCC 33790 (formerly *Arthrobacter* sp. ATCC 33790), *S. chlorophenolica* sp. nov. RA2 (formerly *Pseudomonas* sp. RA2) and *S. chlorophenolica* sp. nov. SR3 (formerly *Pseudomonas* sp. SR3) (Nohynek *et al.*, 1995; Karlson *et al.*, 1995) were isolated and described as being able to utilise PCP as a sole source of carbon and energy (Steiert *et al.*, 1987; Schenk *et al.*, 1989; Radehaus and Schmidt, 1992). *S. chlorophenolica* sp. nov. ATCC 39723 is able to mineralise other chlorinated phenols and the PCP-degradation enzymes are inducible (Steiert *et al.*, 1987). This strain has also been studied in great detail for its growth characteristics in relation to degradation of PCP, the specific biochemical reactions and the genes encoding the enzymes (Steiert *et al.*, 1987; Xun and Orser, 1991; Xun *et al.*, 1992a, b, c; Orser and Lange, 1994; Lee and Xun, 1997). The pathway of PCP degradation has been characterised for *S. chlorophenolica* sp. nov. ATCC 39723 (Xun *et al.*, 1992c) and much molecular analysis of PCP degradation by this organism in pure culture has been carried out (Xun *et al.*, 1992a, b, c; Orser and Lange, 1994). *Sphingomonas* spp. have been tested for growth at PCP concentrations up to 200mg/l (Topp and Hanson, 1990; Xun and Orser, 1991; Saber and

Crawford, 1985). The enzyme, PCP 4-monooxygenase, is involved in the initial attack of *S. chlorophenolica* sp. nov. ATCC 39723 on PCP. It has a very broad substrate range and dehalogenates iodo- and bromophenols as well as chlorinated phenols (Xun and Orser, 1991; Xun *et al.*, 1992a, b). The PCP degradation pathway of *S. chlorophenolica* sp. nov. ATCC 39723 has been studied using chemically derived mutants (Xun and Orser, 1991). This pathway is discussed in detail in Section 2.5.

The survival and activity of the PCP-degrading *S. chlorophenolica* sp. nov. ATCC 39723, inoculated into a variety of soils, has been examined (Topp and Hanson, 1990). The feasibility of inoculating this organism at high cell densities into heavily polluted soils for the purpose of rapid decontamination has been investigated, however additional toxic compounds and natural flora in the contaminated environment hindered growth (Topp and Hanson, 1990). The potential application of *S. chlorophenolica* sp. nov. ATCC 39723 in pure and mixed culture for bioremediation of PCP has been investigated (Yu and Ward, 1996). *S. chlorophenolica* has also been studied in a consortium and manifested the highest PCP degradation capacity (Yu and Ward, 1996).

2.3.3 ACTINOMYCETES

Actinomycetes are a very large group of Gram-positive bacteria mostly found in soils where they are part of the heterotrophic microflora responsible for the degradation of organic material (Noyhnek *et al.*, 1993). Actinomycetes are also known for their ability to transform and degrade xenobiotics. Degradation of different polychlorinated phenolic compounds has been reported for the actinomycetal genera *Arthrobacter* (Stanlake and Finn, 1982) and *Rhodococcus* (Apajalahti and Salkinoja-Salonen, 1986; Häggblom *et al.*, 1988). The related mycobacteria, comprising the genus *Mycobacterium*, are aerobic, acid fast staining non-motile slow-growing rod-shaped actinomycetes. Representatives of the genus *Mycobacteria* are easily confused with those of *Rhodococcus* and often *Nocardia* and *Corynebacterium* (Wayne and Kubica, 1984). Two *Mycobacterium* spp. have been characterised for their ability to degrade PCP.

Mycobacterium chlorophenolicum PCP-1, formerly *Rhodococcus chlorophenolicus* strain PCP-1 (Häggblom *et al.*, 1995) was isolated from a PCP-mineralizing culture developed from a lake sediment (Apajalahti and Salkinoja-Salonen, 1984; Valo *et al.*, 1985; Apajalahti *et al.*, 1986). *M. chlorophenolicum* PCP-1 metabolises PCP ultimately to inorganic end products via tetrachlorohydroquinone (Uotila *et al.*, 1995). *M. chlorophenolicum* PCP-1 was the organism used for the first demonstration of reductive aromatic dechlorination by bacterial enzymes (Apajalahti and Salkinoja-Salonen, 1987b) and has been tested for an ability to degrade PCP in soil and liquid cultures (Apajalahti and Salkinoja-Salonen, 1986; Apajalahti *et al.*, 1986, 1987a, b; Middeldorp *et al.*, 1990). *M. chlorophenolicum* PCP-1 has been grown on and totally degraded PCP at concentrations up to 600mg/kg soil (Middeldorp *et al.*, 1990).

M. chlorophenolicum PCP-1 is Gram-positive, catalase positive, strictly aerobic and rod shaped organism becoming coccoid upon prolonged incubation (Apajalahti *et al.*, 1986). Genetic studies of mycobacteria have been hampered by their typically slow cell doubling times, ranging (by species) from 2 to more than 20 hours (Wayne and Kubica, 1984). The colour of the colonies is yellow or orange, but rarely pink, and is usually due to carotenoid pigments. The G+C content of the DNA ranges from 62 to 70 mol% (Wayne and Kubica, 1984). Studies on the physiology of a mixed bacterial culture including *M. chlorophenolicum* PCP-1 were carried out by Valo *et al.* (1985). The optimum pH for PCP degradation is 6.4 - 7.2 and the optimum temperature 28°C (Valo *et al.*, 1985). PCP degradation by *M. chlorophenolicum* PCP-1 depends on organic matter present and oxygen availability, however addition of other carbon sources did not influence PCP degradation (Valo *et al.*, 1985). *M. chlorophenolicum* PCP-1 oxidises PCP to carbon dioxide and also oxidises many polychlorinated phenols and intermediates along the degradation pathway. The enzyme system for degradation of PCP is inducible by exposure to PCP or the various polychlorinated phenols (Apajalahti and Salkinoja-Salonen, 1986, 1987a). *M. chlorophenolicum* PCP-1 also metabolises chloroguaiacols (Häggblom *et al.*, 1986) and *o*-methylates halogenated phenols as an alternative to biodegradation (Allard *et al.*, 1987). The degradation pathway and substrate specificity of *M. chlorophenolicum* PCP-1 has been widely investigated

(Apajalahti and Salkinoja-Salonen, 1986, 1987a, b; Häggblom *et al.*, 1988, 1989; Uotila *et al.*, 1991, 1995).

Mycobacterium sp. strain CG-2 is a Gram-positive, acid fast staining actinomycete that was isolated from chlorophenol-contaminated soil from a sawmill timber-treating facility and enriched on tetrachloroguaiacol (Häggblom *et al.*, 1988; Noyhnek *et al.*, 1993). This strain mineralises PCP and degrades and *o*-methylates various polychlorinated phenolic compounds (Noyhnek *et al.*, 1993). Two other bacterial strains isolated independently from the sludge were *Rhodococcus* sp. strain CG-1 enriched on tetrachloroguaiacol, and *Rhodococcus* sp. strain CP-2, enriched on pentachlorophenol (Häggblom *et al*, 1988).

All three bacterial strains readily mineralised PCP and removed several other polychlorinated phenols, 2-methoxyphenols (guaiacols), and 2, 6-dimethoxyphenols (syringols) (Häggblom *et al.*, 1988). The three strains degraded chlorophenols, chloroguaiacols and chlorosyringols at micromolar concentrations and were sensitive to the toxic effects of pentachlorophenol (Häggblom *et al.*, 1988). All three strains initiated the degradation of polychlorinated phenols by *para*-hydroxylation into chlorinated hydroquinones. *Rhodococcus* sp. strains CG-1 and CP-2 were sensitive to the toxic effects of PCP, but the same concentration of the *o*-methylation product pentachloroanisole (PCA) was nontoxic (Häggblom *et al.*, 1988). *Mycobacterium* sp. strain CG-2 was however, sensitive to both PCP and PCA.

The isolates had the characteristics of the nocardioform actinomycetes and were assigned to the genera of R*hodococcus* (CG-1 and CP-2) and *Mycobacterium* (CG-2). Strains CG-1 and CP-2 exhibited cyclic changes in morphology of cocci to rods to cocci during growth and formed yellowish orange, mucoid colonies on DSM-65 agar. Strain CG-2 also exhibited a coccus to rod to coccus cycle of growth, but formed white, wrinkled colonies on DSM-65 agar (Häggblom *et al.*, 1988). Colonies are usually off-white or cream coloured (Wayne and Kubica, 1984).

2.3.4 OTHER AEROBIC PCP DEGRADING STRAINS

Streptomyces rochei 303 was isolated in 1993 from soil contaminated with chloroorganic pesticides and has been shown to be able to degrade mono-, di-, tri-, and tetrachlorophenols and PCP (Zaborina *et al.*, 1995). The hydroxyquinol dioxygenase enzyme, catalysing a step of central importance in the degradation of chlorophenols via the chlorohydroquinone pathway, has been characterised for *S. rochei* 303 and has a high degree of similarity to the analogous enzyme in *Azotobacter* sp. strain GP1 (Zaborina *et al.*, 1995). *Azotobacter* sp. strain GP1 was isolated from soil and can degrade different chlorophenols with a preference for trichlorophenols (Zaborina *et al.*, 1995). *Azotobacter* spp. are Gram-negative nitrogen fixers and range from rods to coccoid cells. They are aerobic but can grow under decreased oxygen tensions. The pH range for growth is 4.8-8.5, the optimum for growth and nitrogen fixation being pH 7.0-7.5. They occur in soil and water and one species occurs in association with plant roots (Tchan and New, 1984).

A microorganism has been isolated from PCP-polluted soil (Hussein *et al.*, 1996) and has been tentatively identified by 16sRNA sequencing as a *Bradyrhizobium* sp. and designated *Bradyrhizobium* sp. strain ET01 (Yu, 1997). *Bradyrhizobium* spp. are slow growing, Gram negative rods. In young culture the cells are short rods, but in older cultures or under adverse growth conditions including low concentrations of calcium or magnesium, the cells are commonly pleomorphic (Jordan, 1984). They are aerobic, possessing a respiratory type of metabolism with oxygen as the terminal electron acceptor. They are motile, by one polar or subpolar flagellum and the optimum temperature for growth is between 25 and 30°C. The optimum growth pH is between 6 and 7, although a lower optimum may be exhibited by strains from acid soils. Colonies are circular, opaque, rarely translucent, white and convex, and tend to be granular in texture. They do not exceed 1 mm in diameter within 5-7 days incubation on yeast-mannitol-mineral salts agar. Only moderate turbidity develops after 3-5 days or longer in agitated broth. Faster growing strains are uncommon (Jordan, 1984). The organisms are characteristically able to invade the root hairs of tropical-zone and some temperate-zone leguminous plants (family Leguminoseae) and induce the production of root

nodules wherein the bacteria occur as intracellular symbionts (Jordan, 1984). The bacteria are present in root nodules as swollen forms which are normally involved in fixing atmospheric nitrogen into combined forms utilisable by the host plant. There is usually no requirement for vitamins with the rare exception of biotin, which also may be inhibitory to some strains (Jordan, 1984). Some strains fix nitrogen in the free-living state when examined under special conditions (Jordan, 1984).

2.3.5 ANAEROBIC PCP DEGRADERS

Prior to 1992 single organisms that could anaerobically degrade PCP had not been isolated (Bryant *et al.*, 1991). Shelton and Tiedje (1984) isolated and partially characterised an anaerobic consortium, isolated from sewage sludge, able to use 3-chlorobenzoic acid as its sole energy and carbon source. Seven bacteria were isolated and included one dechlorinating bacterium (strain DCB-1), one benzoate-oxidising bacterium (strain BZ-2), two butyrate-oxidising bacteria (strains SF-1 and NSF-2) two H_2-consuming methanogens (*Methanospirillum hungatei* PM-1 and *Methanobacterium* sp. strain PM-2) and a sulphate-reducing bacterium (*Desulfovibrio* sp. strain PS-1) (Shelton and Tiedje, 1984). A mutualism among the dechlorinating, benzoate-oxidising, and methane-forming members was required for utilisation of the substrate (Shelton and Tiedje, 1984). Mikesell and Boyd (1985) observed reductive dechlorination of PCP in several anaerobic sludges and also observed that PCP degradation in the sludges could be enhanced when the microorganisms were first adapted to dechlorinate a mixture of monochlorophenols. No isolation or characterisation of the microorganisms responsible was attempted. Degradation of PCP by anaerobic organisms in consortia is commonly described (Mikesell and Boyd, 1986; Bryant *et al.*, 1991; Nicholson *et al.*, 1992). Nicholson *et al.* (1992) described a methanogenic consortium that, after acclimation to 0.9mg/l PCP, removed chlorines from PCP and its reductive dechlorination products.

Desulfomonile tiedjei DCB-1 was the first anaerobic microorganism isolated in pure culture that degrades PCP (Mohn and Kennedy, 1992). The organism dehalogenates PCP and other chlorophenols in the *meta* position, but needs 3-chlorobenzoate as an inducer of the enzymes for the degradation pathway (Mohn and Kennedy, 1992).

Desulfitobacterium dehalogenans gen. nov. was isolated and characterised by Utkin *et al.* (1994). *D. dehalogenans* sp. nov. JW/IU-DC1 is a Gram-positive anaerobic bacterium that reductively dechlorinates chlorophenolic compounds (Utkin *et al.*, 1994). Another organism isolated from a methanogenic consortium which dechlorinates PCP anaerobically is *Desulfitobacterium frappieri* sp. nov. (Bouchard *et al.*, 1996). *D. frappieri* PCP-1 is the type strain of the species and is a spore-forming rod-shaped bacterium that is non-motile, asaccharolytic and Gram stain negative. A 16S rRNA analysis revealed that *D. frappieri* PCP-1 exhibits 95% homology with *D. dehalogenans* JW/IU-DC1 (Bouchard *et al.*, 1996).

Anaerobic metabolism of PCP and the microorganisms responsible have been studied to a far lesser extent than have the aerobic ones. It is clear from the limited literature existing on anaerobic degradation of PCP that aerobic biodegradation is a more commonly described and more extensively studied process. This may not be due to the inherent difficulties in working with anaerobes rather than reflecting the contribution of anaerobic degradation processes to PCP removal.

2.3.6 PCP DEGRADING FUNGI

Some of the first organisms characterised for PCP degradation were fungi obtained from unseasoned western hemlock sapwood (Cserjesi, 1967). Three strains of an unidentified *Trichoderma* sp. grown in liquid culture degraded PCP over a 12 day period at a concentration, usually toxic to a basidiomycete, of 10mg/l (Cserjesi, 1967). More recently the lignicolous fungus *Lentinula edodes* and the white rot fungus *Phanerochaete chrysosporium* have demonstrated the ability to carry out extensive degradation of PCP (Mileski *et al.*, 1988; Okeke *et al.*, 1996).

The white rot fungus *Phanerochaete chrysosporium* has been shown to degrade a wide variety of environmentally persistent organopollutants (Mileski *et al.*, 1988). This ability has been shown to be dependent on the nonspecific and nonstereoselective lignin-degrading system which is expressed by this microorganisms under nutrient (nitrogen, carbon, or sulfur) -limiting conditions. The lignin-degrading system consists

in part of ligninases which are able to catalyse the initial oxidation of a number of environmentally persistent xenobiotics. By allowing the fungus to establish a mycelial mat before adding PCP the fungus was able to grow and mineralise PCP at concentrations as high as 500mg/l (Mileski *et al.*, 1988). Purification and characterisation of intracellular enzymes of *Ph. chrysosporium* involved in the PCP degradation pathway has been carried out (Rieble *et al.*, 1994) and the ability of the ligninolytic basidiomycete *Ph. chrysosporium* BKM 1767 to metabolise PCP is now well documented (Mileski *et al.*, 1988; Okeke *et al.*, 1996). Less is known about the process in the shiitake mushroom *Lentinula edodes*, strains of which can effectively degrade PCP (Okeke *et al.*, 1994). The influence of environmental parameters on PCP biotransformation in soil by *Ph. chrysosporium* and *L. edodes* has been clarified and optimal parameters for temperature, soil moisture potential and pH have been elucidated (Okeke *et al.*, 1996). Fungi, therefore, may be useful in the biodegradation of hazardous organochemical wastes in some waste treatment systems (Mileski *et al.*, 1988).

2.3.7 PCP DEGRADING MICROALGAE

There is some evidence for metabolism of PCP by plants (Tikoo *et al.*, 1997). Since microalgae have fundamental metabolic similarities to higher plants it is possible that these organisms can detoxify or metabolise pesticides and herbicides to the same extent (Tikoo *et al.*, 1997). Microalgae are unicellular microscopic, photosynthetic organisms which typically inhabit aquatic environments, soil surfaces and other exposed locations. Microalgae are located at the base of food chains. A microalga (VT-1) was isolated from PCP treated water and its growth and PCP tolerance was compared with two known strains of *Chlorella*. The ability of VT-1 to degrade PCP was tested using uniformly labeled PCP, and $^{14}CO_2$ was released, indicating mineralisation (Tikoo *et al.*, 1997). Algae have the advantage over bacteria and fungi in that they are easily grown, needing only light and CO_2. Further investigation of the uptake and degradation of PCP by VT-1 is being studied (Tikoo *et al.*, 1997).

2.4 GROWTH KINETICS

Growth conditions and nutritional limitations influence the sensitivity of bacteria to chlorinated phenols and other anti-microbial agents. PCP binds to and changes the configuration of proteins and it may competitively inhibit enzymes having adenine-containing cofactors or utilizing adenine-containing substrates (Topp *et al.*, 1988). PCP is an inhibitor of oxidative phosphorylation and may inhibit cell division (Radehaus and Schmidt, 1992). Although PCP is toxic to a variety of organisms (Mileski *et al.*, 1988), several PCP-mineralising organisms have been isolated and characterised (Chu and Kirsch, 1972; Watanabe, 1973; Edgehill and Finn, 1982; Stanlake and Finn, 1982; Saber and Crawford, 1985; Apajalahti *et al.*, 1986; Mileski *et al.*, 1988; Radehaus and Schmidt, 1992).

Complete mineralisation of PCP to CO_2 and HCl is the desired outcome of biodegradation (Smith, 1994). PCP degradation has been monitored by radioactive chloride release, the theoretical mole ratio of which is 5 moles for every mole of PCP degraded (Chu and Kirsch, 1972; Watanabe, 1973; Edgehill and Finn, 1983; Radehaus and Schmidt, 1992). Apajalahti and Salkinoja-Salonen (1986) incubated washed, PCP adapted cells with radioactive PCP and measured a variety of metabolic parameters with time. PCP uptake was rapid and was followed by a rapid discharge of chloride and a slower discharge of carbon dioxide. Oxygen uptake was rapid and excessive, indicating that this was not an accurate parameter of biodegradation of PCP. Approximately 67% of the PCP was respired as shown by carbon dioxide accumulation, and 33% was assimilated in a resting cell preparation (Apajalahti and Salkinoja-Salonen, 1986). Biomass formation, measured by optical density (Stanlake and Finn, 1982; Topp *et al.*, 1988; Gu and Korus, 1995), cell counting (Radehaus and Schmidt, 1992) and disappearance of PCP measured by HPLC (Saber and Crawford, 1985), have also been used. A variety of organisms have been isolated that degrade PCP. Growth experiments have been conducted testing many different parameters to maximise the rate of PCP degradation (Valo *et al.*, 1985; Seech *et al.*, 1990; Okeke *et al.*, 1996).

2.4.1 PARAMETERS AFFECTING GROWTH

2.4.1.1 INITIAL PCP CONCENTRATION

Pentachlorophenol concentration has a significant effect on the acclimation phase for PCP mineralisation (Radehaus and Schmidt, 1992). The initial concentration of PCP in the culture medium is an important factor as bacteria capable of mineralizing PCP are themselves susceptible to PCP toxicity. A significant lag occurs before PCP mineralisation commences and the length of the lag period is directly proportional to the PCP concentration (Stanlake and Finn, 1982).

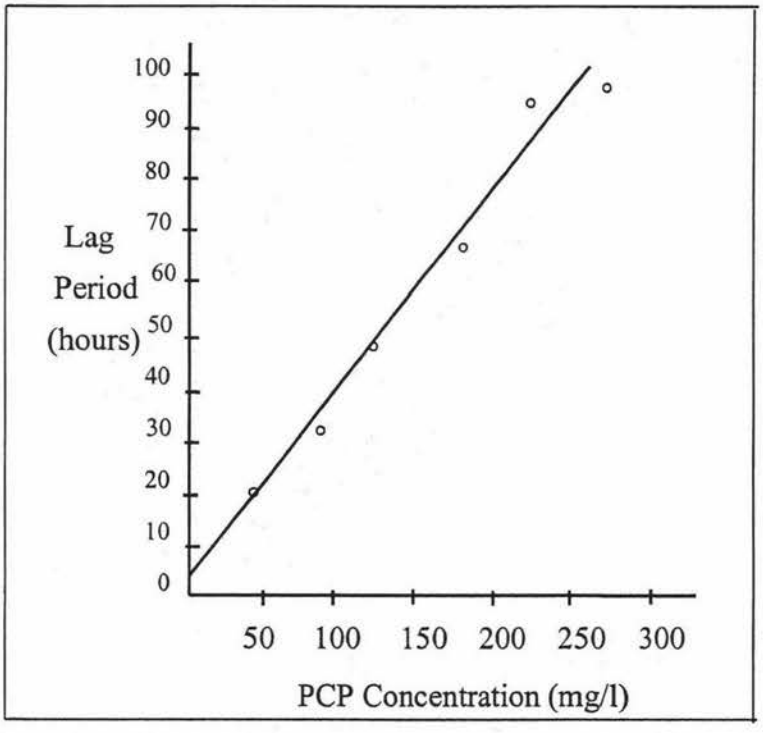


Figure 2.1: The effect of initial PCP concentration on lag time for *Arthrobacter* sp. strain NC in batch culture (Stanlake and Finn, 1982).

Because of the lag time, Stanlake and Finn (1982) suggested continuous culture methods would be more suitable to PCP biodegradation than batch culture methods. Klecka and Maier (1985) conducted experiments on batch and continuous-flow cultures growing on PCP as sole carbon and energy source and concluded that there was no difference in the

resulting kinetic parameters, thereby justifying the use of batch cultures for further studies. Yields are low when bacteria utilise PCP as the sole carbon source. This may necessitate the sporadic or continuous addition of PCP adapted (log phase in PCP) cells to a treatment system (Stanlake and Finn, 1982). A substrate concentration-controlled continuous culture was used to grow a PCP-degrading microorganism designated strain P5 (Rutgers *et al.*, 1993). With PCP concentrations between 120-204mg/l a maximum specific growth rate of $0.142h^{-1}$ was observed (Rutgers *et al.*, 1993). Compared with the growth rates of fed-batch cultures and chemostat cultures the growth rate of strain P5 in the PCP nutristat was not significantly higher.

Micro-organisms which are able to degrade PCP can be killed or experience growth inhibition if they are exposed to a sufficiently high PCP concentrations (Klecka and Maier, 1985; Stanlake and Finn, 1982; Gonzalez and Hu, 1995). Increases in PCP substrate concentration resulted in directly proportional increases in lag time (see Fig. 2.1) for *Arthrobacter* strain NC (Stanlake and Finn, 1982).

2.4.1.2 INOCULUM

The inoculum size of a culture can affect the PCP degradation rate (Middeldorp *et al.*, 1990). Middeldorp *et al.* (1990) incubated *R. chlorophenolicus* in peaty soil and found 500 cells/g soil at 630mg PCP/kg soil gave no significant degradation whereas 1×10^8cells/g inoculum resulted in a significant increase of PCP mineralisation (Middeldorp *et al.*, 1990). Klecka and Maier (1985) also found the rate of degradation of PCP was proportional to the concentration of cell mass used as an inoculum. Klecka and Maier (1985) noted the rate of PCP degradation increased with time, due to growth of micro-organisms.

The apparent lag (Fig 2.1) prior to measurable PCP removal is a function of time required for acclimation and the accumulation of sufficient biomass to observe measurable specific growth rates (Topp *et al.*, 1988). Stanlake and Finn (1982) reported, however, that the size

of the initial inoculum (0.12, 0.24 or 0.34mg/ml) had no discernable effect on the length of the lag period with *Arthrobacter* strain NC (Stanlake and Finn, 1982).

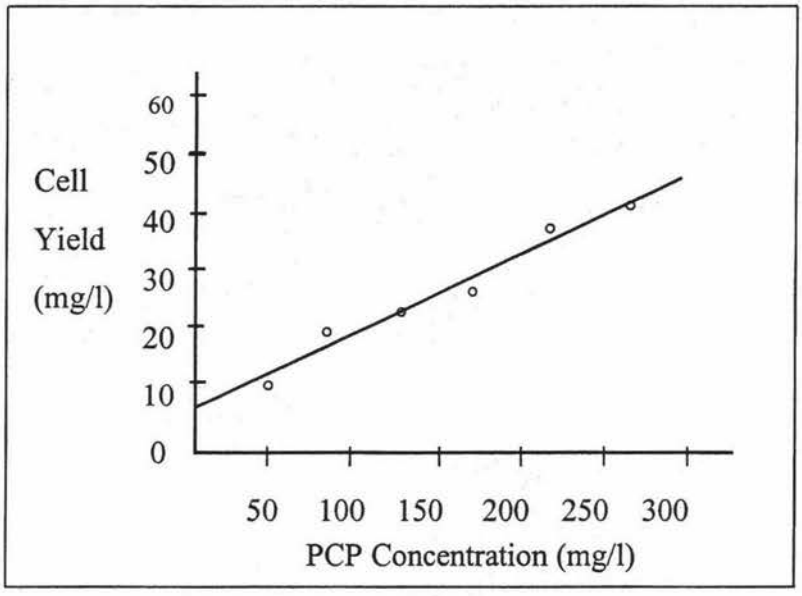


Figure 2.2: Cell yield (dry weight) produced at various PCP concentrations for *Arthrobacter* sp. strain NC in batch culture (Stanlake and Finn, 1982)

Total biomass production for *S. chlorophenolica* RA2 was proportional to PCP concentration at all PCP concentrations degraded (Radehaus and Schmidt, 1992). Stanlake and Finn (1982) also observed that total biomass production was proportional to PCP concentration in batch culture (Fig 2.2). In contrast, final cell density of *S. chlorophenolica* RA2 was reduced to below expected values at PCP concentrations greater than 100mg/l. These results indicate that, in addition to its effect as an uncoupler of oxidative phosphorylation, PCP may also inhibit cell division in *S. chlorophenolica* sp. strain RA2 (Radehaus and Schmidt, 1992).

The exposure of induced *Flavobacterium* cells to PCP alone at different concentrations showed a decrease of viability for those cultures exposed to PCP concentrations higher than the PCP concentration during induction. All cultures showed a lag in degradation of at least a few hours (Gonzalez and Hu, 1995). Of all the cells inoculated in a PCP containing

medium, only a fraction are able to grow and degrade the PCP at the concentration to which they are exposed (Gonzalez and Hu, 1995).

2.4.1.3 CARBON SUPPLEMENTATION

There are no clear indications in the literature concerning the effectiveness of supplementary carbon on the PCP degradation rate. Use of supplementary carbon sources can increase the rate of PCP degradation by either maintaining or increasing the active population and by facilitating repair of cell damage caused by PCP. Degradation rates of PCP for *S. chlorophenolica* sp. strain ATCC 39723, isolated from PCP-contaminated freshwater sediment, were found to increase upon the addition of cellobiose and the organism was capable of tolerating and degrading higher concentrations of PCP (Brown *et al.*, 1986). The supplementary carbon source nourishes the cells and increases the proportion of viable cells thereby increasing the number of metabolically active bacterial cells (Brown *et al.*, 1989). Gu and Korus (1995) observed an increase in the growth rate for *S. chlorophenolica* sp. ATCC 39723 when glutamate was used as an additional carbon source. However, if the supplementary carbon source is too high, i.e. 80mg/l to 4000mg/l, the organisms may utilise it to the exclusion of PCP (Topp *et al.*, 1988; Radehaus and Schmidt, 1992). Furthermore, PCP metabolism of *Flavobacterium* sp. was subject to repression in the presence of significant concentrations of supplementary carbon substrates including glutamate plus either glucose or cellobiose (Topp *et al.*, 1988).

Supplementary carbon was found to decrease acclimation (lag) time and facilitated PCP metabolism by glucose- or glutamate-grown *Flavobacterium* cells. Once cells were acclimated to growth on PCP alone, their specific activity was higher than that of cells metabolising PCP in the presence of supplementary carbon such as glutamate (Topp *et al.*, 1988). Radehaus and Schmidt (1992) found PCP significantly enhanced the metabolism of glucose by *S. chlorophenolica* strain RA2 (Nohynek *et al.*, 1995). Glucose and PCP were simultaneously mineralised by the strain but glucose had no effect on the rate of PCP mineralisation (Radehaus and Schmidt, 1992).

Yeast extract accelerated PCP degradation by a *Pseudomonas* sp. and 100mg/l glucose suppressed the degradation (Watanabe, 1973). Valo *et al.* (1985) studied the effect of nutrient supplementation on PCP and found supplementation with 0.21g/l ammonium chloride (NH_4Cl) reduced the degradation time by two thirds. The metabolism of PCP by the *Flavobacterium* sp. was facilitated by, but did not require, supplementary carbon (Topp *et al.*, 1988). Supplementary carbon sources such as phenol, hydroxybenzoic acids or complex nutrients did not affect the biodegradation, but the presence of ammonium salts enhanced the rate of PCP degradation without affecting the yield of CO_2 (Valo *et al.*, 1985).

Cellular damage of a *Pseudomonas* sp. was apparent at 120mg/ml PCP, as the sole carbon source and there was a reduction in viability of the cells and recovery on nutrient agar took longer (Topp *et al.*, 1988). Survivors adapted to PCP as the sole carbon source and metabolised the compound with removal rates higher than those of cells growing under mixed-substrate conditions.

Kuwatsuka and Igarashi (1975) have studied the degradation pathway of PCP in soil by indigenous bacteria. Degradation rates of PCP were found to increase upon the addition of cellobiose and the micro-organisms were capable of tolerating and degrading higher concentrations of PCP. They reported that the PCP degradation was related to the organic matter content in the soils and rose as the organic matter content rose. Apajalahti and Salkinoja-Salonen (1984) reported that bark chips absorbed PCP reversibly thus detoxifying the medium and allowing degradation to proceed at higher concentration of PCP (above 53mg/l).

2.4.1.4 TEMPERATURE

Temperature can effect PCP degradation. *S. chlorophenolica* sp. ATCC 39723 showed significant removal of PCP between 24 and 35°C, but was ineffective below 12°C or above 40°C (Crawford and Mohn, 1985). Radehaus and Schmidt (1992) reported that *S. chlorophenolica* sp. growing at 22°C and transformation of PCP was shown to proceed at

41°C, but not at 44°C for *Rhodococcus chlorophenolicus* (Apajalahti and Salkinoja-Salonen, 1987a). Valo *et al.* (1985) reported that the degradation organisms in a mixed culture were prokaryotic mesophiles and that no degradation occurred at temperatures below +8°C and above +50°C (Valo *et al.*, 1985). The most common temperature range for incubation is 28-30°C (Knackmuss and Hellwig, 1978; Stanlake and Finn, 1982; Valo *et al.*, 1985; Apajalahti and Salkinoja-Salonen, 1986; Yu and Ward, 1995; Rutgers *et al.*, 1996).

2.4.1.5 pH

Changes in pH affect PCP toxicity so pH should be maintained at the highest level possible commensurate with adequate bacterial growth (Stanlake and Finn, 1982). For both batch and continuous cultures, pH control is especially important because of the large amount of chloride ion released during the metabolism of PCP (5 mol of HCl per mol of PCP) (Edgehill and Finn, 1983). Neutral or slightly acidic conditions favour PCP degradation, the optimum being between 6.4 and 7.2. At a higher pH value (8.4) degradation is inhibited more severely than at a lower pH (5.6) (Valo *et al.*, 1985). Degradation of PCP by *S. chlorophenolica* sp. ATCC 33790 (formerly *Arthrobacter* strain ATCC 33790, Karlson *et al.*, 1995) ceased when the pH decreased to 6.15 and resumed when pH was adjusted to 7.1 Stanlake and Finn (1982). They also found that the lag phase increased as pH decreased.

2.4.1.6 PLASMIDS

Flavobacterium spp. able to mineralise 100-200mg/l of PCP were isolated by Saber and Crawford (1985) using selective enrichment from PCP-contaminated soils. All isolates metabolised PCP as the sole source of carbon and energy although some strains were more efficient than others. All strains possessed an 80 to 100 kilobase plasmid and further evidence suggested the presence of a larger (>200 kilobases) plasmid (Saber and Crawford, 1985). Hussein *et al.* (1996) isolated three organisms growing in the presence of PCP of which two were tentatively classified as *Pseudomonas* spp. They each possessed plasmids

of size between 20-30kb and Yu *et al.* (1996) hypothesised that it is likely that these plasmids encode genes which are involved in the microbial degradation of PCP. The roles of the plasmids are currently being investigated (Yu *et al.*, 1996).

2.4.2 GROWTH RATES

Table 2.2 gives a summary of the specific growth rates attained with aerobic pure and mixed bacterial cultures are grown on PCP as the sole source of carbon and energy.

Table 2.2: Degradation rates of PCP by pure cultures of bacteria.

Organism	**[PCP] (mg/l)**	**Specific Growth Rate (hr^{-1})**	**Reference**
Sphingomonas chlorophenolica sp. ATCC 39723	120	0.1	Topp *et al.*, 1988
	140	0.12	Gu and Korus, 1995
Sphingomonas chlorophenolica sp. strain ATCC 33790	135	0.1	Edgehill and Finn, 1983
Sphingomonas chlorophenolica sp. strain RA2	40-150	0.09-0.05	Radehaus and Schmidt, 1992
Sphingomonas sp. strain P5	10-44	0.142	Rutgers *et al.*, 1996
Pseudomonas sp. strain B13	39	0.4	Knackmuss and Hellwig, 1978
Arthrobacter sp. strain NC	0.013-0.3	0.05-0.28	Stanlake and Finn, 1982

Knackmuss and Hellwig (1978) observed a maximum growth rate of $0.4h^{-1}$ in continuous culture with *Pseudomonas* sp. strain B13 at PCP concentrations between 2.6mg/l and 39mg/l. The organism was grown continuously in 200ml of mineral salts medium with varying PCP concentrations at 30°C in a magnetically stirred chemostat (Knackmuss and

Hellwig, 1978). The growth rates observed with different species of *Sphingomonas* are similar (Table 2.2).

McAllister *et al.* (1991) reviewed and summarised PCP degradation by pure and mixed cultures of aerobic bacteria, anaerobic microbial cultures and fungi. They include information such as source of inoculum, experimental design and conditions, acclimation, the extent of mineralisation and dechlorination and if adsorption of PCP onto organic matter had been reported. Specific growth rates are not reported for every culture and only those comparable with the results of this project have been presented here.

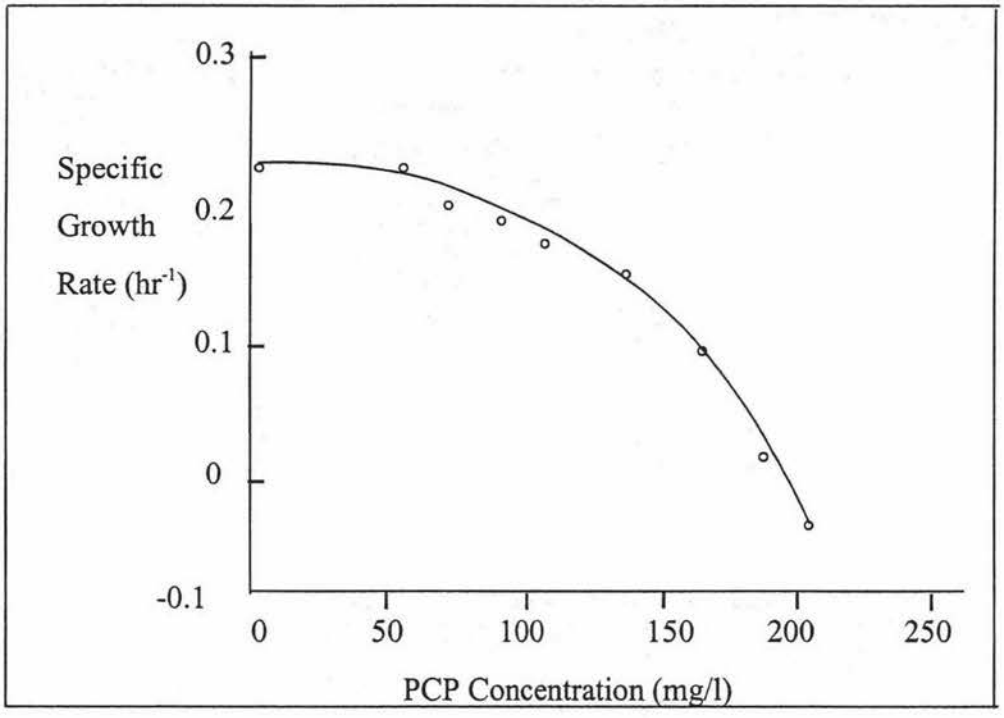


Figure 2.3: Effect of PCP concentration on specific growth rate (Gu and Korus, 1995)

Gu and Korus (1995) investigated the effect of PCP concentration, in media with only PCP as a substrate, on a *S. chlorophenolica* sp. ATCC 39723. As PCP concentration increased the specific growth rate decreased (Fig.2.3.). At a PCP concentration of about 140mg/l the specific growth rate ($0.12h^{-1}$) was only half that observed in the absence of PCP ($0.22h^{-1}$). The organism had a maximum PCP degradation rate at a PCP concentration of approximately 60mg/l. PCP had a stronger effect on the cell numbers than on the PCP

degradation rate. The degradation rate at 180mg/l was approximately 70% of the maximum, while the specific growth rate, calculated from changes in cell population numbers, was nearly zero.

Edgehill and Finn (1983) measured the specific growth rate of *Sphingomonas chlorophenolica* sp. strain ATCC 33790 and found it was constant between a PCP concentration range of 10 and 135mg/l. The culture was tolerant of PCP thus specific growth rates of approximately $0.1h^{-1}$ (7 hour doubling time) occurred at concentrations as high as 300mg/l of PCP (Edgehill and Finn, 1983).

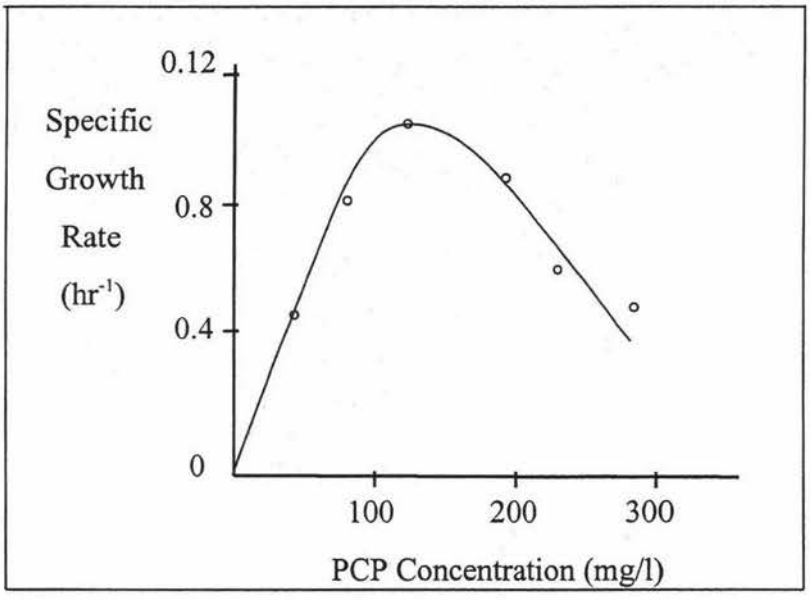


Figure. 2.4 Specific growth rate of *Arthrobacter* sp. strain NC (Stanlake and Finn, 1982).

S. chlorophenolica sp. strain RA2 was able to mineralize a high concentration of PCP (160mg/l) although at a PCP concentration of 200mg/l, cell growth was completely inhibited and PCP was not degraded. However an active population of *S. chlorophenolica* sp. strain RA2 was still present in the cultures after 2 weeks. The highest specific growth rate ($\mu = 0.09hr^{-1}$) was reached at a PCP concentration of 40mg/l but decreased at higher or lower PCP concentrations, with the lowest detectable value ($\mu=0.05hr^{-1}$) occurring at 150mg/l (Radehaus and Schmidt, 1992). Stanlake and Finn (1982) measured the specific

growth rate of *Arthrobacter* sp. strain NC at PCP concentrations between 50 to 300 mg/l. The optimum growth rate was $0.1hr^{-1}$ at a PCP concentration of 130mg/l (Fig. 2.4).

Using a mixed culture from industrial sewage sludge Klecka and Maier (1985) analysed the kinetic parameters with regard to PCP degradation. The maximum specific growth rate was low ($\mu=0.074h^{-1}$). The concentration range tested for the consortium was two orders of magnitude lower than the concentrations used by other investigators. Klecka and Maier (1985) used the Haldane modification of the Monod equation to describe the relationship between the specific growth rate and initial substrate concentration. The Monod constant K_s was 0.06mg/l indicating a high affinity of the micro-organism for the substrate. However, increased substrate concentrations were shown to be inhibitory (K_i = 1.375 mg/l) for metabolism and growth (Klecka and Maier, 1985).

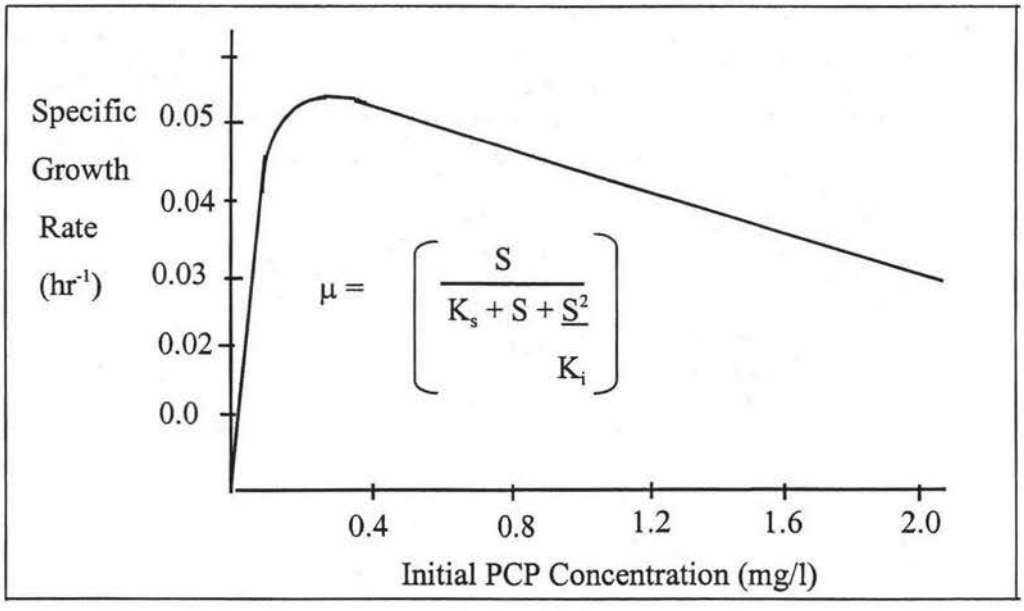


Figure 2.5: The relationship between initial PCP concentration and specific growth rate calculated by using the Haldane modification of the Monod equation. Values of the parameters used for the calculation were; μ_{max} = $0.074h^{-1}$, K_s = 0.060mg/l and K_i = 1.375mg/l (Klecka and Maier, 1985).

Although the Haldane model is valid for describing the kinetics of substrate removal at low growth rates (and hence low effective concentrations) it does not permit evaluation of

system performance over a wider range of operating conditions. However, the rate of PCP degradation increased with time due to growth of micro-organisms. Increased concentration of PCP (0.8-1.6mg/l) was inhibitory for growth as rates of substrate utilisation were greater at low initial concentrations (0.16-0.4mg/l). The relationship between growth rate and PCP concentration was shown to deviate from classical Monod kinetics in that substrate inhibition becomes a rate-limiting factor at increased substrate concentrations (Klecka and Maier, 1985).

Bradyrhizobium sp. strain ET01 was isolated from a bacterial consortium enriched from PCP contaminated soil by Hussein *et al.* (1996). Three isolates of the consortium were tested for their PCP degradation capability in pure and mixed cultures. Only the *Bradyrhizobium* sp. strain ET01 isolate was found to degrade 63ppm PCP within 7 days with 100% chloride release. Degradation of PCP was slower at concentrations in excess of 100mg/l (Hussein *et al.*, 1996). Higher PCP concentrations i.e.125mg/l and above required longer times for degradation or were not degraded at all. *Bradyrhizobium* sp. strain ET01 accounted for 63% of the consortium population (Hussein *et al.*, 1996).

2.5 BIOCHEMISTRY OF PCP BIODEGRADATION

Understanding the mechanisms that microorganisms use to mineralise pentachlorophenol (PCP) is necessary in order to develop bioremediation procedures to remove this compound from contaminated environments. To metabolise or cometabolise aromatic halogen compounds bacteria must possess enzymes that either cleave the ring in the presence of the halogen substituents or catalyze the removal of the halogen substituents prior to the dearomatizing reactions (Apajalahti and Salkinoja-Salonen, 1987b).

Pentachlorophenol (PCP) degrades slowly in the environment by chemical, microbiological and photochemical processes. This degradation is affected by numerous chemical, physical and biological factors. As this study is concerned with the biological degradation of PCP by microorganisms, the chemical and photochemical degradation of PCP will not be explored. Some products of the photochemical degradation will be presented as these exist in the environment where microorganisms utilise PCP.

Figure 2.6 shows the three biological mechanisms involved in PCP degradation. Oxygenolytic dehalogenation reactions are catalysed by monooxygenases (or dioxygenases) which incorporate one (or two) atoms of molecular oxygen into the substrate (Fetzner and Lingens, 1994). Molecular oxygen is required not only as the terminal electron acceptor during respiration but also for insertion into the aromatic compound during ring-activating hydroxylation and ring cleavage (Reineke and Knackmuss, 1988). Hydrolytic dehalogenation reactions are catalysed by halidohydrolases and the halogen substituent is replaced in a nucleophilic substitution reaction by a hydroxyl group which is derived from water (Fetzner and Lingens, 1994; McAllister *et al.*, 1996). Reductive dehalogenation reactions are catalysed by a dehalogenase and replace the halogen with a hydrogen atom (McAllister *et al.*, 1996).

Several pathways of degradation in soil and in microbial consortia in the laboratory have been identified and numerous degradation products have been isolated. This section explores the biological pathways of PCP degradation. The numbering of the compounds in the various

degradation pathways, Fig 2.6-2.10, is consistent with the composite diagram showing the relationships of the major degradation pathways in Fig 2.11.

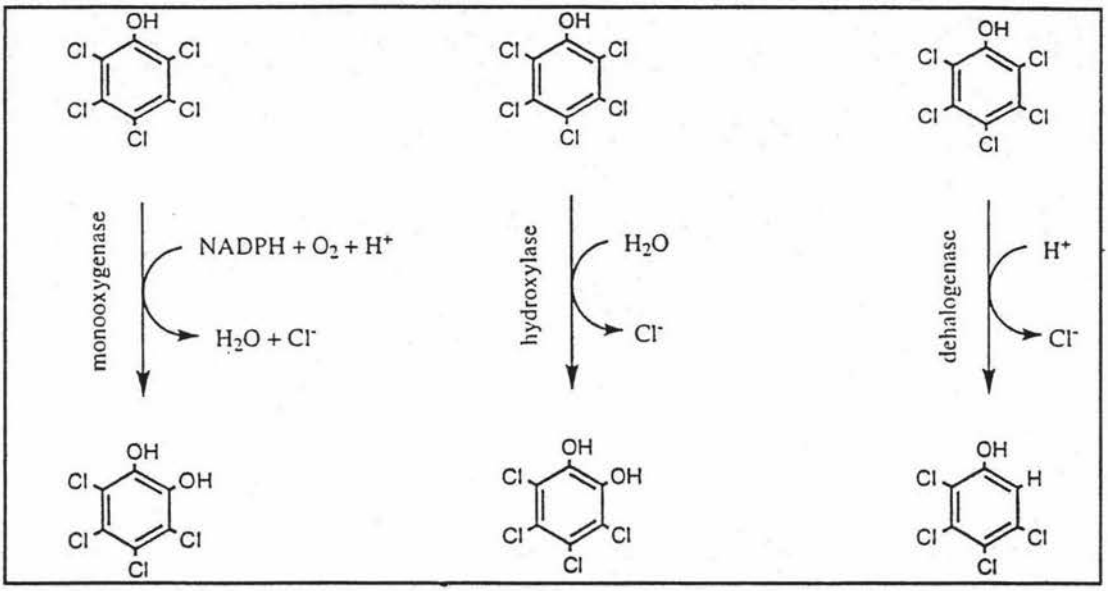


Figure 2.6 Dehalogenation mechanisms involved in the degradation of PCP (McAllister *et al.*, 1996).

Microbial degradation of PCP can proceed under both aerobic and anaerobic conditions. Under aerobic conditions the initial degradation step is catalysed by oxygen followed by hydroxylation and reductive dechlorination steps (Häggblom *et al.*, 1988). Under anaerobic conditions the chlorine atoms of PCP can be removed reductively (Nicholson *et al.*, 1992). Degradation of mono- and dichlorinated phenols, which are intermediates in some PCP degradation pathways, has also been shown to proceed by oxygenation to form chlorocatechols with dechlorination occurring only after ring cleavage of the chlorocatechol (Bollag *et al.*, 1968; Knackmuss, 1983; Knackmuss and Hellwig, 1978).

2.5.1 AEROBIC DEGRADATION OF PCP

Two different pathways of aerobic PCP degradation have been identified. The first is that proposed for *Sphingomonas chlorophenolica* ATCC 39723 (Orser and Lange, 1994; Xun *et al.*, 1992a, b, c; Xun and Orser, 1991; Nohynek *et al.*, 1995; Karlson *et al.*, 1995; Lee and Xun, 1997). Since the latter part of the pathway has not yet been elucidated for *S. chlorophenolica*,

and for completion of the pathway the reactions leading into the tricarboxylic acid (TCA) cycle as deduced from other microorganisms have been included. The second pathway for aerobic degradation of PCP is the pathway elucidated for *Mycobacterium chlorophenolicum* strain PCP-I (Apajalahti *et al.*, 1986; Apajalahti and Salkinoja-Salonen, 1987a, b; Häggblom *et al.*, 1988, 1989, 1995; Uotila *et al.*, 1991, 1995). These two pathways are described below.

2.5.1.1 THE *SPHINGOMONAS* SPP. PATHWAY

The *Sphingomonas* spp. pathway of aerobic PCP degradation involves the degradation of PCP through subsequently dechlorinated hydroquinones. PCP is oxidatively dehalogenated to form the first intermediate, tetrachloro-*p*-hydroquinone. Tetrachloro-*p*-hydroquinone is successively dechlorinated three times to form chlorohydroquinone. This pathway has been deduced from *Sphingomonas* spp. and appears to be similar in *Azotobacter* spp. and *Streptococcus* spp.. In the *Azotobacter* spp. and *Streptococcus* spp. pathways the aromatic ring is then cleaved, forming 2-chloromaleylacetate, and then the final chlorine atom is removed to produce maleylacetate. Maleylacetate then enters into the tricarboxylic acid (TCA) cycle via four steps. Described below are the individual steps in the reaction sequence from PCP to the TCA cycle, these are illustrated in Figure 2.7.

All reports on aerobic degradation propose *para*-hydroxylation of PCP [I] to give tetrachloro-*p*-hydroquinone (TeCHQ) [II] as the first intermediate in the pathway. There has been some argument as to whether the reaction is hydrolytic or oxidative (Apajalahti and Salkinoja-Salonen, 1987a; Schenk *et al.*, 1990; Xun and Orser, 1991; Xun *et al.*, 1992b). Experiments using labelled ^{18}O confirmed that the enzyme PCP 4-monooxygenase incorporates ^{18}O from $^{18}O_2$ but not from $H_2{}^{18}O$, thus the mechanism of PCP conversion to TeCHQ by *S. chlorophenolica* ATCC 39723 is an oxidative dehalogenation (Xun *et al.*, 1992b). PCP 4-monooxygenase is a monomeric (63-kDa) NADPH-dependent flavoprotein monooxygenase containing FAD (Xun *et al.*, 1992b). It is a membrane-associated cytochrome P-450 enyzme (Uotila *et al.*, 1991; Uotila *et al.*, 1992) and the optimal pH is close to neutral (Xun *et al.*, 1992c). Two moles of NADPH and 1 mol of O_2 are consumed for every 1 mol of PCP

hydroxylated (Xun and Orser, 1991; Zeng, 1997). The gene encoding this enzyme has been isolated from *S. chlorophenolica* ATCC 39723 and designated *pcpB* (Xun and Orser, 1991). PCP 4-monooxygenase has been demonstrated to have a broad substrate range, it is capable of hydroxylating the *para* position and removing halogen, nitro, amino, and cyano groups to produce halide, nitrite, hydroxylamine, and cyanide, respectively (Xun *et al.*, 1992a). Southern hybridisation of PCP-4-monooxygenase (*pcpB*) gene from *S. chlorophenolica* ATCC 39723 (Karlson *et al.*, 1995; Orser *et al.*, 1993) with genomic DNA from two other *S. chlorophenolica* strains revealed a similar sized 3.0kb EcoR1 fragments in all, whereas there was no positive hybridisation with genomic DNA from *Mycobacterium chlorophenolicum* strain PCP-I (Häggblom *et al.*, 1995).

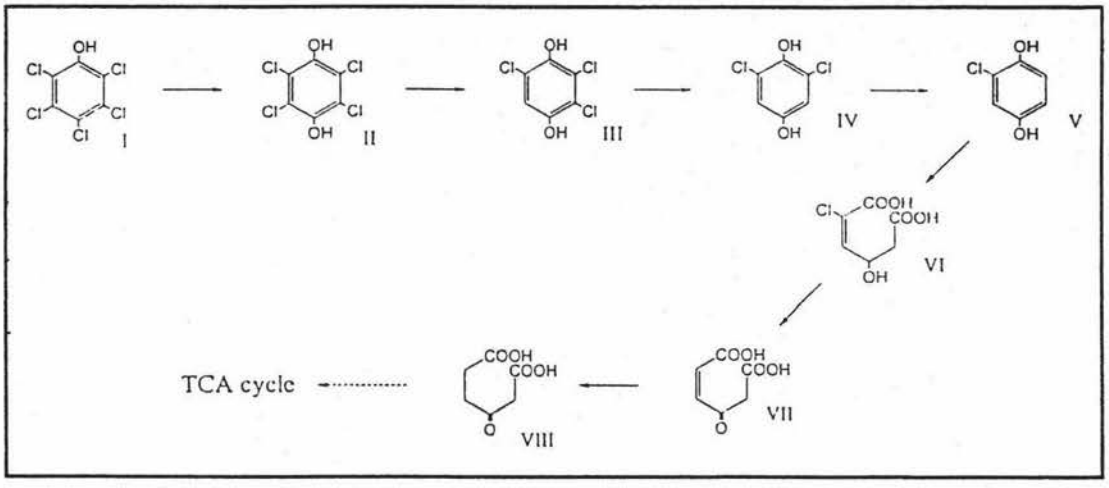


Figure 2.7 Summary of the aerobic *Sphingomonas* spp. pathway with steps from VI on derived from *Azotobacter* and *Streptococcus*; (I) pentachlorophenol; (II) tetrachloro-*p*-hydroquinone; (III) 2,3,6-trichlorohydroquinone; (IV) 2, 6-dichlorohydroquinone; (V) 6-chlorohydroxyquinol; (VI) 2-chloromaleylacetate; (VII) maleylacetate; (VIII) 3-oxoadipate.

The next three steps in the degradation pathway are thought to be catalysed by the same enzyme, a glutathione-dependent reductive dehalogenase, designated tetrachloro-*p*-hydroquinone reductive dehalogenase (TeCHQ reductive dehalogenase) encoded by *pcpC* in *S. chlorophenolica* strain (Fetzner and Lingens, 1994). In contrast to *pcpB*, the gene encoding PCP 4-monooxygenase, the gene *pcpC* producing the glutathione-dependent TeCHQ reductive dehalogenase is expressed constitutively (Fetzner and Lingens, 1994). The product of the

para-hydroxylation of PCP, TeCHQ is further *ortho*-hydroxylated to 2,3,6-trichlorohydroquinone (TCHQ) [III] (Fetzner and Lingens, 1994). TCHQ is then dechlorinated to form 2, 6-dichlorohydroquinone (DCHQ) [IV], also by TeCHQ reductive dehalogenase (Xun *et al.*, 1992c) and then DCHQ to 6-chlorohydroxyquinol (6-CHQ) [V].

The second, third and fourth reactions in this aerobic *Sphingomonas* spp. degradation pathway are catalysed by TeCHQ reductive dehalogenase (Fetzner and Lingens, 1994; Crawford, 1997) purified and characterised from *S. chlorophenolica* (Crawford, 1997). It is a homodimeric glutathione *S*-transferase (GST) with a subunit molecular mass of 30kDa (Fetzner and Lingens, 1994) and an optimal pH close to neutral (Xun *et al.*, 1992c). Glutathione *S*-transferases (GSTs) are a group of enzymes capable of recognising a wide spectrum of substrates but the mode of catalytic attack throughout the group is the same. GSTs activate the attack by glutathione on an electrophilic center, usually electrophilic carbon sites (Xun *et al.*, 1992c).

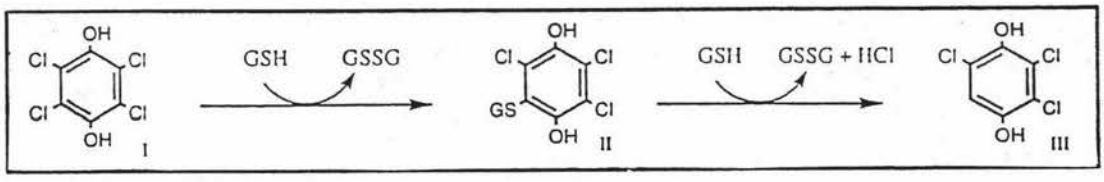


Figure 2.8 Degradation of tetrachloro-*p*-hydroquinone (TeCHQ) to trichlorohydroquinone (TCHQ) by tetrachloro-*p*-hydroquinone reductive halogenase via the *S*-chloromethyl glutathione conjugate; (II) tetrachloro-*p*-hydroquinone; (IIIa) *S*-chloromethyl glutathione conjugate; (III) 2,3,6-trichlorohydroquinone.

The TeCHQ reductive dehalogenase utilises glutathione as the reducing agent as illustrated in Figure 2.8. There is transient formation of an intermediate as the enzyme catalyzes the formation of a *S*-chloromethyl glutathione conjugates with a concomitant dechlorination taking place. The reaction is a thiolytic dehalogenation (Fetzner and Lingens, 1994). TCHQ and the intermediate formed from the previous reaction serve as substrates for TeCHQ reductive dehalogenase (Apajalahti and Salkinoja-Salonen, 1987a). The hydroxyl group forms in position 4 on the intermediate whether or not a substrate has a chlorine substituent in this position. This indicates that the enzyme only functions fortuitously as a dehalogenating

enzyme. The *para*-hydroxylation, although considered a hydrolase reaction, requires the presence of molecular oxygen (Reineke and Knackmuss, 1988). When DCHQ [IV] is converted to 6-CHQ [V] by TeCHQ reductive dehalogenase, 1 mol of molecular oxygen, 3 hydrogen atoms and 4 electrons are required to proceed with the concomitant release of the chloride atom and 1 mol of water (Zeng, 1996).

Lee and Xun (1997) propose that the conversion of DCHQ [IV] to 6-CHQ [V] is catalysed by a different mechanism. They have purified and characterised 2, 6-DCHQ chlorohydrolase from *S. chlorophenolica.* The enzyme requires Fe^{2+} for activity and utilises one mol of water with the release of one mol of hydrochloric acid. It does not require oxygen but is not inhibited in the presence of oxygen. The optimal pH for the chlorohydrolase is 7.0 (Lee and Xun, 1997). This enzyme, 2, 6-DCHQ chlorohydrolase, and chlorophenol 4-monooxygenase of *Burkholderia cepacia* AC1100 are the only two enzymes reported so far to catalyse the conversion of DCHQ to CHQ. They are however different types of enzymes. The former is a single-component hydrolase while the latter is a two-component oxygenase (Xun, 1996).

No further information is available in the degradation pathway of *S. chlorophenolica*, however the ring cleavage enzymes for 6-CHQ [V] have been studied in an *Azotobacter* spp. and a *Streptomyces* spp. (Zaborina *et al.*, 1995; Lee and Xun, 1997). These enzymes are active on both 6-CHQ and hydroxyquinol (Lee and Xun, 1997). It is not yet known whether 6-CHQ, a product of 2,6-DCHQ chlorohydrolase, is subject to ring cleavage in *S. chlorophenolica* or is converted to hydroxyquinol before ring cleavage (Lee and Xun, 1997).

The fifth step in the pathway (Figure 2.7) is the cleavage of the 6-CHQ aromatic ring by 6-chlorohydroxyquinol 1,2-dioxygenase (6-CHQ 1,2-dioxygenase), from *Streptomyces rochei* 303 (Zaborina *et al.*, 1995), forming 2-chloromaleyacetate (ClMAL) [VI]. The enzyme has a high substrate specificity and 6-CHQ and hydroxyquinol are the only compounds utilised forming chloromaleylacetate and maleylacetate [VII] respectively (Zaborina *et al.*, 1995). It is active from pH 6.2 to 7.6, with optimal activity at 6.6 and is active over a temperature range of 20 to 55EC. The molecular weight of 6-CHQ 1,2-dioxygenase was estimated to be

61,000kDa. The enzyme is a homodimer (Zaborina *et al.*, 1995). 2-Chloromaleylacetate enters the TCA cycle via three reactions in *Pseudomonas* sp. strain B13 (Kaschabek and Reineke, 1992) and *Alcaligenes eutrophus* JMP134 (Vollmer *et al.*, 1993), these are illustrated in Figure 2.9. In the first reaction maleylacetate reductase converts 2-chloromaleylacetate [VI] to 3-oxoadipate [VIII] with temporary occurrence of maleylacetate [VII] (Kaschabek and Reineke, 1992). During chloride elimination of 2-chloromaleylacetate to maleylacetate, 2-chloro-4-oxoadipate [VIIIa] is formed and during this conversion, 1 mol of chloride is released and 2 mol of NADH are consumed. Maleylacetate reductase is encoded for on chromosomal DNA (Vollmer *et al.*, 1993). 3-oxoadipate is cleaved to form acetyl-CoA and succinate (Kaschabek and Reineke, 1992) which enter the TCA cycle.

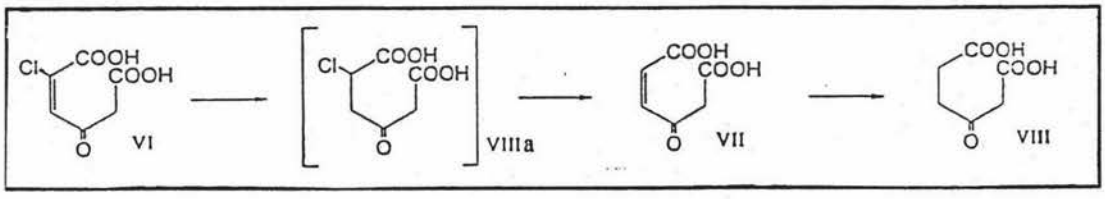


Figure 2.9. Reaction sequence of 2-chloromaleylacetate catalysed by maleylacetate reductase producing 3-oxoadipate (Kaschabek and Reineke, 1992; Vollmer *et al.*, 1993); (VI) 2-chloromaleylacetate; (VIIIa) (Cl-oxo) 2-chloro-4-oxoadipate; (VII) maleylacetate; (VIII) 3-oxoadipate.

2.5.1.2 THE *MYCOBACTERIUM* SPP. PATHWAY

The *Mycobacterium* spp. pathway of aerobic PCP degradation involves the degradation of PCP through subsequently dechlorinated trihydroxybenzenes or hydroxyhydroquinones. PCP is oxidatively dehalogenated to form the first intermediate, tetrachloro-*p*-hydroquinone. Tetrachloro-*p*-hydroquinone is hydroxylated and dechlorinated to form dichlorotrihydroxybenzene. The aromatic ring is cleaved after complete dechlorination forming maleylacetate. Maleylacetate then enters into the tricarboxylic acid (TCA) cycle via the same four steps as described for the *Sphingomonas* spp. pathway. Described below are the individual steps in the reaction sequence from PCP to the TCA cycle (Fig. 2.10). This pathway has been deduced from *Mycobacterium chlorophenolicum* PCP-I (Häggblom *et al.*, 1995; Uotila *et al.*, 1995) and appears to be similar to that described in *Streptomyces rochei* 303 (Zaborina *et al.*, 1995) and *Mycobacterium fortuitum* CG-2 (Uotila *et al.*, 1992).

The first step of PCP [I] degradation for the *Mycobacterium* spp. pathway is the same as for the *Sphingomonas* spp. pathway as described above. Whereby tetrachloro-*p*-hydroquinone (TeCHQ) [II] is formed (Apajalahti and Salkinoja-Salonen, 1987a, b; Uotila *et al.*, 1991, 1995). *Sphingomonas* spp. and *Mycobacterium* spp. appear to use unrelated enzymes for this catalytic step. The specific *Sphingomonas* sp. strain ATCC39723 gene showed a similarity to this gene in other *Sphingomonas* spp. but not to the equivalent gene of *Mycobacterium* sp. strain PCP-I using Southern hybridisation analysis (Orser *et al.*, 1993).

In the next step of this pathway TeCHQ is converted to dichlorotrihydroxybenzene (DCTHB) [X] (Fig 2.10) in reactions involving both *ortho*-hydroxylation and reductive dechlorination (Apajalahti and Salkinoja-Salonen, 1987b; Schenk *et al.*, 1989; Uotila *et al.*, 1992). Trichlorotrihydroxybenzene (TCTHB) [IX] (Fig 2.10), a hypothetic product of hydrolytic dechlorination of TeCHQ, may exist as a short-lived intermediate in this reaction but is not detected (Apajalahti and Salkinoja-Salonen, 1987b; Uotila *et al.*, 1992). The enzyme that catalyses the *ortho*-hydroxylation and reductive dechlorination of TeCHQ is a soluble halohydroquinone dehalogenase which has a broad substrate specificity. Fluorine and bromine atoms, as well as chlorine atoms, have been demonstrated to be removed from the respective halohydroquinones (Uotila *et al.*, 1992). The soluble halohydroquinone dehalogenase has a non-specific requirement for reducing power and is more active in the absence than in the presence of molecular oxygen (Uotila *et al.*, 1995). TCTHB is a poor substrate for *R. chlorophenolicus* enzymes suggesting that it is not a true intermediate in TeCH metabolism. Furthermore, TCTHB was enzymatically converted to 2, 3-dichlorohydroquinone (2, 3-DCHQ) not to DCTHB (Apajalahti and Salkinoja-Salonen, 1987b).

DCTHB [X] (Fig 2.10) is subsequently reductively dechlorinated producing monochlorotrihydroxybenzene (MCTHB) [XI] (Apajalahti and Salkinoja-Salonen, 1987b; Häggblom *et al.*, 1989; Schenk *et al.*, 1989). MCTHB is again reductively dechlorinated producing 1,2,4-trihydroxybenzene (THB) [XII](Apajalahti and Salkinoja-Salonen, 1987b;

Häggblom *et al.*, 1989). The same soluble halohydroquinone dehalogenase catalyses all reactions from TeCHQ through to THB (Uotila *et al.*, 1992; Uotila *et al.*, 1995).

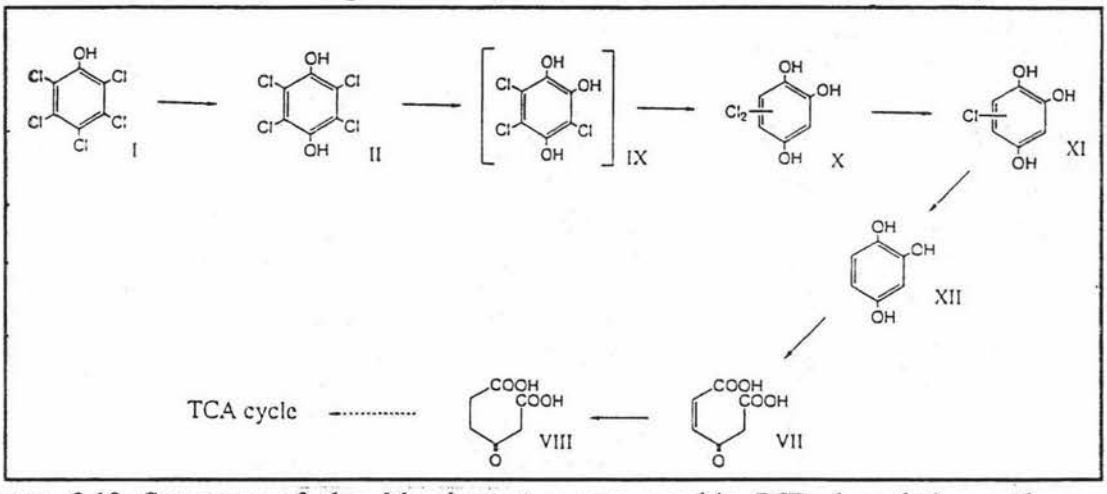


Figure 2.10 Summary of the *Mycobacterium* spp. aerobic PCP degradation pathway; (I) pentachlorophenol; (II) tetrachloro-*p*-hydroquinone; (IX) trichlorotrihydroxybenzene; (X) dichlorotrihydroxybenzene; (XI) chlorotrihydroxybenzene; (XII) trihydroxybenzene; (VII) maleylacetate; (VIII) 3-oxoadipate.

Maleylacetate [VII] (Fig 2.9) is formed in *M. chlorophenolicus* PCP-I from the cleavage of 1,2,4-trihydroxybenzene (THB) by 1,2,4-trihydroxybenzene 1,2-dioxygenase (THB 1,2-dioxygenase) (Uotila *et al.*, 1995). THB 1,2-dioxygenase has a molecular mass of 90kDa and a subunit mass of 45kDa. The enzyme catalyses an intradiol cleavage of the substrate=s aromatic ring to produce maleylacetate. Labelled $^{18}O_2$ incorporation studies demonstrate that molecular oxygen is a cosubstrate in the reaction. The enzyme exhibits a high substrate specificity (Rieble *et al.*, 1994). THB is also cleaved by catechol 1,2-dioxygenase and 6-CHQ 1,2-dioxygenase (Zaborina *et al.*, 1995), it is known to be degraded by aerobic bacteria and also by yeasts (Apajalahti and Salkinoja-Salonen, 1987b). Maleylacetate enters the TCA cycle via 3-oxoadipate, as described in the *Sphingomonas* spp. pathway.

2.5.2 ANAEROBIC DEGRADATION OF PCP

Anaerobic biodegradation of chlorophenols occurs by reductive dechlorination, a process by which chlorine atoms are replaced with hydrogen (Nicholson *et al.*, 1992). The reductive

dechlorination process is of environmental importance because the less chlorinated metabolic products of PCP are generally less toxic and degraded more easily by aerobic bacteria than the more highly chlorinated parent compounds (Mikesell and Boyd, 1985). Under anaerobic conditions the formation of some isomers is more likely than that of others as chlorine atoms in positions *ortho* to the hydroxyl group are removed more readily than those in the *meta* and *para* positions (Mikesell and Boyd, 1985, 1986; Reineke and Knackmuss, 1988; Bryant *et al.*, 1991). The removal of the *para*-chlorine of PCP leads to the formation of chlorinated intermediates that are significantly less mutagenic than PCP (Bryant *et al.*, 1991). However, the opposite is true if the *ortho*-chlorine is removed first (Bryant *et al.*, 1991).

Desulfomonile tiedjei DCB-1 (Mohn and Kennedy, 1992) was the first anaerobe isolated capable of degrading PCP in pure culture. The first three steps in the anaerobic degradation pathway of PCP, illustrated in Figure 2.11 have been determined from *D. tiedjei* DCB-1, the remainder of the degradation pathway has been determined from mixed cultures and as such there are numerous possible interrelating reactions. Nicholson *et al.* (1992) have summarised in their review all previously observed chlorophenol reductive dechlorination pathways and the anaerobic consortia responsible.

In an anaerobic mixed culture and in *D. tiedjei* PCP [I] was observed to be *ortho*-dechlorinated to produce 3,4,5-trichlorophenol (TCP) [XIV] (Rott *et al.*, 1979; Mikesell and Boyd, 1985; Reineke and Knackmuss, 1988; Nicholson *et al.*, 1992; Mohn and Kennedy, 1992) while tetrachlorophenol (TeCP) [XIII] appeared only transiently (Nicholson *et al.*, 1992; Mohn and Kennedy, 1992). However if insufficient reductant or a small amount of oxygen was present, TeCP was detected as an additional product suggesting the dehalogenation of PCP to TCP was accomplished in two separate steps (Mohn and Kennedy, 1992). There are three possible isomers of TeCP depending on where the initial dechlorination takes place i.e., in the *ortho*, *meta* or *para* position. These three isomers are further *ortho*-dechlorinated to form the respective TCPs and 2,3,4,6-TeCP can be *meta*-dechlorinated to the corresponding TCP (Nicholson *et al.*, 1992). Dehalogenase enzymes are responsible for the dechlorinations in the

anaerobic PCP degradation pathway though no purification or characterisation has been reported to date.

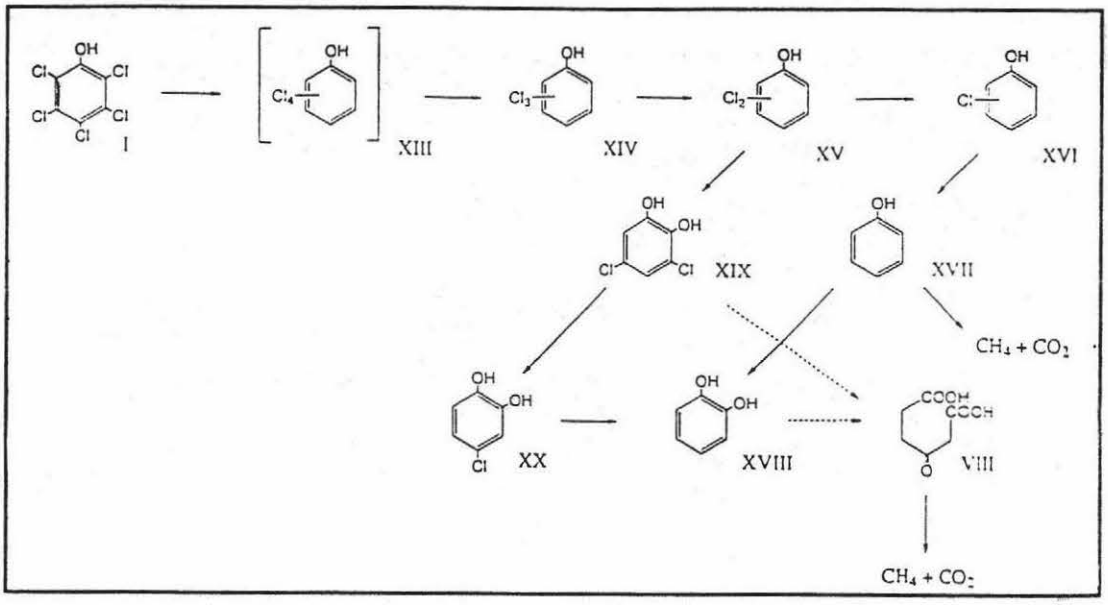


Figure 2.11 Summary of the anaerobic pathway of PCP degradation by pure and mixed cultures; (I) pentachlorophenol; (XIII) tetrachlorophenol; (XIV) trichlorophenol; (XV) dichlorophenol; (XVI) chlorophenol; (XVII) phenol; (XVIII) catechol; (XIX) 3,5-dichlorocatechol; (XX) 4-chlorocatechol; (VIII) 3-oxoadipate.

The various TCP isomers are dechlorinated to their corresponding dichlorophenols (DCP) [XV] with 3,5-DCP being the most common (Mikesell and Boyd, 1986; Bryant *et al.*, 1991; Nicholson *et al.*, 1992; McAllister *et al.*, 1996), although 3,4- and 2,4-DCP also appear (Rott *et al.*, 1979; Schenk *et al.*, 1989; Kennes *et al.*, 1996; McAllister *et al.*, 1996).

The DCPs are then further dechlorinated to monochlorophenols (MCP) [XVI]. 3,5-DCP is *meta*-dechlorinated to form 3-MCP (Mikesell and Boyd, 1986; Bryant *et al.*, 1991; Nicholson *et al.*, 1992; McAllister *et al.*, 1996) and 2,4-DCP and 3,4-DCP are *ortho* and *meta*-dechlorinated respectively to form 4-MCP (Bollag *et al.*, 1968, Mikesell and Boyd, 1985; Kennes *et al.*, 1996). 3-MCP can then be converted to phenol [XVII] (Mikesell and Boyd, 1986; Bryant *et al.*, 1991) which is subsequently converted to catechol [XVIII] (Doelle, 1969). 4-MCP is converted anaerobically to phenol (Kennes *et al.*, 1996).

2,4-DCP can alternatively be converted to 3,5-dichlorocatechol (DCC) [XIX] (Bollag *et al.*, 1968), NADPH and oxygen are required for this reaction suggesting it is catalysed by a mixed-function oxidase (Bollag *et al.*, 1968). DCC is converted to 4-chlorocatechol [XX] by dehalogenation (Knackmuss and Hellwig, 1978) and 4-chlorocatechol can be dechlorinated to catechol [XVIII] (Knackmuss and Hellwig, 1978).

There exists an anaerobic type of aromatic ring metabolism characteristically seen with the methane fermentation of benzoate (Evans, 1963). In this pathway catechol is converted to unidentified oxo-acids (Evans, 1963). Phenol is further degraded to methane and carbon dioxide (Kennes *et al.*, 1996).

2.5.3 COMPOSITE PCP DEGRADATION PATHWAYS

Microorganisms degrade PCP in both mixed and pure culture. Those degrading PCP in a consortium may metabolise intermediates at various stages of PCP degradation (Nicholson *et al.*, 1992; McAllister *et al.*, 1996) or have specialised functions to reduce the toxicity of PCP, such as *o*-methylation (Allard *et al.*, 1987; Häggblom *et al.*, 1988). Biotransformation pathways vary with the characteristics of the microbial consortium. Acclimated microbial consortia may yield biotransformation pathways different from those of unacclimated consortia (Nicholson *et al.*, 1992). There are many additional reactions linking intermediates from the aerobic and anaerobic pathways elucidated from experimental work involving soils and sludges and liquid cultures. PCP is also degraded by light and several photodegradation products exist (Kaufman, 1978). Degradation pathways in soil systems also differ from those in liquid culture as some intermediates are adsorbed onto soil particles reducing the toxicity. The two aerobic and the one anaerobic pathways discussed and how they interlink is illustrated in Figure 2.12. The *o*-methylation of PCP that occurs with various microorganisms is also shown (Allard *et al.*, 1987; Häggblom *et al.*, 1988). The compounds in composite pathways are numbered consistently with their occurrence in Figures 2.6 to 2.11. McAllister *et al.* (1996) have summarised available information on PCP degradation, mineralisation and dechlorination by

aerobic and anaerobic cultures from different environments and the degradative pathways and intermediates found in aerobic and anaerobic microbial communities.

There are links between the anaerobic and aerobic pathways. Chlorophenols may be converted to chlorohydroquinones and chlorohydroquinones to chlorotrihydroxybenzenes. TeCP [XIII] may also be converted to TCHQ [III] (Apajalahti and Salkinoja-Salonen, 1987a; Zaborina *et al.*, 1995). TeCP [XIII] may be *para*-hydroxylated to TeCHQ [II] in the presence of molecular oxygen (Häggblom *et al.*, 1988). Chlorophenols are not transformed to chlorophydroquinones in anaerobic conditions although further metabolism of the hydroquinones can proceed in anaerobiosis (Apajalahti and Salkinoja-Salonen, 1987a). TCP [XIV] can be *para*-hydroxylated to form TCHQ [III] again in the presence of molecular oxygen (Häggblom *et al.*, 1988) and can also be converted to DCHQ [IV] (Apajalahti and Salkinoja-Salonen, 1987b; Zaborina *et al.*, 1995; Zeng, 1997). DCP [XV] can be *para*-hydroxylated to DCHQ [IV] and can also be hydroxylated and dechlorinated to form CHQ [V] (Zaborina *et al.*, 1995). 4-MCP can be converted aerobically to 4-chlorocatechol [XX] by a 1,2-dioxygenase, also known as pyrocatechase II (Knackmuss and Hellwig, 1978) and also to CHQ [V] by *para*-hydroxylation (Zaborina *et al.*, 1995). 4-Chlorocatechol can enter the TCA cycle without first being converted to catechol. It is converted by a 1,2-dioxygenase enzyme to chloro-muconic acid and then chloro-muconolactone before being dechlorinated to 4-carboxymethyl 3-butenolide (Knackmuss and Hellwig, 1978). Maleylacetate reductase converts 4-carboxymethyl 3-butenolide to 3-oxoadipate (Doelle, 1969; Vollmer *et al.*, 1993) which then enters the TCA cycle as described for the aerobic *Sphingomonas* spp. pathway.

Chlorophenols can be converted to chlorohydroxyquinols by the introduction of a third hydroxy group to a chlorohydroquinone. Dichlorohydroquinone, formed from tri- and dichlorophenol, and monochlorohydroquinone, formed from di- and monochlorophenol can be converted, by addition of an hydroxyl group, to monochlorotrihydroxybenzene (MCTHB) [XI] and trihydroxybenzene (THB) [XII] respectively.

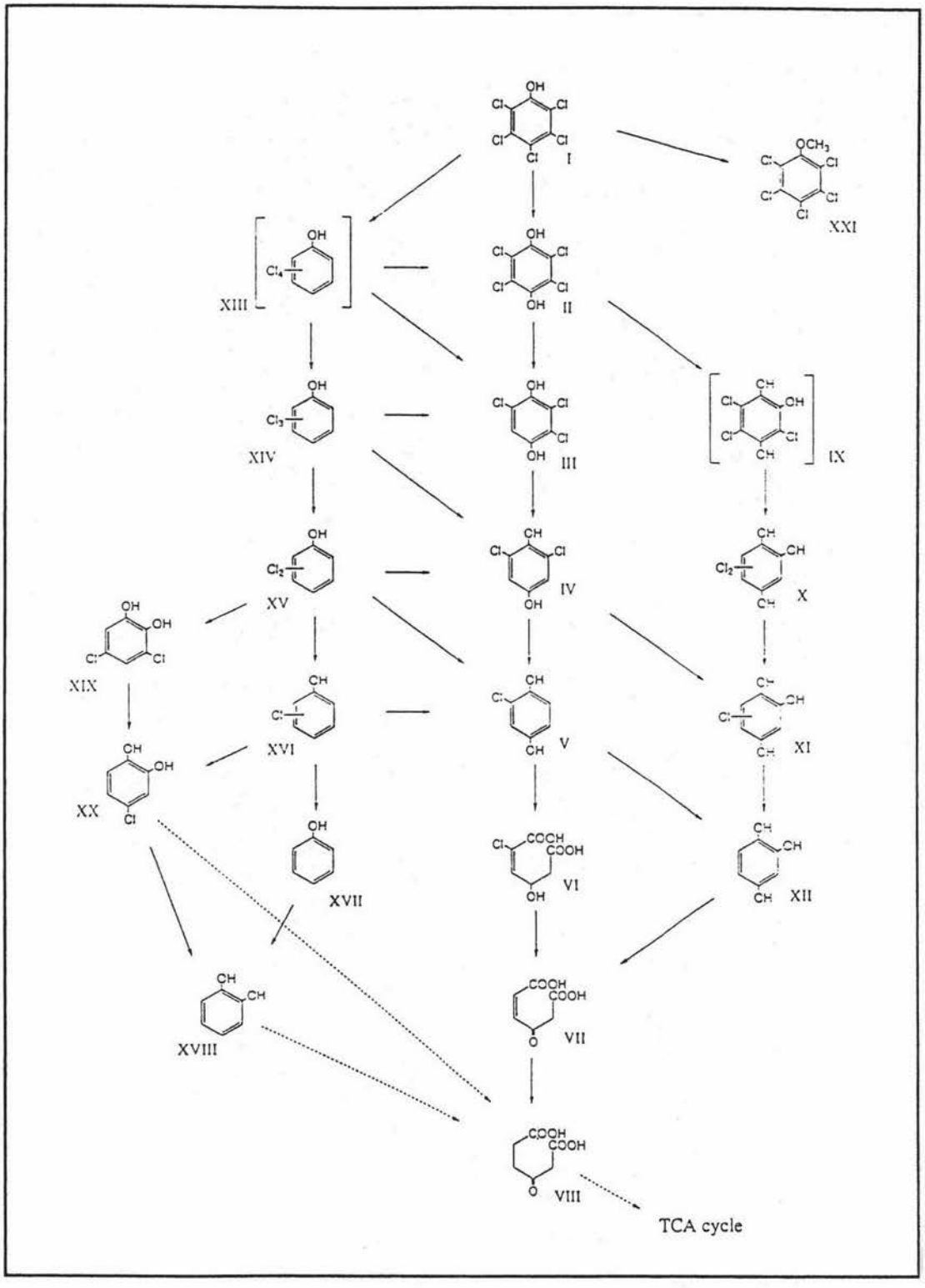


Figure 2.12 A composite diagram illustrating the links between the anaerobic and aerobic PCP degradation pathways.; (I) pentachlorophenol; (II) tetrachloro-*p*-hydroquinone; (III) 2,3,6-trichlorohydroquinone; (IV) 2, 6-dichlorohydroquinone; (V) 6-chlorohydroxyquinol; (VI) 2-chloromaleylacetate; (VII) maleylacetate; (VIII) 3-oxoadipate; (IX) trichlorotrihydroxybenzene; (X) dichlorotrihydroxybenzene; (XI) chlorotrihydroxybenzene; (XII) trihydroxybenzene; (XIV) trichlorophenol; (XV) dichlorophenol; (XVI) chlorophenol; (XVII) phenol; (XVIII) catechol; (XIX) 3,5-dichlorocatechol; (XX) 4-chlorocatechol; (XXI) pentachloroanisole.

THB [XII] is a central intermediate in the degradation of chlorophenols through the chlorohydroquinone pathway. It has been shown to be the intermediate of pentachlorophenol degradation by *Mycobacterium chlorophenolicus* PCP-1 (Apajalahti and Salkinoja-Salonen, 1987b; Häggblom *et al.*, 1995) and by *R. chlorophenolicus* CP-2 and *Mycobacterium fortuitum* and of 2-chlorophenol and 2,4-dichlorophenol degradation by *Streptomyces rochei* 303 (Zaborina *et al.*, 1995).

2.5.3.1 *O*-METHYLATION OF PCP

PCP is often *o*-methylated to form pentachloroanisole (PCA) (I to XXI in Figure 2.12), this occurs in both aerobic and anaerobic soils (Murthy *et al.*, 1979; Häggblom *et al.*, 1988). The preferred substrates of the *o*-methylating enzymes are those with the hydroxyl group flanked by two chlorine substituents. It is a slow reaction compared with degradation but is constitutively expressed, whereas degradation of chlorinated phenolic compounds is inducible (Häggblom *et al.*, 1988). *o*-Methylation may function as a detoxification mechanism as strains sensitive to the toxic effect of PCP were not affected by the same concentration of the methylation product, PCA (Häggblom *et al.*, 1988). *o*-Methylation is an important and significant reaction apart from biodegradation (Allard *et al.*, 1987). Although PCA represents a metabolic modification of PCP it is an unlikely intermediate in its degradation since PCA seems to be more resistant to biological and chemical degradation that PCP (Reiner *et al.*, 1978). PCP is not the only chlorophenol to be *o*-methylated, chlorophenols at each stage of dechlorination may be *o*-methylated as well (Crosby, 1981; Allard *et al.*, 1987).

2.5.3.2 PHOTODEGRADATION OF PCP

In addition to microbiological and chemical degradation processes, PCP is also degraded by photochemical processes, i.e. photolysis. Surface water streams commonly contain micrograms per liter concentration of PCP. Abiotic degradation of PCP processes (primarily photolysis) has been described (Pignatello *et al.*, 1983).

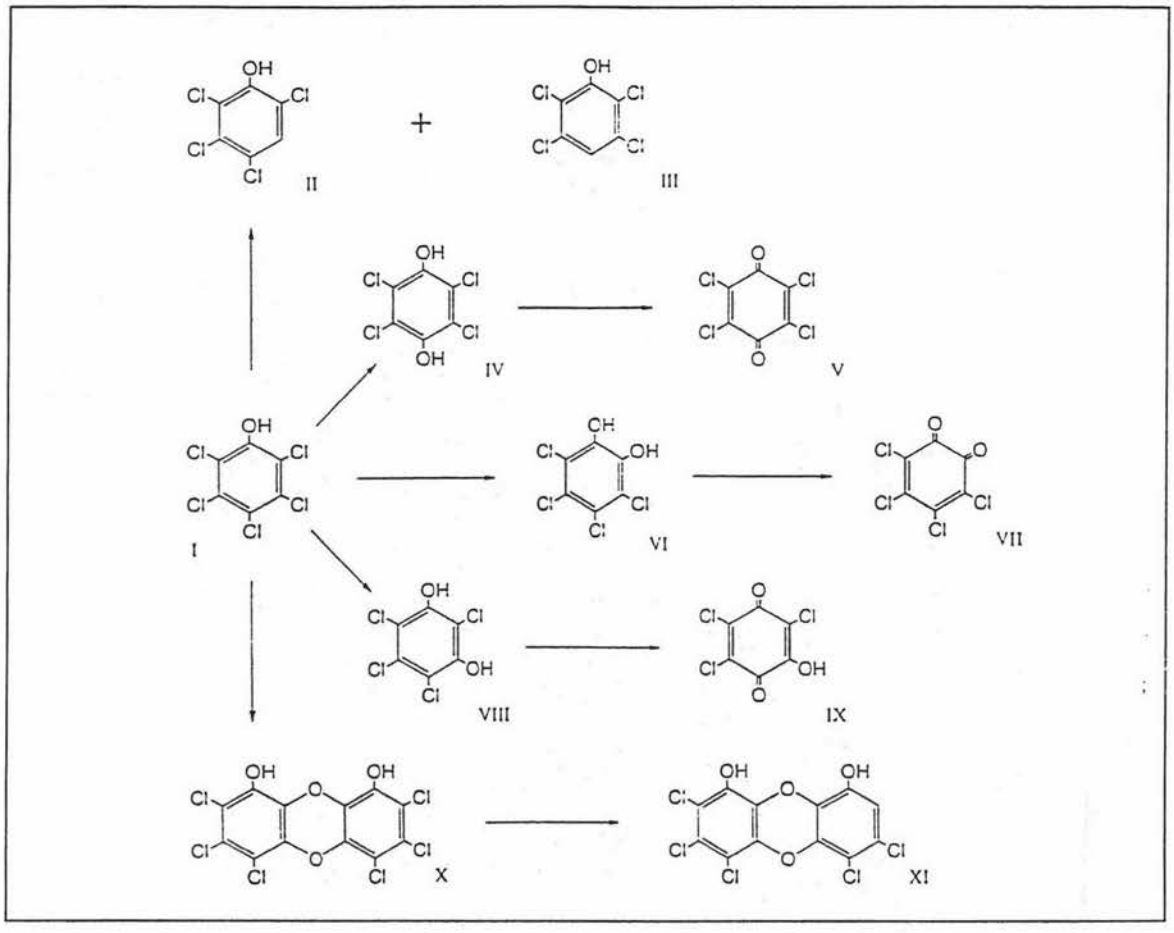


Figure 2.13 The products of photodegradation of PCP (Kaufman, 1978; McAllister *et al.*, 1996); (I) pentachlorophenol; (II) 2,4,5,6-tetrachlorophenol; (III) 2,3,5,6-tetrachlorophenol; (IV) tetrachloro-*p*-hydroquinone; (V) chloroanil; (VI) tetrachlorocatechol; (VII) 1,3-dihydroxytetrachlorophenol; (VIII) 2,4,5,6-tetrachlororesorcinol; (IX) chloranilic acid; (X) hexachlorodibenzodioxin; (XI) heptachlorodibenzodioxin; (XII) 2,5-dichloro-3-hydroxy-6-pentachlorophenoxy-*p*-benzoquinone; (XIII) 3,4,5-trichloro-6-(2',3',4',5',-tetrachloro-6'-hydroxyphenoxy)-*p*-benzoquinone; (XIV) 2,5-dichloro-3-hydroxy-6-(2',4',5',6'-tetrachloro-3'-hydroxyphenoxy)-*p*-benzoquinone; (XV) 3, 5-dichloro-4-(2',3',4',5'-tetrachloro-4-hydroxy)-6-(3,4,5,6-tetrachloro-2-hydroxyphenoxy)-*o*-benzoquinone; (XVI) 1,2,3,4,6,7,8,9-octachlorodibenzo-*p*-dioxin; (XVII) tetrachloromuconic acid; (XVIII) -hydroxytrichloromuconic acid.

Photolysis of PCP in the near surface waters is initially the primary mechanism of PCP removal (Pignatello *et al.*, 1983). Photolysis of PCP contributes to PCP removal to a lesser extent as the average depth of a stream or river increases due to the extinction of light (Pignatello *et al.*, 1983). After a period of weeks the aquatic microflora become adapted to PCP

mineralisation and supplant photolysis as the major PCP removal process. The products of PCP photodegradation are shown in Fig 2.13.

Despite the wealth of knowledge of the degradative capabilities and mechanisms of various microorganisms further research is needed. Further research would help gain a better understanding of the basic mechanisms underlying enhancement and inhibition of microbial degradation of high concentrations of toxic compounds. There is also the possibility that new isolates may have improved degradative characteristics than previously isolated strains. Establishing the degradative capabilities of micro-organisms isolated in New Zealand (in pure culture and in a consortium) may also aid in the development of clean-up strategies for areas in New Zealand with dispersed PCP pollution.

3. MATERIALS AND METHODS

All chemicals used for analytical work and media preparation were of Analytical Grade supplied by BDH Ltd., Poole, England, unless otherwise specified. Yeast extract was supplied by BBL, Becton Dickson Microbiology Systems, Cockeysville, U.S.A.. All analytical equipment is supplied by Alltech Association Incorporated, Auckland, New Zealand. All solutions were sterilised at 121°C and 15kPa for 15 minutes in a Burns and Ferral autoclave.

3.1 CULTURES

The three cultures used in this project were isolated from PCP contaminated New Zealand soil as a consortium (Hussein *et al.*, 1996). Three distinct bacterial isolates, distinguished by colony morphology on minimal mineral salts (MMS) agar plates containing yeast extract, methanol and PCP, constituted the consortium. These were isolated and identified in pure culture by repeatedly subculturing the different colony types on MMS agar (see below) and nutrient agar (NA) plates (Hussein *et al.*, 1996).

Hussein *et al.* (1996) tentatively identified two of the isolates, using API20NE system and other supplementary tests as *Pseudomonas putida* designated ET02 and *Pseudomonas aureofaciens* designated ET03 (formerly *Pseudomonas* spp. strains F and S respectively). The third isolate has been tentatively classified by 16sRNA sequencing as a *Bradyrhizobium* sp. strain and designated ET01 (formerly designated at isolation as *Pseudomonas* strain SS) (pers. com. Yu, 1997). Of the three isolates only ET01 was found to degrade in pure culture PCP (Hussein *et al.*, 1996). Agarose gel electrophoresis results indicated that all three strains possessed large plasmids (Yu *et al.*, 1996), although the function of these plasmids has not been investigated further.

Bradyrhizobium sp. (strain ET01), *Pseudomonas putida* (ET02) and *Pseudomonas aureofaciens* (ET03) will be referred to as ET01, ET02 and ET03 respectively.

3.2 GROWTH AND MAINTENANCE OF CULTURES

3.2.1 INOCULUM

The three cultures used for this project (see Section 3.1) were from the Massey University Institute of Technology and Engineering (formerly the Process and Environmental Technology Department) culture collection. The isolates were stored at 4°C on MMS agar slopes containing 20mg/l PCP, 50mg/l methanol and 200mg/l yeast extract. ET02 and ET03 were revived by subculture onto fresh agar slopes of the same medium and incubated at 30°C. ET01 was subcultured onto agar slopes containing 25mg/l yeast extract and 25mg/l PCP and also incubated at 30°C. After 7 days visible growth of ET02 and ET03 appeared and the surface growth of each was aseptically washed with 2x1ml aliquots of MMS broth containing 25mg/l PCP, 25mg/l yeast extract, 2mg/l methanol, and 2mg/l *L*-glutamic acid and then inoculated into 98ml of MMS broth containing PCP, yeast extract, methanol and *L*-glutamic acid at the same concentrations. Visible growth of ET01 on the agar slopes appeared after 10 days. Each slope was washed after growth appeared with 2x1ml aliquots of MMS broth containing 25mg/l yeast extract and 50mg/l PCP and the pooled washings used to inoculated into a conical flask containing 98ml of MMS Broth again with 25mg/l yeast extract and 50mg/l PCP. The flasks were incubated on a Gallenkamp orbital shaker at 130rpm, to provide the growth medium with sufficient oxygen, at 30°C in the dark. After 3 days incubation the optical density of the three isolates reached 0.02-0.04 when tested on a Hitachi v-2000 spectrophotometer at 600nm. The cultures were used at this stage as inocula for experimental work. The inoculum was prepared in this same way for each experiment.

For experiments requiring washed cells free from yeast extract 2x10ml aliquots of the inoculum were taken and centrifuged at 15,102g for 10 minutes. The supernatant was decanted and the sample resuspended in a 10ml aliquot of MMS broth. This was repeated three times and the resuspended cells were then used as inoculum.

For the experiments using culture combinations the method of inoculation was as follows. The inoculum was prepared as above. For the culture combination experiments all flasks

received a 10% (v/v) inoculum (10ml). For flasks containing pure culture 10ml of that culture was inoculated into the flask. Those in combination received a 10ml inoculum in a 1:1 ratio for two cultures, 5ml each of ET01 and ET02 and ET01 and ET03, or 1:1:1 ratio for all three (3.33ml of each). ET02 and ET03 were not trialed in combination because neither displayed the ability to remove PCP in pure culture. The cell concentration of ET01 when in pure culture in 100ml of media was 2×10^7 cells (2×10^6 cells/ml), in the binary experiments (5ml inoculum of ET01) the cell concentration was 1×10^7 cells and the ET01 cell concentration was 6.7×10^6 cells in the ternary experiment. The ratio of ET01 cell concentration in pure culture, in the binary and ternary experiments was 200:100:67 respectively.

The estimated ratio of ET01:ET02:ET03 in the natural environment from which they were isolated, determined by plate counts on media containing PCP, was 63:25:12 (Hussein *et al.*, 1996). The volumetric equivalent of this ratio was not tested.

Minimal Mineral Salts (MMS) Broth and Agar: Na_2HPO_4, 2.4g: KH_2PO_4, 2g: NH_4NO_3, 1g: $MgSO_4.7H_2O$, 0.01g: $CaNO_3$, 0.01g. Each ingredient was added in the above order to 1l of deionised water after the previous one had fully dissolved with stirring (Hussein *et al.*, 1996). When yeast extract was added to the MMS Broth, it was to a final concentration of 25ppm. MMS Broth was solidified by addition of 1.5g/l Oxoid Agar (Oxoid, Basingstoke, Hampshire, England). After autoclaving this was cooled to 50°C and PCP was added from the stock solution of 10, 000mg/l to give the desired concentration. The stock solution was an aqueous solution of PCP (Product 1045, Sigma, St. Louis, USA) as the sodium salt kept in the dark. The molten MMS Agar was poured into sterile 25ml bottles for slopes and sterile petri dishes.

3.2.2 CULTURE GROWTH

Conical flasks were filled with between 88.5 and 89.8ml of MMS Broth, with or without yeast extract, and were autoclaved. After autoclaving different volumes of PCP were added

to the flasks from a stock solution of 10, 000mg/l to gain the desired concentration. The final pH of the medium was 6.8.

The prepared conical flasks containing 90ml of MMS Broth with or without yeast extract and with the required concentration of PCP were inoculated with 10ml of the required culture. Flasks were incubated on a Gallenkamp orbital shaker at 130rpm in the dark at 30°C. A 500µl sample for HPLC and chloride analysis was taken immediately after inoculation and if necessary, a 5-200µl sample, depending on the dilution factor required, was taken for initial cell counts. Samples were taken daily thereafter. Optical density was trialed for measuring of cell density but as the optical density of the cultures never rose above 0.04, direct epifluorescence staining was used for total cell enumeration instead (see Section 3.5).

3.3 HPLC ANALYSIS

Two different High Performance Liquid Chromatographs were used for PCP analyses.

High performance liquid chromatography (HPLC) analysis was performed using a Waters Inc., Milwaukee (USA) system composed of a Waters 600E system controller, Waters U6K injector and Waters 486 tuneable absorbance detector set to 308nm. A Biorad Radial-Pak column (length 11.5cm, internal diameter 0.8cm) was used. Culture samples were refrigerated at 4°C if analysis was not immediate and never kept for longer than 7 days. A two point standard calibration (5mg/l and 250mg/l) was used to calculate the concentration of PCP (mg/l). Samples were centrifuged at 15,102g for 7 minutes and the supernatant used for analysis. The HPLC syringe was rinsed three times in distilled methanol and twice with the sample before manual injection of a 50µl sample. A mobile phase of distilled methanol: deionised water: glacial acetic acid (90:9.9:0.1) was vacuum filtered and degassed for 15 minutes and used at a flow rate of 4ml/minute.

Additionally, HPLC analyses was also performed using a Jasco system composed of a Jasco PU-980 Intelligent HPLC Pump, a Rheodyne model 7125 injector unit and Jasco UV-975 Intelligent UV/VIS Detector set at 308nm. A Brownlee Column SPHERI-5 ODS 5 micron (length 22cm, internal diameter 0.46cm) was used with a Brownlee RP-18 guard cartridge fitted to the top of the column. The volume of the injection loop was 20µl and a 100µl sample injection was washed through the injection loop. A two point standard calibration (5mg/l and 250mg/l) was used to calculate the concentration of PCP (mg/l). Samples were centrifuged at 15,102g for 7 minutes and the supernatant used for analysis. The syringe was rinsed three times in distilled methanol between samples and twice with the sample before manual injection of a 100µl sample. A mobile phase of distilled methanol: deionised water: glacial acetic acid (90:9.9:0.1) was vacuum filtered and degassed for 15 minutes and used at a flow rate of 2ml/minute.

A comparison of the PCP measurements was made between the two chromatographs at regular intervals and the measured PCP, at a variety of concentrations, was within 4mg/l for each reading. The standard deviation for a sample (triplicate analysis) was 2%.

3.4 CHLORIDE ANALYSIS

Chloride analysis was performed using a Buchler Digital Chloridometer. This is a coulometric titrator that calculates the milli-equivalents in a solution from the electric current between two silver electrodes ($Ag^+ + Cl^- = AgCl$). As the equivalence point is reached the current flowing between the two indicator electrodes increases and this is detected at a certain preset indicator current and the incremental counter is stopped automatically.

Chloridometer Acid Solution: Concentrated nitric acid (Ajax Chemicals Ltd., Sydney, Australia) 6.4ml; glacial acetic acid (Ajax Chemicals Ltd., Sydney, Australia), 100ml. The concentrated nitric acid and glacial acetic acid were added to 893.6ml of deionised water.

Gelatin Reagent: The gelatin for the chloridometer gelatin reagent was food grade (Davis Gelatin Co., Christchurch, New Zealand) and 10g of gelatin was placed in a 500ml conical flask with approximately 450ml of deionised water, stirred and wash 10 times in deionised water. The gelatin solution was made to a concentration of 1% (w/v) in deionised water and stored at 4°C. One solution was made and lasted for the duration of the project.

To ascertain the concentration of chloride in each sample, 1.5ml of sample or sample diluted with deionised water made up to 1.5ml were placed in a vial. Chloridometer acid solution and gelatin reagent were added, 0.5ml and 2 drops respectively each from a plastic disposable pasteur pipette. The meter was set on low.

A blank reading was taken first using deionised water as the sample and then to calibrate the chloridometer a 20mg/l chloride solution was tested. The milli-equivalent reading from the chloridometer was converted to mg/l of chloride by the equation below.

$$\text{mg/l chloride} = (\text{reading mEq/l} - \text{blank reading mEq/l}) \times \frac{35.45}{150}$$

Chloride comprises 66.7% of the total molecular weight of PCP thus the maximum mg/l of chloride calculated is 66.7% of the initial PCP concentration. The molar ratio of chloride released to PCP consumed is 5.0, i.e. 5 moles of chloride are released for each mole of PCP consumed.

3.5 EPIFLUORESCENT STAINING

Acridine Orange (AO) and CTC (5-cyano-2,3-ditolyl tetrazolium chloride) were used for epifluorescent staining to distinguish between inactive and metabolically active cells respectively (Yu *et al.*, 1995). A review of fluorescence microscopy staining techniques can be found in Appendix A. The filter unit used was a Sartorius model SM 16306, with a

surface area of $3cm^2$. The frits of this unit were glass to ensure particularly even distribution of the cells on the surface of the membrane. All reagents used for epifluorescent staining were filtered through a 0.22μm pore diameter filter membrane (Millipore, USA) and kept at 4°C until required for use. Reagents were brought to room temperature immediately before use. When using fluorochromes for direct enumeration of total bacteria Kepner and Pratt (1994) recommended that the information, as presented here in Table 3.1, be included when reporting bacterial densities.

Table 3.1 Summary of staining procedure information as recommended by Kepner and Pratt (1994).

Staining Parameters	AO preparation	CTC preparation	Sequential CTC/AO stain
Stain type	AO	CTC	CTC and AO
Stain concentration	0.01%w/v	50mM	0.01%w/v and 50mM respectively
Staining time	7 minutes	1 hour	1 hour and 7 minutes respectively
Wavelength	530nm	590nm	530nm
Filter	0.22μm pore size black polycarbonate membrane filter		
Vacuum	100mm mercury column		
Counting	10 counting areas per filter, duplicates of filters done		
Magnification	x1000 (50μm = 11 eye piece divisions)		
Preservative	None		

3.5.1 ACRIDINE ORANGE STAINING

3.5.1.1 REAGENTS

Acridine Orange: 10mg of Acridine Orange (BDH, Poole, England) was mixed with 100ml 100mM phosphate buffer (pH 7.2) to give a 0.01% w/v solution (pH 7.4). The solution was

filter sterilised through a 0.22μm pore diameter filter membrane (Millipore, USA) and kept wrapped in foil in the dark at 4°C until required for use.

Phosphate buffer, 100mM (pH 7.2): Mixed 9.7ml solution A (KH_2PO_4, 68.05g/l) and 13.4ml solution B ($Na_2 HPO_4$, 70.99g/l) with 476.9ml of deionised water. The solution was dispensed into 100ml Duran bottles and stored at 4°C until required for use.

Non-Phosphate Wash Solution (NPWS): NaCl, 8.50g; $MgCl_2.6H_2O$, 1.01g. The ingredients were mixed with 1l of autoclaved filter sterilised, through a 0.22μm pore diameter filter membrane (Millipore, USA), deionised water. The solution was dispensed into 100ml Duran bottles and stored at 4°C until required for use.

Formaldehyde Solution (40%w/v): Formaldehyde, 40ml. The formaldehyde was added to 60ml of deionised water. The solution was filtered through a 0.22μm pore diameter filter membrane (Millipore, USA) to removed any particulate matter that may have stained with AO and subsequently fluoresced on the membrane inhibiting accurate counting. The solution was dispensed into 10ml bottles and stored at 4°C until required for use.

All reagents used for AO staining were filter sterilised through a 0.22μm pore diameter filter membrane (Millipore, USA) and kept at 4°C until required for use. Reagents were brought to room temperature immediately before use.

3.5.1.2 STAINING PROCEDURE AND CELL COUNTS

Acridine orange (AO) was used in this study to enumerate the total cell numbers by direct count epifluorescence microscopy and the method was very similar to that documented by Bitton *et al.* (1993). The sample was diluted and stained in an Eppendorf tube rather than on the filter. A sample of 1ml, or the sample diluted with non-phosphate wash solution (NPWS) and made up to 1ml, was placed in a sterile 1.5ml Eppendorf vial. A sample containing 1ml of NPWS only was used as a control (zero count) to check the sterility of

reagents and accuracy of cell counts. Formaldehyde was added, 0.05ml at 40% (w/v), the Eppendorf tubes vortexed for 3 seconds and then incubated for 15 minutes at room temperature to kill the cells. A 115µl aliquot of 0.01% w/v AO was then added to the Eppendorf tube. The 1.165ml samples were mixed on a vortex for 3 seconds before being incubated in the dark for 7 minutes at room temperature.

A 25cm diameter membrane filter unit, with a 201.1mm^2 inner surface area, was assembled with a 0.22µm pore size black polycarbonate membrane filter (Poretics, U.S.A. or Millipore, U.S.A). The entire sample was pipetted into the filter unit prior to the vacuum being applied. Before the last part of the sample had passed through the filter a 1ml aliquot of double filtered sterile deionised water was used to rinse the sides of the filter unit. The vacuum, equivalent of Hg 100mm was applied until all the liquid had gone through. The vacuum was disconnected and turned off and the filter removed with forceps and placed on a clean glass slide to dry at room temperature for 10 minutes.

A drop of Olympus (Japan) non-fluorescent non-drying oil was spread on a glass slide and when the membrane was dry it was placed on the slide in a manner that excluded air between the slide and the membrane. Another drop of Olympus (Japan) non-fluorescent non-drying oil was placed on top of the membrane and a coverslip placed on top. Another drop of Olympus (Japan) non-fluorescent non-drying oil was placed on the coverslip.

The membrane was examined under oil immersion at x1000 magnification with an Olympus epifluorescence microscope with a BH2-RFL fluorescent light attachment. An excitation filter (BP-490) selected the light of the required wavelength (450nm) for excitation from the mercury lamp source. The slides were examined immediately after staining but when stored in the dark retained their fluorescence for up to 1 week, although counts were not taken again after the storage period. Each sample was prepared in duplicate and numbers of cells observed in ten randomly chosen fields at x1000 magnification were recorded per filter membrane.

Cell count (Number/ml) = (Number of cells counted per field – zero count) x Dilution Factor

The effect of the dilution factor (x) on the count of cell numbers (y) was tested to ensure a linear relationship existed between x and y. One sample was enumerated at different dilutions and the end result of the enumeration and calculation was identical. For dilutions up to 100 fold the relationship between x and y was shown to be linear.

3.5.2 CELL NUMBER CONVERSION

A conversion factor of 1.6×10^{-9}mg/cell for the weight of an individual cell (Langfeldt, 1997) was used to change cell numbers per ml to mg of cells per ml.

3.5.3 CTC STAINING

Experiments were conducted to explore the potential value of CTC as a means of detecting cellular metabolic activity. This data is presented in Appendix A8.2.

3.5.3.1 REAGENTS

CTC Solution: CTC, 100mg. A 50mM solution of CTC in deionised double filtered autoclaved water was used, 100mg of the powdered CTC was added to 6.6ml of deionised water. The redox dye CTC was synthesised by Dr. R. Chong at the Massey University Institute of Technology and Engineering (formerly the Process and Environmental Technology Department). The solution was filtered through a 0.22μm pore diameter filter membrane (Millipore, USA) and kept wrapped in foil in the dark at 4°C until required for use. Using the CTC made at Massey University was validated when it compared favourably with a commercial CTC preparation (see Appendix A8.2).

R2A Medium (Reasoner and Geldreich, 1985): Yeast extract, 0.5g; Difco pepteose peptone no.3, 0.5g; casamino acids, 0.5g; glucose, 0.5g; soluble starch, 0.5g; sodium pyruvate, 0.3g;

K_2PO_4, 0.3g; $MgSO_4.7H_2O$, 0.05g. The ingredients were added to 1L of deionised double filtered autoclaved water and the pH was adjusted to 7.2 using K_2HPO_4 or KH_2PO_4. The solution was filtered through a 0.22μm pore diameter filter membrane (Millipore, USA) and stored at 4°C until required for use.

Luria Broth: Difco Tryptone (casein hydrolysate), 10g; Peptone No. 140 (Gibco BRL. Life Technologies, Paisley, UK); NaCl, 10g (Univar analytical reagent, Ajax Chemicals, New South Wales, Australia); BBL yeast extract, 5g (Becton Dickson Microbiology Systems, Cockeysville, U.S.A.). The ingredients were added to 1L of deionised double filtered autoclaved water. The solution was filtered through a 0.22μm pore diameter filter membrane (Millipore, USA) and stored at 4°C until required for use.

Casamino Acids Solution (3% w/v): Casamino acids, 0.3g (Catalogue No. 0231-17-2, Difco Laboratories, Detroit, Michigan, USA). The casamino acids were added to 10ml of deionised double filtered autoclaved water, filtered through a 0.22μm pore diameter filter membrane (Millipore, USA) and stored at 4°C until required for use.

Glucose Stock Solution (500mM): Glucose, 0.901g (Catalogue No. 15023-021, Gibco BRL. Life Technologies, Paisley, UK). The glucose was added to 10ml of deionised double filtered autoclaved water. The solution was filtered through a 0.22μm pore diameter filter membrane (Millipore, USA) and stored at 4°C until required for use.

Nutrient Broth: 13g Dehydrated nutrient broth (Catalogue No. 152-03600, Gibco, Life Technologies Ltd., Paisley, UK). The nutrient broth dehydrated powder was dissolved in 1l of deionised water and sterilised. pH 7.4 ∀ 0.2.

Brain Heart Infusion (BHI) Broth: 37g (Catalogue No. 10680-098 Gibco BRL. Life Technologies, Paisley, UK). The BHI powder was dissolved in 1l of deionised water and sterilised at pH 7.4 ∀ 0.2.

3.5.3.2 STAINING PROCEDURE

Two 1ml samples were taken from each conical flask and cell suspensions prepared by centrifuging at 15,100g for 5 minutes. The supernatant (0.9ml) was removed and the solids washed in a 0.9ml aliquot of non-phosphate wash solution (NPWS). This was repeated twice more. A sample of the prepared cell suspension was dispensed into a sterile 1.5ml Eppendorf tube depending on the dilution required and 0.1ml of the CTC (5-cyano-2,3-ditolyl tetrazolium chloride) stock solution was then added. For the blank a volume of deionised autoclaved water, filtered through a 0.22μm pore size membrane filter was treated as a sample. The volume was made up to 1.0ml with NPWS. After each addition the Eppendorf tubes were vortexed for 3 seconds. The tubes were then incubated in the dark on a Gallenkamp orbital shaker at 130rpm for 1 hour at 30°C. The final CTC concentration was 5.0mM. A 25mm diameter membrane filter unit, with a 201.1mm^2 inner surface, was assembled with a 0.22μm pore size black polycarbonate membrane filter (Poretics, U.S.A. or Millipore, U.S.A).

After incubation the sample was pipetted into the filter unit so the sample completely covered the membrane and the vacuum applied. Before the last part of the sample had passed through the filter a 1.065ml aliquot of double filtered autoclaved deionised water was used to rinse the sides of the filter unit. The vacuum was applied until all the liquid had gone through. The vacuum was disconnected and turned off and the filter removed with membrane forceps and placed on a clean glass slide to dry at room temperature for 10 minutes.

A drop of Olympus (Japan) non-fluorescent non-drying oil was spread on a glass slide and when the membrane was dry it was placed on the slide in a manner that excluded air between the slide and the membrane. Another drop of Olympus (Japan) non-fluorescent non-drying oil was placed on top of the membrane and a coverslip placed on top. Another drop of Olympus (Japan) non-fluorescent non-drying oil was placed on the coverslip and the membrane was examined under oil immersion at x1000 magnification with an Olympus

epifluorescence microscope with a BH2-RFL fluorescent light attachment. At x1000 magnification under oil immersion 50μm was equal to 11 eye piece divisions. An excitation filter (BP-490) selected the light of the required wavelength (450nm) for excitation from the mercury lamp source.

3.5.3.3 STAINING PARAMETERS INVESTIGATED

The effect of various parameters, listed below, in the CTC staining procedure on the respiring cells' fluorescence was investigated. The results are presented in the Appendix Section A8.2.1. These parameters were tested because the actively respiring cells could not be seen clearly when viewed under the epifluorescence microscope. The staining method described in section 3.5.3.2 was used as a control method when each parameter was tested.

A supplementary carbon source was added to the CTC-cell suspension to enhance the respiration activity of the microorganisms utilising the PCP. The addition of 0.2ml of R2A (defined in Section 3.5.3.1), a micro-nutrient medium, to the CTC-cell suspension was tested as were the addition of 0.2ml of Difco Luria broth (defined in Section 3.5.3.1), 0.2ml of Difco casamino acids (defined in Section 3.5.3.1) and 0.2ml of Gibco BRL glucose stock solution (defined in Section 3.5.3.1).

The final CTC concentration in the CTC-cell suspension was tested over a range of 2 to 10mM and at incubation times of 1 hour, 2 hours, 4 hours, 8 hours, and 24 hours.

E. coli was grown on Gibco BRL nutrient broth (NB) and Gibco BRL BHI broth at 37°C for 12 hours to ensure cells were in the exponential growth phase and actively respiring. The staining method described in section 3.5.3.2 above was used. *E. coli* from the nutrient and BHI broths were combined in ratios 1:3, 1:1 and 3:1 with formaldehyde killed *E. coli* and stained as in section 3.5.3.2.

The incubation times and temperatures were tested in combination with ET01 as per section 3.5.3.2. The incubation temperature tested were at room temperature, 30°C, 37°C and 42°C for incubation times of 1 hour, 2 hours, 4 hours, 8 hours and 24 hours.

3.5.4 SEQUENTIAL CTC/ AO STAINING

For the sequential CTC/AO stain the CTC staining procedure was followed and after the hour incubation with CTC, 0.115ml of AO (0.01%w/v) solution was added to the Eppendorf tube. The tube was left to incubate at room temperature for 7 minutes in the dark. The filtration and slide mounting procedures were as for the individual staining methods. The slides were viewed under an Olympus epifluorescence microscope with a BH2-RFL fluorescent light attachment. An excitation filter (BP-490) selected the light of the required wavelength (450nm) for excitation from the mercury lamp source. Wavelengths above 530nm or 590nm, as required for AO or CTC count, respectively, were cut off by the application of a barrier filter. The final magnification was x1000 under oil immersion with Olympus (Japan) non-fluorescing non-drying oil. At x1000 magnification under oil immersion 50μm was equal to 11 eye piece divisions.

The results from the CTC staining are presented in the Appendix Section A8.2.1, CTC Staining.

3.6 VITAMIN EXPERIMENT

The vitamin experiment aimed at determining which of three vitamin solutions, each containing a selection of vitamins (see below), would allow the three isolates, in pure culture and in combination, to grow and degrade PCP without yeast extract in the medium. Each vitamin solution was prepared separately, filter sterilised through a 0.22μm pore diameter filter membrane (Millipore, USA). The solutions were stored at 4°C, wrapped in aluminium foil.

Six conical flasks containing 90ml of MMS were set up for each culture in pure culture and in combination. Each of the cultures and culture combinations had flasks containing the separate vitamin solutions, a flask containing 25mg/l yeast extract and a control with no added yeast extract or vitamins. PCP was added to a concentration of 50mg/l and the flasks were inoculated with 10ml of the appropriate cultures. The method of inoculation is described in the Methods, Section 3.2.1. Flasks were incubated at 30°C on a Gallenkamp orbital shaker (130rpm) in the dark. Samples of 0.5ml were taken daily over a period of 14 days and if not analysed immediately were stored at 4°C for up to a week until analysed by HPLC.

The following vitamin solutions were used to ascertain the vitamin requirements for the isolates. Each vitamin solution was prepared separately and stored at 4°C, wrapped in aluminium foil.

Vitamin solution 1: Biotin, 0.020g (B4501 98% pure, Sigma, St. Louis, USA) and cyanocobalamin, 0.003g (C3607 USP[1], Sigma, St. Louis, USA) were added to 200ml of deionised water before being filter sterilised.

Vitamin solution 2: Biotin, 0.0020g (B4501 98% pure, Sigma, St. Louis, USA); Cyanocobalamin, 0.0006g (C3607 USP[1], Sigma, St. Louis, USA); pantothenic acid 0.0800g (44074Y 98% pure, BDH, Poole, England); Cysteine 0.0100g (11035-011 USP[1], Gibco, Life Technologies Ltd., Paisley, UK) were added to 200ml of deionised water before being filter sterilised.

Vitamin solution 3: Biotin 0.002g (B4501 98% pure, Sigma, St. Louis, USA); cyanocobalamin 0.010g (C3607 USP[1], Sigma, St. Louis, USA); thiamine 0.010g (440055N 98.5% (ex.N) BDH, Poole, England); pyridoxine 0.050g (P4722 USP[1] Sigma, St. Louis, USA); riboflavin 0.010g (R7649 USP[1], Sigma, St. Louis, USA); nicotinic acid (0.020g, AR grade, Sigma, St. Louis, USA); *p*-aminobenzoic acid (0.005g, AR grade, Sigma, St. Louis,

[1] USP is the symbol of products that meet the standards published in the US Pharmacopeia XXIII

USA); pantothenic acid 0.010g (44074Y 98% pure, BDH, Poole, England) were added to 200ml of deionised water and filter sterilised through a 0.22μm pore diameter filter membrane (Millipore, USA).

The vitamin solutions were added to separate flasks of MMS medium, without yeast extract, at 0.10ml per 100ml medium. Cells were washed before inoculation as the carry over of yeast extract from the inoculum gave degradation rates comparable to the medium containing 25mg/l yeast extract. Each of the three cultures were washed three times in sterile centrifuge tubes. The cultures were centrifuged at 15,102g, the supernatant removed and the cells washed with a 50ml aliquot of MMS containing neither yeast extract or PCP. The cells were resuspended by vortexing for 5 seconds before the process was repeated.

3.7 RATE DETERMINATION

The PCP removal and chloride release rates were determined by fitting a regression line through the linear part of the growth and chloride release curves. Three or more points were used in calculating the rate of PCP removal and chloride release.

3.8 CALCULATION OF SPECIFIC GROWTH RATE

The specific growth rates were calculated using the slope of a linear regression of the data from the exponential phase as found from a curve of the natural log of the cell counts versus time. At each point in time 20 cell counts were used for the linear regression. The graphs from which the regression equations were derived are presented in the Appendix Section A8.11.

3.9 CALCULATION OF INHIBITION CONSTANT

To distinguish the inhibition constant (K_I) from K_s it is defined as the highest substrate concentration at which the specific growth rate (μ) is reduced to half the maximum value (Radehaus and Schmidt, 1992). The K_I for PCP removal was obtained by using the highest

specific growth rate divided by two and extrapolating the line across to where it intersected with the descending portion of the curves presented in the Results, Section 4.5 (Figure 4.15). A perpendicular line was drawn at the point where half the maximum specific growth rate and where it intersected the curve the relative PCP concentration was deemed the K_i.

3.10 CALCULATION OF 95% CONFIDENCE INTERVALS

T tables were used to obtain the 95% confidence intervals for the specific growth rates of all cultures at all initial PCP concentrations. The standard deviation of the specific growth rate was multiplied by the T value with the appropriate degrees of freedom and the result deemed the 95% confidence interval. All values were reported to 3 decimal places and not 4 because of the variation of the 95% confidence intervals.

The yield was reported to 3 significant figures. To obtain the 95% confidence intervals for the yield the standard deviation of the biomass value multiplied by 2 (for 2 standard deviations i.e. 95%) was expressed as a fraction of the biomass value. This fraction was transferred to the yield value. The possible error occurring from the weight measurement of the PCP into the flask was not added because the combined error of the measuring cylinder and the analytical balance weighing to 4 decimal places was considered to be less than 1%, negligible when compared to the 95% confidence interval of the biomass counts.

4. RESULTS

4.1 ISOLATE GROWTH AND CHARACTERISTICS

The bacterial strains ET01 (tentatively identified as *Bradyrhizobium* sp. strain ET01, formerly *Pseudomonas* sp. strain SS), ET02 (formerly *Pseudomonas putida* strain F) and ET03 (formerly *Pseudomonas aureofaciens* strain S) isolated by Hussein *et al.* (1996) were grown as described in the Methods, Section 3.2.2. Isolate ET01 was a pin-point beige mucoid colony after 10 days incubation on MMS agar with 50mg/l PCP and 25mg/l yeast extract. Isolate ET02 formed a larger colony, entire, mucoid and white with a diameter of between 1 and 3mm after 10 days growth on MMS agar with 25mg/l PCP, 25mg/l yeast extract, 5mg/l methanol and 5mg/l L-glutamic acid. Isolate ET03 exhibited similar colony characteristics to ET01 when incubated on MMS agar with 25 mg/l PCP, 25mg/l yeast extract, 5mg/l methanol and 5mg/l L-glutamic acid. A line drawing of each of the three Gram-negative isolates' morphology is presented in Table 4.1 below.

Table 4.1: Line drawing of each isolates' cellular morphology and cell size.

Bradyrhizobium sp. strain ET01	*Pseudomonas putida* strain ET02	*Pseudomonas aureofaciens* strain ET03
Stationary phase 1µm Exponential phase	1µm	1µm

Biochemical tests were performed on each of the isolates using a BBL Oxiferm® tube, the results are shown in Table 4.2. When incubated as per the manufacturer's instructions all the results were negative for ET01. The manufacturer's instructions suggest that tubes are left for 48 hours. Table 4.2 indicates the biochemical characteristics of each isolate and those of ET02 and ET03 are consistent with *Pseudomonas* sp.. The results for ET01 are inconclusive therefore the identification of ET01 by Yu (pers. comm., 1997) as *Bradyrhizobium* sp. was adopted.

Table 4.2: Biochemical characteristics of ET01, ET02 and ET03.

	ET01	ET02	ET03
Gram reaction	-	-	-
Oxidase	+	+	+
Motile	+	+	+
Anaerobic glucose	-	-	-
Arginine dihydrolase	-	+	+
Lysine	-	-	-
Lactose	-	-	-
N_2 gas production	-	+	+
Sucrose	-	-	-
Indole	-	-	-
Xylose	-	-	-
Aerobic glucose	-	+	+
Maltose	-	-	-
Mannitol	-	-	-
P. A.	-	-	-
Urea	-	-	-
Citrate	-	+	+

4.2 INITIAL PCP REMOVAL

Isolate ET01 was tested for the ability to remove PCP from the growth medium (MMS broth) at a range of initial PCP concentrations with and without 25mg/l yeast extract. The initial PCP concentrations tested were 20mg/l, 60mg/l and 137mg/l, with and without yeast extract (Fig 4.1). Isolate ET01 was tested with and without yeast extract to determine if yeast extract was necessary for PCP removal. The inoculum was not washed for the initial experiments in media without yeast extract (Fig 4.1b). For subsequent experiments the inoculum for media without yeast extract was washed as described in the Methods, Section 3.2.1. The importance of a washed inoculum can be seen by comparing Fig 4.1b and Fig 4.2b. The small amount of yeast extract carried over in the inoculum is sufficient to allow removal of the lower concentrations of PCP (Fig 4.2b) although the time taken for removal

was lengthened. The minimum amount of yeast extract required for PCP removal was not investigated further and the concentration of yeast extract supplementing the MMS broth was maintained at 25mg/l throughout the course of the experiments. Table 4.3 presents the rates of PCP removal for the graphs presented in Figure 4.1 below (see Appendix A8.4, Table A8.1 for statistical details).

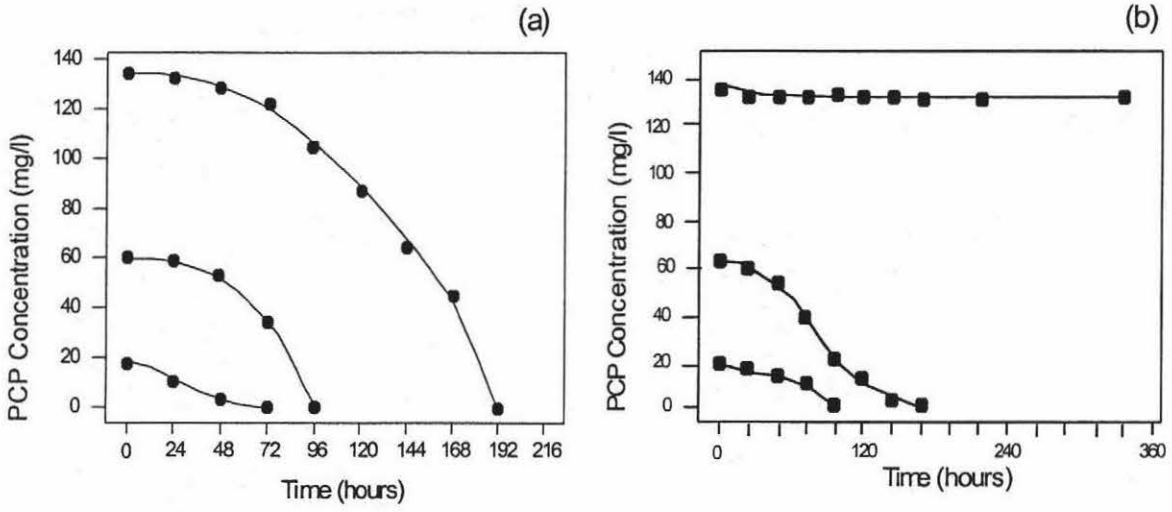


Figure 4.1: PCP removal by ET01 at three initial PCP concentrations (a) with 25mg/l yeast extract; (b) without 25mg/l yeast extract.

Table 4.3: PCP removal rates at three initial PCP concentrations with and without 25mg/l yeast extract.

Initial PCP concentration (mg/l)	**PCP Removal rate (mg/l/hr)**	
	With 25m/l yeast extract	**Without 25mg/l yeast extract**
20	0.16	0.24
60	1.22	0.80
137	1.10	0.01

The PCP removal rates for ET01 in media containing yeast extract were greater than in media without yeast extract for the two higher initial PCP concentrations. The PCP removal rate was measured as per the Materials and Methods, Section 3.7. The time taken for ET01 to remove the PCP was longer in media without yeast extract at all initial PCP concentrations. No PCP was removed by ET01 at an initial PCP concentration of 137mg/l in media without yeast extract. After the initial testing of ET01, an increased number of

initial PCP concentrations was tested to ascertain the concentration at which the highest removal rate was achieved and the highest concentration at which PCP removal would occur. Figure 4.2 below shows the removal of PCP at 5 different initial PCP concentrations and Table 4.4 presents the PCP removal rates for the graphs presented in Figure 4.2 below.

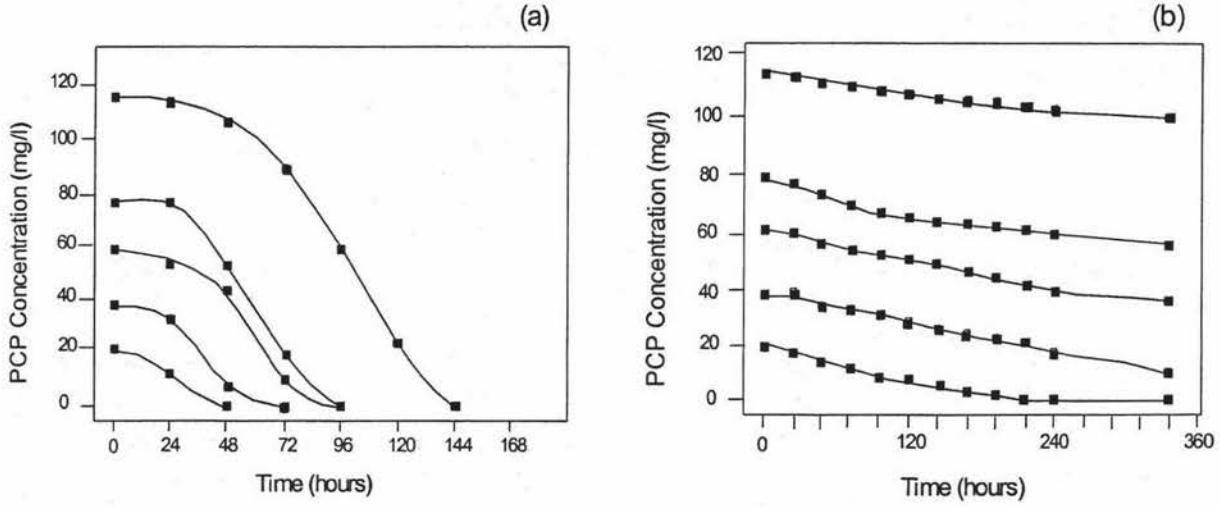


Figure 4.2: PCP removal by ET01 at varying initial PCP concentrations (a) with 25mg/l yeast extract; (b) without 25mg/l yeast extract.

From Figure 4.2 it can be seen that the addition of 25mg/l yeast extract to the medium induces a faster rate of PCP removal by ET01. The inoculum for the flasks without yeast extract consisted of washed cells, the preparation of which is described in the Methods, Section 3.2.1. The removal rates without yeast extract were very slow (see Table 4.4 for removal rates) and only the initial concentration of 20mg/l PCP was completely removed over a 14 day incubation period (360 hours).

Table 4.4: PCP removal rates at various initial PCP concentrations.

Initial PCP concentration (mg/l)	**PCP Removal rate (mg/l/hr)**	
	With 25mg/l yeast extract	**Without 25mg/l yeast extract**
20	0.442	0.086
40	0.575	0.078
60	1.021	0.086
80	1.358	0.044
115	1.375	0.051

The PCP removal rates presented in Table 4.4 are the maximum removal rates (measured as per the Materials and Methods, Section 3.7) for each initial PCP concentration. The rate of PCP removal was obtained by linear regression for the linear part of the curve (regression equations are presented in the Appendix, Section A8.4, Table A8.2). The PCP removal rates in media containing 25mg/l added yeast extract are substantially higher than those without yeast extract. With yeast extract and over the range of initial PCP concentrations tested the maximum PCP removal rate occurred at an initial PCP concentration between 80 and 115mg/l. The PCP removal rates by ET01 in media without yeast extract are approximately 14 fold lower than in media containing yeast extract.

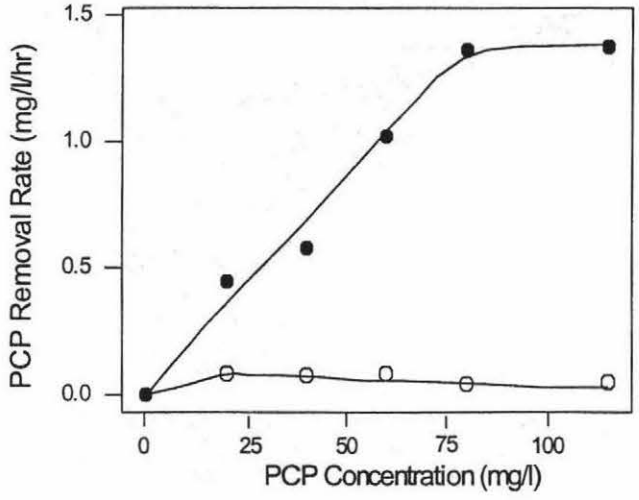


Figure 4.3: PCP removal rates by ET01 at various initial PCP concentrations with (●) and without (O) 25mg/l yeast extract.

The rate of PCP removal by ET01 increased as the initial PCP concentration increased up to 115mg/l in media containing yeast extract (Fig 4.3). The difference in PCP removal rates for ET01 in media with and without yeast extract is marked. Enriching the media with yeast extract provides an environment in which ET01 can remove completely 115mg/l PCP. The removal rate increased with increasing initial PCP concentration up to 80mg/l and beyond this concentration no further rate increases were observed.

4.2.1 LAG PERIOD FOR ET01 IN PURE CULTURE

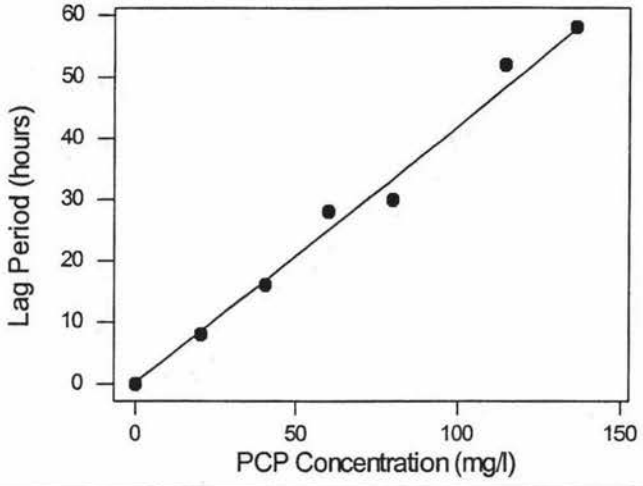
The lag period is defined here as the period of time after inoculation when no PCP removal is detected and in Figure 4.2 can be identified as the flat portion of the growth curve before PCP removal begins. The lag periods from the graphs presented in Figures 4.1 and 4.2 were estimated by using the regression equation slopes (presented in the Appendix, Section A8.4). The slope of the regression line was extrapolated at the relevant slope and the time at which the initial concentration intersected the extrapolated line was estimated to be the lag period. Table 4.5 below presents the lag periods for the various initial PCP concentrations tested in media containing yeast extract for ET01 and derived from Figures 4.1 and 4.2. Figure 4.4 illustrates the relationship between PCP concentration and lag period and the regression equation is presented below. No meaningful value for lag period could be calculated from those experiments without yeast extract. Removal of PCP by ET01 in media without yeast extract was very slow and the lag period, as defined above, did not end. Only lag periods for media containing yeast extract are presented.

Table 4.5: Lag periods for a range of initial PCP concentrations for ET01with yeast extract.

Initial PCP Concentration (mg/l)	Lag Period (hours)
20	<10
40	16
60	36
80	30
115	52
137	58

From these initial PCP removal experiments it was noted that the higher the initial concentration of PCP the longer the lag period, that is the time between inoculation and the beginning of PCP removal. The relationship between the lag period (hours) and the initial PCP concentration (mg/l) is directly proportional and for ET01, the lag period (hours) equals 0.43 of the initial PCP concentration (in mg/l) less 30 minutes as represented in

Figure 4.4. The possibility of using the regression equation as a predictive tool was not explored.



Lag period (hours) = - 0.52 + 0.43 [PCP] R-sq = 0.99 (N=6)

Figure 4.4: Lag period versus initial PCP concentration for ET01.

4.3 PCP REMOVAL AND CHLORIDE RELEASE

The initial kinetic experiments were performed with ET01 in pure culture. The PCP removal rates over a range of initial PCP concentrations with and without 25mg/l yeast extract were examined because, in the initial experiments, ET01 was able to remove PCP from an initial PCP concentration of 137 mg/l in media containing yeast extract. Higher initial concentrations were tested to ascertain at which concentration ET01 could no longer initiate PCP removal. Higher initial concentrations of 150 and 175 mg/l were tested. When PCP is degraded, chloride ions are released. The release of chloride from the PCP was measured to confirm that ET01 was degrading PCP. The theoretical ratio of chloride released to PCP consumed ratio is 5.0. That is, for every one mole of PCP consumed 5 moles of chloride are released. Chloride comprises 67% of the molecular weight of PCP so therefore theoretical chloride release is also 67% of the initial PCP concentration. PCP removal and chloride release for 20mg/l initial PCP concentration with and without 25mg/l yeast extract are presented for ET01 in pure culture in Figure 4.5. Theoretical chloride release from 20mg/l PCP is 13.4mg/l.

The graphs of PCP removal and concomitant chloride release for the range of PCP concentrations tested (40 mg/l – 175mg/l) with and without 25mg/l yeast extract are presented in the Appendix, Section A8.5. The regression equations from which the PCP removal and chloride release rates were obtained are presented in the Appendix, Sections A8.6 and A8.7 respectively.

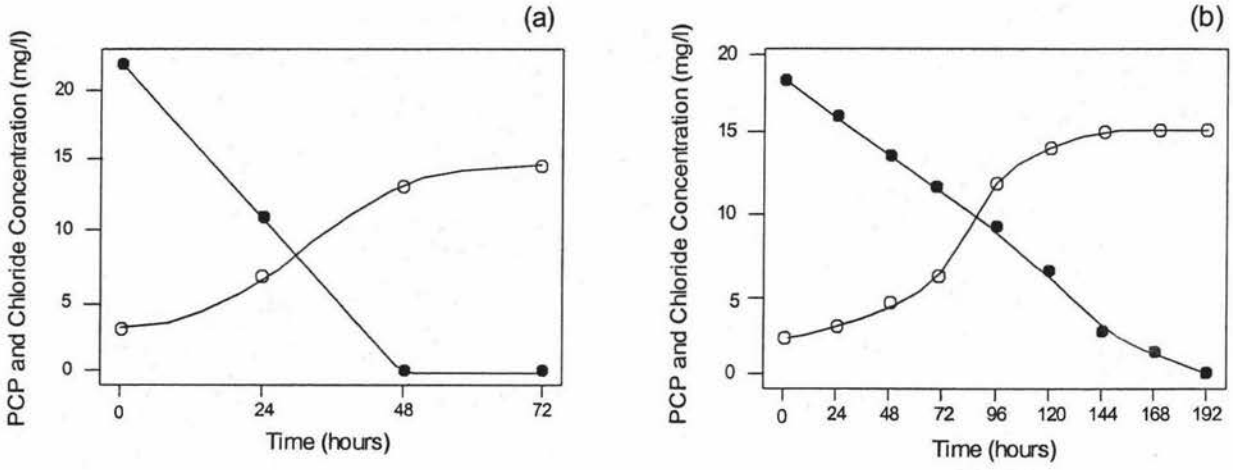


Figure 4.5: Removal of PCP (●) by ET01 at an approximate initial concentration of 20mg/l and concomitant chloride release (O) in media (a) with 25mg/l yeast extract; (b) without 25mg/l yeast extract.

It can be seen from Fig 4.5 that at an initial PCP concentration of 20mg/l in the presence and absence of yeast extract (25mg/l) PCP was completely removed over 48 and 192 hours respectively. The corresponding (theoretical) amount of chloride was released when PCP was completely removed from media with and without yeast extract at 20mg/l PCP. PCP was completely removed from media, containing yeast extract, at all concentrations up to and including 150mg/l and the final amount of chloride released was the theoretical amount of chloride in most cases. The values in Table 4.6 indicate the rate of chloride release between the end of the chloride release lag period and complete PCP disappearance and not the total amount of chloride released for each initial PCP concentration. For media without yeast extract, PCP was not completely removed, although chloride release corresponded to the amount of PCP that had been removed from the media. Neither complete PCP removal nor complete chloride release was observed in media without yeast extract at concentrations

above 20mg/l, even after 14 days incubation (Figs A8.4 – A8.7 in the Appendix, Section A8.5 illustrate this).

In media without yeast extract the chloride release rate was highest at the lowest PCP concentration tested, 20mg/l. This was the only PCP concentration in media without yeast extract where complete PCP removal and complete chloride release occurred. The rates for PCP removal and chloride release at the higher PCP concentrations without yeast extract are not the true rates as complete removal of PCP and release of chloride did not occur. By extrapolating the PCP removal line on the graphs presented in the Appendix, Section A8.5 it is possible to estimate the length of time ET01 would take to remove PCP without yeast extract. On average the time it would take for ET01 to remove PCP from media without yeast extract was seven (7) times longer than in media containing 25mg/l yeast extract.

Table 4.6 shows the rate of PCP removal and chloride release for ET01 at various initial PCP concentrations with and without yeast extract. The PCP consumed and chloride released values are presented in the Appendix, Section A8.6 and A8.7 respectively. Complete chloride release was not achieved in media without yeast extract except at 20mg/l. The chloride release rate for 20mg/l is calculated from an unusual curve (see Fig 4.5b) as most of the chloride was detected suddenly in a short time span thereby giving the impression of an elevated rate. The total amount of chloride released at the end of growth was, however, the theoretically expected value. The ratio was very high at 175mg/l PCP in media containing yeast extract (7.0) and this may be due to measurement variations in rates as very little PCP was removed from the medium and therefore chloride release was minimal.

If chloride release were synchronous with PCP removal the expected ratio of chloride released to PCP removed would be 4.8-5.0. The ratios presented in Table 4.6 are scattered around the expected ratio and are calculated by using the total chloride released and the PCP consumed. For media without yeast extract where complete PCP removal and chloride release did not occur the ratio of the chloride released to PCP consumed at 10 days (240 hours) was used. The values below the expected value suggest that chloride release lags

PCP disappearance. This is because chloride release comes from the transformation of intermediates. At the end of PCP removal the theoretical amount of chloride had been released. Also PCP removal was complete 12 - 48 hours before all the chloride was released in media containing yeast extract (this can be seen from the graphs in the Appendix, Section A8.5) thus suggesting that chlorinated intermediates were still being dechlorinated.

Table 4.6: PCP removal and chloride release rates for ET01 at various initial PCP concentrations with and without 25mg/l yeast extract.

Initial PCP Concentration (mg/l)	PCP Removal Rate (mg/l/hr)	Chloride Release Rate (mg/l/hr)	Ratio of total chloride released to PCP consumed
Without yeast extract			
20	0.09	0.13	4.98
40*	0.09	0.03	4.10
50*	0.12	0.04	3.88
60*	0.09	0.08	5.72
80*	0.08	0.05	5.97
With 25mg/l yeast extract			
20	0.46	0.21	5.41
40	0.80	0.54	5.22
50	1.18	0.60	5.07
60	1.02	0.52	4.85
80	1.41	0.80	4.66
100	1.94	1.31	5.22
120	2.10	1.25	5.29
150	1.48	0.86	3.98
175*	0.01	0.01	0.00

*Neither complete PCP removal nor complete chloride release occurred.

From Table 4.6 it can also be seen that the maximum rate of PCP removal by ET01 was obtained in media containing yeast extract at an initial PCP concentration of 120mg/l. As the

initial PCP concentration rises above 120mg/l the rate of PCP removal decreased sharply (see Fig 4.6). The data in Tables 4.4 and 4.6 are from different experiments and show a correlation where the maximum PCP removal rate occurs at 100mg/l and 120mg/l respectively. At an unidentified initial concentration between 150mg/l and 175mg/l ET01 ceases to remove PCP from the media. The PCP removal rates in media without yeast extract are similar for all the initial PCP concentrations. This suggests the threshold limit for ET01 in media containing PCP with no yeast extract is less than 20mg/l PCP as opposed to between 150 and 175mg/l for media containing yeast extract. The PCP removal rate increased for all PCP concentrations in media with yeast extract in latter experiments compared with these rates observed during the initial PCP removal experiments. The rate of removal at 150mg/l initial PCP concentration improved from 1.48mg/l/hr to 1.85mg/l/hr. The rate of removal at 120mg/l initial PCP concentration improved from 1.38mg/l/hr to a maximum of 2.10mg/l/hr. This suggested that the organism (ET01) was becoming more adapted to the increased concentrations of PCP.

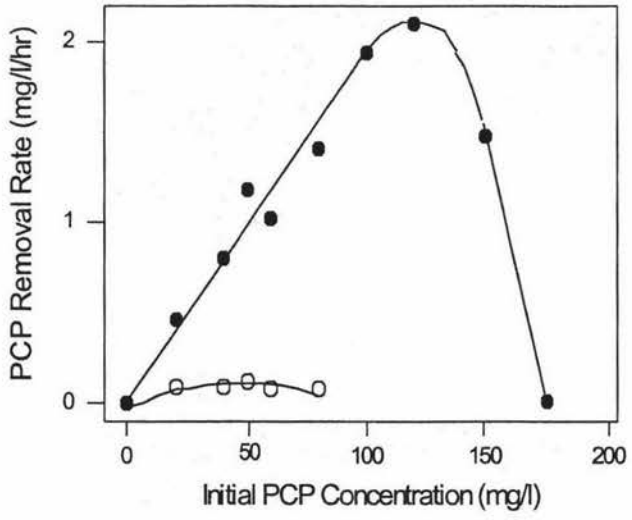


Figure 4.6: PCP removal rates for ET01 at various initial PCP concentrations with (●) and without (O) 25mg/l yeast extract.

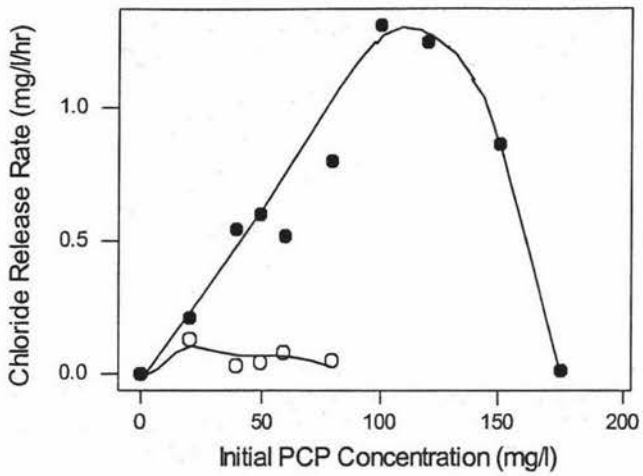


Figure 4.7: Chloride release rates for ET01 at various initial PCP concentrations with (●) and without (O) 25mg/l yeast extract.

Figures 4.6 and 4.7 illustrate how the PCP removal and chloride release rates vary with the initial PCP concentration for ET01. The rates presented are maximum rates measured over a limited period as explained in the Materials and Methods, Section 3.7. It can be seen from these graphs that the maximum rates for PCP removal and chloride release occur at initial PCP concentrations of 120mg/l and 100mg/l respectively in the presence of yeast extract (25mg/l).

4.3.1 THEORETICAL AND ACTUAL CHLORIDE RELEASE

The theoretical ratio of chloride released to PCP removed is 5.0. The theoretical weight ratio is 0.67 or 67%. The graphs presented in Fig 4.8 show how actual release of chloride in the culture medium compared with the theoretical chloride release for ET01 growing on 20mg/l PCP as a sole carbon and energy source.

Figure 4.8 shows that at 20mg/l initial PCP concentration the actual chloride released was lower than theoretical in media containing yeast extract. The reason for this is unclear and warrants further investigation. However, for media without yeast extract the total amount of

chloride released also deviated from theoretical. The released amount of chloride exceeded the utilised unit of PCP. Chloride release in fact exceeded the theoretical release when only 9mg/l PCP has been removed and contamination of equipment by chloride or instrumental error may have occurred in this case. Dechlorination of intermediates may proceed more rapidly without yeast extract in the media than with it as a component of yeast extract could inhibit the dechlorinating enzymes while augmenting the first enzyme (for PCP degradation). Chloride was released more rapidly in media without yeast extract for all initial PCP concentrations tested.

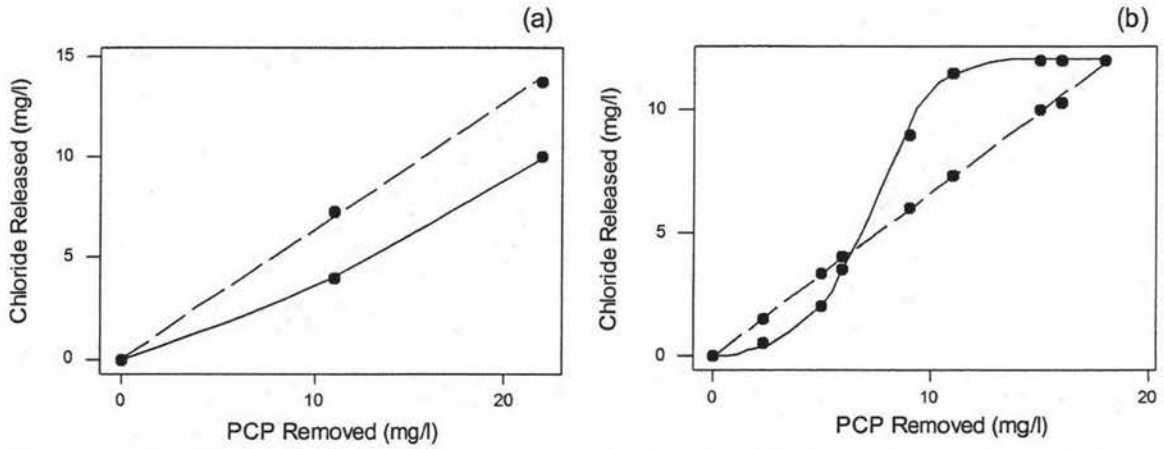


Figure 4.8: Theoretical (●---●) and actual (●—●) chloride release for ET01 at an approximate initial PCP concentration of 20mg/l (a) with 25mg/l yeast extract; (b) without 25mg/l yeast extract.

The graphs of theoretical and actual chloride release for ET01 at the higher initial PCP concentrations, tested with and without yeast extract, are presented in the Appendix, Section A8.8. The rate at which the chloride is actually released in the culture medium differs from theoretical release in that the chloride is not released at a constant rate throughout the experiment. From Figure 4.8 graph (b) the chloride release ratio is less than the theoretical at low initial PCP concentration as would be expected as chloride release comes from transformation of the intermediates but then increases rapidly indicating that the release of more chloride than has been consumed in the form of PCP. The observed chloride release compared well with theoretical values at the higher initial PCP concentrations. Again the reason for this is unclear and may warrant further investigation.

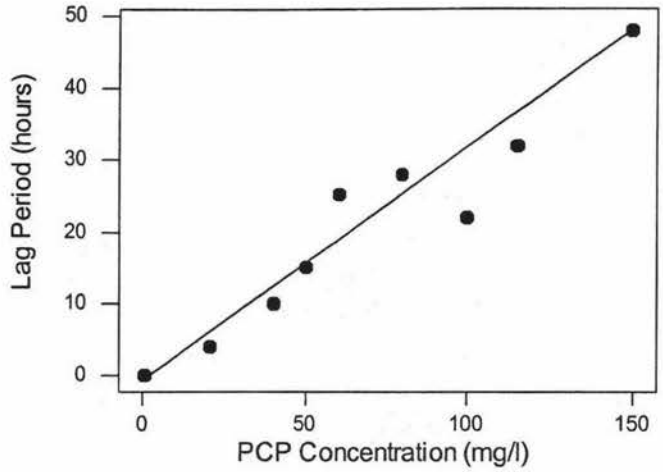
However, given that some highly erroneous chloride readings were obtained, a likely explanation is contaminated glassware. Only at the initial PCP concentrations of 80, 100 and 150mg/l does the actual chloride release closely emulate theoretical. No chloride was released at the highest PCP concentration tested (175mg/l with yeast extract) as there was no PCP removal, even after 14 days. No comparison can therefore be made for the theoretical chloride release from PCP removed at 175mg/l for ET01. Further investigation into the mechanisms of chloride release during PCP degradation by this particular bacterium is necessary.

4.3.2 LAG PERIOD

Table 4.7 presents the lag periods for ET01 at various initial PCP concentrations. Figure 4.9 illustrates the relationship between PCP concentration and lag period, the regression equation is presented below. The equation suggests that the lag period (hours) is 0.3 of the numerical value of the initial PCP concentration. As previously stated, removal of PCP by ET01 in media without yeast extract was very slow and the lag period did not end therefore only lag periods for media containing yeast extract are presented.

Table 4.7: Lag periods for a range of initial PCP concentrations for ET01.

Initial PCP Concentration (mg/l)	Lag Period (hours)
20	4
40	10
50	15
60	33
80	36
100	22
115	32
150	48
175	∞



Lag period (hours) = 0.30 [PCP] R-sq = 0.70

Figure 4.9: Lag period versus PCP concentration for ET01.

As the initial concentration of PCP was increased, the lag period prior to PCP removal (and chloride release) increased and the time taken for the PCP to be completely removed was longer. The lag period was affected by the addition of yeast extract and was substantially shorter in its presence than in media without it. The lag period for ET01 decreased from 0.433 of the initial PCP concentration in the initial PCP removal experiment to 0.30 of the initial PCP concentration in this latter experiment. The inoculation volume for ET01 was constant (10% v/v) throughout the experiments. Again, the regression equations was not used as a predictive tool because the R-squared value was significant at the 95% level.

4.4 SYNERGISM EXPERIMENTS

Strain ET01 was originally isolated from a PCP degrading bacterial consortium (Hussein *et al.*, 1996). The consortium comprised of three strains of bacteria designated ET01, ET02 and ET03. Consequently the possible beneficial effect of the cultures in combination in terms of PCP removal and chloride accumulation was examined. As can be seen from Figure 4.10, ET01 was the only strain which was capable of removing PCP in pure culture and 50mg/l was completely removed in 72 hours. Isolates ET02 and ET03 although part of

the initial PCP degrading consortium could not degrade 50mg/l PCP in pure culture (Fig 4.10). The inoculum used to test the cultures in combination was a 1:1 volumetric ratio for the binary experiments, ET01 with each of the other two, and a 1:1:1 volumetric ratio for the ternary experiments (the three isolates in combination). The inoculum was 10% (v/v) throughout the experiments. The method of inoculation is detailed in the Materials and Methods, Section 3.2.1. ET02 and ET03 were not trialed in combination without ET01.

The PCP removal for the pure cultures and culture combinations for an initial PCP concentration of 50mg/l is shown is Fig 4.10. All media contained 25mg/l yeast extract. As ET02 and ET03 could not remove PCP from 50mg/l with yeast extract in pure culture, they were not tested at higher initial PCP concentrations. It was assumed because of this that only ET01 cells were actively removing PCP from the media and that the lag period, PCP removal rate and time taken to remove PCP were proportional to the ET01 cell concentration. The ratio of ET01 cell concentration in pure culture, in the binary and ternary experiments was 200:100:67 respectively. As the cell concentration of the PCP removing strain was reduced in the binary and ternary experiments the expected PCP removal rate was half and one third of that achieved by ET01 in pure culture.

As can be seen from Fig 4.10 the time taken to remove PCP completely from the initial PCP concentration of 50mg/l was (72 hours) for ET01 in pure culture. This was also the case for all three initial PCP concentrations tested (50, 100 and 150mg/l). The time for each culture combination to remove PCP from the medium was greater than for ET01 in pure culture, though lower than the calculated value if the ET01 cell concentrations are taken into consideration. For the binary experiments at 50mg/l PCP the calculated time for complete PCP removal was twice that of ET01 in pure culture, i.e. 144 hours as the ET01 cell numbers had been reduced by half. The observed time for PCP removal for the binary experiments was 120 and 96 hours for ET01 with ET02 and ET03 respectively, indicating a possible synergism. The presence of the strains unable to metabolise PCP in the medium with ET01 prolonged the latter's ability to remove the substrate although not to the extent calculated. With the ternary combination the calculated time for complete PCP removal from 50mg/l was 216 hours, three times the

observed time for ET01 alone. However, the observed time was 144 hours. Again this is suggestive evidence for synergism.

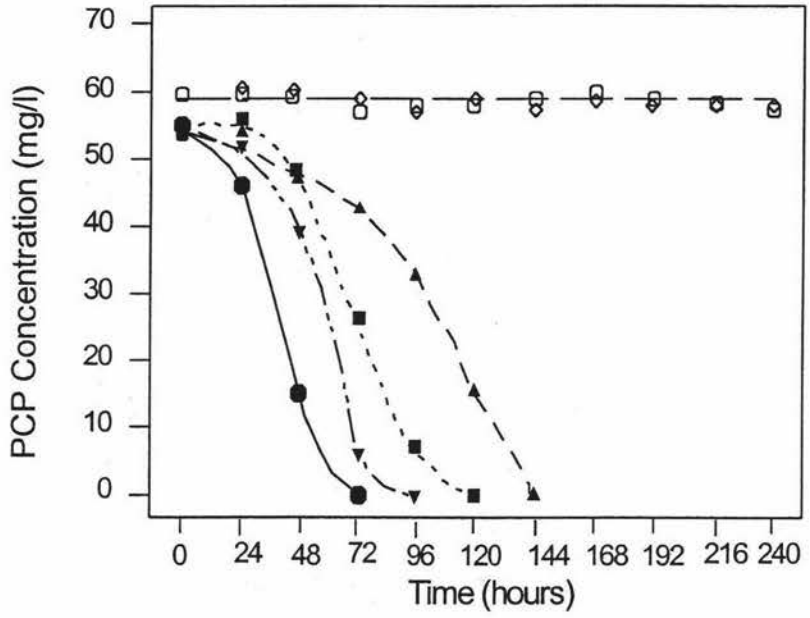


Figure 4.10: PCP removal from 50mg/l initial PCP concentration by culture combinations as indicated (see legend) in a minimal mineral salts (MMS) medium with 25mg/l yeast extract.

Key: —●— ET01; - - -■- - - ET01 and ET02; --▼-- ET01 and ET03; —▲— ET01, ET02 and ET03; —□— ET02; —◊— ET03

The graphs of PCP removal by the culture combinations at higher initial PCP concentrations, 100mg/l and 150mg/l, are presented in the Appendix, Section A8.9. At 100mg/l both the binary combinations exhibited the calculated PCP removal time of 144 hours, twice that of ET01 in pure culture (72 hours) showing no synergistic effect. The ternary combination at 100 mg/l removed the PCP in 144 hours compared with the calculated time of 216 hours. At 150mg/l the binary combinations exhibited a PCP removal time lower than that calculated, approximately 220 hours compared with a predicted 280 hours. For the higher initial PCP concentrations tested the ternary combination exhibited the synergistic trend for the time taken to remove PCP. At 150mg/l the calculated time of PCP removal for the ternary combination was 432 hours as opposed to the observed time of 240 hours.

Table 4.8: PCP removal rates for the culture combinations at three initial PCP concentrations in a minimal mineral salts (MMS) medium with 25mg/l yeast extract..

Culture Combination	PCP Removal Rate (mg/l/hr)					
	50mg/l PCP		100mg/l PCP		150mg/l PCP	
	Observed	Calculated*	Observed	Calculated*	Observed	Calculated*
ET01	**0.95**	-	**2.03**	-	**1.85**	-
ET01/ET02	**0.88**	0.48	**1.08**	1.02	**1.54**	0.93
ET01/ET03	**1.04**	0.48	**1.38**	1.02	**1.09**	0.93
ET01/ET02/ET03	**0.61**	0.32	**1.07**	0.68	**1.13**	0.62

*Calculated values are half (for binary combinations) and one third (for ternary combination) of the rate observed with ET01 in pure culture as the cell concentration of ET01 is lower in the combined experiments.

The calculated and observed PCP removal rates for ET01 in pure culture and the culture combinations at the three initial PCP concentrations are presented in Table 4.8. The rates of PCP removal were calculated using the regression equations presented in the Appendix, Section A8.10. The values were calculated using the 95% confidence intervals. The rate of PCP removal slows as the experiment progresses and the concentration of PCP approaches zero. This can be seen from Figure 4.10 and the figures presented in the Appendix, Section A8.9.

From Table 4.8 it can be seen that where cultures are combined the majority of cases show evidence of synergism. ET01 exhibited the highest PCP removal rate at the two higher initial PCP concentrations tested, 100 and 150 mg/l. At the lowest initial PCP concentration tested the PCP removal rates observed for the binary combinations closely resemble that of ET01 in pure culture and are twice the calculated values. Only the ET01/ET02 combination at an initial PCP concentration of 100mg/l does not show the trend of synergism with the observed PCP removal rate 106% of that calculated. The rate of PCP removal for the combination of ET01/ET02 at 150mg/l PCP is closest to that of ET01 in pure culture than the other culture combinations (Table 4.8). The PCP removal rate for the ET01/ET02 combination and the ternary combination increased as the initial PCP concentration increased. Maximum PCP removal rate occurred at 100 mg/l for the ET01/ET03 combination. The former combinations may not have reached

the highest possible removal rate and require further testing at higher initial PCP concentrations.

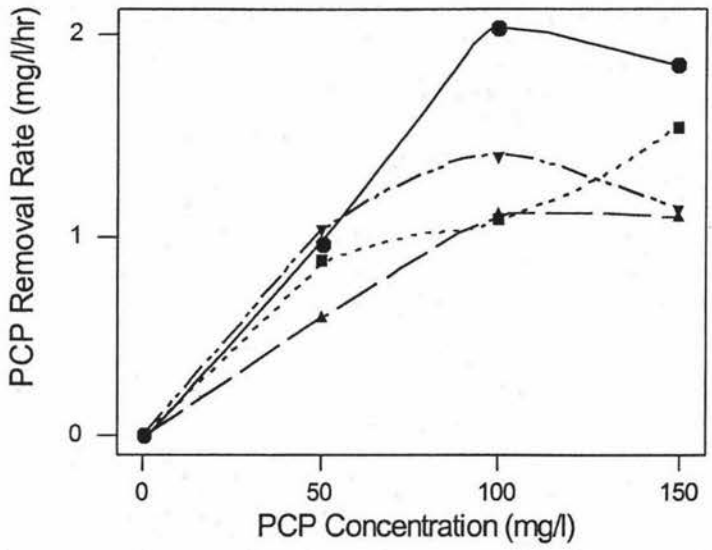


Figure 4.11: PCP removal rates for the culture combinations at three initial PCP concentrations (see legend) in a minimal mineral salts (MMS) medium with 25mg/l yeast extract.

Key: ET01; ET01 and ET02; ET01 and ET03; ET01, ET02 and ET03

The PCP removal rate for ET01 in pure culture at 150mg/l increased from 1.48mg/l/hr in earlier experiments (Appendix, Section A8.6) to 1.85mg/l/hr for this experiment (Table 4.8). Figure 4.11 illustrates the observed PCP removal rates presented in Table 4.8 and shows that at the higher PCP concentrations tested the mixed cultures do not perform as well as ET01 in pure culture even though there was evidence of synergism. Repeated subculturing under the selective pressure of PCP as a sole carbon and energy source at increasing concentrations has probably produced these higher rates of PCP removal. Higher PCP removal rates from initial concentrations above 150mg/l may occur with subculturing from a culture(s) acclimatised to high PCP concentrations.

4.4.1 LAG PERIOD FOR CULTURE COMBINATIONS

The lag periods for ET01 and the culture combinations, i.e. the time during which no PCP utilisation occurred, were estimated by using the PCP removal rate for the graphs

in Figure 4.10 and Appendix Section A8.9. The slope of the regression line was extrapolated at the relevant slope and the time at which the initial PCP concentration intersected the extrapolated line was estimated to be the lag period.

Table 4.9: Lag periods for ET01 and culture combinations at three initial PCP concentrations in a minimal mineral salts (MMS) medium with 25mg/l yeast extract.

Culture Combination	**Lag Period (hours)**					
	50mg/l PCP		**100mg/l PCP**		**150mg/l PCP**	
	Observed	Calculated*	**Observed**	Calculated*	**Observed**	Calculated*
ET01	**10**	-	**24**	-	**48**	-
ET01/ET02	**24**	20	**52**	48	**124**	96
ET01/ET03	**24**	20	**50**	48	**96**	96
ET01/ET02/ET03	**48**	30	**48**	72	**120**	144

*Calculated values are twice (for binary combinations) and three times (for ternary combination) the lag period observed with ET01 in pure culture as the cell concentration of ET01 is lower in the combined experiments.

Table 4.9 presents the observed and calculated (expected) lag periods for the culture combinations at the three initial PCP concentrations tested. The observed lag periods for two culture combinations did not differ appreciably from the calculated values nor did they suggest any synergistic effect between strains on the PCP removal rates. Only observed lag periods for the ternary combination differed from those calculated. At 50mg/l PCP the lag period was much longer than calculated and at the two higher initial PCP concentrations tested the observed lag period was less than the calculated.

The lag period at an initial PCP concentration of 50mg/l increased as the proportion of ET01 in the inoculum was reduced. The lag period was more accentuated at the highest initial PCP concentration than at lower initial PCP concentrations. Over the range of initial PCP concentrations tested the culture combinations took twice as long to remove the PCP as did ET01 in pure culture due mainly to the extended lag period at the higher initial PCP concentrations. This enforces the hypothesis that lag period is proportional to the cell concentration of ET01.

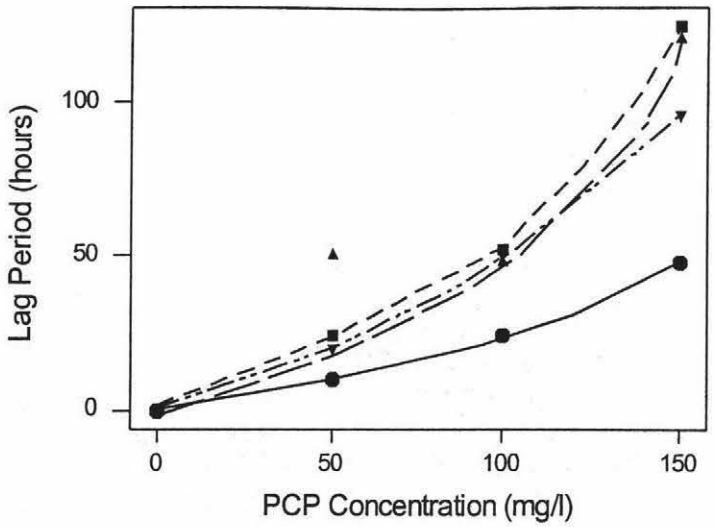


Figure 4.12: The effect of initial PCP concentration on the lag period for ET01 and culture combinations (see legend) in minimal mineral salts (MMS) broth with 25mg/l yeast extract.

Key: —●— ET01; - - -■- - - ET01 and ET02; --▼-- ET01 and ET03; — -▲— ET01, ET02 and ET03

Figure 4.12 illustrates the relationship between PCP concentration and lag period. No linear relationships are apparent for the binary and ternary combinations. There is a marked increase in lag period for the culture combinations when compared with ET01 in pure culture at each initial PCP concentration especially at the two higher concentrations. The reduced cell concentration of ET01, the only strain able to remove PCP from a minimal mineral salts (MMS) broth, was half and one third of that when inoculated in pure culture, therefore the proportion of PCP-removing cell numbers was reduced. The lag period does not reflect this reduction in PCP-removing cell numbers for some cases of combined cultures suggesting evidence of a synergistic effect.

4.5 PCP REMOVAL AND ORGANISM GROWTH

The medium used in these experiments was the minimal mineral salts (MMS) broth with 25mg/l added yeast extract as per the Materials and Methods, Section 3.2.1. Fig 4.13 shows the removal of PCP and the subsequent increase in cell numbers for ET01 in pure culture. Cells numbers were enumerated by AO counts as per the Materials and Methods, Section 3.5.1.2. The graphs of PCP removal and increase in cell numbers, by ET01 in pure culture, for PCP concentrations above 20mg/l are presented in the

Appendix, Section A8.11, as are the graphs for the culture combinations at 50, 100 and 150mg/l PCP. All media contained 25mg/l yeast extract. The bars represent the 95% confidence interval of the natural log value. The 95% confidence interval was calculated as per the Methods 3.1.

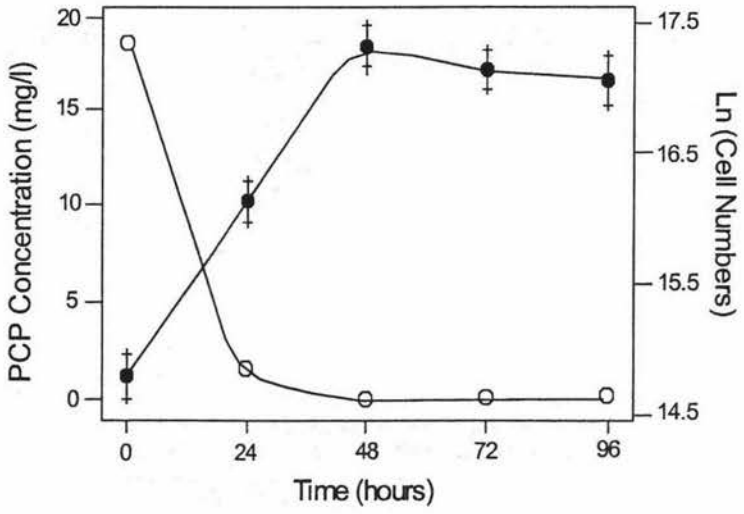


Figure 4.13: Increase in ET01 cell numbers at an initial PCP concentration of 20mg/l.

Table 4.10 presents the yields and specific growth rates (hr^{-1}) for ET01 in pure culture and the observed and calculated values for yield and specific growth rate for the culture combinations. The graphs of ET01 and the culture combinations and regression equations from which the specific growth rates were obtained are presented in the Appendix, Section A8.11 and A8.12 respectively. The graphs and regression equations from which the yields were obtained are presented in the Appendix Sections A8.13 and A8.14 respectively.

For ET01 the yield increased up to 50mg/l PCP then decreased as the initial PCP concentration was increased further although most values were above the calculated value. Only in the ternary combination at 50mg/l and 150mg/l did the yield closely resemble the calculated values, however the ternary yield at 100mg/l gave the highest ratio of observed to calculated yield. The specific growth rates for the ternary combinations at all PCP concentrations were much higher than calculated with the rate at 150mg/l five times the calculated rate.

Table 4.10: Yields and specific growth rates for ET01 and culture combinations in MMS broth containing 25mg/l yeast extract.

Culture Combination	**Yield (mg biomass produced per mg PCP used)**		**Specific Growth Rates (hr^{-1})**	
ET01 20mg/l PCP	0.001		0.053	
ET01 40mg/l PCP	0.002		0.055	
ET01 60mg/l PCP	0.001		0.054	
ET01 80mg/l PCP	0.001		0.054	
50mg/l PCP	**Observed**	Calculated*	**Observed**	Calculated*
ET01	**0.003**	-	**0.054**	-
ET01/ET02	**0.003**	0.002	**0.045**	0.027
ET01/ET03	**0.004**	0.002	**0.059**	0.027
ET01/ET02/ET03	**0.001**	0.001	**0.049**	0.018
100mg/l PCP				
ET01	**0.001**	-	**0.038**	-
ET01/ET02	**0.002**	0.0005	**0.032**	0.019
ET01/ET03	**0.001**	0.0005	**0.051**	0.019
ET01/ET02/ET03	**0.002**	0.0003	**0.047**	0.013
150mg/l PCP				
ET01	**0.001**	-	**0.025**	-
ET01/ET02	**0.003**	0.0005	**0.036**	0.013
ET01/ET03	**0.001**	0.0005	**0.037**	0.013
ET01/ET02/ET03	**0.0002**	0.0003	**0.041**	0.008

*Calculated values are half (for binary combinations) and one third (for ternary combination) the yield and specific growth rate observed with ET01 in pure culture as the cell concentration of ET01 is lower in the combined experiments.

The observed yields and specific growth rates for the binary combinations exceeded the calculated values. It is not clear whether ET02 or ET03 actually contribute to the biomass although both grow in the presence of PCP. Further investigation into the

ability of ET02 and ET03 to grow on intermediates in the PCP degradation pathway is required. Evidence for synergism was indicated in the ET01/ET03 combination at 50 mg/l and 100mg/l, the ternary combination at 100 mg/l, and all culture combinations at 150 mg/l. The observed values exceeded the specific growth rate obtained by ET01 in pure culture. The yield of these organisms on PCP, as a substrate, was particularly low and this compromised the quality of the results, the variation was as high as 67%. The yield results are an indirect measurement and can only be used to indicate a trend or the cultures' potential responses.

The ET01/ET03 combination exhibited the highest specific growth rates at the two lower PCP concentrations. At the highest PCP concentration tested the observed specific growth rate for the ternary combination was the highest value. The possibility that the three isolates may compliment each other, especially at higher PCP concentrations, warrants further investigation. While every measure was taken to ensure results were as robust as possible, on some occasions there was considerable variation in the data due to the inherent variation in the procedure.

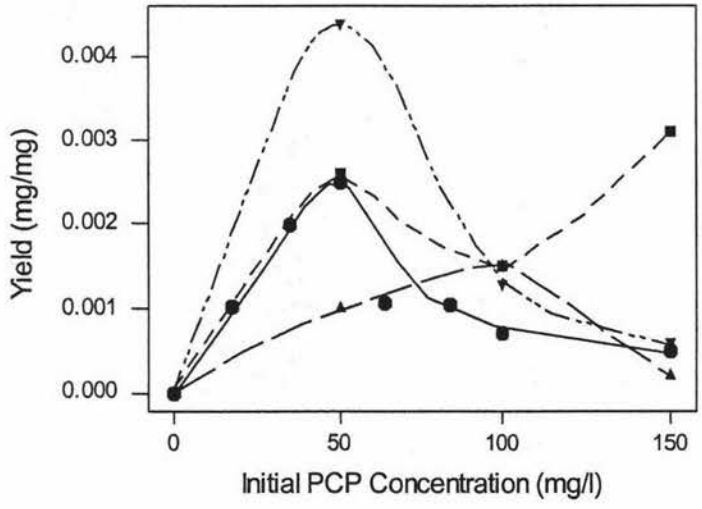


Figure 4.14: Yield for ET01 in pure culture and culture combinations (see legend) in a minimal mineral salts (MMS) medium with 25mg/l yeast extract..

Key: —●— ET01; - - -■- - - ET01 and ET02; --▼-- ET01 and ET03; _ _▲_ _ ET01, ET02 and ET03

Figures 4.14 and 4.15 illustrate the observed yield and specific growth rate data as functions of initial PCP concentrations as calculated for the culture combinations in

Table 4.10. The combination of ET01/ET03 exhibited a maximum yield at the lowest PCP concentration tested, 50mg/l. Both ET01/ET02 and the ternary combination exhibited a maximum yield at 100mg/l and ET01/ET02 exhibited their highest yield at 150mg/l initial PCP concentration. The increased yield (Fig 4.14) does not reflect an increased PCP removal rate.

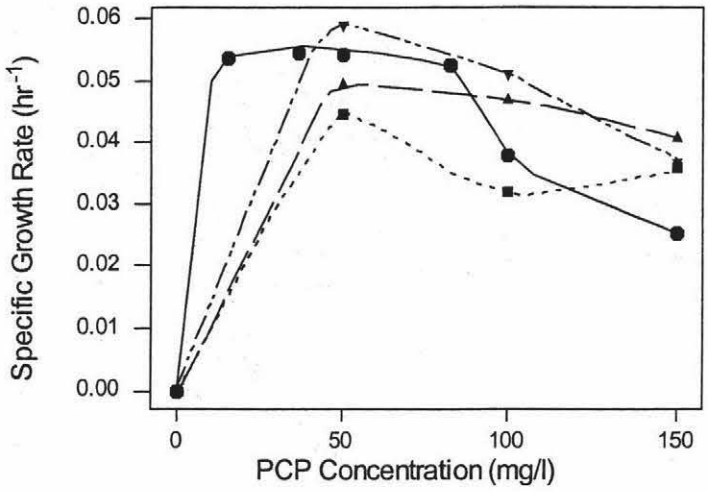


Figure 4.15: Specific growth rates for ET01 in pure culture and culture combinations (see legend) in a minimal mineral salts (MMS) medium with 25mg/l yeast extract..

Key: ET01; ET01 and ET02; ET01 and ET03; ET01, ET02 and ET03

The highest specific growth rates occurred at the lowest PCP concentration tested for ET01 in pure culture and the all culture combinations. The specific growth rates decreased steadily for ET01 as the initial PCP concentration rose to 150mg/l. ET01/ET02 was the combination that exhibited the lowest specific growth rate at 100mg/l. The specific growth rates for the other combinations were lowest at the highest PCP concentration tested, 150mg/l. All observed specific growth rates are markedly higher than the calculated values when the reduced ET01 cell concentration is taken into account for the binary and ternary experiments. There was a suggestion of a synergistic relationship between the isolates, with the three isolates in combination exhibiting a stronger growth response than ET01 in combination with only one of the other isolates. This was evident by comparing the observed specific growth rates with that observed for ET01 in pure culture at each PCP concentration tested.

The inhibition constant (K_i) values for ET01 and the culture combinations growing in the PCP medium are presented in Table 4.12. K_i is the maximum substrate concentration at which the specific growth rate (μ) is reduced to half the maximum value (Radehaus and Schmidt, 1992).

Table 4.11: K_i values for ET01 and the culture combinations.

Culture Combination	Inhibition Constant (K_i) (mg/l)
ET01	100
ET01/ET02	95
ET01/ET03	95
ET01/ET02/ET03	105

The K_i was very similar for ET01 in pure culture and the culture combinations tested. The similarity of the K_i values (they vary 4% around the mean 98.8) attests to the fact that the K_i may be being measured for the same organism in each case. This then suggests that ET01 is the key strain in the consortium. Further subculturing of ET01 in pure culture and with the other isolates under the selective pressure of PCP could yield higher rates of removal and higher concentrations of PCP at which the organism(s) will grow.

4.6 VITAMIN EXPERIMENT

The vitamin experiments were conducted to determine which vitamin combination(s) would allow ET01 in pure culture, and in combination with ET02 and ET03, to grow and remove PCP from media without yeast extract. Three vitamin solutions designated solutions 1, 2 and 3 were tested. The components of each vitamin solution are listed in the Methods Section 3.6. The effect of the 3 vitamin solutions and no vitamin supplementation on the rate of removal of 50mg/l PCP by ET01 and each of the culture combinations was tested and compared with the same cultures supplemented with 25mg/l added yeast extract. The resulting PCP removal curves are presented in Figures 4.16 - 4.19. The PCP removal rates obtained by each of the culture combinations with different vitamin supplementation are presented in Table 4.13. The Figures (4.16 – 4.19) show the effect of the various vitamin supplementation on PCP removal rate for each culture combination. All rates were based upon the linear parts of the PCP removal curve. The regression equations are presented in the Appendix, Section A8.16.

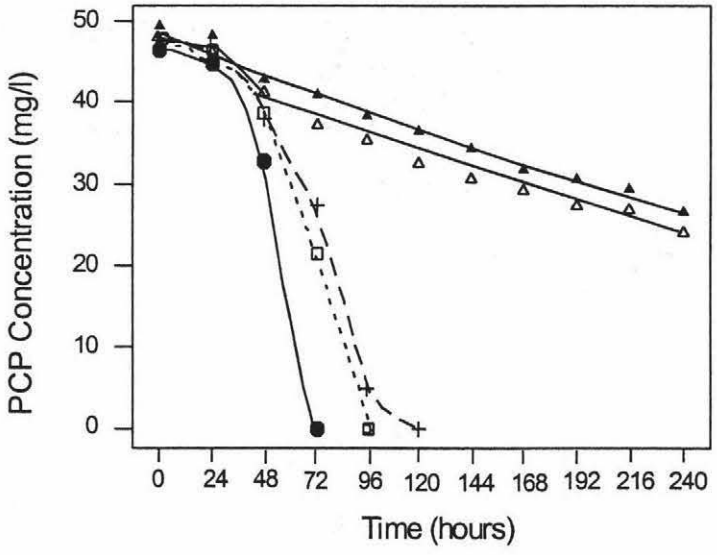


Figure 4.16: Removal of 50mg/l PCP by ET01 in pure culture with different vitamin supplementation (see legend).

Key: —●— 25mg.l yeast extract; —△—Vitamin solution 1; - -+- -Vitamin solution 2; —⊟— Vitamin solution 3; —▲—No vitamin supplementation.

Figure 4.16 shows ET01 removed 50mg/l PCP from media containing 25mg/l yeast extract in the shortest time of 72 hours when compared to media containing vitamin

solutions 2 and 3. The media containing 25mg/l yeast extract gave the highest removal rate with ET01 in pure culture, ET01/ET02 and ET01/ET03. When the three cultures were combined media containing vitamin solution 3 gave the highest PCP removal rate. Vitamin solution 1 had a similar effect on PCP removal as no vitamin supplementation for ET01 in pure culture and the culture combinations.

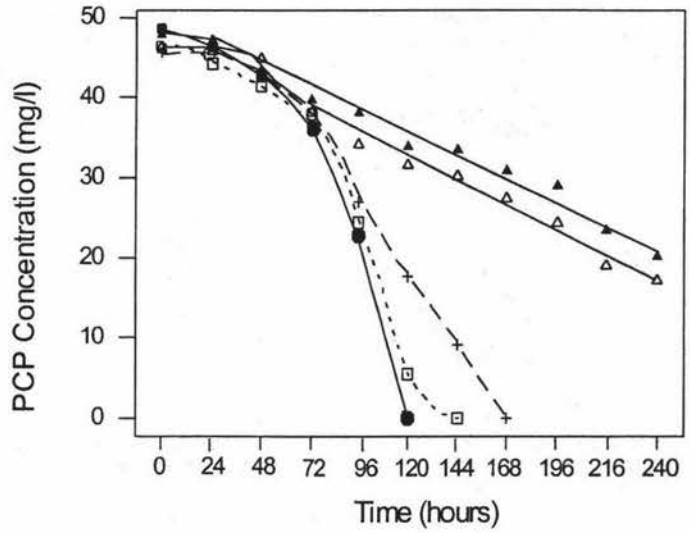


Figure 4.17: Removal of 50mg/l PCP by ET01 and ET02 in combination with different vitamin supplementation (see legend).

Key: —●— 25mg.l yeast extract; —△—Vitamin solution 1; - -+- -Vitamin solution 2; —⊟— Vitamin solution 3; —▲— No vitamin supplementation.

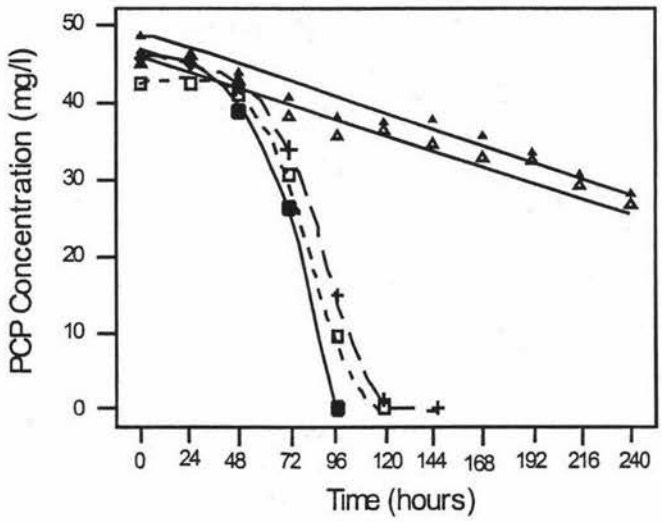


Figure 4.18: Removal of 50mg/l PCP by ET01 and ET03 in combination with different vitamin supplementation (see legend).

Key: —●— 25mg.l yeast extract; —△—Vitamin solution 1; - -+- -Vitamin solution 2; —⊟— Vitamin solution 3; —▲— No vitamin supplementation.

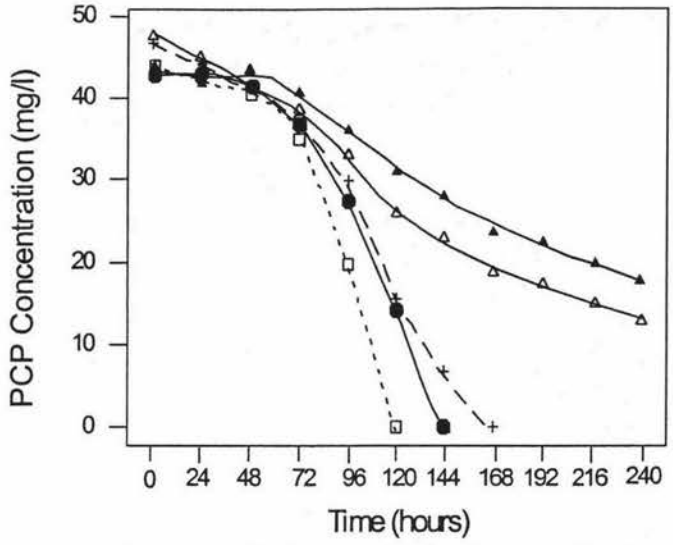


Figure 4.19: Removal of 50mg/l PCP by ET01, ET02 and ET03 in combination with different vitamin supplementation (see legend).

Key: —●— 25mg.l yeast extract; —△—Vitamin solution 1; - -+- -Vitamin solution 2; —⊟— Vitamin solution 3; —▲— No vitamin supplementation.

The removal rates in media containing vitamin solution 1 were marginally lower than without vitamins suggesting the components in vitamin solution 1 neither enhance nor facilitate biological removal of PCP. However the effect of synergism on the rate of PCP removal in media with no added vitamin supplementation was most evident for ET01/ET02 and the three cultures in combination. In media containing vitamin solution 3 ET01/ET03 and ternary combinations exhibited PCP removal rates in excess of that observed for ET01 in pure culture. The PCP removal rates obtained by the ET01/ET03 combination in media containing yeast extract, and ET01/ET02 in media containing vitamin solution 2 compared most favourably with the observed PCP removal rate by ET01 in pure culture (Table 4.13).

With 25mg/l added yeast extract, ET01 in pure culture exhibited the highest PCP removal rate at 50mg/l initial PCP concentration (Fig 4.19). The PCP removal rate for ET01/ET03 was a higher rate than for ET01/ET02, with yeast extract and vitamin solutions 2 and 3, although the lag period (discussed in Results Section 4.6.1) and therefore time taken to remove the PCP was longer.

Table 4.12: PCP removal rates for culture combinations with different vitamin supplementation (MMS medium containing an initial PCP concentration of 50mg/l).

Culture Combination and Vitamins	PCP Removal Rate (mg/l/hr)		R-Squared value (%)	N (number of points)
No Vitamins	**Observed**	Calculated*		
ET01	**0.12**	-	99.9	4
ET01/ET02	**0.18**	0.06	99.8	5
ET01/ET03	**0.11**	0.06	99.2	3
ET01/ET02/ET03	**0.18**	0.04	99.0	7
25mg/l YE				
ET01	**0.96**	-	94.3	3
ET01/ET02	**0.76**	0.48	98.0	3
ET01/ET03	**0.81**	0.48	95.8	3
ET01/ET02/ET03	**0.62**	0.32	99.9	3
Vitamin Solution 1				
ET01	**0.11**	-	98.0	5
ET01/ET02	**0.11**	0.06	99.4	5
ET01/ET03	**0.09**	0.06	99.8	5
ET01/ET02/ET03	**0.16**	0.04	95.7	6
Vitamin Solution 2				
ET01	**0.80**	-	99.6	3
ET01/ET02	**0.65**	0.40	98.4	3
ET01/ET03	**0.64**	0.40	95.0	3
ET01/ET02/ET03	**0.48**	0.27	97.8	3
Vitamin Solution 3				
ET01	**0.42**	-	95.9	3
ET01/ET02	**0.39**	0.21	99.8	5
ET01/ET03	**0.60**	0.21	99.6	4
ET01/ET02/ET03	**0.73**	0.24	99.5	3

*Calculated values are half (for binary combinations) and one third (for ternary combination) of the rate observed with ET01 in pure culture as the cell concentration of ET01 is lower in the combined experiments.

The removal rates of the culture combinations in media containing vitamin solutions 2 and 3 compared most closely with media containing yeast extract. Vitamin solution 2

contained a higher concentration of pantothenic acid than vitamin solution 3, reported to support growth of ET01 on PCP (Shepherd, 1997). The rate of PCP removal for ET01 in pure culture with vitamin solution 2 was 83% of the removal rate with yeast extract. Vitamin solution 1 data compared closely with those data from experiments with no added vitamin supplementation for all culture combinations tested. Despite the low PCP removal rates, media containing vitamin solution 1 exhibited the most noticeable effects of synergism after no vitamin supplementation, especially with the ternary combination.

The observed PCP removal rates for ET01/ET03 and the ternary combination with vitamin solution 3 supplementation were far greater than the calculated values and 75% and 118% of the PCP removal rates obtained with yeast extract respectively. The addition of vitamin solution 3 to the media produced removal rates between two and three times those calculated for the culture combinations. The calculated rates were 50% of the ET01 rate for the binary combinations and 33% for the ternary combination. The addition of vitamin solution 3 to the media gave strong evidence of synergism, as the PCP removal rates for ET01/ET03 and ternary combination were substantially higher than the observed rate for ET01 in pure culture. The addition of vitamin solution 3 to the media produced PCP removal rates between 72% - 116% of those obtained in media containing yeast extract.

The PCP removal rate obtained by the ternary combination with vitamin solution 2 was 77% of that with yeast extract. For both binary combinations PCP removal in media containing 50mg/l PCP and vitamin solution 2 took 24 hours longer than those containing yeast extract. For the three strains combined with vitamin solution 2 total PCP removal occurred in 24 hours less than for yeast extract. Apart from in media containing yeast extract ET01, in pure culture, exhibited the highest PCP removal rate in media containing vitamin solution 2. Only the ternary combination produced a better result than the cultures with yeast extract in media containing vitamin solution 3.

Removal of PCP was fastest when the medium was supplemented with 25mg/l yeast extract. Complete PCP removal took place in media containing yeast extract and vitamin solutions 2 and 3. Biotin was present in vitamin solutions 2 and 3 at the same concentration. Vitamin solution 2 contained pantothenic acid at a greater concentration than in vitamin solution 3.

4.6.1 LAG PERIOD FOR VITAMIN EXPERIMENT

The lag periods for ET01 and the culture combinations were estimated as described in the Results, Section 4.2.1. Table 4.13 below presents the observed and calculated lag periods for the culture combinations in media containing different vitamin supplementation. The lag periods for media containing no yeast extract and vitamin solution 1 did not end (as can be seen in Figs 4.16 – 4.19) according to the definition of the lag period as used in this study and presented in the Results Section 4.2.1; hence the infinity symbol.

Table 4.13: Lag periods for culture combinations with different vitamin supplementation (MMS medium containing an initial PCP concentration of 50mg/l).

Culture Combination	Lag Period (hours)							
	No Vits	25mg/l YE		Vit Sol 1	Vit Sol 2		Vit Sol 3	
		O*	C**		O*	C**	O*	C**
ET01	∞	30	-	∞	40	-	36	-
ET01/ET02	∞	60	60	∞	72	80	60	72
ET01/ET03	∞	42	60	∞	54	80	48	72
ET01/ET02/ET03	∞	60	90	∞	72	120	60	108

O* Observed lag period

C** Calculated values are twice (for binary combinations) and three times (for ternary combination) the lag period observed with ET01 in pure culture as the cell concentration of ET01 is lower in the combined experiments.

The data in Table 4.13 clearly shows ET01 in pure culture exhibited the shortest lag period with each of the vitamin supplements tested and yeast extract gave the shortest lag period with each culture combination. The ET01/ET03 combination showed the next shortest lag periods, which were less than the calculated values. The ET01/ET02 and ternary combinations had the most lengthy lag periods. Only the binary combination of ET01/ET02 in media containing yeast extract matched the observed lag period with the calculated lag period.

With regard to the total time of PCP removal, media containing vitamin solution 3 took 48 hours longer than media containing yeast extract for all culture combinations, mainly due to the extended lag period. Complete PCP removal with all cultures with vitamin solution 3 was slower than removal in media containing yeast extract and vitamin solution 2. Vitamin solutions 2 and 3 both contained pantothenic acid whereas vitamin solution 1 did not. Further experimentation into the effect of pantothenic acid as vitamin supplementation in media containing PCP is desirable. If the lag period is lengthy no matter how fast the rate of PCP removal the total time taken to remove PCP is increased. Although the PCP removal rates for the tested vitamin solutions compare favourably with yeast extract the lag period increased the total time taken. In all cases (except ET01/ET02 in 25mg/l yeast extract) where supplementation was used, the presence of a second and third organism has resulted in the shortening of the lag phase by as much as 48 hours and, on average, 24 hours. Further investigation into parameters that shorten the lag period is necessary.

5. DISCUSSION

5.1 INTRODUCTION

PCP has often been considered relatively resistant to biodegradation due to the high chlorine content of the molecule. However, numerous reports have appeared in the literature describing the microbial degradation of PCP in soil, water and in systems simulating aerobic wastewater treatment (Murthy *et al.*, 1979; Pignatello *et al.*, 1983; Valo and Salkinoja-Salonen, 1986; Sato, 1987; Seech *et al.*, 1990; Topp and Hanson, 1990;). In addition, bacteria isolated from a variety of sources have been shown to utilise PCP as a sole carbon source (Chu and Kirsch, 1972; Watanabe, 1973; Stanlake and Finn, 1982; Hussein *et al.*, 1996). This suggests that PCP-degrading bacteria are widely distributed in the environment. Because of the toxicity of PCP to micro-organisms however, high concentrations are likely to inhibit cell growth and consequently the degradation processes (Watanabe, 1973; Radehaus and Schmidt, 1992). The persistence of PCP in the environment could then be attributed to such factors as lack of micro-organisms in the habitat capable of decomposing PCP, too high or too low a PCP concentration, pH, temperature or anaerobic conditions (Stanlake and Finn, 1982). At high concentrations (up to 3000mg/l), PCP induces PCP degrading organisms or increased degradation activity in organisms that can degrade PCP (Litchfield, 1991).

Pentachlorophenol has been shown to be extensively degraded in both laboratory and full-scale systems and studies have been conducted to evaluate the basic parameters which describe the kinetics of PCP utilisation (Stanlake and Finn, 1985; Klecka and Maier, 1985; Gonzalez and Hu, 1995; Gu and Korus, 1995; Rutgers *et al.*, 1996). Since the efficient operation of biological treatment systems is largely dependent on the kinetic properties of the microbial population, determination of these parameters is essential for the development of operational strategies for the optimum removal of PCP during wastewater treatment (Klecka and Maier, 1985). Establishing the kinetic parameters of micro-organisms associated with PCP utilisation, such as specific growth rate, length of the lag period and toxicity, may aid in the development of clean-up strategies for areas with dispersed pollution (Stanlake and Finn, 1982).

5.2 ISOLATES

The isolates used in this study (ET01, ET02, ET03) were isolated as a consortium from PCP contaminated soil by Hussein *et al.* (1996). The degradative capacities of these individual strains in pure culture, as combinations of two (ET01 & ET02; ET01 & ET03) and the three isolates together were examined in a minimal mineral salts (MMS) medium. Of the individual strains, only ET01 manifested the ability to remove PCP from a MMS broth with yeast extract supplementation although the isolates ET02 and ET03 exhibited growth in liquid media containing PCP, methanol and *L*-glutamic acid (measure by optical density), and produced colonies on MMS agar containing PCP but could not remove 25mg/l PCP in pure culture from MMS broth.

Tentatively classified as a *Bradyrhizobium* sp. (Yu pers. comm., 1997) ET01 was Gram-negative and exhibited colony morphology similar to that described for *Bradyrhizobium* sp. (Jordan, 1984). Colonies were circular, opaque, beige and pin point, not exceeding 1mm in diameter, after 10 days incubation on MMS agar with 50mg/l PCP and 25mg/l yeast extract at 30°C. ET01 exhibited very slow growth developing only moderate turbidity after 3-5 days in agitated broth. Cells of ET01 became unusually long (up to four times normal cell length) during the exponential growth phase when viewed under a fluorescence microscope. The ET01 cells were rod shaped, 2x1μm, at stationary phase. Radehaus and Schmidt (1992) reported that some cells of *Pseudomonas* sp. strain RA2 became up to 15-fold normal cell length during the same phase and only coccoidal cells were observed in the stationary phase. This characteristic is also typical of *Bradyrhizobium* sp. (Jordan, 1984). The pH of the MMS broth remained between 6 and 7 throughout the growth phase, the optimum range for *Bradyrhizobium* sp. (Jordan, 1984). Isolate ET02 formed a larger entire white colony, 1-3mm in diameter. Isolate ET03 exhibited similar colony morphology to ET01 although the colonies were white to straw in colour. Isolates ET02 and ET03 were designated *Pseudomonas putida* and *Pseudomonas aureofaciens* respectively (Hussein *et al.*, 1996) and exhibited colony morphologies and biochemical characteristics similar to those described for *Pseudomonas* spp. (Palleroni, 1984) when incubated aerobically at 30°C. The optimum growth temperature for *Pseudomonas* spp. is 28°C (Palleroni, 1984). ET02 and ET03 were Gram-negative, oxidase and catalase positive and motile. The

characteristics exhibited by ET02 and ET03 are also similar to those described for *Sphingomonas* spp. (Balkwill *et al.*, 1997). *Sphingomonas* spp. have broad catabolic capabilities and are able to degrade PCP unlike ET02 and ET03. *Bradyrhizobium* spp., *Sphingomonas* spp. and *Pseudomonas* spp. are ubiquitous in soil (Jordan, 1984; Palleroni, 1984; Balkwill *et al.*, 1997). A definitive taxonomic analysis of ET02 and ET03 is required to enable placement of these strains in one of these two possible genera.

5.3 PCP REMOVAL

Strain ET01 could grow in media containing PCP as a sole source of carbon and energy and removed PCP from the media at concentrations up to and including 150mg/l with 25mg/l yeast extract. ET01 in pure culture exhibited the ability to remove PCP from an initial concentration of 120mg/l in 5 days from a MMS medium with 25mg/l added yeast extract. The addition of 25mg/l yeast extract accelerated the removal of PCP considerably when compared to PCP removal in media without yeast extract. Hussein *et al.* (1996) used a greater amount of yeast extract, 200mg/l, in the batch culture media for the isolates than was used throughout these experiments. Isolate ET01 was originally found to remove 63mg/l PCP within 7 days with 100% chloride release in media containing 200mg/l yeast extract (Hussein *et al.*, 1996). The maximum rate of PCP removal for ET01 in pure culture obtained in the presently described experiments was twice that obtained by Hussein *et al.* (1996) at twice the initial PCP concentrations and with one eighth of the yeast extract concentration. This may be due to repeated subculturing and cellular adaption under the selective pressure of PCP.

Hussein *et al.* (1996) reported that when ET01 was first isolated it could remove PCP at a concentration of less than 100mg/l within a 7 day incubation period. The concentration of PCP that ET01 could remove was much higher, 250mg/l, in a bioreactor operating in continuous mode than that achieved in batch shake flask culture (Hussein *et al.*, 1996). Because of the lag period, Stanlake and Finn (1982) suggested continuous culture methods would be more suitable to PCP biodegradation than batch culture methods. Klecka and Maier (1985) conducted experiments on batch and

continuous-flow cultures growing on PCP as sole carbon and energy source and concluded that there was no difference in the resulting kinetic parameters, thereby justifying the presently described use of batch cultures for these further studies.

From the initial experiment, (Results Section 4.2) the cells used to inoculate media without yeast extract were not washed. The inoculum was grown in media containing 25mg/l yeast extract. The PCP removal rates at the lower initial PCP concentrations tested in media without yeast extract were similar to those in media containing 25mg/l yeast extract. The presumed carry over of yeast extract from the inoculum of unwashed cells at an unknown concentration to media without yeast extract indicates that considerably less than 25mg/l yeast extract may be required to facilitate PCP removal, at least at the low initial PCP concentrations at which this phenomenon occurred. This indicates that increasing the yeast extract concentration above 25mg/l may not necessarily increase the rate of PCP removal even at low initial PCP concentrations. Yeast extract concentration above 25mg/l did not increase PCP removal rates as the results of Hussein *et al.* (1996) show, where 200mg/l yeast extract was added to the MMS broth, although significant subculturing under the selective pressure of PCP has occurred since the organism's initial isolation. ET01 has substantially increased its effectiveness at removing PCP with regard to both rate of removal and the initial PCP concentration from which it will remove PCP, with a significantly lower concentration of yeast extract supplementation, since it was first isolated.

The rate of PCP removal by ET01 at high initial PCP concentrations increased throughout the course of the experiments. The rate of removal of 150mg/l PCP improved from 1.48 mg/l/hr to 1.85mg/l/hr, although no removal occurred at an initial PCP concentration of 175mg/l. During the course of these experiments strain ET01 has manifested a capacity to remove 50.4mg/l PCP per day from an initial PCP concentration of 120mg/l in batch shake flask culture. This suggests that continued subculturing under the selective pressure of PCP may increase further the ability of ET01 to remove this toxic compound, with yeast extract supplementation at or below 25mg/l.

When media without yeast extract was inoculated with washed cells ET01 took, on average, 7 times longer to remove the same amount of PCP than with 25mg/l yeast

extract. Jordan (1984) states that *Bradyrhizobium* sp. do not usually require vitamins, with the rare exception of biotin, which may also be inhibitory to some strains. Biotin was used to supplement the MMS media in combination with other compounds to ascertain the specific vitamin(s) responsible for the facilitation of PCP removal for ET01 and the culture combinations. The role of vitamin supplementation in PCP removal is discussed below (see Section 5.9). The removal of PCP by ET01 and the culture combinations was enhanced by the addition of yeast extract compared to very low removal rates in media without yeast extract.

Watanabe (1973) found 100mg/l yeast extract accelerated the PCP removal rate at an initial PCP concentration of 100mg/l with an isolate characterised as a pseudomonad. At 100mg/l PCP concentration, the growth of the pseudomonad on the PCP medium was poorer than that at 40mg/l and the bacteria were less effective for PCP removal. At 200mg/l PCP concentration, neither growth nor removal were observed (Watanabe, 1973). The toxicity of PCP to bacteria at 200mg/l concentration is well documented, the pseudomonad described by Watanabe (1973) and *Pseudomonas* sp. strain RA2 (Radehaus and Schmidt, 1992) both could mineralise PCP at a concentration of 160mg/l but not at 200mg/l. In batch cultures, ET01 was capable of completely mineralising PCP at a concentration of 150mg/l with yeast extract in the medium but was unable to mineralise PCP at a concentration of 175mg/l, also with added yeast extract. No concentrations of PCP between 150mg/l and 175mg/l were tested.

An active population of *Pseudomonas* sp. strain RA2 was still present in the medium containing 200mg/l PCP after 2 weeks (Radehaus and Schmidt, 1992). This indicated that *Pseudomonas* sp. strain RA2 cells exhibited the ability to remain viable in media where the PCP concentration was too high for removal to begin. ET01 was subcultured from media containing 150 - 175mg/l PCP in the early stages of the experimental work. ET02 and ET03 exhibited the ability to remain viable in media containing PCP at low and high concentrations but they were not able to remove PCP even at low concentrations. Continued subculturing was performed at the same PCP concentration of 25mg/l to induce the ability of ET02 and ET03 to remove PCP but this strategy was not successful. All three isolates used in this project contained plasmids, the roles of which are unclear (Hussein *et al.*, 1996).

The highest PCP removal rate for ET01, in MMS broth containing 25mg/l yeast extract was 2.1mg/l/hr and occurred at an initial PCP concentration of 120mg/l. The removal rate decreased at higher or lower PCP concentrations, with the lowest removal rate of 0.01mg/l/hr at 175mg/l PCP. For *Pseudomonas* sp. strain RA2 the highest PCP removal rate of 4.1mg/l/hr was reached at a PCP concentration of 150mg/l however the lag period was 72 hours and total degradation occurred after 144 hours (Radehaus and Schmidt, 1992). At 150mg/l PCP ET01 had a 48 hour lag period and also took 144 hours to degrade 150mg/l, the PCP removal rate was 1.85mg/l/hr.

5.4 CHLORIDE RELEASE

ET01 grew in media containing PCP and was capable of removing this compound as indicated by stoichiometric release of chloride, depletion in the concentration of PCP and increased cell numbers. Chloride was released into the medium by ET01 in an amount which accounted for 100% of the chlorine added as PCP. To metabolise PCP bacteria must possess enzymes that either cleave the aromatic ring in the presence of the halogen substituents, in this case chlorine, or catalyse the removal of the chlorine substituents prior to dearomatization. Chloride release and PCP depletion are not simultaneous, the chloride release lags because the free chlorine atoms are sequentially released from transformation of the reaction intermediates. Once the chloride molecules are removed the aromatic ring is much easier to degrade (Reineke and Knackmuss, 1988).

When the actual chloride release for ET01 was compared with the theoretical chloride release the rates were similar. Five moles of chloride are released per mole of PCP. The measured ratio of actual chloride released to PCP removed was 4.4 for ET01 in media with yeast extract and 4.9 in media without. The total amount of chloride released corresponded closely with the initial amount of PCP in the media although the release pattern was not always in accordance with theory. Dechlorination of intermediates may proceed more rapidly without yeast extract in the media than with it as a component of yeast extract could inhibit the dechlorinating enzymes while augmenting the first enzyme (for PCP degradation). Chloride is released more rapidly in media without yeast

extract for all initial PCP concentrations tested. PCP was not the only peak seen throughout the growth phase when HPLC analysis was done. One other much smaller and less polar peak was observed but was not identified. Radehaus and Schmidt (1992) stated that stoichiometric release of chloride and proportional increase in cell biomass indicated that *Pseudomonas* sp. strain RA2 could completely mineralise PCP. The same can then be said for ET01. These results also suggest that PCP is mineralised without the release of measurable amounts of toxic metabolites or other end products although such a suggestion needs confirmation by a toxicity test of the final solution using e.g. Microtox ® or Daphnia magna. Neither ET02 nor ET03 exhibited the ability to degrade PCP and therefore would not contribute to chloride release.

5.5 LAG PERIOD

The existence of a lag period before a toxic substance is metabolised by micro-organisms is not an unusual phenomenon. PCP concentration has a significant effect on the lag or acclimation period of micro-organisms prior to PCP mineralisation (Stanlake and Finn, 1982; Gonzalez and Hu, 1995). For ET01 the lag period was directly proportional to the initial substrate concentration, the higher the PCP concentration the longer the time interval between inoculation and initiation of PCP removal and it was shown that every mg/l of PCP extends the lag period by 1 hour at initial PCP concentrations over 50mg/l. Radehaus and Schmidt (1992) observed a 48 hour lag period at an initial PCP concentration of 150mg/l with *Pseudomonas* sp. strain RA2, a considerably shorter lag period (for the initial concentration) than the present experiment would predict. The onset of logarithmic growth was delayed at high initial PCP concentrations as compared with the onset of growth at lower concentrations. Topp *et al.* (1988) reported that the apparent lag period prior to measurable PCP removal is a function of the time required for acclimation and the accumulation of sufficient biomass to get measurable removal rates. This has important implications in the remediation of contaminated sites where the concentration of PCP might be in excess of 100mg/ml of leachate. This would require acclimatisation periods of in excess of 43 hours if the factor measured in this research is used.

Bacteria capable of mineralising PCP are themselves susceptible to PCP toxicity (Stanlake and Finn, 1982). Gonzalez and Hu (1995) found that with a *Flavobacterium* sp. all cultures showed a lag in degradation of at least a few hours. Gonzalez and Hu (1995) assumed only a fraction of all the inoculated cells is resistant to the level of PCP encountered. The proportion of viable ET01 cells capable of growing on PCP was not ascertained in the current research project. Gonzalez and Hu (1995) found that *Flavobacterium* cells acclimatised to PCP showed a decrease of viability for PCP concentrations higher than the PCP level during acclimation. All flask cultures exhibited a lag for PCP degradation of about 10 hours to 25 hours. The flasks of 99 and 154mg/l of PCP also show loss of viability during the same period. Growth resumed after the lag in all PCP containing flasks (Gonzalez and Hu, 1995). Lag and loss of viability were more noticeable at PCP concentrations above 50mg/l (Gonzalez and Hu, 1995).

The concentration of PCP also affected the lag period before the onset of mineralisation by *Pseudomonas* sp. strain RA2 (Radehaus and Schmidt, 1992). Stanlake and Finn (1982) found the length of the lag period was directly proportional to the PCP concentration for *Arthrobacter* sp. strain NC and a significant lag period of 10 or more hours occurred before PCP mineralisation commenced. Lag periods were longer in media containing PCP when *Arthrobacter* sp. strain NC was maintained in media without PCP (Stanlake and Finn, 1982). The effect of PCP concentration on the length of lag period and growth rate from *Arthrobacter* sp. strain NC catabolising PCP was characteristic of toxic substrate catabolism (Stanlake and Finn, 1982).

Stanlake and Finn (1982) found the size of the initial inoculum had no discernible effect on the length of the lag period, however Topp *et al.* (1988) and Radehaus and Schmidt (1992) found the opposite to be true. The relationship between lag period and inoculum size was not investigated thoroughly though when ET01 was tested for PCP removal in combination with the other two isolates the lag period increased with the reduction of ET01 cell numbers in the inoculum. This suggests that the inoculum size of ET01 affects the lag period. The lag period for ET01 in pure culture (determined as described in the Results Section 4.2.1) was less than 48 hours for PCP concentrations less than 80mg/l and between 48 and 60 hours for initial PCP concentrations of 100mg/l to 150mg/l. The lag period decreased over the course of the experiments, it was initially 40

hours for an initial PCP concentration of 100mg/l then decreased to 20 hours for the same initial PCP concentration.

Increasing the pH from 6.8 to 7.8 decreased the length of the lag period for growth of *Arthrobacter* sp. strain NC on PCP (Stanlake and Finn, 1982). No effect of pH change on PCP removal was studied for ET01 and all experiments were conducted at pH 6.8 – 7.0.

The results of present investigations confirm that the length of the lag period depends on the concentration of the toxic substance to be degraded and that culture acclimation is a useful method for minimising this period of time.

5.6 CELL DENSITY AND YIELD

The concentration of the cell inoculum into a medium containing PCP has an effect on the lag period and the rate of degradation (Klecka and Maier, 1985; Radehaus and Schmidt, 1992). Klecka and Maier (1985) found that the rate of degradation was proportional to the concentration of initial cell mass used in the experiment. In all experiments conducted by Radehaus and Schmidt (1992) with *Pseudomonas* sp. strain RA2 the initial bacterial density was $2x10^6ml^{-1}$. The initial cell density for ET01 in all pure ET01 culture experiments was approximately $2x10^6ml^{-1}$ and a 10% (v/v) inoculum was used. A higher population density can withstand a higher initial PCP concentration because the relative concentration of PCP per cell is less (Radehaus and Schmidt, 1992), which suggests an adsorption effect. Measurable PCP removal was observed when the cell density of ET01 reached approximately $1x10^7$cells/ml. Radehaus and Schmidt (1992) found the viable cell number in the culture with 200mg/l of PCP slowly decreased from $2x10^6$ cells per ml to approximately 10^4 cells per ml over a period of 16 days. In contrast, high concentrations of PCP had only a moderate effect on cell dry weight and yield (Radehaus and Schmidt, 1992).

ET01 was susceptible to the toxicity of PCP and did not exhibit growth at 175mg/l PCP. A larger inoculum was not tested but may have allowed ET01 to remove PCP at this

high concentration. Investigation of the inoculum size on the removal of PCP, lag period and cell growth is warranted. Changes in pH affect PCP toxicity (Stanlake and Finn, 1982). No effect of pH (6.8-7.8) on cell yield at a PCP concentration of 130mg/l was observed for *Arthrobacter* sp. strain NC (Stanlake and Finn, 1982).

The final number of cells for ET01 for initial PCP concentrations over the range of 20mg/l to 150mg/l was approximately $5.5x10^7$ cells per ml (0.09mg/ml). Brown *et al.* (1986) studied an epilithic microbial consortia and measured cell density at various initial PCP concentrations. The final number of PCP degraders for initial PCP concentrations over the range of 20mg/l to 220mg/l was $5.3x10^6$ cells/ml (0.0085mg/ml). Total biomass production was proportional to PCP concentration at all PCP concentrations degraded by *Pseudomonas* sp. strain RA2 (Radehaus and Schmidt, 1992). At an initial PCP concentration of 40mg/l Radehaus and Schmidt (1992) obtained a final cell density of $2.0x10^7$ cells/ml (0.032mg/ml)[1]. These workers found that at an initial PCP concentration of 140mg/l the final cell density was $7x10^8$ cells/ml (1.12mg/ml). Radehaus and Schmidt (1992) found though that final cell density was reduced to below expected values at PCP concentrations greater than 100mg/l.

The elongation of cells during growth on PCP for *Pseudomonas* sp. strain RA2 and for ET01 is not consistent with the assumption that PCP acts only as an uncoupler of oxidative phosphorylation because an uncoupler should reduce biomass as well as cell number (Radehaus and Schmidt, 1992). Radehaus and Schmidt (1992) proposed that the unusually long cells observed during exponential growth indicated that *Pseudomonas* sp. strain RA2 had the potential to grow without dividing. Rutgers *et al.* (1996) state that besides the uncoupling of oxidative phosphorylation substituted phenols are also known to inhibit enzymes of the respiratory chain and ATPase activity. This type of inhibition by PCP, rather than uncoupling reactions, can possibly explain the lower growth rate in the cultures at high PCP concentrations (Rutgers *et al.*, 1996). Cells up to four times greater in length were observed by fluorescence microscopy during the growth phase of ET01 as compared to cells from the stationary phase (2x1μm).

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[1] These numbers were converted according to Langfeldt (1996) using $1.6x10^{-9}$mg/cell.

Gu and Korus (1995) stated that PCP concentration had a stronger effect on the cell growth rate than on the degradation rate. This is evident for ET01, and the culture combinations tested. The cell growth rate did not increase like the removal rate did as the initial PCP concentration rose. The amount of biomass is obtained from the total cell numbers counted by fluorescence microscopy and differs from yield which is the amount of cells produced (mg) per mg of substrate consumed. Yields are low when bacteria utilise PCP as the sole carbon source and based on cell yield, PCP proved to be a poor substrate for the growth of *Arthrobacter* sp. strain NC (Stanlake and Finn, 1982).

Changes in PCP concentration affected growth rate and length of the lag period but not cell yield for *Arthrobacter* sp. strain NC (Stanlake and Finn, 1982). For ET01 the yield was 0.001mg/mg at 20mg/l PCP, with a specific growth rate of $0.05hr^{-1}$, 0.003mg/mg at 50mg/l PCP with a specific growth rate of $0.05hr^{-1}$ and 0.001mg/mg at 100mg/l PCP with a specific growth rate of $0.04hr^{-1}$. The yield decreased as the initial PCP concentration increased to 50mg/l PCP but decreased at higher concentrations. Likewise specific growth rates also diminished at PCP concentrations in excess of 50mg/l. The latter observation will be examined in the next section.

5.7 SPECIFIC GROWTH RATE

Organisms growing on PCP exhibit substrate inhibition; that is an increasing initial substrate concentration will eventually inhibit the organisms' growth. PCP exhibited a substrate inhibition effect on *S. chlorophenolica* sp. strain ATCC 39723 (Gu and Korus 1995). Rutgers *et al.* (1996) derived inhibition kinetics for PCP from non-steady state cultures (generally batch cultures). Rutgers *et al.* (1996) found that adaptation of the cells to toxic conditions is very slow, so that growth inhibition in batch culture is more profound than that found in continuous culture. As the kinetics of PCP degradation appear to involve substrate inhibition Klecka and Maier (1985) suggested that adaptation of bacterial populations to PCP can best be achieved by continuous exposure to low substrate concentrations.

Specific growth rates were related to the initial PCP concentration for ET01 and the culture combinations; high initial substrate concentrations (>175mg/l) were inhibitory and the specific growth rates were greater at low concentrations (50-100mg/l). These observations indicate that the relationship between the specific growth rate and PCP concentration deviates from the classical hyperbolic function described by the Monod equation. Klecka and Maier (1985) described the same phenomenon with two mixed bacterial cultures isolated from industrial sewage capable of utilising PCP as a sole carbon source, though the PCP concentrations used by Klecka and Maier (1985) were only a fraction of the PCP concentrations used in this experiment. High PCP concentrations (0.8-1.6mg/l) were inhibitory and the removal rates were greater at low concentrations (0.16-0.4mg/l).

Gu and Korus (1995) studied the kinetics of PCP degradation by *Sphingomonas* sp. strain ATCC 39723 and the organism was capable of degrading 150mg/l PCP in 4 hours. The specific growth rate was $0.12hr^{-1}$ at 140mg/l and decreased as the initial PCP concentration increased (Gu and Korus, 1995). Topp *et al.* (1988) obtained a specific growth rate of 0.1 hr^{-1} at 120mg/l initial PCP concentration with the same organism. The maximum specific growth rate for *Sphingomonas* sp. strain ATCC 39723 was at a PCP concentration of 60mg/l (Gu and Korus, 1995). The specific growth rate of ET01 increased as the initial PCP concentration increased to 80mg/l where the specific growth rate reached a maximum of $0.05hr^{-1}$. At initial PCP concentrations higher than 80mg/l the specific growth rate decreased as the effect of substrate inhibition became more pronounced.

Stanlake and Finn (1982) obtained a specific growth rate of $0.3hr^{-1}$ at an initial PCP concentration of 0.013mg/l PCP with *Arthrobacter* sp. strain NC. The specific growth rate decreased to $0.05hr^{-1}$ when the PCP concentration was 0.30mg/l. In a continuous culture at 600mg/l the growth rate for *Sphingomonas chlorophenolica* sp. nov. ATCC 39723 (formerly *Flavobacterium* sp. ATCC 39723) was $0.045hr^{-1}$ (Nohynek *et al.*, 1995; Brown *et al.*, 1986). In batch culture *S. chlorophenolica* sp. nov. ATCC 39723 exhibited specific growth rates of $0.19hr^{-1}$, $0.21hr^{-1}$ and $0.14hr^{-1}$ at 25mg/l, 50mg/l and 100mg/l PCP respectively (Nohynek *et al.*, 1995; Gu and Korus, 1995). Edgehill and Finn (1983) obtained a specific growth rate of $0.1hr^{-1}$ at an initial PCP concentration of 135mg/l for the same organism. For ET01 the at 20mg/l, 50mg/l and 100mg/l PCP the

specific growth rates were $0.053hr^{-1}$, $0.054hr^{-1}$ and $0.038hr^{-1}$ respectively. For ET01 the specific growth rates at initial PCP concentrations below 80mg/l were higher than at initial PCP concentrations over 80mg/l. At 100mg/l and 150mg/l initial PCP concentration the specific growth rates decreased and were $0.038hr^{-1}$ and $0.025hr^{-1}$ respectively. The specific growth rate of ET01 appears to be one third to one quarter as fast as *S. chlorophenolica* sp. nov. ATCC 39723. The specific growth rate of ET01 is more similar to that obtained by Radehaus and Schmidt (1992) with *Sphingomonas chlorophenolica* sp. strain RA2. A specific growth rate of 0.05 hr^{-1} was obtained at an initial PCP concentration of 150mg/l (Radehaus and Schmidt, 1992).

The inhibition constant (K_i)[2] for PCP removal for ET01 in pure culture was 100mg/l. For the culture combinations of ET01 with each of the other two isolates the K_i was 95mg/l. The K_i for *Pseudomonas* sp. strain RA2 was 155mg/l (Radehaus and Schmidt, 1992). Rutgers *et al.* (1996) obtained a K_i of 0.62mg/l for strain P5. Edgehill and Finn (1982) studied the relationship of PCP concentration and growth rate for *Sphingomonas* sp. ATCC 33790 K_i of 1.3mg/l. These values indicate that ET01 is more tolerant of PCP and more able to withstand high initial PCP concentrations than P5 and *Sphingomonas* sp. ATCC 33790, but less tolerant than *Pseudomonas* sp. strain RA2 was 155mg/l.

5.8 CULTURE COMBINATIONS

As ET01 was initially isolated from a consortium which has PCP degrading characteristics. Consequently the possible beneficial effect of the three bacterial strains growing together was investigated. ET01 was combined respectively with ET02 and ET03 and then the three strains were combined and the rate of PCP removal for the combinations compared to that of ET01 in pure culture. When ET01 was used in combination with either of the other two strains the observed lag period increased and the rate of PCP removal was inferior to that observed with ET01 alone. The inoculum concentration of ET01 cell numbers when combined with either of the other two isolates was reduced by 50% thus leading to a lag period increase when the initial PCP

[2] K_i is the inhibition constant defined as the highest inhibitor concentration at which the specific growth rate (μ) equals half the maximum (Radehaus and Schmidt, 1992).

concentration was 50mg/l. PCP removal may have been delayed until ET01 cells numbers reached a sufficient concentration to allow detectable degradation, usually at about $1x10^7$cells/ml. In the medium containing all three strains (inoculated in a 10% (v/v), 1:1:1 volumetric ratio) the lag period increased further and the PCP removal rate was again inferior to that observed with ET01 in pure culture.

There was experimental evidence that strains ET02 and ET03 enhanced the ability of strain ET01 to remove PCP. Brown *et al.* (1986) found that specific PCP degradation rates measured in pure culture can be obtained and maintained indefinitely in epilithic microbial consortia acclimatised to PCP. However because neither ET02 nor ET03 could remove PCP from a MMS broth containing yeast extract in pure culture that only ET01 cells were actively removing PCP from the medium in the strain combination experiments. The ratio of ET01 cell concentration in pure culture, in the binary and ternary experiments was 200:100:67 respectively. As the cell concentration of the PCP removing strain, i.e., ET01, was reduced in the binary and ternary experiments, the expected PCP removal rate was half and one third of that achieved by ET01 in pure culture. The lag period was expected to double and triple for the binary and ternary combinations respectively as was the total time taken to remove PCP from the medium. Yield was also expected to be proportional to the ET01 cell concentration again expected to reduce by half and then one third for the binary and ternary combinations. Yu and Ward (1996) experienced the same reduction in removal rate when the cell concentration of the most efficient PCP degrading strain was decreased. Contrary to their study and the expectations arising, all three cultures used by Yu and Ward (1996) had PCP degrading capacity and consequently PCP degradation was more efficient when the three cultures were combined.

To identify a synergistic effect the observed results of the culture combinations had to exceed those obtained by ET01 in pure culture. Over the range of parameters tested the three isolates in combination gave the most evidence of a synergistic relationship. For PCP removal rate and specific growth rate the observed values for the ternary combination far exceeded the expected values at all PCP concentrations tested. The observed lag periods were far less than the expected values for the ternary combination, again at all PCP concentrations tested. For the yield values, each combination performed best at one PCP concentration. ET01 and ET03 combined gave the highest

ratio of observed to expected yield at the lowest PCP concentration tested (50mg/l). The ternary combination gave the highest ratio at 100mg/l PCP and ET01 and ET02 gave the highest ratio of observed to expected yield at the highest PCP concentration tested (150mg/l PCP).

Further experimentation into ET01 and ET02 in combination at higher PCP concentrations than those tested in this project may prove worthwhile as there was an increase in PCP removal rate as the initial PCP concentration increased. At an initial PCP concentration of 50, 100 and 150mg/l the removal rate for ET01 and ET02 in combination was 0.88mg/l/hr, 1.08mg/l/hr and 1.54mg/l/hr respectively. The results gained from testing the culture combinations clearly illustrate that PCP removal is more efficient when ET01 is in pure culture although the observed values for the culture combinations far exceeded the expected values in all but a few instances. The ability of each of the strains to remove PCP was tested individually and in combination. Rates of PCP removal by individual isolates were very different than those observed for culture combinations. Yu and Ward (1996) found the rates of PCP degradation by individual isolates to be lower than those observed for their three isolates combined. Mixed microbial consortia often manifest higher rates of removal of recalcitrant compounds (Yu and Ward, 1996), however for ET01, ET02 and ET03 this was not the case.

5.9 VITAMINS

Various vitamins were chosen as supplementation for the MMS broth containing PCP as a sole source of carbon and energy. Three vitamin solutions were compared with yeast extract and no vitamin supplementation to ascertain the specific vitamin(s) or components of yeast extract responsible for the facilitation of PCP removal for ET01 and the culture combinations. Of the three vitamin solutions tested only two facilitated the removal of PCP. The other one, vitamin solution 1, compared almost identically to no vitamin supplementation. Vitamin solution 1 contained biotin and cyanocobalamin only. Jordan (1984) states that *Bradyrhizobium* sp. do not usually require vitamins, with the rare exception of biotin, which may also be inhibitory to some strains. Shepherd (1997) found pantothenic acid to be the vitamin that supported growth of ET01 on PCP

in combination with biotin. Vitamin solutions 2 and 3 gave comparable results to yeast extract in the medium. Vitamin solution 2 contained pantothenic acid and cysteine in addition to those compounds in vitamin solution 1. Vitamin solution 3 also contained pantothenic acid, at a lesser concentration than in vitamin solution 2, and biotin (as well as other compounds listed in the Methods Section 3.2.6).

Defining more precisely what is the useful component(s) in vitamin solution 2 for degradation of PCP may give an economical means for bioremediation of large volumes of PCP contaminated leachate with the addition of gram quantities of the identified components. The lack of knowledge may be counter-productive to bioremediation of waste material. Further experimentation into the specific vitamin requirements of ET01 and the culture combinations and the synergistic effects is desirable. Modification of the concentration of components in vitamin solution 2 may yield the same result as for yeast extract. Deletion and addition of certain components would further define the organisms' requirements. Yeast extract gave the fastest removal rates and shortest lag periods suggesting that micro traces of metals in the yeast extract may have a role in the facilitation of PCP removal. This investigation of yeast extract would be an appropriate starting point for the next series of experiments in this area of study.

6. CONCLUSIONS

From the experimental work conducted on the isolates ET01, ET02 and ET03 isolated by Hussein *et al.* (1996) the following conclusions were drawn.

ET01, ET02 and ET03 were designated as *Bradyrhizobium* sp. (Yu, 1998), *Pseudomonas putida* and *Pseudomonas aureofaciens* respectively (Hussein *et al.*, 1996) and exhibited colony morphologies and biochemical characteristics consistent with these designations. The biochemical tests conducted in this research gave results consistent with the previous designations.

A minimal mineral salts medium supplemented with 25mg/l yeast extract provided an environment in which ET01 could completely remove up to and including 150mg/l PCP. Bacteria capable of mineralising PCP are susceptible to the toxicity of PCP and PCP removal by ET01 ceased at a concentration of 175mg/l. The threshold limit for ET01 in media containing PCP with no yeast extract was less than 20mg/l as the removal rates did not increased above this initial concentration. PCP removal by ET01 ceased at a concentration between 150mg/l and 175mg/l for media containing yeast extract. The actual PCP concentration between 150 and 175mg/l at which PCP removal ceases was not ascertained.

The highest specific growth rate for ET01, $0.06hr^{-1}$, occurred in media containing yeast extract at an initial PCP concentration of 40mg/l. The highest specific growth rates for culture combinations with 50mg/l initial PCP concentration and 25mg/l yeast extract were $0.05hr^{-1}$, $0.06hr^{-1}$ and $0.05hr^{-1}$ for ET01 and ET02, ET01 and ET03 and the three isolates together respectively. The specific growth rates were lower for the culture combinations at higher initial PCP concentrations.

The capacity of ET01 to remove PCP increased with continued subculturing under the selective pressure of PCP. The PCP removal rate increased for ET01 in pure culture through the course of the experiments. The rate of removal at 150mg/l initial PCP concentration improved from 1.48mg/l/hr to 1.85mg/l/hr. The rate of removal at 120mg/l initial PCP concentration improved from 1.38mg/l/hr to a maximum of

2.10mg/l/hr. ET01 was grown in pure culture on MMS broth with 25mg/l added yeast extract.

The higher the initial PCP concentration the longer the lag period for ET01 and the culture combinations. The length of the lag period was directly proportional to the initial PCP concentration for ET01. The PCP removal rate increased and the lag period decreased with continued subculturing of ET01 under the selective pressure of PCP as the sole carbon and energy source in media containing yeast extract. The shortest lag period was 4 hours for ET01 in pure culture on 20mg/l initial PCP concentration. The lag period was equal to 0.30 of the initial PCP concentration for ET01 in pure culture. The size of the inoculum of ET01 had an effect on the lag period and the rate of PCP removal. When the ET01 inoculum was reduced from 10% volumetric ratio to 5% volumetric ratio and then 3% the lag period increased. The effect of inoculum size/cell concentration on lag period and PCP removal rate for ET01 needs to be investigated further.

Depletion of PCP, stoichiometric release of chloride and proportional increase in cell biomass indicated that ET01 can completely mineralise PCP. PCP was not the only peak observed during HPLC analysis indicating ET01 may not degrade PCP without the release of measurable amounts of toxic metabolites. Confirmation of mineralisation using ^{14}C-labelled PCP as substrate is recommended.

Changes in PCP concentration affected the PCP removal rate and length of the lag period but not cell yield for ET01 or the culture combinations. Cell yield was low for ET01 and the culture combinations at all initial PCP concentrations tested. The highest cell yield was 0.004mg/mg for the ET01/ET03 combination at 50mg/l initial PCP concentration. Measurable PCP removal was observed when the cell density of ET01 reached $1x10^7$ cells/ml. The final cell number for ET01 for initial PCP concentrations over the range of 20mg/l to 150mg/l was approximately $5.5x10^7$ cells/ml. For the culture combinations the final cell number for the PCP concentrations tested was approximately $6.5\ x10^7$ cells/ml.

Yeast extract was the most effective vitamin supplement although when specific vitamins were tested pantothenic acid together with biotin yielded results similar to

contained yeast

those obtained with yeast extract. Standardisation of vitamin concentrations and yeast extract components may facilitate the deduction of the precise vitamins enhancing PCP removal by ET01. Ascertaining the concentrations of the components that make up yeast extract and their effectiveness in facilitating PCP removal for ET01 would narrow the vitamin requirements and provide useful information for further maximisation of the PCP removal rate. However, due to the complexity of yeast extract this would be a major undertaking.

Yeast extract at a concentration of less than 25mg/l is required to facilitate the removal of PCP by ET01. Different concentrations of yeast extract lower than 25mg/l could be tested to ascertain lowest yeast extract concentration at which removal is maximum. On average the time taken for ET01 to degrade PCP in a minimal minerals salts media with no vitamin supplementation was seven times longer than in media with yeast extract. This value was ascertained by extrapolating the PCP removal curve in media without yeast extract and comparing it to the time taken for PCP removal curve in media containing yeast extract.

The highest PCP removal rate obtained with no vitamin supplementation, i.e. MMS broth with no yeast extract or vitamins, was with ET01 and ET02 in combination and with the three isolates together, 0.18mg/l/hr for both, possibly indicative of the consortium in the environment from which they were isolated. For lag period, PCP removal rate and specific growth rate the ternary combination exhibited the most significant evidence of synergism. For yield each combination performed best at one PCP concentration. Further investigation into the relationship between ET01 and the other two isolates in binary and ternary combination at higher initial concentrations of PCP is desirable. Characterisation and classification of all three isolates would also be beneficial.

An understanding of the kinetics of PCP removal provides a foundation for process analysis and design for optimum removal of the compound in waste water treatment systems. For the biological treatment of PCP contaminated soil and water to be successful, it is necessary to use an organism or microbial population enriched and acclimated to this recalcitrant substance. ET01 was shown to remove relatively high concentrations of PCP and future work with this organism should be directed at

selecting strains that can degrade even higher concentrations of PCP. Further work could also involve growth and removal of PCP by ET01 in soil. Confirmation of mineralisation using ^{14}C-labelled PCP as substrate is recommended.

To make bioremediation a more effective technology, further research is needed to gain a better understanding of the basic mechanisms underlying enhancement and inhibition of microbial degradation of high concentrations of toxic compounds. Establishing the kinetic parameters of micro-organisms associated with PCP utilisation, such as specific growth rate, length of the lag period and toxicity aids in the development of bioremediation strategies for areas polluted with PCP.

7. LITERATURE CITED

Aislabie, J., Smith, D., Wickstrom, M., Lloyd-Jones, G. and B. Huser. 1997. Bioremediation - Treatment Option of Contaminated Sites. Landcare Research. Environment Waikato.

Allard, A-S., Remberger, M. and A. H. Neilson. 1987. Bacterial *O*-Methylation of Halogen-Substituted Phenols. Applied and Environmental Microbiology, **53**: 839-845.

Alleman, B. C., Logan, B. E. and R. L. Gilbertson. 1992. Toxicity of Pentachlorophenol to Six Species of White Rot Fungi as a Function of Chemical Dose. Applied and Environmental Microbiology, **58**: 4048-4050.

Anderson, T. A., Guthrie, E. A. and B. T. Walton. 1993. Bioremediation in the Rhizosphere; Plant Roots and Associated Microbes Clean Contaminated Soil. Environmental Science and Technology, **27**: 2630-2636.

Apajalahti, J. H. A. and M. S. Salkinoja-Salonen. 1984. Absorption of Pentachlorophenol by Bark Chips and Its Role in Microbial Pentachlorophenol Degradation. Microbial Ecology, **10**: 359-367.

Apajalahti, J. H. A., Kärpänoja, P. and M. S. Salkinoja-Salonen. 1986. *Rhodococcus chlorophenolicus* sp. nov., a Chlorophenol-Mineralising Actinomycete. International Journal of Systematic Bacteriology, **36**: 246-251.

Apajalahti, J. H. A. and M. S. Salkinoja-Salonen. 1986. Degradation of polychlorinated phenols by *Rhodococcus chlorophenolicus*. Applied Microbiology and Biotechnology, **25**: 62-67.

Apajalahti, J. H. A. and M. S. Salkinoja-Salonen. 1987a. Dechlorination and *para*-Hydroxylation of Polychlorinated Phenols by *Rhodococcus chlorophenolicus*. Journal of Bacteriology, **169**: 675-681.

Apajalahti, J. H. A. and M. S. Salkinoja-Salonen. 1987b. Complete Dechlorination of Tetrachlorohydroquinone by Cell Extracts of Pentachlorophenol-Induced *Rhodococcus chlorophenolicus*. Journal of Bacteriology, **169**: 5125-5130.

Balkwill, D. L., Drake, G. R., Reeves, R. H., Fredrickson, J. K., White, D. C., Ringelberg, D. B., Chandler, D. P., Romine, M. F., Kennedy, D. W. and C. M. Spadoni. 1997. Taxonomic Study of Aromatic-Degrading Bacteria from Deep-Terrestrial-Subsurface Sediments and Description of *Sphingomonas aromaticivorans* sp. nov., *Sphingomonas subterranea* sp. nov., and *Sphingomonas stygia* sp. nov. International Journal of Systematic Bacteriology, **47**: 191-201.

Barhart, M. and J. Hyzy. 1993. Bringing in the Expert Microbes; Quantitative Determination of Degradation Rate Potentials. Reprinted from Soils Magazine by Wastestream Technology, Buffalo, New York.

Bergström, I., Heinänen, A. and K. Salonen. 1986. Comparison of Acridine Orange, Acriflavine, and Bisbenzimide Stains for Enumeration of Bacteria in Clear and Humic Waters. Applied and Environmental Microbiology, **51**: 664-667.

Bingham, A. G. 1992. National Task Group on Site Contamination Task Brief 6 - Background Paper on PCP Related Issues. Department of Health, New Zealand.

Bitton, G., Koopman, B., Jung, K., Voiland, G. and M. Kotob. 1993. Modification of the Standard Epifluorescence Microscopic Method for Total Bacterial Counts in Environmental Samples. Water Research, **27**: 1109-1112.

Bollag, J. -M., Helling, C. S. and M. Alexander. 1968. 2, 4- D Metabolism: Enzymatic Hydroxylation of Chlorinated Phenols. Journal of Agricultural and Food Chemistry, **16**: 826-828.

Bollag, J. -M., Briggs, G. G., Dawson, J. E. and M. Alexander. 1968. 2, 4- D Metabolism: Enzymatic Degradation of Chlorocatechols. Journal of Agricultural and Food Chemistry, **16**: 829-833.

Bouchard, B., Beaudet, R., Villemur, R., McSween, G., Lépine, F. and J.-G. Bisaillon. 1996. Isolation and Characterisation of *Desulfitobacterium frappieri* sp. nov., an Anaerobic Bacterium Which Reductively Dechlorinates Pentachlorophenol to 3-Chlorophenol. International Journal of Systematic Bacteriology, **46**: 1010-1015.

Brown, E. J., Pignatello, J. J., Martinson, M. M. and R. L Crawford. 1986. Pentachlorophenol Degradation: a Pure Bacterial Culture and an Epilithic Microbial Consortium. Applied and Environmental Microbiology, **52**: 92-97.

Bryant, F. O., Hale, D. D. and J. E. Rogers. 1991. Regiospecific Dechlorination of Pentachlorophenol by Dichlorophenol-Adapted Microorganisms in Freshwater, Anaerobic Sediment Slurries. Applied and Environmental Microbiology, **57**: 2293-2301.

Chu, J. P. and E. J. Kirsch. 1972. Metabolism of Pentachlorophenol by an Axenic Bacterial Culture. Applied Microbiology, **23**: 1033-1035.

Compeau, G. C., Mahaffey, W. D. and L. Patras. 1991. Full-Scale Bioremediation of Contaminated Soil and Water, in Environmental Biotechnology for Waste Treatment. Plenum Press, New York, **41**: 91-109.

Crawford, R. L. 11 March, 1997. http://www.uidaho.edu/~crawford/refs.html

Crosby, D. G. 1981. Environmental Chemistry of Pentachlorophenol. Pure and Applied Chemistry, **53**: 1051-1080.

Doelle, H. W. 1969. Bacterial Metabolism. Academic Press, New York and London, pp. 352-401.

Edgehill, R. U. and R. K. Finn. 1983. Microbial Treatment of Soil to Remove Pentachlorophenol. Applied and Environmental Microbiology, **45**: 1122-1125.

Evans, W. C. 1963. The Microbiological Degradation of Aromatic Compounds. Journal of General Microbiology, **32**: 177-184.

Fetzner, S. and F. Lingens. 1994. Bacterial Dehalogenases: Biochemistry, Genetics and Biotechnological Applications. Microbiological Reviews, **58**: 641-685.

Gerhardt, P., Murray, R. G. E., Wood, W. A. and N. R. Krieg. 1994. Methods for General and Molecular Bacteriology. American Society for Microbiology, Washington D.C.

Gifford, J. S., McFarlane, P. N., Judd, M. C. and S. M. Anderson. 1994. The Historic Use of Pentachlorophenol by the Solid Wood Processing Industry and Its Impacts on Aquatic Environments. Environmental Research Group. New Zealand Forest Research Institute, Rotorua, New Zealand.

Glaser, J. A. and R. T. Lamar. 1995. Lignin-Degrading Fungi as Degraders of Pentachlorophenol and Creosote in Soil. Soil Science Society of America, American Society of Agronomy, and Crop Science Society. Madison, U.S.A. Bioremediation: Science and Applications.

Gonzalez, J. F. and W-S, Hu. 1995. Pentachlorophenol Biodegradation: Simple Models. Environmental Technology, **16**: 287-293.

Gu, Y. and R. A. Korus. 1995. Kinetics of Pentachlorophenol Degradation by a *Flavobacterium* sp.. Applied Microbiology and Biotechnology, **43**: 374-378.

Häggblom, M. M., Apajalahti, J. and M. S. Salkinoja-Salonen. 1986. Metabolism of Chloroguaiacols by *Rhodococcus chlorophenolicus*. Applied Microbiology and Biotechnology, **24**: 397-404.

Häggblom, M. M., Nohynek, L. J. and M. S. Salkinoja-Salonen. 1988. Degradation and *O*-Methylation of Chlorinated Phenolic Compounds by *Rhodococcus* and *Mycobacterium* Strains. Applied and Environmental Microbiology, **54**: 3043-3052.

Häggblom, M. M., Janke, D. and M. S. Salkinoja-Salonen. 1989. Hydroxylation and Dechlorination of Tetrachlorohydroquinone by *Rhodococcus* sp. Strain CP-2 Cell Extracts. Applied and Environmental Microbiology, **55**: 516-519.

Häggblom, M. M., Nohynek, L. J., Palleroni, N. J., Kronqvist, K., Nurmiaho-Lassila, E-L, Salkinoja-Salonen, M. S., Klatte, S and R. M. Kroppenstedt. 1994. Transfer of Polychlorophenol-Degrading *Rhodococcus chlorophenolicus* (Apajalahti *et al.*, 1986) to the Genus *Mycobacterium* as *Mycobacterium chlorophenolicum* comb. nov.. International Journal of Systematic Bacteriology, **44**: 485-493.

Harms, C.. 1996. Biodegradation of Pentachlorophenol in Soil. Department of Process and Environmental Technology. Massey University, Palmerston North, New Zealand.

Haughland, R. P. 1996. Handbook of Fluorescent Probes and Research Chemicals. Sixth Edition. Molecular Probes Inc., The Netherlands.

Hayes, C. L. 1996. Characterisation of Key Processes in Bioremediation of PCP Contaminated Soil. A Thesis Presented in Partial Fulfillment of the Requirements for the Degree of Master of Technology in Environmental Engineering at Massey University. Palmerston North, New Zealand.

Heinemann Publishers (New Zealand) Ltd. Eton Statistical and Math Tables, Fourth Edition. Reed Publishing (New Zealand) Ltd, Auckland, New Zealand.

Herbert, R. A. 1990. Methods for Enumerating Microorganisms and Determining Biomass in Natural Environments. Methods in Microbiology, **22**: 1-39.

Hobbie, J. E., Daley, R. J. and S. Jasper. 1977. Use of Nucleopore Filters for Counting Bacteria by Fluorescence Microscopy. Applied and Environmental Microbiology, **33**: 1225-1228.

Hughes, M. N. and R. K. Poole. 1989. Metals and Microorganisms. Chapman and Hall, London, New York.

Hussein, H. M., Bhamidimarri, R., Manderson, G. J. and P. L. Yu. 1996. Isolation and Characterisation of Pentachlorophenol-Degrading Pseudomonas Species. Department of Process and Environmental Technology. Massey University, Palmerston North, New Zealand. Unpublished data.

Jordan, D. C. 1984. Genus II. *Bradyrhizobium* Jordan 1982, 137^{VP}, in Bergey's Manual of Systematic Bacteriology. Editor in Chief - Holt, J. G.. Williams and Wilkins. Baltimore/ London. Volume 1, pp. 242 - 244.

Jackman, G. 1992. The Deadly Legacy: A report on the toxic contamination of New Zealand by the indiscriminate use of pentachlorophenol (PCP). Greenpeace New Zealand Inc., New Zealand.

Karlson, U., Rojo, F., Van Elsas, J. D. and E. Moore. 1995. Genetic and Serological Evidence for the Recognition of Four Pentachlorophenol-Degrading Bacterial Strains as a Species of the Genus *Sphingomonas*. Systematic and Applied Microbiology, **18**: 539-548.

Karns, J. S., Kilbane, S., Duttagupta, S. and A. M. Chakrabarty. 1983. Metabolism of Halophenols by 2,4,5-Trichlorophenoxyacetic Acid-Degrading *Pseudomonas cepacia*. Applied and Environmental Microbiology, **46**: 1176-1181.

Kaschabek, S. R. and W. Reineke. 1992. Maleylacetate reductase of *Pseudomonas* sp. Strain B13: Dechlorination of Chloromaleylacetates, Metabolites in the Degradation of Chloroaromatic Compounds. Archives of Microbiology, **158**: 412-417.

Kaschabek, S. R. and W. Reineke. 1993. Degradation of Chloroaromatics: Purification and Characterisation of Maleylacetate Reductase from *Pseudomonas* sp. Strain B13. Journal of Bacteriology, **175**: 6075-6081.

Kaufman, D. D. 1978. Degradation of Pentachlorophenol in Soil, and by Soil Microorganisms, in Pentachlorophenol: Chemistry, Pharmacology, and Environmental Toxicology. K. Ranga Rao. Plenum Press, New York and London, pp. 27-39.

Kennes, C., Wu, W. -M., Bhatnagar, L. and J. G. Zeikus. 1996. Anaerobic Dechlorination and Mineralisation of Pentachlorophenol and 2,4,6-Trichlorophenol by Methanogenic Pentachlorophenol-Degrading Granules. Applied Microbiology and Biotechnology, **44**: 801-806.

Kepner, R. L. and J. R. Pratt. 1994. Use of Fluorochromes for Direct Enumeration of Total Bacteria in Environmental Samples: Past and Present. Microbiological Reviews, **58**: 603-615.

Klecka, G. M. and W. J. Maier. 1985. Kinetics of Microbial Growth on Pentachlorophenol. Applied and Environmental Microbiology, **49**: 46-53.

Knackmuss, H. -J. 1983. Xenobiotic Degradation in Industrial Sewage: Haloaromatics as Target Substrates, in Biochemical Society Symposium no. 48: Biotechnology. C. F. Phelps and P. H. Clarke (Eds.). The Biochemical Society, London, pp. 173-190.

Knackmuss, H. -J. and M. Hellwig. 1978. Utilisation and Co-oxidation of Chlorinated Phenols by *Pseudomonas* sp. B13. Archives of Microbiology, **117**: 1-7.

Kodak. 1990. Laboratory Chemicals, No. 54. Eastman Kodak Fine Chemicals, U.S.A.

Kogure, K., Simidu, U. and N. Taga. 1979. A Tentative Direct Microscopic Method for Counting Living Marine Bacteria. Canadian Journal of Microbiology, **25**: 415-420.

Koser, S. A. 1968. Vitamin Requirements of Bacteria and Yeasts. Charles C. Thomas, Illinois.

Kuwatsuka, S. and M. Igarashi. 1975. Degradation of Pentachlorophenol in Soils. Soil Science and Plant Nutrition, **21**: 405-414.

Langfeldt, J. 1997. Biodegradation of 2-Methyl-4-Chlorophenozyacetic Acid and Related Compounds - Growth and Activity of a Bacterial Consortium. Masterate Thesis. Massey University, Palmerston North, New Zealand.

Lee, J-Y. and L. Xun. 1997. Purification and Characterisation of 2, 6-Dichloro-p-Hydroquinone Chlorohydrolase from *Flavobacterium* sp. Strain ATCC 39723. Journal of Bacteriology, **179**: 1521-1524.

Litchfield, C. D. 1991. Practices, Potential, and Pitfalls in the Application of Biotechnology to Environmental Problems, in Environmental Biotechnology for Waste Treatment. G. Sayler, R. Fox and J. Blackburn (Eds.), Plenum Press, New York, **41**: 147-157.

McAllister, K. A., Lee, H. and J. T. Trevors. 1996. Microbial Degradation of Pentachlorophenol. Biodegradation, **7**: 1-40.

McCarthy, D. L., Navarrete, S., Willett, W. S., Babbitt, P. C. and S. D. Copley. 1996. Exploration of the Relationship between Tetrachlorohydroquinone Dehalogenase and the Glutathione *S*-Transferase Superfamily. Biochemistry, **35**: 14634-14642.

McCarthy, D. L., Claude, A. A and S. D Copley. 1997. In Vivo Levels of Chlorinated Hydroquinones in a Pentachlorophenol-Degrading Bacterium. Applied and Environmental Microbiology, **63**: 1883-1888.

Melin, E. S., Ferguson, J. F. and J. A. Puhakka. 1997. Pentachlorophenol Biodegradation Kinetics of an Oligotrophic Fluidized-Bed Enrichment Culture. Applied Microbiology and Biotechnology, **47**: 675-682.

Middeldorp, P. J. M., Briglia, M. and M. S. Salkinoja-Salonen. 1990. Biodegradation of Pentachlorophenol in Natural Soil by Inoculated *Rhodococcus chlorophenolicus*. Microbial Ecology, **20**: 123-139.

Miethling, R. and U. Karlson. 1996. Accelerated Mineralisation of Pentachlorophenol in Soil upon Inoculation with *Mycobacterium chlorophenolicum* PCP1 and *Sphingomonas chlorophenolica* RA2. Applied and Environmental Microbiology, **62**: 4361-4366.

Mikesell, M. D. and S. A. Boyd. 1985. Reductive Dechlorination of Pesticides 2, 4-D, 2, 4, 5-T, and Pentachlorophenol in Anaerobic Sludges. Journal of Environmental Quality, **14**: 337-340.

Mikesell, M. D. and S. A. Boyd. 1986. Complete Reductive Dechlorination and Mineralisation of Pentachlorophenol by Anaerobic Microorganisms. Applied and Environmental Microbiology, **52**: 861-865.

Mikesell, M. D. and S. A. Boyd. 1988. Enhancement of Pentachlorophenol Degradation in Soil Through Induced Anaerobioses and Bioaugmentation with Anaerobic Sewage Sludge. Environmental Science and Technology, **22**:1411-1414.

Mileski, G. J., Bumpus, J. A., Jurek M. A. and S. D. Aust. 1988. Biodegradation of Pentachlorophenol by the White Rot Fungus *Phanerochaete chrysosporium*. Applied and Environmental Microbiology, **54**: 2885-2889.

Mohn, W. W. and K. J. Kennedy. 1992. Reductive Dehalogenation of Chlorophenols by *Desulfomonile tiedjei* DCB-1. Applied and Environmental Microbiology, **58**: 1367-1370.

Morton, J.. 1997. Personal Communication. The Doctors, 27 Linton Street, Palmerston North, New Zealand.

Murthy, N. B. K., Kaufman, D. D. and G. F. Fries. 1979. Degradation of Pentachlorophenol in Aerobic and Anaerobic Soil. Journal of Environmental Science and Health (B), **14**: 1-14.

Nadebaum, P., Gifford, J. and A. Bingham. 1992. Pentachlorophenol Risk Assessment Pilot Study. National Task Group Study Team Report. Camp Scott Furphy. New Zealand.

National Task Group. 1992. Pentachlorophenol Risk Assessment Pilot Study. Camp Scott Furphy, New Zealand Forest Research Institute, DSIR. New Zealand.

Nicholson, D. K., Woods, S. L., Istok, J. D. and D. C. Peek. 1992. Reductive Dechlorination of Chlorophenols by a Pentachlorophenol-Acclimated Methanogenic Consortium. Applied and Environmental Microbiology, **58**: 2280-2286.

Nohynek, L. J., Häggblom, M. M., Palleroni, N. J., Kronqvist, K., Nurmiaho-Lassila, E-L. and M. S. Salkinoja-Salonen. 1993. Characterisation of a *Mycobacterium fortuitum* Strain Capable of Degrading Polychlorinated Phenolic Compounds. Systemic and Applied Microbiology, **16**: 126-134.

Nohynek, L. J., Suhonen, E. L., Nurmiaho-Lassila, E., Hantula, J. and M. Salkinoja-Salonen. 1995. Description of Four Pentachlorophenol-Degrading Bacterial Strains as *Sphingomonas chlorophenolica* sp. nov.. Systematic and Applied Microbiology. **18**: 527-538.

Nohynek, L. J., Nurmiaho-Lassila, E., Suhonen, E. L., Busse, H-J., Mohammadi, M., Hantula, J., Rainey, F. and M. S. Salkinoja-Salonen. 1996. Description of Chlorophenol-Degrading *Pseudomonas* sp. Strains KF1^{T}, KF3, and NKF1 as a New

Species of the Genus *Sphingomonas*, *Sphingomonas subarctica* sp. nov.. International Journal of Systematic Bacteriology, **46**: 1042-1055.

Okeke, B. C., Smith, J. E., Paterson, A. and I. A. Watson-Craik. 1996. Influence of Environmental Parameters of Pentachlorophenol Biotransformation in Soil by *Lentinula edodes* and *Phanerochaete chrysosporium*. Applied Microbiology and Biotechnology, **45**: 263-266.

O'Reilly, K. T. and R. L. Crawford. 1989. Degradation of PCP by Polyurethane-Immobilized *Flavobacterium* Cells. Applied and Environmental Microbiology, **55**: 2113-2118.

Orser, C. S., Lange, C. C., Xun, L., Zahrt, T. C. and B. J. Schneider. 1993. Cloning, Sequence Analysis, and Expression of the *Flavobacterium* Pentachlorophenol-4-Monooxygenase Gene in *Escherichia coli*. Journal of Bacteriology, **175**: 411-416.

Orser, C. S., Dutton, J., Lange, C., Jablonski, P., Xun, L. and M. Hargis. 1993. Characterisation of a *Flavobacterium* Glutathione *S*-Transferase Gene Involved in Reductive Dehalogenation. Journal of Bacteriology, **175**: 2640-2644.

Palleroni, N. J. 1984. Family I. Pseudomonadaceae Winslow, Broadhurst, Buchanan, Krumwiede, Rogers and Smith 1917, 555^{AL}, in Bergey's Manual of Systematic Bacteriology. Editor in Chief - Holt, J. G.. Williams and Wilkins. Baltimore/ London. Volume 1.

Pignatello, J. J., Martinson, M. M., Steiert, J. G., Carlson, R. E. and R. L. Crawford. 1983. Biodegradation and Photolysis of Pentachlorophenol in Artificial Freshwater Streams. Applied and Environmental Microbiology, **46**: 1024-1031.

Pignatello, J. J., Johnson, L-A. K., Martinson, M. M., Crawford, R. E. and R. L. Crawford. 1985. Response of the Microflora in Outdoor Experimental Streams to

Pentachlorophenol: Environmental Factors. Canadian Journal of Microbiology, **32**: 38-46.

Pyle, B. H., Broadway, S. C. and G. A. McFeters. 1995. A Rapid, Direct Method for Enumerating Respiring Enterohemorrhagic *Escherichia coli* O157:H7 in Water. Applied and Environmental Microbiology, **61**: 2614-2619.

Radehaus, P. M. and S. K. Schmidt. 1992. Characterisation of a Novel *Pseudomonas* sp. that Mineralizes High Concentrations of Pentachlorophenol. Applied and Environmental Microbiology, **58**: 2879-2885.

Reasoner, D. J. and E. E. Geldreich. 1985. A New Medium for the Enumeration and Subculture of Bacteria from Potable Water. Applied and Environmental Microbiology, **49**: 1-7.

Reineke, W. and H. -J. Knackmuss. 1988. Microbial Degradation of Haloaromatics. Annual Reviews of Microbiology, **42**: 263-287.

Reiner, E. A., Chu, J. and E. J. Kirsch. 1978. Microbial Metabolism of Pentachlorophenol, in Pentachlorophenol: Chemistry, Pharmacology, and Environmental Toxicology. K. Ranga Rao (Ed.). Plenum Press, New York and London, pp. 67-81.

Riebel, S., Joshi, D. K. and M. H. Gold. 1994. Purification and Characterisation of a 1,2,4-Trihydroxybenzene 1,2-Dioxygenase from the Basidiomycete *Phanerochaete chrysosporium*. Journal of Bacteriology, **176**: 4838-4844.

Robinson, F. A. 1966. The Vitamin Co-Factors of Enzyme Systems. Pergamon Press, London.

Rodriguez, G. G., Phipps, D., Ishiguro, K. and H. F. Ridgway. 1992. Use of a Fluorescent Redox Probe for Direct Visualization of Actively Respiring Bacteria. Applied and Environmental Microbiology, **58**: 1801-1808.

Rost, F. W. D. 1992. Fluorescence Microscopy V1. University Press, Cambridge.

Rott, B., Nitz, S. and F. Korte. 1979. Microbial Decomposition of Sodium Pentachlorophenolate. Journal of Agricultural and Food Chemistry, **27**: 306-310.

Ruckdeschell, G. and G. Renner. 1986. Effects of Pentachlorophenol and Some of Its Known and Possible Metabolites on Fungi. Applied and Environmental Microbiology, **51**: 1370-1372.

Rutgers, M., Bogte, J. J., Breuer, A. M. and J. G. vanAndel. 1993. Growth and Enrichment of Pentachlorophenol-Degrading Micro-organisms in the Nutristat, a Substrate Concentration Continuous Culture. Applied and Environmental Microbiology, **59**: 3373-3377.

Rutgers, M., Gooch, D. D., Breure, A. M. and J. G. Van Andel. 1996. Assessment of Inhibition Kinetics of the Growth of Strain P5 on Pentachlorophenol Under Steady-State Conditions in a Nutristat. Archives of Microbiology, **165**: 194-200.

Saber, D. L. and R. L. Crawford. 1985. Isolation and Characterisation of *Flavobacterium* Strains That Degrade Pentachlorophenol. Applied and Environmental Microbiology, **50**: 1512-1518.

Sato, K.. 1987. Effect of a Pesticide Pentachlorophenol on Soil Microflora. III. Growth Rates as an Index of Pesticide Resistance of Bacterial Groups Isolated From Soil. Canadian Journal of Microbiology, **33**: 819-822.

Schaule, G., Flemming, H. -C. and H. F. Ridgway. 1993. Use of 5-Cyano-2,3-Ditolyl Tetrazolium Chloride for Quantifying Planktonic and Sessile Respiring Bacteria in Drinking Water. Applied and Environmental Microbiology, **59**: 3850-3857.

Schenk, T., Müller, R., Mörsberger, F., Otto, M. K. and F. Lingens. 1989. Enzymatic Dehalogenation of Pentachlorophenol by Extracts from *Arthrobacter* sp. Strain ATCC 33790. Journal of Bacteriology, **171**: 5487-5491.

Schenk, T., Müller, R., and F. Lingens. 1990. Mechanism of Enzymatic Dehalogenation of PCP by *Arthrobacter* sp. strain ATCC 33790. Journal of Bacteriology, **172**: 7272-7274.

Seech, A. G., Trevors, J. T. and T. L. Bulman. 1990. Biodegradation of Pentachlorophenol in Soil: The Response to Physical, Chemical, and Biological Treatments. Canadian Journal of Microbiology, **37**: 440-444.

Shelton, D. R. and J. M. Tiedje. 1984. Isolation and Partial Characterisation of Bacteria in an Anaerobic Consortium That Mineralises 3-Chlorobenzoic Acid. Applied and Environmental Microbiology, **48**: 840-848.

Shepherd, J. 1997. Unpublished results. Massey University, Palmerston North, New Zealand.

Smith, R. F. 1990. Microscopy and Photomicroscopy: A Working Manual. CRC Press Inc. Boca Raton, Florida, U.S.A.

Smith, A. 1994. Pentachlorophenol Bacterial Biodegradation. Chemical Technology Research Project. Massey University, Palmerston North, New Zealand.

Stanlake, G. J. and R. K. Finn. 1982. Isolation and Characterisation of a Pentachlorophenol-Degrading Bacterium. Applied and Environmental Microbiology, **44**: 1421-1427.

Steiert, J. G., Pignatello, J. J. and R. L. Crawford. 1987. Degradation of Chlorinated Phenols by a Pentachlorophenol-Degrading Bacterium. Applied and Environmental Microbiology, **53**: 907-910.

Szabo, M.. 1993. New Zealand's Poisoned Paradise: Toxic Waste has Seeped into New Zealand's Soil and Water, Tarnishing the Country's Pristine Image. New Scientist, **139**: 29.

Tchan, Y-T. and P. B. New. 1984. Genus I. *Azotobacter* Beijerinck 1901, 567^{AL}, in Bergey's Manual of Systematic Bacteriology. Editor in Chief - Holt, J. G.. Williams and Wilkins. Baltimore/ London. Volume 1.

Tikoo, V., Scragg, A. H. and S. W. Shales. 1997. Degradation of Pentachlorophenol by Microalgae. Journal of Chemical Technology and Biotechnology, **68**: 425-431.

Toivari, J., Wirtanen, G. and T. Mattila-Sandholm. 1996. Metabolic Indicators in the Assessment of Biofilms on Equipment Surfaces. American Society for Microbiology. Conference on Microbial Biofilms.

Topp, E., Crawford, R. L. and R. S. Hanson. 1988. Influence of Readily Metabolizable Carbon on Pentachlorophenol Metabolism by a Pentachlorophenol-Degrading *Flavobacterium* sp.. Applied and Environmental Microbiology, **54**: 2452-2459.

Topp, E. and R. S. Hanson. 1990. Factors Influencing the Survival and Activity of a Pentachlorophenol-Degrading *Flavobacterium* sp. in Soil Slurries. Canadian Journal of Soil Science, **70**: 83-91.

Uotila, J. S., Apajalahti, J. H. A. and M. S. Salkinoja-Salonen. 1991. Dechlorination of Pentachlorophenol by Membrane Bound Enzymes of *Rhodococcus chlorophenolicus* PCP-1. Biodegradation, **2**: 25-31.

Uotila, J. S., Kitunen, V. H., Saastamoinen, T., Coote, T., Häggblom, M. M. and M. S. Salkinoja-Salonen. 1992. Characterisation of Aromatic Dehalogenases of *Mycobacterium fortuitum* CG-2. Journal of Bacteriology, **174**: 5669-5675.

Uotila, J. S., Kitunen, V. H., Coote, T., Saastamoinen, T., M. S. Salkinoja-Salonen and J. H. A. Apajalahti. 1995. Metabolism of Halohydroquinones in *Rhodococcus chlorophenolicus* PCP-1. Biodegradation, **6**: 119-126.

Utkin, I., Woese, C. and J. Wiegel. 1994. Isolation and Characterisation of *Desulfitobacterium dehalogenans* gen. nov.. sp. nov., an Anaerobic Bacterium Which Reductively Dechlorinates Chlorophenolic Compounds. International Journal of Systematic Bacteriology, **44**: 612- 619.

Valo, R., Apajalahti, J. and M. Salkinoja-Salonen. 1985. Studies on the Physiology of Microbial Degradation of Pentachlorophenol. Applied Microbiology and Technology, **21**: 313-319.

Valo, R. and M. Salkinoja-Salonen. 1986. Bioreclamation of Chlorophenol-Contaminated Soil by Composting. Applied Microbiology and Biotechnology, **25**: 68-75.

Van Rhee, A. B.. 1996. Biodegradation of Pentachlorophenol in Soil. Department of Process and Environmental Technology, Massey University, Palmerston North, New Zealand.

Vollmer, M. D., Stadler-Fritzche, K. and M. Schlömann. 1993. Conversion of 2-Chloromaleyacetate in *Alcaligenes eutrophus* JMP134. Archives of Microbiology, **159**: 182-188.

Walker, P. M. B. 1988. Chambers Science and Technology Dictionary. W & R Chambers Ltd. and Cambridge University Press, Cambridge.

Walker, G. M. 1994. The Roles of Magnesium in Biotechnology. Critical Reviews in Biotechnology, **14**: 311-354.

Watanabe, I. 1973. Isolation of Pentachlorophenol Decomposing Bacteria from Soil. Soil Science and Plant Nutrition, **19**: 109-116.

Wayne, L. G. and G. P. Kubica. 1984. Genus *Mycobacterium* Lehmann and Neumann 1896, 363^{AL}, in Bergey's Manual of Systematic Bacteriology. Editor in Chief - Holt, J. G.. Williams and Wilkins. Baltimore/ London. Volume 2.

White, D. C., Sutton, S. D. and D. R. Ringelberg. 1996. The Genus *Sphingomonas*: Physiology and Ecology. Current Opinion in Biotechnology, **7**: 301-306.

World Health Organisation (WHO). 1989. Pentachlorophenol Health and Safety Guide No. 19. WHO.

Xun, L. 1996. Purification and Characterisation of Chlorophenol 4-Monooxygenase from *Burkholderia cepacia* AC1100. Journal of Bacteriology, **178**: 2645-2649.

Xun, L. and C. S. Orser. 1991. Purification and Properties of Pentachlorophenol Hydroxylase, a Flavoprotein from *Flavobacterium* sp. Strain ATCC 39723. Journal of Bacteriology, **173**: 4447-4453.

Xun, L., Topp, E. and C. S. Orser. 1992a. Diverse Substrate Range of a *Flavobacterium* Pentachlorophenol Hydroxylase and Reaction Stoichiometries. Journal of Bacteriology, **174**: 2898-2902.

Xun, L., Topp, E. and C. S. Orser. 1992b. Confirmation of Oxidative Dehalogenation of Pentachlorophenol by a *Flavobacterium* Pentachlorophenol Hydroxylase. Journal of Bacteriology, **174**: 5745-5747.

Xun, L., Topp, E. and C. S. Orser. 1992c. Purification and Characterisation of a Tetrachloro-*p*-Hydroquinone Reductive Dehalogenase from a *Flavobacterium* sp.. Journal of Bacteriology, **174**: 8003-8007.

Xun, L. 1996. Purification and Characterisation of Chlorophenol 4-monooxygenase from *Burkholderia cepacia* AC1100. Journal of Bacteriology, **174**: 2645-2649.

Yu, P-L. 1997. Personal Communication. Massey University, Palmerston North, New Zealand.

Yu, J. and O. P. Ward. 1994. Studies on Factors Influencing the Biodegradation of Pentachlorophenol by a Mixed Bacterial Culture. Elsevier Science Limited. International Biodeterioration and Biodegradation, 209-221.

Yu, J. and O. P. Ward. 1996. Investigation of the Biodegradation of Pentachlorophenol by the Predominant Bacterial Strains in a Mixed Culture. Elsevier Science Limited. International Biodeterioration and Biodegradation, 181-187.

Yu, W., Dodds, W. K., Banks, M. K., Skalsky, J. and E. A. Strauss. 1995. Optimal Staining and Sample Storage Time for Direct Microscopic Enumeration of Total and Active Bacteria in Soil with Two Fluorescent Dyes. Applied and Environmental Microbiology, **61**: 3367-3372.

Yu, P-L., Heap, R. and H. H. Hussein. 1996. Growth and Plasmid Characterisation of Pentachlorophenol-Degrading *Pseudomonas* Soil Isolates, in "International Environmental Biotechnology". Massey University, Palmerston North, New Zealand, pp. 210-215.

Zaborina, O., Latus, M., Eberspächer, J., Golovleva, L. A. and F. Lingens. 1995. Purification and Characterisation of 6-Chlorohydroxyquinol 1,2-Dioxygenase from *Streptomyces rochei* 303: Comparison with an Analogous Enzyme from *Azotobacter* sp. Strain GP1. Journal of Bacteriology, **177**: 229-234.

Zeng, Y.. 26 September, 1996. BBDMaster@simvax.labmed.umn.edu (http://dragon.labmed.umn.edu/~lynda/pcp/pcp_map.html)

Zimmerman, R., Iturriaga, R. and J. Becker-Birck. 1978. Simultaneous Determination of the Total Number of Aquatic Bacteria and the Number Thereof Involved in Respiration. Applied and Environmental Microbiology, **36**: 926-935.

A8. APPENDIX

A8.1 FLUORESCENCE MICROSCOPY STAINING TECHNIQUES

Quantification of bacterial numbers and biomass is important in understanding the ecological role of bacteria in any environment (Kepner and Pratt, 1994). Bacteria have been enumerated in several ways, with many of the same techniques being applied in diverse systems (Kepner and Pratt, 1994). There are five different methods to enumerate microorganisms: (i) direct counts by microscope with stains specific to biological molecules to enumerate total and active bacteria; (ii) colony counts using diluents, spread, layered and pour plates; (iii) most probable number techniques; (iv) biomass measurement using wet or dry weights or densities, water content and volume; and (v) light scattering or turbidimetry (Gerhardt *et al.*, 1994), particle counts using electronic detection e.g. Coulter Particle Counter.

Epifluorescence microscopy was the method chosen to enumerate bacteria in this study of PCP degrading organisms because enumeration of the total and active bacteria on different concentrations of PCP was to be studied. Total numbers were enumerated using epifluorescence microscopy as the bacteria were less easily seen under a brightfield microscope. Growth of the culture on PCP and nutrient agar was slow and direct counts under brightfield microscope have been shown to significantly underestimate numbers (Kepner and Pratt, 1994). The use of a spectrophotometer as a method to ascertain cell suspension density was of no use as culture optical density reached a maximum of 0.04, at 600nm, at the exhaustion of the carbon source.

A8.1.1 DIRECT EPIFLUORESCENCE MICROSCOPY

Epifluorescence microscopy allows a direct-count method that involves staining the microorganisms in a sample with a fluorescent dye, termed a fluorochrome, collecting the cells on a membrane filter, followed by counting using an epifluorescence microscope (Hobbie *et al.*, 1977; Herbert, 1990). In fluorescence microscopy short

wavelength regions of the spectrum, ranging from the invisible (to humans) to the green portion of the spectrum, are used to excite dyes, fluorochromes, or naturally fluorescent specimens into self-luminous objects (Smith, 1990). Epifluorescence microscopy has a number of advantages over other forms of microscopy, offering high sensitivity and specificity. Different fluorochrome dyes are available to stain bacterial cells in contrasting colours according to the cells' metabolic activity (Langfeldt, 1997). Epifluorescence microscopy is a reflected light system where the objective lens serves as it's own condenser (Smith, 1990). In an epifluorescence microscope the specimen is illuminated with light of a short wavelength, e.g. ultraviolet or blue. Part of this light is absorbed by the specimen and re-emitted as fluorescence. The light used for excitation is filtered out by a barrier filter between the specimen and the observer's eye. This filter, in principle, should be fully opaque at the wavelength used for excitation and fully transparent at longer wavelengths so as to transmit the fluorescence (Rost, 1992). The fluorescent object is seen as a bright image on a dark background (Rost, 1992).

Epifluorescent staining methods are considerably more rapid than traditional cultivation methods. Moreover, the number of viable cells may be underestimated with plate counts due to the viable but nonculturable state of bacteria caused by cell stress or injury (Toivari *et al.*, 1996). Direct counts are used in studies of bacterial population densities rather than traditional spread plate methods which have been shown to significantly underestimate numbers (Kepner and Pratt, 1994). In epifluorescence microscopy both viable and total bacterial numbers are estimated from the enumeration result derived by the usage of different fluorescent dyes (Langfeldt, 1997).

Several dyes have been used to enumerate total bacterial numbers with epifluorescent direct counting (Yu *et al.*, 1995). An advantage of fluorescence microscopic methodology is the opportunity it provides to observe smaller amounts of colour than is usually possible with transmitted light microscopy and consequently it is possible to visualise both small specimens and specimens on opaque surfaces, attributes not commonly available in bright field microscopy (Langfeldt, 1997). Furthermore, the optical responses of fluorescent dyes are much more readily detected than those of nonfluorescent dyes (Schaule *et al.*, 1993).

There are several precautions that can be identified if reproducible and meaningful results are to be obtained from epifluorescent direct counts. The examiner has to have experience in the examination of bacteria using microscopes (Bergstrom, 1986; Kepner and Pratt, 1994). Small cells are masked by larger ones particularly in mixed cultures and because of the higher content of dye in the larger cells will exhibit brighter fluorescence (Bergstrom *et al.*, 1986). Small cells fade relatively faster than the larger cells due to the lower concentration of dye in the smaller cells. The numbers of organisms in the sample will be extrapolated from the aliquot, hence, the accuracy of the aliquot volume is critically important. Cell clumping and floc formation are problems because small aliquots are examined it is important that a representative and homogeneous sample is used. The number of bacteria in the sample is estimated by visual count which is multiplied by a factor of about 10^5, depending on the system used. This multiplication factor adds a further five orders of magnitude of error to the result. Together with the error associated with the dilution factor, the total error associated with this and any other method of microscopic counting can be very large.

Frequent verification of the method over a range of counts in combination with control, blanks and replicates seems necessary to determine any possible deviation in the order of magnitude of error for a particular set of assays. The total volume of liquid sample filtered through the membrane is also important, as it affects the evenness of distribution of bacteria on membrane surfaces. In addition to appropriate cell dispersion, a minimum volume of 2ml is recommended for the most commonly used 25mm-diameter membrane filters. Samples are easily diluted with particle-free (0.2μm pore size filtered) water prior to staining and filtration. However the volume of acridine orange (AO) solution volumes used to flood the specimen should be adjusted accordingly to maintain an adequate stain concentration (Kepner and Pratt, 1994).

Rapid direct methods of enumerating respiring organisms in an environmental sample are developing continually. Pyle *et al.* (1995) have developed a rapid, direct filtration-incubation procedure which facilitates the assessment of respiratory activity concurrently with the detection of specific bacteria in water and wastewater. A fluorescent viability probe has been tested to ascertain the efficacy of cleansing procedures in a meat works (Pyle *et al.*, 1995). In the images produced using viability

probes, viable cells emit green fluorescence and nonviable cells emit red fluorescence (Korber *et al.*, 1997). Studies on the *Bac*Light probe in soil system biofilms indicated that there is an underestimation of viable counts (Korber *et al.*, 1997).

A8.1.2 FLUORESCENT DYES

A8.1.2.1 TOTAL BACTERIAL COUNTS

Several dyes have been used to enumerate total bacterial numbers (Kodak, 1990; Kepner and Pratt, 1994). The two fluorescent dyes, or fluorochromes, most often used in direct counting are 9-isothiocyanatoacridine (acridine orange, AO) and 4',6-diamidino-2-phenylindole (DAPI). With these fluorochromes bacteria are identified on the basis not only of colour but also of size and shape (Kepner and Pratt, 1994). A major breakthrough in the development of epifluorescent counts was the first use of polycarbonate Nucleopore membrane filters, prestained with irgalan black (IB) to reduce background autofluorescence (Hobbie *et al.*, 1977). These filters were not always available during the course of this study.

The Acridine Orange (AO) direct count procedure is a popular method for determining microbial numbers in environmental samples via epifluorescence microscopy (Bergstr m *et al.*, 1986; Bitton *et al.*, 1993; Kepner and Pratt, 1994). Acridine Orange (AO) binds to both DNA and RNA. The excitation maximum for the dye is approximately 470nm and emits green fluorescence when bound to double-stranded nucleic acids and red fluorescence when bound to single-stranded nucleic acids. This property has been exploited in methods for simultaneously analyzing the DNA and RNA content of a cell culture (Haughland, 1996).

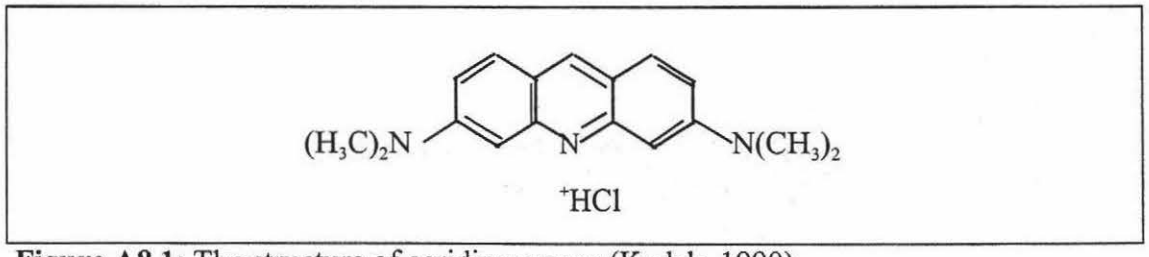

Figure A8.1: The structure of acridine orange (Kodak, 1990).

At low concentrations of AO, bacteria growing at high growth rates will fluoresce red-orange because of the predominance of cellular RNA while moribund bacteria have mostly DNA and will fluoresce green (Hobbie *et al.*, 1977; Yu *et al.*, 1995). AO direct counting is only a crude indicator of metabolic activity of natural and undefined bacterial communities because the RNA/DNA ratio in a cell and thus the AO colour reaction could be affected by growth media, AO staining concentration and procedure, and the method of cell fixation (if any), as well as the organism's taxonomy (Yu *et al.*, 1995).

The level of moisture on the membrane also changes the percentage of green and red fluorescing cells (Bitton *et al.*, 1993). Since the level of moisture can vary across the filter and is difficult to control, the use of colour as a basis for inferring cell activity is not justified (Bitton *et al.*, 1993). The distribution of dead, metabolically inactive but living (moribund), and actively respiring cells cannot be determined by the standard technique of either AO or DAPI staining because DNA retains its staining properties even in nonviable cells (Kepner and Pratt, 1994). DAPI binds to the minor groove of DNA at AT-rich sequences (Haughland, 1996) and is rapidly replacing AO as the bacterial stain of choice (Kepner and Pratt, 1994). DAPI is a non-intercalating, DNA-specific stain which at or above 390nm fluoresces blue or bluish-white (Kepner and Pratt, 1994). DAPI counts have been shown to underestimate those obtained with AO, averaging 70% of the AO total (Kepner and Pratt, 1994).

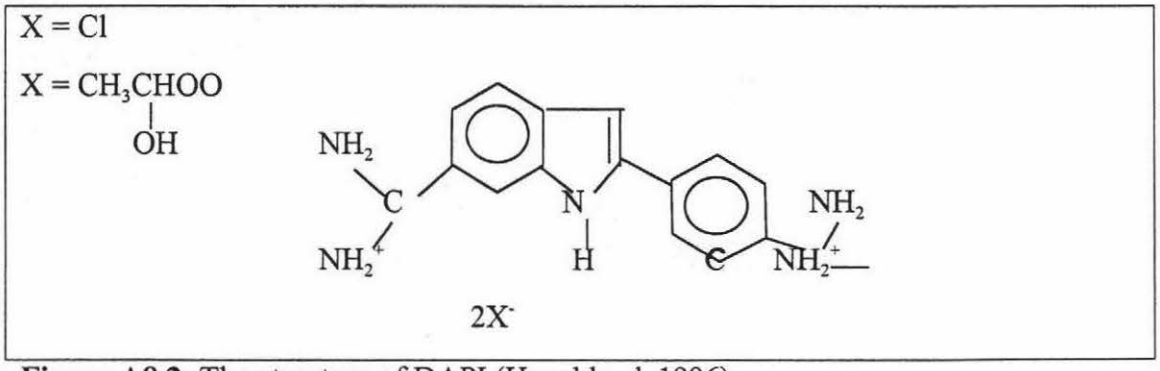

Figure A8.2: The structure of DAPI (Haughland, 1996).

The above limitations notwithstanding, epifluorescence microscopy offers methods for enumerating both active and total cells. This is the major advantage, in being able to compare results derived by one principal method and this may outweigh disadvantages

generally associated with using microscopic enumeration techniques alone (Langfeldt, 1997).

A8.1.2.2 METABOLICALLY ACTIVE BACTERIAL COUNTS

Enumeration of metabolically active bacteria in environmental samples is frequently required to estimate ecosystem productivity, biomass turnover, or substrate utilisation potentials (Rodriguez *et al.*, 1992). Several methods have been established for enumeration of active bacteria (Kogure *et al.*, 1979; Zimmerman *et al.*, 1978; Rodriguez *et al.*, 1992). Respiratory activity measured by reduction of tetrazolium salts has become a popular criterion for determining the viability of bacterial cells (Langfeldt, 1997). The INT [2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride] and CTC (5-cyano-2,3-ditolyl tetrazolium chloride) staining techniques are currently popular (Rodriguez *et al.*, 1993; Schaule *et al.*, 1993; Pyle *et al.*, 1995; Yu *et al.*, 1995).

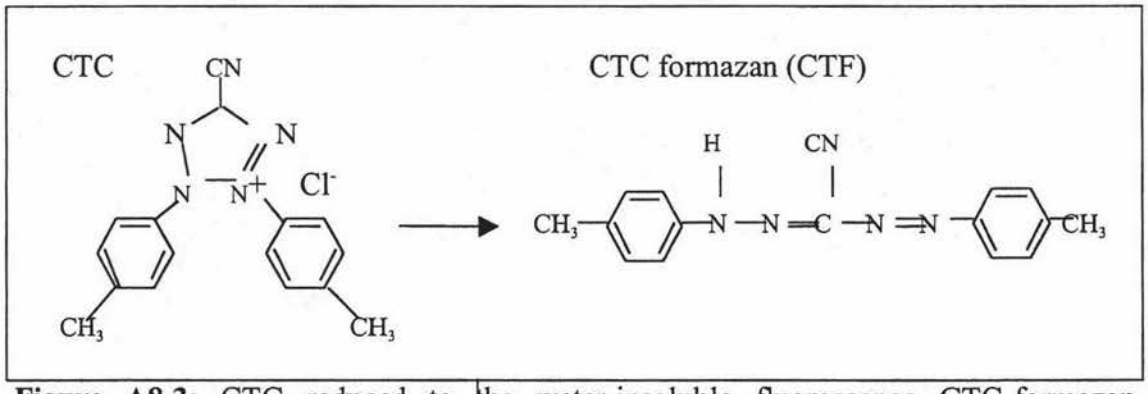

Figure A8.3: CTC reduced to the water-insoluble fluorescence CTC-formazan (Haughland, 1996).

CTC was used in this study. It is readily reduced to insoluble, highly fluorescent, and intracellularly accumulated CTC-formazan through bacterial respiration. Water-soluble CTC is transported inside the cell where it is readily reduced to the water-insoluble fluorescent CTC-formazan by dehydrogenases associated with the microbial electron transport system and thus indicates respiratory activity (Rodriguez *et al.*, 1992). Fluorescence emission of CTC-formazan is primarily in the red region, with excitation at 450nm and emission at 630nm (Yu *et al.*, 1995). Thus, actively respiring bacteria

can be distinguished from nonrespiring bacteria and abiotic material that typically emit in the blue or blue-green regions (490nm).

Also, INT can be reduced to insoluble INT-formazan crystals by the electron transport system of respiring organisms and observed microscopically by bright-field microscopy as an opaque red intracellular deposit (Yu *et al.*, 1995). Intracellular deposition of the CTC-formazan appears to be similar or identical to the deposition of INT-formazan however, CTC-formazan may be more stable and because of its red fluorescence is more easily detected than INT-formazan (though CTC-formazan still fades in UV light). The CTC technique is thus superior to INT staining (Yu *et al.*, 1995). The fraction of respiring bacteria in the total population is variable and unpredictable, depending on the particular sampling environment. Staining of cells with CTC (Yu *et al.*,1995) and enumeration under fluorescence microscope allows the determination of the number of active bacteria within the sample. The extent to which the determination of actively respiring and total bacteria reflects reality in the ecosystem is dependent on the staining technique and physicochemical characteristics, as well as individual investigator's biases (Kepner and Pratt, 1994).

The CTC method of fluorescence staining was used in this study to ascertain whether any respiratory activity was occurring when micro-organisms were subjected to a toxic compound as a sole source of carbon and energy. The CTC method of fluorescence staining has been suggested as providing a convenient and rapid approach for quantifying the effect of a biocide in a given system and may also be applicable to monitoring inhibitory effects in bioreactors and activated sludge treatment processes (Schaule *et al.*, 1993).

Carbon supplementation (with R2A medium or glucose) of environmental samples seems to be required to maximise the number of bacteria that can actively reduce CTC in the selected time frame (Rodriguez *et al.*, 1992). The enhancement effect of nutrient supplemented CTC microscopic counts was compared with the R2A plate count method. The direct microscopic count yielded a result 4 orders of magnitude higher than the plate count thus providing a more sensitive indicator of actively respiring bacteria (Rodriguez *et al.*, 1992). Nutrient supplementation is one of the most critical

aspects in the CTC staining method and its influence on the results can not be clearly defined. There may be a minor population present that does not degrade the inhibitor but readily metabolises the additional nutrients supplied as R2A medium so unwanted growth of cells at this step is clearly possible. To avoid preferential stimulation of inactive bacteria when enumeration of active bacteria in a population is desired, there should be no nutrient supplementation (Yu *et al.*, 1995).

Counterstaining of CTC-treated samples with either AO or DAPI allows enumeration of active and total bacterial subpopulations within the same preparation (Rodriguez *et al.*, 1992; Kepner and Pratt, 1994).

A8.2 CTC VALIDATION EXPERIMENT

The redox dye CTC was synthesised by Assoc. Prof. R. Chong at the Massey University Institute of Technology and Engineering (formerly the Process and Environmental Technology Department). The synthesised CTC was compared with a commercial preparation and validated by Dr. R. Chong.

Both the commercial preparation and the synthesised CTC were placed on a slide and reduced by adding sodium dithionite. Both slides fluoresced red under UV light at 630nm thereby validating the synthesised CTC. The red fluorescence indicated that the CTC was being reduced to CTC-formazan thereby validating the use of the synthesized CTC.

A8.2.1 CTC STAINING

CTC staining parameters were tested and supplementary nutrients added to the staining media as described in the Methods Section 3.5.2.3. A combination of parameters were tested to optimise CTC staining of moribund and actively respiring cells. Due to technical difficulties no cell enumeration results were obtained from testing the various parameters although the best staining procedure was determined. The CTC staining was to be used in the enumeration of actively respiring cells when grown on a toxic carbon and energy source, namely PCP. This in turn would have provided a ratio of active cells to moribund/dead cells for each initial PCP concentration tested. The specifications of the filters on the Olympus microscope were correct for visualisation of the reduced CTC-formazan however in practice this was not the case. The initial staining method was validated when tested on another microscope and the parameters to optimise the staining procedure were determined. Slides of the AO/CTC stained cells were stored in the dark for 2 months. The accuracy of the slides for enumeration purposes after this period was not determined, however moribund cells were clearly visible. For the enumeration of total cells the method of AO staining was used successfully as described in the Methods, Section 3.5.1. The sequential AO/CTC staining method is described in the Methods Section 3.5.4. It would be highly

advantageous to utilise the AO/CTC staining for micro-organisms grown on an inhibitory substrate. The method would give great insight into the cellular activity during growth and provide a clearer picture of how micro-organisms, in pure culture and consortia, react to inhibitory substrates.

A8.3 MINERALS AND VITAMINS

Degradation of PCP by the isolates in pure culture and in combination was tested in media containing various vitamin supplementation to determine which vitamin solution and then which specific vitamin would allow the isolates to grow and degrade PCP without yeast extract. The functions of the various vitamins and minerals tested in the experiment are described below

A8.3.1 MINERALS

The cations potassium (K^+) and magnesium (Mg^{2+}) are bulk intracellular ionic species, while sodium (Na^+) and calcium (Ca^{2+}) are essential metals, although they are present only at trace or ultratrace levels (Hughes and Poole, 1989). These cations are known to be involved in the stabilization of a range of biological structures, from cell walls to protein conformations. They are often highly effective catalysts of a range of diverse biochemical processes and in some cases are able to trigger, moderate or inhibit metabolic reactions (Hughes and Poole, 1989).

A small number of enzymes, have a specific requirement for sodium cations. Little is known of the function of Na^+ but it is possible that Na^+ binds to enzymes and controls their conformation (Hughes and Poole, 1989).

Many enzymes are specifically activated by the potassium cation (K^+). The potassium cation K^+ activates the enzymes involved in protein synthesis, other biosynthetic enzymes and catabolic enzymes, although very little is known about the precise mechanisms by which K^+ exerts these catalytic effects (Hughes and Poole, 1989).

As a direct nutrient, Mg^{2+} is absolutely essential for maintaining functions of all organisms (Walker, 1994). Furthermore, the strict Mg^{2+} dependence of phosphate transfer enzymes, particularly those involving ATP, and of enzymes involving the synthesis, expression, and translation of genetic information serves to highlight the biological essentiality of this metal (Walker, 1994). The physiological role of Mg^{2+} is primarily intracellular, where it serves as a cofactor for over 300 enzymes, maintains conformation of nucleic acids, stabilizes ribosomes and generally maintains the structural integrity of membranes in cells and organelles (Walker, 1994). Magnesium is involved in almost every metabolic and bioenergetic pathway in the cell. In contrast to calcium which activates many cellular processes, Mg^{2+} requires no intermediary to exert its coordinate control but can produce the broad metabolic and growth effects based on its own intrinsic properties. Calcium is recognized as a metabolic regulatory cation (Walker, 1994).

Magnesium and potassium are regarded as "bulk" cations in establishing the required ionic environment of the cell. Their uptake is strongly influenced by cell growth rate. Their absolute concentrations, as well as their relative ratio, play an important role in intracellular structure maintenance and in enzyme action. The primary cellular functions of potassium are in the control of the osmotic balance of the cell and as a charge compensator for small anions (Walker, 1994).

The interactions between Ca^{2+} and Mg^{2+} are complex and one proposal to explain biological antagonism is that Ca^{2+} outperforms Mg^{2+} in binding to ATP and nucleic acids because of its stronger affinity. It has also been suggested that one of the major biological functions of Mg^{2+} is to modulate the binding and activity of Ca^{2+} (Walker, 1994). It should be remembered that cells actively exclude Ca^{2+} (and Na^{+}) and actively include Mg^{2+} (and K^{+}) (Walker, 1994).

A8.3.2 VITAMINS

Vitamins are organic substances required in relatively small amounts by both eukaryotic and prokaryotic cells for the proper functioning of the organism (Walker, 1988). Presented below are some of the key features of the vitamins used to

supplement the minimal mineral salts medium for the growth of ET01, ET02 and ET03. Full reviews of the vitamins and their functions can be found in Robinson (1966) and Koser (1968).

Thiamine (Vitamin B_1) is effective as a growth promoting agent. It is made up of pyrimidine and thiazole and functions as a coenzyme of various enzymes as thiamine pyrophosphate (Koser, 1968).

Riboflavin (Vitamin B_2) forms part of the prosthetic group of flavoproteins (Walker, 1988) and is important in nutrition of microorganisms as a growth factor (Koser, 1968). There are two coenzyme forms of riboflavin, riboflavin-5'-phosphate and flavin adenine dinucleotide. Enzymes containing the coenzymes of riboflavin serve as one of the links in the oxidation of many metabolic products of carbohydrate and protein (Koser, 1968).

Nicotinic acid (Niacin) is a component of the coenzyme nicotinamide adenine dinucleotide (NAD) and the chemically related coenzyme nicotinamide adenine dinucleotide phosphate (NADP) (Koser, 1968). Nicotinic acid serves as a growth factor for bacteria and NAD and NADP are essential for hydrogen and electron transport in the process of biological oxidation (Koser, 1968).

Pyridoxal is one of the three compounds, and their phosphates, in the vitamin B_6 group (Koser, 1968). Pyridoxal phosphate acts as a coenzyme for transaminases (Walker, 1988).

Pantothenic acid is a carboxylic acid and it's functional form is coenzyme A (CoA) (Koser, 1968). Both moieties of the pantothenic acid molecule function as growth factors, β-alanine for some microorganisms and pantoic acid for others (Robinson, 1966). For some microorganisms a part of the pantothenic acid molecule suffices for growth while other microorganisms must be supplied the whole pantothenic acid molecule (Koser, 1968).

Biotin is a member of the vitamin B complex and an important growth factor for a number of microorganisms (Robinson, 1966). It is a component of yeast extract and a carboxylic acid. The gamut of activities attributed to biotin include some aspects of the metabolism of carbohydrate, amino acids, protein, nucleic acids or their components, and lipids (Koser, 1968). Biotin is also the prosthetic group of the enzyme carboxylase (Walker, 1988).

Cyanocobalamin is a permissive name for vitamin B_{12} (Koser, 1968) and is a growth promoter for a variety of microorganisms. Cyanocobalamin is involved in a number of enzymatic reactions (Koser, 1968) and takes part in enzymatic interconversions of acyl CoA and methylations (Walker, 1988).

Cysteine is an amino acid found in proteins. It is one of the 20 basic organic chemical units from which proteins and peptides are synthesized.

p-Aminobenzoic acid (PABA) is utilised for biosynthesis of folic acid, donor of 1-carbon fragments for several biosyntheses (Walker, 1988), and is also highly active as a growth factor (Koser, 1968). PABA also participates in the synthesis of cyanocobalamin and pantothenic acid (Koser, 1968).

A8.4 INITIAL PCP REMOVAL REGRESSION EQUATIONS

PCP removal rates have been calculated using linear regression from the graphs in the Results Section 4.2, Fig 4.1. The rates are the maximum rates of PCP removal for a given experiment and were based on the steepest part of the PCP removal curve. The high R-squared values indicate the significance of a linear relationship between the two parameters. The possibility of using these equations as a predictive tool has not been explored.

Table A8.1: Regression equations for PCP removal at various initial PCP concentrations with and without 25mg/l added yeast extract.

Initial PCP Concentration (mg/l)	Regression Equation	PCP Degradation Rate (mg/l/hr)*	R-Squared value (%)	N (no. of points)
With 25mg/l YE				
20	PCP = 26.0 – 0.16 hours	0.16 ± 0.05	98.8	4
60	PCP = 148 – 1.22 hours	1.22 ± 0.57	99.0	3
137	PCP = 209 – 1.10 hours	1.10 ± 0.08	91.8	5
Without 25mg/l YE				
20	PCP = 16.8 – 0.24 hours	0.24 ± 0.08	99.3	4
60	PCP = 80.3 – 0.80 hours	0.80 ± 0.08	96.7	5
137	PCP = 139 – 0.01 hours	0.01 ± 0.00	90.2	10

* These are ± the 95% confidence intervals.

The regression equations presented in Table A8.2 below are also from initial PCP removal experiments though at a greater range of initial PCP concentrations. The PCP removal rate has been calculated as per the method described above.

Table A8.2: Regression equations for PCP removal at various initial PCP concentrations with and without 25mg/l added yeast extract.

Initial PCP Concentration (mg/l)	Regression Equation	PCP Degradation Rate (mg/l/hr)*	R-Squared value (%)	N (no. of points)
Without 25mg/l YE				
20	PCP = 17.6 – 0.086 hours	0.086 ± 0.011	97.9	8
40	PCP = 39.4 – 0.078 hours	0.078 ± 0.018	94.7	8
60	PCP = 60.0 – 0.086 hours	0.086 ± 0.005	99.9	5
80	PCP = 74.4 – 0.044 hours	0.044 ± 0.029	92.2	4
115	PCP = 115 – 0.051 hours	0.051 ± 0.004	99.1	10
With 25mg/l YE				
20	PCP = 21.5 – 0.442 hours	0.442 ± 0.129	99.5	3
40	PCP = 40.0 – 0.575 hours	0.575 ± 0.371	92.4	4
60	PCP = 99.2 – 1.021 hours	1.021 ± 0.430	99.0	3
80	PCP = 112 – 1.358 hours	1.358 ± 1.175	96.1	3
115	PCP = 189 – 1.375 hours	1.375 ± 0.243	99.8	3

* These are ± the 95% confidence intervals.

A8.5 PCP REMOVAL AND CHLORIDE RELEASE

The following graphs relate to Results Section 4.3 in which PCP removal rates and chloride release rates for ET01 have been calculated from these graphs and presented in Table 4.6. The theoretical chloride release is indicated on the left axis of each graph by the horizontal line (,).

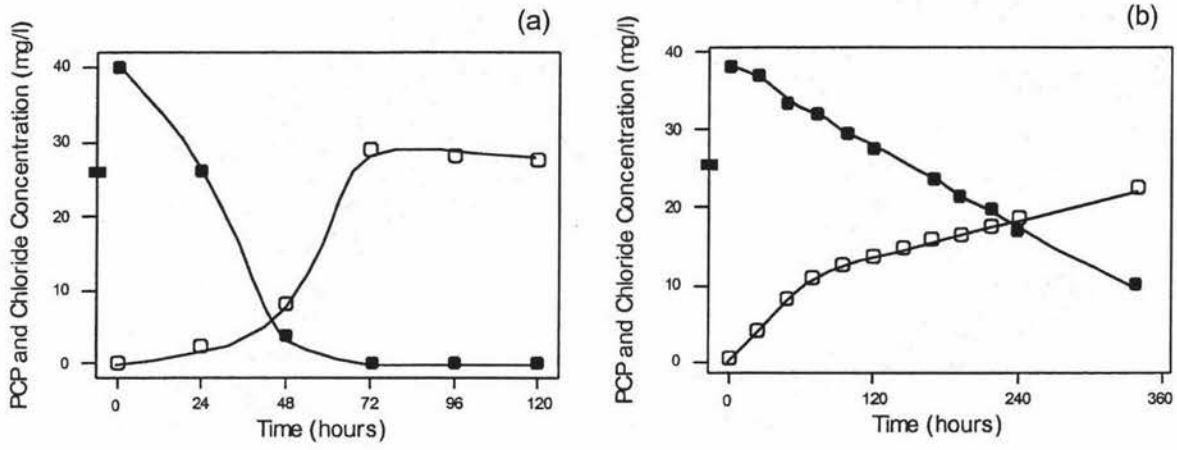

Figure A8.4: Removal of PCP (■) at an initial concentration of 40mg/l and concomitant chloride release () (a) with 25mg/l yeast extract added; (b) without 25mg/l yeast extract added. Theoretical chloride release from 40mg/l PCP is 26.8mg/l (,).

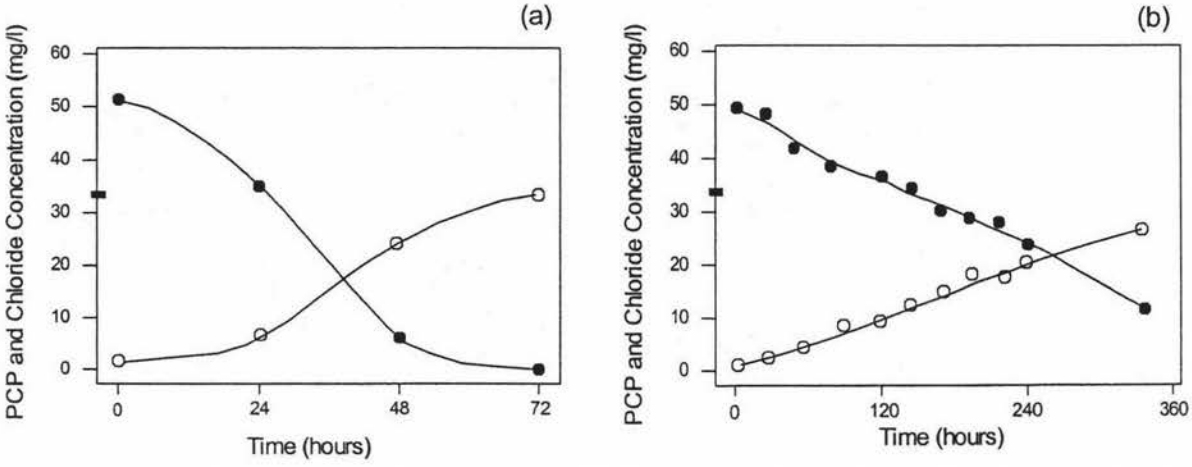

Figure A8.5: Removal of PCP (■) at an initial concentration of 50mg/l and concomitant chloride release ( ) (a) with 25mg/l yeast extract added; (b) without 25mg/l yeast extract added. Theoretical chloride release from 50mg/l PCP is 33.5mg/l (,).

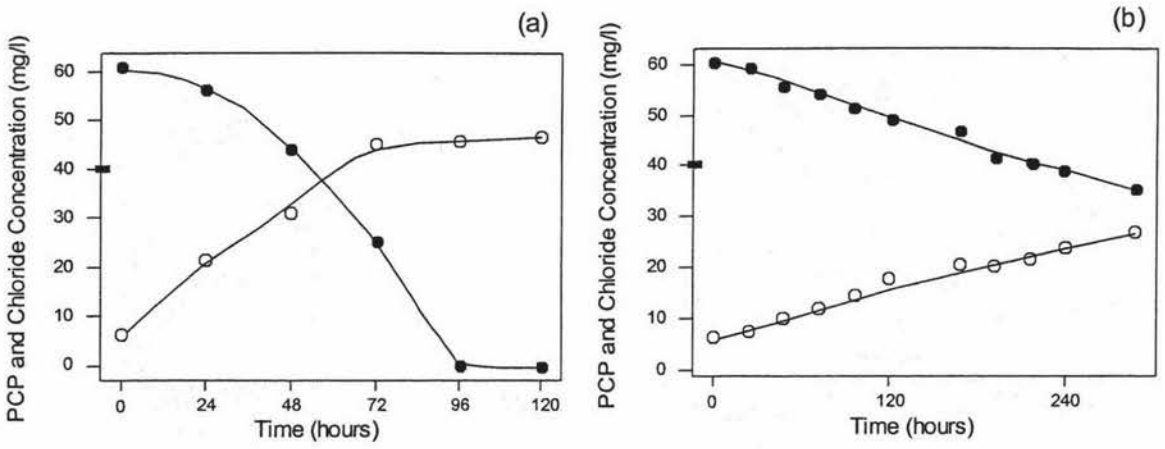

Figure A8.6: Removal of PCP (■) at an initial concentration of 60mg/l and concomitant chloride release () (a) with 25mg/l yeast extract added; (b) without 25mg/l yeast extract added. Theoretical chloride release from 60mg/l PCP is 40.2mg/l (,).

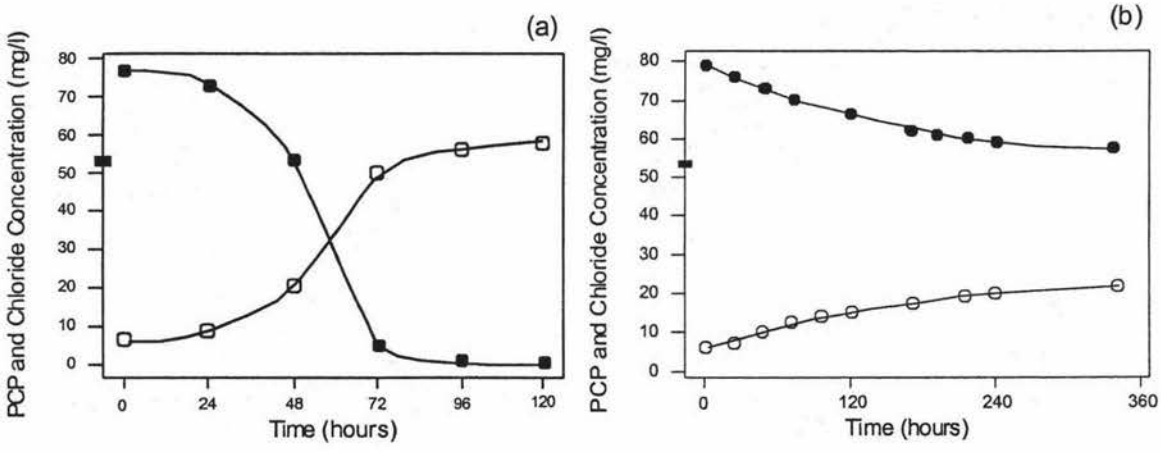

Figure A8.7: Removal of PCP (■) at an initial concentration of 80mg/l and concomitant chloride release () (a) with 25mg/l yeast extract added; (b) without 25mg/l yeast extract added. Theoretical chloride release from 80mg/l PCP is 53.6mg/l (,).

The following graphs (Figs. A8.8, A8.9, A8.10 & A8.11) only show media containing 25mg/l supplementary yeast extract. The removal rates without yeast extract were very slow at all PCP concentrations. Removal ceased at 175mg/l in media containing yeast extract as shown in Figure A8.11.

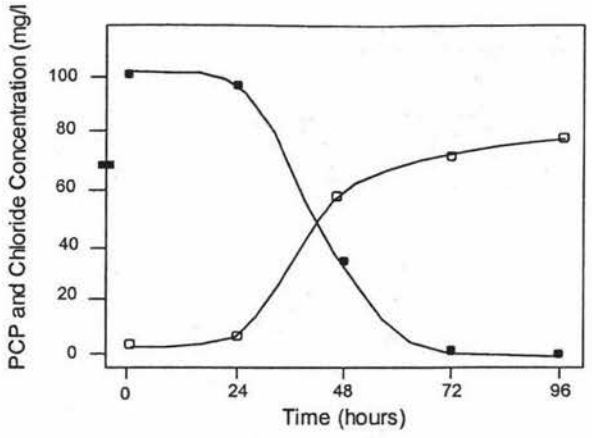

Figure A8.8: Removal of PCP (■) and concomitant release of chloride () at an initial concentration of 100mg/l PCP. Theoretical chloride release from 100mg/l PCP is 67.7mg/l (,).

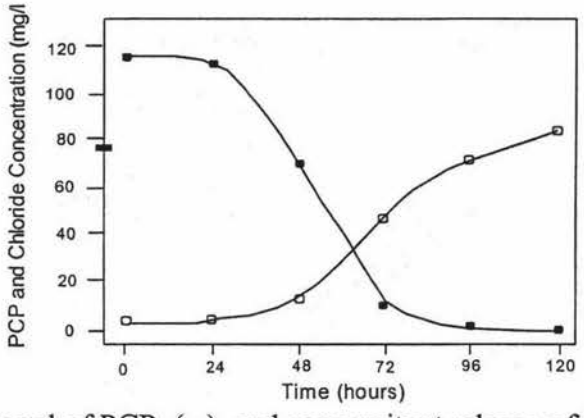

Figure A8.9: Removal of PCP (■) and concomitant release of chloride () at an initial concentration of 120mg/l PCP. Theoretical chloride release from 120mg/l PCP is 80.4mg/l (,).

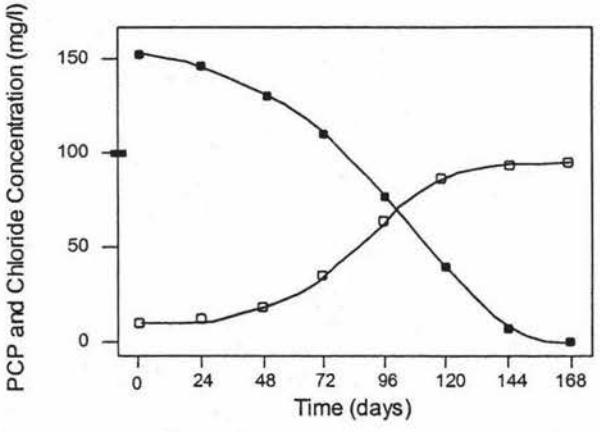

Figure A8.10: Removal of PCP (■) and concomitant release of chloride () at an initial concentration of 150mg/l PCP. Theoretical chloride release from 150mg/l PCP is 100mg/l (,).

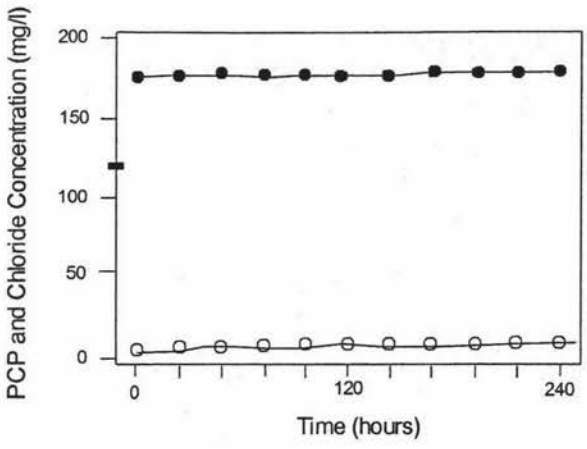

Figure A8.11: Removal of PCP (■) and concomitant release of chloride () at an initial concentration of 175mg/l PCP. Theoretical chloride release from 175mg/l is 116.7mg/l.

A8.6 PCP REMOVAL REGRESSION EQUATIONS

PCP removal rates have been calculated using linear regression from the graphs in the Results Section 4.3 and the Appendix Section A8.5. Not all the points on each graph were used to fit the regression line. Only those points in the steepest phase of removal were used. The equations and R-squared values are presented in the table below.

Table A8.3: Regression equations for PCP removal at various initial PCP concentrations with and without 25mg/l added yeast extract.

Initial PCP Concentration (mg/l)	Regression Equation	PCP Removal Rate (mg/l/hr)*	R-Squared value (%)	N (No. of points)
Without 25mg/l YE				
20	PCP = 18.0 – 0.09 hours	0.09 ± 0.03	95.8	8
40	PCP = 38.2 – 0.09 hours	0.09 ± 0.02	99.2	11
50	PCP = 50.9 – 0.12 hours	0.12 ± 0.02	95.5	10
60	PCP = 60.4 – 0.09 hours	0.09 ± 0.02	98.4	10
80	PCP = 77.4 – 0.08 hours	0.08 ± 0.02	97.4	7
With 25mg/l YE				
20	PCP = 21.9 – 0.46 hours	0.46 ± 0.13	99.6	3
40	PCP = 47.2 – 0.80 hours	0.80 ± 0.22	96.2	3
50	PCP = 71.1 – 1.18 hours	1.18 ± 0.16	98.4	3
60	PCP = 99.3 – 1.02 hours	1.02 ± 0.19	98.9	3
80	PCP = 109.0 – 1.41 hours	1.41 ± 0.35	94.9	3
100	PCP = 138.0 – 1.94 hours	1.94 ± 0.45	96.9	3
120	PCP = 165.0 – 2.10 hours	2.10 ± 0.38	98.9	3
150	PCP = 226.0 – 35.3 hours	1.48 ± 0.37	99.0	4
175	PCP = 177.0 – 0.2 hours	0.01 ± 0.00	65.8**	11

* These are ± the 95% confidence intervals **Not significant at the 0.05 level (95%)

A8.7 CHLORIDE RELEASE REGRESSION EQUATIONS

Chloride release rates have been calculated using linear regression from the graphs in the Results Section 4.3 and the Appendix Section A8.5. Not all the points on each graph were used to fit the regression line. Those points in the steepest part of release curve were used. The equations and R-squared values are presented in the table below. These equations indicate the rate of chloride release by ET01 in media with and without yeast extract at various initial PCP concentrations.

Table A8.4: Regression equations for chloride release at various initial PCP concentrations with and without 25mg/l added yeast extract.

Initial PCP Concentration (mg/l)	Regression Equation	Chloride Release Rate (mg/l/hr)*	R-Squared value (%)	N (No. of points)
Without 25mg/l YE				
20	Cl = -0.63 + 0.13 hours	0.13 ± 0.04	99.6	3
40	Cl = 10.4 + 0.09 hours	0.09 ± 0.03	94.7	9
50	Cl = 1.82 + 0.08 hours	0.08 ± 0.02	99.3	8
60	Cl = 6.11 + 0.08 hours	0.08 ± 0.03	98.5	10
80	Cl = 9.53 + 0.05 hours	0.05 ± 0.02	97.7	7
With 25mg/l YE				
20	Cl = 2.70 + 0.21 hours	0.21 ± 0.06	98.7	3
40	Cl = -12.5 + 0.54 hours	0.54 ± 0.17	90.0	3
50	Cl = -5.70 + 0.60 hours	0.60 ± 0.21	97.4	3
60	Cl = 7.00 + 0.52 hours	0.52 ± 0.15	99.4	4
80	Cl = -13.0 + 0.80 hours	0.80 ± 0.32	93.7	3
100	Cl = -18.0 + 1.31 hours	1.31 ± 0.39	88.7**	3
120	Cl = -45.0 + 1.25 hours	1.25 ± 0.26	99.1	3
150	Cl = -17.2 + 0.86 hours	0.86 ± 0.11	99.7	4
175	Cl = 7.32 + 0.01 hours	0.01 ± 0.00	99.8	11

* These are ± the 95% confidence intervals **Not significant at the 0.05 level (95%)

A8.8 THEORETICAL VERSUS ACTUAL CHLORIDE RELEASE

The actual chloride concentration released by ET01 has been compared with the theoretical value for all concentrations of PCP tested with and without yeast extract. The graphs below illustrate the differences between the rate of observed and theoretical chloride release. When the observed release line is below the theoretical the observed chloride release is incomplete. When the observed release is above the theoretical line, observed chloride release has exceeded the theoretical release. The most important feature of the graphs is that the actual and theoretical lines terminate in approximately the same point up to 100mg/l PCP. Above this, deviations from theoretical yields of chloride occur. Uptake and release are incomplete in the absence of yeast extract.

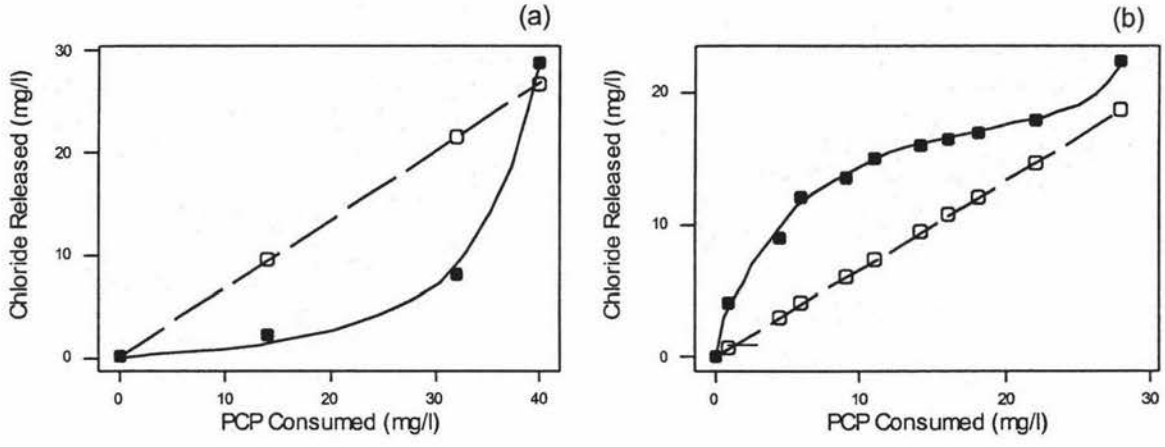

Figure A8.12: Theoretical (---) and actual chloride ( )—release for PCP at an initial concentration of 40mg/l (a) with 25mg/l yeast extract; (b) without 25mg/l yeast extract.

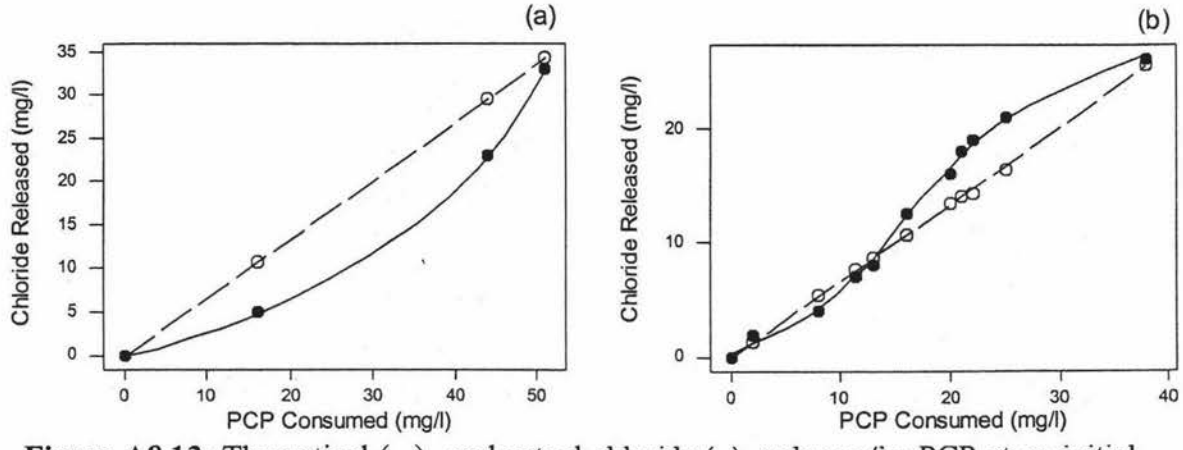

Figure A8.13: Theoretical (---) and actual chloride ( )—release for PCP at an initial concentration of 50mg/l (a) with 25mg/l yeast extract; (b) without 25mg/l yeast extract.

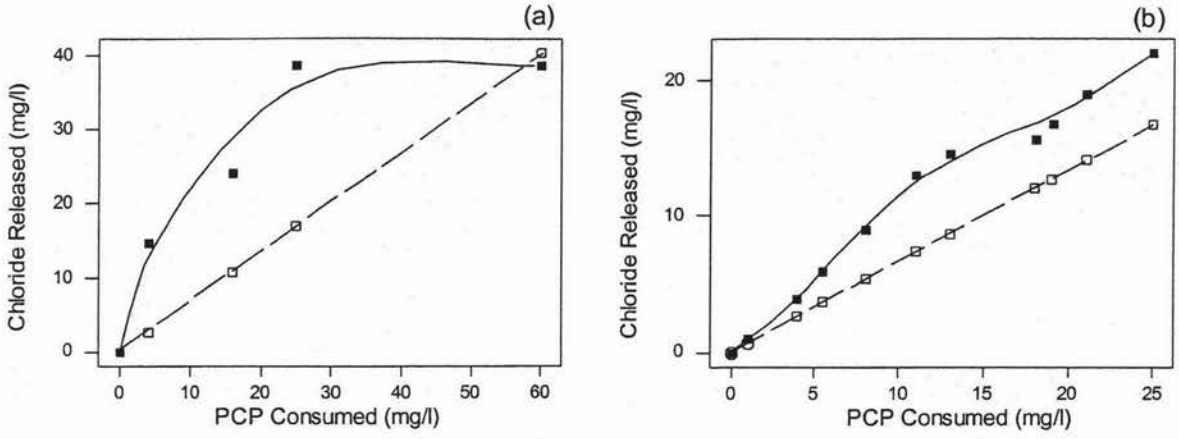

Figure A8.14: Theoretical (---) and actual chloride ( ) release for PCP at an initial concentration of 60mg/l (a) with 25mg/l yeast extract; (b) without 25mg/l yeast extract.

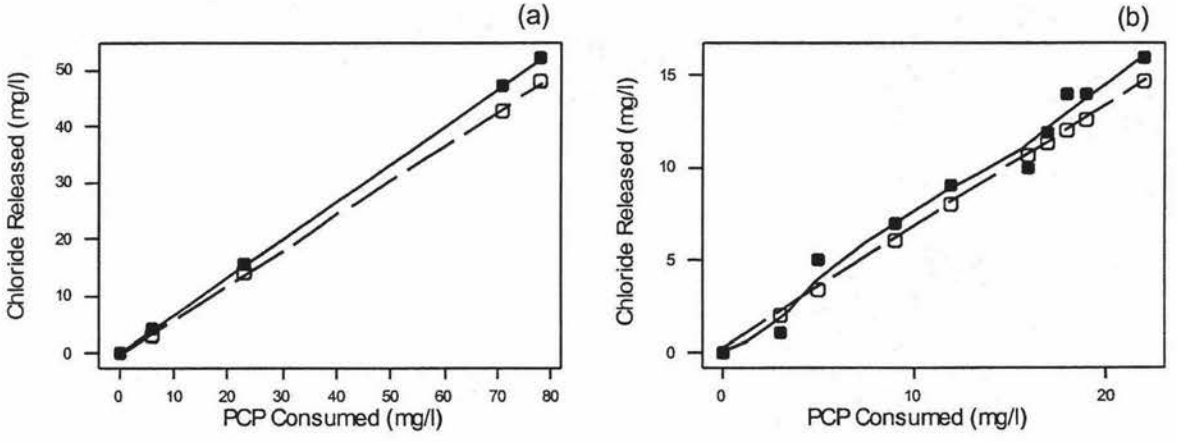

Figure A8.15: Theoretical (---) and actual chloride ( ) release for PCP at an initial concentration of 80mg/l (a) with 25mg/l yeast extract; (b) without 25mg/l yeast extract.

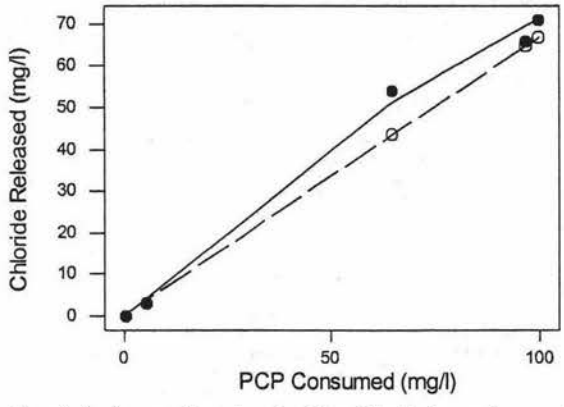

Figure A8.16: Theoretical (---) and actual chloride ( ) release for PCP at an initial concentration of 100mg/l with 25mg/l yeast extract.

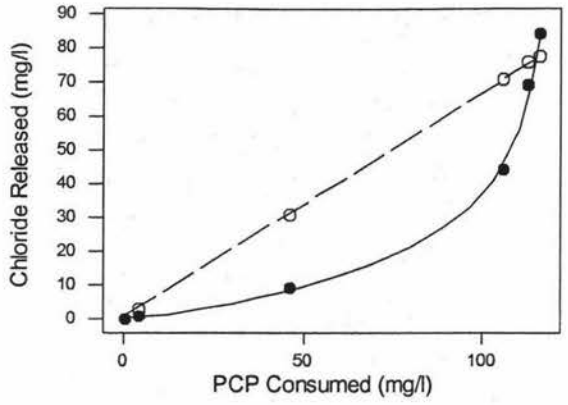

Figure A8.17: Theoretical (---) and actual chloride ( )—release for PCP at an initial concentration of 120mg/l with 25mg/l yeast extract.

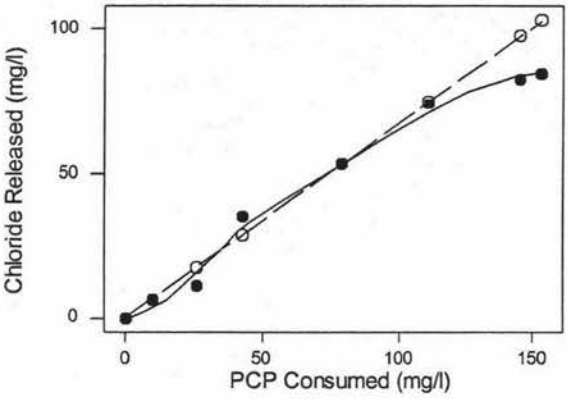

Figure A8.18: Theoretical (---) and actual chloride ( )—release for PCP at an initial concentration of 150mg/l with 25mg/l yeast extract.

There is no graph presented for chloride release for the initial PCP concentration of 175mg/l with 25mg/l yeast extract as there was no removal of PCP observed and therefore no chloride released.

A8.9 CULTURE COMBINATION PCP REMOVAL

The graphs presented below show the removal of PCP by ET01 in pure culture, ET01 with ET02 and ET03 respectively and all three isolates together. Removal of PCP is shown from initial PCP concentrations of 100mg/l and 150mg/l. The media contains 25mg/l yeast extract. Removal of PCP at an initial concentration of 50mg/l is shown in the Results Section 4.4.

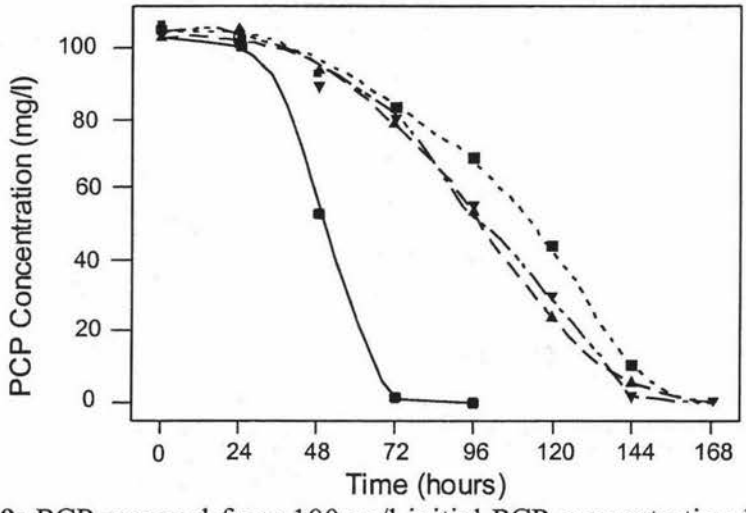

Figure A8.19: PCP removal from 100mg/l initial PCP concentration by three bacterial cultures in pure culture and in combinations as indicated (see legend).

Key: —●— ET01; - - -■- - - ET01 and ET02; --▼-- ET01 and ET03; _ —▲— ET01, ET02 and ET03.

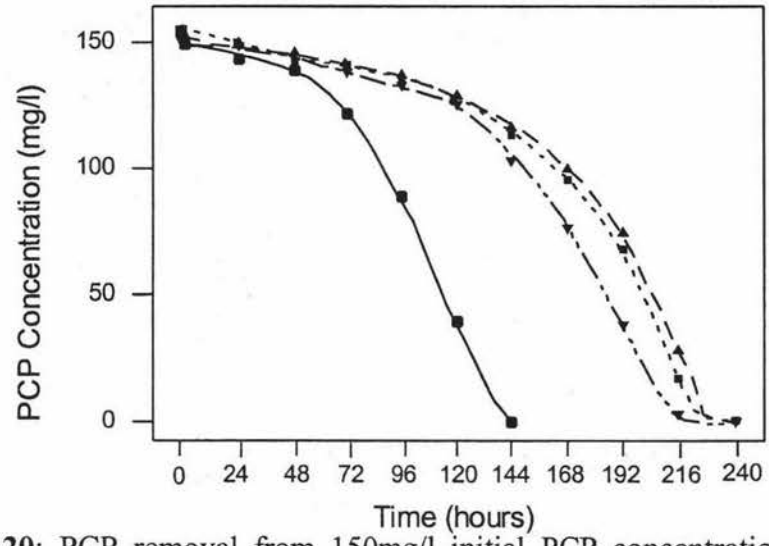

Figure A8.20: PCP removal from 150mg/l initial PCP concentration by ET01 and culture combinations as indicated (see legend).

Key: —●— ET01; - - -■- - - ET01 and ET02; --▼-- ET01 and ET03; _ —▲— ET01, ET02 AND ET03.

A8.10 CULTURE COMBINATION PCP REMOVAL REGRESSION EQUATIONS

Table A8.4 below presents the regression equations for the graph in the Results Section 4.4 and the graphs in the Appendix Section A8.9. The regression equations indicate the maximum observed rate of PCP removal in a Minimal Mineral Salts (MMS) medium with 25mg/l yeast extract. The regression equations may also be used as a predictive tool for further experimentation.

Table A8.5: Regression equations for PCP removal by culture combinations at 50, 100 and 150mg/l PCP.

Culture Combination	Regression Equation	PCP Removal Rate (mg/l/hr)*	R-Squared value (%)	N (No. of points)
50mg/l PCP				
ET01	PCP = 66.0 - 0.954 hours	0.954 ± 0.849	95.9	3
ET01/ET02	PCP = 91.2 - 0.881 hours	0.881 ± 0.383	99.7	3
ET01/ET03	PCP = 83.9 - 1.040 hours	1.040 ± 0.998	95.2	3
ET01/ET02/ET03	PCP = 86.5 - 0.607 hours	0.607 ± 0.274	99.0	4
100mg/l PCP				
ET01	PCP = 148 - 2.03 hours	2.030 ± 0.050	99.8	3
ET01/ET02	PCP = 156 - 1.08 hours	1.080 ± 0.267	99.7	4
ET01/ET03	PCP = 204 - 1.38 hours	1.380 ± 0.870	97.9	3
ET01/ET02/ET03	PCP = 156 - 1.07 hours	1.070 ± 0.217	99.9	3
150mg/l PCP				
ET01	PCP = 265 - 1.85 hours	1.850 ± 0.559	99.5	3
ET01/ET02	PCP = 346 - 1.54 hours	1.540 ± 1.080	91.1	4
ET01/ET03	PCP = 255 - 1.09 hours	1.090 ± 0.306	98.8	5
ET01/ET02/ET03	PCP = 279 - 1.13 hours	1.130 ± 0.567	96.3	5

* These are ± the 95% confidence intervals.

A8.11 PCP REMOVAL AND ORGANISM GROWTH

The following graphs which relate to the Results Section 4.5 and growth rates of ET01 and the culture combinations at each PCP concentration tested have been calculated by linear regression from these graphs. The bars on each graph show the 95% confidence interval for each point.

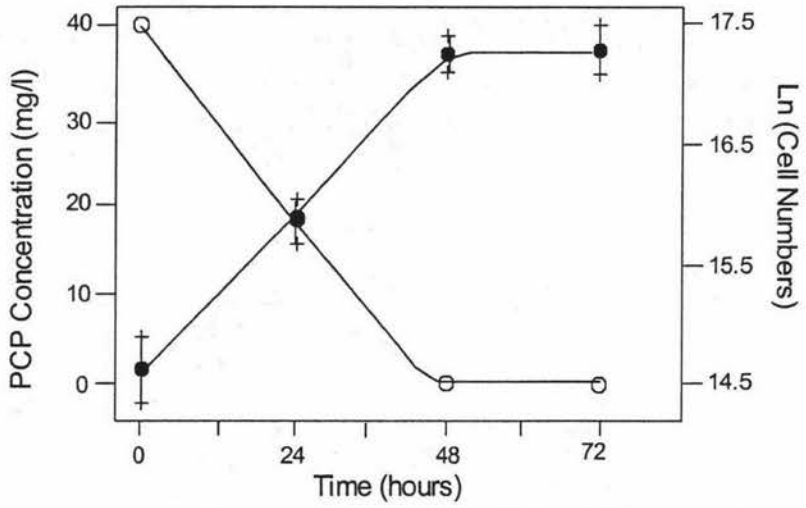

Figure A8.21: Increase in ET01 cell numbers as PCP is depleted from an initial PCP concentration of 40mg/l; O PCP concentration (mg/l); ● Natural log of cell numbers.

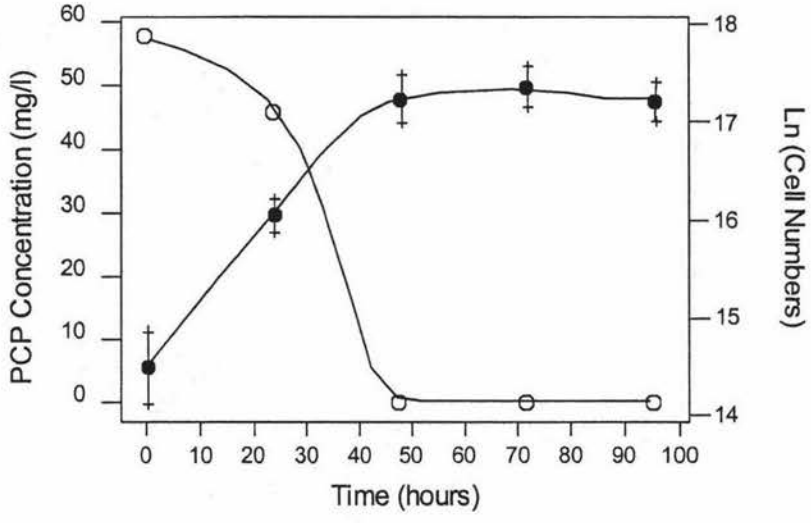

Figure A8.22: Increase in ET01 cell numbers as PCP is depleted from an initial PCP concentration of 60mg/l; O PCP concentration (mg/l); ● Natural log of cell numbers.

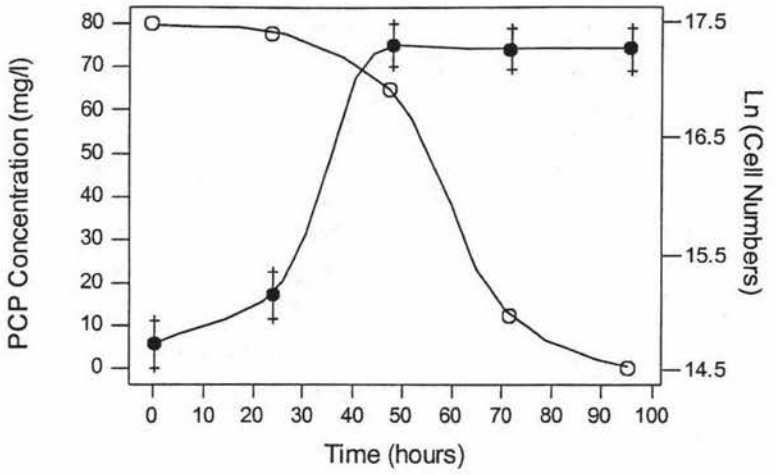

Figure A8.23: Increase in ET01 cell numbers as PCP is depleted from an initial PCP concentration of 80mg/l; O PCP concentration (mg/l); ● Natural log of cell numbers.

The following graphs are presented with ET01 in pure culture, ET01 and ET02, ET01 and ET03, and then the three cultures combined at 50, 100 and 150mg/l initial PCP concentration. The inoculum for the combined culture was a 1:1 volumetric ratio for ET01 with ET02 and ET03 and a 1:1:1 volumetric ratio for the three cultures together. The numbers of cells were estimated using the Acridine Orange (AO) fluorescent staining technique described in the Methods Section 3.5.1.2. No attempt to differentiate between the strains during growth and cell enumeration was made as the cell morphology was similar at certain stages of growth. The lack of lag phase at the beginning of the growth curve could be due to the inoculum being induced into exponential growth phase.

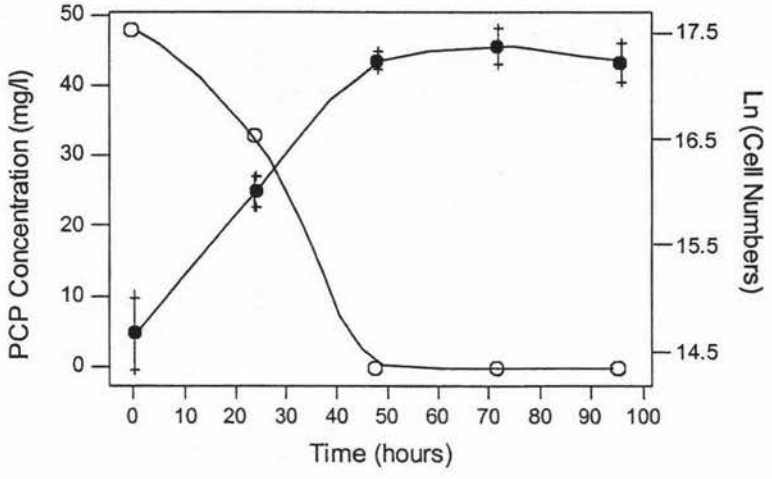

Figure A8.24: Increase in ET01 cell numbers as PCP is depleted from an initial PCP concentration of 50mg/l; O PCP concentration (mg/l); ● Natural log of cell numbers.

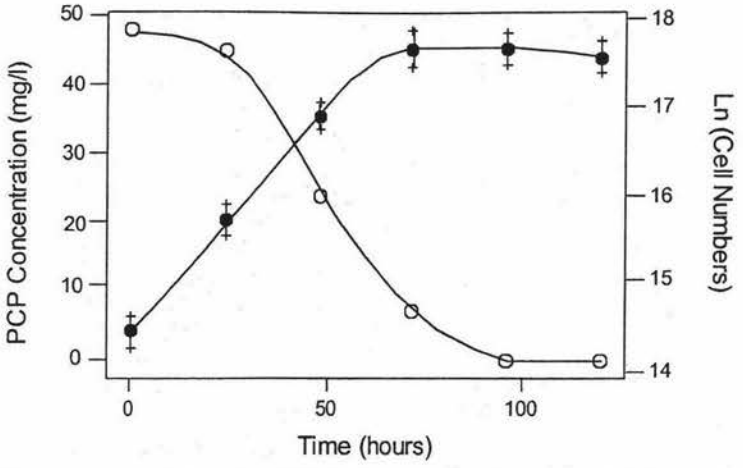

Figure A8.25: Increase in ET01 and ET02 cell numbers as PCP is depleted from an initial PCP concentration of 50mg/l; O PCP concentration (mg/l); ● Natural log of cell numbers.

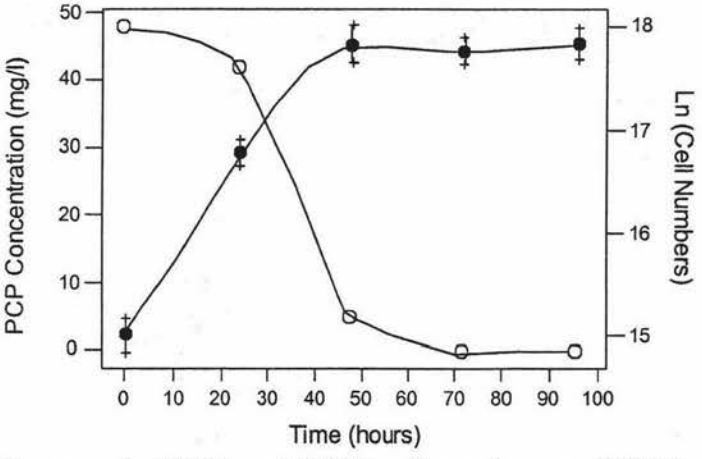

Figure A8.26: Increase in ET01 and ET03 cell numbers as PCP is depleted from an initial PCP concentration of 50mg/l; O PCP concentration (mg/l); ● Natural log of cell numbers.

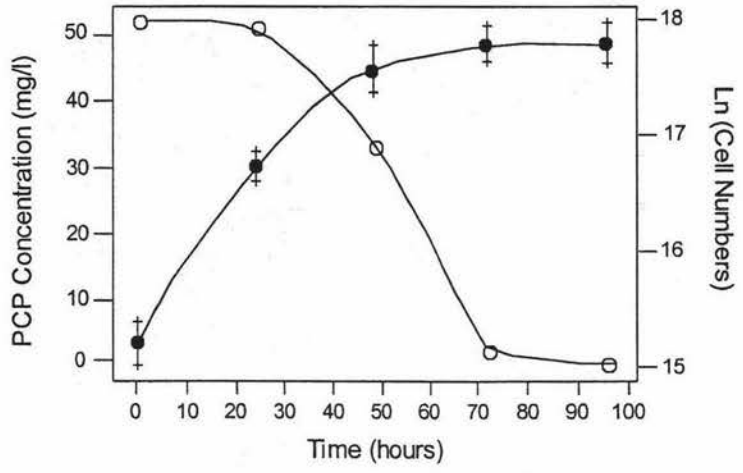

Figure 8.27: Increase in ET01, ET02 and ET03 cell numbers as PCP is depleted from and initial PCP concentration of 50mg/l; O PCP concentration (mg/l); ● Natural log of cell numbers.

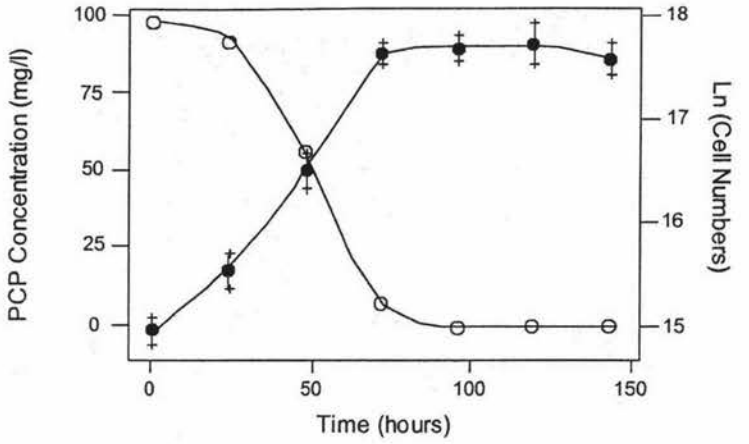

Figure A8.28: Increase in ET01 cell numbers as PCP is depleted from an initial PCP concentration of 100mg/l; O PCP concentration (mg/l); ● Natural log of cell numbers.

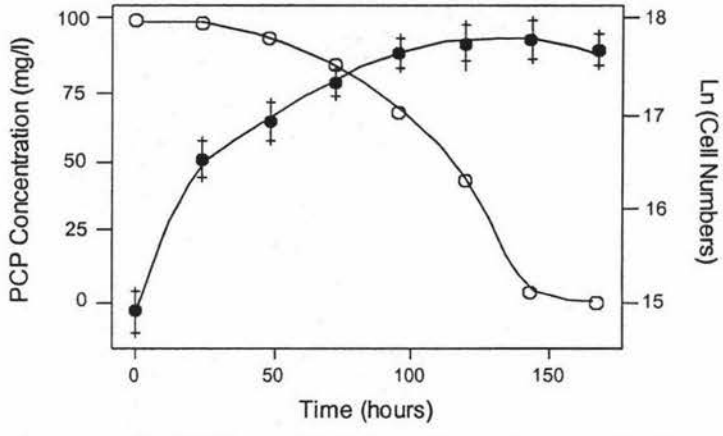

Figure A8.29: Increase in ET01 and ET02 cell numbers as PCP is depleted from an initial PCP concentration of 100mg/l; O PCP concentration (mg/l); ● Natural log of cell numbers.

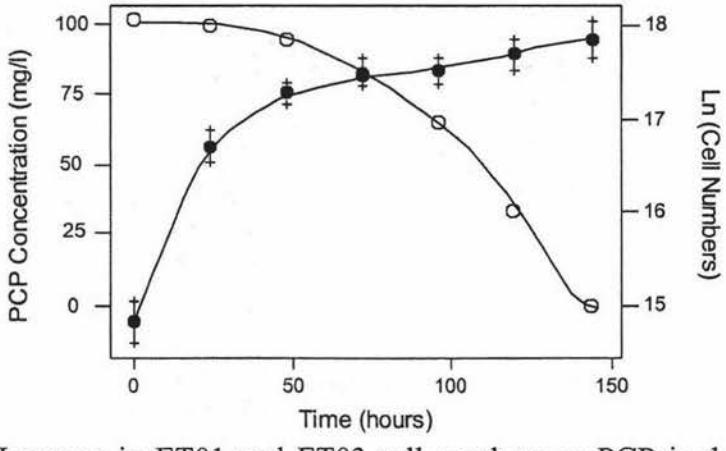

Figure A8.30: Increase in ET01 and ET03 cell numbers as PCP is depleted from an initial PCP concentration of 100mg/l; O PCP concentration (mg/l); ● Natural log of cell numbers.

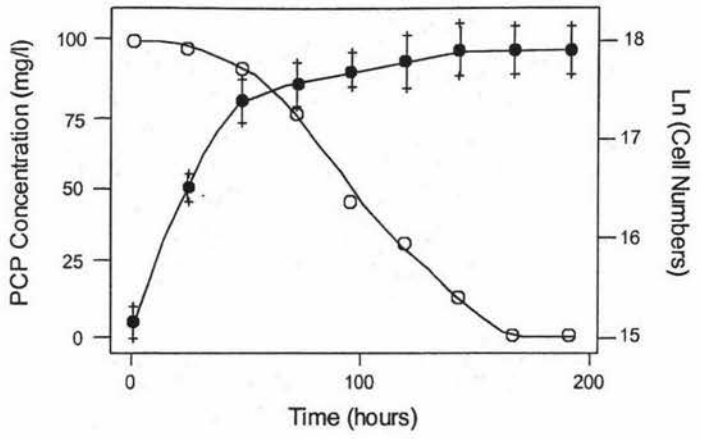

Figure A8.31: Increase in ET01, ET02 and ET03 cell numbers as PCP is depleted from an initial PCP concentration of 100mg/l; O PCP concentration (mg/l); ● Natural log of cell numbers.

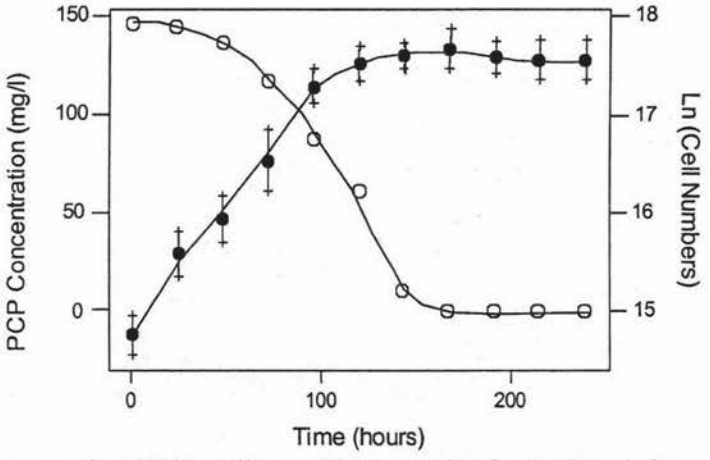

Figure A8.32: Increase in ET01 cell numbers as PCP is depleted from an initial PCP concentration of 150mg/l; O PCP concentration (mg/l); ● Natural log of cell numbers.

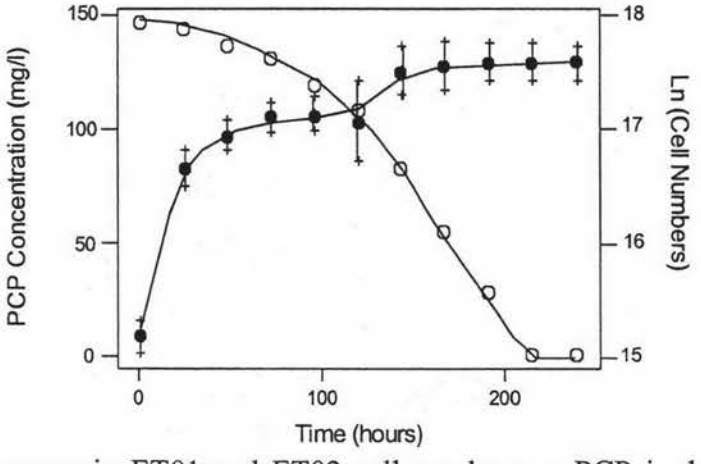

Figure A8.33: Increase in ET01 and ET02 cell numbers as PCP is depleted from an initial PCP concentration of 150mg/l; O PCP concentration (mg/l); ● Natural log of cell numbers.

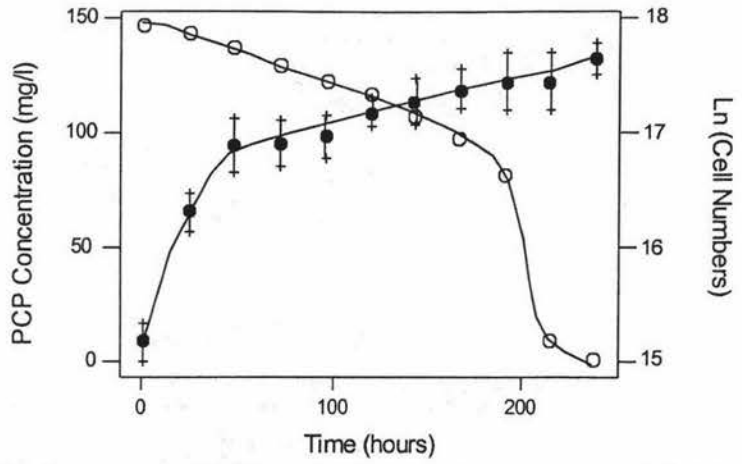

Figure A8.34: Increase in ET01 and ET03 cell numbers as PCP is depleted from an initial PCP concentration of 150mg/l; O PCP concentration (mg/l); ● Natural log of cell numbers.

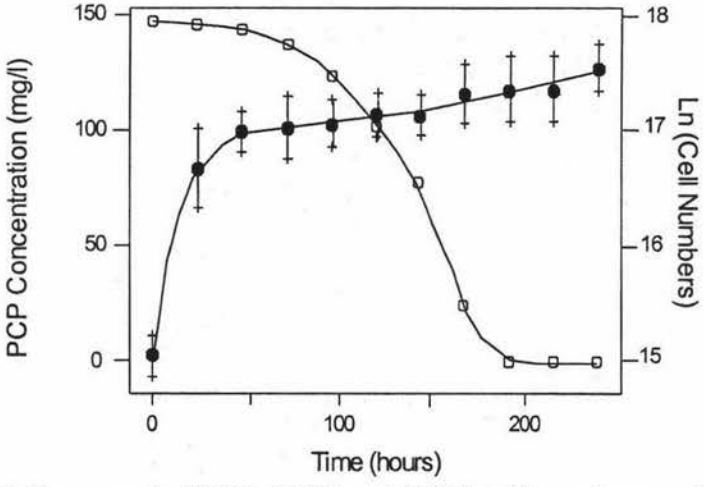

Figure A8.35: Increase in ET01, ET02 and ET03 cell numbers as PCP is depleted from an initial PCP concentration of 150mg/l; O PCP concentration (mg/l); ● Natural log of cell numbers.

A8.12 SPECIFIC GROWTH RATES FOR ET01 AND CULTURE COMBINATIONS

The regression equations in Table A8.5 are for the graphs presented in the Results Section 4.5 and the Appendix Section A8.11. The regression line was fitted to the steepest part of the graph.

Table A8.6: Regression equations for cell growth, ln (cell numbers), of ET01 at various PCP concentrations and culture combinations at 50, 100 and 150mg/l PCP.

Initial PCP Concentration (mg/l)	Regression Equation	Specific Growth Rate (hr^{-1}) *	R-Squared value (%)	N (no. of points)
ET01 20mg/l	Ln cell no. = 14.8 + 0.0529 hours	0.0529 ± 0.0073	99.9	3
ET01 40mg/l	Ln cell no. = 14.6 + 0.0552 hours	0.0552 ± 0.0063	99.9	3
ET01 60mg/l	Ln cell no. = 14.5 + 0.0574 hours	0.0574 ± 0.0148	99.6	3
ET01 80mg/l	Ln cell no. = 14.4 + 0.0536 hours	0.0536 ± 0.0037	86.9	3
50mg/l PCP				
ET01	Ln cell no. = 14.7 + 0.0540 hours	0.0540 ± 0.0054	99.9	3
ET01/ET02	Ln cell no. = 14.6 + 0.0445 hours	0.0445 ± 0.0120	98.1	4
ET01/ET03	Ln cell no. = 15.1 + 0.0588 hours	0.0588 ± 0.0378	97.8	3
ET01/ET02/ET03	Ln cell no. = 15.3 + 0.0493 hours	0.0493 ± 0.0357	97.2	3
100mg/l PCP				
ET01	Ln cell no. = 14.4 + 0.0439 hours	0.0439 ± 0.0066	99.8	3
ET01/ET02	Ln cell no. = 15.0 + 0.0298 hours	0.0298 ± 0.0177	98.1	3
ET01/ET03	Ln cell no. = 15.1 + 0.0488 hours	0.0488 ± 0.0325	97.7	3
ET01/ET02/ET03	Ln cell no. = 15.2 + 0.0468 hours	0.0468 ± 0.0364	98.5	3
150mg/l PCP				
ET01	Ln cell no. = 14.8 + 0.0253 hours	0.0253 ± 0.0048	98.6	5
ET01/ET02	Ln cell no. = 15.2 + 0.0161 hours	0.0161 ± 0.0016	99.9	3
ET01/ET03	Ln cell no. = 15.2 + 0.0173 hours	0.0173 ± 0.0072	99.1	3
ET01/ET02/ET03	Ln cell no. = 15.0 + 0.0139 hours	0.0139 ± 0.0036	99.6	3

*These are ± the 95% confidence intervals.

A8.13 YIELD FOR ET01 AND CULTURE COMBINATIONS

The amount of biomass produced (mg/l) at each point in time was plotted against the substrate removed at the same point. The biomass was calculated from the raw cell numbers by multiplying the biomass produced (cells/ml) by $1.6x10^{-9}$mg/cell (Langfeldt, 1996). The graphs below represent these points with the 95% confidence intervals shown by error bars. The regression equation for each graph is presented in the Appendix Section A8.14.

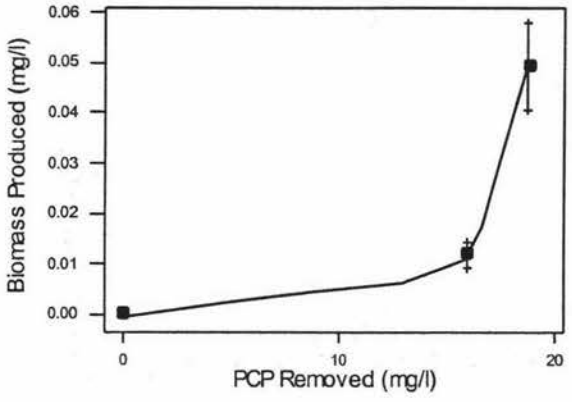

Figure A8.36: Biomass produced for PCP removed for ET01 at 20mg/l initial PCP concentration.

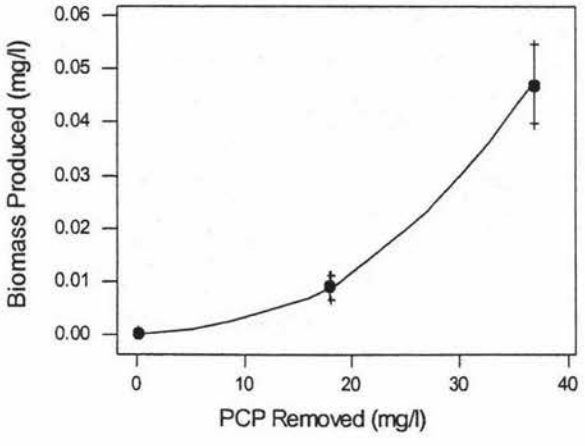

Figure A8.37: Biomass produced for PCP removed for ET01 at 40mg/l initial PCP concentration.

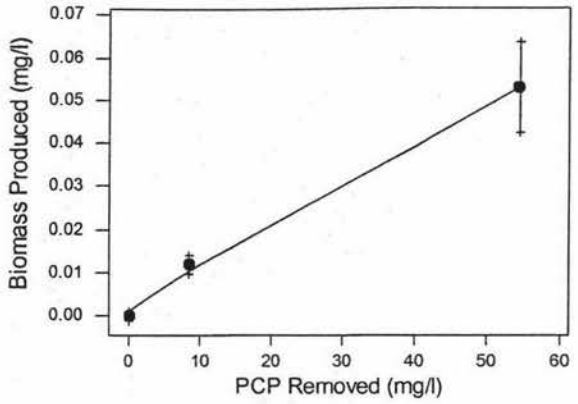

Figure A8.38: Biomass produced for PCP removed for ET01 at 60mg/l initial PCP concentration.

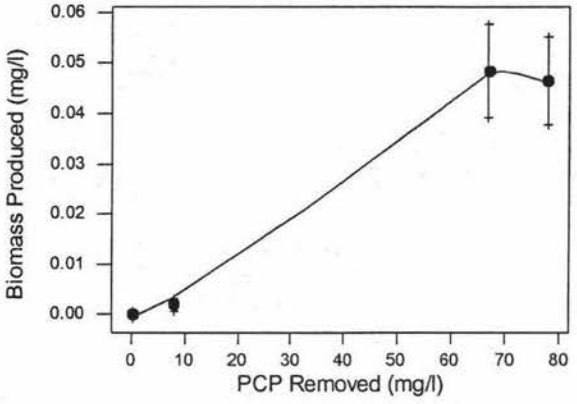

Figure A8.39: Biomass produced for PCP removed for ET01 at 80mg/l initial PCP concentration.

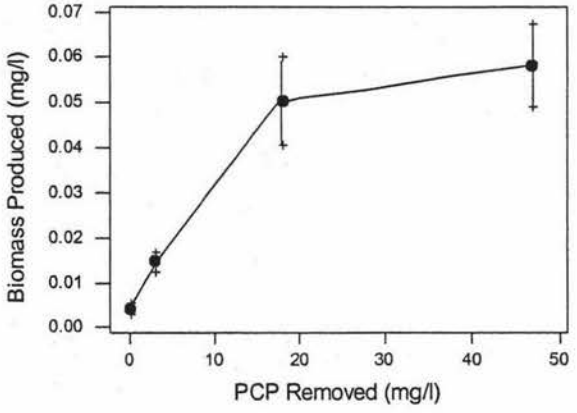

Figure A8.40: Biomass produced for PCP removed for ET01 at 50mg/l initial PCP concentration.

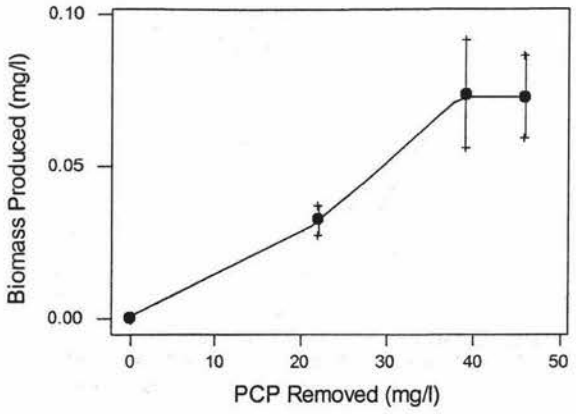

Figure A8.41: Biomass produced for PCP removed for ET01 and ET02 in combination at 50mg/l initial PCP concentration.

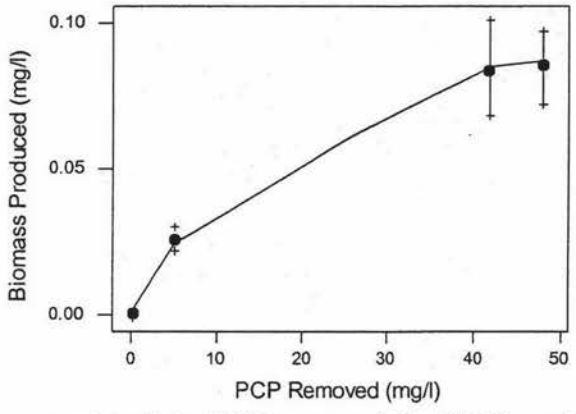

Figure A8.42: Biomass produced for PCP removed for ET01 and ET03 in combination at 50mg/l initial PCP concentration.

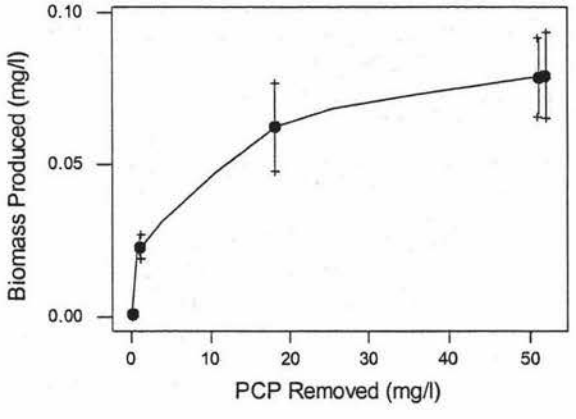

Figure A8.43: Biomass produced for PCP removed for ET01, ET02 and ET03 in combination at 50mg/l initial PCP concentration.

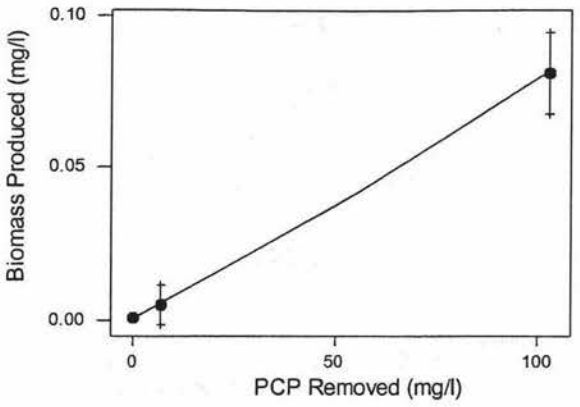

Figure A8.44: Biomass produced for PCP removed for ET01 at 100mg/l initial PCP concentration.

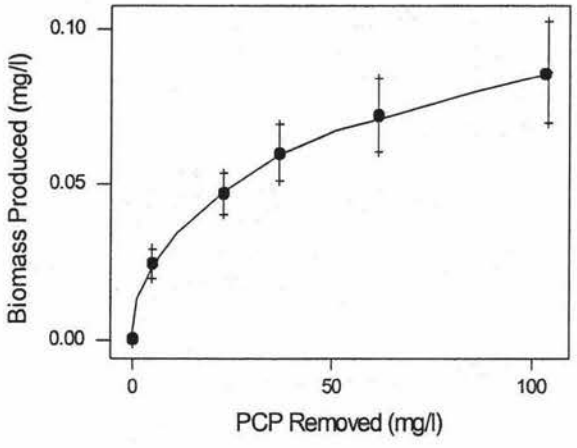

Figure A8.45: Biomass produced for PCP removed for ET01 and ET02 in combination at 100mg/l initial PCP concentration.

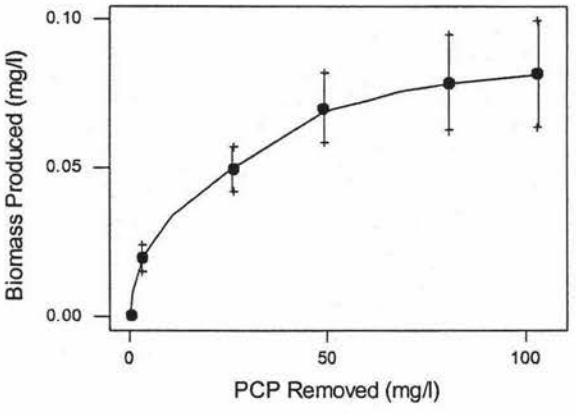

Figure A8.46: Biomass produced for PCP removed for ET01 and ET03 in combination at 100mg/l initial PCP concentration.

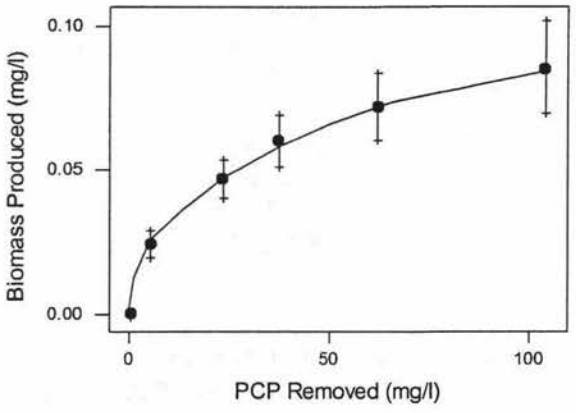

Figure A8.47: Biomass produced for PCP removed for ET01, ET02 and ET03 in combination at 100mg/l initial PCP concentration.

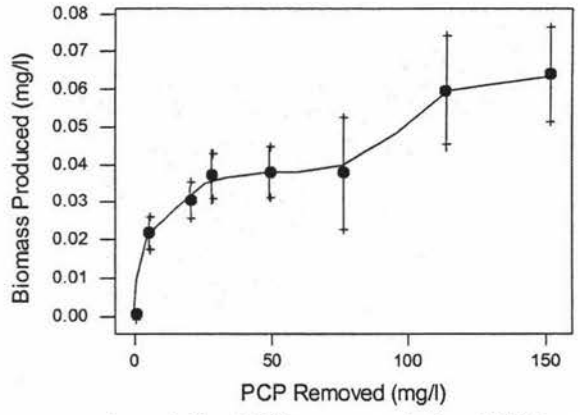

Figure A8.48: Biomass produced for PCP removed for ET01 at 150mg/l initial PCP concentration.

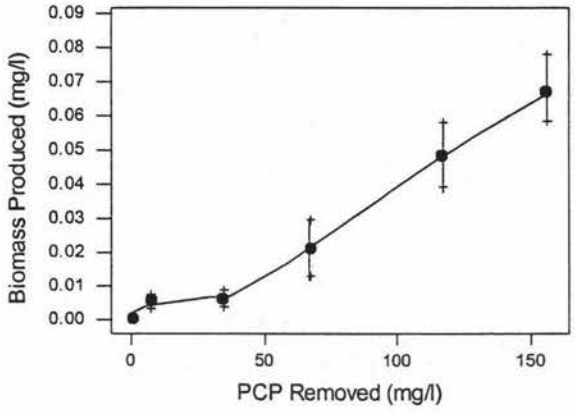

Figure A8.49: Biomass produced for PCP removed for ET01 and ET02 in combination at 150mg/l initial PCP concentration.

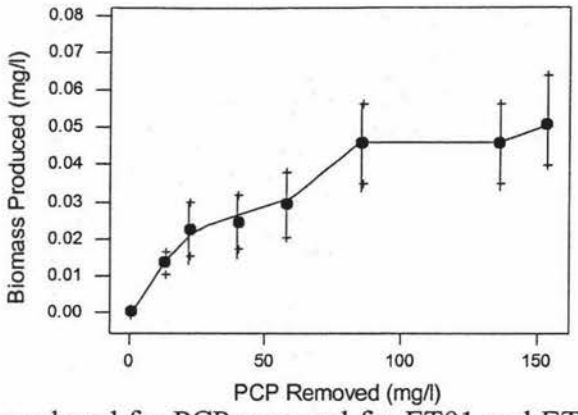

Figure A8.50: Biomass produced for PCP removed for ET01 and ET03 in combination at 150mg/l initial PCP concentration.

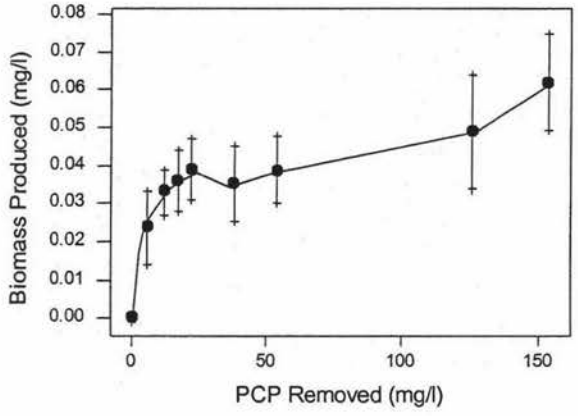

Figure A8.51: Biomass produced for PCP removed for ET01, ET02 and ET03 in combination at 150mg/l initial PCP concentration.

A8.14 YIELD REGRESSION EQUATIONS

The cells produced versus the substrate removed was plotted and the slope of the regression line gave the yield. The R-squared values are presented and indicate the fit of the regression line. The regression lines giving the highest R-squared values possible were used.

Table A8.7: Biomass yield for ET01 and culture combinations.

Culture Combination	Regression equation	Yield (mg/mg) *	R-squared (%)	N (no. of points)
ET01 20mg/l PCP	Biomass = 0.0014 PCP removed - 0.0053	0.0014 ± 0.0008	88.2**	3
ET01 40mg/l PCP	Biomass = 0.0021 PCP removed - 0.0022	0.0021 ± 0.0014	61.1**	3
ET01 60mg/l PCP	Biomass = 0.0010 PCP removed + 0.0018	0.0010 ± 0.0006	94.7	3
ET01 80mg/l PCP	Biomass = 0.0019 PCP removed - 0.0026	0.0019 ± 0.0001	99.5	3
50mg/l PCP				
ET01	Biomass = 0.0025 PCP removed + 0.0053	0.0025 ± 0.0010	99.6	3
ET01/ET02	Biomass = 0.0026 PCP removed + 0.0019	0.0026 ± 0.0004	98.0	3
ET01/ET03	Biomass = 0.0044 PCP removed + 0.0044	0.0044 ± 0.0018	99.0	3
ET01/ET02/ET03	Biomass = 0.0010 PCP removed + 0.0310	0.0010 ± 0.0002	86.7**	4
100mg/l PCP				
ET01	Biomass = 0.0007 PCP removed + 0.0067	0.0007 ± 0.0004	100.0	3
ET01/ET02	Biomass = 0.0015 PCP removed + 0.0087	0.0015 ± 0.0001	91.7	4
ET01/ET03	Biomass = 0.0013 PCP removed + 0.0091	0.0013 ± 0.0001	93.9	4
ET01/ET02/ET03	Biomass = 0.0015 PCP removed + 0.0087	0.0015 ± 0.0001	91.7	4
150mg/l PCP				
ET01	Biomass = 0.0005 PCP removed - 0.0072	0.0005 ± 0.0001	96.5	5
ET01/ET02	Biomass = 0.0031 PCP removed + 0.0027	0.0031 ± 0.0005	99.1	3
ET01/ET03	Biomass = 0.0006 PCP removed + 0.0071	0.0006 ± 0.0001	86.7**	4
ET01/ET02/ET03	Biomass = 0.0002 PCP removed + 0.0265	0.0002 ± 0.0001	91.7	4

* These values are the 95% confidence intervals **Not significant at the 0.05 level (95%)

A8.15 COMPOSITE TABLE OF PCP REMOVAL, CHLORIDE RELEASE, YIELD AND SPECIFIC GROWTH RATES

Table A8.8: PCP removal rate, chloride release rate, yield and specific growth rate for ET01 in pure culture with yeast extract.

Initial PCP Concentration (mg/l)	**PCP Removal Rate (mg/l/hr)**	**Chloride Release Rate (mg/l/hr)**	**Yield (mg/mg)**	**Specific Growth Rate (hr^{-1})**
20	0.460	0.210	0.001	0.053
40	0.800	0.540	0.002	0.055
50	1.180	0.600	0.003	0.054
60	1.020	0.520	0.001	0.057
80	1.410	0.800	0.001	0.054
100	1.940	1.310	0.001	0.044
150	1.480	0.860	0.001	0.025

A8.16 VITAMIN EXPERIMENT REGRESSION EQUATIONS

PCP removal and chloride release rates have been calculated using linear regression from the graphs in the Results Section 4.6. The equations and R-squared values are presented in Table A8.8 below.

Table A8.9: Regression equations for the culture combinations with different vitamin supplementation.

Culture Combination	Regression Equation	PCP Removal Rate (mg/l/hr)*	R-Squared value (%)	N (number of points)
No Vitamins				
ET01	PCP = 50.8 - 0.120 hours	0.120 ± 0.010	99.9	4
ET01/ET02	PCP = 50.6 - 0.180 hours	0.180 ± 0.010	99.8	5
ET01/ET03	PCP = 50.0 - 0.110 hours	0.110 ± 0.020	99.2	3
ET01/ET02/ET03	PCP = 49.3 - 0.180 hours	0.180 ± 0.020	99.0	7
25mg/l YE				
ET01	PCP = 72.1 - 0.960 hours	0.960 ± 0.040	94.3	3
ET01/ET02	PCP = 93.0 - 0.760 hours	0.760 ± 0.020	98.0	3
ET01/ET03	PCP = 80.1 - 0.810 hours	0.810 ± 0.520	95.8	3
ET01/ET02/ET03	PCP = 89.1 - 0.620 hours	0.620 ± 0.040	99.9	3
Vit Sol. 1				
ET01	PCP = 47.1 - 0.110 hours	0.110 ± 0.030	98.0	5
ET01/ET02	PCP = 48.0 - 0.110 hours	0.110 ± 0.010	99.4	5
ET01/ET03	PCP = 47.5 - 0.090 hours	0.090 ± 0.010	99.8	5
ET01/ET02/ET03	PCP = 54.7 - 0.160 hours	0.160 ± 0.050	95.7	6
Vit Sol. 2				
ET01	PCP = 77.9 - 0.800 hours	0.800 ± 0.230	99.6	3
ET01/ET02	PCP = 84.7 - 0.650 hours	0.650 ± 0.230	98.4	3
ET01/ET03	PCP = 75.0 - 0.640 hours	0.640 ± 0.010	95.0	3
ET01/ET02/ET03	PCP = 75.0 - 0.480 hours	0.480 ± 0.010	97.8	3
Vit Sol. 3				
ET01	PCP = 57.3 - 0.420 hours	0.420 ± 0.100	99.7	3
ET01/ET02	PCP = 65.5 - 0.390 hours	0.390 ± 0.030	99.8	5
ET01/ET03	PCP = 72.5 - 0.600 hours	0.600 ± 0.090	99.6	4
ET01/ET02/ET03	PCP = 88.2 - 0.730 hours	0.730 ± 0.220	99.5	3

*These are ± the 95% confidence intervals.