Mono-chlorophenol Degradation by *Pseudomonas putida* CP1 and a Mixed Microbial Population.

A thesis submitted to Dublin City University in fulfilment of the requirements for the award of the degree of Doctor of Philosophy.

by

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D. is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Date : 26-7-2000

"Everything is everywhere, the environment selects"

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Abstract

Mono-chlorophenol degradation by *Pseudomonas putida* CP1 and a mixed microbial population.

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A commercial mixed culture, Biolyte HAB, degraded mono-chlorophenols using a *meta*- cleavage pathway. 2- and 3-chlorophenol degradation was incomplete, leading to the accumulation of dead-end metabolites. Biolyte HAB was capable of the complete degradation of 2.34 mM 4-chlorophenol, via the intermediate 5-chloro-2-hydroxymuconic semialdehyde, using the *meta*- cleavage pathway.

Pseudomonas putida CP1 degraded mono-chlorophenols to completion via an *ortho*-cleavage pathway. The ability of *P. putida* CP1 to degrade mono-chlorophenols was in the order of 4-chlorophenol > 2-chlorophenol > 3-chlorophenol. 2- and 3-chlorophenol were degraded at concentrations of 1.56 mM, while 4-chlorophenol was degraded at concentrations of 2.34 mM. Growth of *P. putida* CP1 on the mono-chlorophenols resulted in the formation of large clumps of cells in the culture medium.

When *P. putida* CP1 was added to Biolyte HAB, biodegradation of mono-chlorophenols was significantly enhanced. Degradation occurred via the *ortho*-cleavage pathway. Although the mixture displayed similar degradative abilities to *P. putida* CP1, higher concentrations of chlorophenol were degraded and at increased rates. Addition of increasing concentrations of Biolyte HAB to *P. putida* CP1 resulted in increased rates of chlorophenol degradation. Times of degradation and lag periods decreased proportionately with increasing Biolyte HAB inocula sizes. The beneficial effect of Biolyte HAB on the degradation of mono-chlorophenols by *P. putida* CP1 was greatest for 3-chlorophenol, followed by 2-chlorophenol and finally 4-chlorophenol.

Generation of a rifampicin mutant of *P. putida* CP1 allowed for its survival in the mixed culture to be studied. *P. putida* CP1 was capable of surviving following its addition to the mixed culture. Total bacterial numbers, chlorophenol degrading numbers and *P. putida* CP1 numbers were found to increase with substrate removal. Increases in *P. putida* CP1 numbers were greater with increasing concentrations of Biolyte HAB.

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1. Introduction

1.1 Sources of chemical pollution.

Before man began large scale industrial activities, the concentrations of organic chemicals in the environment remained more or less constant with biosynthesis and biodegradation being held in equilibrium by the consorted activities of plants, animals and microbes. However the increase in the usage and production of man-made chemicals has led to an increase in the levels of pollutants being generated.

Environmental pollutants or xenobiotics may be seen as chemicals of natural or synthetic origin that are released into the environment by man's activities, where they have an undesirable effect on the environment or on man via their permanent or transitory accumulation in the environment. The entry of organic chemicals into the environment can be described as outlined in Figure 1 (Leisinger, 1983). In this figure, four streams of organic chemicals leading to pollution are described :

1. Chemicals whose use leads to their entry into the environment, e.g., aerosols, propellants, pesticides and fertilisers.
2. Chemicals entering the environment in the effluents of municipal sewage treatment systems, e.g., hard detergents, solvents.
3. Chemicals resistant to biological degradation in industrial waste treatment systems, e.g., chlorobenzenes, aminonapthal sulphonic acids and aromatic hydrocarbons.
4. Direct discharges, losses, spills and accidents leading to the entry of chemicals from production sites into the environment.

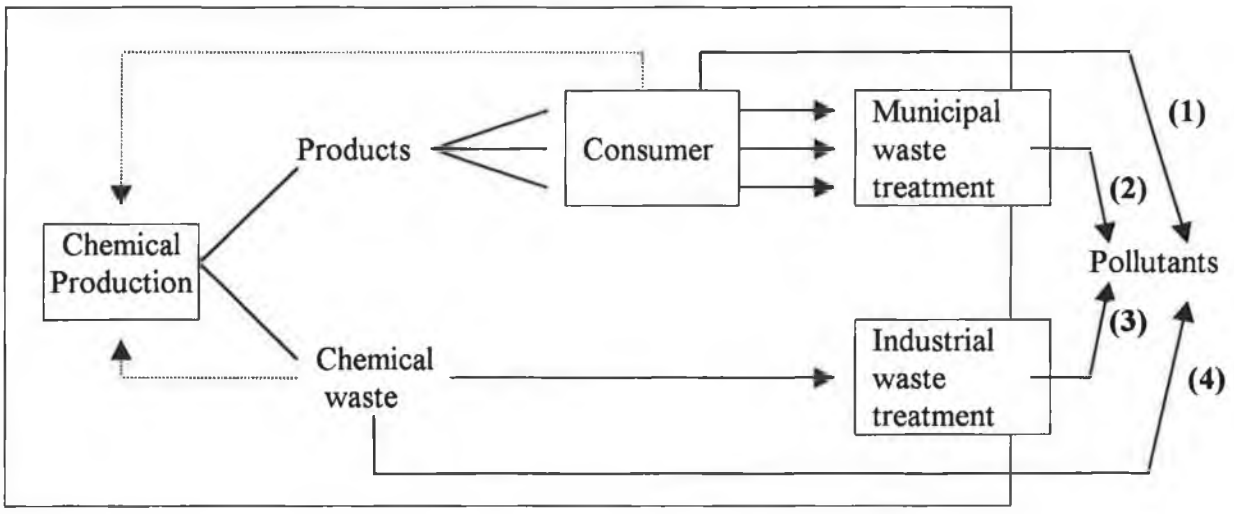

Figure 1. Release of organic chemicals into the environment (Leisinger, 1983).

1.2 Chloroaromatics.

The entry of such chemical pollutants into the environment has increased dramatically throughout the world with increased industrialisation. One group of man-made chemical pollutants are the chloroaromatics. Their unusual substitution, coupled with their highly condensed aromatic rings and excessive molecular size results in their persistence in the environment (Atlas and Bartha, 1992). Organohalogen compounds and substances which may form such compounds in the aquatic environment are named on the "Black list" compounds of the European Union (McClure *et al.*, 1991b).

Chloroaromatics constitute a wide range of chemicals such as chlorophenols, chlorobenzoic acids, chlorobenzenes, chlorobiphenyls, chloroanilines and chlorotoluenes. Of these, chlorophenolic compounds are one of the most studied. Chlorophenols are commonly found in the environment due to their use as wide spectrum biocides in both industry and agriculture. They are also formed following the bleaching of pulp with chlorine, the incineration of organic material and during the chlorination of water (Puhakka and Melin, 1996). As a result, both soils and waters have become contaminated with chlorophenols. Chlorophenols are organic compounds formed by the substitution of phenol with one or more atoms of chlorine.

Nineteen different forms of chlorophenol are possible, ranging from mono-chlorophenols to pentachlorophenols (Table 1). The mobility and bioavailability of chlorophenols, and thus their biodegradation are affected by their chemical and physical properties. The solubility of chlorophenols in water decreases with increasing numbers of chlorine substitution. Increasing the number of chlorine substitutions increases the lipophilicity of chlorophenols, thus increasing their tendency to bioaccumulate. The decreased lipophilicity and increased water solubility of mono-chlorophenols makes them the most suitable of the chlorinated phenols for bioremediation studies.

Table 1. Various forms of chlorinated phenols.

Mono-chloro	Di-chloro	Tri-chloro	Tetra-chloro	Penta-chloro
2-CP	2,3-DCP	2,3,4-TCP	2,3,4,5-TeCP	PCP
3-CP	2,4-DCP	2,3,5-TCP	2,3,4,6-TeCP	
4-CP	2,5-DCP	2,3,6-TCP	2,3,5,6-TCP	
	2,6-DCP	2,4,5-TCP		
	3,4-DCP	2,4,6-TCP		
	3,5-DCP	3,4,5-TCP		

Two of the mono-chlorophenols are of significance in industry. 2-chlorophenol is produced as an intermediate during the production of phenols, phenol resins and dyes, and is used as a bactericide and fungicide, while 4-chlorophenol is produced as an intermediate in the production of 2,4-dichlorophenol, trichlorophenol and tetrachlorophenol, and is also used as a solvent for the mineral oil industry (Wiesmann and Libra, 1999).

Information on the levels of hazardous waste quantities in Ireland is available in the National Waste Database Report for 1995 (EPA, 1996) and is shown in Table 2. As can be seen from this Table, chloroaromatics which are found in chlorinated organic

solvents, pesticides and PCB waste, constitute a large proportion of the chemical waste generated in Ireland.

Table 2. Hazardous waste generation in Ireland in 1995 by waste type.

Waste Type	**Total Quantity Generated** (tonnes)
Organic and chlorinated organic solvents	86,404.80
Other pharmaceutical waste (mixed)	49,467.00
Paint/ink/varnish sludges	1,205.20
Paint/ink/varnish waste	823.30
Pesticides	330.00
PCB waste	71.10

Increased regulation in the release of recalcitrant and toxic chemicals into the environment, on grounds of public and ecological health, in addition to the greater environmental awareness of the public at large, has led to increased pressure being placed on industry to place more emphasis on the ecological impact of their processes.

1.3 Treatment of chemical wastes.

Several non-biological treatments have been effective in reducing exposure of the environment to hazardous wastes. These physical/chemical methods include use of long-term storage, landfills, incineration and air stripping (Levin and Gealt, 1993). Long-term storage and landfilling have similar advantages and disadvantages. Since there is no manipulation of the toxic material, cost is generally minimal as long as a reasonably close locale can be identified for the location of a storage locker or landfill site. The major cost involved, is the removal and transport of the hazardous material. The cost can be extreme if the distance is very great. Further complication is frequently encountered because of public resistance to the development of long-term storage facilities for such toxic wastes. The possibility of insufficient maintenance followed by possible leaching into soil or groundwater has increased the public concern. With

increasing concerns has come increased regulation and legislation on storage and landfill design, security, containment, financing against liability risks and the near perpetual care in the post-closure period, all of which have led to ever increasing costs.

Incineration and air stripping of wastes have the advantage of decreasing the amount of toxic material. However, incineration consumes energy and may lead to the production of additional toxic materials, e.g., dioxin, which requires costly scrubbing before release into the atmosphere. Air stripping also results in the environmental release of small amounts of toxics, and depending on the method used, may also involve the transfer of toxic materials onto activated carbon, which will then require treatment.

All these factors have resulted in a growing interest in biological solutions to chemical problems, as a cleaner and more environmentally friendly option. Application of such biological solutions for the clean-up of sites contaminated with chemical pollutants is encompassed by the term bioremediation (Alexander, 1994b). Bioremediation addresses the limitations of more conventional techniques by bringing about the actual destruction of many organic contaminants at reduced costs. Biological technologies are generally lower in cost because the destructive reactions, which are mediated in general by naturally occurring biocatalytic activities, can occur relatively quickly at ambient temperatures, thus reducing energy costs. Consequently, interest in bioremediation techniques has grown over the past 20 years from a virtually unknown technology to a technology that is considered for the cleanup of a wide range of contaminating compounds in a variety of differing matrices (Walter and Crawford, 1997). Application of microbial degradation and removal of undesirable constituents in industrial and municipal wastewaters is not a new concept. There is now a wide range of biological treatment systems in current use for the decontamination of wastewaters. As the awareness of the chemical contamination of the environment has greatly increased, much research on biological degradation of toxic chemicals has occurred. Of the many agents involved in the biological treatment of waste chemicals, bacteria are

thought to be the most important. Bacteria are ubiquitous and their size, specific growth rates and metabolic versatility makes them eminently suitable for this function.

1.4 Biodegradation.

Biodegradation can be defined as the "biologically catalysed reduction in complexity of chemicals" (Alexander, 1994a) and frequently, although not necessarily, leads to the conversion of much of the C, N, P, S, and other elements in the original organic compound to inorganic products. When studies into the biodegradability of a particular compound are being carried out, consideration must be given not only to the concentration at which a particular compound is released into the environment, but to the chemical and toxicological properties of the chemical. Since degradation of chemicals is generally microbially mediated, any structural feature of a chemical precluding or retarding its attack by microbes will lead to its accumulation in the environment, i.e. to recalcitrance of the particular compound (Leisinger, 1983). Recalcitrance is a term applied to any xenobiotic that is attacked slowly or not at all by microbial enzymes. Studies carried out have shown that relatively small changes in chemical structure can appreciably alter a chemical's susceptibility to degradation. The following molecular features generally increase the recalcitrance of a xenobiotic to degradation (Boethling, 1993) :

1. Branching, especially tertiary (N) and quaternary (C)
2. Polymerisation
3. Presence of halogen, nitro, nitroso, aryl sulphonate, aryl amino and azo substitutions (especially when there is a multiple substitution)
4. Aliphatic ether linkages
5. Polycyclic residues, especially with more than three fused rings
6. Heterocyclic residues

There are several responses by a microbial population when confronted with a xenobiotic compound :

1. It may be essentially unreactive (neither toxic to the micro-organism, nor degraded by them).
2. It may be toxic to the micro-organisms.
3. Complete mineralization of the compound may occur immediately due to the presence of suitable enzymes or following a period of acclimation.
4. It may be degraded by co-oxidation/cometabolism.
5. Partial transformation of the compound leading to incomplete degradation (Biotransformation).

   (Grady Jr., 1985).

Cometabolism is the "transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound" (Grady Jr., 1985). As can be seen from the definition two compounds are required, first a non-growth substrate which cannot serve as the sole source of carbon and energy, and a second substrate which acts as an energy source allowing the non-growth substrate to be transformed in parallel. Cometabolism results in the production of metabolic products which may or may not be further metabolised by micro-organisms.

Complete mineralization of a compound is the most desirable of the processes as it generates carbon and energy for microbial growth and leads to the disappearance of the xenobiotic compound. If enzymes capable of xenobiotic degradation are present, degradation will occur rapidly. However sometimes a period of acclimation is necessary before degradation may take place. Acclimation is a period of physiological, morphological and genetic adaptation of micro-organisms to a new environment. It is thought to involve one or more of the following :

1. Induction of the enzymes required for degradation to occur.
2. Growth of an initially low population of degrading micro-organisms within a community.

3. Acquisition of new catabolic capabilities by gene transfer or mutation (Boethling, 1993)

In order for complete mineralization of a particular compound to occur, suitable enzymes must be present to catalyse the reactions necessary for degradation to occur. Enzyme mediated degradation of organics is a highly specific event, even for naturally occurring material. Evolutionary pressures in nature have resulted in micro-organisms being capable of the synthesis of enzymes responsible for the degradation of natural organic compounds under most environmental conditions. Nevertheless, human activities have produced numerous structures never before seen or at least infrequently encountered in nature (Grady Jr., 1985).

A phenomenon known as "fortuitous" or "gratuitous" metabolism allows the degradation of man-made xenobiotics, the recent entry of which into the environment has not exerted long term selective evolutionary pressures on the genetic capability of a bacterium, required to express specific enzymes which are synthesised in response to the presence of organic substrates. This phenomenon is attributable to the fact that the degradative enzymes of bacteria are not always absolutely specific for their natural substrates (Knackmuss, 1981). Some enzymes can be quite tolerant of substrate modification. Consequently, it is not uncommon for them to bind analogs of the natural substrate, which contain xenobiotic functional groups. If the functional group does not greatly alter the active site of the particular enzyme, then it can be possible for the catalytic properties to remain unaltered and to be carried out on the xenobiotic substrate.

Gratuitous metabolism of organic compounds may or may not result in the build up of metabolites in the medium. If the transformation product is more toxic than the original substrate, or if no suitable enzyme capable of its degradation is present, then it is likely to accumulate. Accumulation may lead to a bioconcentration of hazardous chemicals on or within micro-organisms and may lead to their entry into the food chain. However, if the transformation product is benign and can be acted upon by another

enzyme, it will be further transformed and the process will continue until an intermediate of the normal metabolic pathways of the bacteria is produced (Grady Jr., 1985).

The ability of micro-organisms to degrade an organic chemical is governed not only by the chemical structure, but also by the environment in which it is found. Many variables contribute to the ability of micro-organisms to degrade any particular compound. These variables may be considered as being either physical/chemical related or organism related.

Among the physical/chemical factors which may affect the microbial degradation of xenobiotics, the following are thought to be most important :

1. Concentration of the xenobiotic chemical
2. Temperature
3. Nutrients
4. Concentration of oxygen
5. Salinity/Hydrostatic pressure
6. pH

Biodegradation of recalcitrant compounds will only take place if the above environmental factors are to the liking of the degradative bacteria. Micro-organisms will not grow sufficiently unless the required nutrients (macronutrients such as nitrogen and phosphorous and micronutrients such as essential trace elements and sometimes growth factors) are present at a suitable temperature/pH/salinity, in the presence of oxygen (for aerobic micro-organisms), in order for biodegradation to occur. The concentration at which the chemical is found also can affect biodegradation. At very low substrate concentrations, degradation can sometimes not occur or may occur more slowly than expected due to the failure of the chemical to induce sufficient levels of enzyme activity. At high concentrations, biodegradation may also fail to take place due to the toxicity of the chemical to the micro-organisms.

Organism-related factors include, whether degradation is being carried out by a pure culture or a microbial community, the concentrations of viable micro-organisms present, whether the micro-organisms have been acclimated to the particular compound and the intra- and interspecies interactions such as predation and mutualism. Clearly degradation is more likely to take place if an acclimated organism is present at high cell numbers, than in the presence of a non-acclimated culture at low cell numbers. It is also known that degradation is more likely to result in complete mineralization when carried out by mixed cultures than by pure cultures (Boethling, 1993).

1.5 Degradation of mono-chlorophenols and other aromatics.

Chloroaromatics are toxic to most organisms due to their ability to uncouple oxidative phosphorylation. Despite this, many organisms have developed mechanisms to detoxify chlorophenols. Chlorinated aromatics can be degraded by both aerobic and anaerobic micro-organisms. Little is known about the degradation of chloroaromatics in anaerobic environments, and no pure bacterial culture capable of their degradation has been isolated (Häggblom, 1990). Aerobic degradation however has been demonstrated, and several bacteria have been isolated capable of chloroaromatic degradation. A critical feature of aerobic catabolic routes is the channelling of structurally diverse substrates into a limited number of central pathways. This is brought about by the activity of a large number of enzymes that catalyse the initial reactions, funnelling degradative pathways to form a central intermediate through which degradation continues using one central pathway.

1.5.1 The degradation of catechol.

The initial conversion steps for the aerobic degradation of aromatic hydrocarbons are carried out by a series of different enzymes that funnel degradative pathways to a

limited number of central intermediates such as protocatechuate and more importantly catechol (Fig. 2).

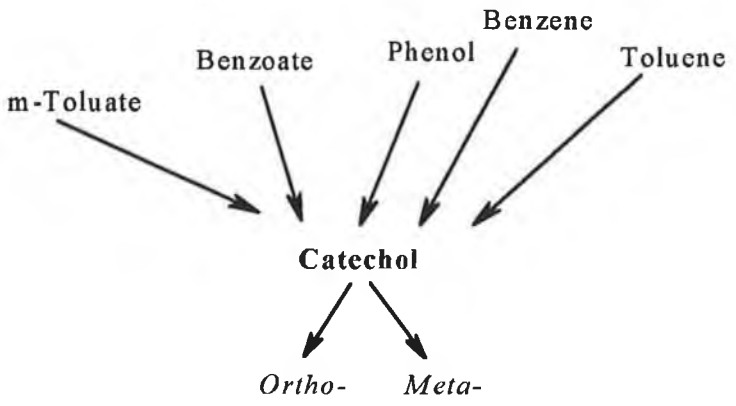

Figure 2. The central role of catechol in the oxidation of aromatic compounds by *Pseudomonas* spp. (Feist and Hegeman, 1969).

These dihydroxylated intermediates are then channelled into one of two possible pathways, either a *meta-* cleavage pathway or an *ortho-* cleavage pathway. *Meta-* cleavage, or extradiol cleavage, occurs between two adjacent carbon atoms, one carrying a hydroxyl group and the other being unsubstituted, while *ortho-* cleavage (intradiol cleavage) occurs between adjacent carbon atoms substituted with hydroxyl groups. Both types of pathways lead to intermediates of central metabolic routes, such as the tricarboxylic acid cycle (TCA cycle).

1.5.1.1 The *meta-* cleavage pathway.

The *meta-* cleavage of catechol results in the ultimate production of pyruvate and acetaldehyde, both of which are intermediates of the central TCA cycle of the micro-organism. The first step in the *meta-* cleavage of catechol is catalysed by the enzyme catechol 2,3-dioxygenase (*meta*-pyrocatechase) and results in the production of 2-hydroxymuconic semialdehyde, which is then further metabolised by either a dehydrogenative or a hydrolytic route, both of which converge later in the metabolic

pathway. Dehydrogenative degradation of 2-hydroxymuconic semialdehyde catalysed by the enzyme hydroxymuconic semialdehyde dehydrogenase results in the enol form of 4-oxalocrotonate which is then converted by isomerase activity to form the keto form. The keto form of 4-oxalocrotonate is then metabolised by the enzyme 4-oxalocrotonate decarboxylase to produce 2-hydroxypent-2,4-dienoate which is converted by the enzyme 4-hydroxy-2-oxovalerate to produce the TCA intermediates, pyruvate and acetaldehyde. In the hydrolytic route, 2-hydroxymuconic semialdehyde is broken down by a hydrolase to form 2-oxopent-4-enoate which can be converted by 2-oxopent-4-enoate hydratase to form 2-hydroxypent-2,4-dienoate, thus rejoining the dehydrogenative pathway.

The *meta-* cleavage pathway for the degradation of catechol is plasmid encoded. The most comprehensively studied *meta-* cleavage pathway is that of the TOL plasmid. TOL plasmids are self-transmissible and encode for the enzymes involved in the degradation of methyl benzenes such as toluene, xylenes and 1,2,4-trimethylbenzene (van der Meer *et al.*, 1992). On the TOL plasmid, pWW0, which encodes for toluene degradation in *P. putida*, one operon (*xyl*CMABN) codes for the "upper pathway" enzymes, which are responsible for the conversion of toluene and xylenes to the corresponding benzoates (Harayama *et al.*, 1986) while a separate operon (*xyl*EFGHIJK) codes for the "lower pathway" enzymes, which are responsible for the conversion of benzoates, via catechol, to the central metabolites pyruvate and acetaldehyde (Harayama *et al.*, 1984).

1.5.1.2 The *ortho-* cleavage pathway.

The *ortho-* cleavage of catechol results in the ultimate production of succinate and acetyl coA, both of which feed into the TCA cycle. The initial step in the *ortho-* cleavage pathway is catalysed by the enzyme catechol 1,2-dioxygenase (pyrocatechase Type I) and results in the production of *cis,cis*-muconate. Muconate lactonizing enzyme then converts *cis,cis*-muconate into muconolactone, which is then converted via the enzyme muconolactone isomerase to form 3-oxoadipate enol-lactone. 3-

oxoadipate enol-lactone is metabolised by 3-oxoadipate enol-lactone hydrolase to form 3-oxoadipate which is further transformed by a transferase enzyme to 3-oxoadipate coA. Finally, 3-oxoadipate coA is converted to succinate and acetyl coA which enter the TCA cycle.

The *ortho-* cleavage pathway for the degradation of catechol is chromosomally encoded. The structural genes for muconate lactonizing enzyme (*cat*B) and muconolactone isomerase (*cat*C) have been shown to lie in close proximity on the chromosome. The synthesis of these enzymes is co-ordinately induced by *cis,cis*-muconate. This compound also induced *cat*A, the structural gene for catechol 1,2-dioxygenase. 3-oxoadipate enol-lactone hydrolase is encoded by the structural gene *cat*D (Schlömann, 1994).

1.5.2 Chlorophenol degradation.

The presence of a chlorine group makes chloroaromatics more persistent in the environment. Biodegradation may only be considered as being complete once the carbon skeleton is converted into intermediates of central metabolic pathways and its organic halide is converted to its free mineral state. For a bacterium to achieve the complete mineralization of chlorophenols, it must be capable of removing the chlorine substituents at an early stage of metabolism or have enzymes capable of dehalogenation following cleavage of the aromatic ring (Häggblom, 1990). The aerobic degradation of chlorophenols generally occurs using the latter pathway, with dehalogenation taking place after ring cleavage. Aerobic mineralization of chlorinated aromatic compounds appears to follow similar principles to those seen for the degradation of non-halogenated aromatics. For many of the chloroaromatic compounds, degradative pathways converge at chlorocatechols as central intermediates (Fig. 3).

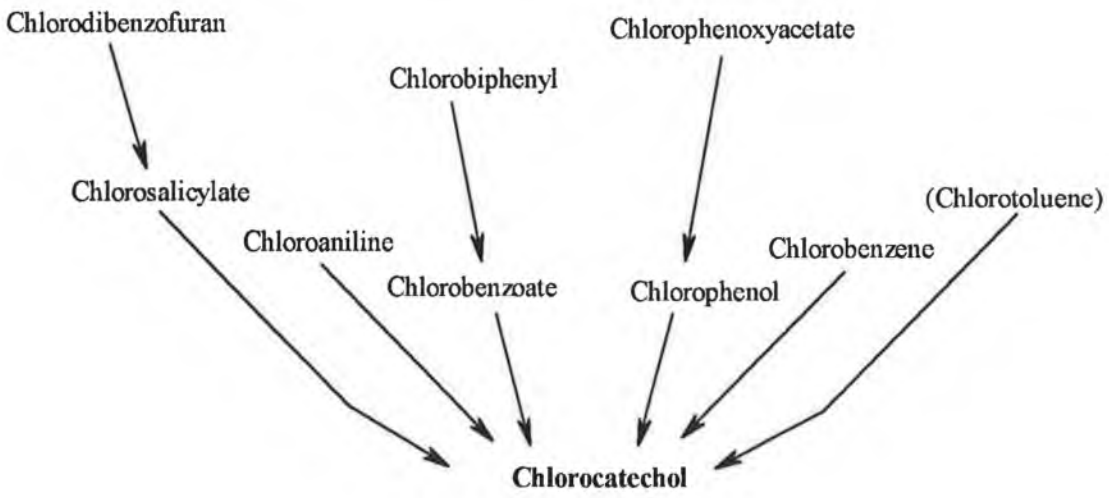

Figure 3. The central role of chlorocatechols in the oxidation of chlorinated aromatic compounds (Schlömann, 1994).

1.5.2.1 Hydroxylation.

The initial step in the aerobic degradation of mono-chlorophenols is their transformation to the central metabolites, chlorocatechols. This step may be carried out by either a hydroxylase or a dioxygenase enzyme system. Degradation of 2- and 3-chlorophenol generally results in the production of 3-chlorocatechol, while metabolism of 4-chlorophenol leads to the production of 4-chlorocatechol.

Pseudomonas sp. B13, an organism shown to be capable of the degradation of 3-chlorobenzoate and 4-chlorophenol as sole carbon source, and also of the co-metabolism of 2- and 3-chlorophenol (Knackmuss and Hellwig, 1978), was shown to carry out hydroxylation to the corresponding chlorocatechols using a broad spectrum phenol hydroxylase. Conjugation of *Alcaligenes* sp. strain A7, a strain capable of growth on benzoate and phenol, with *Pseudomonas* sp. B13 resulted in a transconjugant of the *Alcaligenes* sp., strain A7-2, capable of growth on all three mono-chlorophenols, a property not possessed by either the donor or recipient strains (Schwein and Schmidt, 1982). The increased ability of the transconjugant strain, *Alcaligenes* A7-2, to grow on mono-chlorophenols over *Pseudomonas* sp. B13 can be

attributed to the presence of a more rapidly inducible non-specific phenol hydroxylase, which correlates with the greater level of resistance to the mono-chlorophenols.

Transformation of mono-chlorophenols to chlorocatechols was demonstrated in *P. putida* F1 (Spain and Gibson, 1988). Cells pre-grown in toluene were studied for their ability to degrade many substituted aromatics. Degradation of 2- and 3-chlorophenol resulted in an accumulation of 3-chlorocatechol in the medium, while growth on 4-chlorophenol led to the transient accumulation of 4-chlorocatechol. It was suggested that a toluene dioxygenase was responsible for the transformation of chlorophenols to chlorocatechols as generation of mutants of *P. putida* F1, lacking toluene dioxygenase activity, failed to transform chlorophenols.

When the degradation of 4-chlorophenol by *P. putida* PpG4 was studied, it was found that transformation of 4-chlorophenol could only occur following growth of the bacterium on phenol (Saez and Rittman, 1991). This suggested that degradation could only occur following induction of a phenol hydroxylase which catalysed the first step in the degradation of 4-chlorophenol.

The phenol-degrading strain *P. putida* EKII was found to be capable of the degradation of mono-chlorophenols both by co-metabolism with phenol and as sole carbon source with cells pre-grown on phenol (Hinteregger *et al.*, 1992). Degradation of the chlorophenols was considerably faster in the presence of phenol than in its absence. Although activity of the broad spectrum phenol hydroxylase was not detectable in cell free extracts, it was detectable in whole cells.

The transformation of chlorophenols to chlorocatechols by a phenol hydroxylase in the yeast *Rhodotorula glutinis* was demonstrated (Katayama-Hirayama *et al.*, 1994). Degradation of mono-chlorophenols was improved following the addition of phenol to the medium, suggesting the induction of phenol hydroxylase by phenol. The order of biodegradability was 4-chlorophenol > 3-chlorophenol > 2-chlorophenol suggesting

that the substrate specificity of phenol hydroxylase may be related to the position of the hydroxyl group in the chlorophenol molecule.

1.5.2.2 Ring cleavage.

The critical step in the aerobic degradation of chlorophenols is cleavage of the aromatic ring. As is the case during the degradation of non-halogenated aromatics, ring cleavage may take place using either the *ortho-* or the *meta-* pathway. Aromatic compounds found naturally in the environment, such as phenol and benzene, are typically broken down via the *meta-* cleavage pathway. While methyl- substituted aromatic compounds are also successfully degraded via the *meta-* cleavage pathway, chlorinated aromatics are generally broken down via the *ortho-* pathway.

1.5.2.2.1 The *ortho-* cleavage pathway.

Normal 1,2-dioxygenases involved in catechol metabolism exhibit low activities for halogenated substrates (Schmidt and Knackmuss, 1980). Compared with ordinary dioxygenases, enzymes with broad substrate specificities that have a high affinity for chloroaromatic substrates have been identified (Dorn and Knackmuss, 1978).

In *Pseudomonas* sp. B13, catechol and chlorocatechol were assimilated via two separate *ortho-* cleavage pathways. Correspondingly, two types of isofunctional enzymes for ring fission were found. Catechol 1,2-dioxygenase (pyrocatachase Type I) was present in cells grown on benzoate and was highly specific for catechol, while chlorocatechol 1,2-dioxygenase (pyrocatechase Type II) was induced when 3-chlorobenzoate was the growth substrate. Chlorocatechol 1,2-dioxygenase had relaxed specificities and high activities for chlorinated aromatic compounds (Knackmuss and Hellwig, 1978). Chlorocatechols interfere with the oxygen-binding of the normal catechol 1,2-dioxygenase due to the steric and inductive effects of the chloride atom. This results in the accumulation of the chlorocatechols which polymerise due to auto-oxidation causing a brown/black coloration of the culture medium.

Transformation of chlorocatechols via the "modified" *ortho-* cleavage pathway by chlorocatechol 1,2-dioxygenase yields chloromuconates (Fig. 4) (Schlömann, 1994). The chlorocatechol is oxidised at both carbon atoms carrying hydroxyl substituents, yielding two carboxylic acid (-COOH) groups at the end of a chain. Ring cleavage occurs between the hydroxyl-substituted carbons. *Ortho-* cleavage of 3-chlorocatechol yields 2-chloro-*cis,cis*-muconate while cleavage of 4-chlorophenol results in the production of 3-chloro-*cis,cis*-muconate.

1.5.2.3 Cycloisomerisation.

Following ring cleavage via the modified *ortho-* pathway, the products produced undergo cycloisomeration as described by Schmidt and Knackmuss (1980). The chloro-substituted *cis,cis*-muconates are transformed resulting in the production of dienelactones (4-carboxymethylenebut-2-en-4-olides). This is a critical step in the degradation of chlorophenols as it results in the elimination of the chloro-substituent (Fig. 4). The enzyme involved, chloromuconate cycloisomerase (cycloisomerase Type II) is homologous to muconate cycloisomerase (cycloisomerase Type I) involved in the degradation of catechol via the *ortho-* pathway.

Both cycloisomerases catalyse similar reactions and differ with respect to their substrate specificities and product formation. Cycloisomerase II has been shown to exhibit higher activities with substituted-muconates than with unsubstituted substrates (Schmidt and Knackmuss, 1980). Cycloisomerase I responsible for the conversion of *cis,cis*-muconate to muconolactone also converts 2-chloro-*cis,cis*-muconate to mixtures of 2- and 5-chloromuconolactone by carrying out both 1,4 and 3,6-cycloisomerisations of the substrate (Vollmer *et al.*, 1994) and 3-chloro-*cis,cis*-muconate to form the bacteriotoxic protoanemonin (Blasco *et al.*, 1995). Cycloisomerase I is unable to further metabolise 2- or 5-chloromuconolactone. Cycloisomerase II however converts 2-chloro-*cis,cis*-muconate to the intermediate 5-chloromuconolactone and then dehalogenates the metabolite to form *trans-*

dienelactone, while 3-chloro-*cis,cis*-muconate is converted to *cis*-dienelactone (Vollmer *et al.*, 1998).

The final steps in the degradation of chlorophenols is the conversion of dienelactones produced following cycloisomeration into metabolites of the normal metabolism of the bacterium. This is achieved by the conversion of *cis*- and *trans*-dienelactone, by the enzyme dienelactone hydrolase, to produce maleylacetate which is readily converted to intermediates of the TCA cycle, and therefore may be utilised for the production of biomass, energy, carbon dioxide and water, resulting in complete degradation.

In contrast to the ordinary *ortho*- cleavage pathway, the genes for the modified *ortho*-pathway are generally located on catabolic plasmids (van der Meer *et al.*, 1992). The most extensively studied genes encoding the modified *ortho*- cleavage pathway are the *clc*ABD operon of *P. putida* (pAC27), the *tfd*CDEF operon of *Alcaligenes eutrophus* JMP134 (pJP4) and the *tcb*CDEF of *Pseudomonas* sp. strain P51 (p51). It has been shown that the chlorocatechol 1,2-dioxygenase genes appeared to be linked to the genes for the rest of the pathway in a single operon and that the chlorocatechol 1,2-dioxygenase and chlorocycloisomerase genes of these operons are significantly homologous to the counterparts of the ordinary *ortho*- cleavage pathway genes, while the relationship between dienelactone hydrolase and 3-oxoadipate enol-lactone hydrolase is more distant (Schlömann, 1994).

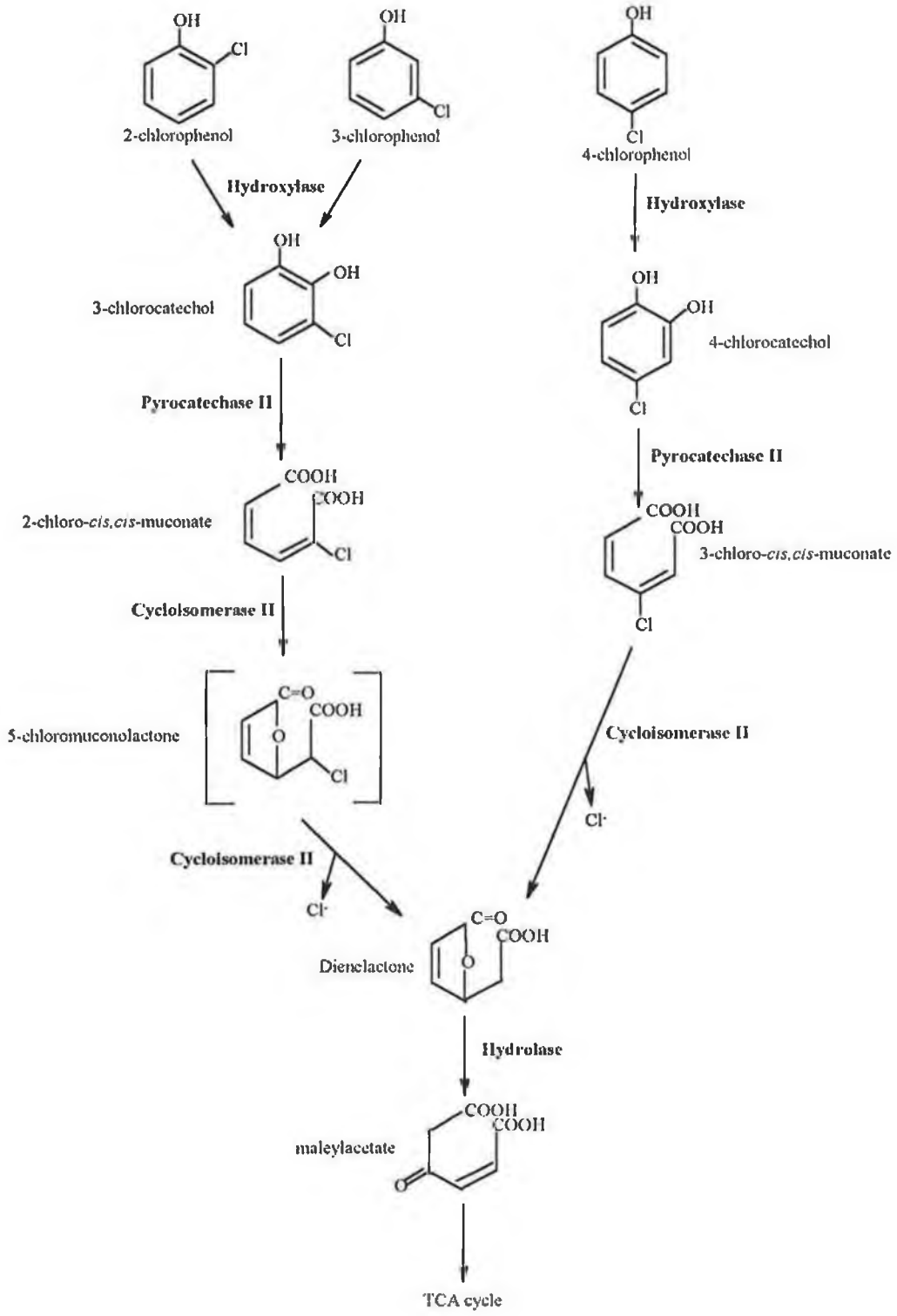

Figure 4. The degradation of mono-chlorophenols via the modified *ortho*- pathway (Knackmuss, 1981).

1.5.2.4 The *meta*- cleavage pathway.

The alternative route to *ortho*- cleavage of chlorocatechols, is ring cleavage via the *meta*- cleavage pathway by the enzyme catechol 2,3-dioxygenase. In general, *ortho*-cleavage is required to bring about complete mineralization of chlorophenols as *meta*-cleavage can result in dead-end pathways. Dead-end pathways result in two ways, generation of a metabolite which may not be degraded further or generation of a metabolite which acts as a specific enzyme inhibitor. The latter is called "lethal synthesis" and can result from competitive inhibition of an essential enzyme, removal of an essential metal co-factor from the enzyme's active site by a chelating agent, or from an irreversible form of inhibition (Bartels *et al.*, 1984).

Generally *meta*- cleavage of 3-chlorocatechol, produced following hydroxylation of 2- and 3-chlorophenol, results in the inactivation of catechol 2,3-dioxygenase. Two mechanisms have been proposed by which 3-chlorocatechol may interfere with the activity of catechol 2,3-dioxygenase. The first way is by 3-chlorocatechol itself, acting as a chelating compound (Klecka and Gibson, 1981). 3-chlorocatechol may chelate the iron co-factor required for the catabolic activity of catechol 2,3-dioxygenase causing a large build up of chlorocatechols. Following chelation, enzyme activity may be reactivated by treatment with ferrous iron and a reducing agent. Alternatively, *meta*-cleavage of 3-chlorocatechol can lead to the production of a highly reactive acyl chloride, 5-chloroformyl-2-hydroxypenta-2,4-dienoic acid, which has been proposed to act as a suicide compound, binding irreversibly to catechol 2,3-dioxygenase with a subsequent release of chloride, destruction of metabolic activity and an accumulation of chlorocatechols (Fig. 5) (Bartels *et al.*, 1984). In both cases the accumulated chlorocatechols then polymerise due to auto-oxidation resulting in the production of brown/black coloured chlorocatechol polymers.

The complete degradation of chloro-aromatics via 3-chlorocatechol by a *meta*-cleavage pathway has been demonstrated in the organism *P. putida* GJ31 (Mars *et al.*, 1997) but is extremely rare. The proposed pathway by which *P. putida* GJ31 degrades

3-chlorocatechol is shown in Figure 6. *P. putida* GJ31 appears to possess a catechol 2,3-dioxygenase which is able to convert 3-chlorocatechol to 2-hydroxy-*cis,cis*-muconic acid, an intermediate of the *meta*- cleavage of catechol, avoiding the rapid inactivation of the enzyme by the highly reactive acyl chloride usually associated with the *meta*- cleavage of catechol 2,3-dioxygenase. 2-hydroxy-*cis,cis*-muconic acid can then be degraded by the normal *meta*- cleavage pathway involved in the degradation of catechol.

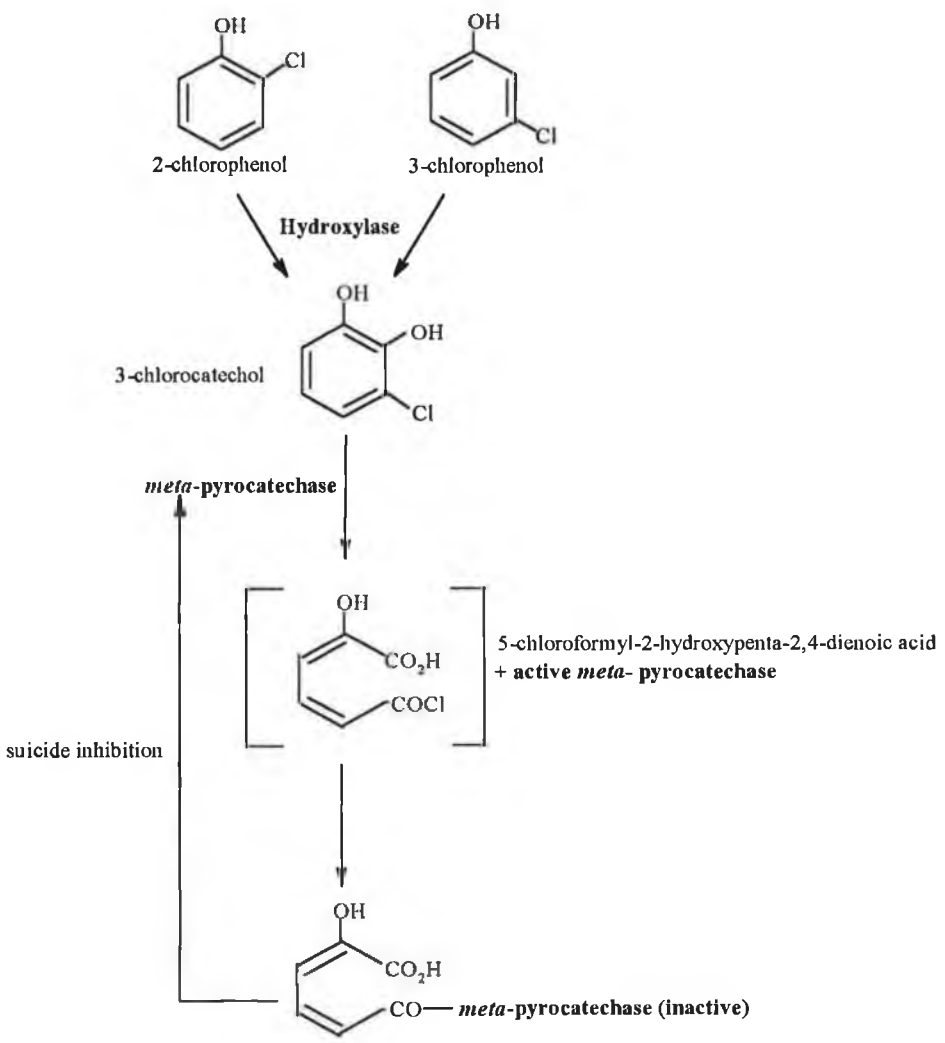

Figure 5. The *meta*- cleavage of 3-chlorocatechol (Bartels *et al.*, 1984).

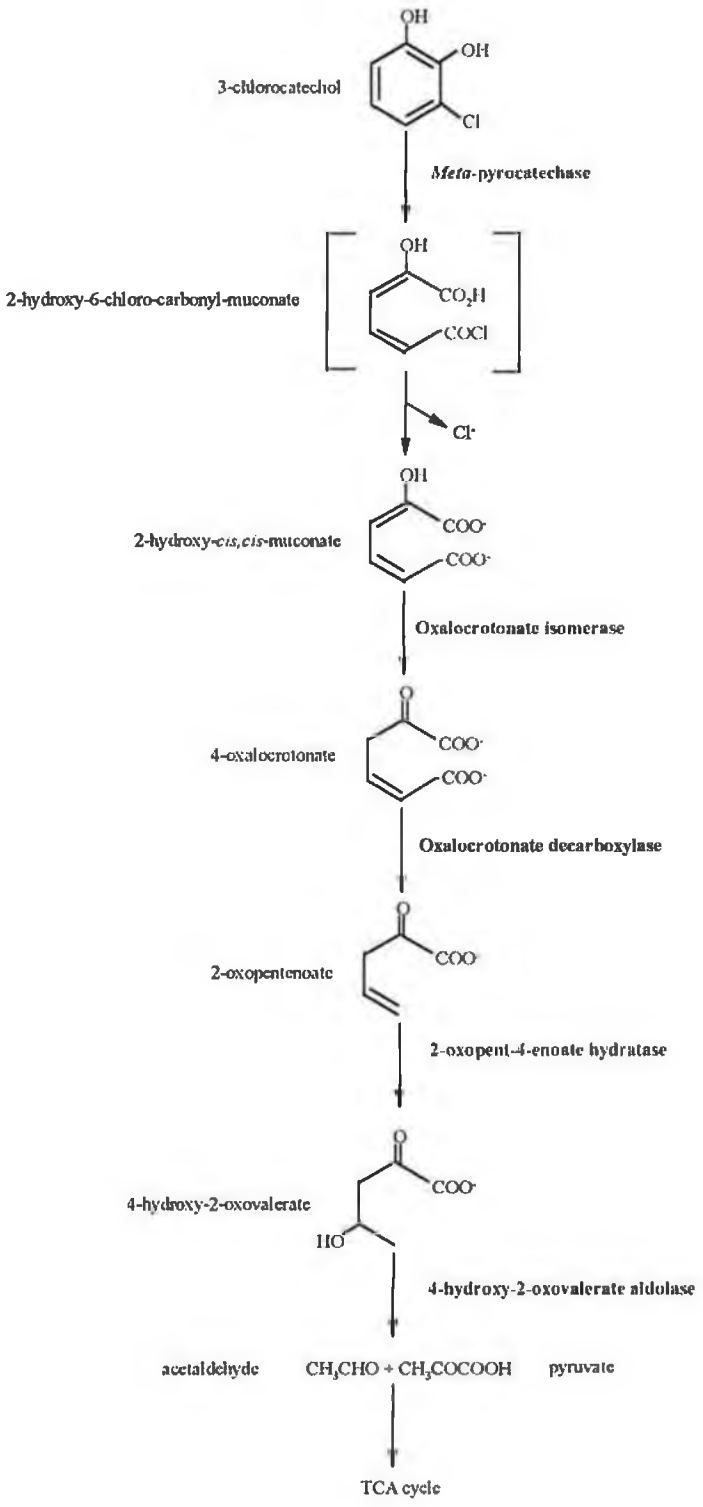

Figure 6. The *meta*- cleavage of 3-chlorocatechol by *Pseudomonas putida* GJ31 (Mars *et al*., 1997).

The *meta-* cleavage of 4-chlorocatechol, produced following metabolism of 4-chlorophenol, results in the production of a chlorinated aliphatic compound, 5-chloro-2-hydroxy-muconic semialdehyde (Weiser *et al.*, 1994) which has been widely reported as being a dead-end metabolite (Reineke *et al.*, 1982; Westmeier and Rehm, 1987). Accumulation of this compound, resulting from the fact that this product ordinarily cannot be further metabolised, causes an intense lime/yellow coloration of the culture medium (Knackmuss, 1981).

However recent reports have shown that further metabolism of 5-chloro-2-hydroxymuconic semialdehyde may occur, resulting in complete degradation of 4-chlorophenol via a *meta-* cleavage pathway (Sung Bae *et al.*, 1996; Hollender *et al.*, 1997). Although the exact mechanism by which 4-chlorophenol is degraded via 4-chlorocatechol by a *meta-* cleavage pathway is unclear, it is likely to be similar to a proposed mechanism as described by Arnesdorf and Focht (1995) for the degradation of 4-chlorobenzoate (Fig. 7).

5-chloro-2-hydroxymuconic semialdehyde is transformed to 5-chloro-2-hydroxymuconic acid and 5-chloro-2-hydroxy-pent-2,4-dienoic acid (enol) by the enzymes 2-hydroxymuconic semialdehyde hydrolase, 4-oxalocrotonate isomerase and 4-oxalocrotonate decarboxylase. 5-chloro-2-hydroxy-pent-2,4-dienoic acid is then transformed to 5-chloro-4-hydroxy-2-oxopentanoic acid which is further transformed to chloroacetic acid and pyruvic acid. Chloroacetic acid may then be dehalogenated to form glycolate, which may be utilised along with pyruvic acid in the TCA cycle (McCullar *et al.*, 1994) leading to the production of biomass, energy, carbon dioxide and water.

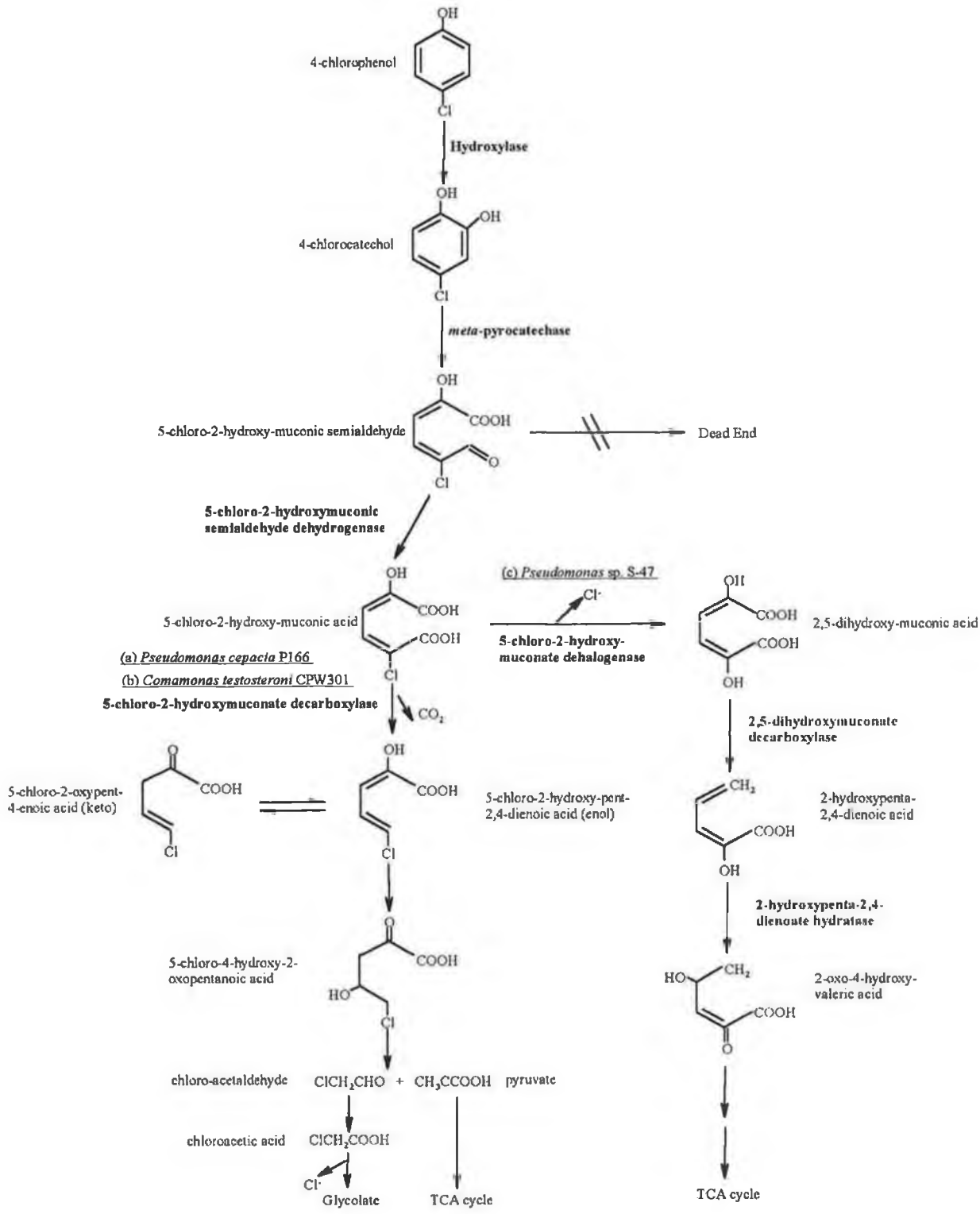

Figure 7. The *meta*- cleavage of 4-chlorophenol ((a) Arnesdorf and Focht, 1995; (b) Hollender *et al.*, 1997; (c) Seo *et al.* 1998).

Similarly, Seo *et al.* (1997 & 1998) reported the complete degradation of 4-chlorobenzoate, via 4-chlorocatechol, using a *meta-* cleavage pathway by a *Pseudomonad*, sp. S-47 (Fig. 7). They described how 4-chlorocatechol was initially transformed to 5-chloro-2-hydroxymuconic semialdehyde and then 5-chloro-2-hydroxymuconic acid. *Pseudomonas* sp. S-47 appeared to dehalogenate at this stage, converting 5-chloro-2-hydroxymuconic acid to 2-hydroxypenta-2,4-dienoic acid via 2,5-dihydroxy-muconic acid. 2-hydroxypenta-2,4-dienoic acid is a normal metabolite following the degradation of catechol via the *meta-* pathway and was easily metabolised to form pyruvate and acetaldehyde which may be utilised by the TCA cycle.

Despite these examples, the *meta-* cleavage of chloroaromatics usually results in dead-end pathways, resulting in large accumulations of polychlorinated catechol polymers. The destruction of *meta-* cleavage activity by the chlorocatechols and their inefficient oxidation by unmodified *ortho-* cleavage enzymes explains the instability and dark coloration of activated sludges suddenly loaded with chloroaromatics (Bartels *et al.*, 1984). Consequently the degradation of chloroaromatics in wastewaters generally requires the action of the modified *ortho-* cleavage pathway for their efficient removal from wastewaters.

1.6 Micro-organisms reported to degrade chloroaromatic compounds.

A variety of micro-organisms have been shown to be capable of the aerobic degradation of chloroaromatic compounds. Degradation of the mono-chlorophenols has been demonstrated using both pure cultures and mixed cultures.

1.6.1 Pure cultures.

The tendency of microbiologists to work with pure strains has led to the majority of chloroaromatic biodegradation studies to be carried out using isolated pure strains. Of

the strains used, the most common genus used is *Pseudomonas*. It appears that *Pseudomonas* strains have a remarkable potential to evolve entire catabolic sequences for specific degradative pathways. These organisms are ubiquitous in nature, being found in large numbers in all major natural environments, terrestrial, freshwater and marine. They are also known to have a remarkable nutritional versatility and are capable of utilising a wide range of organic compounds as growth substrates.

Many *Pseudomonas* strains have been shown capable of degrading a wide range of chlorinated aromatic compounds both as sole carbon source via the *meta-* and *ortho-* cleavage pathways. *Pseudomonas* sp. B13 is the most famous chloroaromatic degrader. It was isolated for the degradation of 3-chlorobenzoate and has been shown to be capable of the degradation of 4-chlorophenol as sole carbon source and the cometabolism of 2- and 3-chlorophenol (Knackmuss and Hellwig, 1978). Degradation takes place via the modified *ortho-* pathway which is generally required for complete degradation. This *ortho-* cleavage ability was transferred to a strain of *Alcaligenes*, sp. A-7 possessing a broad spectrum phenol hydroxylase, resulting in a transconjugant strain of *Alcaligenes*, sp. A 7-2, capable of the degradation of 2- and 4-chlorophenol and the cometabolism of 3-chlorophenol (Schwein and Schmidt, 1982).

A strain of *P. putida*, CLB250, has been shown to be capable of the degradation of 2-chlorobenzoate as sole carbon source, with stoichiometric releases of chloride using the *ortho-* cleavage pathway (Engesser and Schulte, 1989).

Pseudomonas pickettii LD1 (now *Rhalstonia pickettii*) was shown to be capable of the complete degradation of 2-, 3- and 4-chlorophenol as sole carbon source. Chlorophenol degradation was accompanied by a stoichiometric release of chloride and the absence of any chlorocatechol accumulations in the culture medium suggesting that metabolism was via the *ortho-* cleavage pathway (Fava *et al.*, 1995).

Pseudomonas strains capable of the degradation of chloroaromatics via the *meta-* cleavage pathway have also been isolated. Spain and Gibson (1988) showed the

transformation of mono-chlorophenols to their corresponding chlorocatechols by *P. putida* F1. However, ring cleavage was via the *meta-* cleavage pathway, leading to an accumulation of the chlorocatechols.

P. putida GJ31 has been shown to be capable of growth simultaneously on toluene and chlorobenzene and possesses a *meta-* cleavage pathway capable of the complete degradation of 3-chlorobenzoate via the intermediate 3-chlorocatechol (Mars *et al.*, 1997).

Arnesdorf and Focht (1994) have reported a *Pseudomonas cepacia* strain, P166, capable of the degradation of mono-chlorobiphenyls via the *meta-* cleavage pathway. Seo *et al.* (1997) have shown the degradation of 4-chlorobenzoate via a *meta-* cleavage pathway by *Pseudomonas* sp. S-47. Degradation of 2- and 3-chlorobiphenyl resulted in a dead-end pathway, while complete degradation of 4-chlorobiphenyl was demonstrated.

Pseudomonads are not the only genus for which degradation of chloroaromatics has been demonstrated. Sung Bae *et al.* (1996) showed the complete degradation of 4-chlorophenol via a *meta-* cleavage pathway by *Comamonas testosteroni* CPW301. Similarly Hollender *et al.* (1997), also demonstrated total metabolism of 4-chlorophenol by *Comamonas testosteroni* JH5 via a *meta-* cleavage pathway.

A strain of *Rhodococcus opacus*, GM-14, was shown to be capable of the complete degradation of all three mono-chlorophenols, with a stoichiometric release of chloride (Zaitsev *et al.*, 1995) while immobilisation allowed a strain of *Pseudomonas testosteroni* to degrade low concentrations of 3- and 4-chlorophenol to completion (Lu *et al.*, 1996).

Degradation of mono-chlorophenols has also been demonstrated by a strain of yeast, *Rhodotorula glutinis* (Katayama-Hirayama *et al.*, 1994). Degradation was via the *ortho-* cleavage pathway, leading to complete degradation of 3- and 4-chlorophenol.

The non-specific ligninolytic activities of some fungi, particularly white rot fungi, allows then to degrade complex mixtures of pollutants, including substituted aromatics. *Phanerochaete chrysosporium* has been shown capable of degrading 2-chlorophenol (Lewandowski *et al.*, 1990; Wang and Ruckenstein, 1994). Rubio Pérez *et al.* (1997) reported the degradation of 2-, 3- and 4-chlorophenol by *Phanerochaete chrysosporium*. The extraordinary ability of micro-organisms to metabolise xenobiotics was demonstrated by the isolation of a thermophilic *Bacillus* sp., A2, capable of the biotransformation of 2-chlorophenol to 3-chlorocatechol at 60 °C (Reinscheid *et al.*, 1996).

1.6.1.1 *Pseudomonads.*

The term *Pseudomonad* is used to describe strictly aerobic, Gram-negative, non-sporulating bacteria. They are oxidase positive, non-acid fast rods, which are generally straight but maybe slightly curved, 0.5 - 1 μm in diameter and 1.5 - 5 μm in length (Bergey's Manual of Systematic Bacteriology, 1984). They are generally motile, with polar flagellae and generally do not ferment carbohydrates, do not fix nitrogen and are not photosynthetic. The optimum growth temperature for most strains is 28° C but many are capable of growth in the range of 4 - 43° C.

Members of the genus *Pseudomonas* are free-living organisms and occupy a dominant position in the biosphere in terms of variety of habitats and the number of species in a given habitat. *Pseudomonas* species are subdivided on the basis of rRNA homology into five similarity groups. Group I is the largest, including scientifically relevant strains such as *P. aeruginosa*, *P. fluorescens* and *P. putida*. Group I can be further divided into the fluorescent and non-fluorescent species.

One of the most striking properties of members of this genus is their remarkable nutritional versatility. They play an important role in decomposition, biodegradation and the carbon and nitrogen cycles. Organic compounds such as alcohols, aliphatic acids, amides, amines, amino acids, aromatic compounds, carbohydrates and

hydrocarbons are all readily used by *Pseudomonas* species as growth substrates. Of these different compounds, the aromatic compounds are particularly interesting due to the biochemical intricacies of the various pathways by which they are metabolised (Palleroni, 1986). The ability of *Pseudomonads* to utilise such a wide range of compounds makes them a vital component of any biodegradative system, particularly those involved in the treatment of wastewaters derived from the chemical industry.

1.6.2 Pure cultures versus mixed cultures.

While microbiologists are more likely to work with pure cultures, communities having only one species are rare in nature except those monospecific communities associated with extreme environments where only a few species can survive or proliferate and where biodegradation is less likely to occur. Pure cultures are extremely useful for elucidating biodegradation pathways that might be environmentally significant, but the biodegradative capacity of microbial communities, both quantitatively and qualitatively, as a general rule is much greater than that of pure cultures, particularly where xenobiotics are concerned (Grady Jr, 1985). Toxicity is also less likely to influence degradation, as there is a greater chance that a micro-organism capable of detoxifying the chemical will be present in a mixed culture. Although little is known in a mechanistic sense about microbial interactions, it is known that the complete degradation of a compound may require the participation of several microbial species or strains, since the genetic capability to mineralise the compound may not lie within a single micro-organism.

Mixed populations of micro-organisms are difficult to avoid in wastewater treatment facilities. Because wastewater flow rates are usually relatively large, management of the microbial population in terms of species present would be extremely difficult. Wastewaters are also normally open to the environment and therefore easily contaminated with organisms from the air, soil and other sources. Thus treatment systems are constantly open to contamination with opportunistic organisms and

therefore the usage of pure cultures in the treatment of wastewaters is unusual in field applications.

Mixed cultures are also known to play an important role in the natural evolution of metabolic pathways. In order for the genetic information of a pure culture to change, mutations must take place, and successful mutations are rare events. Successful mutations usually only increase the activity of a single enzyme by slight changes in conformation or regulation. However, degradative pathways involve a series of enzymes, so a single mutation is unlikely to result in the evolution of whole pathways. Mixed cultures contain a vastly larger genetic pool of information than a pure culture which can be exchanged via gene transfer from organism to organism. If the diverse genetic information needed to construct a complete metabolic pathway could be transferred from the various members of the population into a single organism, a new complete pathway would have evolved.

1.6.3 Plasmids and biodegradation.

Plasmids are genetic elements found outside the chromosome within the cell (Maniatis *et al.*, 1982). They can replicate autonomously independent of the chromosome, and although considered non-essential for the cell, they often perform secondary functions that are vital to the cell under certain conditions. These might include drug resistance, heavy metal resistance or degradative capabilities. Catabolic plasmids are widespread in nature and may confer ecologically advantageous characteristics on an organism.

The degradation of many xenobiotics has been shown to be dependent upon genetic information contained on catabolic plasmids. The majority of catabolic plasmid-bearing strains studied are *Pseudomonads* or *Pseudomonas*-like species (Sayler *et al.*, 1990). Plasmids responsible for the degradation of a variety of organic xenobiotic compounds have been isolated from nature. These include the TOL plasmid responsible for the degradation of toluene, the NAH plasmid responsible for naphthalene degradation, pAC25, linked to the degradation of 3-chlorobenzoate and pB13 responsible for 4-

chlorobenzoate degradation (Sayler *et al.*, 1990). Many larger plasmids carry genes allowing for plasmid transfer between organisms via conjugation. As a result degradative genes contained on a plasmid within a mixed microbial community may be exchanged throughout the members of the community. This can result in the assembling of degradative pathways, possibly within single organisms and distributing degradative capabilities throughout the mixed culture. This has been demonstrated with the transfer of a plasmid, pD10, from a *Pseudomonas* strain, UWC1, encoding for 3-chlorobenzoate degradation to indigenous members of an activated sludge population (McClure *et al.*, 1989).

1.6.4 Mixed cultures.

In the natural environment, communities having only one species are rare, and biodegradation is almost certainly carried out by mixed cultures. Despite this, the greater majority of biodegradation studies carried out, have used pure cultures. However, the degradation of chloroaromatics using mixed cultures from a wide variety of sources has been demonstrated.

A limited number of reports have been published describing the degradation of mono-chlorophenols by commercial bioaugmentation products. Three commercial preparations were tested for their ability to degrade phenol, 2-chlorophenol and 2,4-dichlorophenoxyacetic acid in aerated biological batch reactors at room temperature (Lewandowski *et al.*, 1986). The products used, Hydrobac produced by Polybac Corporation, BI-CHEM DC-1006/7 produced by Sybron Corporation and Liquid Live Micro-organisms (LLMO) produced by General Environmental Sciences were compared to a mixed culture from a municipal wastewater treatment plant. The municipal population was found to perform better than any of the commercial populations. Performance of the municipal population however was improved following addition of the products at a ratio of 1 (commercial product) : 20 (municipal population). However, the level of addition required for enhancement was considered as being commercially unfeasible.

Goulding *et al.* (1988) studied the biodegradation of a range of substituted aromatics using an unnamed commercial bioaugmentation product. The product consisted of five strains of *Pseudomonad*, one *Klebsiella*, four *Rhodococci* and two fungal strains. They reported the effective removal of many chlorinated aromatics including the removal of 2-, 3- and 4-chlorophenol within 100 hrs. It was not specified in the publication whether removal resulted in complete metabolism of the xenobiotics.

The degradation of mono-chlorophenols by natural mixed cultures has also been described. A defined, two-species mixed culture was shown to be capable of the degradation of 3-chlorobenzoate and benzoate (Knackmuss, 1982). When the benzoate degrader, *Alcaligenes eutrophus* ATCC 17697 was subjected to the benzoate/3-chlorobenzoate mixture, growth of the pure strain was severely inhibited by 3-chlorobenzoate. However, when a two-membered mixed culture consisting of *Alcaligenes eutrophus* and the chlorinated aromatic degrader, *Pseudomonas* sp. B13, was used, both benzoate and 3-chlorobenzoate were completely degraded. In addition to *Pseudomonas* sp. B13 completely degrading 3-chlorobenzoate and *Alcaligenes eutrophus* degrading benzoate, *Pseudomonas* B13 transformed benzoate into *cis,cis*-muconate which was then degraded by *Alcaligenes eutrophus*, while *Alcaligenes eutrophus* transformed 3-chlorobenzoate into 3-chlorocatechol, which was completely degraded by *Pseudomonas* sp. B13. This demonstrated the mutualistic relationship that exists between members of a mixed culture during degradation of xenobiotics.

A synthetic sewage, consisting of a mixed culture of *Pseudomonas extorquens* and *Alcaligenes* sp. A7, phenol, acetone and alkanols plus 4-chlorophenol was studied for its ability to degrade mono-chlorophenols (Schmidt *et al.*, 1983). Degradation of mono-chlorophenols by the two-membered mixture resulted in an accumulation of dead-end products following *meta*- cleavage. However, when *Pseudomonas* sp. B13 was added to the mixture, the initial *meta*- cleavage activity of the three-membered population was replaced with *ortho*- cleavage activity, leading to the complete degradation of mono-chlorophenols by the defined mixed culture.

The degradation of chlorophenols in undefined mixed cultures has also been studied. A mixed culture capable of the degradation of phenol and solvents was isolated from soil and sludge samples and examined for its ability to degrade mono-chlorophenols (Schmidt, 1987). The mixed culture was found to degrade mono-chlorophenols via the unproductive *meta-* cleavage pathway, again leading to dead-end metabolites. However, when the undefined mixed culture was supplemented with *Pseudomonas* sp. B13, the *meta-* cleavage activity was switched off, and replaced with *ortho-* cleavage activity, leading to complete degradation.

The ability of activated sludges to degrade chlorophenols have been studied by many authors including Arbuckle and Kennedy (1989) who studied the effect of the addition and removal of 4-chlorophenol on activated sludge and Ettala *et al.* (1992) who studied the degradation of chlorophenols in wastewater using municipal sewage.

1.7 Activated sludge.

Bioremediation is the result of the biological, mainly microbial, breakdown or biodegradation of contaminating compounds into less complex compounds and ultimately to water and either carbon dioxide or methane depending on whether degradation is aerobically or anaerobically mediated. In general biodegradation of most organic pollutants will occur at a faster rate under aerobic conditions. The most widely used aerobic biological waste treatment process for the decontamination of waste streams is the activated sludge process. The activated sludge process was originally developed by Ardern and Lockett (1914) at the Manchester sewage works. Initially wastewater was treated simply by aeration and after a certain period of time, they stopped aeration, let the flocs settle, decanted the supernatant, filled in wastewater, and repeated the cycle again and again. The settled sludge was termed "Activated Sludge". The initial process had to be operated manually and encountered a lot of operational problems (Kayser, 1999). Since then the process has been improved

through a number of engineering and operation developments, while the underlying theory remains the same.

The activated sludge process is commonly used for the treatment of both domestic sewage and industrial wastewater. In Ireland most chemical plants treat their waste effluents in aerobic biological treatment systems (O'Flaherty, 1989). The active biological component of activated sludge is not a pure culture, but an association of bacteria, yeast, fungi, protozoa and higher organisms. These organisms grow on the incoming waste and interact with one another. The activated sludge community is determined by the waste characteristics and process operation parameters of the sludge system, but is normally dominated by organotrophic bacteria. The biological treatment process depends on the principle that bacteria are ubiquitous and that the most effective species of micro-organisms for a given environment will eventually establish themselves in that environment (Zachopoulis and Huag, 1990). The predominant microbes in activated sludge processes are chemoheterotrophic bacteria. The microbes best suited are those with the ability to grow as either microbial aggregates or those able to become directly associated with such aggregates so as to be recycled. Although the concept of "microbial community" is generally accepted in microbial ecology, little insight has been obtained on how rapidly communities evolve and what their principle characteristics are.

1.8 Bioaugmentation

The inability of micro-organisms present in natural environments such as fresh and marine waters, sewage and soils to perform biodegradative processes on a given molecule can be due to various causes. Compounds which are ordinarily readily degradable may not be biodegraded due to physical/chemical conditions such as oxidation-reduction conditions, availability of carbon, nitrogen and phosphorus and environmental conditions such as pH, salinity and water content. However, under ideal degradative conditions, degradation may not occur due to the absence of pre-existing enzymes evolved for the degradation of xenobiotic compounds making the compound

inherently resistant to degradation. Even where the degradative capacity exists within a wastewater treatment facility, degradation of xenobiotics must be completed during the residence time in the treatment system. Thus, merely the presence of a degradation potential within a wastewater ecosystem is not sufficient, but degradation rates must be high and degradation must be faster than the residence time of bacteria in suspended systems. Problems with micro-organisms limiting contaminant biodegradation may be overcome by (a) isolating or genetically engineering better degrading strains or consortia, (b) improving formulation and inoculation methods for introduced micro-organisms and/or (c) improving degradative activity by indigenous micro-organisms in a process known as bioaugmentation (Providenti *et al.*, 1993).

Bioaugmentation is the application of indigenous or allochthonous wild-type or genetically-modified organisms to polluted hazardous waste sites or bioreactors in order to accelerate the removal of undesired compounds (Van Limbergen *et al.*, 1998). Bioaugmentation fortifies biomass with micro-organisms that have been selected to degrade specific compounds and allows the treatment facility to better deal with certain situations such as shock loading and to tackle contaminants not broken down by the indegenous population, resulting in improved treatment.

Bioaugmentation can increase the biological diversity and activity of a population. An increase in bacterial diversity increases the gene pool available to the population in times of stress. The ability of the introduced organisms to transfer genetic information to the indigenous population may be of equal or greater importance than the survival of the organism itself and has been reported to play a significant role in the adaptation of populations to the presence of recalcitrant compounds (Sayler *et al.*, 1990; van der Meer *et al.*, 1992). As many metabolic pathways for the degradation of xenobiotic compounds are plasmid encoded, bioaugmentation with such strains could lead to an exchange of such information throughout a mixed population, increasing the number of strains capable of degradation, thus improving biodegradation.

Bioaugmentation has been demonstrated to :

1. Reduce process instability caused by fluctuations in organic loadings.
2. Improve the degradation of target substrates.
3. Improve removal efficiencies for biological oxygen demand (BOD) and chemical oxygen demand (COD).
4. Speed recovery from plant upsets, or prevent upsets.
5. Reduce the inhibitory effects of toxic substrates.
6. Improve mixed liquor flocculation.
7. Reduce sludge and scum from aerobic and anaerobic digesters and lagoon deposits.
8. Induce or stabilise nitrification and recover nitrates. (Huban and Plowman, 1997)

All of the above result in a more efficient treatment process thus lowering operational and energy costs.

There are three basic methods by which a micro-organism or group of micro-organisms (consortium) capable of degrading a specific compound can be obtained : (1) selective enrichment, (2) the use of commercial products and (3) genetic engineering. Selective enrichment is designed to increase the population of a specific micro-organism(s) relative to the initial inoculum. Inocula can be obtained from a variety of sources including, sludge, contaminated groundwater or soils that have demonstrated degradative ability. The strategy involves providing conditions under which micro-organisms capable of degrading the compound of interest may grow. By repeated subculturing into systems containing the xenobiotic as sole carbon source, the selective pressure under which micro-organism(s) might become established can be maintained.

If time constraints or facilities negate selective enrichment, it is possible to purchase highly adapted micro-organisms. Micro-organisms are isolated from sites which are highly contaminated with organic pollutants and by means of selection or mutation, an increase in the degradative activities of these isolates is achieved. Pure strains are

preserved by air or freeze-drying techniques and then combined into mixed cultures for use in bioaugmentation (Walter, 1997).

The use of modern molecular biology techniques has allowed for the construction of genetically modified microbes capable of the degradation of toxic recalcitrant compounds. Portions of metabolic pathways which are genetically encoded within a number of different organisms may be fitted together into a single plasmid allowing for a single, complete metabolic pathway to be contained within a single isolate. However, the addition of such a genetically engineered micro-organism (GEM) to a treatment plant constitutes a release of such organisms into the environment, as most plants are open systems with final effluents commonly being discharged into local waterways. Unfortunately, the lack of knowledge on the fate and effects of GEMs following their introduction into the environment is hindering progress (Singleton, 1994). Studies into the persistence of introduced organisms, their rate of gene transfer to indigenous populations, their escape from the original application site to surrounding areas and their effect on ecosystems must be carried out before their usage can be considered. There is considerable public and legislative opposition to the release of engineered organisms which must be considered and therefore commercial products for bioaugmentation do not contain GEMs.

The introduction of bacteria with degradative capabilities into a variety of mixed cultures has been studied. The addition of pure strains of bacteria to activated sludge for the degradation of phenol has been the focus of a number of recent studies. Watanabe *et al.* (1996) studied the effect of adding *Alcaligenes* sp. R5, *Acromobacter* sp. E1 and *Alcaligenes* sp. E2 into three separate activated sludge systems for the degradation of phenol. The addition of strains E1 and E2 increased the rate of phenol degradation from 10 days in the uninoculated activated sludge to 2 and 3 days respectively. Strain R5 did not increase the rate of phenol degradation.

Selvaratnam *et al.* (1997) studied the effect of the addition of *Pseudomonas putida* ATCC 11172, a phenol degrading strain, to activated sludge for the degradation of

phenol. Augmentation again resulted in increased phenol degradation over that which was seen in uninoculated control sludges.

The augmentation of activated sludge with the GEM, *Pseudomonas putida* BH (pS10-45), for the degradation of phenol was studied by Soda *et al.* (1998). *Pseudomonas putida* BH contains the genetically engineered plasmid (pS10-45) which encodes all the genes required for the degradation of phenol. Shock loading experiments carried out showed that the phenol removal efficiency of the activated sludge inoculated with *Pseudomonas putida* BH (pS10-45) was greatly enhanced in comparison to an uninoculated control activated sludge for as long as the GEM remained within the sludge.

Bioaugmentation has also proved successful for the degradation of chloroaromatics. The bioaugmentation of a synthetic sewage consisting of *Pseudomonas extorquens* and *Alcaligenes* A7 with the strain *Pseudomonas* sp. B13 was studied by Schmidt *et al.* (1983). Augmentation of the mixed culture, which previously degraded mono-chlorophenols using the unproductive *meta-* cleavage pathway, resulted in the complete degradation of the chlorophenols using the modified *ortho-* cleavage pathway.

Pseudomonas sp. B13 has also been used to augment undefined mixed cultures. A mixed culture isolated from soil and sludge samples was augmented for the degradation of phenol, chlorophenols and cresol (Schmidt, 1987). Prior to augmentation, the mixed culture degraded aromatic organics via the *meta-* cleavage pathway. However, following addition of *Pseudomonas* sp. B13, the more productive *ortho-* cleavage pathway was induced, leading to complete degradation. Population analysis showed that members of the indigenous population had acquired the degradative capacity of *Pseudomonas* sp. B13. These isolates demonstrated an ability to degrade a greater range of aromatic compounds than did *Pseudomonas* sp. B13.

Genetically modified forms of *Pseudomonas* sp. B13 have also been used in bioaugmentation studies. Nüßlein *et al.* (1992) used two genetically modified micro-organisms, *Pseudomonas* sp. B13 FR1 (pFRC20P) (FR120) and *Pseudomonas putida* KT2440 (pWWO-EB62) (EB62) to augment activated sludge. Strain FR120 is a genetically modified version of *Pseudomonas* sp. B13 which allows for the degradation of 3-chlorobenzoate and 4-methylbenzoate via a hybrid *ortho*- cleavage pathway, while strain EB62 is a modification of *Pseudomonas putida* KT2440 (pWWO) which allows for the degradation of 4-ethyl benzoate via a modified toluate degradation pathway. Augmentation of the activated sludge for the degradation of 3-chlorobenzoate and 4-methylbenzoate with FR120 allowed for faster and more complete degradation than was observed without augmentation. FR120 also displayed a protective effect on the indigenous population against the toxicity of 3-chlorobenzoate and 4-methylbenzoate at higher concentrations. Augmentation of the activated sludge with KT2440 for the degradation of 4-ethylbenzoate was less successful with degradation rates only slightly increased over the uninoculated activated sludge system.

FR120 was also used to inoculate freshwater microcosms and sediment microcosms for the degradation of 3-chlorobenzoate and 4-methylbenzoate (Heuer *et al.*, 1995). Inoculation of water microcosms with FR120 allowed for degradation of both 3-chlorobenzoate and 4-methylbenzoate which was not achieved by uninoculated wastewater. Similarly FR120 was able to survive in sediment microcosms, bringing about complete degradation of 3-chlorobenzoate and 4-methylbenzoate.

The effects of inoculating another form of *Pseudomonas* sp. B13, SN45RE, into activated sludge for the degradation of mixtures of chlorophenols and methylphenols was studied by Erb *et al.* (1997). *Pseudomonas* sp. B13 SN45RE is a genetic modification of B13 and also allows for the simultaneous degradation of chloro- and methylaromatics via an *ortho*- cleavage pathway. SN45RE differs from FR120 in that all the genes responsible for degradation have been integrated into the chromosome, while the genes remain plasmid encoded for FR120. Following addition into the activated sludge, SN45RE managed to establish itself as part of the indigenous

population following an initial drop in numbers. Addition also significantly increased the rate of 4-chlorophenol/4-methylphenol and 3-chlorophenol/4-methylphenol degradation by the activated sludge. In the absence of SN45RE, degradation was via the *meta-* cleavage pathway leading to the accumulation of toxic metabolites. Following addition of SN45RE, degradation occurred using the *ortho-* cleavage pathway. Augmentation also resulted in a protective effect on the indigenous population. Without addition of the GEM, shock loads of substituted phenols resulted in sharp decreases in indigenous heterotrophic bacteria and the elimination of protozoa and metazoa found in the sludge. Augmentation of the sludge with SN45RE protected the sludge from the toxic effects of the shock loads as there were only small drops in indigenous cell numbers, protozoa numbers and metazoa numbers following addition of high concentrations of substituted aromatics.

Wilderer *et al.* (1991) described how the addition of *Pseudomonas putida* PRS 2015 (pAC27), a strain capable of the complete degradation of 3-chlorobenzoate, to activated sludge, brought about improved degradation of 3-chlorobenzoate. The supplemented activated sludge system degraded 3-chlorobenzoate at considerably higher rates than were achieved by the unsupplemented system.

In contrast, the addition of bacteria of degradative capacity to mixed cultures does not always lead to improved degradation as was noted for phenol degradation by *Alcaligenes* sp. R5 (Watanabe *et al.*, 1996). McClure *et al.* (1989) reported that the introduction of a strain that harboured a plasmid carrying a gene encoding for the degradation of 3-chlorobenzoate, *P. putida* UWC1 (pD10), did not enhance degradation in a laboratory scale activated sludge unit. Although *P. putida* UWC1 survived within the activated sludge unit and plasmid pD10 was stably maintained within the host bacterium, addition to the activated sludge did not enhance the degradation of 3-chlorobenzoate. Plasmid pD10, however, was transferred to autochthonous members of the activated sludge, demonstrating horizontal transfer of genetic material between members of a mixed culture. Two of the indigenous bacteria which were isolated, strain AS2 and a transconjugant, *Pseudomonas putida* 2.8, now

containing the plasmid pD10, were reinoculated into the activated sludge unit and survived at higher numbers than was observed for *P. putida* UWC1. Strain AS2 and *P. putida* 2.8 enhanced the degradation of 3-chlorobenzoate (McClure *et al.*, 1991a).

These studies demonstrate that the ability to metabolise a chemical is a necessary but not a sufficient condition for the organism to effect the transformation of xenobiotics in a natural environment. Reasons suggested for the failure of introduced micro-organisms to degrade pollutants include :

1. The concentration of the compound in nature may be too low to support the growth of the inoculated species.
2. The natural environment may contain substances that are toxic or inhibitory to the growth and activity of the added organisms.
3. The growth rate of the organism may be slower than the rate of predation for example by protozoa, so that the rate of activity of the predators reduces the cell density of the inoculated species.
4. The introduced species are exposed to intense competition with indigenous micro-organisms better adapted to environmental conditions.
5. Wastewaters normally contain a complex spectrum of more easily assimilated carbon sources than xenobiotics and so organisms do not express genes for xenobiotics.
6. Inoculated strains, serving as a "gene pool" survive, but others are not competent for gene transfer.
7. The organisms may fail to flocculate and can be washed out of the system resulting in increased turbidity of the effluent. Non-flocculated organisms are also more susceptible to grazing by protozoa.

   (Goldstein *et al.*, 1985 and Gallert and Winter, 1999)

In spite of this, the approach of using bacterial inocula to destroy pollutants may be feasible if the correct strains are selected for inoculation into the natural environment.

1.9 Flocculation and its importance in bioaugmentation.

Aerobic wastewater treatment relies heavily on the ability of micro-organisms to aggregate, allowing a straight forward separation of the formed microbial biomass and the effluent in the final settling tank (Van Limbergen *et al.*, 1998). As a result the selection of bacteria which possess the ability to flocculate or incorporate themselves into sludge flocs not only improves the aerobic treatment process, but also can improve the chances of successful bioaugmentation. When introduced micro-organisms are incorporated into flocs, they can stay in the treatment system for longer periods of time, thus helping to maintain the degradative capacity in the mixed culture.

It is not quite clear exactly why cellular aggregation occurs. However, there are a number of possible reasons why flocculation might be beneficial to the survival and growth of micro-organisms. Cells might aggregate in order to increase substrate acquisition. In many waste streams, organic wastes can be found in particulate form. By attaching themselves to the particulate waste, cells may degrade these macromolecules effectively increasing the concentration of substrates in the vicinity of the floc available for degradation. Flocs are not uniformly dense structures, but rather channel-rich conglomerates. Through the process of advective flow around and through these channels, attached bacteria might experience, up to a factor of two, a higher substrate availability compared to free swimming bacteria (Bossier and Verstraete, 1996).

Another theory proposed for the flocculation of micro-organisms is aggregation in response to physical stress such as unfavourable environmental growth conditions or a response to chemical stress such as toxic shock loading of chemicals. It is reasonable to suggest that micro-organisms growing in aggregates are sheltered from such stressful conditions. However, there is very little evidence available indicating that environmental stress leads to cellular aggregation (Bossier and Verstraete, 1996). Cellular aggregation has been shown to occur in cases of slow growth or starvation due to depletion of substrate. A strain of *Flavobacterium*, an organism typically found

in activated sludge systems has been shown to flocculate during slow growth in the early stationary phase of batch cultures (Hantula and Bamford, 1991).

Furthermore, flocs provide a protective environment for bacteria against predation from protozoa which feed mainly on free suspended micro-organisms (McClure *et al.*, 1989). Protozoa are not able to reach the bacteria which are located in the inner part of a floc. Therefore, the selective pressure exerted on activated sludge micro-organisms by predatory protozoa makes floc formation more likely. It is likely that flocculation occurs as a result of predation and not due to bacteria sensing the presence of protozoa (Bossier and Verstraete, 1996).

Flocculation of activated sludge occurs in a three stage process (Verstraete and van Vaerenbergh, 1986). Firstly ionic bridges formed by polyvalent cations such as Fe^{3+} and Ca^{2+} bind individual bacterial cells. Next, gluey substances, consisting of polysaccharides and DNA/RNA from dead cells, make up a matrix in which cells are embedded and by which they stick to one another. Finally, the matrix of cells attach themselves to filamentous micro-organisms (sometimes fungi, but particularly bacteria) forming the flocs found in activated sludge.

Selection of bacteria which flocculate, or those which may incorporate themselves into sludge flocs, for use as microbial supplements stand a greater chance of survival following their introduction into mixed cultures, thus improving bioaugmentation.

1.10 Aims of project.

As a result of the persistence and accumulation of chlorophenols and the difficulty with which they are removed from the environment, the microbial degradation of mono-chlorophenols was studied. The aims of the project were :

- To study the degradation of mono-chlorophenols, when supplied as sole carbon source, by a commercial bioaugmentation product, Biolyte HAB, and by *Pseudomonas putida* CP1, in aerobic shaking batch cultures.

- To investigate the biochemical pathways involved in mono-chlorophenol degradation for each inoculum.

- To study the degradation of the mono-chlorophenols when Biolyte HAB was augmented with *Pseudomonas putida* CP1.

2. Materials and methods.

2.1 Materials.

2.1.1 Organisms.

Biolyte HAB

The mixed culture used was a commercially produced bioaugmentation product, Biolyte HAB and was obtained from International Biochemicals Ltd., Dublin. This mixture contained at least eleven micro-organisms comprising bacteria belonging to the genera *Pseudomonas* and *Actinomycetes*, together with a fungus, *Trichoderma harazanium*. Surfactants, cryoprotectants and growth media were also present along with the sterile cereal base. The product was specially formulated to degrade a variety of substituted aromatic compounds.

Pseudomonas putida CP1

P. putida CP1 was obtained from Dr. Fabio Fava, University of Bologna, Italy, who isolated it from nature. The strain was separately maintained on 2-chlorophenol (1.56 mM), 3-chlorophenol (0.78 mM) or 4-chlorophenol (1.56 mM) at 4 °C.

2.1.2 Media.

All media unless otherwise stated were obtained from Oxoid and were sterilised by autoclaving at 121°C for 15 minutes.

Arginine Agar

Peptone	1g
NaCl	5g
K_2HPO_4	0.3g
Phenol red, 1.0% aq. soln.	1 ml
L(+) arginine hydrochloride	10g
Agar	3g
Distilled water	1000 ml

The medium was dissolved, the pH adjusted to 7.2 and distributed in 3.5 ml volumes into glass universals before sterilisation (Cowan and Steel's Manual for the Identification of Medical Bacteria, 1993).

Growth media

Degradation studies were carried out in *Pseudomonas* minimal medium which was prepared as described by Goulding *et al.* (1988). The trace salts solution was prepared separately in distilled water and was stored in a dark bottle for 6 - 8 weeks.

Pseudomonas minimal medium

K_2HPO_4	4.36g
NaH_2PO_4	3.45g
NH_4Cl	1.0g
$MgSO_4.6H_2O$	0.912g
Distilled water	1000ml
Trace salts solution*	1ml
pH 7.0	

Trace Salts Solution*	**g/100ml**
$CaCl_2.2H_2O$	4.77
$FeSO_4.7H_2O$	0.37
$CoCl_2.6H_2O$	0.37
$MnCl_2$	0.10
$NaMoO_4$	0.02

Mono-chlorophenols and chlorocatechols were added to the *Pseudomonas* minimal medium following sterilisation. A range of mono-chlorophenol concentrations from 0.78 mM to 3.12 mM were used.

Chlorophenol agars were prepared by the addition of bacteriological agar, at a concentration of 1% (w/v) to the minimal medium. Following sterilisation, the agar was allowed to cool and the chlorophenol was added to the agar to give the appropriate final concentration.

Hugh and Leifsons' Medium

Peptone	2g
NaCl	5g
K_2HPO_4	0.3g
Agar	3g
Distilled Water	1000 ml
Bromothymol blue, 0.2% w/v aq. soln.	15 ml

The solids were dissolved by heating in water and the pH was adjusted to 7.1 with 2M NaOH. The indicator dye was then added and the medium was sterilised. Following sterilisation, a sterile solution of glucose was added aseptically to give a final concentration of 1%. The medium was then mixed and distributed aseptically in 10 ml volumes into sterile test tubes (Cowan and Steel's Manual for the Identification of Medical Bacteria, 1993).

Malonate-phenylalanine medium

$(NH_4)_2SO_4$	2g
K_2HPO_4	0.6g
KH_2PO_4	0.4g
NaCl	2g
Sodium malonate	2g
DL-phenylalanine	3g
Yeast extract	2g
Distilled water	1000 ml
Bromothymol blue, 0.2% w/v aq. soln.	12.5 ml

The solids were dissolved in the water, filtered and the indicator solution was added before sterilisation (Cowan and Steel's Manual for the Identification of Medical Bacteria, 1993).

Rifampicin Agar

Plate count agar, prepared according to the manufacturer's instructions was allowed to cool following sterilisation and rifampicin (100 μg/ml), which had been dissolved in a minimal amount of methanol, was added to the agar.

Tween 80 agar

Tween 80	10g
Peptone	10g
NaCl	5g
$CaCl_2.2H_2O$	0.1g
Agar	20g
Distilled Water	1000 ml
pH 7.4	

The solids were dissolved in water and sterilised (Cowan and Steel's Manual for the Identification of Medical Bacteria, 1993).

2.1.3 Buffers.

Dilution Buffer (for resuscitation of freeze-dried bacteria)

The dilution buffer was prepared by adding 1.25ml of stock buffer solution (0.1M K_2HPO_4, pH 7.2) and 1ml of 10% (v/v) Triton-X-100 solution to 1 litre of distilled water.

Sodium Phosphate Buffer

Sodium phosphate buffer (0.01M) was prepared by dissolving Na_2HPO_4 (0.01M) and NaH_2PO_4 (0.01M) in distilled water. The pH of the resulting solution was then adjusted to pH 7.0.

Potassium Phosphate Buffer

Potassium phosphate buffer was prepared by dissolving K_2HPO_4 (104.5 g/L) and KH_2PO_4 (72.3 g/L) in distilled water as outlined in Standard Methods for the Examination of Water and Wastewater (1992). The pH of the resulting solution should be 6.8.

Tris-HCl Buffer

Tris-HCl buffer (0.033M) was prepared by dissolving Tris (0.033M) in distilled water and adjusting the pH to 7.6 using 2 M HCl.

2.1.4 Source of Chemicals.

Chemicals were obtained from a number of sources including Reidel-de-Haen, BDH, Sigma and Aldrich.

2.2 Methods.

2.2.1 Measurement of growth.

Growth of the mixed culture on the mono-chlorophenols was followed by measurement of the optical density at 660nm and by performing cell counts on Oxoid plate count. The numbers of chlorophenol degrading organisms were determined by plating onto chlorophenol agar. The numbers of *P. putida* CP1, following its addition to the mixed culture, were enumerated by plating onto plate count agar containing rifampicin (100 μg/ml). All plates were incubated at 30^0 C for 48 hours.

2.2.2 Resuscitation of freeze-dried micro-organisms of Biolyte HAB.

The mixed culture, supplied in the form of a buff/brown freeze-dried granular powder, was rehydrated in sterile dilution buffer (10g in 90ml), shaken for 1 hr at 30 °C. Following agitation, the cereal base carrier was allowed to settle out for 5 mins. The resulting supernatant fluid (10ml) was centrifuged at 5,000 rpm for 10 min, the pellet was then washed twice with 10 ml 0.01M sodium phosphate buffer, pH 7.0, removing any additional growth substrates contained in the mixed culture, and used to inoculate chlorophenol or chlorocatechol flasks at a variety of inocula sizes. The OD 660nm of the resultant inoculum was approximately 2.8.

2.2.3 Preparation of *Pseudomonas putida* CP1.

P. putida CP1, taken from chlorophenol agar plates, was grown up overnight in nutrient broth, washed twice with 0.01 M sodium phosphate buffer, pH 7.0, resuspended to give an optical density at 660nm of approximately 0.7 and used to inoculate chlorophenol flasks.

2.2.4 Growth conditions and biodegradation studies.

Biodegradation experiments were performed in 250ml conical flasks containing a final culture volume of 100ml following inoculation and addition of substrate. Substrates were added as outlined in section 2.2.2. Flasks were inoculated with either Biolyte HAB (10% v/v), *P. putida* CP1 (0.5% v/v or 5% v/v) or a combination of Biolyte HAB and *P. putida* CP1 ((% v/v Biolyte HAB : % v/v *P. putida* CP1) 10% : 5%, 9.5%: 0.5% , 5% : 5% or 1% : 5%). The cultures were incubated aerobically at 150 rpm and 30 °C. Samples were aseptically removed at regular intervals and analysed for pH, cell density, chlorophenol concentration and free chloride concentration. Uninoculated control flasks were incubated in parallel. The wild type strain of *P. putida* CP1 was used in biodegradation studies, while a rifampicin mutant of *P. putida* CP1 was used only in growth studies.

2.2.5 Generation of Rifampicin mutants of *Pseudomonas putida* CP1.

Spontaneous mutants of *P. putida* CP1, capable of growth in the presence of rifampicin, were generated by plating high density cultures of *P. putida* CP1 onto plate count agar containing 100 μg/ml rifampicin. Any resulting colonies were restreaked onto chlorophenol/rifampicin agar to confirm that any spontaneous mutants retained the ability to grow on chlorophenol.

2.2.6 Data analysis.

The results presented were the mean of duplicate experiments. All experiments were repeated to confirm the data obtained. In all cases, the standard deviations between runs were found to be less than 5%.

Rates of degradation were calculated in terms of substrate degraded per unit time from the end of the lag period (L) to the time of substrate disappearance (Td). The lag period was defined as follows : in a plot of chlorophenol concentration versus time, the

straight line was extrapolated to the initial chlorophenol concentration (S_0) and the intercept on the time axis was taken to be the length of the lag period.

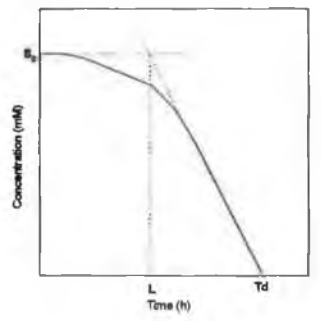

Figure 8. Definition of lag period, L.

The relationship between times of degradation/lag period and Biolyte HAB inoculum size was studied using regression analysis. Regression coefficients and slopes were calculated using a Sigma Plot computer package (Jandel Corporation).
Statistical analysis of cell counts was carried out using a SPSS computer package (SPSS, Inc.).

2.2.7 Tests used to identify *Pseudomonas putida* CP1.

Gram reaction

The Gram stain was carried out on 18-24 hour cultures according to the Hucker method (Collins and Lyne, 1985).

Spore stain

A smear of the organism was prepared from a 48 hour nutrient broth culture and heat fixed. The smear was stained with 5% (w/v) aqueous malachite green and kept steaming for over 5 minutes, renewing the stain as it evaporated. It was then washed for 30 seconds with distilled water and counterstained with 0.25% (w/v) safranin for 1 minute.

Motility test

An overnight culture of the organism was examined in "hanging drop" preparations, using a high-powered objective (X100) and reduced illumination. A "hanging drop" slide was prepared by placing a loopful of the bacterial suspension onto the centre of a coverslide. A depression slide onto which a ring of vaseline had been spread around the concavity was lowered onto the coverslip, with the concavity facing down over the drop. When a seal had formed, the hanging drop slide was turned over and examined under a microscope.

Catalase activity

A loopful of culture was emulsified with a loopful of 3% (v/v) hydrogen peroxide. Effervescence, caused by the liberation of free oxygen as gas bubbles, indicated a positive result.

Oxidase activity

Filter paper was impregnated with a 1% (w/v) aqueous solution of tetramethyl-p-phenylene-diamine (with 0.1% ascorbic acid to prevent auto-oxidation). Bacterial cultures were smeared across the filter paper with a glass rod. The formation of a purple colour within 5-10 seconds indicated oxidase positive cultures.

Oxidation-Fermentation test

Two tubes of Hugh and Leifsons' medium were stab inoculated with a straight wire containing the test culture. One tube was covered with sterile mineral oil to a depth of about 3 cm above the medium to seal it from air and the two tubes were incubated at 30 °C for up to 14 days. Acid production was indicated by a change in the colour of the medium from blue/green to yellow. Fermentative organisms produced acid in both tubes resulting in a yellow colour in both the open and sealed tubes, while oxidative

produced acid only in the open tube, resulting in a yellow colour appearing in the open tube and usually only at the surface.

Arginine hydrolysis

Arginine agar was stab-inoculated and a layer of sterile mineral oil was pipetted onto the surface to a depth of about 1 cm. The tubes were incubated at 30 °C for 5 days. A positive reaction was shown by a colour change of the indicator to red.

Malonate hydrolysis

Malonate-phenylalanine medium was inoculated with the test culture and incubated for 24 - 48 hours at 30 °C. Malonate hydrolysis was indicated by a deep blue colour around the growth, while negative reactions were indicated by the unchanged greenish colour of the medium.

Starch hydrolysis

Nutrient agar containing 0.2% soluble starch was inoculated by streaking the test organism across the surface of the medium. Plates were incubated at 30 °C for 3 -4 days and flooded with Grams' iodine to stain the starch. The medium turned blue where starch had not been hydrolysed, while clear colourless zones around the growth indicated starch hydrolysis.

Tween 80 hydrolysis

A poured, dried plate of Tween 80 agar was inoculated by streaking once across the surface. Plates were incubated at 30 °C for 1 - 7 days. Opaque zones surrounding the inoculum consisting of calcium salts of free fatty acids indicated hydrolysis of the Tween.

API Test

The API 20NE, for non-enteric Gram negative rods, was used to identify the chlorophenol degrading isolate, *P. putida* CP1. The identification system was carried out according to the manufacturers' instructions (bioMérieux sa, Marcy-l'Etoile, France). The inocula were prepared as follows: an overnight nutrient broth culture (10ml) was harvested in a Labofuge 6000 bench-top centrifuge (5,000 rpm for 10 minutes) and washed once with sterile 0.01 M sodium phosphate buffer (Section 2. 1. 3). The pellet was resuspended in 0.85% (w/v) NaCl (10ml) and used to inoculate a portion of the tests. For assimilation tests, 200µl of this suspension was used to inoculate auxiliary medium supplied by the manufacturer and this was then used to inoculate the remaining tests.

Biolog Test

Identification was also carried out by a Biolog microlog system (BIOLOG Inc., Hayward, Calif., USA) according to the manufacturers' instructions. GN microplates, for Gram negative organisms were used. An isolated colony was transferred to Biolog inoculating fluid using sterile swabs to give the correct cell density required by the system. Cell densities were compared against GN-NENT (Gram negative Non-enteric) turbidity standards supplied by the company. 150µl of the resulting inoculum was added to each of the 96 test wells in the microtitre plate. The plates were incubated for 16 - 24 hours at 30 °C before results were interpreted using Biolog automated microlog software. Identification is obtained by comparison of the results obtained with those of database patterns supplied in the software. Similarity values must be > 0.5 after 16 – 24 hours, while the distance value must be < 5.0 for a valid identification to occur.

2.2.8 Measurement of chlorophenol concentrations.

Chlorophenol concentrations were measured using a 4-aminoantipyrene colorimetric method based on the procedure detailed in Standard Methods for the Examination of Water and Wastewater (1992). Samples were centrifuged at 5,000 rpm for 10 min and the resulting supernatants were diluted to bring the concentration into the range of the standard curve. The standards prepared were in the range 0 - 0.05 mg of chlorophenol in 10ml distilled water. The samples and standards were treated by placing 10ml in a test tube and adding 0.25ml 0.5N NH_4OH. The pH was then adjusted to 7.9 ± 0.1 with approximately 200µl potassium phosphate buffer (pH 6.8). 100µl of 2% (w/v) 4-aminoantipyrene solution was added and the tubes mixed well. The 100µl of 8% (w/v) potassium ferricyanide was added and the tubes mixed well. The tubes were allowed to stand for 15 min at room temperature. The absorbance was read at 500nm and the concentrations were calculated from the standard curve.

Sample standard curves for the mono-chlorophenols are presented in Figure 9.

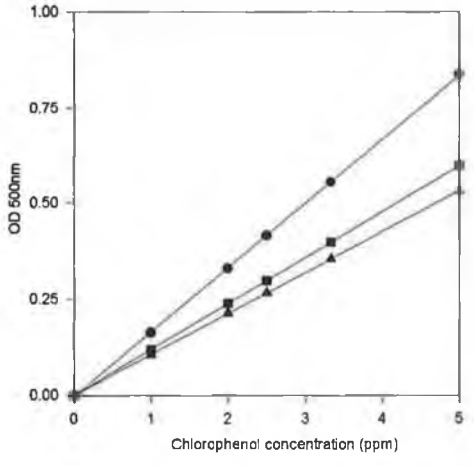

Figure 9. Standard curve for mono-chlorophenols using colorimetric assay. Symbols : ● 2-chlorophenol, ■ 3-chlorophenol and ▲ 4-chlorophenol.

2.2.9 Measurement of chlorocatechol concentrations.

Chlorocatechol concentrations were quantified by the method of Arnow (1937). Samples were centrifuged at 5,000 rpm for 10 min to remove cells. 1ml of standard/sample was placed in a test-tube (1/10 dilution sample used). Standards were prepared in the range of 0 - 0.020 mg/ml chlorocatechol. To each test-tube 1ml 0.5 N HCl was added. Tubes were mixed well and to this 1ml nitrite-molybdate reagent was added, resulting in a yellow colour. Nitrite-molybdate reagent was prepared by dissolving 1g each of sodium nitrite and sodium molybdate in 10 mls of water. After mixing, 1ml of 1 N NaOH was added, resulting in a red colour. To this 1ml distilled water was added. Following mixing, the absorbance was read at 510nm and concentrations were calculated from the standard curve.
Sample standard curves for the chlorocatechols are presented in Figure 10.

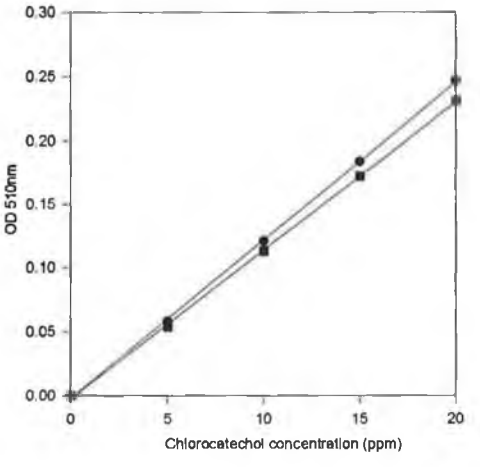

Figure 10. Standard curve for chlorocatechols using Arnow's colorimetric assay. Symbols : ● 3-chlorocatechol and ■ 4-chlorocatechol.

2.2.10 Identification of metabolites.

Identification of chlorocatechols was carried out using HPLC. Samples were centrifuged to remove cells; the resulting supernatant (5ml) was acidified to pH 2.0 with 2M HCl, and extracted with ethyl acetate, (2ml) in 3 successive extractions. The resulting organic phase was assayed immediately or stored at 4 °C for 2-3 days. A sample was loaded into a

Reodyne injection loop and a Shimadzu LC-9A solvent delivery unit automatically injected 20µl of sample onto the Novapak C18 column (Waters, 4µm particle size, 3.9mm x 150mm stainless steel column). The mobile phase used was 0.075M acetic acid / Acetonitrile (70:30) at a flow rate of 0.8ml/min. Detection of chlorocatechols was at 270nm using a Shimadzu SPD-6AV UV/Vis detector. An Axiomm Chromatography data acquisition package was used to monitor and process the data.

The production of brown/black chlorocatechol polymers was followed by measurement of the optical density at 600nm following removal of cells by centrifugation (Haller and Finn, 1979).

The production of the *meta-* cleavage product of 4-chlorocatechol, 5-chloro-2-hydroxymuconic semialdehyde, was followed by measurement of the optical density at 380nm following removal of cells by centrifugation (Sala-Trepat and Evans, 1971).

The drop in 5-chloro-2-hydroxymuconic semialdehyde concentrations due to spontaneous decomposition was examined by incubation of the compound following removal of the microbial inoculum. 5-chloro-2-hydroxymuconic semialdehyde was produced by incubation of Biolyte HAB with 4-chlorophenol for 24 hours following which the culture medium was filter sterilised (0.22 µm filter). The resulting supernatant was transferred to a sterile flask and incubated under the same conditions used in degradation trials.

Colour production due to the accumulation of degradative intermediates was photographed using a Nikon F50 camera.

2.2.11 Chloride assay.

Chloride release was followed with an Orion chloride specific electrode (model 9417). The electrode was calibrated with reference to NaCl standards and chloride concentrations were calculated using the direct readout capability of an Orion benchtop pH/ISE meter (model

920A). Samples and standards were diluted with 2% 5M $NaNO_3$ ionic strength adjusting solution.

2.2.12 Measurement of pH.

The pH was measured using an Orion Triode™ pH electrode Model 91-57BN connected to an Orion benchtop pH/ISE meter (model 920A).

2.2.13 Enzyme Assays.

Preparation of cell-free extracts

Cells, grown up on 1.56 mM mono-chlorophenol, were harvested by centrifugation (5,000 rpm, 10 mins), and washed twice in 0.033 M Tris-HCl buffer (pH 7.6). The cells were broken by sonication for 3 min. (30 sec. on, 30 sec. off) at 16 amplitude microns using a Labsonic U (B. Braun). The cell suspension was kept on ice throughout sonication. Whole cells and debris were removed by centrifugation at 20,000 rpm at 0 - 4 °C for 20 mins. The cell free extract was kept on ice and assayed as soon as possible for catechol dioxygenase activity.

Catechol 1,2-dioxygenase activity

Catechol 1,2-dioxygenase activity was measured by following the formation of *cis,cis*-muconic acid, the *ortho*- cleavage product of catechol using the method of Dorn and Knackmuss (1978). The following reagents were added to a quartz cuvette :

2 ml 50 mM Tris-HCl buffer (pH 8.0)
0.7ml distilled water
0.1ml 100 mM 2-mercaptoethanol
0.1ml cell-free extract.

The contents of the cuvette were mixed by inversion and 0.1ml catechol (1 mM) was then added and the contents mixed again. *Cis,cis*-muconic acid formation was followed by an increase in the absorbance at 260nm over a period of 5 min.

Chlorocatechol 1,2-dioxygenase activity

Chlorocatechol 1,2-dioxygenase activity was measured by following the formation of 2-chloromuconic acid, the *ortho*- cleavage product of 3-chlorocatechol. The procedure used was as for Type I activity, with 3-chlorocatechol (1 mM) being used in the place of catechol (1 mM).

Catechol 2,3-dioxygenase activity

Catechol 2,3-dioxygenase activity was measured by following the formation of 2-hydroxymuconic semialdehyde, the *meta*- cleavage product of catechol, using the method of Feist and Hegeman (1969). The following reagents were added to a plastic cuvette :

2ml 50 mM Tris-HCl buffer (pH 7.5)
0.6ml distilled water
0.2ml cell free extract.

The contents were mixed by inversion and 0.2ml catechol or 4-chlorocatechol (100 mM) was added and mixed with the contents. The production of 2-hydroxymuconic semialdehyde was followed by increase in absorbance at 375nm over a period of 5 min.

Calculation of enzyme activity

Activities were calculated using the following extinction coefficients for each reaction product : catechol at 260nm = 16,800 l/mol/cm, 3-chlorocatechol at 260nm = 17,100 l/mol/cm (Schmidt, 1987) and catechol at 375nm = 36,000 l/mol/cm (Sung Bae *et al.*,

1996). One unit of enzyme activity was defined as the amount of enzyme that catalysed the formation of 1 µmol of product formed per min in 1 ml reaction mixture and was calculated using the following formula :

Activity (U/min.) = [(Δ OD/ Δ T) / ε] x V x 1000

where Δ OD / Δ T = increase in optical density per minute

ε = molar extinction coefficient ($l.mol^{-1}cm^{-1}$)

V = reaction volume (3 ml)

(John R.A., 1995)

One unit of enzyme activity results in an increase of 5.6 absorbance units at 260nm for catechol 1,2-dioxygenase, 5.7 absorbance units at 260nm for chlorocatechol 1,2-dioxygenase and 12 absorbance units at 375nm for catechol 2,3-dioxygenase activity.

Specific activities were expressed as units per mg of protein. The protein concentrations in cell free extracts were determined by the method of Lowry *et al.* (1951).

$$\text{Specific activity (U / mg)} = \frac{\text{Activity}}{\text{Total protein}}$$

Protein determination

The protein concentration in the cell-free extract was determined by the method of Lowry *et al.*, (1951). Reagent A (2.5ml) was added to 0.5ml of suitably diluted sample or standard. The tubes were left for 10 minutes in the dark at room temperature. Reagent B (0.125ml) was then added and the tubes were left for 25 minutes at room temperature in the dark. The tubes were then mixed by vortexing and left for a further 5 minutes. The absorbance was read at 600nm. Standards were prepared with bovine serum albumin (0 - 1 $mg.ml^{-1}$).

Reagent A: 50ml 0.2% Na_2CO_3 in 0.1M NaOH

0.5ml 1% $CuSO_4$

0.5ml 2% NaK tartarate

Reagent B: 50% (v/v) Folins - Ciocalteau in water

A sample standard curve is presented in Figure 11.

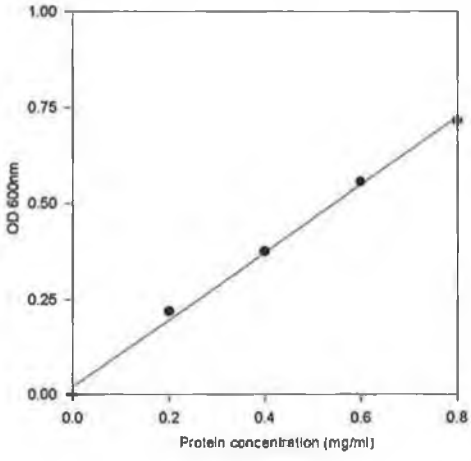

Figure 11. Standard curve for protein using the Lowry assay.

3. Results.

3.1 The degradation of mono-chlorophenols by Biolyte HAB.

The aerobic degradation of 2-, 3- and 4-chlorophenol by Biolyte HAB (10% inoculum) was investigated. In studying the degradation of the mono-chlorophenols, a number of parameters were studied including substrate removal at a variety of concentrations, the production of metabolites, growth of the mixed culture and the key enzymes involved in chlorophenol degradation.

3.1.1 The degradation of various concentrations of mono-chlorophenols by Biolyte HAB.

The degradation of mono-chlorophenols by Biolyte HAB was studied at a range of concentrations between 0.78 mM (100 ppm) and 3.12 mM (400 ppm). In control flasks, no loss of 3- or 4-chlorophenol occurred. Losses due to volatilisation, were detected in 2-chlorophenol control flasks. The average loss in 2-chlorophenol concentrations due to volatilisation was found to be 4.65 μM/hr and this was taken into account during the calculations.

Biolyte HAB was found to be capable of metabolising up to 2.33 mM (300 ppm) 2-, 3- and 4-chlorophenol. Degradation of 2- and 3-chlorophenol was incomplete however, as indicated by the unstoichiometric releases of chloride (Table 3). Despite 100% 3-chlorophenol (0.78 mM) being removed by Biolyte HAB, metabolism resulted in the release of only 30% free chloride, while removal of 65% 2-chlorophenol (1.56 mM, 200 ppm) resulted in the release of 30% chloride. During the degradation of both 2- and 3-chlorophenol, a brown/black colour accumulated in the culture medium (Fig. 12).

4-chlorophenol was the most readily removed of the mono-chlorophenol isomers (Table 3). Incubation of Biolyte HAB with 4-chlorophenol resulted in complete removal of 1.56 mM within 48 hours and 2.33 mM within 72 hours. Degradation was complete as indicated by the stoichiometric releases of chloride. Metabolism of 4-chlorophenol resulted in a lime/yellow coloration accumulating in the culture fluid (Fig. 12).

Table 3. The degradation of various concentrations of mono-chlorophenols by Biolyte HAB.

Substrate and concentration (mM)	Percentage chlorophenol removal	Percentage chloride release
2-chlorophenol		
1.56 mM	65 %	30 %
2.33 mM	21 %	17 %
3.12 mM	0 %	0 %
3-chlorophenol		
0.78 mM	100 %	30 %
1.56 mM	80 %	21 %
2.33 mM	25 %	3 %
4-chlorophenol		
1.56 mM	100 %	100 %
2.33 mM	100 %	100 %
3.12 mM	0 %	0 %

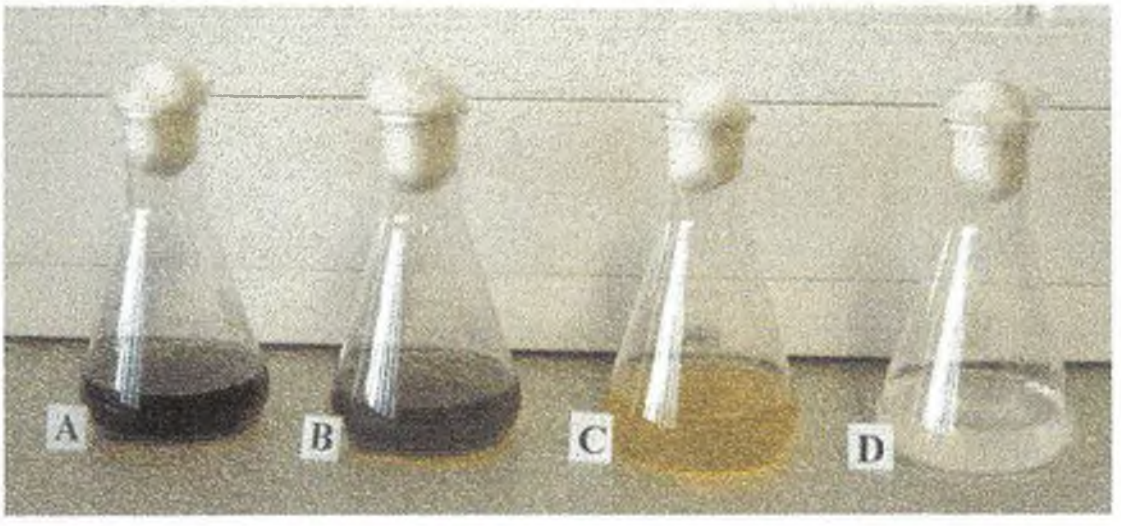

Figure 12. Colour production following the degradation of mono-chlorophenols (1.56 mM) by Biolyte HAB. Symbols : **A** 2-chlorophenol, **B** 3-chlorophenol, **C** 4-chlorophenol and **D** control.

3.1.2 Investigations into the degradation of 2- and 3-chlorophenol by Biolyte HAB.

Preliminary experiments carried out into the degradation of 2- and 3-chlorophenol by Biolyte HAB had shown the accumulation of brown/black coloured intermediates in the culture medium and non-stoichiometric releases of chloride, indicating that metabolism of the chlorophenols was not complete. Further investigations were carried out to identify the intermediates accumulated following 2- and 3-chlorophenol degradation.

3.1.2.1 Identification of accumulated metabolites resulting from the degradation of 2- and 3-chlorophenol by Biolyte HAB.

The degradation of 1.56 mM 2- and 3-chlorophenol by Biolyte HAB was studied and degradation curves typical of their degradation are shown in Figures 13 and 14. 2-chlorophenol degradation appeared to cease after 48 hours (Fig. 13), while metabolism of 3-chlorophenol by Biolyte HAB continued resulting in 100% removal after 238

hours (Fig. 14). However, degradation of the chlorophenols was incomplete as indicated by the non-stoichiometric releases of chloride. The pattern of chloride release for both 2- and 3-chlorophenol mirrored the pH drop observed for both chlorophenols. The initial chloride release was accompanied by a corresponding drop in pH of approximately 0.2 pH units which reached a plateau as the chloride release also reached completion.

2- and 3-chlorophenol metabolism caused an accumulation of a degradative intermediate in the culture medium (Figs. 13 & 14). Using HPLC analysis, this intermediate was identified as being 3-chlorocatechol. As 2- and 3-chlorophenol metabolism continued, 3-chlorocatechol concentrations as measured by Arnow's colorimetric assay and HPLC reached a peak at 48 hours and began to drop, corresponding with the development of a brown/black colour which remained in the medium.

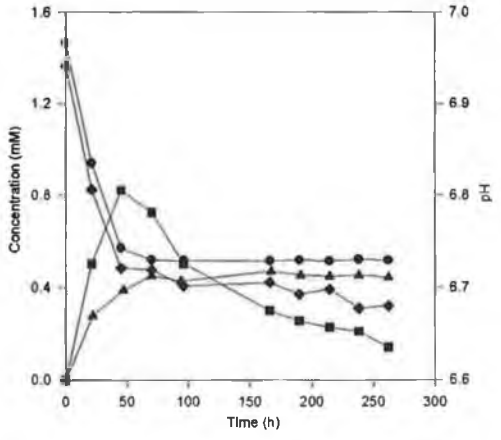

Figure 13. The degradation of 2-chlorophenol (1.56 mM) by Biolyte HAB. Symbols ● 2-chlorophenol, ■ 3-chlorocatechol, ▲ chloride release and ◆ pH.

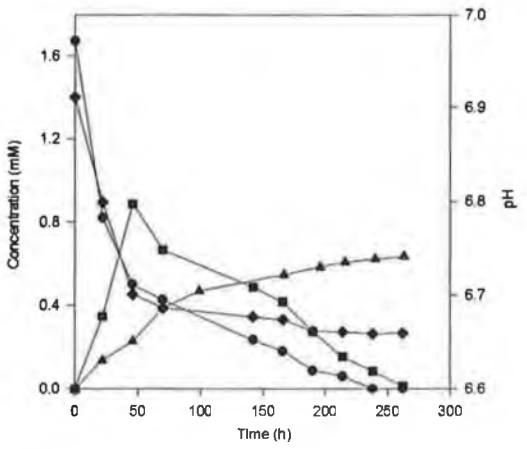

Figure 14. The degradation of 3-chlorophenol (1.56 mM) by Biolyte HAB. Symbols ● 3-chlorophenol, ■ 3-chlorocatechol, ▲ chloride release and ◆ pH.

As 3-chlorocatechol had been identified as an intermediate in the degradation of 2- and 3-chlorophenol, 3-chlorocatechol (0.69 mM and 1.38 mM) degradation by Biolyte HAB was investigated (Fig. 15). Degradation was incomplete as indicated by the unstoichiometric releases of chloride and the accumulation of a brown/black colour in the culture media. The reduction in 3-chlorocatechol concentrations in flasks containing Biolyte HAB was similar to that observed in control flasks incubated in parallel (Fig. 15). This showed that the reduction in the measured chlorocatechol concentrations observed during the degradation of 2- and 3-chlorophenol and 3-chlorocatechol by Biolyte HAB was due to autooxidation and not due to further microbial metabolism. Autooxidation of 3-chlorocatechol in the presence and in the absence of Biolyte HAB resulted in a brown/black coloration of the culture medium. The production of these coloured polymers in the absence of any microbial inoculum resulted in an increase in the optical density at 660nm (Fig. 16).

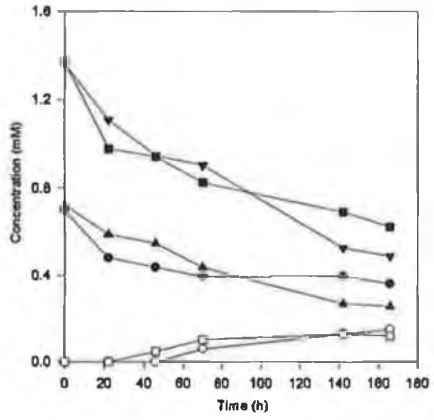

Figure 15. The degradation of 3-chlorocatechol (0.69 mM and 1.38 mM) by Biolyte HAB. Symbols : ● 3-chlorocatechol (0.69 mM), ▲ control (0.69 mM), O chloride release (0.69 mM); ■ 3-chlorocatechol (1.38 mM), ▼ control (1.38 mM) and □ chloride release (1.38 mM).

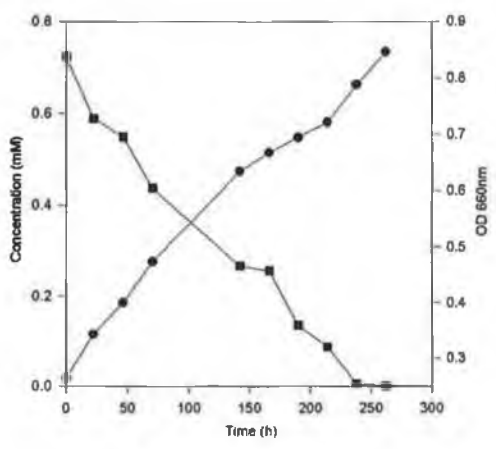

Figure 16. Drop in 3-chlorocatechol concentrations and development of brown/black coloration due to autooxidation in sterile minimal medium. Symbols : ■ 3-chlorocatechol and ● OD 660nm.

3.1.2.2 Measurement of the growth of Biolyte HAB during the degradation of 2- and 3-chlorophenol.

The growth of Biolyte HAB on 2- and 3-chlorophenol was monitored by measurement of the optical density at 660nm and by carrying out plate counts. However, while the optical density continued to rise during the degradation of both 2- and 3-chlorophenol, suggesting continued cell growth, viable cell counts showed that cell numbers reached a maximum after approximately 48 hours during growth on 2-chlorophenol and 72 hours on 3-chlorophenol, following which cell numbers began to decrease (Fig. 17 & 18).

Although the measurement of OD 660nm suggested similar amounts of cell growth, cell numbers showed significantly more growth on 3-chlorophenol than on 2-chlorophenol. The accumulation of dark coloured chlorocatechol polymers appeared to interfere with the optical density around 660nm. An increase in optical density measurements at 660nm corresponding to the development of a brown/black colour in the culture fluid was previously shown to occur in flasks containing 3-chlorocatechol in the absence of any microbial inoculum (Fig. 16).

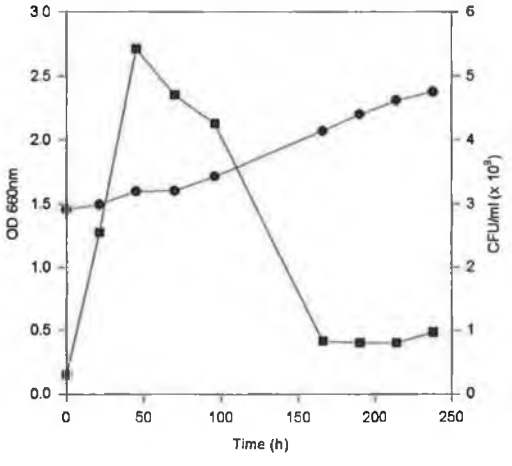

Figure 17. Growth of Biolyte HAB on 2-chlorophenol as measured by ● OD 660nm and ■ viable cell counts.

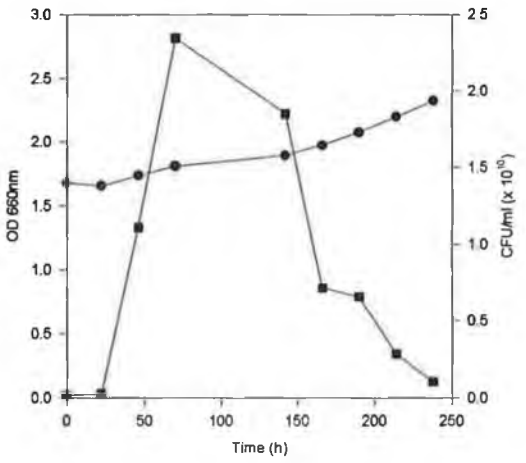

Figure 18. Growth of Biolyte HAB on 3-chlorophenol as measured by ● OD 660nm and ■ viable cell counts.

The development of the brown/black colour in flasks containing 3-chlorocatechol is ordinarily followed by measurement of the optical density at 600nm following removal of biomass. As the brown/black colour developed during the degradation of 2- and 3-chlorophenol, resulting in an increase in the optical density at 660nm, a similar increase in the optical density at 600nm was noted (Figs. 19 & 20).

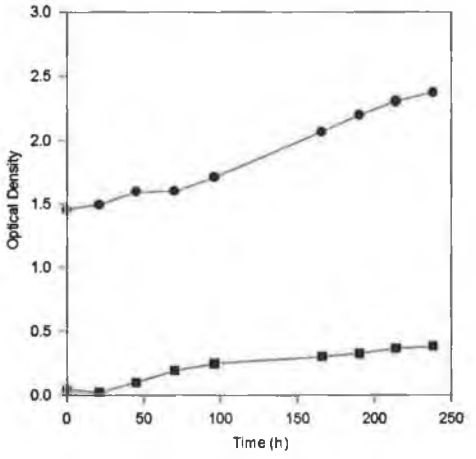

Figure 19. Development of brown/black coloration following degradation of 2-chlorophenol by Biolyte HAB as measured by OD 600nm, ■, and OD 660nm, ●.

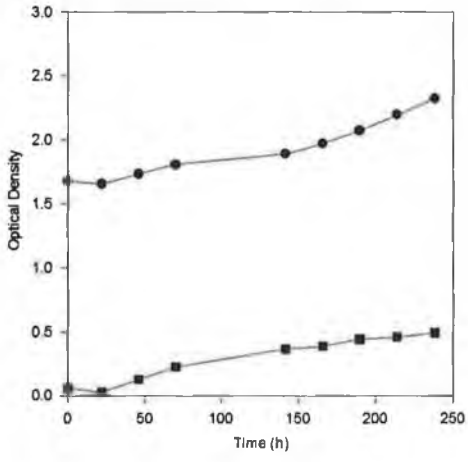

Figure 20. Development of brown/black coloration following degradation of 3-chlorophenol by Biolyte HAB as measured by OD 600nm, ■, and OD 660nm, ●.

3.1.2.3 Ring cleavage enzyme activities during the degradation of 2- and 3-chlorophenol by Biolyte HAB.

The degradation of 2- and 3-chlorophenol by Biolyte HAB resulted in the accumulation of chlorocatechol polymers in the culture fluid and the unstoichiometric release of chloride. These phenomena were typical of the ring cleavage of 3-chlorocatechol by the *meta-* cleavage pathway (Bartels *et al.*, 1984). In order to confirm this, the key enzymes involved in catalysing ring fission (catechol 1,2-dioxygenase, chlorocatechol 1,2-dioxygenase and catechol 2,3-dioxygenase) were assayed (Table 4 & 5). Enzyme assays confirmed that degradation was via the *meta-* cleavage pathway. Low levels of catechol 2,3-dioxygenase activities were detected during 2- and 3-chlorophenol degradation, as activity is irreversibly inactivated by 3-chlorocatechol. No *ortho-* cleavage activities were detected during chlorophenol metabolism by Biolyte HAB. The low levels of catechol 2,3-dioxygenase activity detected, decreased quickly on accumulation of 3-chlorocatechol after 48 hours, demonstrating the toxicity of 3-chlorocatechol metabolism to the *meta-* cleavage enzyme. Even after the *meta-* cleavage capability of Biolyte HAB was destroyed by the

accumulation of 3-chlorocatechol, no *ortho-* cleavage activities were detected, indicating the absence of any *ortho-* cleavage capability in Biolyte HAB.

Table 4. Enzyme activities during the degradation of 2-chlorophenol (1.56 mM) by Biolyte HAB.

Enzyme Assayed and assay substrate	Specific Activity (U/mg)			
	T = 24 hrs	T = 36 hrs	T = 48 hrs	T = 72 hrs
Catechol 2,3- dioxygenase				
Catechol	0.47	0.01	< 0.001	< 0.001
Catechol 1,2-dioxygenase				
Catechol	< 0.001	< 0.001	< 0.001	< 0.001
3-chlorocatechol	< 0.001	< 0.001	< 0.001	< 0.001

Table 5. Enzyme activities during the degradation of 3-chlorophenol (1.56 mM) by Biolyte HAB.

Enzyme Assayed and assay substrate	Specific Activity (U/mg)			
	T = 24 hrs	T = 48 hrs	T = 72 hrs	T = 96 hrs
Catechol 2,3- dioxygenase				
Catechol	0.63	0.02	< 0.001	< 0.001
Catechol 1,2-dioxygenase				
Catechol	< 0.001	< 0.001	< 0.001	< 0.001
3-chlorocatechol	< 0.001	< 0.001	< 0.001	< 0.001

3.1.3 Investigations into the degradation of 4-chlorophenol by Biolyte HAB.

The aerobic degradation of 4-chlorophenol by Biolyte HAB at concentrations of 1.56 mM and 2.33 mM was shown to be complete as indicated by the total removal of

chlorophenol and the stoichiometric releases of chloride (Table 3). 4-chlorophenol metabolism by Biolyte HAB resulted in the accumulation of a lime/yellow colour in the culture fluid. Further investigations into the degradation of 4-chlorophenol were carried out in order to establish the degradative pathway used and the intermediates responsible for the coloration of the culture fluid.

3.1.3.1 The degradation of 4-chlorophenol by Biolyte HAB.

The degradation of 4-chlorophenol (1.56 mM) by Biolyte HAB was studied and a typical degradation curve is shown in Figure 21. Incubation of Biolyte HAB with 4-chlorophenol resulted in complete removal of the chlorophenol within 48 hours with a stoichiometric release of chloride within 100 hours. The degradation of 4-chlorophenol resulted in a greater drop in pH than was observed during the degradation of 2- and 3-chlorophenol. The pH dropped by over 0.3 pH units after approximately 48 hours, after which the pH rose by over 0.1 pH unit. No 4-chlorocatechol accumulation was detected during the degradation of 4-chlorophenol by Biolyte HAB.

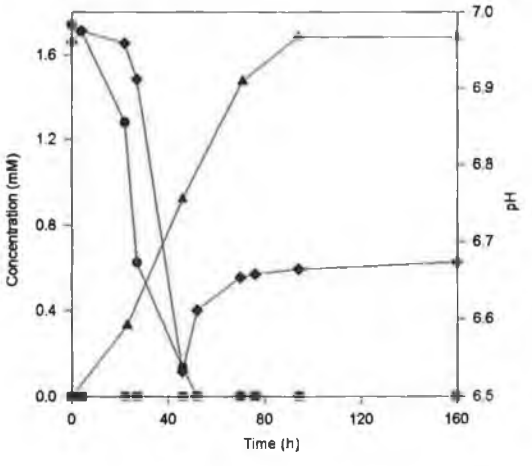

Figure 21. The degradation of 4-chlorophenol (1.56 mM) by Biolyte HAB. Symbols ● 4-chlorophenol, ■ 4-chlorocatechol, ▲ chloride release and ◆ pH.

3.1.3.2 The degradation of 4-chlorocatechol by Biolyte HAB.

Although no chlorocatechols were detected during the degradation of 4-chlorophenol by Biolyte HAB, the ability of Biolyte HAB to degrade 4-chlorocatechol as an intermediate of 4-chlorophenol degradation was demonstrated by incubation of 4-chlorocatechol with Biolyte HAB (Fig. 22). Degradation of 1.38 mM 4-chlorocatechol by Biolyte HAB was incomplete, with 83% being removed and 80% of the total chloride being released within 180 hours. However growth of Biolyte HAB on 0.69 mM 4-chlorocatechol resulted in complete removal within 120 hours and a stoichiometric release of chloride within 160 hours. Removal of 4-chlorocatechol was at a rate in excess of that observed due to polymerisation in control flasks and resulted in stoichiometric releases of chloride demonstrating Biolyte HAB's ability to completely metabolise 4-chlorocatechol as an intermediate of 4-chlorophenol degradation.

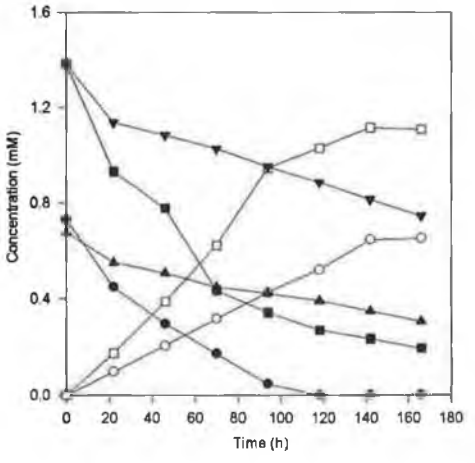

Figure 22. The degradation of 4-chlorocatechol (0.69 mM and 1.38 mM) by Biolyte HAB. Symbols : ● 4-chlorocatechol (0.69 mM), ▲ control (0.69 mM), O chloride release (0.69 mM); ■ 4-chlorocatechol (1.38 mM), ▼ control (1.38 mM) and □ chloride release (1.38 mM).

3.1.3.3 Investigations into the lime/yellow coloration of the culture fluid following the degradation of 4-chlorophenol by Biolyte HAB.

During the degradation of 4-chlorophenol and 4-chlorocatechol, a lime/yellow colour accumulated in the medium. However, unlike the brown/black colour produced during 2- and 3-chlorophenol degradation, this lime/yellow colour did not remain in the medium. During the degradation of 4-chlorophenol, the lime/yellow colour became visible within 16 hours, developed to form a straw yellow colour and finally disappeared after 48 hours, suggesting further metabolism of the yellow intermediate. The accumulated lime/yellow intermediate possessed all the characteristics of 5-chloro-2-hydroxymuconic semialdehyde, known to be the *meta*- cleavage product of 4-chlorocatechol. The lime/yellow colour with an absorption maximum of 379nm at pH 7.0 and 334nm at pH 2.0 (Fig. 23) disappeared reversibly upon acidification.

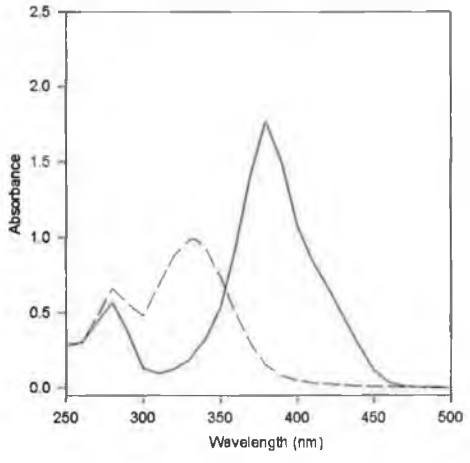

Figure 23. UV spectrum, at pH 7.0 —— and pH 2.0 – – – resulting from the development of lime/yellow coloration of the culture medium following degradation of 4-chlorophenol by Biolyte HAB.

The development of 5-chloro-2-hydroxymuconic semialdehyde following the degradation of 4-chlorophenol by Biolyte HAB was monitored by measurement of the optical density at 380nm and is shown in Figure 24. Optical density measurements rose

sharply in the initial 24 hours, reached a peak and began to drop corresponding to the development and disappearance of the yellow colour and the release of chloride into the culture medium. The further metabolism of 5-chloro-2-hydroxymuconic semialdehyde was demonstrated by the fall in OD 380nm measurements, the disappearance of the lime/yellow colour and the stoichiometric releases of chloride in the presence of Biolyte HAB. When 5-chloro-2-hydroxymuconic semialdehyde was incubated following the removal of Biolyte HAB cells by filtration, its disappearance from the culture fluid was significantly lower (Fig. 24), while the yellow colour remained in the culture medium indicating that removal in the presence of Biolyte HAB was microbially mediated and not due to spontaneous decomposition.

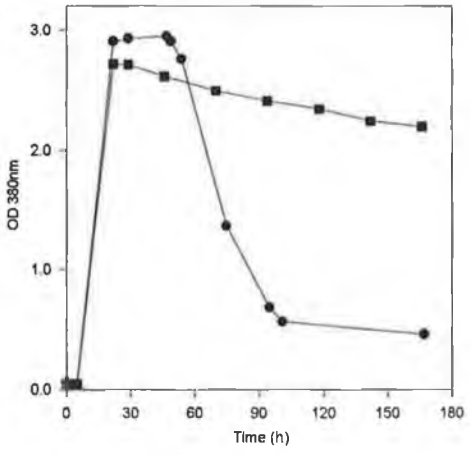

Figure 24. Development and disappearance of 5-chloro-2-hydroxymuconic semialdehyde during the degradation of 4-chlorophenol by Biolyte HAB ● and its disappearance in the absence of any microbial inoculum ■.

3.1.3.4 Growth of Biolyte HAB during the degradation of 4-chlorophenol.

The production of the lime/yellow coloured intermediate did not interfere with the optical density readings at 660nm and hence absorbance readings could be used as an indication of growth on 4-chlorophenol (Fig. 25). The growth of Biolyte HAB as measured by turbidity readings and plate counts continued to rise during 4-

chlorophenol degradation. Cell numbers only began to decrease as the complete metabolism of 4-chlorophenol neared completion after approximately 100 hours. Viable cell counts showed a greater increase in cell numbers during the degradation of 4-chlorophenol, which was as expected due to its complete mineralization by Biolyte HAB, followed by 3-chlorophenol and 2-chlorophenol (Figs. 17 & 18) indicating the relative toxicities of the mono-chlorophenols to Biolyte HAB.

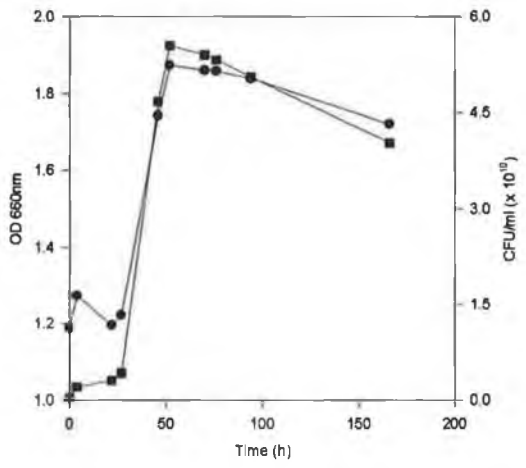

Figure 25. Growth of Biolyte HAB on 4-chlorophenol as measured by ● OD 660nm and ■ viable cell counts.

3.1.3.5 Ring cleavage enzyme activities during the degradation of 4-chlorophenol by Biolyte HAB.

As was shown for 2- and 3-chlorophenol, degradation of 4-chlorophenol by Biolyte HAB was shown to be via the *meta*- cleavage pathway. Higher levels of catechol 2,3-dioxygenase activity were detected following growth on 4-chlorophenol (Table 6) than were observed during 2- and 3-chlorophenol degradation by Biolyte HAB (Tables 4 & 5). Similarly, no *ortho*- activities, catechol 1,2-dioxygenase or chlorocatechol 1,2-dioxygenase, were detected during 4-chlorophenol metabolism. The levels of catechol 2,3-dioxygenase activity detected disappeared only after 4-chlorophenol was completely degraded by the mixture.

Table 6. Enzyme activities during the degradation of 4-chlorophenol (1.56 mM) by Biolyte HAB.

Enzyme Assayed and assay substrate	Specific Activity (U/mg)			
	T = 24 hrs	T = 36 hrs	T = 48 hrs	T = 72 hrs
Catechol 2,3- dioxygenase				
Catechol	2.75	1.31	0.72	0.01
Catechol 1,2-dioxygenase				
Catechol	< 0.001	< 0.001	< 0.001	< 0.001
3-chlorocatechol	< 0.001	< 0.001	< 0.001	< 0.001

3.2 The degradation of mono-chlorophenols by *Pseudomonas putida* CP1.

An isolate, CP1, capable of the degradation of mono-chlorophenols was obtained from Dr. Fabio Fava, University of Bologna, Italy. The organism was identified and tested for its ability to degrade the mono-chlorophenols as sole carbon source.

3.2.1 Identification of CP1 strain.

The cell characteristics and Gram reaction were noted together with its response to a range of biochemical tests (Tables 7 & 8). CP1 was found to be a Gram negative, non-spore forming, motile rod. The isolate was shown to be an oxidative organism and was both oxidase and catalase positive. CP1 gave positive results for arginine hydrolysis, malonate utilisation, growth on cetrimide agar and fluorescence on *Pseudomonas* agar F. The strain was not capable of starch hydrolysis and did not possess lipolytic activity. These tests were selected as conventional phenotypic tests used to identify *Pseudomonas* organisms (Costas *et al.*, 1992). The tests selectively identified CP1 as being a *Pseudomonas putida* strain.

Table 7. Cell characteristics of *P. putida* CP1.

	P. putida CP1
Gram +/-	-
Shape	Rod
Spores +/-	-
Motility	+

Table 8. Biochemical properties of *P. putida* CP1. **A** - Hugh and Leifson, Oxidative (Ox) and Fermentative (Fer); **B** - Oxidase; **C** - Catalase; **D** - Thornley Arginine Dihydrolase; **E** - Starch Hydrolysis; **F** - Malonate utilisation; **G** - Growth on Cetrimide agar; **H** - Tween 80 hydrolysis; **I** - Fluorescence on *Pseudomonas* agar F.

	A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**
P. putida CP1	Ox	+	+	+	-	+	+	-	+

In addition to the biochemical tests carried out, identification was carried out using Biolog and API 20NE identification systems. Identification using a Biolog GN system identified CP1 as being a *Pseudomonas putida* strain, similarity value - 0.633, distance - 4.15 (Table 9), while identification using API 20NE tests gave a 99.9% i.d., t = 0.98; very good identification *Pseudomonas putida* strain (Table 10).

On the basis of the biochemical tests, in addition to the results of the Biolog and API identifications, CP1 was identified as being a *Pseudomonas putida* strain.

Table 9. Results of Biolog GN identification for strain CP1.

Assimilation Test		Assimilation Test	
Water	-	i-erythritol	-
α-cyclodextrin	-	D-fructose	+
Dextrin	+	L-fucose	-
Glycogen	+	D-galactose	-
Tween 40	+	Gentiobiose	-
Tween 80	+	α-D-glucose	+
N-acetyl-D-galactosamine	-	m-inositol	-
N-acetyl-D-glucosamine	-	α-D-lactose	-
Adonitiol	-	Lactulose	-
L-arabinose	+	Maltose	-
D-arabitol	-	D-mannitol	-
Cellobiose	-	D-mannose	+
D-melibiose	-	Acetic acid	+
ß-methyl D-glucoside	-	*cis*-aconitic acid	+
D-psicose	+	Citric acid	+
D-raffinose	-	Formic acid	+
L-rhamnose	-	D-galactonic acid lactone	+
D-sorbitol	-	D-galacturonic acid	+
Sucrose	-	D-gluconic acid	+
D-trehalose	-	D-glucosaminic acid	-
Turanose	-	D-glucuronic acid	+
Xylitol	-	α-hydroxybutyric acid	+
Methyl pyruvate	+	ß-hydroxybutyric acid	+
Mono-methyl succinate	+	γ-hydroxybutric acid	-
p-hydroxyphenylacetic acid	-	Bromo succinic acid	+
Itaconic acid	-	Succinamic acid	+
α-keto butyric acid	+	Glucuronamide	+

α-keto glutaric acid	+	Alaninamide	+
α-keto valeric acid	+	D-alanine	+
D,L-lactic acid	+	L-alanine	+
Malonic acid	+	L-alanyl-glycine	+
Propionic acid	-	L-asparagine	+
Quinic acid	+	L-aspartic acid	+
D-saccharic acid	+	L-glutamic acid	+
Sebacic acid	-	Glycyl-L-aspartic acid	-
Succinic acid	+	Glycyl-L-glutamic acid	+
L-histidine	+	Urocanic acid	+
Hydroxyl L-proline	+	Inosine	+
L-leucine	+	Uridine	+
L-ornithine	+	Thymidine	-
L-phenylalanine	+	Phenyl ethylamine	+
L-proline	+	Putrescine	+
L-pyroglutamic acid	+	2-amino ethanol	+
D-serine	+	2,3-butanediol	+
L-serine	+	Glycerol	+
L-threonine	+	D,L-α-glycerol phosphate	-
D,L-carnitine	+	glucose-1-phosphate	-
γ-amino butyric acid	+	glucose-6-phosphate	-

Table 10. Results of API 20NE identification for strain CP1.

Test	CP1
Reduction of nitrates	-
Indole production	-
Glucose acidification	-
Arginine Dihydrolase	+
Urease	-
Esculin Hydrolysis	-
Gelatine hydrolysis	-
ß-galactosidase	-
Glucose assimilation	+
Arabinose assimilation	+
Mannose assimilation	-
Mannitol assimilation	-
N-Acetyl-Glucosamine assimilation	-
Maltose assimilation	-
Gluconate assimilation	+
Caprate assimilation	+
Adipate assimilation	-
Malate assimilation	+
Citrate assimilation	+
Phenyl-acetate assimilation	+
Cyctochrome oxidase	+
Identification (After 48 hrs.)	*Pseudomonas putida* i.d. = 99.9%; t = 0.98; V. Good Identification

3.2.2 The degradation of mono-chlorophenols by *Pseudomonas putida* CP1.

The degradation of a range of mono-chlorophenol concentrations by *P. putida* CP1 (5% inoculum) was studied. Degradation studies were carried out under the same incubation conditions that were used in degradation trials by Biolyte HAB. The degradation of the mono-chlorophenols was studied at a range of concentrations between 0.78 mM and 3.12 mM.

3.2.2.1 The degradation of various concentrations of 2-chlorophenol by *Pseudomonas putida* CP1.

The degradation of 2-chlorophenol by *P. putida* CP1 is shown in Figs. 26 - 28. 1.56 mM 2-chlorophenol was degraded within 94 hours, with a stoichiometric release of chloride indicating complete degradation. Degradation of 2.33 mM 2-chlorophenol by *P. putida* CP1 resulted in 18% removal and 15% total chloride release within 180 hours. *P. putida* CP1 was unable to even partially metabolise 3.12 mM 2-chlorophenol. Degradation of 1.56 mM resulted in a drop in pH of 0.12 units.

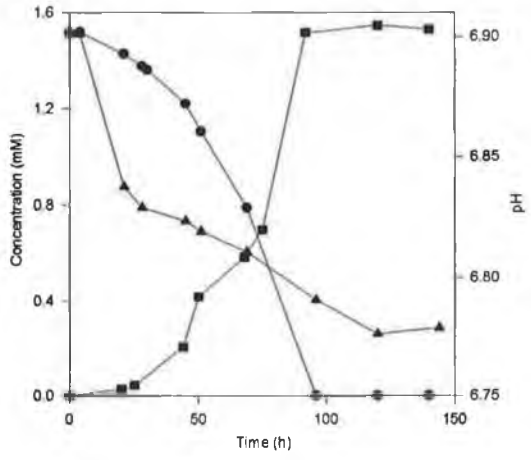

Figure 26. The degradation of 2-chlorophenol (1.56 mM) by *P. putida* CP1. Symbols: ● 2-chlorophenol, ■ chloride release and ▲ pH.

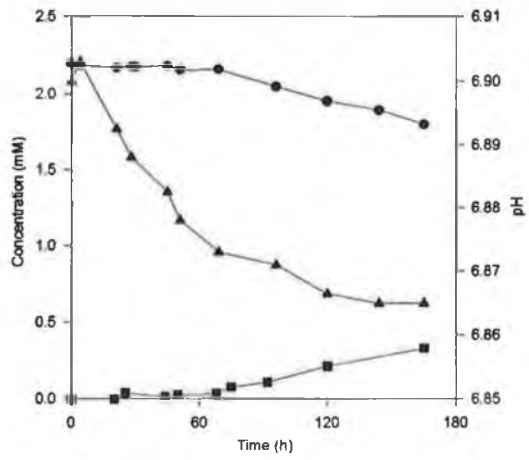

Figure 27. The degradation of 2-chlorophenol (2.33 mM) by *P. putida* CP1. Symbols: ● 2-chlorophenol, ■ chloride release and ▲ pH.

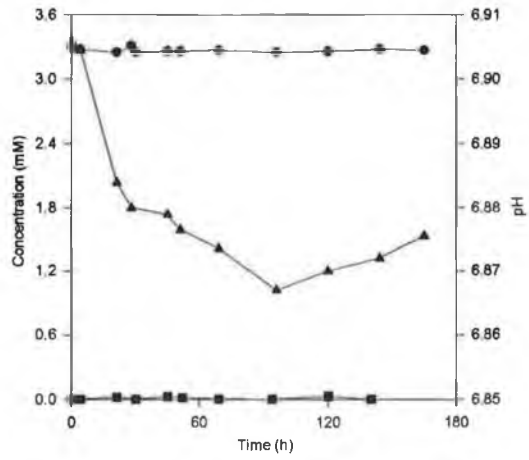

Figure 28. The degradation of 2-chlorophenol (3.12 mM) by *P. putida* CP1. Symbols: ● 2-chlorophenol, ■ chloride release and ▲ pH.

3.2.2.2 The degradation of various concentrations of 3-chlorophenol by *Pseudomonas putida* CP1.

3-chlorophenol was less readily degraded by *P. putida* CP1 than 2-chlorophenol. *P. putida* CP1 was capable of the complete degradation of 0.78 mM 3-chlorophenol within 80 hours and 1.56 mM within 240 hours (Fig. 29 & 30). Degradation resulted in

a stoichiometric release of chloride which was concurrent with chlorophenol removal. *P. putida* CP1 was unable to degrade 2.33 mM 3-chlorophenol (Fig. 31). Metabolism of 0.78 mM 3-chlorophenol resulted in a pH drop of 0.08 units, while degradation of 1.56 mM resulted in a pH drop of 0.16 units.

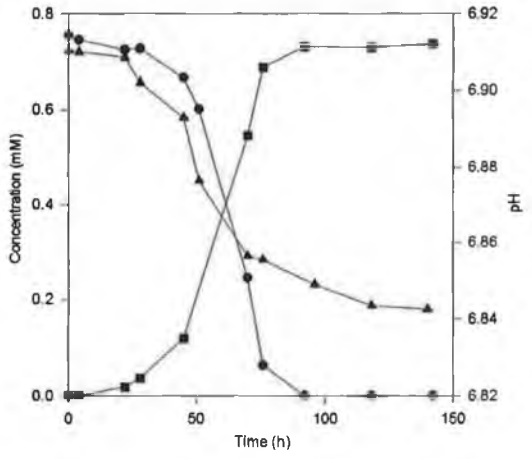

Figure 29. The degradation of 3-chlorophenol (0.78 mM) by *P. putida* CP1. Symbols: ● 3-chlorophenol, ■ chloride release and ▲ pH.

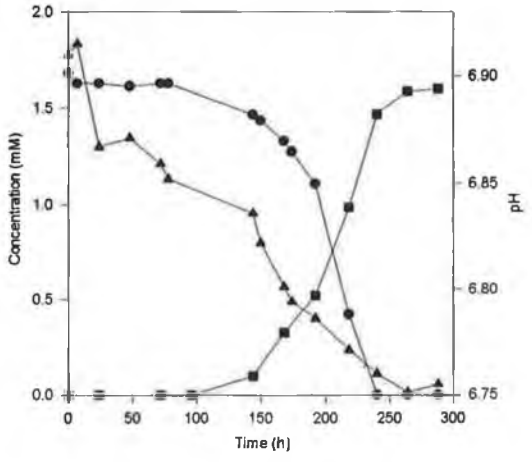

Figure 30. The degradation of 3-chlorophenol (1.56 mM) by *P. putida* CP1. Symbols: ● 3-chlorophenol, ■ chloride release and ▲ pH.

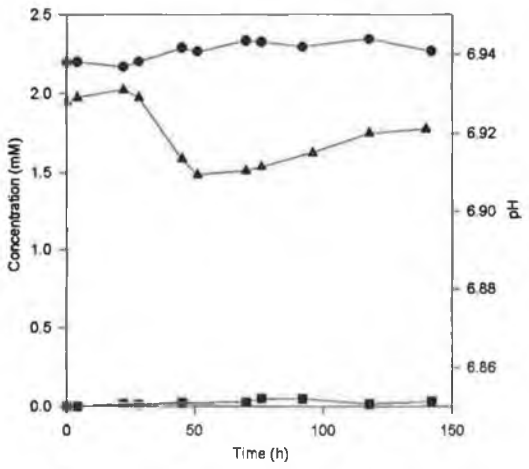

Figure 31. The degradation of 3-chlorophenol (2.33 mM) by *P. putida* CP1. Symbols: ● 3-chlorophenol, ■ chloride release and ▲ pH.

3.2.2.3 The degradation of various concentrations of 4-chlorophenol by *Pseudomonas putida* CP1.

Of the mono-chlorophenol isomers, 4-chlorophenol degradation by *P. putida* CP1 was the most successful. *P. putida* CP1 completely degraded 1.56 mM 4-chlorophenol within 33 hours, while 2.33 mM 4-chlorophenol was degraded within 80 hours (Figs. 32 & 33). Chlorophenol removal was mirrored by stoichiometric releases of chloride indicating complete degradation of 1.56 mM and 2.33 mM 4-chlorophenol. *P. putida* CP1 was unable to degrade 3.12 mM 4-chlorophenol (Fig. 34). Degradation of 1.56 mM resulted in a pH drop of 0.11 units, while 2.33 mM degradation resulted in a pH drop of 0.14 units.

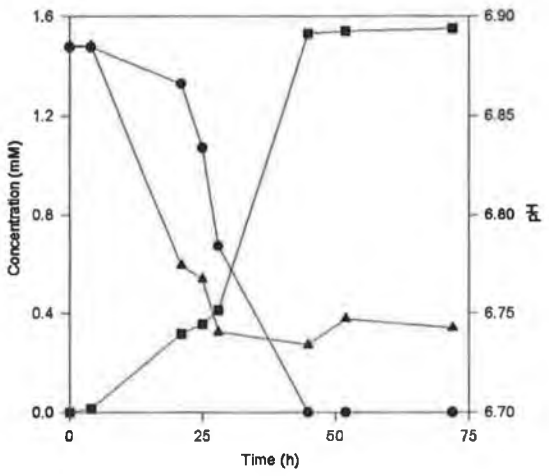

Figure 32. The degradation of 4-chlorophenol (1.56 mM) by *P. putida* CP1. Symbols: ● 4-chlorophenol, ■ chloride release and ▲ pH.

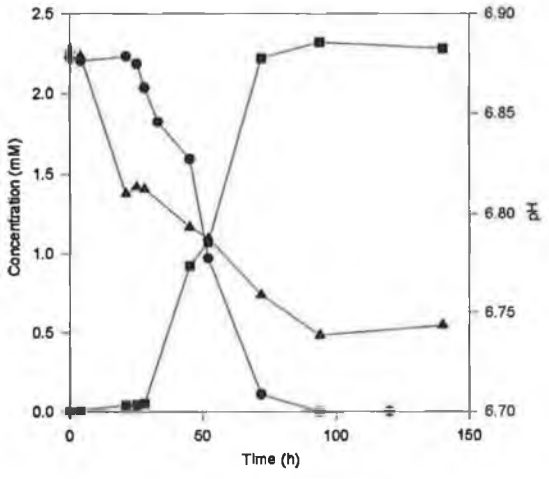

Figure 33. The degradation of 4-chlorophenol (2.33 mM) by *P. putida* CP1. Symbols: ● 4-chlorophenol, ■ chloride release and ▲ pH.

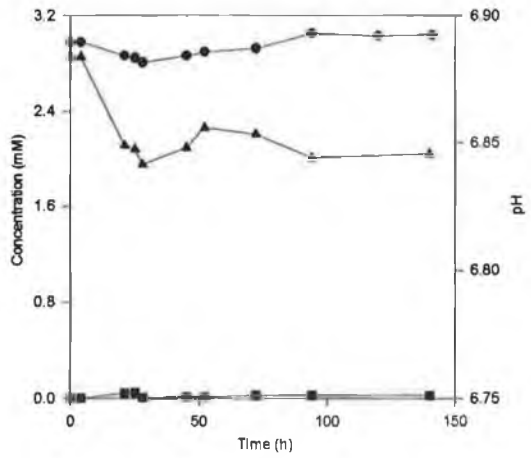

Figure 34. The degradation of 4-chlorophenol (3.12 mM) by *P. putida* CP1. Symbols: ● 4-chlorophenol, ■ chloride release and ▲ pH.

During the degradation of each of the three mono-chlorophenols, no chlorocatechol accumulations were detected in the culture fluid. However, the concurrent release of chloride following the degradation of each of the chlorophenols suggested that any intermediates produced in the degradative pathway were metabolised immediately and therefore would not be detected in the culture fluid. As a result of this, no colour production typical of the accumulation of metabolites was observed during chlorophenol degradation.

3.2.3 The degradation of 3- and 4-chlorocatechol by *Pseudomonas putida* CP1.

Although no chlorocatechols were detected during the degradation of 2, 3- and 4-chlorophenol by *P. putida* CP1, 3-chlorocatechol is a known intermediate of the degradation of 2- and 3-chlorophenol, while 4-chlorocatechol is produced during the degradation of 4-chlorophenol (Häggblom, 1990). Therefore it was of interest to study the degradation of chlorocatechols by *P. putida* CP1. Cells capable of the degradation of 2-chlorophenol were tested for their ability to degrade 3-chlorocatechol, while cells capable of degrading 4-chlorophenol were used to study 4-chlorocatechol degradation.

The degradation of 3- and 4-chlorocatechol (0.13 mM [20 ppm], 0.35 mM [50 ppm] and 0.69 mM [100 ppm]) by *P. putida* CP1 was studied under identical incubation conditions to those used during chlorophenol degradation studies.

P. putida CP1 was shown to be capable of degrading 0.13 mM and 0.35 mM 3-chlorocatechol within 5 hours, and 0.69 mM 3-chlorocatechol within 20 hours (Fig. 35). 3-chlorocatechol degradation resulted in a concurrent release of chloride which was stoichiometric for 0.13 mM and 0.35 mM. During the degradation of 0.69 mM 3-chlorocatechol by *P. putida* CP1, a faint brown colour accumulated in the culture medium, indicating polymerisation of the chlorocatechol. Due to the polymerisation of the chlorocatechol, only 82% total chloride release was observed during degradation of 0.69 mM 3-chlorocatechol.

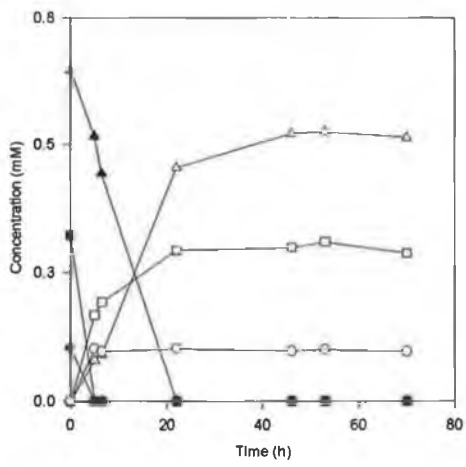

Figure 35. The degradation of various concentrations of 3-chlorocatechol (0.13 mM, 0.35 mM and 0.69 mM) by *P. putida* CP1. Symbols : ● 3-chlorocatechol (0.13 mM), O chloride release (0.13 mM); ■ 3-chlorocatechol (0.35 mM), □ chloride release (0.35 mM); ▲ 3-chlorocatechol (0.69 mM) and Δ chloride release (0.69 mM).

Degradation of 4-chlorocatechol was slower than was observed for 3-chlorocatechol degradation. *P. putida* CP1 was also capable of degrading 0.13 mM 4-chlorocatechol within 10 hours, 0.35 mM 4-chlorocatechol within 46 hours and 0.69 mM 4-chlorocatechol within 53 hours (Fig. 36). 4-chlorocatechol degradation also resulted in

stoichiometric releases of chloride for 0.13 mM and 0.35 mM. Polymerisation was also observed in flasks containing 0.69 mM 4-chlorocatechol resulting in a slight brown coloration of the culture medium. As a result, degradation of 0.69 mM 4-chlorocatechol resulted in only 90% total chloride release.

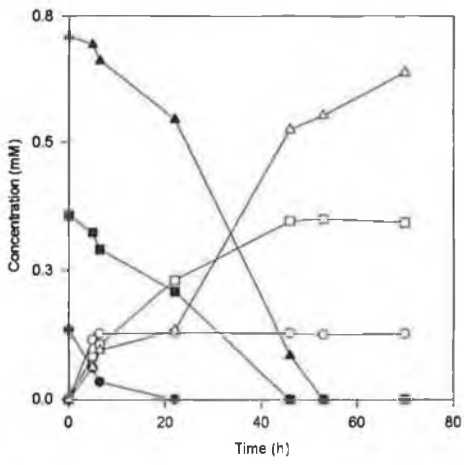

Figure 36. The degradation of various concentrations of 4-chlorocatechol (0.13 mM, 0.35 mM and 0.69 mM) by *P. putida* CP1. Symbols : ● 4-chlorocatechol (0.13 mM), O chloride release (0.13 mM); ■ 4-chlorocatechol (0.35 mM), □ chloride release (0.35 mM); ▲ 4-chlorocatechol (0.69 mM) and Δ chloride release (0.69 mM).

3.2.4 Morphology of *Pseudomonas putida* CP1 during growth on mono-chlorophenols.

The growth of *P. putida* CP1 on each of the mono-chlorophenol isomers resulted in the formation of large clumps of cells in the culture medium (Fig. 37). Growth of *P. putida* CP1 on 4-chlorophenol resulted in smaller clumps throughout the culture medium, while growth on 2- and 3-chlorophenol resulted in larger, less numerous flocs. Flocculation occurred during the lag period of chlorophenol degradation and chlorophenol degradation did not occur until *P. putida* CP1 had formed clumps in the culture medium (Fig. 37). No clumping was not during growth of *P. putida* CP1 in nutrient broth.

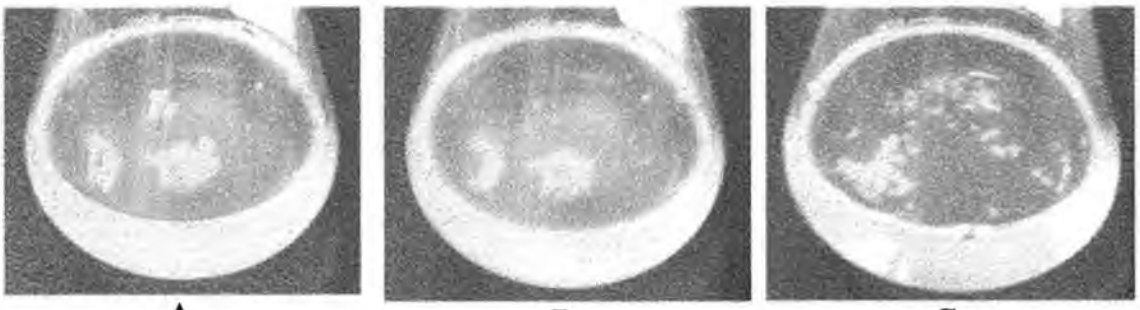

Figure 37. Clump formation following 48 hours growth of *P. putida* CP1 on mono-chlorophenols. Symbols : **A** 2-chlorophenol (1.56 mM), **B** 3-chlorophenol (0.78 mM) and **C** 4-chlorophenol (1.56 mM).

3.2.5 Ring cleavage enzyme activities during the degradation of mono-chlorophenols by *Pseudomonas putida* CP1.

The absence of any colours in the culture medium and the stoichiometric release of chloride following the degradation of each of the chlorophenols by *P. putida* CP1 suggested that degradation occurred using the *ortho-* cleavage pathway. Enzyme assays carried out confirmed this (Tables 11 - 13). No *meta-* cleavage activity was detected during the degradation of the mono-chlorophenols. *Ortho-* cleavage activities were detected throughout the degradation of the mono-chlorophenols and only became undetectable after degradation was complete. *Ortho-* cleavage activities towards both catechol and chlorocatechol were detected during the degradation of the mono-chlorophenols. Enzyme levels were at their highest during the period of maximum chlorophenol degradation. *Ortho-* cleavage activities for 2-chlorophenol degradation (1.56 mM) were greatest at 72 hours corresponding to the period of maximum chlorophenol degradation between 60 and 90 hours (Fig. 26). Similarly, *ortho-* cleavage activities were greatest at 72 hours for 3-chlorophenol (0.78 mM) and 24 hours for 4-chlorophenol (1.56 mM), corresponding to the periods of maximum chlorophenol degradation between 60 and 90 hours for 3-chlorophenol (Fig. 29) and 20 to 40 hours for 4-chlorophenol degradation (Fig. 32).

Table 11. Enzyme activities during the degradation of 2-chlorophenol (1.56 mM) by *P. putida* CP1.

Enzyme Assayed and assay substrate	Specific Activity (U/mg)			
	T = 24 hrs	T = 48 hrs	T = 72 hrs	T = 96 hrs
Catechol 2,3- dioxygenase				
Catechol	< 0.001	< 0.001	< 0.001	< 0.001
Catechol 1,2-dioxygenase				
Catechol	< 0.001	1.90	5.92	1.65
3-chlorocatechol	< 0.001	0.78	4.06	1.34

Table 12. Enzyme activities during the degradation of 3-chlorophenol (0.78 mM) by *P. putida* CP1.

Enzyme Assayed and assay substrate	Specific Activity (U/mg)			
	T = 24 hrs	T = 48 hrs	T = 72 hrs	T = 96 hrs
Catechol 2,3- dioxygenase				
Catechol	< 0.001	< 0.001	< 0.001	< 0.001
Catechol 1,2-dioxygenase				
Catechol	0.63	0.36	5.55	1.56
3-chlorocatechol	0.41	0.24	3.35	1.62

Table 13. Enzyme activities during the degradation of 4-chlorophenol (1.56 mM) by *P. putida* CP1.

Enzyme Assayed and assay substrate	Specific Activity (U/mg)			
	T = 12 hrs	T = 24 hrs	T = 48 hrs	T = 72 hrs
Catechol 2,3- dioxygenase				
Catechol	< 0.001	< 0.001	< 0.001	< 0.001
Catechol 1,2-dioxygenase				
Catechol	5.60	6.63	1.34	< 0.001
3-chlorocatechol	5.43	5.55	2.29	< 0.001

3.3 Degradation of mono-chlorophenols by Biolyte HAB augmented with *Pseudomonas putida* CP1.

Biolyte HAB was shown to degrade mono-chlorophenols using the *meta*- cleavage pathway (Section 3.1). The *meta*- cleavage of chloro-aromatics is known to generally result in dead-end pathways. While Biolyte HAB possessed a *meta*- cleavage pathway which allowed for the complete degradation of 4-chlorophenol, *meta*- cleavage of 2- and 3-chlorophenol was incomplete. In order for Biolyte HAB to degrade the mono-chlorophenol isomers to completion, induction of the *ortho*- cleavage pathway would be necessary.

P. putida CP1 however was capable of degrading all three of the mono-chlorophenols to completion using an *ortho*- cleavage pathway (Section 3.2). As very few studies have evaluated the introduction of degradative bacteria into mixed cultures, in order to enhance biodegradation of xenobiotics with any success, it was of interest to add *P. putida* CP1 to Biolyte HAB and to study the effect of its addition on the overall degradative capacity of the mixed culture. Degradation of the mono-chlorophenols by a 9.5% (v/v) inoculum of Biolyte HAB augmented with 0.5% (v/v) *P. putida* CP1 was examined. Whereas previously a 10% (v/v) inoculum of Biolyte HAB resulted in the incomplete degradation of 2- and 3-chlorophenol by the *meta*- cleavage pathway, it

was hoped that the addition of 0.5% (v/v) *P. putida* CP1 to 9.5% (v/v) Biolyte HAB would bring about complete degradation of all three mono-chlorophenol isomers.

3.3.1 The degradation of mono-chlorophenols by *Pseudomonas putida* CP1 (0.5% inoculum).

While the degradation of 2-, 3- and 4-chlorophenol by a 5% inoculum of *P. putida* CP1 was described previously (Section 3.2), it was of interest to study mono-chlorophenol degradation by a 0.5% inoculum as a pure culture prior to its addition to Biolyte HAB. Mono-chlorophenol degradation by *P. putida* CP1 (0.5% inoculum) is shown in Figures 38 - 40. Chlorophenol degradation resulted in no colour production, indicating that degradation was via the *ortho*- cleavage pathway and was accompanied by stoichiometric releases of chloride. *P. putida* CP1 (0.5% inoculum) degraded up to 1.56 mM 2-chlorophenol and 0.78 mM 3-chlorophenol within 118 hours (Figs. 38 & 39). Degradation of 4-chlorophenol by *P. putida* CP1 was more successful with 1.56 mM degraded within 74 hours (Fig. 40). Degradation of the mono-chlorophenols by the 0.5% inoculum was slower than by a 5% inoculum, which better degraded 1.56 mM 2-chlorophenol within 96 hours (Fig. 26), 0.78 mM 3-chlorophenol within 78 hours (Fig. 29) and 1.56 mM 4-chlorophenol within 32 hours (Fig. 32). 2.33 mM 2- and 4-chlorophenol and 1.56 mM 3-chlorophenol were not degraded by 0.5% *P. putida* CP1 during the period allowed for these experiments. Growth of *P. putida* CP1 on each of the mono-chlorophenols again resulted in the formation of clumps of cells in the culture medium as was seen during the degradation of mono-chlorophenols by a 5% inoculum.

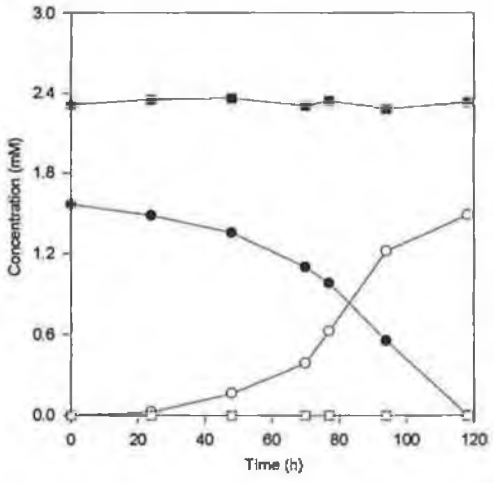

Figure 38. The degradation of 2-chlorophenol by *P. putida* CP1 (0.5% inoculum). Symbols : ● 2-chlorophenol (1.56 mM), O chloride release (1.56 mM), ■ 2-chlorophenol (2.33 mM) and □ chloride release (2.33 mM).

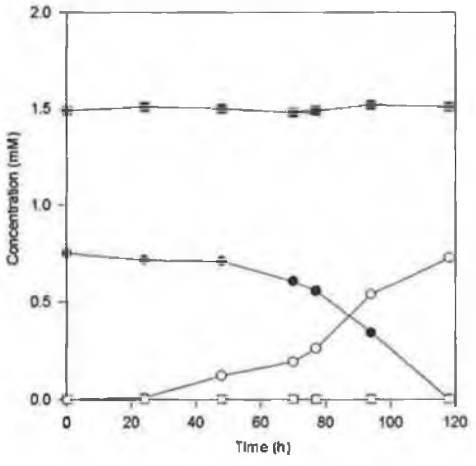

Figure 39. The degradation of 3-chlorophenol by *P. putida* CP1 (0.5% inoculum). Symbols : ● 3-chlorophenol (0.78 mM), O chloride release (0.78 mM), ■ 3-chlorophenol (1.56 mM) and □ chloride release (1.56 mM).

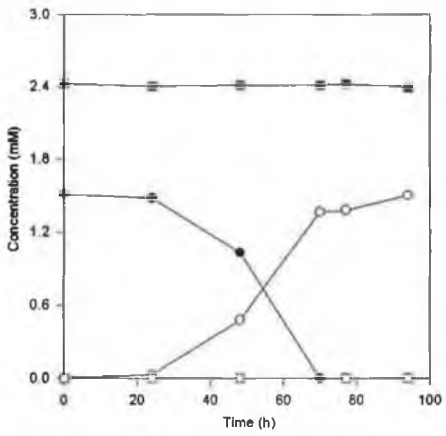

Figure 40. The degradation of 4-chlorophenol by *P. putida* CP1 (0.5% inoculum). Symbols : ● 4-chlorophenol (1.56 mM), O chloride release (1.56 mM), ■ 4-chlorophenol (2.33 mM) and □ chloride release (2.33 mM).

3.3.2 The degradation of mono-chlorophenols following addition of *Pseudomonas putida* CP1 to Biolyte HAB.

Addition of *P. putida* CP1 to Biolyte HAB resulted in improved degradation of the mono-chlorophenols. Whereas previously Biolyte HAB was unable to degrade 2-chlorophenol to completion and 0.5% *P. putida* CP1 degraded up to 1.56 mM within 118 hours, the augmented mixed culture could now degrade 1.56 mM within 45 hours and 2.33 mM within 90 hours (Fig. 41). Chlorophenol degradation was accompanied by stoichiometric releases of chloride resulting in a pH drop of 0.16 pH units for 1.56 mM and 0.20 for 2.33 mM.

It was also shown previously that Biolyte HAB was unable to degrade 3-chlorophenol to completion, while 0.5% *P. putida* CP1 degraded 0.78 mM within 118 hours. The augmented mixed culture however could now degrade up to 1.56 mM 3-chlorophenol. 0.78 mM 3-chlorophenol was degraded within 35 hours and 1.56 mM 3-chlorophenol within 60 hours (Fig. 42). The resultant stoichiometric releases of chloride resulted in a drop in pH of 0.13 pH units for 0.78 mM and 0.17 pH units for 1.56 mM.

While 0.5% *P. putida* CP1 degraded up to 1.56 mM 4-chlorophenol within 74 hours and Biolyte HAB (10% inoculum) degraded 1.56 mM within 44 hours and 2.33 mM within 72 hours, the augmented mixed culture degraded 1.56 mM 4-chlorophenol within 30 hours, while 2.33 mM 4-chlorophenol was degraded within 58 hours (Fig. 43). The stoichiometric release of chloride following the degradation of the mono-chlorophenols caused the pH to drop by 0.19 pH units and 0.21 pH units for 1.56 mM and 2.33 mM 4-chlorophenol respectively.

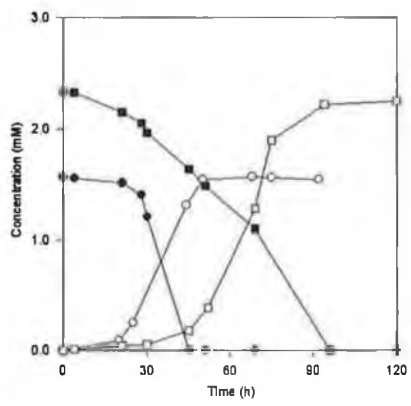

Figure 41. The degradation of 2-chlorophenol by the augmented mixed culture. Symbols : ● (1.56 mM) & ■ (2.33 mM) 2-chlorophenol, O (1.56 mM) & □ (2.33 mM) differential chloride concentration.

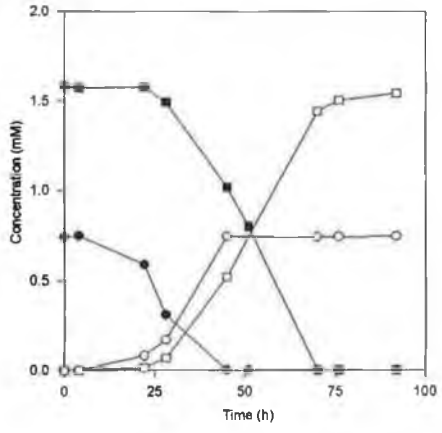

Figure 42. The degradation of 3-chlorophenol by the augmented mixed culture. Symbols : ● (0.78 mM) & ■ (1.56 mM) 3-chlorophenol, O (0.78 mM) & □ (1.56 mM) differential chloride concentration.

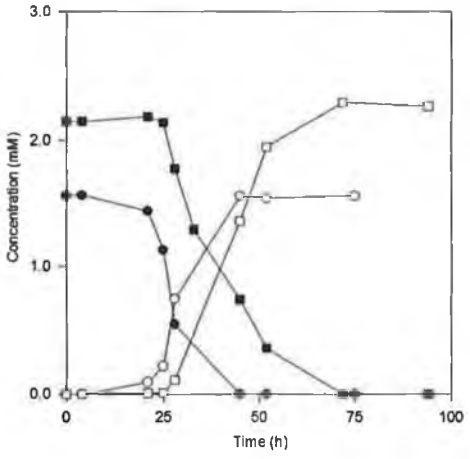

Figure 43. The degradation of 4-chlorophenol by the augmented mixed culture. Symbols : ● (1.56 mM) & ■ (2.33 mM) 4-chlorophenol, O (1.56 mM) & □ (2.33 mM) differential chloride concentration.

While the augmented mixed culture settled very quickly in the absence of agitation during the degradation of all three mono-chlorophenols, the characteristic formation of large aggregates observed in pure culture was not observed for the augmented mixed culture.

3.3.3 Ring cleavage enzyme activities during the degradation of mono-chlorophenols by Biolyte HAB/*Pseudomonas putida* CP1 (9.5%:0.5%).

The stoichiometric releases of chloride and the absence of colour production in flasks following the degradation of each of the mono-chlorophenols by the augmented mixed culture, suggested that metabolism had occurred using the *ortho*- cleavage pathway. In order to confirm this, the key enzymes involved in the ring cleavage of chlorinated aromatics, chlorocatechol 1,2-dioxygenase, and catechol 2,3-dioxygenase were assayed following growth of the Biolyte HAB/*P. putida* mixture CP1 on 2-chlorophenol (1.56 mM), 3-chlorophenol (0.78 mM) and 4-chlorophenol (1.56 mM).

The enzyme assays confirmed that metabolism did take place using the *ortho-* cleavage pathway. No *meta-* cleavage activity (catechol 2,3-dioxygenase) was found following growth of the augmented mixture on the three mono-chlorophenol isomers. The *meta-* cleavage activity, previously observed during the degradation of mono-chlorophenols by Biolyte HAB was no longer present.

However, *ortho-* cleavage activity towards catechol and chlorocatechol was found following growth on each of the mono-chlorophenols (Table 14 - 16). Addition of *P. putida* CP1, which had previously been shown to express *ortho-* cleavage activity following growth on mono-chlorophenols, to Biolyte HAB had switched the metabolic pathway from the *meta-* cleavage pathway to the more productive *ortho-* cleavage pathway. This allowed the augmented mixed culture to degrade all three of the mono-chlorophenols to completion. Levels of chlorocatechol 1,2-dioxygenase were greatest at 36 and 48 hours for 2-chlorophenol, 24 and 48 hours for 3-chlorophenol and 12 and 24 hours for 4-chlorophenol, corresponding to the periods of maximum chlorophenol degradation for each of the mono-chlorophenols. Whereas no catechol 2,3-dioxygenase activity was detected, catechol 1,2-dioxygenase activity was detected throughout the degradation of the mono-chlorophenols by the augmented mixed culture. Levels of catechol 1,2-dioxygenase only decreased after each of the mono-chlorophenols had been completely degraded.

Table 14. Enzyme activities during the degradation of 2-chlorophenol (1.56 mM) by Biolyte HAB/ *P. putida* CP1 (9.5% : 0.5%).

Enzyme Assayed and assay Substrate	Specific Activity (U/mg) T = 24 hrs	T = 36 hrs	T = 48 hrs	T = 72 hrs
Catechol 2,3- dioxygenase				
Catechol	< 0.001	< 0.001	< 0.001	< 0.001
Catechol 1,2-dioxygenase				
Catechol	2.14	2.89	3.82	0.13
3-chlorocatechol	0.41	2.63	4.67	0.19

Table 15. Enzyme activities during the degradation of 3-chlorophenol (0.78 mM) by Biolyte HAB/ *P. putida* CP1 (9.5% : 0.5%).

Enzyme Assayed and assay Substrate	Specific Activity (U/mg) T = 24 hrs	T = 48 hrs	T = 72 hrs	T = 96 hrs
Catechol 2,3- dioxygenase				
Catechol	< 0.001	< 0.001	< 0.001	< 0.001
Catechol 1,2-dioxygenase				
Catechol	3.31	2.48	0.76	0.43
3-chlorocatechol	2.67	3.26	1.46	0.57

Table 16. Enzyme activities during the degradation of 4-chlorophenol (1.56 mM) by Biolyte HAB/ *P. putida* CP1 (9.5% : 0.5%).

Enzyme Assayed and assay Substrate	Specific Activity (U/mg) T = 12 hrs	T = 24 hrs	T = 48 hrs	T = 72hrs
Catechol 2,3- dioxygenase				
Catechol	< 0.001	< 0.001	< 0.001	< 0.001
Catechol 1,2-dioxygenase				
Catechol	4.37	1.77	1.39	0.31
3-chlorocatechol	3.69	2.02	2.02	0.39

3.3.4 Survival of *Pseudomonas putida* CP1 following its addition to Biolyte HAB.

The addition of *P. putida* CP1 to Biolyte HAB resulted in the pathway of mono-chlorophenol degradation being shifted from the *meta-* cleavage pathway to the more productive *ortho-* cleavage pathway. It was of interest to know whether the introduced strain responsible for the metabolic shift survived in the mixed culture and whether the relative populations of the mixed culture changed during chlorophenol degradation.

3.3.4.1 The degradation of mono-chlorophenols by a rifampicin mutant of *Pseudomonas putida* CP1 (5% inoculum).

The generation of a rifampicin mutant of *P. putida* CP1 allowed for its survival in Biolyte HAB to be followed by plating onto rifampicin plate count agar plates. When Biolyte HAB was plated on rifampicin plate count agar, no colonies resulted. The rifampicin mutant displayed the same chlorophenol degrading capabilities as the wild type strain (Figs. 26, 29 & 32). The degradation of 2-chlorophenol (1.56 mM), 3-chlorophenol (0.78 mM) and 4-chlorophenol (1.56 mM) by the rifampicin mutant of *P. putida* CP1 is shown in Figure 44.

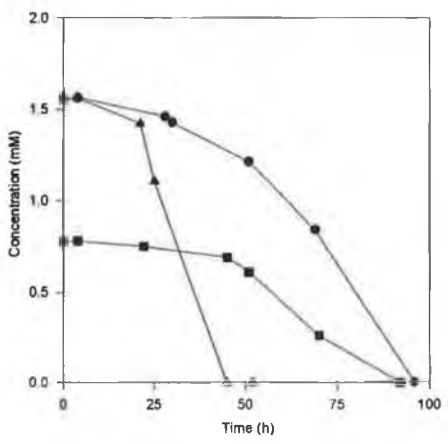

Figure 44. The degradation of mono-chlorophenols by *P. putida* CP1 (Rifampicin mutant, 5% inoculum). Symbols : ● 2-chlorophenol (1.56 mM), ■ 3-chlorophenol (0.78 mM) & ▲ 4-chlorophenol (1.56 mM).

3.3.4.2 Microbiological analysis of the augmented mixed culture during the degradation of mono-chlorophenols.

During the degradation of the mono-chlorophenols by the augmented mixed culture microbiological analysis was carried out to determine the total bacterial numbers, the

total chlorophenol degrading numbers and the numbers of *P. putida* CP1 in the mixed culture. The survival of *P. putida* CP1 in the mixed culture is shown in Figure 45.

During the degradation of all three mono-chlorophenols by the augmented mixed culture, *P. putida* CP1 established itself as part of the mixture and its numbers increased during the degradation of the mono-chlorophenols. As *P. putida* CP1 numbers rose, so too did the overall numbers enumerated on plate count agar and chlorophenol degraders enumerated on chlorophenol agar.

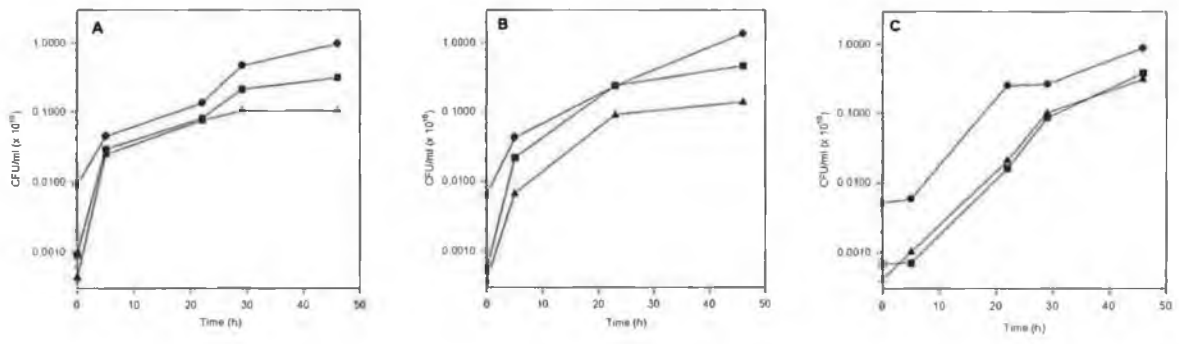

Figure 45. Growth of augmented mixed culture (9.5% Biolyte HAB : 0.5% *P. putida* CP1) on **A** 2-chlorophenol (1.56 mM), **B** 3-chlorophenol (0.78 mM) and **C** 4-chlorophenol (1.56 mM). Symbols : ● Total counts, ■ Chlorophenol degraders and ▲ *P. putida* CP1 counts.

3.4 The influence of Biolyte HAB on the degradation of mono-chlorophenols by *Pseudomonas putida* CP1.

The role of *P. putida* CP1 in the degradation of mono-chlorophenols by a mixture of Biolyte HAB and *P. putida* CP1 was clear, as in its absence degradation of 2- and 3-chlorophenol was not possible. Biolyte HAB which was shown to degrade mono-chlorophenols using a *meta*- cleavage pathway, degraded all three mono-chlorophenols to completion using an *ortho*- cleavage pathway following addition of *P. putida* CP1. To investigate the role of Biolyte HAB in the degradation of mono-chlorophenols by

the Biolyte HAB/*P. putida* CP1 mixture, varying inocula sizes of Biolyte HAB were added to *P. putida* CP1 (5% inoculum) for the degradation of mono-chlorophenols. The inocula sizes studied were (% v/v Biolyte HAB : % v/v *P. putida* CP1) 0% : 5%, 1% : 5%, 5% : 5% and 10% : 5%.

3.4.1 The degradation of mono-chlorophenols by various ratios of Biolyte HAB/*Pseudomonas putida* CP1.

Addition of Biolyte HAB to *P. putida* CP1 improved chlorophenol degradation for each of the isomers. As was seen during the degradation of the mono-chlorophenols by *P. putida* CP1 in isolation (Section 3.2) and 9.5% Biolyte HAB : 0.5% *P. putida* CP1 (Section 3.3), degradation of the mono-chlorophenols was accompanied by stoichiometric releases of chloride (data not shown). The release of chloride, indicating complete degradation of the mono-chlorophenols, resulted in small drops in pH of the culture medium no greater than 0.25 pH units. Similar to the degradation of mono-chlorophenols by Biolyte HAB augmented with 0.5 % *P. putida* CP1, no flocculation was observed during degradation by the various Biolyte HAB/*P. putida* CP1 combinations, although cultures settled quickly in the absence of agitation. The degradation of the mono-chlorophenol isomers by *P. putida* CP1 and the augmented mixed cultures is detailed in Figures 46 - 48.

3.4.1.1 The degradation of 2-chlorophenol by various ratios of Biolyte HAB/*Pseudomonas putida* CP1.

The degradation of 2-chlorophenol (1.56 mM and 2.33 mM) by the various ratios of Biolyte HAB and *P. putida* CP1 is detailed in Figures 46, 48 and 50. No colours typical of metabolite accumulation were produced during the degradation of 2-chlorophenol by the Biolyte HAB/*P. putida* CP1 mixtures (Fig. 47). While previously Biolyte HAB was shown not to be capable of the complete degradation of 2-chlorophenol (Section 3.1) and *P. putida* CP1 degraded up to 1.56 mM in isolation

(Section 3.2), the various Biolyte HAB/*P. putida* CP1 mixtures degraded up to 2.33 mM 2-chlorophenol. 3.12 mM 2-chlorophenol was not degraded by the Biolyte HAB/*P. putida* CP1 mixtures.

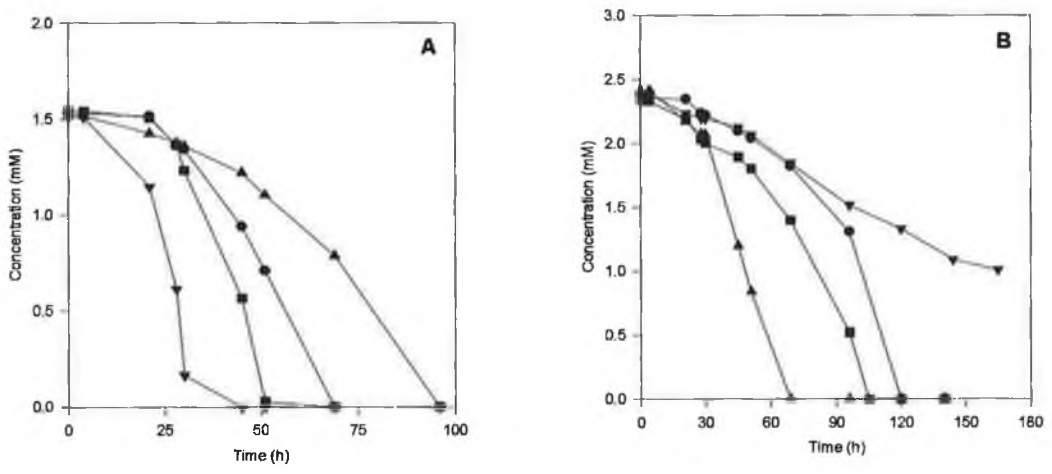

Figure 46. The degradation of 2-chlorophenol (**A** 1.56 mM, **B** 2.33 mM) by Biolyte HAB/*P. putida* CP1. Symbols : (Biolyte HAB : *P. putida* CP1) ● 1% : 5%, ■ 5% : 5%, ▼ 10% : 5%, ▲ 0% : 5%.

Figure 47. Colour production following the degradation of 2-chlorophenol (1.56 mM) by Biolyte HAB/*P. putida* CP1. Symbols : **A** Biolyte HAB (10%), **B** *P. putida* CP1 (5%), **C** 10% : 5% (Biolyte HAB : *P. putida* CP1), **D** 9.5% : 0.5%, **E** 5% : 5%, **F** 1% : 5% and **G** control.

Addition of Biolyte HAB to 5% *P. putida* CP1 enhanced the degradation of 2-chlorophenol. Addition of 1% Biolyte HAB reduced the time required for 1.56 mM 2-chlorophenol degradation from 96 hours to 69 hours (Table 17). Subsequent increases

in Biolyte HAB inocula sizes resulted in further reductions in the times of degradation, resulting in the degradation of 1.56 mM 2-chlorophenol within 51 and 30 hours following the addition of 5% and 10% Biolyte HAB respectively. Similar reductions in degradation times with increasing Biolyte HAB inocula sizes were observed at the higher concentration of 2.33 mM 2-chlorophenol (Table 18). Addition of Biolyte HAB reduced the time required for degradation of 2.33 mM from 120 hours by 1% Biolyte HAB : 5% *P. putida* CP1, to 102 and 62 hours following addition of 5% and 10% Biolyte HAB respectively.

Reductions in degradation times resulted from decreases in lag periods and increases in rates of degradation. As can be seen from Tables 17 and 18, lag periods decreased for the degradation of both 1.56 mM and 2.33 mM with increasing Biolyte HAB inocula sizes. The lag period for the degradation of 1.56 mM 2-chlorophenol was reduced from 35 hours for *P. putida* CP1 in isolation to 33, 25 and 19 hours following the addition of 1%, 5% and 10% Biolyte HAB respectively. Similarly, lag times reduced from 76 hours for the degradation of 2.33 mM 2-chlorophenol by 1% Biolyte HAB: 5% *P. putida* CP1 to 64 and 28 hours following the addition of 5% and 10% Biolyte HAB respectively.

At both concentrations (1.56 mM and 2.33 mM) the maximum rate of 2-chlorophenol degradation increased with increasing Biolyte HAB inocula size (Tables 17 & 18). The rate of 1.56 mM 2-chlorophenol degradation was increased from 0.043 mM/hr for 1% Biolyte HAB : 5% *P. putida* CP1 to 0.059 and 0.138 mM/hr following addition of 5% and 10% Biolyte HAB. Similarly 2.33 mM degradation rates increased from 0.054 mM/hr (1%:5%) to 0.062 mM/hr (5%:5%) and 0.071 mM/hr (10%:5%).

Table 17. The degradation of 2-chlorophenol (1.56 mM) by Biolyte HAB/*P. putida* CP1.

Inoculum	Lag time (h)	Time of Degradation (h)	Rate of Degradation (mM/h)
0% : 5%	35	96	0.025
1% : 5%	33	69	0.043
5% : 5%	25	51	0.059
10% : 5%	19	30	0.138

Table 18. The degradation of 2-chlorophenol (2.33 mM) by Biolyte HAB/*P. putida* CP1.

Inoculum	Lag time (h)	Time of Degradation (h)	Rate of Degradation (mM/h)
0% : 5%[a]	——	——	——
1% : 5%	76	120	0.054
5% : 5%	64	102	0.062
10% : 5%	28	62	0.071

a - Degradation not complete, from Section 3.2.

3.4.1.2 The degradation of 3-chlorophenol by various ratios of Biolyte HAB/*Pseudomonas putida* CP1.

The degradation of 3-chlorophenol (0.78 mM and 1.56 mM) by the various ratios of Biolyte HAB and *P. putida* CP1 is detailed in Figure 48. No colour production was observed following the degradation of 3-chlorophenol by the Biolyte HAB/*P. putida* CP1 mixtures (Fig. 49). Biolyte HAB was previously shown to be unable to degrade 3-

chlorophenol to completion (Section 3.1), while *P. putida* CP1 degraded up to 1.56 mM in isolation (Section 3.2).

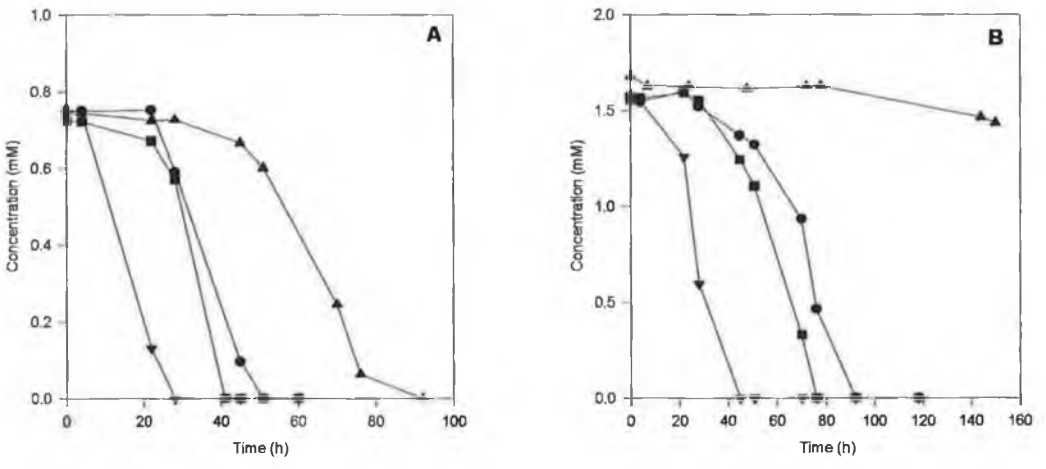

Figure 48. The degradation of 3-chlorophenol (**A** 0.78 mM, **B** 1.56 mM) by Biolyte HAB/*P. putida* CP1. Symbols : (Biolyte HAB : *P. putida* CP1) ● 1% : 5%, ■ 5% : 5%, ▼ 10% : 5%, ▲ 0% : 5%.

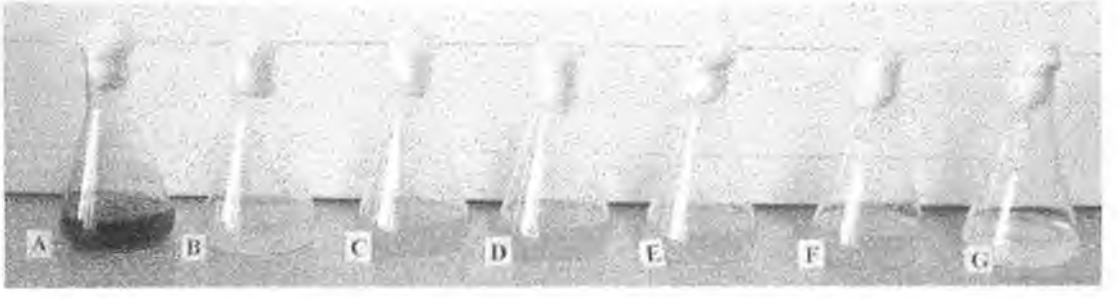

Figure 49. Colour production following the degradation of 3-chlorophenol (0.78 mM) by Biolyte HAB/*P. putida* CP1. Symbols : **A** Biolyte HAB (10%), **B** *P. putida* CP1 (5%), **C** 10% : 5% (Biolyte HAB : *P. putida* CP1), **D** 9.5% : 0.5%, **E** 5% : 5%, **F** 1% : 5% and **G** control.

Addition of Biolyte HAB to 5% *P. putida* CP1 enhanced the degradation of 3-chlorophenol. Although addition of various inocula sizes of Biolyte HAB did not increase the concentration range over which 3-chlorophenol could be degraded, times of degradation were reduced following addition of the mixed culture. Addition of 1%

Biolyte HAB reduced the time required for 0.78 mM 3-chlorophenol degradation from 78 hours to 49 hours (Table 19). Subsequent increases in Biolyte HAB inocula sizes resulted in further reductions in times of degradation, resulting in the degradation of 0.78 mM 3-chlorophenol within 41 and 24 hours following the addition of 5% and 10% Biolyte HAB respectively. Similar reductions in degradation times with increasing Biolyte HAB inocula sizes were observed at the higher concentration of 1.56 mM 3-chlorophenol (Table 20). Addition of 1% Biolyte HAB reduced the time of degradation from 240 hours for *P. putida* CP1 in isolation to 88 hours. Subsequent increases in Biolyte HAB concentrations reduced degradation times to 73 and 36 hours following addition of 5% and 10% Biolyte HAB respectively.

As was seen for 2-chlorophenol, reductions in degradation times resulted from decreases in lag periods and increases in rates of degradation. Tables 19 and 20 show lag periods decreased for degradation of both 0.78 mM and 1.56 mM with increasing Biolyte HAB inocula sizes. The lag period for the degradation of 0.78 mM 3-chlorophenol was reduced from 42 hours for *P. putida* CP1 in isolation to 25, 23 and 10 hours following the addition of 1%, 5% and 10% Biolyte HAB respectively. Similarly lag times reduced from 170 hours for the degradation of 1.56 mM 3-chlorophenol by *P. putida* CP1 in isolation to 52, 43 and 20 hours following the addition of 1%, 5% and 10% Biolyte HAB respectively.

At both concentrations (0.78 mM and 1.56 mM) the maximum rate of 3-chlorophenol degradation increased with increasing Biolyte HAB inocula size (Tables 19 & 20). The rate of 0.78 mM 3-chlorophenol degradation was increased from 0.032 mM/hr for 1% Biolyte HAB : 5% *P. putida* CP1 to 0.038 and 0.053 mM/hr following addition of 5% and 10% Biolyte HAB. Similarly 1.56 mM degradation rates increased from 0.048 mM/hr (1%:5%) to 0.052 mM/hr (5%:5%) and 0.098 mM/hr (10%:5%).

Table 19. The degradation of 3-chlorophenol (0.78 mM) by Biolyte HAB/*P. putida* CP1.

Inoculum	Lag time (h)	Time of Degradation (h)	Rate of Degradation (mM/h)
0% : 5%	42	78	0.021
1% : 5%	25	49	0.032
5% : 5%	23	41	0.038
10% : 5%	10	24	0.053

Table 20. The degradation of 3-chlorophenol (1.56 mM) by Biolyte HAB/*P. putida* CP1.

Inoculum	Lag time (h)	Time of Degradation (h)	Rate of Degradation (mM/h)
0% : 5%	170	240	0.023
1% : 5%	52	88	0.048
5% : 5%	43	73	0.052
10% : 5%	20	36	0.098

3.4.1.3 The degradation of 4-chlorophenol by various ratios of Biolyte HAB/*Pseudomonas putida* CP1.

The degradation of 4-chlorophenol (1.56 mM and 2.33 mM) by the various ratios of Biolyte HAB and *P. putida* CP1 is detailed in Figure 50. No colour production was observed following the degradation of 4-chlorophenol by the Biolyte HAB/*P. putida* CP1 mixtures (Fig. 51). Both Biolyte HAB and *P. putida* CP1 were shown to be capable of the complete degradation of up to 2.33 mM 4-chlorophenol (Sections 3.1 & 3.2).

Addition of Biolyte HAB to 5% *P. putida* CP1 enhanced the degradation of 4-chlorophenol. Although addition of various amounts of Biolyte HAB did not increase the concentration range over which 4-chlorophenol could be degraded, times of degradation were reduced following addition of Biolyte HAB. Addition of 1% Biolyte HAB reduced the time required for 1.56 mM 4-chlorophenol degradation from 32 hours for 5% *P. putida* CP1 to 26 hours (Table 21). Subsequent increases in Biolyte HAB inocula sizes resulted in further reductions in times of degradation, resulting in the degradation of 1.56 mM 4-chlorophenol within 13 and 8 hours following the addition of 5% and 10% Biolyte HAB respectively. Similar reductions in degradation times with increasing Biolyte HAB inocula sizes were observed at the higher concentration of 2.33 mM 4-chlorophenol (Table 22). Addition of 1% Biolyte HAB reduced the time of degradation for degradation of 2.33 mM 4-chlorophenol from 75 hours for 5% *P. putida* CP1 to 35 hours. Subsequent increases in Biolyte HAB concentrations reduced degradation times to 29 and 17 hours following addition of 5% and 10% Biolyte HAB respectively.

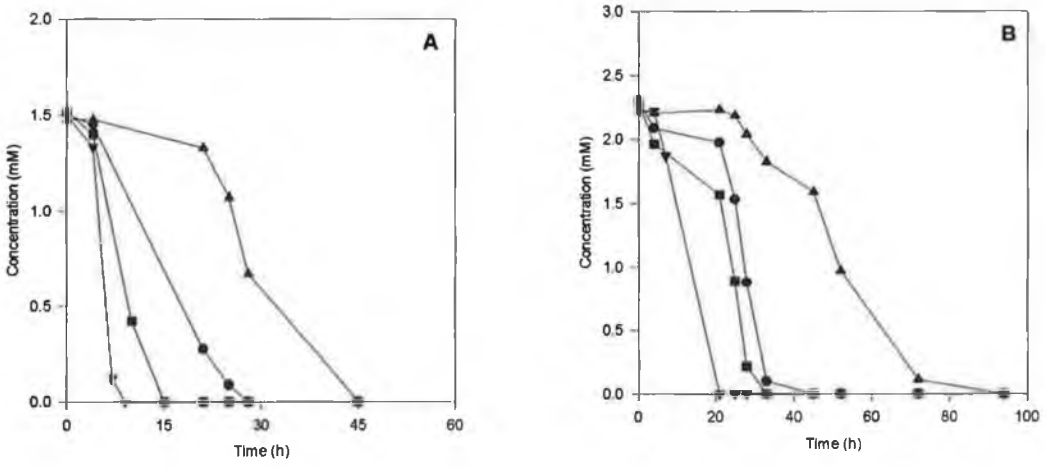

Figure 50. The degradation of 4-chlorophenol (**A** 1.56 mM, **B** 2.33 mM) by Biolyte HAB/*P. putida* CP1. Symbols : (Biolyte HAB : *P. putida* CP1). ● 1% : 5%, ■ 5% : 5%, ▼ 10% : 5%, ▲ 0% : 5%.

Figure 51. Colour production following the degradation of 4-chlorophenol (1.56 mM) by Biolyte HAB/*P. putida* CP1. Symbols : **A** Biolyte HAB (10%), **B** *P. putida* CP1 (5%), **C** 10% : 5% (Biolyte HAB : *P. putida* CP1), **D** 9.5% : 0.5%, **E** 5% : 5%, **F** 1% : 5% and **G** control.

Again reductions in degradation times resulted from decreases in lag periods and increases in rates of degradation. As can be seen from Tables 21 and 22, lag periods decreased for degradation of both 1.56 mM and 2.33 mM with increasing Biolyte HAB inocula sizes. The lag period for the degradation of 1.56 mM 4-chlorophenol was reduced from 23 hours for 5% *P. putida* CP1 to 6, 4 and 2 hours following the addition of 1%, 5% and 10% Biolyte HAB respectively. Similarly lag times reduced from 25 hours for the degradation of 2.33 mM 4-chlorophenol by *P. putida* CP1 in isolation to 20, 18 and 7 hours following the addition of 1%, 5% and 10% Biolyte HAB respectively.

At both concentrations (1.56 mM and 2.33 mM) the maximum rate of 4-chlorophenol degradation increased with increasing Biolyte HAB inocula size (Tables 21 & 22). The rate of 1.56 mM 4-chlorophenol degradation was increased from 0.075 mM/hr for 1% Biolyte HAB : 5% *P. putida* CP1 to 0.168 and 0.250 mM/hr following addition of 5% and 10% Biolyte HAB. Similarly 2.33 mM degradation rates increased from 0.153 mM/hr (1%:5%) to 0.207 mM/hr (5%:5%) and 0.233 mM/hr (10%:5%).

When the rates of mono-chlorophenol degradation for each inoculum ratio for each of the chlorophenol isomers were compared at a single concentration, 1.56 mM (Tables 17, 20 & 21) rates of degradation were found to be in the order of 4-chlorophenol > 2-

chlorophenol > 3-chlorophenol mirroring the degradative abilities of *P. putida* CP1 in isolation.

Table 21. The degradation of 4-chlorophenol (1.56 mM) by Biolyte HAB/*P. putida* CP1.

Inoculum	Lag time (h)	Time of Degradation (h)	Rate of Degradation (mM/h)
0% : 5%	23	32	0.164
1% : 5%	6	26	0.075
5% : 5%	4	13	0.168
10% : 5%	2	8	0.250

Table 22. The degradation of 4-chlorophenol (2.33 mM) by Biolyte HAB/*P. putida* CP1.

Inoculum	Lag time (h)	Time of Degradation (h)	Rate of Degradation (mM/h)
0% : 5%	25	75	0.045
1% : 5%	20	35	0.153
5% : 5%	18	29	0.207
10% : 5%	7	17	0.233

3.4.2 Analysis of the influence of Biolyte HAB inoculum size on lag times for the degradation of mono-chlorophenols by Biolyte HAB/*Pseudomonas putida* CP1.

It was noted previously that increasing the inoculum density of Biolyte HAB added to *P. putida* CP1 reduced the lag phase required before chlorophenol degradation began for each of the mono-chlorophenols. This reduction in lag times was directly related to

the concentration of Biolyte HAB added to *P. putida* CP1 as indicated by the linear relationship (regression coefficients not less than 0.891) resulting from plots of lag times versus Biolyte HAB inoculum size (Fig. 52). The slopes of these graphs represent the net reduction in lag periods per percentage increase in Biolyte HAB inoculum added to *P. putida* CP1. The slopes and regression coefficients from these plots are shown in Table 23.

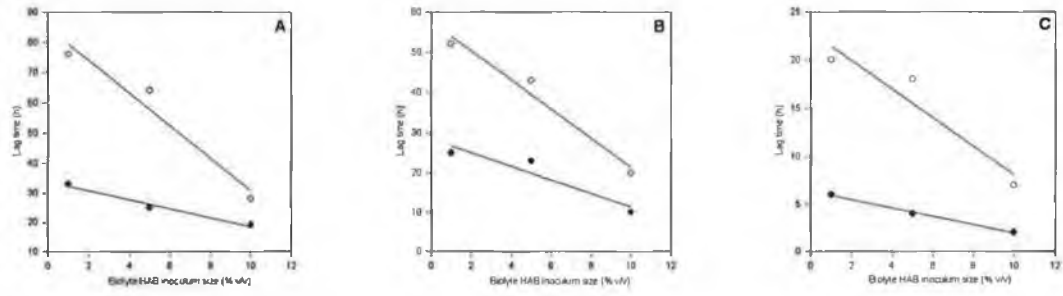

Figure 52. Effect of Biolyte HAB inoculum density on the lag period for the degradation of mono-chlorophenols by various ratios of Biolyte HAB/*P. putida* CP1. Symbols **A** 2-chlorophenol ● 1.56 mM, O 2.33 mM, **B** 3-chlorophenol ● 0.78 mM, O 1.56 mM and **C** 4-chlorophenol ● 1.56 mM, O 2.33 mM.

For every percentage increase in Biolyte HAB added to *P. putida* CP1, the was a reduction of the lag period of 1.5 hours (1.56 mM) and 5.4 hours (2.33 mM) for 2-chlorophenol degradation, 1.7 hours (0.78 mM) and 3.6 hours (1.56 mM) for 3-chlorophenol degradation and 0.4 hours (1.56 mM) and 1.5 hours (2.33 mM) for 4-chlorophenol degradation. The reduction in lag times per percentage increase of Biolyte HAB for each of the chlorophenols at a single concentration (1.56 mM) was greatest for 3-chlorophenol, followed by 2-chlorophenol and finally 4-chlorophenol. This was the reverse of the degradative abilities of *P. putida* CP1 in isolation. Also, reductions in lag times per percentage increase of Biolyte HAB were greater at higher concentrations of chlorophenol.

Table 23. Effect of Biolyte HAB inoculum density on the lag period for the degradation of mono-chlorophenols by Biolyte HAB/*P. putida* CP1.

Substrate and concentration	Slope (mM/percent)	Regression Coefficient
2-chlorophenol		
1.56 mM	-1.54	0.979
2.33 mM	-5.41	0.954
3-chlorophenol		
0.78 mM	-1.70	0.891
1.56 mM	-3.59	0.967
4-chlorophenol		
1.56 mM	-0.44	0.996
2.33 mM	-1.48	0.903

3.4.3 Analysis of the influence of Biolyte HAB inoculum size on times of degradation for the degradation of mono-chlorophenols by Biolyte HAB/*Pseudomonas putida* CP1.

When times of degradation were plotted against Biolyte HAB inoculum size, regression analysis showed a direct relationship (regression coefficients not less than 0.904) between the reduction in degradation times and the concentration of Biolyte HAB added to *P. putida* CP1 (Fig. 53). The slopes of these graphs represent the net reduction in degradation times per percentage increase in Biolyte HAB inoculum added to *P. putida* CP1. The slopes and regression coefficients from these plots are shown in Table 24.

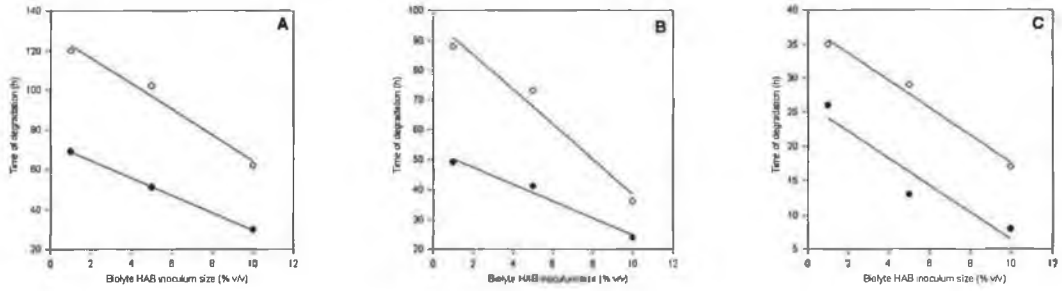

Figure 53. Effect of Biolyte HAB inoculum density on the time of degradation for the degradation of mono-chlorophenols by various ratios of Biolyte HAB/*P. putida* CP1. Symbols **A** 2-chlorophenol ● 1.56 mM, O 2.33 mM, **B** 3-chlorophenol ● 0.78 mM, O 1.56 mM and **C** 4-chlorophenol ● 1.56 mM, O 2.33 mM.

Table 24. Effect of Biolyte HAB inoculum density on the time of degradation for the degradation of mono-chlorophenols by Biolyte HAB/*P. putida* CP1.

Substrate and concentration	Slope (mM/percent)	Regression Coefficient
2-chlorophenol		
1.56 mM	-4.33	0.999
2.33 mM	-6.51	0.977
3-chlorophenol		
0.78 mM	-2.80	0.980
1.56 mM	-5.84	0.969
4-chlorophenol		
1.56 mM	-1.94	0.904
2.33 mM	-2.02	0.984

Addition of Biolyte HAB to *P. putida* CP1 resulted in a reduction in the time of degradation for 2-chlorophenol in the order of 4.3 hours (1.56 mM) and 6.5 hours (2.33 mM) per percentage increase in Biolyte HAB, 2.8 hours (0.78 mM) and 5.8

hours (1.56 mM) per percentage increase for 3-chlorophenol degradation and 1.9 hours (1.56 mM) and 2.0 hours (2.33 mM) per percentage increase for 4-chlorophenol. As was shown for lag times, the reduction in degradation times per percentage increase of Biolyte HAB for each of the chlorophenols at a single concentration (1.56 mM) was greatest for 3-chlorophenol, followed by 2-chlorophenol and finally 4-chlorophenol. This was the reverse of the degradative capabilities of *P. putida* CP1 in isolation. In addition, reductions in degradation times per percentage increase of Biolyte HAB were greater at higher concentrations of chlorophenol.

3.4.4 Ring cleavage enzyme activities during the degradation of mono-chlorophenols by various ratios of Biolyte HAB/*Pseudomonas putida* CP1.

No colour production was observed during the degradation of each of the mono-chlorophenols by Biolyte HAB/*P. putida* CP1, at any of the ratios tested. The stoichiometric releases of chloride and absence of any colour production in the culture medium suggested that metabolism occurred using the *ortho-* cleavage pathway. Enzyme assays carried out confirmed this. Enzyme activities were similar to those observed during the degradation of mono-chlorophenols by Biolyte HAB/*P. putida* CP1 (9.5% v/v : 0.5% v/v). No *meta-* cleavage activity was found during chlorophenol degradation, while *ortho-* cleavage activity towards catechol and chlorocatechol was detected. Enzyme levels detected are shown in Tables 25 - 33. The *meta-* cleavage activities found during the degradation of mono-chlorophenols by Biolyte HAB were no longer present during degradation by the various Biolyte HAB/*P. putida* CP1 ratios. For each inoculum ratio, enzyme levels were highest at the time of maximum chlorophenol degradation for each of the mono-chlorophenols.

Table 25. Enzyme activities during the degradation of 2-chlorophenol (1.56 mM) by Biolyte HAB/ *P. putida* CP1 (1% : 5%).

Enzyme Assayed and assay Substrate	Specific Activity (U/mg) T = 24 hrs	T = 48 hrs	T = 62 hrs	T = 72 hrs
Catechol 2,3- dioxygenase				
Catechol	< 0.001	< 0.001	< 0.001	< 0.001
Catechol 1,2-dioxygenase				
Catechol	5.53	8.22	5.56	1.29
3-chlorocatechol	4.99	7.64	5.34	2.80

Table 26. Enzyme activities during the degradation of 3-chlorophenol (0.78 mM) by Biolyte HAB/ *P. putida* CP1 (1% : 5%).

Enzyme Assayed and assay Substrate	Specific Activity (U/mg) T = 24 hrs	T = 40 hrs	T = 48 hrs	T = 72 hrs
Catechol 2,3- dioxygenase				
Catechol	< 0.001	< 0.001	< 0.001	< 0.001
Catechol 1,2-dioxygenase				
Catechol	11.14	19.02	11.94	0.50
3-chlorocatechol	6.42	8.67	3.10	0.68

Table 27. Enzyme activities during the degradation of 4-chlorophenol (1.56 mM) by Biolyte HAB/ *P. putida* CP1 (1% : 5%).

Enzyme Assayed and assay Substrate	Specific Activity (U/mg)			
	T = 16 hrs	T = 24 hrs	T = 36 hrs	T = 48 hrs
Catechol 2,3- dioxygenase				
Catechol	< 0.001	< 0.001	< 0.001	< 0.001
Catechol 1,2-dioxygenase				
Catechol	1.15	3.62	2.96	1.99
3-chlorocatechol	1.85	3.25	2.91	2.24

Table 28. Enzyme activities during the degradation of 2-chlorophenol (1.56 mM) by Biolyte HAB/ *P. putida* CP1 (5% : 5%).

Enzyme Assayed and assay substrate	Specific Activity (U/mg)			
	T = 24 hrs	T = 36 hrs	T = 48 hrs	T = 72 hrs
Catechol 2,3- dioxygenase				
Catechol	< 0.001	< 0.001	< 0.001	< 0.001
Catechol 1,2-dioxygenase				
Catechol	8.49	6.59	4.54	0.24
3-chlorocatechol	0.35	4.86	3.24	0.37

Table 29. Enzyme activities during the degradation of 3-chlorophenol (0.78 mM) by Biolyte HAB/ *P. putida* CP1 (5% : 5%).

Enzyme Assayed and assay substrate	Specific Activity (U/mg)			
	T = 24 hrs	T = 48 hrs	T = 72 hrs	T = 96 hrs
Catechol 2,3- dioxygenase				
Catechol	< 0.001	< 0.001	< 0.001	< 0.001
Catechol 1,2-dioxygenase				
Catechol	0.19	6.48	0.84	0.58
3-chlorocatechol	0.22	5.76	1.55	1.05

Table 30. Enzyme activities during the degradation of 4-chlorophenol (1.56 mM) by Biolyte HAB/ *P. putida* CP1 (5% : 5%).

Enzyme Assayed and assay substrate	Specific Activity (U/mg)			
	T = 12 hrs	T = 24 hrs	T = 48 hrs	T = 72 hrs
Catechol 2,3- dioxygenase				
Catechol	< 0.001	< 0.001	< 0.001	< 0.001
Catechol 1,2-dioxygenase				
Catechol	2.81	3.72	1.87	0.40
3-chlorocatechol	3.14	3.59	2.15	0.67

Table 31. Enzyme activities during the degradation of 2-chlorophenol (1.56 mM) by Biolyte HAB/ *P. putida* CP1 (10% : 5%).

Enzyme Assayed and assay substrate	Specific Activity (U/mg)			
	T = 16 hrs	T = 24 hrs	T = 40 hrs	T = 48 hrs
Catechol 2,3- dioxygenase				
Catechol	< 0.001	< 0.001	< 0.001	< 0.001
Catechol 1,2-dioxygenase				
Catechol	9.88	14.83	2.64	2.13
3-chlorocatechol	6.94	8.54	2.75	2.14

Table 32. Enzyme activities during the degradation of 3-chlorophenol (0.78 mM) by Biolyte HAB/ *P. putida* CP1 (10% : 5%).

Enzyme Assayed and assay substrate	Specific Activity (U/mg)			
	T = 16 hrs	T = 24 hrs	T = 40 hrs	T = 48 hrs
Catechol 2,3- dioxygenase				
Catechol	< 0.001	< 0.001	< 0.001	< 0.001
Catechol 1,2-dioxygenase				
Catechol	4.11	7.45	0.80	0.71
3-chlorocatechol	1.28	3.70	0.94	1.51

Table 33. Enzyme activities during the degradation of 4-chlorophenol (1.56 mM) by Biolyte HAB/ *P. putida* CP1 (10% : 5%).

Enzyme Assayed and assay substrate	Specific Activity (U/mg) T = 16 hrs	T = 24 hrs	T = 36 hrs	T = 48 hrs
Catechol 2,3- dioxygenase				
Catechol	< 0.001	< 0.001	< 0.001	< 0.001
Catechol 1,2-dioxygenase				
Catechol	5.60	6.63	1.03	1.34
3-chlorocatechol	5.43	5.55	1.81	2.29

3.4.5 Microbiological analysis carried out during the degradation of mono-chlorophenols by Biolyte HAB/*Pseudomonas putida* CP1 mixtures.

Analysis of the microbial content of the Biolyte HAB/*P. putida* CP1 mixtures (1%:5%, 5%:5% and 10%:5% Biolyte HAB : *P. putida* CP1) was carried out during the degradation of the mono-chlorophenols (2- and 4-chlorophenol 1.56 mM, 3-chlorophenol 0.78 mM). The survival of *P. putida* CP1 in the mixed culture is shown in Figures 54 - 56. As can been seen from these Figures, *P. putida* CP1 numbers, total bacterial counts and chlorophenol degrading numbers increased during the degradation of each of the mono-chlorophenol isomers by each Biolyte HAB/*P. putida* CP1 inoculum ratio.

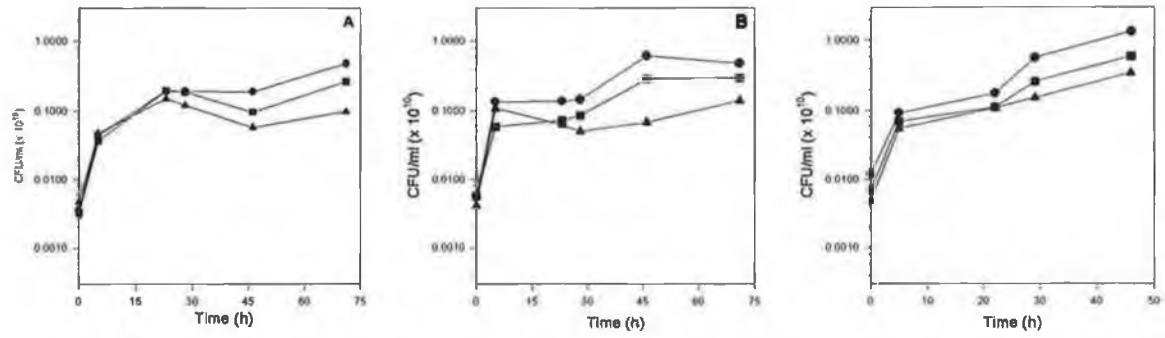

Figure 54. Population analysis during the degradation of 2-chlorophenol (1.56 mM) by Biolyte HAB/*P. putida* CP1, **A** 1%:5%, **B** 5%:5% and **C** 10%:5%. Symbols : ● Total counts, ■ chlorophenol degraders and ▲ *P. putida* CP1.

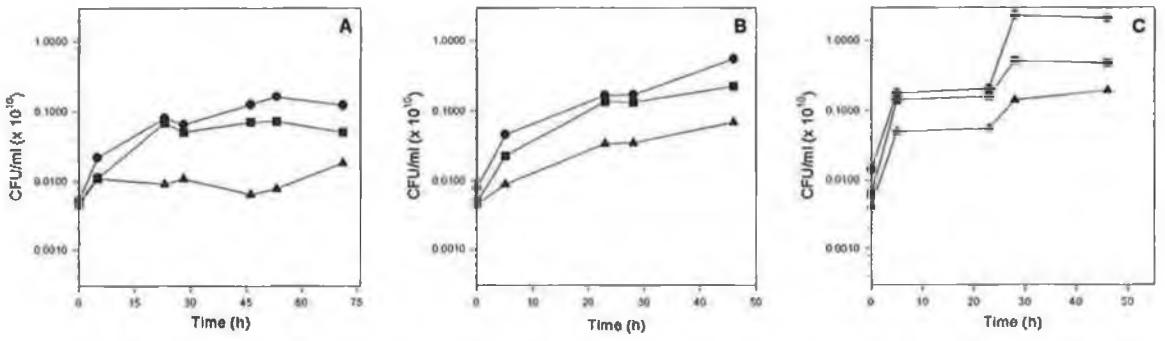

Figure 55. Population analysis during the degradation of 3-chlorophenol (0.78 mM) by Biolyte HAB/*P. putida* CP1, **A** 1%:5%, **B** 5%:5% and **C** 10%:5%. Symbols : ● Total counts, ■ chlorophenol degraders and ▲ *P. putida* CP1.

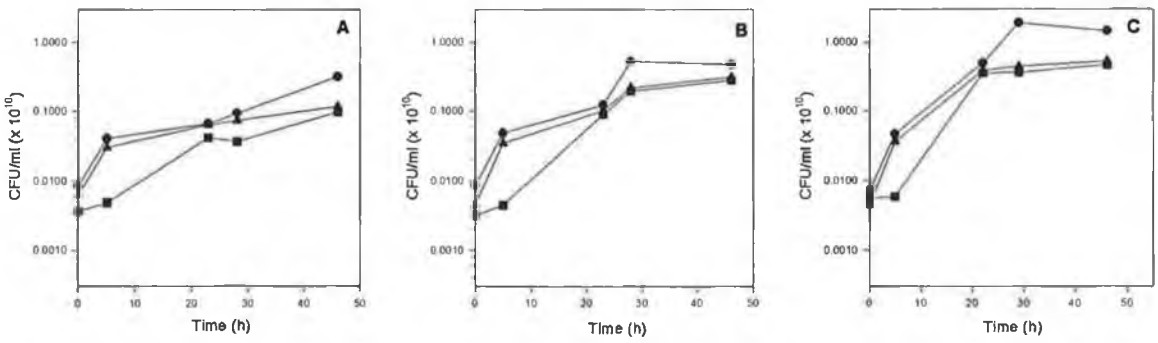

Figure 56. Population analysis during the degradation of 4-chlorophenol (1.56 mM) by Biolyte HAB/*P. putida* CP1, **A** 1%:5%, **B** 5%:5% and **C** 10%:5%. Symbols : ● Total counts, ■ chlorophenol degraders and ▲ *P. putida* CP1.

Statistical analysis showed there was a significant difference between final *P. putida* CP1 numbers following degradation of the mono-chlorophenols by the various inocula ratios (Table 36, Appendix). The increase in *P. putida* CP1 numbers was greater with increasing Biolyte HAB inoculum size in the mixtures (Table 34). There was also a significant difference between the final *P. putida* CP1 numbers following the degradation of each of the mono-chlorophenols (Table 36, Appendix). The increase in *P. putida* CP1 numbers was greatest following the degradation of 4-chlorophenol, followed by 2-chlorophenol and finally 3-chlorophenol, reflecting the degradative abilities of *P. putida* CP1 in isolation.

Table 34. Final *P. putida* CP1 numbers following the degradation of mono-chlorophenols by Biolyte HAB/*P. putida* CP1.

Inoculum	Final *P. putida* CP1 counts (CFU/ml) following growth on		
	2-chlorophenol	3-chlorophenol	4-chlorophenol
1%:5%	9.8×10^8	1.8×10^8	1.2×10^9
5%:5%	1.4×10^9	6.8×10^8	3.1×10^9
10%:5%	3.4×10^9	1.9×10^9	5.3×10^9

Statistical analysis showed that total bacterial counts increased significantly for each of the inocula ratios during the degradation of the mono-chlorophenols (Table 37, Appendix). The greatest increase in cell numbers was observed for the degradation of chlorophenols by 10%: 5% ratio, followed by 5%:5% and finally 1%:5% (Table 35). There was no significant difference in increases in cell numbers for each inoculum ratio between the different mono-chlorophenol isomers (Table 37, Appendix).

Table 35. Final total cell counts following the degradation of mono-chlorophenols by Biolyte HAB/*P. putida* CP1.

Inoculum	Final cell counts (CFU/ml) following growth on		
	2-chlorophenol	3-chlorophenol	4-chlorophenol
1%:5%	4.8×10^9	1.2×10^9	3.1×10^9
5%:5%	6.2×10^9	5.4×10^9	4.7×10^9
10%:5%	1.3×10^{10}	2.1×10^{10}	1.9×10^{10}

4. Discussion.

The ability of Biolyte HAB to degrade mono-chlorophenols as the sole source of carbon and energy for growth was studied. Biolyte HAB (Haloaromatic Biodegradation) was a commercial bioaugmentation product, produced by International Biochemicals Ltd., Ireland, designed for use in conventional aerobic wastewater treatment plants or in the bioremediation of contaminated soils. The formulation was a mixed culture consisting of a minimum of eleven strains of micro-organisms including bacteria belonging to the genera *Pseudomonas* and *Actinomycetes*, together with a fungus, *Trichoderma harzanium*. The product was said to contain the ability to degrade chlorinated aromatics such as chlorobenzenes, polychlorinated biphenyls and structurally related compounds.

In the production of the formulation, micro-organisms were grown up in pure culture, harvested and preserved by freeze-drying, before being blended in the final formulation which also contained surfactants, cryoprotectants, growth media and a cereal base (manufacturers' literature). The degradation of the mono-chlorophenol isomers by Biolyte HAB was studied in the basal salts medium of Goulding *et al.* (1988), which provided a source of nitrogen, phosphate and trace salts essential for the growth of micro-organisms. Prior to introduction of Biolyte HAB to the minimal medium containing the mono-chlorophenol, the inoculum was washed with sodium phosphate buffer. This washing step removed the carry over of nutrients which were contained within the cereal base of the formulation. Previous studies had shown that the presence of the cereal base in degradation studies had a detrimental effect on the degradation of phenol and mono-chlorophenols (O' Sullivan, 1998). Degradation of phenol was shown to occur at a lower rate in the presence of the additional nutrients, while the removal of the mono-chlorophenols was reduced in comparison to their removal by the washed formulation. This inhibition of degradation was thought to be due to the presence of more readily degradable carbon sources in the cereal base shifting the selection pressure away from the degradation of the phenolics.

Chlorophenols have been used for a wide range of domestic, agricultural and industrial purposes for more than 50 years. They are used as wide-spectrum biocides and are formed during paper and pulp bleaching, the incineration of organic material in the presence of chloride and the chlorination of wastewaters. Chlorophenols have a low water solubility which increases with increased chlorine substitution. Xenobiotic compounds with low water solubilities such as chlorophenols are often lipophilic and therefore can bioaccumulate in fatty tissues.

The transformation of chemical compounds by living organisms in nature is one of the most important processes that causes the breakdown of organic compounds. The toxicity of chlorophenols towards micro-organisms results in their resistance to biodegradation and their resultant accumulation in the environment. Their toxicity along with other lipophilic xenobiotics is primarily connected to lipid membrane disruptions. Dissolution of chlorophenols in the cell membrane of micro-organisms disturbs structural integrity and causes permeabilization. This leads to an inhibition of membrane-bound proteins such as Na^+/K^+-ATPase or the glucose transport system (Jensen, 1996).

Despite the toxicity of chlorophenols towards micro-organisms, biodegradation has been demonstrated both aerobically and anaerobically. Aerobic degradation has proved most successful for the degradation of chlorophenols. Theoretically, the aerobic mineralization of mono-chlorophenols follows the reaction stoichiometries (Puhakka and Melin, 1996) :

$$C_6H_4OHCl + 6.5O_2 \longrightarrow 6CO_2 + HCl + 2H_2O$$

Aerobic degradation is initiated by the action of oxygenase enzymes which hydroxylate mono-chlorophenols to their corresponding chlorocatechols. These enzymes, responsible for the introduction of the oxygen atoms indicated in the reaction mechanism above, can be highly specific. As a result, the position of the chlorine atom on the aromatic ring can affect the removal of mono-chlorophenols. This can be seen in the ease of removal pattern seen during the degradation of mono-chlorophenols by

Biolyte HAB. Removal of chlorophenols by Biolyte HAB was in the order of 4-chlorophenol > 3-chlorophenol > 2-chlorophenol, demonstrating the effect of the chloro-substitution on the transformation of mono-chlorophenols.

Enzyme assays carried out showed that degradation of the mono-chlorophenols by Biolyte HAB was via the *meta*- cleavage pathway. This led to incomplete degradation of both 2- and 3-chlorophenol by Biolyte HAB, as indicated by the unstoichiometric releases of chloride. The initial step in the degradation of 2- and 3-chlorophenol was their transformation to the intermediate 3-chlorocatechol. This intermediate was identified using HPLC during the degradation of 2- and 3-chlorophenol by Biolyte HAB. 3-chlorocatechol concentrations peaked after 48 hours after which they began to drop. As chlorocatechol concentrations dropped, a dark brown colour developed in the media. Similarly, a brown coloration of the culture medium resulted when the degradation of 3-chlorocatechol by Biolyte HAB was studied.

The production of brown colours following the degradation of chloroaromatics via 3-chlorocatechol has been widely reported in the literature (Haller and Finn, 1979; Knackmuss, 1982; Fava *et al.*, 1993a). Haller and Finn (1979) reported the production of the brown pigment following the production of 3-chlorocatechol as an intermediate of the degradation of 3-chlorobenzoic acid/benzoate mixtures by sludges. The brown pigment appeared to result from the polymerisation of 3-chlorocatechol to form an melanin-like pigment. 3-chlorobenzoic acid metabolism by a 3-methylbenzoate-utilising culture of *Pseudomonas putida* mt-2 in the absence and in the presence of *Pseudomonas sp.* B13 resulted in the production of brown polymers, due to an accumulation of 3-chlorocatechol (Knackmuss, 1982).

Adams *et al.* (1992) reported the production of a brown-black pigment in flasks following the degradation of 3-chlorobiphenyl by the recombinant *Pseudomonas* sp. strain CB15. When GC-mass spectrometry analysis was carried out on the coloured pigment, it was found that the brown-black material was composed in part of a dimer formed from 3-chlorocatechol.

Fava and Marchettii (1991) reported the production of a brown pigment following the degradation of 3-chlorobenzoate via 3- and 4-chlorocatechol by *Pseudomonas fluorescens* CP30. Studies carried out into the nature of the brown pigment supported the theory that polymerisation of chlorocatechols resulted in the production of what were termed "chlorocatechol melanins" (Fava *et al.*, 1993 a). Further studies by Fava *et al.* (1993 b) suggested that simple autooxidation of chlorocatechols was not the only mechanism by which pigment formation took place. It was suggested that the bacterial cell might take an active part in the process of polymerisation. A peroxidase enzyme and another enzyme activity referred to as a polyphenol oxidase were shown to actively oxidise 4-chlorocatechol to form 4-chloro-1,2-benzoquinone, a highly reactive substrate for pigment formation, during the co-metabolism of 3-chlorobenzoic acid by *Pseudomonas fluorescens* CP30 (Fava *et al.*, 1993 b). Despite the activity of these enzymes, a significant amount of the polymerisation resulted from simple autooxidation of the chlorocatechols.

Chlorophenols can be fortuitously metabolised by the catabolic enzymes of various pathways which have broad substrate specificity (Knackmuss and Hellwig, 1978; Spain and Gibson, 1988). The oxidation of both 2- and 3-chlorophenol results in the formation of 3-chlorocatechol which may or may not accumulate. Chlorocatechol accumulation results from the inefficient metabolism of chlorocatechols by the *ortho*-cleavage pathway or from the negative effect 3-chlorocatechol has on the *meta*-cleavage enzyme, catechol 2,3-dioxygenase, either by acting as a chelating agent, binding to the iron core of catechol 2,3-dioxygenase, resulting in reversible inactivation (Klecka and Gibson, 1981) or due to the production of a highly reactive acyl halide, 5-chloroformyl-2-hydroxy-penta-2,4-dienoic acid (Bartels *et al.*, 1984) which has been proposed to act as a suicide compound, binding irreversibly to catechol 2,3-dioxygenase with a subsequent release of chloride and a destruction of metabolic activity. As 2- and 3-chlorophenol metabolism by Biolyte HAB by the *meta*- cleavage pathway led to some chloride release causing the slight drop in pH observed, it was assumed that the build up of 3-chlorocatechol resulted from the latter mechanism.

The polymerised 3-chlorocatechol produced during degradation of 2- and 3-chlorophenol by Biolyte HAB resulted in the drop in chlorocatechol concentrations as measured by Arnow's colorimetric assay or HPLC. The drop in chlorocatechol concentrations was not due to further microbial degradation, as was confirmed by control experiments containing 3-chlorocatechol in sterile minimal medium. Abiotic removal due to autooxidation occurred at a rate similar to that observed during the degradation of 2- and 3-chlorophenol. Polymerisation in 3-chlorocatechol control flasks also resulted in a brown coloration of the culture medium. This would suggest that colour production following degradation or 2- and 3-chlorophenol by Biolyte HAB was due to simple autooxidation and not in the main due to the activity of peroxidase and oxidase enzymes.

Degradation of 4-chlorophenol by Biolyte HAB also took place using the *meta*-cleavage pathway. Metabolism of 4-chlorophenol resulted in a lime/yellow coloration of the culture medium typical of the *meta*- cleavage pathway. However, unlike the brown colour that resulted from 2- and 3-chlorophenol metabolism by Biolyte HAB, the yellow colour did not remain in the culture medium. It is known that degradation of 4-chlorophenol via the *meta*- cleavage pathway results in the formation of 5-chloro-2-hydroxymuconic semialdehyde, which accumulates to form a yellow coloration of culture fluids (Knackmuss, 1981). The accumulated intermediate which resulted from the degradation of 4-chlorophenol by Biolyte HAB had all the characteristics of 5-chloro-2-hydroxymuconic semialdehyde, the *meta*- cleavage product of 4-chlorocatechol, with an absorption maximum of 379nm at pH 7.0 and 334nm at pH 2.0 as was reported by Morris and Barnsley (1981), while the yellow colour disappeared reversibly upon acidification as described by Weiser *et al.* (1994).

This *meta*- cleavage product was widely reported to be a dead-end metabolite (Reineke *et al.*, 1982; Balfanz and Rehm, 1991; Weiser *et al.*, 1994). However, complete degradation of 4-chloroaromatics via a *meta*- cleavage pathway has been reported in the literature. The complete metabolism of 4-chlorobenzoate by *P. cepacia*

P166 via a *meta-* cleavage pathway was described by Arnesdorf and Focht (1994). 5-chloro-2-hydroxymuconic semialdehyde was identified as one of the metabolites of degradation and was shown to be further degraded, ultimately resulting in complete removal (Arnesdorf and Focht, 1995). Sung Bae *et al.* (1996) reported the production of 5-chloro-2-hydroxymuconic semialdehyde following the degradation of 4-chlorophenol by *Comamonas testosteroni* CPW301 via the *meta-* cleavage pathway. Further metabolism ensued, resulting in the disappearance of the yellow colour. Hollender *et al.* (1997) described how 4-chlorophenol was degraded to completion via a *meta-* cleavage pathway by *Comamonas testosteroni* JH5. Again, 5-chloro-2-hydroxymuconic semialdehyde was identified as being an intermediate of degradation, which was further metabolised with a stoichiometric release of chloride, indicating complete degradation. Most recently, Seo *et al.* (1998) reported the complete degradation of 4-chlorobenzoate via 5-chloro-2-hydroxymuconic semialdehyde by *Pseudomonas* sp. S-47 with a stoichiometric release of chloride.

During degradation of 4-chlorophenol by Biolyte HAB, the accumulated yellow colour disappeared and the optical density at 380nm decreased, suggesting further metabolism of 5-chloro-2-hydroxymuconic semialdehyde. Chloride analysis showed stoichiometric releases of chloride, confirming further metabolism and showing complete degradation of 4-chlorophenol via a *meta-* cleavage pathway. Weiser *et al.* (1994) described how the concentrations of 5-chloro-2-hydroxymuconic semialdehyde reached a peak following degradation of 4-chlorophenol by *Azotobacter* sp. GP1 after approximately 10 hours, remained constant for 1 day, following which a slow decrease was observed. This decrease was at a rate corresponding to the spontaneous decomposition rate of 5-chloro-2-hydroxymuconic semialdehyde suggesting that any removal was not microbially mediated. This was confirmed by viable cell counts which showed cell numbers to remain constant. Microbial metabolism of 5-chloro-2-hydroxymuconic semialdehyde should lead to increases in cell numbers. The removal of 5-chloro-2-hydroxymuconic semialdehyde by Biolyte HAB was shown to be microbially mediated by incubation of the *meta-* cleavage product following removal of the cell biomass by filtration. In the absence of Biolyte HAB, there was only a slight decrease in 5-chloro-

2-hydroxymuconic semialdehyde concentrations, significantly less than that which was observed in the presence of Biolyte HAB. In addition, 4-chlorophenol metabolism resulted in increases in cell numbers. As 4-chlorophenol was supplied as sole source of carbon, growth of Biolyte HAB must have resulted from the energy gained from 4-chlorophenol degradation.

Although no chlorocatechol accumulations were detected in the culture medium following the degradation of 4-chlorophenol, 5-chloro-2-hydroxymuconic semialdehyde is known to be the *meta*- cleavage product of 4-chlorocatechol, while 4-chlorophenol is known to be transformed to 4-chlorocatechol during aerobic degradation. However, the degradation of 4-chlorocatechol by Biolyte HAB was demonstrated. Degradation of 4-chlorocatechol resulted in the same characteristics of 4-chlorophenol degradation, the production and disappearance of the yellow colour in the medium and stoichiometric releases of chloride indicating complete metabolism. This would indicate that 4-chlorocatechol was produced at low concentrations and immediately metabolised during the degradation of 4-chlorophenol by Biolyte HAB.

The production of the brown pigment during the degradation of 2- and 3-chlorophenol by Biolyte HAB was followed by measuring the optical density at 600nm following removal of the cell biomass (Haller and Finn, 1979). However, the production of the polymers interfered with the optical density at 660nm, which is routinely used to give an indication of cell growth. Arnesdorf and Focht (1994) described how optical density could not be used as a measure of cell growth resulting from *meta*- cleavage of chloroaromatics via 3-chlorocatechol, as cultures with very low viable-cell counts during the degradation of 2- and 3-chlorobiphenyl routinely gave high optical density readings.

Growth of Biolyte HAB on 4-chlorophenol as measured by viable cell counts was greater than that which was observed following growth on 2- and 3-chlorophenol. This was as expected due to the complete mineralization of 4-chlorophenol by Biolyte HAB. The production of yellow-coloured intermediates did not interfere with the

optical density at 660nm and so cell growth could be monitored using turbidity readings.

Degradation of 4-chlorophenol by Biolyte HAB resulted in a greater drop in pH than was observed during the degradation of 2- and 3-chlorophenol. This drop in pH resulted not only from the greater release of chloride, but due to the production of organic acids as intermediates of the degradation of 4-chlorophenol by the *meta*-cleavage pathway as described by Arnesdorf and Focht (1995). However, unlike the drop in pH following 2- and 3-chlorophenol degradation which reached a plateau, the pH in culture fluids during 4-chlorophenol metabolism began to rise again. This rise in pH followed the further metabolism of the acidic metabolites produced during 4-chlorophenol degradation via a *meta*- cleavage pathway and reached a plateau only after complete metabolism.

Although Biolyte HAB was specially designed to degrade substituted aromatic compounds, degradation of mono-chlorophenols under the conditions used was via the *meta*- cleavage pathway. This led to incomplete degradation of both 2- and 3-chlorophenol. The lack of degradative ability could have resulted from the environmental conditions used to study chlorophenol degradation. Environmental conditions, which play a central role in the performance of any microbiological process, may have prevented growth of a population capable of the degradation of mono-chlorophenols via the *ortho*- pathway, generally required for the successful degradation of mono-chlorophenols.

Previous studies carried out in the laboratory attempted to bring about more successful degradation by modification of the degradative conditions (O' Sullivan, 1998). The addition of more easily metabolised carbon sources to Biolyte HAB and the modification of environmental conditions such as incubation temperature and pH were studied for the degradation of mono-chlorophenols. However, degradation of chlorophenols by Biolyte HAB under a variety of environmental conditions remained

via the *meta-* cleavage pathway, leading to incomplete degradation of 2- and 3-chlorophenol.

Previous studies carried out in the laboratory also showed that no pure bacterial culture capable of even the partial metabolism of 2- and 3-chlorophenol could be isolated from Biolyte HAB (O' Sullivan, 1998). A fungus, *Trichoderma harazanium*, capable of growth on 2-chlorophenol at concentrations up to 1.56 mM could be isolated from Biolyte HAB. Two *Pseudomonas putida* strains were isolated from Biolyte HAB, capable of the degradation of 4-chlorophenol at concentrations of 1.56 mM. Degradation however took place using the *meta-* cleavage pathway, suggesting the absence of any *ortho-* cleavage capability contained within Biolyte HAB.

Despite the acute toxicity of chlorophenols, the aerobic degradation of chlorophenols by pure cultures has been demonstrated. Sung Bae *et al.* (1996) demonstrated the degradation of 4-chlorophenol at concentrations up to 0.78 mM within 340 hours by *Comamonas testosteroni* CPW301 using the *meta-* cleavage pathway. Similarly, the degradation of 4-chlorophenol by *Comamonas testosteroni* JH5 using the *meta-* cleavage pathway was demonstrated by Hollender *et al.* (1997). Degradation was shown to be complete at concentrations up to 1.75 mM. However, metabolism of mono-chlorophenols via a *meta-* cleavage pathway ordinarily results in incomplete degradation.

The successful degradation of 2- or 3-chlorophenol using the *meta-* cleavage pathway has not been demonstrated. However, Mars *et al.* (1997) demonstrated the complete degradation of 3-chlorobenzoate, via 3-chlorocatechol, using a *meta-* cleavage pathway. This shows that degradation of 2- and 3-chlorophenol using the *meta-* cleavage pathway, although not demonstrated to date, might nevertheless be possible.

Despite these reports, successful degradation of chloroaromatics generally requires the *ortho-* cleavage pathway. The complete degradation of mono-chlorophenols using *ortho-* cleavage pathways has been demonstrated. Chitra and Chandrakasan (1996)

demonstrated the degradation of 4-chlorophenol as sole carbon source at concentrations up to 1.56 mM by *Pseudomonas pictorum*.

Menke and Rehm (1992) demonstrated the complete degradation of 2-chlorophenol at concentrations up to 0.3 mM within 80 hours and 4-chlorophenol at concentrations up to 0.45 mM within 25 hours using the constructed strain *Alcaligenes sp.* A7-2. Degradation of mono-chlorophenols took place using the *ortho-* cleavage pathway which had been introduced from the chlorobenzoate degrading strain, *Pseudomonas sp.* B13. *Alcaligenes sp.* A7-2 was unable to degrade 3-chlorophenol as sole carbon source. *Pseudomonas sp.* B13 was capable of degrading 4-chlorophenol as sole carbon source at concentrations up to 0.15 mM within 18 minutes when pre-grown on 4-chlorophenol and within 23 minutes when pre-grown on phenol (Knackmuss and Hellwig, 1978).

Katayama-Harayama *et al.* (1994) demonstrated the complete degradation of 3- and 4-chlorophenol at concentrations up to 0.5 mM by the yeast *Rhodotorula glutinis* using the *ortho-* cleavage pathway. Partial degradation of 2-chlorophenol (22%) was also demonstrated. The order of degradability of chlorophenols was 4-chlorophenol > 3-chlorophenol > 2-chlorophenol.

It can be seen from these examples that the complete degradation of all three mono-chlorophenol isomers by a single degradative system is difficult and has rarely been reported. However the complete degradation of 2-, 3- and 4-chlorophenol at concentrations of 0.08 mM was demonstrated by immobilised cells of *Pseudomonas testosteroni* using the *ortho-* cleavage pathway (Lu *et al.*, 1996). The time of degradation was in the order of 4-chlorophenol < 3-chlorophenol < 2-chlorophenol.

Pseudomonas pickettii LD1 (now *Rhalstonia pickettii*) was shown to be capable of degrading all three mono-chlorophenol isomers at considerably higher concentrations (Fava *et al.*, 1995). The chlorophenol degrading strain was shown to be capable of

degrading 3-chlorophenol at concentrations up to 0.57 mM within 29 hours, 0.75 mM 4-chlorophenol within 38 hours and 1.51 mM 2-chlorophenol within 30 hours.

Similarly, all three mono-chlorophenol isomers were degraded to completion by *Rhodococcus opacus* GM-14 (Zaitsev *et al.*, 1995). 3-chlorophenol was degraded at concentrations up to 0.78 mM, while 2- and 4-chlorophenol were degraded at concentrations up to 1.95 mM.

A chlorophenol degrading isolate, CP1, isolated from the same mixed culture as *Pseudomonas pickettii* LD1 was obtained from Dr. Fabio Fava, University of Bologna, Italy. The strain was found to conform to the traditional definition of aerobic *Pseudomonads* as outlined by Stanier *et al.* (1966), being unicellular, straight rods, motile, Gram negative, non-spore forming, oxidase positive with oxidative metabolism of glucose. Identification of CP1 was confirmed using the commercial identification systems API 20NE and Biolog.

API 20NE identification comprises 21 tests which are performed in cupules on a plastic strip where desiccated contents are reconstituted with a suspension of the test organism. Some tests have to be overlayed with mineral oil to obtain the correct gaseous conditions. Results are available in 24 - 48 hours and are represented as a seven digit profile number which may be read from the Analytical Profile Index. API 20NE provides a quick and simple identification system which is capable of correctly identifying the majority of *Pseudomonas* species. Studies carried out by Costas *et al.* (1992) showed the correct identification of 90.4% of 146 *Pseudomonas* strains used. 5.5% were not identified, while 4.1% were incorrectly identified.

The Biolog identification system is based on tests for the oxidation of 95 substrates in a 96-well microtitre plate. Each well contains a carbon source along with tetrazolium violet which determines colorimetrically the increased respiration that occurs when cells oxidise the carbon source. Reactions are read after 4 and 24 hours, thus providing a rapid identification. Studies carried out by Costas *et al.* (1992) showed the correct

identification of between 74% and 79% of 114 *Pseudomonas* or *Pseudomonas*-like species depending on whether results were read using an automated plate reader or read manually. The application of API 20NE and Biolog identification systems in combination with the range of biochemical tests provided a reliable identification of CP1 as being a *Pseudomonas putida* strain.

Chlorophenol degradation studies by *P. putida* CP1 were carried out using the minimal medium used in Biolyte HAB/chlorophenol studies. *P. putida* CP1 was shown to be capable of the degradation of all three mono-chlorophenols, a phenomenon rarely reported in the literature. Incubation of *P. putida* CP1 resulted in the degradation of up to 1.56 mM 3-chlorophenol within 240 hours, 1.56 mM 2-chlorophenol within 94 hours and 2.33 mM 4-chlorophenol within 80 hours. These concentrations compared favourably with those degraded by *Pseudomonas pickettii* LD1 which degraded up to 0.57 mM 3-chlorophenol, 0.75 mM 4-chlorophenol and 1.51 mM 4-chlorophenol (Fava *et al.*, 1995) and *Rhodococcus opacus* GM-14 which degraded up to 0.78 mM 3-chlorophenol and 1.95 mM 2- and 4-chlorophenol (Zaitsev *et al.*, 1995). Degradation for each chlorophenol isomer was complete as indicated by the stoichiometric releases of chloride. Elimination of chloride and the subsequent formation of HCl resulted in small pH drops corresponding to chlorophenol degradation. No intermediates were detected in the culture fluids during the degradation of the chlorophenols, suggesting that any metabolites produced were degraded immediately.

The absence of any colour production and the detection of stoichiometric releases of chloride indicating complete metabolism of the chlorophenols suggested chlorophenol metabolism by *P. putida* CP1 occurred using the *ortho*- cleavage pathway. When enzyme assays were carried out, metabolism was confirmed to be via the *ortho*-cleavage pathway. *Ortho*- cleavage activities towards both catechol and 3-chlorocatechol were detected throughout the periods of chlorophenol degradation.

Although neither 3- or 4-chlorocatechol were detected during the degradation of 2-, 3- or 4-chlorophenol by *P. putida* CP1, their degradation as intermediates of chlorophenol degradation was confirmed. *P. putida* CP1 was shown to be capable of complete degradation of up to 0.69 mM 3- and 4-chlorocatechol with almost stoichiometric releases of chloride being detected. During the degradation of 0.69 mM 3- or 4-chlorocatechol by *P. putida* CP1, a slight brown colour accumulated in the culture medium, indicating an amount of polymerisation of the chlorocatechols. *P. putida* CP1 appeared to be unable to degrade the chlorocatechol polymers formed due to autooxidation, resulting in the release of almost stoichiometric releases of chloride. 3-chlorocatechol metabolism was quicker than that observed during 4-chlorocatechol degradation, indicating a higher affinity of chlorocatechol 1,2-dioxygenase for 3-chlorocatechol than 4-chlorocatechol. However, degradation of 4-chlorophenol, which occurs via 4-chlorocatechol, was at higher rates than were observed during 2- or 3-chlorophenol degradation where 3-chlorocatechol is produced as an intermediate. This would suggest that the initial step in the degradation of chlorophenols, hydroxylation of chlorophenols to chlorocatechols, was the rate limiting step in the degradation of chlorophenols by *P. putida* CP1. This was confirmed by incubation of lower concentrations of 3- and 4-chlorocatechol (0.13 mM) which were metabolised almost immediately without any lag phase being evident.

Chlorophenol degradation is delayed in comparison to that of phenol due to their substitution with an electron-withdrawing group such as chlorine. The electron-withdrawing effect (-I effect) of the chloro-substituent, diminishes enzymatic transformation and deactivates electrophilic substitution (Menke and Rehm, 1992). Previous studies carried out in the laboratory showed increased degradation of phenol by Biolyte HAB when compared to mono-chlorophenol degradation (O' Sullivan, 1998).

The differences between the degradation of the mono-chlorophenols result from the differing positions of the chlorine atom on the aromatic ring. During the hydroxylation of chlorophenols to chlorocatechols, introduction of the second hydroxyl group is

governed by the electron-donating effect of the initial hydroxyl group and electron-withdrawing effect of the chlorine atom, which both direct subsequent substituents in the *ortho-* and *para-* positions to themselves. Due to the reaction-decelerating effect of the chlorine atom and reaction-accelerating effect of the initial hydroxyl group, theoretically the order of degradability should be 4-chlorophenol > 2-chlorophenol > 3-chlorophenol (Menke and Rehm, 1992).

In cases where the phenol hydroxylase enzyme is highly specific to certain mono-chlorophenol isomers, the order of degradability may not be that which might be expected due to the theoretical effects of the substitutions. This would explain the order of degradability of chlorophenol for *Rhodotorula glutinis* (Katayama-Harayama, 1994) and *Pseudomonas testosteroni* (Lu *et al.*, 1996) which was found to be 4-chlorophenol > 3-chlorophenol > 2-chlorophenol. However, in the strain *Alcaligenes sp.* A7-2, which contained a broad spectrum phenol hydroxylase, the order of degradability followed that predicted by theory (Menke and Rehm, 1992). *Rhodococcus opacus* GM-14, which was shown to be capable of growth on 48 aromatic and haloaromatic compounds, clearly demonstrating a broad spectrum phenol hydroxylase enzyme, also showed an order of degradability for chlorophenols agreeing with that expected by theory (Zaitsev *et al.*, 1995).

Chlorophenol degradation by *P. putida* CP1 was in the order of 4-chlorophenol > 2-chlorophenol > 3-chlorophenol also agreeing with the order predicted by theory, indicating that *P. putida* CP1 contained a broad spectrum phenol hydroxylase. This broad spectrum hydroxylase contributed along with its *ortho-* cleavage ability to allow *P. putida* CP1 to grow on all three mono-chlorophenol isomers.

When incubated in the presence of chlorophenols, *P. putida* CP1 formed large clumps of cells in the culture medium. Clumping of *P. putida* CP1 may have resulted from the toxic effects of the mono-chlorophenols on the micro-organisms, as growth *of P. putida* CP1 on 4-chlorophenol, the least toxic of the mono-chlorophenols, resulted in the formation of smaller clumps than were observed during growth on 2- and 3-

chlorophenol, while no clumping was observed during growth of *P. putida* CP1 on nutrient broth. As chlorophenol removal did not begin until *P. putida* CP1 had formed clumps of cells in the culture medium, it is reasonable to speculate that the ability of *P. putida* CP1 to clump conferred a protective advantage on *P. putida* CP1, allowing for chlorophenol degradation to occur.

Degradation of the mono-chlorophenol isomers by Biolyte HAB was shown to take place using the *meta-* cleavage pathway. Although this allowed for complete degradation of 4-chlorophenol, degradation of 2- and 3-chlorophenol was shown to be incomplete. In cases of incomplete degradation, successful degradation may be brought about by modification of environmental conditions, or by the addition of additional nutrients. Under these conditions, stimulation of the required biodegradative capacities of the indigenous microflora may bring about degradation of recalcitrant wastes.

Another approach to the problem of recalcitrant pollutants is the inoculation of the environment with micro-organisms possessing the appropriate catabolic properties known to metabolise the pollutants readily in a process known as bioaugmentation. As *P. putida* CP1 was shown to possess an *ortho-* degrading capability which allowed for the complete degradation of all three mono-chlorophenols, it was of interest to augment Biolyte HAB with *P. putida* CP1. It was hoped that the addition of *P. putida* CP1 to Biolyte HAB would bring about degradation of chlorophenols using the *ortho-* cleavage pathway, allowing the augmented mixed culture to degrade each of the chlorophenol isomers to completion.

P. putida CP1 was added initially at an inoculum size of 0.5% (v/v) to 9.5% (v/v) Biolyte HAB. When *P. putida* CP1 was added to Biolyte HAB, it was noticed that no coloured metabolites accumulated in the culture medium. Prior to augmentation of Biolyte HAB, metabolism of 2- and 3-chlorophenol resulted in a brown/black coloration of the medium, while a lime/yellow coloration was observed following 4-chlorophenol degradation. The absence of any colour formation following augmentation was a strong indication that addition of *P. putida* CP1 to the mixture had

shifted the metabolism of the mono-chlorophenols by the augmented mixed culture from the *meta-* cleavage pathway to the more productive *ortho-* cleavage pathway which would allow for complete degradation of the mono-chlorophenols. This was confirmed by assay of the key enzyme activities.

Addition of *P. putida* CP1 to Biolyte HAB resulted in complete degradation of each of the mono-chlorophenol isomers. Complete degradation was indicated again by stoichiometric releases of chloride that accompanied chlorophenol removal. The degradative capacities of the augmented mixed culture were greater than *P. putida* CP1 displayed in isolation. Degradation of the mono-chlorophenols by the augmented mixture was shown to be faster than *P. putida* CP1 achieved on its own. Addition of 0.5% *P. putida* CP1 to Biolyte HAB (9.5%) also allowed the mixture to degrade higher concentrations of 2-chlorophenol than *P. putida* CP1 (5% inoculum) could achieve. Up to 2.33 mM 2-chlorophenol could now be degraded, whereas previously only 1.56 mM 2-chlorophenol could be degraded to completion. The capability of the augmented mixed culture to degrade mono-chlorophenols was in the order of 4-chlorophenol > 2-chlorophenol > 3-chlorophenol mirroring that observed for the degradation of chlorophenols by *P. putida* CP1 as a pure culture. Analysis of the population during the degradation of the mono-chlorophenols by the augmented mixed culture showed that the introduced strain was able to establish itself as part of the mixture. *P. putida* CP1 numbers increased as chlorophenol degradation neared completion.

The addition of pure strains of bacteria containing catabolic properties to environments lacking degradative capacities for the degradation of xenobiotics has been described previously. Strains have been added to a number of varying environments such as soil, freshwater and activated sludges. Di Giovanni *et al.* (1996) described how *Alcaligenes eutrophus* JMP134 was added to a soil for the degradation of 2,4-dichlorophenoxyacetic acid (2,4-D). *Alcaligenes eutrophus* JMP134 contained the catabolic plasmid pJP4 which allowed for the degradation of 2,4-D and conferred mercury resistance. When uninoculated soil was incubated with 2,4-D, degradation

was slow and incomplete after 9 weeks incubation. However, inoculation of the soil with *Alcaligenes eutrophus* JPM134 allowed the supplemented soil to degrade 2,4-D to completion within 4 weeks. In addition, significant numbers of the indigenous population were isolated which had received the catabolic plasmid pJP4, allowing for 2,4-D degradation and mercury resistance. As no mercury resistant, 2,4-D degrading members of the uninoculated soil were isolated, it was shown that plasmid transfer from *Alcaligenes eutrophus* JMP134 to indigenous organisms occurred.

The 3-chlorobenzoate degrading organism, *Alcaligenes sp.* BR60 was added to a non-sterile freshwater lake ecosystem (Fulthorpe and Wyndham, 1994). The freshwater was unable to degrade 3-chlorobenzoate in isolation. However, following introduction of *Alcaligenes sp.* BR60 (pBR60), degradation occurred. Population analysis showed that the introduced strain did not survive within the lakewater. However, indigenous members of the lakewater were isolated capable of the degradation of 3-chlorobenzoate. These organisms contained the plasmid pBR60, indicating plasmid transfer from *Alcaligenes sp.* BR60.

The addition of catabolic strains to activated sludges for the degradation of xenobiotics has also been described. The phenol degrading strain, *Pseudomonas putida* ATCC 11172 was added to activated sludge in a sequencing batch reactor (Selvaratnam *et al.*, 1997). Following addition of *Pseudomonas putida* ATCC 11172, phenol removal was increased and maintained at 95% - 100% for over 6 days, while in the uninoculated sludge decreased removal was observed, resulting in an accumulation of phenol within the reactor.

The effects of the addition of pure bacterial strains to activated sludge for the degradation of phenol was also studied by Watanabe *et al.* (1996). Three phenol degrading strains were studied, *Alcaligenes* sp. R5, *Acromobacter* sp. E1 and *Alcaligenes* sp. E2. Activated sludge inoculated with strain R5 showed little improvement in phenol degradation, while strains E1 and E2 shortened degradation time from 10 days to 2 and 3 days respectively. Strains E1 and E2 were found to be

capable of forming the predominant population in the activated sludge systems resulting in successful bioaugmentation.

Bokhamy *et al.* (1997) studied the degradation of p-toluenesulfonate (*p*TS) by activated sludge following the introduction of *Comamonas testosteroni* T-2. T-2 was capable of the complete degradation of *p*TS as sole carbon source. The activated sludge was also shown to be capable of the degradation of *p*TS. However, addition of T-2 to the activated sludge resulted in degradation of *p*TS at rates greater than those achieved by the activated sludge in isolation. The activated sludge was supplemented with differing concentrations of T-2 and it was found that, by increasing the T-2 inoculum used to augment the sludge, the rate of *p*TS degradation increased.

The degradation of chlorinated aromatic compounds by activated sludges following addition of catabolic strains has also been studied. Wilderer *et al.* (1991) studied the effect of the addition of a 3-chlorobenzoate degrading strain, *Pseudomonas putida* PRS 2015 containing the catabolic plasmid pAC27, on the degradation of 3-chlorobenzoate by activated sludge in a sequencing batch reactor. It was found that addition of *P. putida* PRS 2015 resulted in improved degradation by reducing the lag phase or start-up period of degradation. The supplemented activated sludge also better retained its 3-chlorobenzoate degrading abilities following periods of starvation.

Pseudomonas sp. B13 has also been used to augment mixed cultures for the degradation of substituted aromatics. Schmidt *et al.* (1983) added B13 to a synthetic sewage consisting of a methanol degrading strain, *Pseudomonas extorquens* and *Alcaligenes sp.* A7, capable of degrading phenol via the *meta-* cleavage pathway, along with phenol, acetone and alkanols plus 4-chlorophenol. The unsupplemented mixture was found to degrade 4-chlorophenol via a *meta-* cleavage pathway leading to the production of 5-chloro-2-hydroxymuconic semialdehyde, which was found to be a dead-end metabolite. However, when B13 was added to the mixture, degradation was found to take place using an *ortho-* cleavage pathway, leading to complete degradation. B13 had managed to become a stable member of the mixture under the

selective pressure of 4-chlorophenol, leading to complete degradation. These results somewhat mirror those obtained during the present study. When the unsupplemented sewage was subjected to a mixture of the isomeric mono-chlorophenols, the toxicity of the chlorophenols led to the reference culture being washed out. However, following addition of B13 to the mixture, a stable culture was obtained and the chlorophenols were degraded to completion using the *ortho-* cleavage pathway. Analysis of the populations resulting from the degradation of 4-chlorophenol or the isomeric chlorophenol mixture revealed the development of a population of *Alcaligenes sp.* A7 capable of degrading chlorophenol via the *ortho-* cleavage pathway following transfer of catabolic abilities from B13.

Similar results were obtained when *Pseudomonas sp.* B13 was added to an undefined mixed culture for the degradation of chlorophenols (Schmidt, 1987). The mixed culture was found to degrade mono-chlorophenols via the *ortho-* cleavage pathway following addition of B13. The initial *meta-* cleavage activity of the mixed culture was replaced by modified *ortho-* cleavage activity, leading to complete degradation of the chlorophenols. Population analysis demonstrated the establishment of an *ortho-* cleaving population in the mixture other than strain B13, indicating a transfer of catabolic abilities.

In addition to natural bacteria, genetically engineered micro-organisms (GEMs) have been used to augment mixed cultures for the degradation of xenobiotics. Soda *et al.* (1998) described how the GEM *Pseudomonas putida* BH (pS10-45), capable of the degradation of phenol, was used to augment activated sludge in a sequencing batch reactor process to improve phenol degradation. Initially the GEM-augmented activated sludge was better able to deal with shock loadings of phenol resulting in enhanced degradation. However, the GEM was gradually washed out resulting in a deterioration in degradative capabilities.

A genetically modified form of *Pseudomonas sp.* B13 was used to augment activated sludge for the simultaneous degradation of 3-chlorobenzoate (3CB) and 4-

methylbenzoate (4MB) (Nüßlein *et al.*, 1992). The GEM, *Pseudomonas sp.* FR120 (pFRC20P) metabolised 3CB and 4MB using a hybrid *ortho-* cleavage pathway. Inoculation of the activated sludge brought about faster and more complete degradation than in control reactors. Addition of FR120 also had a protective effect on the indigenous population. The indigenous population of the activated sludge was killed due to the toxicity of 3CB and 4MB. However, following augmentation with FR120, the numbers of indigenous organisms was maintained indicating a protective effect exerted by FR120.

Another modified *Pseudomonas sp.* B13, SN45RE, was used by Erb *et al.* (1997) to augment activated sludge for the degradation of 4-chlorophenol/4-methylphenol or 3-chlorophenol/4-methylphenol mixtures. *Pseudomonas sp.* B13 SN45RE allows for simultaneous degradation of chloro- and methylaromatics using an *ortho-* cleavage pathway. Degradation of chlorophenol and methylphenol mixtures by the activated sludge occurred using the *meta-* cleavage pathway. This led to accumulation of dead-end metabolites following substituted aromatic degradation. Following augmentation of the sludge, the metabolic pathway had switched from the *meta-* cleavage pathway to the *ortho-* cleavage pathway leading to complete degradation. Addition of the GEM had a protective effect on the indigenous population of the sludge as numbers of prokaryotes and eukaryotes, which had decreased sharply during degradation by unaugmented sludges, remained relatively constant following introduction of the GEM.

McClure *et al.* (1989) studied the introduction of the GEM, *Pseudomonas putida* UWC1, to activated sludge for the degradation of 3-chlorobenzoate (3CB). The recombinant plasmid pD10, encoding the essential steps in chlorocatechol metabolism, was introduced to *P. putida* UWC1, allowing for the degradation of 3CB via the *ortho-* cleavage pathway. Following introduction into the activated sludge unit, UWC1 numbers declined due to its washout from the sludge. As a result, augmentation of the sludge did not result in enhanced 3CB degradation. A further study by McClure *et al.* (1991a) introduced UWC1 to activated sludge at a higher cell density. However, UWC1 numbers in the activated sludge declined as before, resulting in the lack of 3CB

degradation. As the degradation study continued, natural chlorobenzoate degrading strains other than UWC1 appeared in the activated sludge, bringing about 3CB degradation. The rapid decrease in UWC1 numbers corresponded with the development of this natural population, indicating the decline in UWC1 numbers was due not only to washout, but as a result of competition with bacteria more adapted to the environment. Chlorobenzoate degradation commenced only after the appearance of this natural population and therefore was not a direct result of the introduction of UWC1. The natural chlorobenzoate population consisted of a number of transconjugants which had received pD10 from UWC1 and a natural activated sludge bacterium, strain AS2, not containing the plasmid pD10. These activated sludge derived bacteria were found to degrade 3CB at higher rates than UWC1. The effects of reintroducing two of these bacteria, *Pseudomonas putida* ASR2.8, a transconjugant, and strain AS2 into the activated sludge was studied. Both introduced strains were found to survive in the activated sludge at higher populations than UWC1. Introduction of the bacteria also resulted in 3CB metabolism, commencing within 3 days for AS2 and 4 days for ASR2.8.

Simple addition of pure cultures possessing certain desirable metabolic abilities to mixtures does not guarantee enhanced degradative capabilities. The ability to metabolise a chemical is a necessary but not a sufficient condition for the organism to effect the transformation in a mixed culture. The studies carried out by McClure *et al.* (1989, 1991a) clearly indicate this fact. A number of reasons have been suggested in the literature for the failure of inoculation. Firstly, the introduced strain may not survive in the mixed culture due to predation or parasitism, or competition with indigenous micro-organisms better adapted to environmental conditions. In addition, the concentration of the compound in the new environment may be too low to support growth of the inoculated species or the natural environment may contain substances such as heavy metals inhibiting growth or activity of the added organism. The growth rate of the organism on a low concentration of the chemical of interest in the environment may be slower than the rate of predation by protozoa, thus reducing the inoculum size of the introduced species (Goldstein *et al.*, 1985).

Also, the presence of additional, more easily assimilated carbon sources, commonly found in industrial wastes can inhibit degradation of the pollutant of interest. In the presence of such chemicals, the introduced strain is under no selective pressure to maintain its degradative capabilities, utilising the more easily degradable carbon sources in preference to the more recalcitrant pollutants (McClure *et al.*, 1989).

The problems of maintaining an active xenobiotic-degrading population were described by Westmeier and Rehm (1987) for the degradation of 4-chlorophenol in municipal wastewater. The natural microbial population of the wastewater degraded 4-chlorophenol via the *meta-* cleavage pathway as indicated by the accumulation of 5-chloro-2-hydroxymuconic semialdehyde. This compound was found to be a dead-end metabolite for the natural population and caused a rapid decrease in the metabolic activity of the mixture. It was hoped to bring about successful degradation of 4-chlorophenol by the wastewater following addition of the *ortho-* degrader, *Alcaligenes sp.* A7-2. When *Alcaligenes sp.* A7-2 was added to sterile wastewater, degradation was successful. However, when *Alcaligenes sp.* A7-2 was added to non-sterile wastewater, 4-chlorophenol accumulated in the medium after approximately 20 hours, indicating unsuccessful degradation. This was due to the inability of *Alcaligenes sp.* A7-2 to establish itself as a member of the natural population of the wastewater.

Despite the difficulties involved in the introduction of bacteria with catabolic capabilities to mixed populations, *P. putida* CP1 successfully survived in Biolyte HAB resulting in complete chlorophenol degradation. This may have been due to a number of factors. The absence of any additional carbon sources and the absence of predation by protozoa may have contributed to its success. However, *P. putida* CP1 managed to compete with a natural chlorophenol degrading population during the degradation of 4-chlorophenol which cannot be put down solely to the catabolic properties of *P. putida* CP1.

The ability of the introduced strain to compete may have been due to its ability to flocculate. The ability to flocculate has been proposed to facilitate the survival of introduced bacteria in mixed populations. Soda *et al.* (1998) described how the inability of *Pseudomonas putida* BH (pS10-45) to flocculate resulted in its washout from an activated sludge system, resulting in decreased phenol degradation.

McClure *et al.* (1991a) described how the characteristic flocculation of strain AS2 may have been an important factor in the maintenance of a stable population following its introduction into the activated sludge unit, facilitating 3-chlorobenzoate degradation. Flocculation was also proposed by Watanabe *et al.* (1996) as a reason for the successful augmentation of activated sludge with *Alcaligenes sp.* E2 for the degradation of phenol. E2 did not wash out of the activated sludge system during degradation due to its ability to flocculate, allowing higher phenol degrading populations to survive.

P. putida CP1 formed large clumps of cells which appeared in the lag phase of chlorophenol degradation. Floc formation may have resulted from the toxicity of the chlorophenols towards *P. putida* CP1. Clumping was not as evident during chlorophenol degradation by the *P. putida* CP1/Biolyte HAB mixture. This may be due to the increased chlorophenol degrading capacity of the mixture resulting in decreased toxicity of the chlorophenols. Schmidt *et al.* (1983) described how large aggregates of cells were formed in the early stage of exposure of a defined catabolic mixed culture to mixtures of isomeric mono-chlorophenols. The formation of these clumps may have protected the majority of the microbial population from the toxic effects of the chlorophenol mixtures. Aggregation was not observed during the degradation of 4-chlorophenol by the defined mixture, suggesting that clump formation resulted from the increased toxicity of the chlorophenol mixture.

Addition of *P. putida* CP1 to Biolyte HAB switched the degradative pathway from the *meta-* cleavage pathway to the more productive *ortho-* cleavage pathway resulting in the complete degradation of the chlorophenols. While the role of *P. putida* CP1 in the

mixture is clear, resulting in a metabolic shift, the influence of Biolyte HAB on the degradation of mono-chlorophenols by the mixture is less so. In order to study the influence of Biolyte HAB, increasing concentrations of Biolyte HAB were added to *P. putida* CP1 (5%) and the degradation of the mono-chlorophenols was studied. As was the case with Biolyte HAB/*P. putida* CP1 (9.5%:0.5%), addition of Biolyte HAB to 5% *P. putida* CP1 increased the capacity of *P. putida* CP1 to degrade mono-chlorophenols. Higher concentrations of 2-chlorophenol than were degraded by *P. putida* CP1 in isolation were degraded by the Biolyte HAB/*P. putida* CP1 combinations.

For each of the inocula sizes, degradation of the mono-chlorophenols was complete, with the time of degradation for each of the mono-chlorophenols decreased proportionally with increasing percentages of Biolyte HAB. Addition of Biolyte HAB to *P. putida* CP1 resulted in degradation of the mono-chlorophenols quicker than was achieved by *P. putida* CP1. This contrasts with the study carried out by Bokhamy *et al.* (1997) where degradation of p-toluenesulfonate (pTS) by activated sludge supplemented with the pTS degrader, *Comamonas testosteroni* T-2, was slower for each of the inocula sizes used than was achieved by T-2 in isolation.

The reduction in mono-chlorophenol degradation times resulted from a reduction in the lag period, in addition to an increase in the rate of chlorophenol degradation following the lag period. The decrease in lag period was directly proportional to the concentrations of Biolyte HAB added to *P. putida* CP1. The reduction of lag periods with increasing inocula size has been reported by Greer *et al.* (1990); Balfanz and Rehm (1991) and Comeau *et al.* (1993). The rate of chlorophenol degradation increased with increasing Biolyte HAB inocula size for both 2- and 3-chlorophenol. There appeared to be a competition effect following the addition of Biolyte HAB to *P. putida* CP1 for the degradation of 1.56 mM 4-chlorophenol, as the rate of degradation decreased following the addition of 1% (v/v) Biolyte HAB to *P. putida* CP1. Subsequent increases of the Biolyte HAB inoculum size resulted in increased rates of 4-chlorophenol degradation. This reduction in degradative rates following addition of

1% Biolyte HAB to *P. putida* CP1 was not observed at a concentration of 2.33 mM where *P. putida* CP1 is less able to degrade the higher concentration.

The ability of an organism to degrade any compound is dictated by a number of physiological and environmental factors (Goldstein *et al.*, 1985). Among the factors effecting biodegradation, is the size of the initial inoculum (Ramadan *et al.*, 1990). It is widely understood that the degradation of xenobiotics improves with increasing inocula sizes. Balfanz and Rehm (1991) described how the degradation of 4-chlorophenol in aerobic shake cultures by *Alcaligenes* sp. A 7-2 improved with increasing cell concentrations. Comeau *et al.* (1993) reported the bioaugmentation of soil with increasing concentrations of *Pseudomonas cepacia* strain BRI6001 for the degradation of 2,4-dichlorophenoxyacetic acid (2,4-D). Degradation times for 2,4-D decreased with increasing BRI6001 inocula sizes. The degradation of p-toluenesulfonate (pTS) by activated sludge supplemented with *Comamonas testosteroni* T-2 improved with increasing T-2 inocula sizes (Bokhamy *et al.*, 1997).

At a concentration of 1.56 mM, the decreases in lag periods and in times of degradation following addition of increasing concentrations of Biolyte HAB were in the order of 3-chlorophenol > 2-chlorophenol > 4-chlorophenol. This reflects the degradative capacities of *P. putida* CP1 which degrades chlorophenols in the order of 4-chlorophenol > 2-chlorophenol > 3-chlorophenol. Decreases in lag periods and times of degradation were greater at higher concentrations for each of mono-chlorophenols indicating the increased importance of Biolyte HAB at concentrations near or beyond the limit of *P. putida* CP1's degradative capabilities.

Analysis of the population during degradation of the mono-chlorophenols by the various Biolyte HAB/*P. putida* CP1 mixtures indicated that *P. putida* CP1 was able to establish itself as part of the mixed culture. *P. putida* CP1 numbers within the mixed culture increased as chlorophenol concentrations decreased for each inoculum ratio. Final *P. putida* CP1 numbers increased with increasing Biolyte HAB inoculum size, suggesting a protective effect of Biolyte HAB on *P. putida* CP1 growth. When the

growth of *P. putida* CP1 was compared for each of the mono-chlorophenols, growth on 4-chlorophenol resulted in the greatest increases in *P. putida* CP1 cell numbers, followed by 2-chlorophenol and 3-chlorophenol. This reflected the ability of *P. putida* CP1 to degrade the mono-chlorophenols in pure culture which was in the order of 4-chlorophenol > 2-chlorophenol > 3-chlorophenol.

This study showed the potential of the *P. putida* CP1 strain for use in the treatment of xenobiotic containing wastewaters. As *P. putida* CP1 was naturally isolated, it avoids the problems involved with the deliberate release of GEMs into the natural environment.

5. Conclusions

(i) Biolyte HAB degraded the mono-chlorophenols using a *meta-* cleavage pathway. This led to the complete degradation of 4-chlorophenol at concentrations up to 2.33 mM while degradation of 2- and 3-chlorophenol by Biolyte HAB was incomplete.

(ii) *Pseudomonas putida* CP1 degraded the mono-chlorophenols via an *ortho-* cleavage pathway. This led to the complete degradation of all three mono-chlorophenol isomers at concentrations up to 1.56 mM for 2- and 3-chlorophenol and 2.33 mM for 4-chlorophenol. The ability of *P. putida* CP1 to degrade the mono-chlorophenols was in the order of 4-chlorophenol > 2-chlorophenol > 3-chlorophenol. Growth of *P. putida* CP1 on the mono-chlorophenols resulted in the formation of large clumps of cells in the culture medium.

(iii) Addition of *P. putida* CP1 to Biolyte HAB resulted in the complete degradation of all three mono-chlorophenols via an *ortho-* cleavage pathway. *P. putida* CP1 was capable of surviving in the mixed culture with numbers increasing throughout the degradation of the mono-chlorophenols. The augmented mixed culture displayed increased degradative capabilities, with times of degradation reduced for each of the mono-chlorophenols when compared to those achieved by *P. putida* CP1 in isolation. The augmented mixed culture degraded higher concentrations of 2-chlorophenol (2.33 mM) than were degraded by *P. putida* CP1 while still degrading 1.56 mM 3-chlorophenol and 2.33 mM 4-chlorophenol. The ability of the augmented mixed culture to degrade the mono-chlorophenols was in the order of 4-chlorophenol > 2-chlorophenol > 3-chlorophenol reflecting the ability of *P. putida* CP1 to degrade mono-chlorophenols in isolation.

(iv) Addition of increasing concentrations of Biolyte HAB to *P. putida* CP1 increased the ability of the augmented mixture to degrade mono-chlorophenols. Times of degradation decreased with increasing Biolyte HAB inoculum size, due to decreases in lag periods and increases in rates of degradation.

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7. Appendix.

The results of analysis of variance (ANOVA) for significance testing for the final microbial cell numbers following the addition of varying concentrations of Biolyte HAB to 5% *P. putida* CP1.

Table 36. Test of significance of final *P. putida* CP1 numbers following degradation of mono-chlorophenols by Biolyte HAB/*P. putida* CP1.

Source	Type III Sum of squares	df	Mean Square	F	Significance	Remarks
Corrected Model	4.26e19	8	5.32e18	574.34	0.00	
Intercept	7.39e19	1	7.39e19	7978.36	0.00	
Inoculum	2.38e19	2	1.19e19	1285.53	**0.00**	**Signif.**
Chlorophenol	1.54e19	2	7.72e18	832.58	**0.00**	**Signif.**
Inoc * CP	3.32e18	4	8.30e17	89.62	0.00	
Error	8.34e16	9	9.27e15			
Total	1.17e20	18				
Corrected Total	4.27e19	17				

Table 37. Test of significance of final total cell counts following degradation of mono-chlorophenols by Biolyte HAB/*P. putida* CP1.

Source	Type III Sum of squares	df	Mean Square	F	Significance	Remarks
Corrected Model	6.73e20	8	8.41e19	72.82	0.00	
Intercept	1.17e21	1	1.17e21	1014.58	0.00	
Inoculum	6.02e20	2	3.01e20	260.60	**0.00**	**Signif.**
Chlorophenol	8.62e18	2	4.31e18	3.74	**0.66**	**Not Signif.**
Inoc * CP	6.22e19	4	1.56e19	13.48	0.01	
Error	1.04e19	9	1.15e18			
Total	1.85e21	18				
Corrected Total	6.83e20					