The Degradation of Phenol and Mono-Chlorophenols by a Mixed Microbial Population.

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

by

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This thesis is dedicated to my Dad
who was a constant source of the support, motivation and
encouragement that made it possible

and to the memory of my mother.

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.

Signed: Marianne O'Sullivan

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Abstract.

The Degradation of Phenol and Mono-Chlorophenols by a Mixed Microbial Population.

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The degradation of phenol and the mono-chlorophenol isomers by a bioaugmentation product was investigated. The product comprised a mixed microbial culture which included several bacterial species, including Pseudomonads, and a brown rot fungus. Concentrations of phenol, up to 1300mgl^{-1}, were metabolised by the mixed microbial culture. Complete removal of 4-chlorophenol, when present as the sole carbon source, was achieved at concentrations up to 200mgl^{-1}. Removal of both 3- and 2-chlorophenol was relatively poor, as a result of an unproductive *meta*-cleavage pathway.

Additional carbon, detected in the product formulation, was found to inhibit removal. Phenolic removal was inhibited in the presence of increasing concentrations of glucose, but not fructose. Acclimation was found to influence the rate of removal of the additional carbon and therefore the response of the cells to this carbon. The cells had been previously exposed to glucose in the product formulation and its removal was rapid. In contrast, no acclimation to fructose had taken place and preferential removal of the phenolic from the phenolic/fructose mixtures was observed. Following acclimation of the cells to fructose, which altered the rate of utilisation of the sugar, fructose exerted inhibitory effects on phenolic removal. Inhibition due to the presence of additional carbon was found to be alleviated by incubation at lower temperatures, due to changes in substrate removal patterns. A continuous presence of the sugar, under controlled pH conditions, also facilitated complete phenolic removal. Environmental conditions, particularly temperature and acclimation, were therefore considered fundamental to the successful performance of this mixed microbial culture.

Bacterial species capable of growth on 4-chlorophenol were isolated from the product. Bacteria capable of growth on 3- or 2-chlorophenol could not be isolated in pure culture. The fungus, when grown in pure culture, could not grow on 3-chlorophenol. It was capable of growth on 2-chlorophenol, but did not perform successfully as a member of the mixed culture.

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1. Introduction.

1. 1 Aromatic hydrocarbons in the environment.

The increasing presence of synthetic organic compounds in the environment has become a matter of great concern. Such compounds cause considerable environmental pollution and health problems as a result of their persistence and toxicity. Many xenobiotics are halocarbons. Substitution with a chlorine or other halogen, along with highly condensed aromatic rings and excessive molecular size have been cited as common reasons for persistence (Atlas and Bartha, 1992a). Haloaromatic compounds are produced in vast amounts by the chemical industry for use as solvents, lubricants and plasticisers, as well as for use as herbicides and pesticides.

With the considerable growth in the chemical and pharmaceutical industries, the levels of hazardous waste generated are also on the increase. Information on the hazardous waste quantities generated in Ireland is available in the National Waste Database Report for 1995 (EPA, 1996), which breaks down the raw data of the survey undertaken at the time. The levels of some waste categories are outlined in Table 1.

Table 1: Hazardous waste generation in 1996 by waste type.

Waste Type	**Total Quantity Generated** (tonnes)
Organic and organic chlorinated 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

Chlorinated solvents account for a large proportion of the total waste generated and therefore studies into the removal of such compounds from the environment merit consideration. This is reported data and does not represent the organisations that did not respond to the questionnaire.

Organic compounds can be removed from the environment by biological, physical or chemical methods. Biological processes are generally accepted to play the major role in nature (Painter and King, 1985). Public opposition against traditional methods of toxic waste disposal, such as landfill and incineration, has led to growing interest in biological treatment which is often viewed as the cleaner and more environmentally friendly option. Furthermore, biological methods are often less energy-intensive and more economical than the alternatives. In general, the performance of biological treatment systems depends on the activities of communities of living organisms. The metabolic capabilities of these organisms are harnessed and the ultimate aim of the treatment system is the oxidation of the organic compounds to carbon dioxide and water, or their anaerobic decomposition to carbon dioxide and methane. While these microbial populations are adaptable to many influent changes, the increasing diversity of modern waste streams and in particular the presence of toxic chemicals, often demands the use of bioaugmentation as the regulation of pollution levels becomes increasingly stringent. The potential of bioaugmentation, where specialised microbes are added to a treatment system in order to improve its performance, has been recognised for some time and several commercial bioaugmentation products are currently on the market. A detailed understanding of the physiological and biochemical basis of biodegradation is required for the development of efficient bioaugmentation products and biological waste treatment systems.

Biodegradation can be defined as the biologically catalysed reduction in complexity of chemicals. Alexander (1980) asserted that the use of the word "biodegradation" as a more comprehensive term may obscure the significance of different effects such as mineralisation, detoxification, cometabolism and

defusing. The extent of biodegradation of a compound can vary and the process can only be considered complete with the conversion of the C, N, P, S and other elements in the original compound to inorganic products (e.g. CO_2, H_2O, NO_3^-, NH_4^-) and the formation of new cells (Painter and King, 1985). Such a conversion of an organic substrate to inorganic products is known as mineralisation.

Biodegradability is the capacity of a substance to undergo microbial attack. This property is a function of a number of parameters. These include the chemical structure and the potential of the compound to associate with other organic and inorganic material in the environment, such as humic material or macromolecules such as polysaccharides, proteins, etc. (Neilson *et al*, 1985).

Phenol and mono-chlorophenols are generated from a number of sources including pulp and paper manufacture, the chlorination of water and the breakdown of pesticides such as 2, 4-D and pentachlorophenol. Chlorophenols are toxic to several micro-organisms because of their ability to uncouple oxidative phosphorylation. However, many organisms have developed mechanisms to detoxify such compounds, usually by simple chemical alterations in the structure e.g. alkylation, esterification or nucleophilic displacement of the halogen substituent. Complete biodegradation of aromatic compounds is hindered by the presence of chlorine or other halogen substituents on the aromatic ring. Halogen atoms deactivate the benzene nucleus to electrophilic attack by dioxygen, by withdrawing electrons from the ring. This deactivating effect increases with the number of halogen substituents (Neilson *et al*, 1985).

Phenolic degradation is also hindered by the toxic effects of these compounds on whole cells. Aromatic structures with a hydrophilic substituent, particularly phenols, resemble phospholipids. They consequently dissolve readily in the cell membrane, disturbing its integrity and effecting specific permeabilisation (Heipieper *et al*, 1991). Heipieper *et al* (1992) showed how changes in the fatty acid composition, induced by phenol, affected the permeability of the cell

membrane. They observed a concentration-dependent efflux of K^+ ions upon the addition of phenol. The ability of micro-organisms to tolerate the presence of phenolic compounds has been correlated with membrane fluidity. Micro-organisms react to externally dictated changes in their environment by modifying their membranes to keep them in the same fluidity conditions. Using supplementation with various fatty acids, Keweloh *et al* (1991) recognised how the increase in tolerance of *E.coli* cells to phenol and substituted phenols was determined essentially by the ratio of saturated to unsaturated fatty acids. Studies with *Pseudomonas putida* P8 growing in the presence of sublethal concentrations of phenol, revealed a conversion of *cis*-unsaturated fatty acids to *trans*, in the presence of phenol and 4-chlorophenol (Heipieper *et al*, 1992). The benefit of this isomerisation was based on the steric differences between *cis* and *trans* unsaturated fatty acids. The conversion of *cis* to *trans* unsaturated fatty acids reduced membrane fluidity resulting in an increase in tolerance against toxic compounds. Knowledge of the mechanism of the inhibitory action of lipophilic compounds is helpful in the technical applications of micro-organisms.

1. 2 The pathways of biodegradation of phenols, chlorophenols and other aromatics.

The contamination of the environment with chlorinated aromatic compounds is a global problem and consequently the biodegradation of phenol and chlorophenols has been the focus of extensive work (Häggblom, 1990, Sangodkar *et al*, 1989, Reineke and Knackmuss, 1988). The bacteria associated with the aerobic degradation of chlorophenols can be divided into two groups: those that degrade phenol and the mono-chlorophenols and those that attack only the more highly chlorinated phenols (Häggblom, 1992).

A critical feature of catabolic routes is the channelling of structurally diverse substrates into a limited number of central pathways. This is accomplished through the activity of a large number of enzymes that carry out the initial reactions in the catabolic pathways and that collectively are able to attack a wide

range of substrates. In the aerobic catabolism of aromatic molecules, these reactions generally lead to the formation of dihydroxy intermediates which are key intermediates for the entry into central pathways (Fig. 1). Depending on whether or not the substrate already carries hydroxyl groups on the aromatic ring, the hydroxylation may be carried out by either mono- or dioxygenase enzymes. Catabolism of aromatic substrates with more complex substitutions may involve additional steps that process the substituent group.

1. 2. 1 The degradation of catechol.

A common intermediate of many biodegradative pathways is catechol. This compound plays a central role in the oxidation of phenol, chlorophenols, benzoate and other aromatics. A diverse array of enzymes can be elaborated to convert aromatic compounds to central catecholic intermediates.

The enzymes for benzoate degradation are chromosomally encoded and are ubiquitous in *Pseudomonas* spp. Benzoate is initially attacked by benzoate 1, 2-dioxygenase to form 1, 2-hydro-1, 2-dihydroxybenzoic acid, which is subsequently converted to catechol by the single component enzyme, dihydroxybenzoic acid (DHB) dehydrogenase (Reineke and Knackmuss, 1978). Catechol is metabolised to acetyl co A and succinate via the *ortho*-cleavage pathway. Benzoate can also be attacked by the TOL plasmid-encoded enzymes, toluate 1, 2-dioxygenase and 1, 2-dihydroxy-3, 5-cyclo hexadiene-1-carboxylate dehydrogenase. Toluate dioxygenase has a broad substrate specificity while the benzoate dioxygenase enzyme has a much narrower substrate range. An evolutionary relationship between TOL-encoded toluate 1, 2-dioxygenase and benzoate 1, 2-dioxygenase has been suggested by Harayama and Rekik (1990), who reported the similarity in the molecular weights of the three subunit proteins of both enzymes.

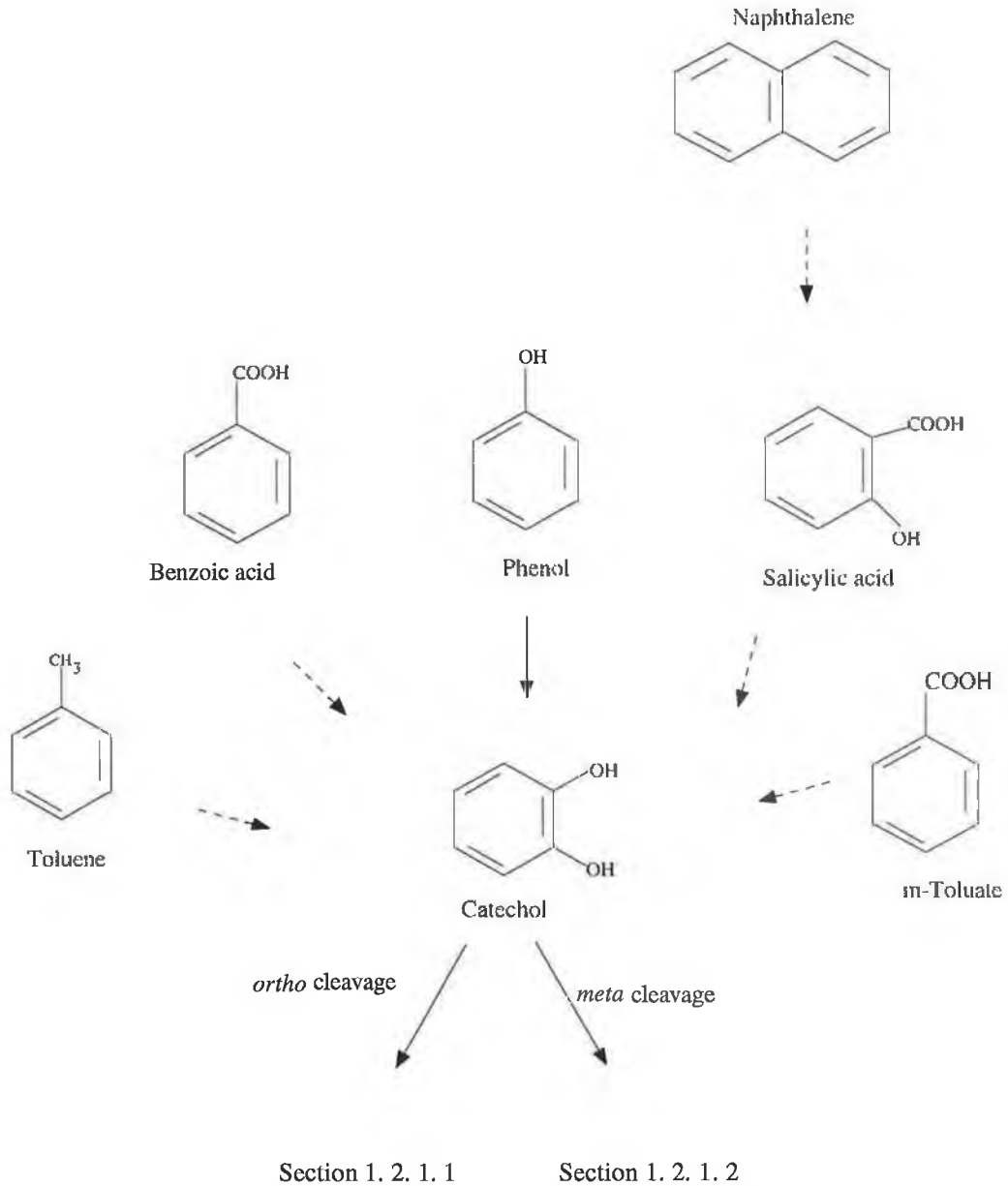


Fig. 1 The central role of catechol in the oxidation of aromatic compounds by *Pseudomonas* sp. (Feist and Hegeman, 1969).

An upper and lower pathway of naphthalene degradation have been elucidated. This substrate is initially acted upon by naphthalene dioxygenase, a multi-component membrane-associated enzyme system (Haigler and Gibson, 1990), to produce *cis*-naphthalene dihydrodiol. This compound is subsequently oxidised to 1, 2-dihydroxynaphthalene by *cis*-naphthalene dihydrodiol dehydrogenase, an enzyme that requires NAD as an electron acceptor (Yen and Serdar, 1988). Cleavage of this compound by a dioxygenase yields 2-hydroxychromene-2-carboxylic acid, which is converted to *cis*-2-hydroxybenzalpyruvate by an isomerase enzyme. A subsequent aldolase-catalysed reaction yields salicylaldehyde and pyruvate. Salicylaldehyde is further oxidised to salicylate by an NAD^+-dependent dehydrogenase. In most cases, the lower pathway of salicylate degradation begins with the conversion of salicylate to catechol by salicylate hydroxylase and concludes with the production of pyruvate and acetaldehyde. The formation of gentisate from salicylate has been proposed during naphthalene metabolism by *Pseudomonas fluorescens* and *Pseudomonas alcaligenes* (Yen and Serdar, 1988, Fuenmayor *et al*, 1998).

A number of plasmids carrying naphthalene catabolic genes have been demonstrated in *Pseudomonas* spp. The best studied naphthalene catabolic plasmid is NAH7 whose degradative genes are arranged into two operons separated by a 7kb fragment. One operon encodes the upper pathway and the other codes for salicylate hydroxylase and the enzymes of the *meta* pathway of catechol degradation. A regulatory gene, *nah* R, is located between the two operons and its product is required for the expression of both operons (Lehrbach and Timmis, 1983).

A number of pseudomonads possess the ability to utilise *m*-toluate and related compounds as their sole carbon and energy source. It is produced during the metabolism of *m*-xylene by the TOL plasmid. The catabolic genes of the archetypal TOL plasmid, pWWO, are organised into two operons, one encoding enzymes for the upper pathway and the other encoding the lower (*meta*) pathway

enzymes. Two regulatory genes, *xyl* R and *xyl* S, have also been located on the TOL plasmid. Substrates for the upper pathway enzymes e.g. toluene, benzyl alcohol are inducers of the pathway when the *xyl* R product is present. The combining of *m*-toluate with the gene product of *xyl* S induces the enzymes responsible for the further metabolism of *m*-toluate via catechol and a *meta* pathway (Worsey *et al*, 1978).

The degradation of catechol itself however, is limited to one of two metabolic alternatives: the *ortho*-cleavage pathway or *meta*-cleavage pathway. The cleavage of catechol between adjacent carbon atoms carrying hydroxyl groups is known as *ortho* cleavage or intradiol cleavage. *Meta* cleavage occurs between two adjacent carbon atoms, one carrying a hydroxyl group and the other being unsubstituted or substituted with any group other than a hydroxyl group.

1. 2. 1. 1 The *ortho*-cleavage pathway.

Steps taken in the *ortho*-cleavage pathway, also known as the β-ketoadipate pathway, result in the complete degradation of catechol to succinate and acetyl coA, via β-ketoadipate.

The initial cleavage of catechol, resulting in the production of *cis*, *cis*-muconate, is catalysed by catechol 1, 2-dioxygenase, an enzyme which is quite sensitive to catabolite repression (Wu *et al*, 1972). Catechol 1, 2-dioxygenase has a single active site and requires ferric iron as a cofactor. Following treatment with SDS, it dissociates into two non-identical subunits (Nozaki, 1979). This enzyme is induced not by its substrate but by its product *cis*, *cis*-muconate. The next two enzymes of the pathway, muconate lactonizing enzyme and muconolactone isomerase are co-ordinately induced by *cis*, *cis*-muconate (Kemp and Hegeman, 1968).

Muconate lactonizing enzyme is a decyclising enzyme, which converts *cis*, *cis*-muconate into muconolactone. This compound did not serve as a growth

substrate for *Pseudomonas aeruginosa* strain PRS104, because of its failure to induce the requisite enzymes (Kemp and Hegeman, 1968). Muconolactone isomerase then converts muconolactone to enol-lactone which is further metabolised to β-ketoadipate by enol-lactone hydrolase. The activity of a transferase enzyme transforms β-ketoadipate into β-ketoadipyl coA. Both the transferase and the hydrolase enzymes are induced by β-ketoadipate or β-ketoadipyl coA. β-ketoadipyl coA is finally converted to succinate and acetyl coA before entry into the TCA cycle (Wheelis and Ornston, 1972).

The *ortho*-cleavage pathway of the degradation of catechol is chromosomally encoded. The structural genes for muconate lactonizing enzyme (catB) and muconolactone isomerase (catC) 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 induces catA, the structural gene for catechol 1, 2-dioxygenase. The synthesis of this latter enzyme, however, is also subject to independent regulation (Wu *et al*, 1972).

Evidence indicating that mutations confined to one region of the chromosomal map of *Ps. putida*, affect the regulation of both catA and catB expression, has been presented by Wheelis and Ornston (1972). They propose that this region to the left of catB, designated catR, has a regulatory function. All point mutations in this area abolished simultaneously the expression of catB and catC. The induction of catechol 1, 2-dioxygenase was not prevented by the mutations in catR. Wu *et al* (1972) concluded that *cis, cis*-muconate is the inducer of the three catechol enzymes and that the catR gene is specifically required for the expression of catB and catC.

1. 2. 1. 2 The *meta*-cleavage pathway.

The *meta*-cleavage pathway produces pyruvate and acetaldehyde as final products of catechol degradation. Cleavage of the aromatic ring of catechol is performed by catechol 2, 3-dioxygenase, a colourless enzyme consisting of four

identical subunits. The ternary complex of the enzyme reacts with oxygen and the organic substrate in this reaction and requires ferrous iron as a cofactor (Nozaki, 1979). The product of this cleavage, 2-hydroxymuconic semialdehyde, can be further metabolised by either a dehydrogenative or a hydrolytic route. Both routes reconverge later.

In the dehydrogenative branch, 2-hydroxymuconic semialdehyde is first converted to the enol form of 4-oxalocrotonate (2-hydroxyhexa-2, 4-diene-1, 6-dionate) by an NAD^+-dependent hydroxymuconic semialdehyde dehydrogenase. This enzyme is also active against the ring fission product of 4-methylcatechol, 2-hydroxy-5-methyl-6-oxohexa-2, 4-dienoate. Both catechol and 4-methylcatechol are generally metabolised via the dehydrogenative route because of the high affinity of the dehydrogenase for their ring fission products.

3-methylcatechol, however, is generally metabolised via the hydrolytic route to 2-oxopent-4-enoate and formate. Following cleavage of its aromatic ring, 2-hydroxy-6-oxohepta-2, 4-dienoate is produced which lacks an oxidisable aldehyde group resulting in the inability of the dehydrogenase to attack this compound (Harayama *et al*, 1989).

The product of dehydrogenase activity on 2-hydroxymuconic semialdehyde is the enol form of 4-oxalocrotonate. This compound is subsequently converted to its keto form (2-hydroxyhexa-4-ene-1, 6-dioate) by isomerase activity. The ensuing activity of 4-oxalocrotonate decarboxylase results in the production of 2-hydroxypent-2, 4-dienoate with the release of carbon dioxide. Mg^{2+} is required for this activity (Bayly and Barbour, 1984).

The specificity of this decarboxylase for the *keto* form of 4-oxalocrotonate was noted by Harayama *et al* (1989), when only the *keto* form was removed from an equilibrium solution. As the concentration of the *keto* form decreased, the *enol* form was transformed to its isomer thus promoting further decarboxylase activity.

2-hydroxypent-2, 4-dienoate and its *keto* isomer are the final products and both pathways reconverge here. A hydratase enzyme converts 2-hydroxypent-2, 4-dienoate to 4-hydroxy-2-oxovalerate. Harayama *et al* (1989) suggested that the decarboxylase and the hydratase form a complex *in vivo*, ensuring the efficient transformation of the chemically unstable 2-hydroxypent-2, 4-dienoate. Finally, 4-hydroxy-2-oxovalerate is converted to pyruvate and acetaldehyde by aldolase activity.

The most comprehensively studied *meta*-cleavage pathway is that of the IncP-9 TOL plasmid pWW0, which encodes a toluene degradation pathway in *Ps. putida*. The *meta*-cleavage pathway genes are located in an operon that encodes the enzymes for conversion of benzoate, via catechol, to central metabolites. A separate operon encodes the enzymes required to convert toluene and xylenes to the corresponding benzoates. The *meta* operon of pWW0 comprises 13 genes: *xyl* E, F, G, H, I, J, K which code for the structural genes of the enzymes responsible for the degradation of catechol to pyruvate and acetaldehyde; *xyl* X, Y, Z which encode the three subunits of toluate 1, 2-dioxygenase and *xyl* L which encodes 1, 2-dihydroxy-3, 5-cyclohexadiene-1-carboxylase. The conversion of toluate or benzoate to catechol is catalysed by the latter two enzymes. The functions of the remaining two genes *xyl*T and *xyl*Q are, as yet, unknown (Harayamana and Rekik, 1990). Expression of the structural genes is controlled by two regulatory proteins encoded by *xyl*R and *xyl*S.

A regulatory model by Worsey *et al* (1978) states that the expression of the upper pathway is induced in the presence of toluene and the *xyl*R gene product. If the organism is grown in the presence of compounds inducing the upper pathway, the lower pathway needs both the *xyl*R and *xyl*S products for induction. In the presence of lower pathway substrates, such as *m*-toluic acid or benzoate, interaction of the *xyl*S gene product with the operator-promoter region of the lower pathway OP2 is sufficient for induction.

In a negatively controlled system, the most common regulatory mutation is to produce constitutivity, and the production of non-inducible mutants is a much rarer event; the converse holds for positive control (Engelsberg and Wilcox, 1974). Evidence for the positive regulation of the *meta*-cleavage pathway is presented by Worsey *et al* (1978). They observed a frequent occurrence of spontaneous deletion mutants in the *xyl*S gene which resulted in the inability to induce the *meta* pathway.

A possibility of a third regulatory locus on the TOL plasmid has been suggested by Cuskey and Sprenkle (1988) following work with *Pseudomonas* species. A mutation in *xyl*S prevented induction of the *meta* pathway by *m*-toluate, but not by unsubstituted compounds such as benzoate or benzyl alcohol. They reported that interaction with OP2, an operator-promoter region of the lower pathway, was sufficient for benzoate induction in the absence of known plasmid regulatory genes. This phenomenon was species specific and suggested the involvement of a chromosomal gene product, possibly a regulatory protein, for the chromosomally encoded benzoate dioxygenase genes.

Shingler *et al* (1992) have studied the plasmid pVI150, which is responsible for the ability of *Pseudomonas* sp. strain CF600 to grow efficiently with phenol, cresols or 3, 4-dimethylphenol as the sole carbon and energy source. The pathway encoded on this IncP-2 plasmid involves hydroxylation followed by a *meta*-cleavage pathway. The 15 genes, encoded in the order *dmp*KLMNOPQBCDEFGHI lie in a single operon. The ring cleavage enzyme, catechol 2, 3-dioxygenase, is coded by *dmp*B. The *dmp*C and *dmp*D genes encode the first enzymes of the branches of the *meta*-cleavage pathway: 2-hydroxymuconic semialdehyde dehydrogenase and 2-hydroxymuconic semialdehyde hydrolase, respectively. *dmp*E encodes 2-oxopent-4-enoate hydratase activity; *dmp*F encodes acetaldehyde dehydrogenase activity; *dmp*G encodes 4-hydroxy-2-oxovalerate aldolase activity. *dmp*H and I encode 4-oxalocrotonate decarboxylase and isomerase activity, respectively.

The previously mentioned tight association of the decarboxylase and the hydratase was also noted with these enzymes from *Pseudomonas* sp. strain CF600 (Shingler *et al*, 1992).

1. 2. 2 Phenol degradation.

The biodegradation of phenols begins with the activity of phenol hydroxylase enzymes, which transform the phenolic substrate to the corresponding catechol. These enzymes are often coded for on catabolic plasmids. Their metabolic function is to introduce a second hydroxyl group into the benzene nucleus, in most cases forming a catechol which then undergoes ring cleavage by a dioxygenase system (Beadle and Smith, 1982).

Growth of *Pseudomonas putida* on phenol, induces the synthesis of phenol hydroxylase and the enzymes of the *meta* pathway of catechol degradation (Feist and Hegeman, 1969). The induction of an entire pathway by the primary substrate, would appear to be a much less specific control compared to the *ortho* pathway which is induced by a product of one of its pathway enzymes, namely *cis-cis*, muconate (Ornston and Stanier, 1966).

Meta cleavage of catechol results in the formation of 2-hydroxymuconic semialdehyde, which can be further metabolised by either the NAD^+-dependent dehydrogenase or a hydrolase (Section 1. 2. 1. 2). In *Ps. putida* strain U, phenol degradation via the dehydrogenase activity seemed to be the preferred route (Wigmore *et al*, 1974). A mutant strain deficient in NAD^+-dependent dehydrogenase activity, was capable of phenol utilisation via the hydrolytic route, although the growth rate was reduced. The observed accumulation of 2-hydroxymuconic semialdehyde was thought to be necessary in order to "drive" the substrate down the hydrolytic pathway.

Pseudomonas strain CF600 is capable of growth on phenol, cresols and 3,4-dimethylphenol via a plasmid-encoded multicomponent phenol hydroxylase and a

subsequent *meta* cleavage pathway (Shingler *et al*, 1989). The entire phenol catabolic pathway of this strain is encoded in a single operon consisting of 15 genes, *dmp* KLMNOPQBCDEFGHI, with the first 6 genes involved in the conversion of phenol to catechol (Shingler *et al*, 1992). The genetic organisation is different to that of *Ps. putida* strain U (Bayly and Barbour, 1984), where a lack of co-ordination between induction of phenol hydroxylase and the other *meta* pathway enzymes, suggested that their structural genes exist in two operons.

1. 2. 3 Chlorophenol degradation.

The presence of a chlorine group on phenol makes the compound more persistent in the environment. The biodegradation of a halogenated compound can be considered complete only when its carbon skeleton is converted into intermediary metabolites and its organic halide is converted to its mineral state. For a bacterium to achieve mineralisation of the chlorophenol, it should be capable of removing the chlorine substituents at an early stage of metabolism or have enzymes with substrate specificities that can cope with the chlorinated substrate. In the latter case, the chlorine group is removed following ring cleavage (Häggblom, 1990). Following hydroxylation, ring cleavage may take place via an *ortho* or a *meta*-cleavage pathway.

1. 2. 3. 1 Hydroxylation.

Degradation of chlorophenols can occur through chlorocatechols, with halogen removal taking place only after ring cleavage (Fig. 2). The production of chlorocatechols from chlorophenols may be catalysed by a hydroxylase or a dioxygenase system. The reaction requires molecular oxygen and usually NADH or NADPH.

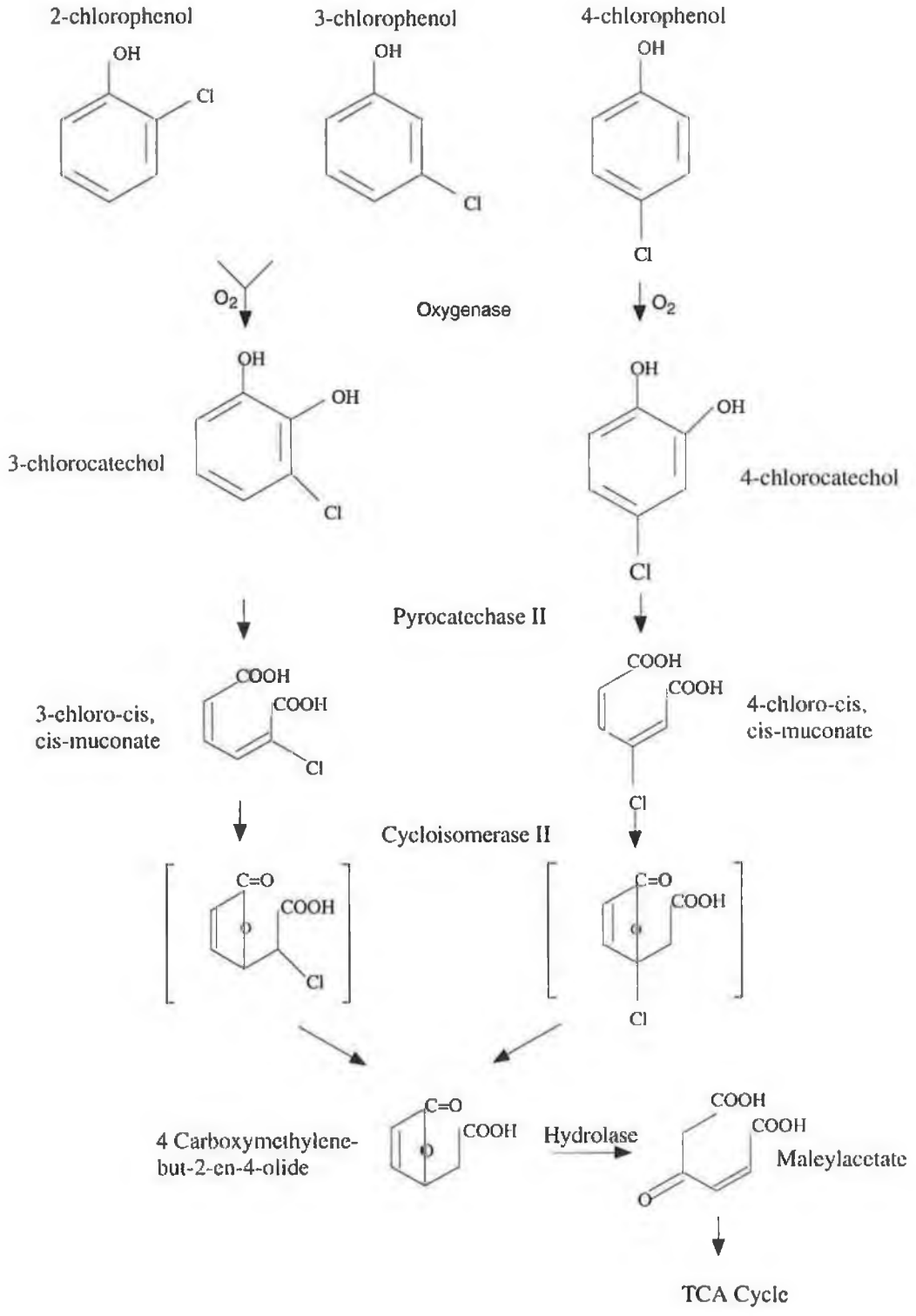


Fig. 2 The degradation of chlorophenol in pseudomonads (Knackmuss, 1981).

Pseudomonas sp. B13 can degrade 3-chlorobenzoate (Dorn *et al*, 1974). Studies on this micro-organism by Knackmuss and Hellwig (1978) showed its ability to utilise 4-chlorophenol as a single carbon source, and also to co-oxidise both 2- and 3-chlorophenol. 2- and 4-chlorophenol produced 3- and 4-chlorocatechol. 3-chlorophenol, however, produced both 3- and 4-chlorocatechol. Following a comparison of the rates of transformation of phenol into catechol, and chlorophenol into chlorocatechol, it was suggested that the enzyme activity was the same; a broad spectrum phenol hydroxylase.

Schwien and Schmidt (1982) used conjugation with *Pseudomonas* sp. B13 to construct a strain of *Alcaligenes* capable of growth on mono-chlorophenols, by means of a non-specific phenol hydroxylase. Further study on biodegradation by the constructed *Alcaligenes* strain revealed the effects of the position of the halogen substituent. At a given concentration, phenol was degraded most rapidly, followed by 4-chlorophenol and then 2-chlorophenol. The ability of the substrates to induce phenol hydroxylase activity followed the same order (Menke and Rehm, 1992).

Saez and Rittman (1991) observed that 4-chlorophenol was transformed without any lag phase by *Pseudomonas putida* PpG4, following growth of the organism on phenol. This suggested that the enzyme carrying out the first step of the transformation of 4-chlorophenol, was phenol hydroxylase.

The broad specificity of the phenol hydroxylase of *Pseudomonas putida* EKII was also demonstrated by Hinteregger *et al* (1992). The hydroxylase activity was detected in the degradation of mono-substituted and 3, 4-disubstituted phenols. Only poor activity was found for 2, 4-disubstituted phenols.

Hydroxylation of chlorinated phenols to chlorocatechols was observed by Spain and Gibson (1988) with toluene-grown *Pseudomonas putida* F1 and *Pseudomonas* sp. JS6. *Pseudomonas putida* F1 metabolised 2- and 3-chlorophenol to 3-chlorocatechol, and 3, 4-dichlorophenol to 3, 4-

dichlorocatechol with the chlorocatechols then accumulating in the medium. This accumulation was thought to be due to the toxicity of the chlorocatechols to the 3-methylcatechol 2, 3-dioxygenase present in strain F1 after growth on toluene. 4- and 3, 5-dichlorocatechol were produced from 4- and 2, 4-dichlorophenol, and accumulated only transiently before being further metabolised. *Pseudomonas* sp. JS6 grown on 1, 4-dichlorobenzene transformed 2, 5-dichlorophenol into 3, 6-dichlorocatechol. Suggestions of the involvement of a toluene dioxygenase in the transformation of chlorophenols into chlorocatechols, in *Pseudomonas putida* F1 and *Pseudomonas* sp. JS6, were made in this paper. Induction and activity of the toluene dioxygenase was shown to be closely correlated with the ability to oxidise phenols. Furthermore, mutant strains deficient in the toluene dioxygenase, failed to transform chlorophenols.

Further studies with *Ps. putida* F1 growing on a variety of methyl-, nitro- and chloro-substituted phenols demonstrated that the reactions were fastest with phenols containing a single group in the *meta* position. This illustrated the influence of steric effects on the enzyme activity (Spain and Gibson, 1988).

1. 2. 3. 2 Ring cleavage.

Cleavage of the aromatic ring, which can be *ortho* or *meta*, is a critical step in the degradation of chlorophenols. All bacterial systems which successfully utilise haloaromatics have developed degradative routes with a modified *ortho* ring cleavage mechanism. Compared to ordinary, rather specific 1, 2-dioxygenases, ring cleavage enzymes with broad substrate specificities are required (Knackmuss, 1981).

Such enzymes have been described for *Pseudomonas* sp. B13. This organism can induce two types of enzymes: highly specific enzymes, induced during growth with aromatic compounds like benzoate and phenol, and enzymes with relaxed specificities and high activities for chloro- and bromo-substituted substrates (Knackmuss and Hellwig, 1978). These latter enzymes are of the 'modified

ortho' pathway and include pyrocatechase II (chlorocatechol 1, 2-dioxygenase) and cycloisomerase II. They are isoenzymes of the corresponding unmodified enzymes and are selected during enrichment on chlorinated substrates. The unmodified enzymes exhibit some activity with chlorinated substrates, suggesting that they are ancestors of the modified enzymes.

Ortho-cleavage of the chlorocatechols is catalysed by pyrocatechase II and produces chloromuconates (Knackmuss and Hellwig, 1978). 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. The products of *ortho*-cleavage of 3- and 4-chlorocatechol are 2- and 3-chloro-*cis, cis*-muconate. 3, 5-dichlorocatechol is metabolised via 2, 4-dichloro-*cis,cis*-muconate (Reineke and Knackmuss, 1988). The *meta*-cleavage pathway is discussed later (Section 1. 2. 3. 4).

1. 2. 3. 3 Cycloisomerisation.

Cycloisomerisation of the products of *ortho* cleavage of 3- and 4-chlorocatechol, namely 2- and 3-chloro-*cis, cis*-muconate, has been described by Schmidt and Knackmuss (1980). This reaction was said to involve the formation of a structural isomer of the muconate, followed by the loss of Cl^- by anti-elimination of HCl from the unstable intermediate, to form butenolides.

In *Pseudomonas* sp. B13 grown of 3-chlorobenzoate, cycloisomerisation has the following sequence of events: 2-chloro-*cis, cis*-muconate is enzymatically transformed into 4-carboxychloromethylbut-2-en-4-olide (5-chloromuconolactone). However, this compound is unstable and is subject to spontaneous anti-elimination of HCl to give the *trans*-dienelactone (*trans*-4-carboxymethylenebut-2-en-4-olide). The enzyme involved, namely chloromuconate cycloisomerase, is said to catalyse the same reaction as its close relative, muconate cycloisomerase. They differ only with respect to their

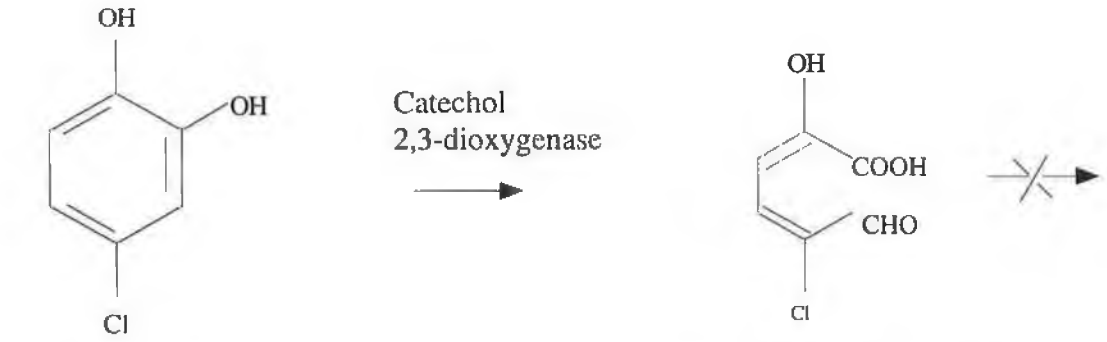
substrate specificities, the latter having higher activity with the unsubstituted substrate (Schmidt and Knackmuss, 1980).

More recent work by Vollmer *et al* (1994) casts doubt on the validity of the conclusions made by Schmidt and Knackmuss (1980) when 5-chloromuconolactone proved to be remarkably stable under acidic and neutral conditions. Furthermore, they argue that the evolution of chloromuconate cycloisomerases from ordinary muconate cycloisomerases was not just a question of altered substrate specificities but that new catalytic capabilities had to be acquired. They show that muconate cycloisomerase produces a mixture of 2- and 5-chloromuconolactone from 2-chloromuconate and that spontaneous elimination of HCl does not occur at a significant rate at physiological pH. No *trans*-dienelactone was detected.

The final steps must lead to normal metabolic intermediates if the compound is to be totally degraded. Hydrolase II catalyses the conversion of 4-carboxymethylenebut-2-en-4-olide to maleylacetate which is further metabolised via the TCA cycle, for the production of biomass, energy, carbon dioxide and water.

1. 2. 3. 4 Dead end pathways.

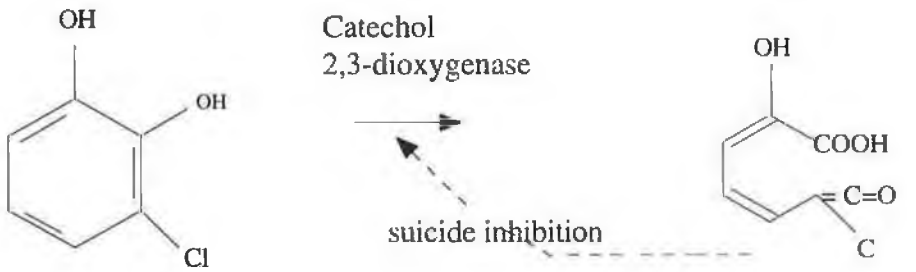
Ring cleavage, a crucial reaction in chlorophenol degradation , can be an *ortho* or *meta* reaction. The modified *ortho* pathway transforms chlorophenols into intermediates of the TCA cycle with the halide being returned to its mineral state. In the *meta* pathway, the chlorophenol proceeds along the metabolic route until it is converted to a molecule which acts as a specific enzyme inhibitor (Figs. 3 and 4). This so called "lethal synthesis" could result from competitive inhibition of an essential enzyme, removal of an essential cofactor from the enzymes active site, or from an irreversible type of inhibition (Bartels *et al*, 1984). The pathway of *meta* fission of chlorophenols is therefore, dead end, with dead end intermediates being accumulated in two ways depending on the position of the



4 chlorocatechol

5 chloro-2-hydroxymuconic semialdehyde

Fig. 3 The *meta*-cleavage of 4-chlorocatechol.



3-chlorocatechol

5 chloroformyl-2-hydroxypenta-2,4-dienoic acid

Fig. 4 The *meta*-cleavage of 3-chlorocatechol.

chlorine on the phenol: by production of an inhibitor of the *meta* cleaving enzyme (catechol 2,3-dioxygenase) or by production of a compound which cannot be further metabolised by the organism.

Chlorophenols are converted by hydroxylation to the corresponding chlorocatechols. These 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 and their black auto-oxidation products in culture supernatants (Bartels *et al*, 1984).

Work by Bartels *et al* (1984) demonstrated that 3-chlorocatechol was a suicide substrate of catechol 2, 3-dioxygenase, due to the formation of 5-chloroformyl-2-hydroxypenta-2, 4-dienoic acid as the actual deactivation species. This inactivation was oxygen dependent. The mechanism of inhibition by 3-chlorocatechol is due to the ability of the substrate to chelate the iron cofactor required for catalytic activity. Samples of inactivated enzyme could be fully reactivated by treatment with ferrous iron and a reducing agent.

During oxidation of 4-chlorocatechol by *Ps. putida* mt-2, 5-chloro-2-hydroxymuconic semialdehyde was produced (Reineke and Knackmuss, 1980). Confirmation of the structure of the *meta* cleavage product of 4-chlorocatechol has been supplied by Wieser *et al* (1994). This product cannot be further metabolised and accumulates in the culture medium, resulting in an intense yellow colour (Knackmuss, 1981).

The final outcome from the *meta* pathway, is the accumulation of chlorocatechol and its auto-oxidation products. The destruction of *meta* cleavage activity by the chlorocatechols and their inefficient oxidation by unmodified *ortho* enzymes explains the instability and dark colour of activated sludge suddenly loaded with chloroaromatics (Bartels *et al*, 1984).

Although plasmids such as the TOL plasmid have been implicated in the initial metabolism of chloroaromatics, few plasmids are known which allow complete degradation of such substrates. Examples include the plasmids pAC25 and pAC31 which encode a pathway for the complete degradation of chlorobenzoate, and pJP2 and pJP4 which encode for the degradation of 2, 4-dichlorophenoxyacetic acid and 3-chlorobenzoate. In nature several plasmids and their gene products may interact to allow the complete degradation of complex haloaromatic compounds. Chromosomally encoded enzymes such as those of the modified *ortho*-cleavage pathway also play a vital role (Karns *et al*, 1984).

1. 3 Micro-organisms reported to degrade phenol and mono-chlorophenols.

A wide variety of micro-organisms participate in the biodegradation of phenol and mono-chlorophenols. For convenience, these micro-organisms have been grouped into three classes: (i) bioaugmentation products, (ii) pure cultures and (iii) mixed cultures. While anaerobic degradation of these compounds has been reported, a review of this literature is beyond the scope of this study and therefore the following information is limited to aerobic degradation processes only.

1. 3. 1 Bioaugmentation products.

While literature is available on the application of various bioaugmentation strategies in wastewater treatment, independent reports on the capabilites of actual commercial bioaugmentation products are rare.

Three commercial products, Hydrobac, BI-CHEM DC-1006/7 and Liquid Live Microorganisms (LLMO) have been studied by Lewandowski *et al* (1986). These products were produced by Polybac Corporation, Sybron Corporation and General Environmental Sciences, respectively. Biodegradation rates for phenol and 2-chlorophenol were determined for the products. A mixed population from

a municipal wastewater treatment plant was found to perform better than all three bioaugmentation products. Improved performance of the municipal mixed culture was noted upon addition of the commercial products. The levels of product required for this enhancement however, were substantial and would not be economically feasible.

Phenol, at concentrations up to 600 and 1000mgl^{-1}, was degraded by the bioaugmentation products, Polybac and Phenobac, respectively (Lallai and Mura, 1989).

1. 3. 2 Pure cultures.

Well established enrichment and isolation techniques, as well as their ease of use for the generation of information on the physiological, biochemical, genetic and molecular aspects of microbial reactions, have resulted in numerous reports on the biodegradation of phenol and mono-chlorophenols by pure cultures.

Among the most widely studied catabolic pathways, are those found in the genus *Pseudomonas*. Pseudomonads appear to have a remarkable potential to evolve entire catabolic sequences for xenobiotic compounds. This evolutionary flexibility may reflect the diversity of enzymes and pathways that have evolved to degrade naturally occurring organic compounds and may also involve genetic rearrangements within and between strains (Clarke and Slater, 1986). A detailed description of the characteristics of members of this genus can be found in Bergey's Manual of Systematic Bacteriology (1984). These organisms are ubiquitous in natural habitats, being found in large numbers in all major natural environments, terrestrial, freshwater and marine, and also in association with plants and animals. Members of the genus *Pseudomonas* occupy a dominant position in the biosphere in terms of variety of habitat and the number of species in a given habitat (Clarke and Slater, 1986). They are renowned for their remarkable nutritional versatility. Organic compounds readily used by *Pseudomonas* species include alcohols, aliphatic acids, amides, amines, amino

acids, aromatic compounds, carbohydrates and hydrocarbons. The biodegradation of such compounds by *Pseudomonas* spp. has been studied at the biochemical and genetic level with a view to understanding and improving the degradation of these compounds in the environment. This ability to utilise a wide range of compounds makes *Pseudomonas* species an important component of activated sludge, particularly that treating wastewater from the chemical industry.

Several *Pseudomonas* species have been reported to degrade phenol. They include strains of *P. putida* (Feist and Hegeman, 1969, Bayly and Wigmore, 1973, Ritchie and Hill, 1995), *P. putida* EKII (Hinteregger *et al*, 1992) and *P. putida* F1 (Spain *et al*, 1989). Particularly high concentrations of phenol, up to $2gl^{-1}$, were degraded by the mutant strain *Pseudomonas pictorum* MU 174, compared to only $1.2gl^{-1}$ by the wild type strain (Chitra *et al*, 1995). Immobilisation has been shown to protect bacteria against the toxicity of phenol (Keweloh *et al*, 1989) and immobilised organisms therefore tend to tolerate even higher phenol concentrations. Immobilised *Pseudomonas* cells degraded phenol up to a concentration of $3gl^{-1}$, compared to $1.5gl^{-1}$ by the free cells (Bettman and Rehm, 1984). *Pseudomonas* cells, adsorbed an activated carbon, remained active following the addition of phenol concentrations up to $15gl^{-1}$ (Ehrhardt and Rehm, 1985). The added phenol disappeared rapidly by adsorption and degradation. Following depletion of the phenol in the medium, the cells continued to grow by degrading the adsorbed phenol. Several established wastewater treatment systems, such as biofilters and fluidised bed processes, utilise immobilised micro-organisms.

Pseudomonas sp. B13 is a typical example of a *Pseudomonas* species capable of mono-chlorophenol degradation (Knackmuss and Hellwig, 1978). The halocatechol-degrading capacity of this strain has been transferred to an *Alcaligenes* strain (Schwien and Schmidt, 1982). This constructed strain was found to be capable of growing on 2- and 4-chlorophenol, and also cometabolised 3-chlorophenol when pre-grown or growing on phenol.

Pseudomonas pickettii LD1 is reported to be the only strain capable of using all mono-chlorophenol isomers as sole carbon sources (Fava *et al*, 1995). Spain and Gibson (1988) studied mono-chlorophenol degradation by *Pseudomonas* sp. JS6 and *Pseudomonas putida* F1. It was suggested that phenol oxidation by the latter two species did not occur via the classical phenol hydroxylase system, as the activity was found to be closely correlated with the induction and activity of the broad spectrum toluene dioxygenase (Spain *et al*, 1989). Chlorophenol transformation, which takes place only in the presence of phenol, has been reported in *Pseudomonas putida* EKII (Hinteregger *et al*, 1992).

While reports on phenolic biodegradation by pseudomonads are numerous, other bacteria are also represented. For example, *Rhodococcus* sp. An117 and An213 (Janke *et al*, 1989), *Rhodococcus opacus* GM-14 (Zaitsev *et al*, 1995), *Arthrobacter* sp. (Bollag *et al*, 1968), *Azotobacter* sp. GP1 (Wieser *et al*, 1994) and the previously mentioned *Alcaligenes* sp. A7-2 (Menke and Rehm, 1992) are all involved in phenolic removal. The pathway of degradation of the phenolics is important in determining whether complete mineralisation of the pollutants is achieved (Section 1. 2. 3). While the *meta*-cleavage pathway of chlorophenol degradation has been suggested to be dead end, *Comamonas testosteroni* CPW301 has been reported to completely degrade 4-chlorophenol, with stoichiometric chloride release, via a *meta* pathway (Bae *et al*, 1996).

Strains of soil yeast, *Trichosporon cutaneum* and *Candida tropicalis*, can be induced to metabolise phenol (Mörtberg and Neujahr, 1985). Biodegradation of both phenol and mono-chlorophenols by a yeast strain of *Rhodotorula glutinis* has also been demonstrated (Katayama-Hirayama *et al*, 1994). The non-specific ligninolytic activities of some fungi, particularly white rot fungi, allows them to degrade even complex mixtures of pollutants. They have been implicated in the degradation of chlorinated compounds. *Phanerochaete chrysosporium* has been shown to degrade 2-chlorophenol (Lewandowski *et al*, 1990, Wang and Ruckenstein, 1994).

1. 3. 3 Mixed cultures.

In nature biodegradation is, in general, realised by microbial communities. Despite this fact, in depth studies of biodegradation by mixed cultures are less readily available compared to those using pure cultures.

The degradation of high concentrations of phenol by mixed cultures, originating from industrial wastes (Lee *et al*, 1992) and activated sludge (Kumaran and Paruchuri, 1997, Yoong *et al*, 1997, Magbanua *et al*, 1994) has been reported. Defined heterogeneous cultures have also been used. Simultaneous degradation of phenol and reduction of Cr(VI) has been observed by a coculture of the phenol-degrader *Pseudomonas putida* DMP-1 and *Escherichia coli* ATCC 33456 (Shen and Wang, 1995). The rate and extent of both phenol degradation and Cr(VI) reduction were significantly influenced by the population composition of the coculture. Phenol degradation by a defined mixed culture of the yeast *Cryptococcus elinovii* H1 and *Pseudomonas putida* P8 has also been observed (Mörsen and Rehm, 1990).

A synthetic sewage containing alkanols, acetone and phenol was readily degraded by a mixed culture of *P. extorquens* and *Alcaligenes* sp. A7 (Schmidt *et al*, 1983). While this community tolerated the presence of chlorophenol, the degradation of this compound was incomplete and resulted in the accumulation of dead end products. Successful chlorophenol degradation was achieved via *ortho*-cleavage however, following the addition of *Pseudomonas* sp. B13 to the community. Progressive replacement of *Pseudomonas* sp. B13 with the *Alcaligenes* strain was noted, following extensive exposure of the community to chlorophenols. The *Alcaligenes* strain acquired new capabilities. The new strain, *Alcaligenes* A7-2, had lost the ability to induce the unproductive *meta*-cleavage pathway and had emerged as the more competitive chlorophenol degrader. Goulding *et al* (1988) reported the degradation of a range of substituted

aromatic compounds by a defined mixed culture, which consisted of five pseudomonads, one klebsiella, four rhodococci and two fungal strains.

Chlorophenol degradation by undefined heterogeneous cultures has been widely documented. Activated sludge has been the source of inoculum for many authors including Ryding *et al* (1994), Ettala *et al* (1992), Sofer *et al* (1990), and Arbuckle and Kennedy (1989). The microbiological decontamination of chlorophenol-contaminated soil (Valo and Salkinoja-Salonen, 1986), groundwaters (Järvinen *et al*, 1994) and landfill leachate (McAllister *et al*, 1991) have also been reported.

The dynamic nature of mixed microbial populations, together with the vast myriad of interactions constantly occurring between its members, make mixed cultures less conducive to accurate and conclusive reporting.

1. 4 Mixed culture interactions.

Xenobiotics in natural ecosystems, and even in waste water treatment systems, are generally present in mixtures and they encounter mixed populations of organisms. Within such mixed populations, metabolic relationships and interactions between individual species are intrinsic. An account of such relationships within a stable community of *Pseudomonas stutzeri*, *Brevibacterium linens* and a *Curtobacterium* sp. growing on orcinol, was given by Slater (1978). Interactions among members of a community, rather than a physiological response of a single population, have been suggested to be a cause of acclimation (Wiggins and Alexander, 1988b).

Possible interactions between microbial populations can be classified as (a) negative interactions such as competition and amensalism, (b) positive interactions such as commensalism, synergism and mutualism, or (c) interactions that are positive for one but negative for an other population such as parasitism and predation. Within a complex natural biological community, all of these

possible interactions will probably occur between different populations (Atlas and Bartha, 1992b).

Commensalism

Commensalism is a unidirectional relationship in which only one population benefits. The unaffected population does not benefit from, nor is it negatively affected by the actions of the other population. The production of growth factors, the conversion of polymers into simpler compounds and the transformation of insoluble componds to soluble forms, can all form the basis of commensal relationships between microbial populations. Cometabolism may also form the basis of such a type of relationship. *Mycobacterium vaccae* is able to cometabolise cyclohexane while growing on propane (Beam and Perry, 1974). The cyclohexane is oxidised to cyclohexanol which can support the growth of other populations. These bacterial populations benefit because they are unable to utilise intact cyclohexane; the *Mycobacterium* is unaffected as it does not utilise the cyclohexane. In a study by Bouchez *et al* (1995) growth of a *Pseudomonas* sp. designated *S Phe Na 1* on phenanthrene was inhibited in the presence of naphthalene. Addition of a second strain, *Pseudomonas stutzeri S Nap Ka 1* which was capable of growth on naphthalene, relieved the inhibition of phenanthrene degradation.

Synergism

A relationship of synergism benefits all populations. Synergism allows microbial populations to perform activities, such as the synthesis of a product, that neither population could perform alone. The synergistic activities of two microbial populations may allow completion of a metabolic pathway that otherwise could not be completed.

Mutualism

Mutualistic associations are similar to synergistic ones in that both populations benefit however, mutualism is an obligatory and highly specific relationship. The relationships between certain algae or cyanobacteria and fungi that result in

lichens are examples of mutualism. One member produces organic compounds that are utilised by its associate, who provides a form of protection and mineral nutrient transport in return. Many biodegradation processes require the cooperation of more than a single species. For example, cyclohexane can be degraded by a mixed population of a *Nocardia* sp. and a *Pseudomonas* sp., but not by either population alone (Slater, 1978). The relationship was based on the ability of the *Nocardia* to metabolise cyclohexane, forming products that were utilised by the *Pseudomonas* species, which in turn produced biotin and growth factors required by the *Nocardia* species. In a study by Wijngaard *et al* (1993) the cultivation of *Xanthobacter autotrophicus* GJ10 in the absence of biotin led to the accumulation of toxic compounds from 1, 2-dichloroethane (DCE). Inoculation with *Pseudomonas* sp. GJ1 fulfilled the biotin requirement of strain GJ10, resulted in degradation of the accumulated toxic compounds and the mixed culture grew stably on DCE.

Competition

Competition is a negative relationship between populations which, for example, utilise the same resource, whether it is space or a limiting nutrient. Available sources of carbon, nitrogen, phosphate, oxygen, growth factors and water are all resources for which micro-organisms may compete. Intrinsic growth rates of competing populations vary under different environmental conditions, explaining the coexistence of populations in the same habitat competing for the same resources. For example, bacterial populations may be excluded from a given habitat when temperature or other environmental factors prevent them from competing successfuly with other populations because of their lower intrinsic growth rates. Under different environmental conditions, these same populations may compete successfully and even dominate.

An understanding of the basis of competitive behaviour is particularly relevant to the biological treatment of wastewater. The use of specific strains in such processes requires knowledge of ways to stimulate the growth of desirable organisms over those with less favourable properties and therefore factors that

determine competitive behaviour are important. Benzylamine mineralisation by *Pseudomonas putida* proceeded in the presence of 100nM phosphorus but not in its absence (Steffensen and Alexander, 1995). Enhancement due to phosphorus addition did not occur however, if the medium also contained caprolactum and a caprolactum-utilising *Pseudomonas aeruginosa*. The effect of one biodegradable substrate on the metabolism of a second was suggested to result from competition between the micro-organisms for inorganic nutrients. Such observations are particularly relevant as biodegradation often occurs in environments containing several substrates and frequently where the supply of one or more inorganic nutrients is less than the demand. Competition for low concentrations of nitrogen and phosphorus has been suggested as a reason for the failure of inoculation in natural ecosystems (Alexander, 1994a). Low concentrations of the target compound result in slow growth of the organism and hinder its effectiveness as a competitor against its rapidly growing neighbours. Competition among indigenous and inoculated 2, 4-D degraders in soil has been studied by Ka *et al* (1994). Factors such as lag time and the presence of a plasmid which encodes for the 2, 4-D pathway, were deemed important in determining the outcome of competitive interactions. A kinetic model which describes competition between two microbial populations for phenol has been described by Dikshitulu *et al* (1993). The authors demonstrated the importance of population dynamics in the scale-up of biodegradation data. The model predicted that the kinetics of biodegradation and the conditions used in the sequencing fed-batch reactor could determine whether neither species, only one species or both species could survive.

Amensalism

Amensalism, or antagonism, is a negative interaction where one population gains the competitive advantage by its production of a substance that is inhibitory to other populations. The production of such inhibitory compounds may allow the producer to modify a given habitat so as to preclude the growth of other populations. Lactic acid production or the consumption/production of oxygen, are examples of activities that are detrimental to certain microbial populations

and consequently allow the proliferation of others. The presence of toxins in soils and waters can affect the survival of a species introduced into an environment in which it is not native (Alexander, 1994a) and can result in the failure of an introduced population to function as desired.

Parasitism

In a host-parasite relationship, the population that benefits, the parasite, normally derives its nutritional requirements from the population that is harmed, the host. Viruses are obligate parasites of bacterial, fungal and other microbial populations which exhibit host cell specificity. Protozoan populations are subject to parasitism by a large number of fungi, bacteria and other protozoa. Such parasitic interactions cause a reduction in host population density, thus providing a means for population control and allowing the renewal of environmental resources that were being utilised by the host. As parasitism is dependent on the density of the host population, a decline in host population also causes the parasite population to diminish, which in turn permits the recovery of the host. Parasitism is therefore a mechanism of negative feedback control, which keeps growth of the host in check and therefore prevents the exhaustion of resources which would lead to possible extinction of the organisms.

Predation

Predation typically occurs when one organism, the predator, engulfs and digests another organism, the prey. Moderate predatory pressure keeps the prey populations from exhausting the carrying capacity of their environment and maintains the prey population in a dynamic state of growth. Coexistence of predator and prey species can occur with periodic population oscillations if the prey species can escape predation pressure and recover.

A number of natural environments and wastewater treatment systems are inhabited by active protozoan populations which prey on the bacterial populations in these environments. Protozoa are important predators of bacteria in activated sludge. Such protozoan grazing has been suggested to account for

the acclimation period prior to the mineralisation of certain organic compounds in sewage. Predation may reduce the number that can mineralise a target chemical and thus delay the onset of rapid degradation until the number of all bacteria becomes low and the intensity of grazing diminishes. In a study by Wiggins and Alexander (1988b), protozoan consumption of bacteria was found to be directly related to the duration of acclimation. The suppression of protozoan populations, by the addition of inhibitors selctive for eucaryotes, resulted in a marked reduction in the acclimation period normally observed prior to 4-nitrophenol mineralisation in sewage. Acclimation is less likely to be attributed to protozoa in environments in which predation is not marked, such as natural waters (Wiggins and Alexander, 1987). The use of small inoculum sizes has been suggested as a reason for the failure of inocula to function as required, as inocula may be eliminated by protozoan grazing (Ramadan *et al*, 1990).

1. 5 The influence of acclimation on microbial transformations.

An important factor, when considering the biodegradation of a recalcitrant compound, is acclimation. It has been defined as the length of time between the addition or entry of a chemical into an environment, and evidence of its detectable loss (Alexander, 1994c). The length of time required for acclimation to various pollutants can range from a few days to several months. As there is little or no mineralisation during acclimation, it is important to minimise the time required. The length of the acclimation period can be affected by the presence of a secondary carbon source or the rarity of the microbes that can degrade certain chemicals. Studies on the adaptation of sludge to degrade *m*-chlorobenzoate or *p*-chlorophenol by Haller (1978) however, concluded that the presence or absence of an easily metabolised carbon source, did not affect the length of time required for the sludge to adapt. The presence of inhibitory compounds or a high enough concentration of a chemical to inhibit those microbes capable of metabolising it, will also affect the time needed for acclimation (Wiggins and Alexander, 1988a). As mentioned earlier in another report by Wiggins and

Alexander (1988b), interactions among community members, such as protozoan consumption of bacteria, may be a further cause of acclimation.

Acclimation includes such processes as selection and adaptation by which a mixed population of micro-organisms develops the ability to degrade a substance, hitherto non-biodegradable. It also covers the situation in which the population develops tolerances to inhibitory substances (Painter and King, 1985). During acclimation by selective enrichment the numbers or proportion of micro-organisms, that can utilise the compound of interest, increase within the community. Adaptation can be either phenotypic or genotypic. In phenotypic adaptation, the genetic information of the cell remains unchanged but the degree of expression of certain genes is altered, with the induction and/or depression of certain enzymes. This may occur during short term fluctuations in the environment. Genetic adaptation involves changes in the genetic information of the cell and two basic methods have been identified: mutation and recombination. Mutation is a change in the nucleotide sequence of the DNA occurring within an individual cell. Recombination is any process whereby genes from two separate cells are brought together into the one cell (e.g. transformation, conjugation, transduction and genetic engineering). Genotypic adaptation may be common during chronic changes in the environment (Senthilnathan and Ganczarzyk, 1989). Plasmid DNA may play a particularly important role in genetic adaptation, in that it represents a highly mobile form of DNA which can be transferred via conjugation or transformation and can impart novel phenotypes to recipient organisms (Leahy and Colwell, 1990). When acclimated organisms are subjected to a different environment, they tend to adapt to the new situation and may lose the ability to grow at the expense of the original substrate. Phenotypic adaptations are temporary and therefore more rapidly lost than genotypic adaptations. This process of adaptation and deadaptation of biomass may influence the biodegradability of organic pollutants which are discharged intermittently (Senthilnathan and Ganczarzyk, 1989). For those industries that produce a variety of products on staggerred schedules, it has been suggested that

a background level of the pollutant in the influent would help to maintain the bacterial population responsible for its mineralisation (Eckenfelder, 1989).

The type of organic materials to which a microbial community has been exposed will play an important role in determining the response of the community to a new compound. In most environments, the organic exposure history of aquatic microbial communities is dominated by naturally occurring substrates, namely amino acids, carbohydrates and fatty acids. Shimp and Pfaender (1985) examined how adaptation to these three substrate classes, affects the ability of the adapted bacteria to degrade *m*-cresol, *m*-aminophenol and *p*-chlorophenol. Enhanced biodegradation of the monosubstituted phenols was observed, following adaptation of the community to increasing concentrations of amino acids, carbohydrates or fatty acids. It was concluded that both the type and concentration of natural materials in the environment can influence the response of the community to new compounds, despite a lack of any structural or biochemical similarity between the new molecule and the natural substrates.

Shimp and Pfaender (1987) reported that the adaptation of a mixed aquatic microbial community to phenol, resulted in adaptation of the community to the structurally related aromatic compounds *m*-cresol, *m*-aminophenol and *p*-chlorophenol. The ability of phenol to affect the biodegradation of related phenolic compounds, might be surmised from their structural similarity, common elements within their biodegradation pathways, or similar cell membrane transport systems. This response may be important when considering the fate of complex mixtures containing a variety of structurally related chemicals.

1. 6 The role of nutrients.

Biochemical studies of the degradation of aromatic compounds by micro-organisms have traditionally involved the presence of the pollutant as a single carbon source. In nature however, mixtures of potential growth substrates occur

and the effects of their presence must be considered, if any application to waste treatment is to be successful.

1. 6. 1 Readily metabolised carbon sources.

The presence of additional readily metabolised carbon sources may be beneficial or detrimental to the degradation of a target hazardous compound. In some cases, additional substrates have enhanced cell growth or adaptation to the utilisation of the target compound, thus improving system performance (Kim and Maier, 1986, Rittman, 1987, Shimp and Pfaender, 1985). However, the converse may also occur where suppression may result from toxicity of a second compound or from competition of the populations for a limiting factor such as phosphorus or oxygen (Alexander, 1994a). The presence of alternative substrates has inhibited or repressed the biodegradation pathway or led to the selection of strains that lack the necessary pathways for degradation (Daugherty and Karel, 1994, Goulding *et al*, 1988, Lu and Speitel, 1991, Møller *et al*, 1995, Schmidt and Alexander, 1985).

If two organic compounds can each serve as sole carbon and energy sources for growth of a bacterial culture, these nutrients are said to be substitutable. Machado and Grady (1989) examined whether concentrations of two substitutable substrates, lysine and 2-chlorophenol, in a chemostat would respond in a systematic manner to their concentration in the feed, when the reactor was operated at a dilution rate low enough to allow their concurrent utilisation. A pure bacterial culture capable of utilising either substrate as a sole carbon source was employed and its response to dual substrate limitation by the two compounds was examined. The effect of two independent variables, dilution rate and feed composition, on lysine concentration was more uniform and consistent than effects on the 2-chlorophenol concentration. Degradation was increased when the feed contained equal quantities of the two substrates or a predominance of 2-chlorophenol. The results observed could not be explained by existing models for dual substitutable substrates. The concentration of the two

substrates in the effluent responded to the two independent variables, in a complex manner which suggests that no generalisations can be made about the behaviour of individual substrates in multicomponent mixtures.

Using nutrient broth as the alternate substrate, Kim and Maier (1986) studied its effect on the acclimation to and the biodegradation of 2,4-D, by activated sludge micro-organisms. The presence of nutrient broth had no effect on the duration of the lag period, however, substrate utilisation by the acclimated culture was enhanced. Both substrates were utilised concurrently.

In a kinetic study using a 2,4-D acclimated culture, consisting of predominantly *Pseudomonas* species, the utilisation of 2,4-D was seen to be inhibited by glucose and vice versa (Papanastasiou and Maier, 1982). However, the larger concentrations of active biomass produced when glucose was available, masked the inhibitory effect on 2,4-D removal. Their concurrent utilisation resulted in an overall increase in the rate of target substrate utilisation and hence a positive result of the presence of glucose.

The presence of a secondary substrate can have significance in practical problems such as groundwater contamination, where the pollutant is present in high enough concentrations to cause concern for consumers, but too low to support growth of any bacteria capable of transforming it. The consumption of the xenobiotic, methylene chloride, present in trace concentrations was possible in the presence of relatively high concentrations of acetate to serve as the primary substrate for growth and maintenance (LaPat-Polasko *et al*, 1984). The rate of methylene chloride degradation was enhanced in the presence of acetate but the presence of the xenobiotic had a negative effect on acetate utilisation. A *Pseudomonas* strain LP was responsible for this transformation.

Another study on the effect of nutrient supply on the mineralisation of phenol in trace concentrations by a natural aquatic microbial community, revealed a carbon source-dependent effect (Rubin and Alexander, 1983). Enhanced phenol

mineralisation was observed in the presence of arginine. In contrast, adenine and glucose did not shorten the lag phase and reduced the subsequent removal rate. Propionate almost completely suppressed mineralisation.

A population of activated sludge micro-organisms acclimated to phenol showed an initial preference for phenol when presented with a mixed phenol/glucose substrate (Rozich and Colvin, 1986). The phenol removal rates were hindered by the presence of the glucose, and the cells, if given sufficient time, switched their substrate preference to the carbon source that could permit more rapid growth i.e. glucose.

A comparison of removal rates of 3-chlorobenzoic acid in minimal and complex media by a microbial mixture has been reported (Goulding *et al*, 1988). Degradation rates were decreased in the presence of the easily metabolisable substrates, but total degradation was achieved.

Studies on the biodegradation of organic compounds at low concentrations, by *Pseudomonas acidovorans*, revealed the inhibition of phenol removal by as little as 70µg acetate per litre (Schmidt and Alexander, 1985).

Styrene metabolism by *Pseudomonas putida* CA-3 was repressed in the presence of nonaromatic carbon sources such as glutamate and citrate (O'Connor *et al*, 1995).

The effectiveness of bioaugmentation is dependent on a number of factors including the presence of easily metabolisable carbon sources. In a study of the clean up of oil polluted soil with a commercial bioaugmentation product, the product formulation was deemed responsible for the inhibition of degradation observed with increasing amounts of added product (Møller *et al*, 1995). The product contained micro-organisms and small amounts of surfactants on a starch-based carrier material. Despite claims of adaptation to grow on various petroleum products, the micro-organisms added to the soil failed to degrade the

oil. The excessive mineralisation rate observed at the highest concentration of bioaugmentation product added (100mg/g soil), was explained by the degradation of the product itself, the starch-based carrier being a preferred source of carbon. The depletion of essential nutrients during the growth, together with O_2 consumption leading to partial anaerobiosis, were suggested to contribute to the inability of the introduced micro-organisms to express their oil degrading capacity. This clearly emphasises the role that readily available carbon plays in yet another aspect of wastewater treatment.

Research has been carried out by Lu and Speitel (1991) on the influence of naturally occurring organic matter (NOM) on the biodegradation of pentachlorophenol (PCP) in columns initially inoculated with a mixed culture acclimated to PCP. The results indicated that the presence of the NOM interfered with PCP degradation. Among the explanations given were the preferential utilisation of NOM, and the displacement of the PCP degraders. The extent of the interference could be diminished by pre-treatment of the influent water with ozone and/or chloramines. Ozone increased the biodegradability of NOM through the production of smaller molecules and incorporation of oxygen into the chemical structure. Chloramines reacted with the NOM to produce chlorinated organic chemicals, which may be thought of as increasing the structural similarity between NOM and PCP.

A detailed molecular study of carbon source-dependent repression at the level of expression of the *xyl* catabolic operons of the *Pseudomonas putida* TOL plasmid, was published by Holtel *et al* (1994). Two *xyl* promoters Pu and Ps were identified as the primary targets of the inhibition. Succinate, citrate, pyruvate, glycerol, fructose and arabinose allowed activation of Pu, while glucose, gluconate, lactate and acetate inhibited its activation. After elimination of the classical system of enteric catabolite repression involving cyclic AMP as the signal for glucose starvation, a number of mechanisms that could possibly bring about the observed effect, were suggested. These mechanisms range from changes in promoter DNA supercoiling which may affect gene expression in

response to nutritional changes, to the possibility that the promoters require the specific RNA polymerase subunit σ^{54} for transcription (Assinder and Williams, 1990). It may thus be that σ^{54} -dependent promoters, either directly or indirectly, are negatively affected in the presence of repressive carbon sources.

Studies on the ability of *Pseudomonas aeruginosa* to preferentially metabolise one carbon source over another, have led to the cloning of a catabolic repression control (CRC) gene (Mac Gregor *et al*, 1991). The gene product is a 30K protein with an expected cytoplasmic location. Its lack of regulation by cAMP and glucose is consistent with the opinion that the molecular mechanism of CRC in *Pseudomonas aeruginosa* is fundamentally different from that in *E. coli* and other enteric bacteria.

The molecular mechanism of catabolite repression of phenol degradation in *Pseudomonas putida* strain H has been reported (Müller *et al*, 1996). The level of activity of phenol catabolic enzymes was decreased in the presence of organic acids and carbohydrates. The genes encoding the catabolic enzymes were contained within a single operon on plasmid pPGH1. Transcription of this operon was activated by the product of *phl*R in the presence of phenol. The authors suggested that PhlR protein was the target for control, and that carbon catabolite repression of phenol degradation was mediated by a negative controlling factor which inhibited the *phl* gene-specific activator PhlR. Inhibition of PhlR, and the resulting lack of transcription of the catabolite operon, was cited as the dominant mechanism of catabolite repression of phenol degradation.

1. 6. 2 Cometabolism.

Numerous micro-organisms are able to oxidise organic substrates without using the compound as a source of energy or of one of the elements necessary for growth. Even a substrate that will, in nature, support growth of micro-organisms may be metabolised by some bacteria in culture, with no incorporation of the C

into their cells (Alexander, 1994b, Schmitt *et al*, 1992). Cometabolism is the term used to describe this type of metabolism, and is an important example of the influence that readily degradable secondary carbon substrates can have on the biodegradation of pollutants, as the secondary carbon source is thought to provide the carbon and energy required for the alteration of the parent compound. The term however, does not infer the presence or absence of growth substrate during the oxidation (Horvath, 1972). Cometabolism does not include any mineralisation of the parent compound, only an alteration in structure to some intermediate. However, in a mixed culture situation, the cometabolic product may be completely utilised by other bacteria which cannot use the intact parent molecule (Shimp and Pfaender, 1985).

The introduction of certain kinds of substituents into aromatic compounds capable of supporting microbial growth, has resulted in an increased resistance of the molecule to degradation. The degree of inhibition of oxygenation by substituted substrate analogues, depends on the character and position of the substituents (Reineke and Knackmuss, 1978). The substituted compound often fails to support microbial growth, yet they can be partially metabolised. This partial metabolism is often carried out by an enzyme, of relaxed specificity, found in the initial step of the degradation pathway of the unsubstituted compound. However, subsequent enzymes are often more dedicated and an accumulation of the metabolite containing the substituent can occur (Horvath and Alexander, 1970(b)). Accumulation of a metabolite, characteristic of cometabolism, could be due to effetcs other than tight substrate specificity. The metabolite itself might be an enzyme inhibitor or, when acted upon by the next enzyme of the sequence, might give rise to a potent "suicide inhibitor". The fact that growth is not sustained by the reactions that formed the metabolite, may be ascribed to the fact that processes available for harnessing catabolic energy (substrate-level phosphorylation and electron-transport phosphorylation) are strictly limited. Hydrolases, hydratases and hydroxylases are examples of catabolic enzymes that function simply to modify structures, so that substrates are provided for subsequent ATP-generating processes. When they act on analogues of their

natural substrates, it is little wonder that they give rise to products that accumulate and are useless to the micro-organisms that attack them (Dagley, 1989).

Pseudomonas sp. B13 was shown to utilise 4-chlorophenol (up to 0.15mM) as the only carbon source. These cells contained high levels of a 1,2-dioxygenase activity for chlorocatechols, an activity crucial for the catabolism of haloaromatics (Reineke and Knackmuss, 1978). Phenol, a growth substrate for strain B13, was only cometabolised by the chlorophenol-adapted cells leading to an accumulation of *cis*, *cis*-muconic acid. This was also seen with 3-chlorobenzoate grown cells, which co-oxidised benzoate to muconic acid. In contrast to the 4-chlorophenol grown cells, phenol grown cells of strain B13 demonstrated incomplete degradation of chlorophenols, with the production of 3- and 4-chlorocatechols. 4-chlorophenol-grown *Pseudomonas* sp. B13 readily degraded 2-, 3- and 4-cresol, while cometabolism of these substrates by the phenol grown cells produced methylcatechols (Knackmuss and Hellwig, 1978).

Pseudomonas putida, when supplemented with chlorobenzene as the sole carbon source, was unable to use this compound for growth but, had the ability to co-oxidise chlorobenzene to 3-chlorocatechol. Further metabolism of 3-chlorocatechol did not occur due to the chelation by the halocatechol of the iron cofactor necessary for activity of the ring fission enzyme (Klecka and Gibson, 1981).

Similarly, an *Arthrobacter* sp. cometabolised 3-chlorobenzoate to 4-chlorocatechol, using an enzyme system also involved in the oxidation of benzoate (Horvath and Alexander, 1970(b)). Furthermore, this report demonstrated that cometabolism was carried out by a number of bacterial genera known to be widespread in nature, including strains of *Bacillus*, *Flavobacterium*, *Micrococcus* and *Xanthomonas*.

As a result of cometabolism, 2,3,6-trichlorobenzoate was converted to 3,5-dichlorocatechol, by a benzoate grown *Brevibacterium* sp. (Horvath and Alexander, 1970(a)).

In the end, cometabolism is merely a special type of microbial transformation with two, immediately evident, environmental consequences. Firstly, cometabolism of synthetic chemicals present in soils and waters, will only be carried out by a limited population of organisms; the transformation will be slow and the rate of conversion will not increase with time. This is in contrast with chemicals used as carbon and energy sources. Secondly, due to the nature of the cometabolic reaction, the accumulation of products results, and these products often tend to persist. This may be overcome if a second species is present that can mineralise the metabolite and is therefore, dependent on the characteristics of the local environment.

A strain of *Enterobacter cloacae* anaerobically dechlorinated the pesticide DDT and formed 4,4'-dichlorodiphenylmethane (DDM) as it grew on and fermented lactose. The coimmobilisation of this bacteria and an *Alcaligenes* sp., that grows aerobically on diphenylmethane but cometabolises DDM, in Ca-alginate was reported by Beunink and Rehm (1988). This partnership provided a means for the synchronous anaerobic and aerobic degradation of the persistent pesticide, DDT, in one reactor vessel.

In recent times, treatment systems have been designed which exploit biodegradation by cometabolising micro-organisms. Alvarez-Cohen and McCarty (1991) designed a two stage bioreactor for trichloroethylene (TCE) transformation, where methane was used for cell growth in one reactor, followed by transfer of the cells to a second reactor where TCE was transformed. Since methanotrophic TCE transformation and methane oxidation both required the same key enzyme, competitive inhibition was avoided by separation of the stages. Another procedure involved an expanded bed consisting of crushed glass on which the microbial consortia grew. The reactor was supplied with a solution of

inorganic nutrients, TCE, either methane or propane as sources of carbon and energy, and the bacteria. These micro-organisms degraded not only TCE, but also vinyl chloride, benzene and toluene (Phelps *et al*, 1991).

Given the biochemical potential of cometabolic processes, additional development is needed to convert many microbiological studiees of cometabolic transformations, into practical methods for bioremediation.

1. 6. 3 Inorganic nutrients.

The availability of inorganic nutrients, such as nitrogen and phosphorus, may also affect the biodegradation of a compound. It has been reported that the addition of inorganic nutrients substantially reduced the acclimation period prior to biodegradation in fresh water (Swindoll *et al*, 1988). The rates of growth, biodegradation and other microbial processes can be influenced by the availability of inorganic nutrients. The addition of phosphorus (P) to lake water markedly enhanced the mineralisation of *p*-nitrophenol (PNP) by *Flavobacterium* sp. (Jones and Alexander, 1988), *P. cepacia* (Ramadan *et al*, 1990) and a *Cornyebacterium* sp. (Zaidi *et al*, 1988). Kuiper and Hanstveit (1984) observed that the biodegradation rates of 4-chlorophenol and 2, 4-dichlorophenol in samples of marine water could be limited by lack of inorganic nutrients.

With the presence, in many environments, of several metabolisable organic substrates and frequently a limited supply of inorganic nutrients, a study was undertaken to determine the effect of one species acting on a substrate, on the biodegradation of a second substrate by a second species at low phosphate concentrations (Steffensen and Alexander, 1995). *Pseudomonas aeruginosa*, a caprolactam degrader and a benzylamine-degrading *Pseudomonas putida* were used in these studies. At limiting P levels, growth of the second species on a compound not used by the first, resulted in a reduction in degradation of the first compound. The decreased degradation appeared to result from competition between the two species for P present in limiting concentrations. In pure culture,

both bacterial populations showed an enhanced mineralisation of their respective substrate following P addition.

The presence of iron (Fe) and calcium (Ca) can affect the availability of phosphate for microbial degradation of organic chemicals (Robertson and Alexander, 1992). 10mM phosphate was shown to increase glucose mineralisation by a *Pseudomonas* sp. and PNP or phenol removal by a *Cornyebacterium* sp. However, the extent of mineralisation was markedly less in P-amended media containing 5mM Ca than in media with lower Ca levels. Fe levels of 0.1mM and 0.2mM reduced the population sizes considerably.

In a study on the degradation of carbon tetrachloride (CT) by Tatara *et al* (1993), results indicated that the transformation was linked to the iron-scavenging functions of *Pseudomonas* sp. strain KC. Addition of iron inhibited CT transformation possibly by competing for a binding site. A copper requirement was noted when omission of only 1µM copper was sufficient to prevent CT transformation.

1. 7 Project Objectives.

In this project, the degradative capabilities of a commercial bioaugmentation product were investigated. The product, Biolyte HAB (Haloaromatic Biodegradation), produced by International Biochemicals Ltd., Ireland, was designed to provide a selection of organisms able to degrade a range of substituted aromatic compounds. The product was designed for use in conventional aerobic waste water treatment plants or in the bioremediation of contaminated soils. The formulation consisted of a minimum of eleven strains of micro-organisms including *Pseudomonads*, *Actinomycetes* and a fungus. The micro-organisms were said to have the capability of degrading chlorosubstituted benzenes, polychlorinated biphenyls and structurally related compounds. They

were grown in pure culture, harvested and preserved by freeze-drying before being blended in the final formulation (manufacturers' literature).

Preliminary studies in the laboratory had suggested that the ability of the product to degrade chlorophenols was limited and that the product formulation could under certain circumstances adversely affect the degradative capabilities of the mixed culture. Therefore, this project was designed to study the ability of the mixed culture to degrade phenol and mono-chlorophenols with particular emphasis on the nutritional status and the microbiology of the system.

2. Materials and methods.

2. 1 Materials.

2. 1. 1 Biolyte HAB.

The bioaugmentation product, Biolyte HAB, was obtained from International Biochemicals Ltd., Sandyford Industrial Estate, Dublin 18. It consisted of a minimum of eleven strains of micro-organisms. These included the genera *Pseudomonas* and *Actinomyces*. A species of brown rot fungus, surfactants, cryo-protectants and growth media were also present along with the sterile cereal base. The product was specially formulated to degrade a range of substituted aromatic compounds.

2. 1. 2 Media.

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

Chlorophenol Agar

Pseudomonas minimal medium was prepared as outlined later. Bacteriological agar, at a concentration of 1%(w/v), was added to the minimal media. Following sterilisation, the agar was allowed to cool. Immediately prior to pouring, chlorophenol was added to the agar to give the appropriate final concentration.

Gelatin Agar

Gelatin was added to nutrient agar before sterilisation to a final concentration of 0.4%(w/v) as outlined in Harrigan and Mc Cance (1976).

Hugh and Leifsons' Medium

The medium was prepared as outlined in Harrigan and Mc Cance (1976). All the ingredients except Bromothymol Blue were dissolved in distilled water. The pH was adjusted to 7.1 with 2M NaOH. The indicator dye was then added. The medium was

dispensed into test tubes in 10ml aliqouts. Following sterilisation, the tubes were rapidly cooled in an iced water bath and inoculated immediately.

	$g.l^{-1}$
Peptone	2.0
NaCl	5.0
K_2HPO_4	0.3
Glucose	10.0
Agar	3.0
Bromothymol Blue (1% w/v aq.soln.)	3.0ml
pH 7.1	

Luria-Bertani Broth and Luria Broth

Luria-Bertani (LB) broth and Luria broth were prepared by combining the ingredients in distilled water, as outlined in Maniatis *et al.* (1982).

LB Broth	$g.l^{-1}$	*Luria Broth*	$g.l^{-1}$
Tryptone	10.0	Tryptone	10.0
Yeast Extract	5.0	Yeast Extract	5.0
NaCl	10.0	NaCl	5.0
pH 7.0		Glucose	1.0
		pH 7.0	

Malt Extract/Chloramphenicol Agar

The malt extract agar was prepared according to the manufacturers' instructions and sterilised by autoclaving at 110°C for 10 minutes. Following sterilisation, the medium was allowed to cool. Chloramphenicol, at a concentration of $1gl^{-1}$, was added to the malt extract agar immediately before pouring. Thorough mixing was necessary for dissolution of the antibiotic throughout the agar.

***Pseudomonas* Minimal Medium**

The ingredients of the medium (Goulding *et al*, 1988) were combined in distilled water and the pH was adjusted to 7.0 with 2M NaOH. The trace salts solution was prepared separately in distilled water and was stored in a dark bottle for 6-8 weeks.

	$g.l^{-1}$
K_2HPO_4	4.36
NaH_2PO_4	3.45
NH_4Cl	1.0
$MgSO_4.6H_2O$	0.912
pH 7.0	

Trace salts solution was added at a concentration of 1ml l^{-1}.

Trace Salts Solution	$g.100ml^{-1}$
$CaCl_2.2H_2O$	4.77
$FeSO_4.7H_2O$	0.37
$CoCl_2.6H_2O$	0.37
$MnCl_2$	0.10
$NaMoO_4$	0.02

Tween 80 Agar

The ingredients were dissolved in distilled water as outlined in Harrigan and Mc Cance (1976) and the pH was adjusted, if necessary, to pH 7.0 - 7.4.

	$g.l^{-1}$
Peptone	10.0
$CaCl_2.H_2O$	0.1
NaCl	5.0
Tween 80	10.0
Agar	15.0
pH 7.0-7.4	

2. 1. 3 Buffers

Citric acid - Na_2HPO_4 (for pH studies)

This wide range buffer [pH 2.6 - 8.0] was prepared by combining x ml of 0.1M citric acid and (100-x) ml 0.2M Na_2HPO_4. The pH of the resulting solution will be dependent on the amount of citric acid added, for example:

x (ml)	81.1	49.0	17.8	2.8
pH	3.0	5.0	7.0	8.0

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.

Phosphate Buffer, Na_2HPO_4 - NaH_2PO_4 (for pH studies)

This buffer [pH 5.8 - 8.0] was prepared by adding x ml 0.2 M Na_2HPO_4 and (50 - x) ml 0.2M NaH_2PO_4 and diluting to 100ml. The pH of the resulting solution will be dependent on the magnitude of x, for example:

x (ml)	4.0	13.25	30.5	47.35
pH	5.8	6.4	7.0	8.0

Potassium Phosphate Buffer

Potassium phosphate buffer was prepared as outlined in Standard Methods for the Examination of Water and Wastewater (1985). The ingredients were combined in distilled water. The pH of the resulting solution should be 6.8.

	$g.l^{-1}$
K_2HPO_4	104.5
KH_2PO_4	72.3

Sodium Phosphate Buffer, NaH_2PO_4 - NaOH (for pH studies)

Sodium phosphate buffer [pH 5.8 - 8.0]was prepared by adding x ml of NaOH (0.2M) to 50 ml of NaH_2PO_4 (0.2M) and diluting to 100 ml. The pH of the resulting solution will be dependent on the amount of NaOH added, for example:

x (ml)	3.5	13	30	47
pH	5.8	6.4	7.0	8.0

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 should be 7.0.

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 was monitored turbidimetrically at 660nm using a Unicam 8625 UV/VIS spectrometer. Biomass values were determined from a calibration curve relating mg dry weight cells per ml to O. D. units.

2. 2. 2 Measurement of pH.

The pH was measured using an Orion 920A pH meter.

2. 2. 3 Addition of aromatic substrates.

m-toluic acid was added to the medium, prior to sterilisation, at concentrations of 5, 10 and 20mM. The same concentrations of benzoic acid and naphthalene were used.

These substrates were added following sterilisation of the media. Phenol was added to the medium before sterilisation. Phenol concentrations ranging from 200mgl^{-1} to 1500 mgl^{-1} were used. The mono-chlorophenols were added to the *Pseudomonas* minimal medium after sterilisation. A range of concentrations of chlorophenol, from 50 mgl^{-1} to 1500 mgl^{-1}, were used.

2. 2. 4 Resuscitation of freeze-dried micro-organisms of Biolyte HAB.

The microbial mixture (10g) was resuscitated by shaking in dilution buffer (90ml) at 30°C for 1 hour. The dilution buffer was prepared by adding 1ml of a 10% triton-X-100 solution and 1.25ml of 0.01M K_2HPO_4 (pH 7.2) to 1 litre of distilled water. Following agitation, the cereal base carrier was allowed to settle for 5 minutes. A washed and an unwashed form of the supernatant were used. The washing procedure was carried out as follows; the supernatant fluid was centrifuged at 5,000 rpm for 10 minutes; the pellet was then washed with 0.01M sodium phosphate buffer (pH7.0) and finally resuspended in the same volume of this buffer before inoculation. In the case of the unwashed inoculum, the unmodified supernatant was added directly to the minimal medium.

2. 2. 5 Growth conditions and biodegradation studies.

Biodegradation experiments, using the mixed microbial population, were performed in 250ml conical flasks containing a final culture volume, following inoculation, of 100ml. Substrates were added as outlined in Section 2. 2. 3. All flasks were inoculated with the appropriate culture, washed or unwashed, at a concentration of 10% (v/v). The cultures were incubated at 150rpm, 30°C and pH7.0, except when the effects of altering these environmental conditions were being investigated. In such cases, the appropriate environmental conditions are specified. Samples were aseptically removed at regular intervals and analysed for growth and pH. The samples were then immediately centrifuged at 5,000rpm for 10 minutes and supernatants were analysed for phenol/chlorophenol and reducing sugar concentrations. Phenol/chlorophenol concentrations were determined using HPLC or the 4-aminoantipyrene method (Section 2. 2. 11). Additional carbon sources were assayed

using the DNS method (Section 2. 2. 13). Uninoculated controls were incubated in parallel in order to monitor abiotic removal.

Additional carbon studies.

Where added carbon effects were being studied, the washed form of the inoculum was always used and concentrated stock solutions of the sugars were filter sterilised prior to addition to sterilised minimal media, to give the appropriate final concentration (0.01-1%).

Temperature studies.

The effects of incubation of the mixed culture at temperatures of 20, 30 and 45°C on substrate removal were examined, using shaking water baths for temperature control. Difficulties were encountered in the maintenance of the incubation temperature of 20°C, because of its proximity to ambient temperatures. The storage of this water bath at temperatures below ambient i.e in a cold room, was necessary in order to facilitate temperature control at 20°C.

pH studies.

Medium pH's of 3, 5, 7 and 8 were used. Prior to sterilisation, the pH of the *Pseudomonas* minimal medium was adjusted using 10M NaOH or 10M HCl, as appropriate. Buffers were not used due to their toxic effects on phenolic removal (Section 3. 2. 3).

Acclimation studies.

In order to acclimate the mixed microbial culture to a substrate (phenol, 4-chlorophenol, fructose), the mixed culture was grown on the substrate (until the depletion of the substrate). The biomass was then removed from the spent medium by centrifugation at 5,000rpm and used to inoculate fresh minimal medium containing the appropriate substrate(s).

Statistical strategy.

Parameters of substrate removal and growth were calculated and used for the comparison of removal efficiency under differing conditions. Rates of substrate removal were calculated following the lag period. Overall removal rates were expressed as mgl^{-1} substrate removed per unit time. Specific rates of substrate removal were expressed as mgl^{-1} substrate removed per unit time per unit biomass. Biomass values were obtained as outlined in Section 2. 2. 1. The plot relating optical density to biomass dry weight is illustrated in Appendix 2.

The lag period was defined as follows (Fig. 5): in a plot of substrate remaining vs. time, the straight line was extrapolated to the initial substrate level (S_0) and the intercept on the time axis was taken to be the length of the lag period (L).

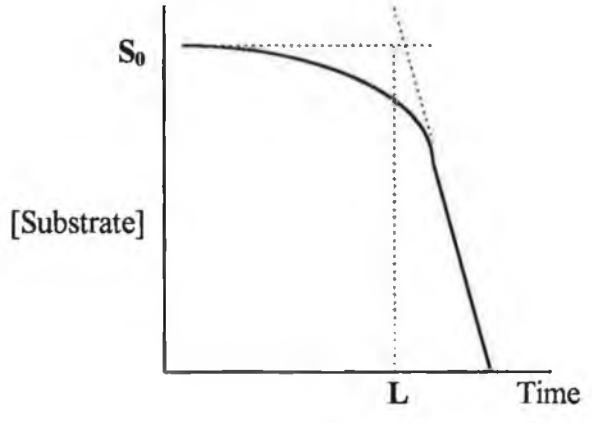


Fig. 5 Definition of lag period, L.

Throughout the study, all experiments were carried out using duplicate flasks and were repeated a minimum of three times. In each case, duplicate control flasks were incubated in parallel. Loss of substrate, if any, from the control flasks was accounted for in the removal calculations. Average results from all the experiments were presented. In all cases, standard error values were calculated and found to be less than 8.5%. This value pertains to all parameters measured, including substrate concentration, optical density, pH, etc.

2. 2. 6 Isolation and maintenance of the fungus.

A well-mixed sample of the mixed culture was serial diluted in Ringers solution and spread-plated (0.1ml) on malt extract/chloramphenicol agar. The plates were

incubated at 25°C. Fungal growth was evident following incubation for 2/3 days. The fungus was maintained on malt extract agar and subcultured periodically.

2. 2. 7 Growth of fungus on phenolic substrates.

Following isolation, plugs of fungus were withdrawn from the malt extract agar plates and transferred to minimal medium agar, which contained phenol or mono-chlorophenol isomers. The cultures were incubated at 25°C for several days. The plugs were also added to flasks containing HAB, minimal medium and chlorophenol.

2. 2. 8 Isolation and maintenance of bacterial cultures.

A well-mixed sample of the mixed culture was serially diluted in 1/4 strength Ringers solution and spread-plated (0.1ml) on both nutrient agar and chlorophenol agar. All plates were incubated at 30°C. Individual cultures were isolated by serial streaking on nutrient agar plates, until they appeared pure. These cultures were maintained on nutrient agar slopes and subcultured every 2-3 months.

2. 2. 9 Growth of isolated bacterial cultures.

Cells from nutrient agar plates were used to inoculate nutrient broths (10ml). Cultures were incubated overnight at 30°C and 150rpm. The cells were harvested in a Labofuge 6000 benchtop centrifuge (5,000rpm for 10 minutes). The cultures were washed in sterile 0.01M sodium phosphate buffer (pH7.0) and the final pellet resuspended in the same buffer. This cell suspension was used to inoculate *Pseudomonas* minimal medium, at a concentration of 2% (v/v). The appropriate carbon source was added (2. 2. 9) and all cultures were incubated at 30°C and 150rpm.

Specific growth rates were calculated using the computer software package Sigma Plot, Jandel Corporation. A simple mathematical transform was used to determine:

$$\text{Ln } (X/X_0)$$

where X = absorbance at time t

X_0 = initial absorbance

A plot of Ln (X/X_0) vs. t was constructed. Regression analysis was performed on the exponential portion of the curve. The resulting slope being equal to the specific growth rate, μ.

2. 2. 10 Tests used in the characterisation of isolated bacteria.

API Tests

The API identification systems API 20NE, for non-enteric Gram negative rods and API 50CH for determining carbohydrate assimilation were used. The identification systems were used according to the manufacturers' instructions (Bio Merieux, 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 the medium supplied by the manufacturer for API 50CH tests. In the case of API 20NE, the pellet was resuspended in 0.85% (w/v) NaCl (10ml) and used to inoculate a portion of the tests. For the API 20NE 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.

The following tests were performed as outlined in Harrigan and McCance (1976) unless otherwise stated.

Casein hydrolysis

Milk agar plates were inoculated by streaking once across the surface. Plates were incubated at 30°C for 2-14 days. Clear zones after incubation were presumptive evidence of casein hydrolysis. This was confirmed by flooding the plates with 1% (v/v) hydrochloric acid which precipitated unhydrolysed protein.

Catalase

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.

DNase activity (Collins and Lyne, 1985)

The organisms were streaked onto DNase agar and incubated at 30°C overnight. The plates were flooded with 1M hydrochloric acid, which precipitated unchanged nucleic acids. Clear zones around the inoculum indicated a positive reaction.

Gelatin hydrolysis

Gelatin agar was inoculated by streaking the test organism once across the surface of the medium. Plates were incubated at 30°C for 2-14 days. Plates were flooded with 8-10ml of mercuric chloride solution 15% (w/v) in 20% (v/v) hydrochloric acid. Unhydrolysed gelatin formed a white opaque precipitate thus a clear zone was recorded as a positive result.

Gram reaction

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

Morphology

The colony morphology was examined following growth on nutrient agar at 30°C for 24 hours. The cell morphology was examined following growth in nutrient broth for 24 hours at 30°C with agitation at 150 rpm.

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 Leifson's medium were stab inoculated with the test culture. One tube was covered with sterile mineral oil and both 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 and oxidative organisms produced acid in only the open tube and usually only at the surface.

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.

Starch hydrolysis

Nutrient agar containing 0.8% soluble starch was inoculated by streaking the test organism once 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. Hydrolysis was indicated by clear zones around the growth. Unhydrolysed starch gave a blue colour.

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 consisted of calcium salts of free fatty acids and were indicative of Tween 80 hydrolysis.

Temperature profiles

The organisms were grown on nutrient agar and incubated as follows:

4°C	7-14 days
20, 25°C	2 - 3 days
30, 37, 42°C	1 - 2 days

2. 2. 11 Assay for phenol compounds.

Colorimetric assay

Phenol and mono-chlorophenol concentrations were determined by a modification of the method detailed in Standard Methods for the Examination of Water and Wastewater (1985).

Cells were removed from samples by centrifugation (5,000rpm for 10 minutes) and the resulting supernatant was diluted if necessary. A range of standards were prepared containing 0 - 0.05mg of phenol or chlorophenol in 10ml of distilled water. The sample or standard was placed in a test tube and 0.5 N NH_4OH (0.25ml) was added. The pH was then adjusted to 7.9 ± 0.1 with potassium phosphate buffer (pH 6.8). 2% (w/v) 4-aminoantipyrene solution (100µl) was added and the tubes mixed well. Finally, 8% (w/v) potassium ferricyanide (100µl) was added and the tubes mixed. The tubes were allowed to stand at room temperature for 15 minutes and the absorbance was read at 500nm.

HPLC assay

Samples were centrifuged (5,000rpm for 10 minutes) to remove cells and the supernatant (5ml) was extracted with ethyl acetate (2ml) in three successive extractions. The resulting organic phase was assayed immediately or stored at 4°C for 2-3 days. Prior to analysis, the organic phase was filtered through a Millipore PTFE filter (0.2µm). A sample (100µl) was loaded onto a readyne injection loop. A Shimadzu LC-9A Solvent Delivery Unit automatically injected 20µl of sample onto the Novapak C_{18} column (Waters; 4µ particle size, 3.9mm x 150mm stainless steel column). The mobile phase used was acetic acid (0.075M)/acetonitrile (67:33) at a flowrate of 0.8ml/min. The Shimadzu SPD-6AV detector was set to 270nm for phenol and mono-chlorophenols, with an absorbance sensitivity of 0.01.

Following analysis, the column was washed with ultrapure water (20ml), to remove the acetic acid, and was then stored in acetonitrile and water (50:50). Following removal of the column, the system was washed with methanol. All solutions used

were HPLC grade and were filtered (Millipore, 0.45µm PTFE filters) and degassed before use.

2. 2. 12 Chemical Oxygen Demand (C. O. D.).

The chemical oxygen demand was determined using a modification of the method outlined in Standard Methods for the Examination of Water and Wastewater (1985).

C. O. D. tubes and caps were washed with 20%(v/v) H_2SO_4. Suitably diluted sample or standard (2.5ml) was added to the tube. Digestion solution (1.5ml) was carefully added. Sulphuric acid reagent (3.5ml) was carefully added down the side of the tube. The caps were screwed on tightly and the tubes were inverted, with care, to mix the contents. The tubes were placed in a preheated digestion block at 150°C for 2 hours. After the elapsed time, the tubes were removed and cooled. The entire contents of each tube was transferred to a 100ml flask and Ferroin indicator (50µl) was added. The contents were titrated against the FAS reagent. The end-point was a sharp colour change from blue/green to reddish brown.

The sensitivity of the assay was in the range of 0-500mgO_2l^{-1}.

The C. O. D. was calculated as follows:

$$\text{C. O. D. } (mgO_2l^{-1}) = \frac{(A-B) \times M \times 8000}{\text{Sample volume}}$$

Where A = volume of FAS used for blank (ml)
B = volume of FAS used for sample (ml)
M = molarity of FAS

$$\text{Molarity of FAS (M)} = \frac{\text{volume in digestion tube}}{\text{volume of FAS used for blank}} \times 0.02$$

Reagents:

Digestion solution (Potassium dichromate solution)

$K_2Cr_2O_7$ (4.913g), previously dried at 103°C for 2 hours, was dissolved in distilled water. Concentrated H_2SO_4 (167ml) and $HgSO_4$ (33.3g) were added, dissolved and the reagent was allowed to cool. The reagent was then diluted to 100ml.

Sulphuric acid reagent

$AgSO_4$ (5.5g) was added to H_2SO_4 (545ml) and allowed to stand for 2-3 hours.

FAS reagent (Ferrous Ammonium Sulphate reagent)

$Fe(NH_4)_2(SO_4)_2.6H_2O$ (0.02M) was dissolved in distilled water. Concentrated H_2SO_4 (20ml) was added and the solution allowed to cool. The reagent was then diluted to 0.02M.

Potassium Hydrogen Phthalate Standard

Potassium hydrogen phthalate (425mg), previously dried at 103°C for 2 hours, was dissolved in 1 litre of distilled water. This solution should give a C. O. D. of 500 mgO_2l^{-1}.

2. 2. 13 Sugar analysis.

Cells were removed from samples by centrifugation (5,000rpm for 10 minutes) before assaying. The reducing sugar (glucose/fructose) concentration was determined by the D.N.S. method (Miller, 1951). 1ml of standard or suitably diluted sample and 1ml of water was placed in a test tube. 2mls of DNS reagent was added. The tubes were capped and placed in a boiling water bath for 10 minutes. The tubes were then rapidly cooled and 10mls of water added to each. Absorbance was read at 540nm.

Reagents

**DNS* : 1g 3,5 dinitrosalicylic acid

30g potassium sodium tartrate (for long term storage)

50ml distilled water

16ml 10% sodium hydroxide

Warm to dissolve (DO NOT BOIL). When dissolved, cool and make up to 100ml with distilled water.

**Reducing sugar stock solution*:

1.5g reducing sugar

0.02% sodium azide (preservative)

Make up to 100ml and store in the fridge.

**Reducing sugar standard solution*:

Dilute 10ml of stock solution to 100ml. This gives a final concentration of 1.5mgml^{-1}.

2. 2. 14 Glucose Assay.

Cells were removed from samples by centrifugation (5,000 rpm for 10 minutes). The glucose concentration in the culture supernatant was determined using a Boehringer Mannheim GOD-PAP kit, in accordance with the manufacturers' instructions. The method was sensitive in the range of 0 - 5mM glucose.

2. 2. 15 Catechol dioxygenase assays.

Preparation of cell-free extracts.

Cells were grown on aromatic substrates (200ml) and harvested in a Sorvall RC-5B high speed centrifuge (5,000rpm for 10 minutes). The resulting pellet was washed in 0.033M Tris-HCl buffer (pH7.6) and the final pellet was resuspended in the same buffer (3ml). The cells were disrupted by sonication for 3 minutes (30 seconds on;30 seconds off) at 16 amplitude microns in an MSE Soniprep 150. The cell suspension was kept on ice throughout sonication. Whole cells and debris were removed by centrifugation at 20,000rpm and 4°C for 20 minutes in the above centrifuge. The cell-free extract was kept on ice and assayed as soon as possible for catechol dioxygenase activity.

Catechol 1,2-dioxygenase activity.

The *ortho*-cleavage of catechol by catechol 1,2-dioxygeanse was measured by following the formation of *cis, cis* muconic acid, the cleavage product. The following reagents were added to a quartz cuvette:

2ml 50mM tris-HCl buffer (pH 8.0)

0.7ml distilled water

0.1ml 100mM 2-mercaptoethanol

0.1ml cell-free extract

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

Catechol 2,3-dioxygenase activity.

Catechol 2,3-dioxygenase activity was determined by following the formation of the *meta*-cleavage product, 2-hydroxymuconic semialdehyde. The following reagents were added to a plastic cuvette:

2.0ml 50mM tris-HCl buffer (pH 7.5)

0.6ml distilled water

0.2ml cell-free extract

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

Calculation of enzyme activity.

The enzyme activity was calculated using the following equation:

$$\textit{Activity}\ (\mu\ \textit{moles product formed}/\text{min}) = \frac{E \times C \times L}{V} \times \frac{\Delta OD}{\text{min}}$$

where E = Molar extinction coefficient ($1 mol^{-1} cm^{-1}$)

C = substrate concentration (moles)

L = path length (cm)

V = reaction volume (L)

- Catechol 1,2-dioxygenase: E_{260} =16,800 $1mol^{-1}cm^{-1}$

$$Activity = \frac{16.800 \times 10^{-7} \times 1}{3 \times 10^{-3}} \times \frac{\Delta\, OD}{min}$$

$$= 0.56 \times \frac{\Delta\, OD}{min}$$

$$= \mu\, moles\ product\ formed/min$$

- Catechol 2,3-dioxygenase: E_{375} = 36,000 $1mol^{-1}cm^{-1}$

$$Activity = \frac{36.000 \times 2 \times 10^{-7} \times 1}{3 \times 10^{-3}} \times \frac{\Delta\, OD}{min}$$

$$= 2.4 \times \frac{\Delta\, OD}{min}$$

$$= \mu\, moles\ product\ formed/min$$

The specific enzyme activity is expressed as µmoles of product formed per minute per mg of protein and was calculated from:

$$Specific\ activity\ (\mu moles / min / mg) = \frac{Activity}{Total\ protein}$$

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 $mgml^{-1}$).

Reagent A: 50ml 0.2% Na_2CO_3 in 0.1M NaOH
0.5ml 1% $CuSO_4$
0.5ml 2% NaK tartrate

Reagent B: 50% (v/v) Folin - Ciolcalteau in water

3. Results

3. 1 The influence of inoculum preparation on the removal of varying concentrations of phenol and mono-chlorophenol by HAB.

In studying the removal of phenol and mono-chlorophenols by the mixed microbial population, two methods of inoculum preparation were used (Section 2. 2. 4). Throughout this study, the two inoculum types will be referred to as the washed and unwashed inocula. The unwashed inoculum contained both nutrients and micro-organisms. The washing step removed carry over of nutrients. Utilisation of the washed inoculum therefore facilitated the study of phenol and mono-chlorophenol removal as the sole carbon sources. The results of analysis of the bioaugmentation product, before and after washing, are shown in Table 2. The presence of additional carbon, at a concentration of 1700mgl^{-1} reducing sugar, was detected in the unwashed inoculum. The washing procedure was seen to remove a significant portion of this carbon. C.O.D. and protein levels were also significantly higher in the unwashed preparation. Throughout the experiments to follow, inoculation with both the washed and unwashed preparations was at a level of 10%(v/v). The concentrations of reducing sugar, protein and C.O.D. in the culture fluids can therefore be expected to be approximately ten times lower than the values given in Table 2.

Table 2: Characterisation of the washed and unwashed bioaugmentation product.

Analysis	**Bioaugmentation Product**	
	Unwashed	***Washed***
Reducing Sugar (mg.l^{-1})	1700	208
Protein (mg.ml^{-1})	8.91	2.12
C.O.D. (mgO_2.l^{-1})	16744	1472

3. 1. 1 The removal of varying concentrations of phenol.

The degradation of a range of phenol concentrations by washed and unwashed HAB was studied. The cultures were incubated at 30°C with agitation at 150rpm until the phenol was degraded and the cultures reached stationary phase. The pattern of removal and growth obtained, with phenol at concentrations ranging from 200 to 1500mgl^{-1}, by both the washed and unwashed mixed microbial populations is illustrated in Fig. 6 and Fig. 7. Phenol could be effectively degraded by both inoculum types at concentrations up to 1300mgl^{-1}. At a concentration of 1500mgl^{-1} no removal was observed and the turbidity of the cultures decreased with time. No phenol removal occurred in the control flasks.

Phenol removal by both populations was always preceded by a lag period. In both cases, the duration of the lag period increased with increasing initial substrate concentration (Fig. 8). The duration of this lag period was however, longer with the unwashed mixed microbial population. Differences between the lag period, with the washed and the unwashed product became less substantial as the phenol concentration increased above 500mgl^{-1} where substrate inhibition exceeded the effects of the additional carbon. Microbial growth by the washed population was also preceded by a lag period (Fig. 6). No such lag was evident with the unwashed population where a diauxic- type growth pattern was observed, with utilisation of the secondary carbon source occurring prior to the onset of phenol removal (Fig. 7). In all cases, growth of the micro-organisms commenced with the onset of substrate removal and the maximum $O.D._{660nm}$ was reached when the substrate was exhausted. A drop in pH from its initial value of 7.0 was observed, with the minimum pH coinciding with exhaustion of the substrate. This drop however, was minor, ranging from 0.07 unit for 500mgl^{-1}, to 0.25 unit for 1300mgl^{-1}.

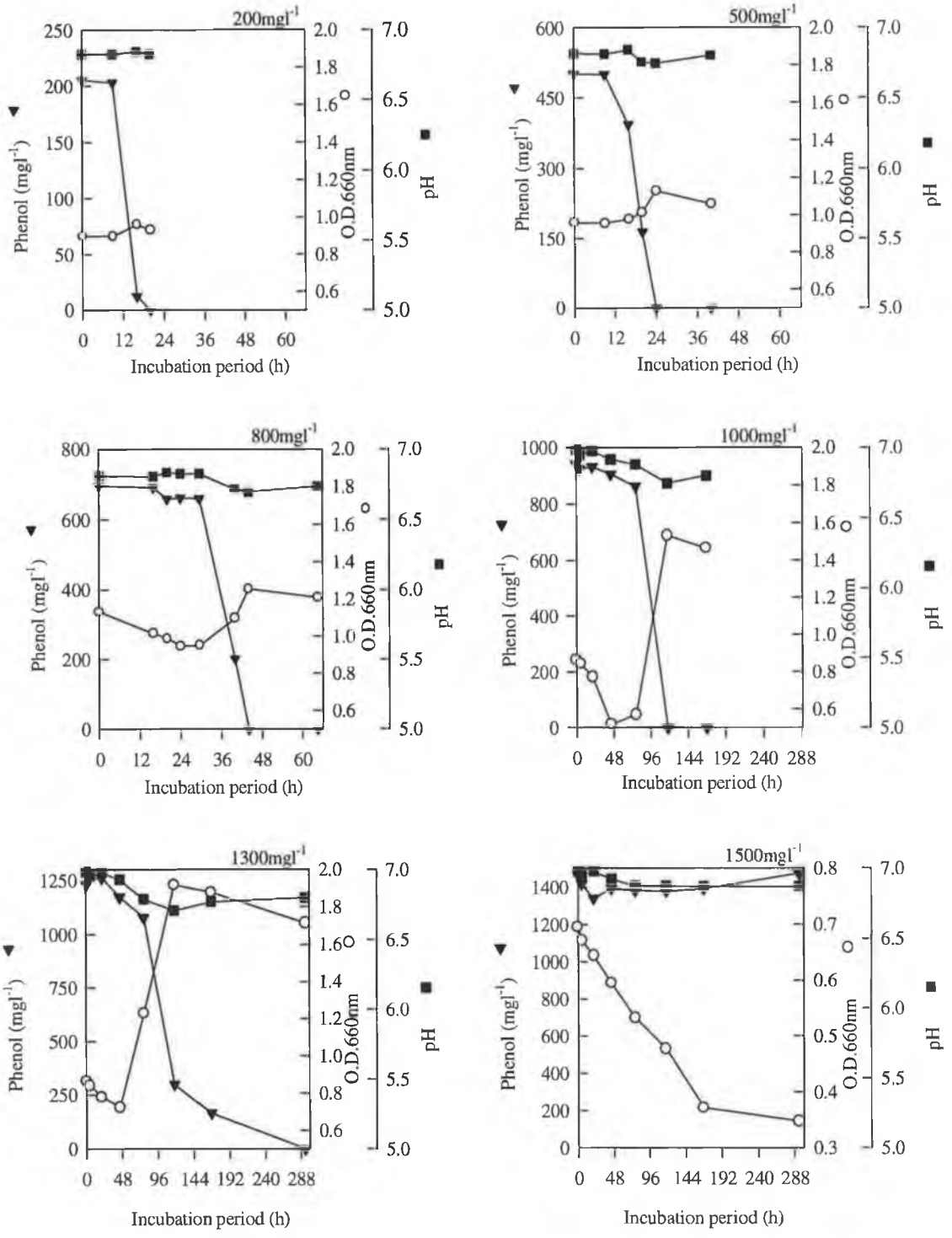


Fig. 6. The removal of various concentrations of phenol (200-1500mgl^{-1}) by the washed mixed microbial population.

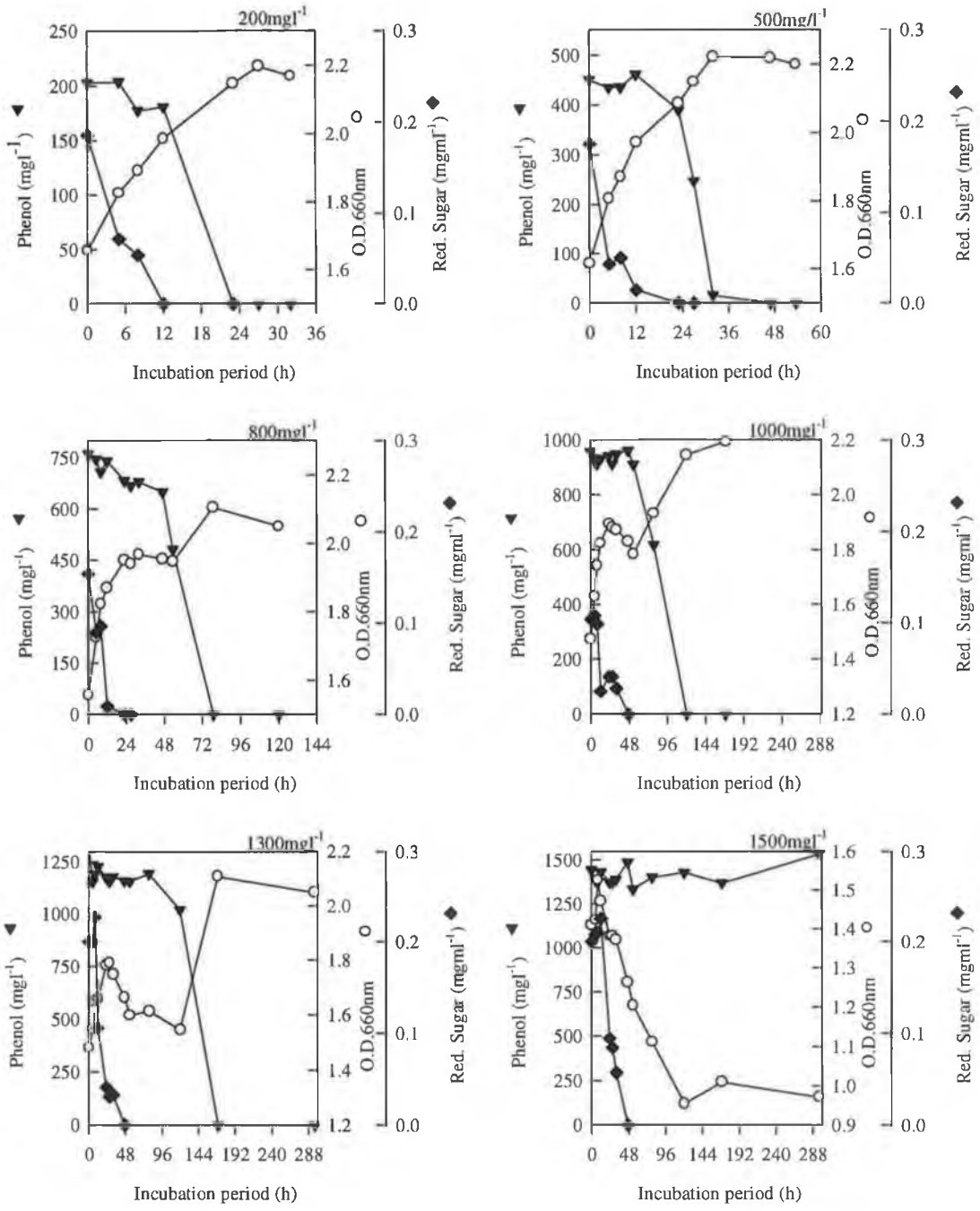


Fig. 7 The removal of various concentrations of phenol (200-1500mgl^{-1}) by the unwashed mixed microbial population.

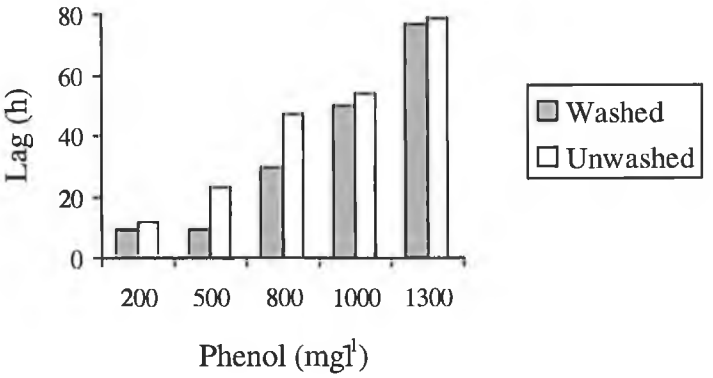


Fig. 8. The influence of inoculum preparation and phenol concentration on the duration of the lag period preceding phenol removal.

At phenol concentrations up to and including 800 mgl^{-1}, the phenol removal rate attained by the washed inoculum, varied little with substrate concentration (Table 3).This was also the case with the unwashed inoculum however, rates of removal of phenol by the washed culture were consistently higher than those of the unwashed culture. In both cases, substrate inhibition became significant at phenol concentrations greater than 800mgl^{-1}, as rates of removal decreased with any further increase in phenol concentration.

Table 3: Rates of substrate removal during in the presence of varying concentrations of phenol by washed and unwashed HAB.

	Washed HAB		**Unwashed HAB**			
Phenol	Phenol Removal		Phenol Removal		Sugar Removal	
(mgl^{-1})	($mgl^{-1}h^{-1}$)	($mgl^{-1}h^{-1}mg^{-1}$)	($mgl^{-1}h^{-1}$)	($mgl^{-1}h^{-1}mg^{-1}$)	($mgl^{-1}h^{-1}$)	($mgl^{-1}h^{-1}mg^{-1}$)
200	27.3	0.26	16.5	0.08	155	0.77
500	34.0	0.29	23.1	0.09	130	0.67
800	31.9	0.25	20.0	0.10	110	0.59
1000	21.0	0.12	13.9	0.07	60	0.32
1300	19.0	0.10	13.4	0.06	70	0.40
1500	No Removal		No Removal		42.5	0.30

The rates of removal of the additional carbon were influenced by phenol concentration (Fig. 9). In the phenol concentration range, 200 to 1000mgl^{-1}, no lag preceded sugar removal and small decreases in the rate of sugar removal were observed with increasing phenol concentrations. In the presence of 1300 and 1500mgl^{-1} phenol however, sugar removal was preceded by a lag period. The duration of this period, as well as the subsequent rates of sugar removal, were influenced by the phenol concentration.

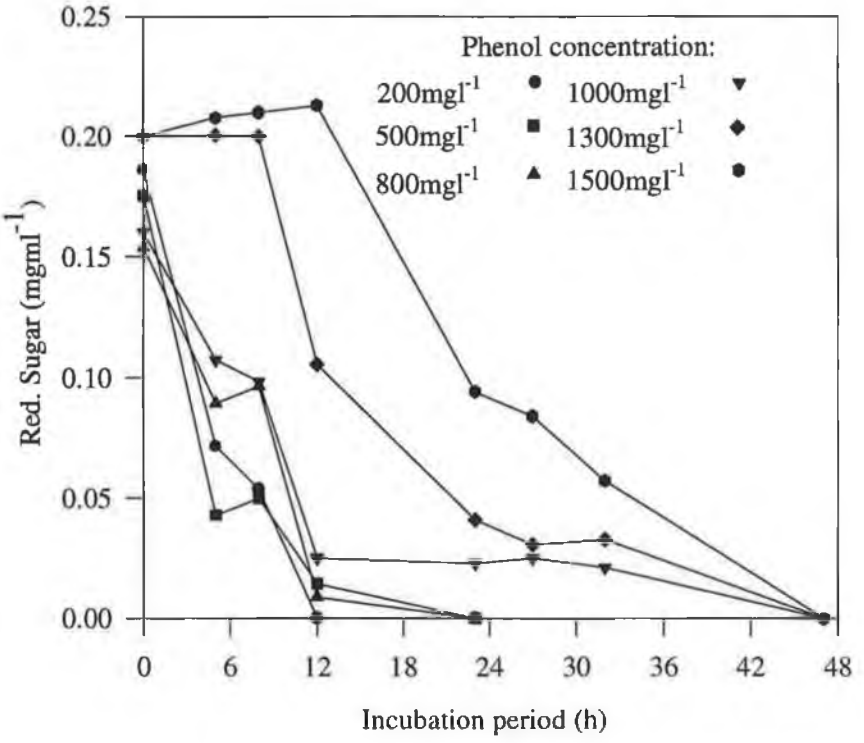


Fig. 9 Reducing sugar removal by the unwashed culture in the presence of increasing phenol concentrations.

3. 1. 2 The removal of varying concentrations of mono-chlorophenol.

The removal of various concentrations of the mono-chlorophenol isomers, from 50 to 1500mgl^{-1}, was studied. The pH, O.D.$_{660nm}$ and chlorophenol concentration were monitored until no further removal was detected. In the control flasks, no loss of 3- or 4-chlorophenol occurred. Some loss, due to volatilisation, was detected with 2-chlorophenol and was taken into account during the calculations. 4-Chlorophenol was the most readily removed of the mono-chlorophenol isomers, with complete removal achieved by the washed culture at concentrations up to 200mgl^{-1} and by the unwashed culture at a concentration of 50mgl^{-1} (Fig. 10). Complete removal of 3-chlorophenol was not achieved in the concentration range examined (Fig. 11). A maximum removal of only 75-80% was achieved at concentrations up to 200mgl^{-1} by the washed culture and at a concentration of 50mgl^{-1} by the unwashed culture. 2-chlorophenol proved most difficult to remove with over 50% always remaining (Fig. 12). No mono-chlorophenol removal was observed at concentrations above 400mgl^{-1}. Where removal did occur, a drop in pH, of no more than 0.2 unit from its initial value of 7.0, was observed.

4-Chlorophenol was successfully removed by the washed culture, at concentrations up to and including 200mgl^{-1} (Fig. 10). The rates of removal varied little with concentration with specific removal rates of 0.02, 0.027 and 0.022mgl^{-1}h^{-1}mg^{-1} at 4-chlorophenol concentrations of 50, 100 and 200mgl^{-1} respectively. The percentage removal by the unwashed inoculum was relatively poor with complete removal achieved only with 50mgl^{-1}, at the slower removal rate of 0.01mgl^{-1}h^{-1} mg^{-1}. In all cases, removal was preceded by a lag period. Where removal was incomplete i.e. above 200 and 50mgl^{-1} for the washed and unwashed inocula, respectively, the duration of the lag period increased significantly and the percentage removals decreased with increases in concentration. Growth of the biomass accompanied substrate disappearance. Where removal was observed, an intense yellow colour developed in the culture

medium in the first 12 to 18 hours, but then disappeared towards the end of the incubation.

Degradation of 3-chlorophenol was more difficult, with only 75-80% removal achieved, at concentrations up to and including 200mgl^{-1} by the washed inoculum (Fig. 11). At corresponding concentrations, removal was even less successful by the unwashed culture. In both cases, further concentration increases resulted in an even poorer degree of removal. 2-chlorophenol proved most difficult to remove with over 50% always remaining (Fig. 12). Removal in the presence of additional carbon sources was even poorer. With concentration increases above 200mgl^{-1}, the percentages removal decreased further. Despite the poor removal of both 3 and 2-chlorophenol, high $O.D._{660nm}$ readings were attained. The increase in $O.D._{660nm}$ did not reflect growth and was attributed to the formation of black compounds in the medium. These black compounds formed in the medium following initial degradation of the chlorophenol.

Mono-chlorophenol removal was inhibited by the presence of additional carbon in the formulation of the unwashed inoculum. Utilisation of this additional carbon however, was also adversely affected by the presence of the mono-chlorophenol (Fig. 13). This effect was concentration dependent with sugar utilisation rates decreasing with increases in mono-chlorophenol concentration.

In the presence of up to 400mgl^{-1} 4-chlorophenol, reducing sugar utilisation was not preceded by a lag period and rapid removal was observed (Fig. 13A). A marked reduction in the rate of utilisation of the reducing sugar was observed in the presence of 500mgl^{-1} 4-chlorophenol. A similar but more marked inhibitory pattern was observed with 3- and 2-chlorophenol (Figs. 13B and 13C). Increasing concentrations of both 3- and 2-chlorophenol had a more substantial negative effect on the removal of additional carbon. At concentrations greater than 200mgl^{-1}, the rates of sugar removal were greatly reduced, with little or no removal occurring at concentrations of 500mgl^{-1}.

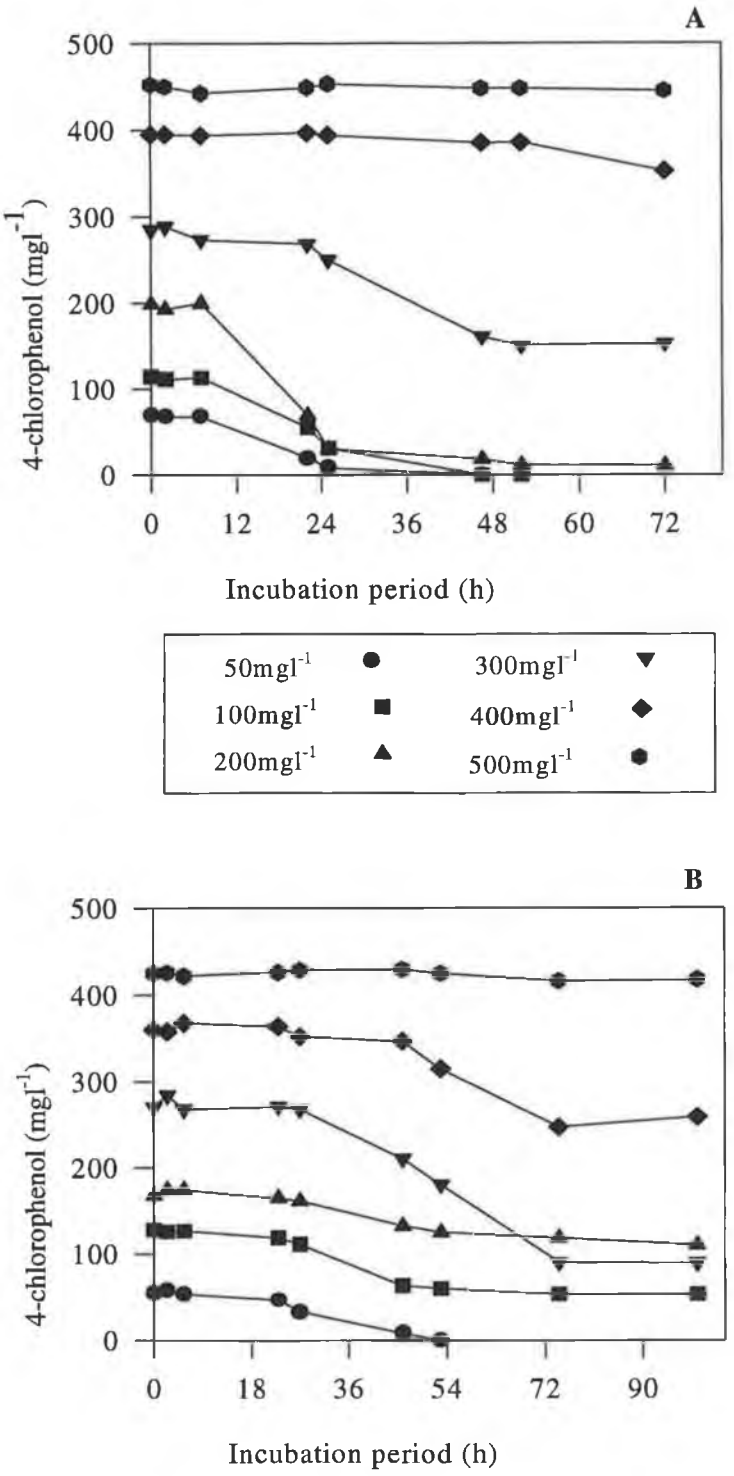


Fig. 10 The removal of various concentrations of 4-chlorophenol (50-500mgl^{-1}) by the washed (A) and unwashed (B) mixed microbial population.

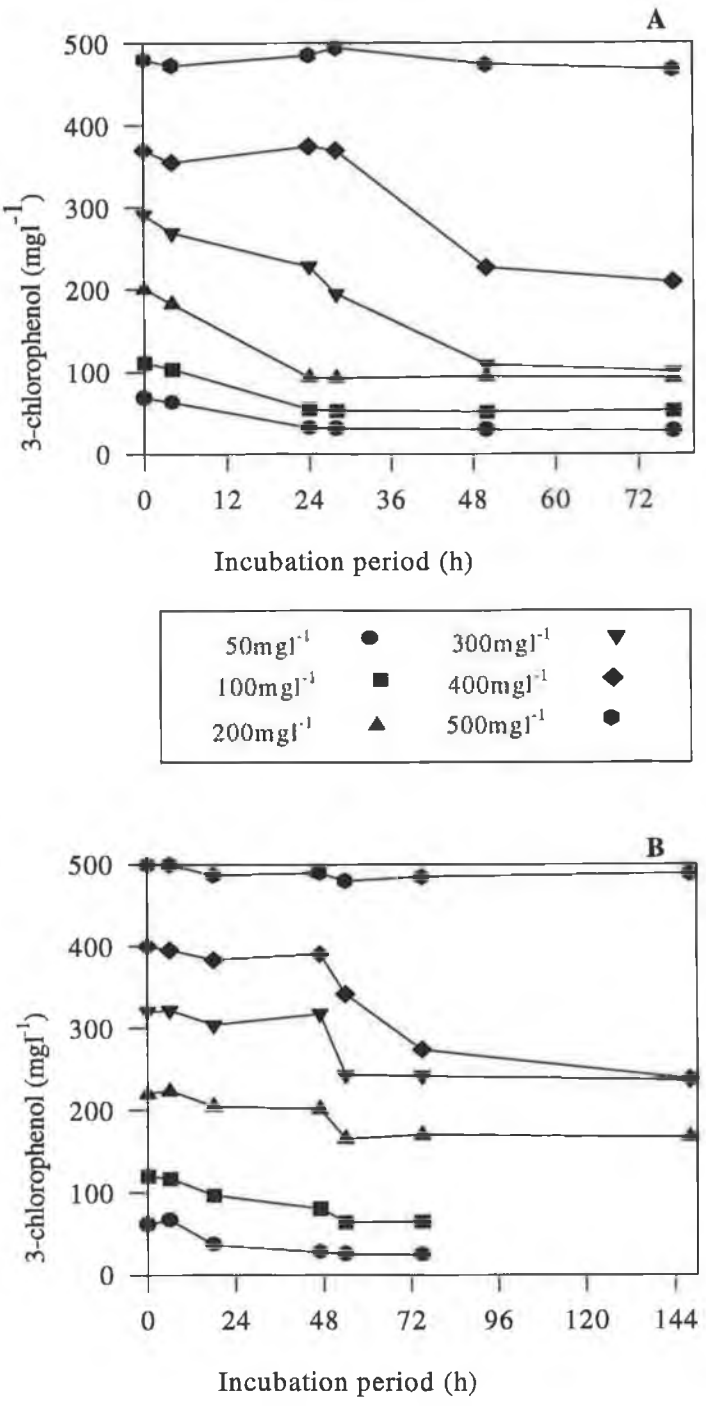


Fig. 11 The removal of various concentrations of 3-chlorophenol (50-500mgl^{-1}) by the washed (A) and unwashed (B) mixed microbial population.

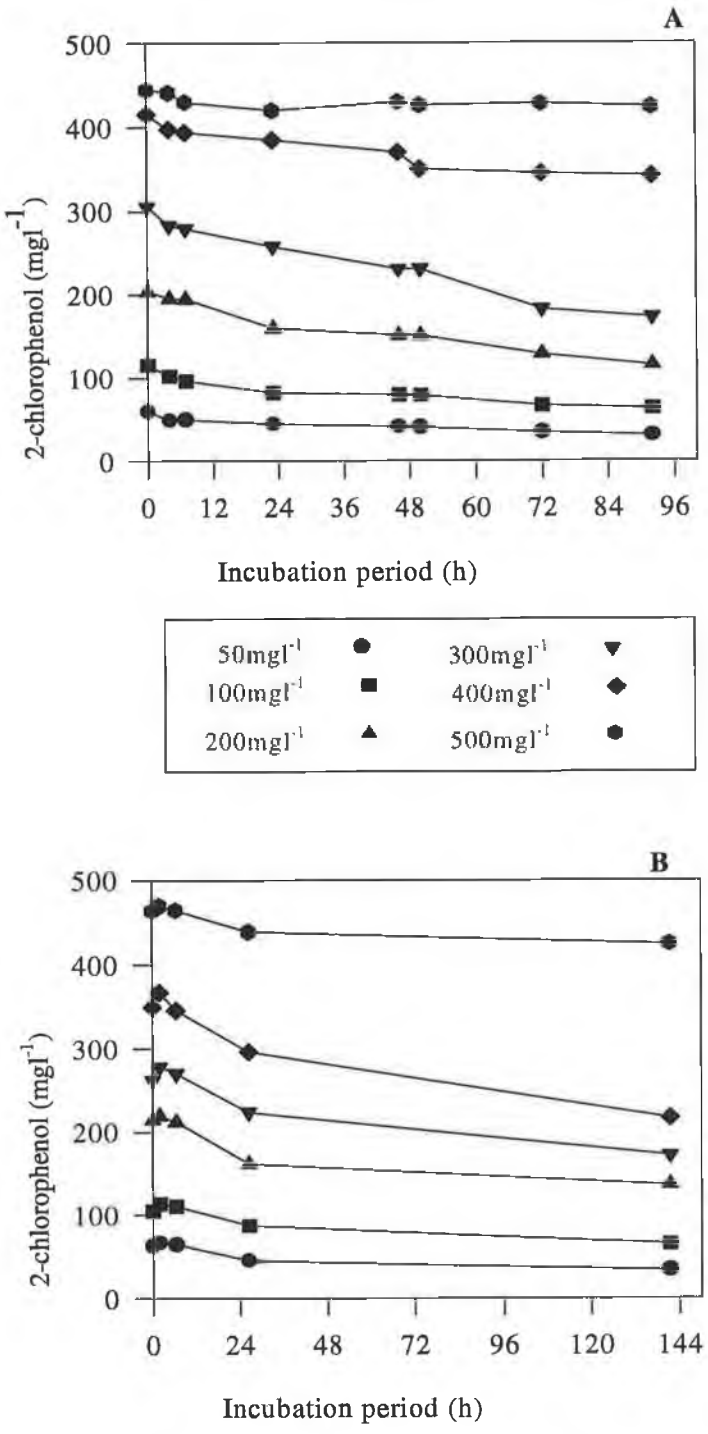


Fig. 12 The removal of various concentrations of 2-chlorophenol (50-500mgl^{-1}) by the washed (A) and unwashed (B) mixed microbial population.

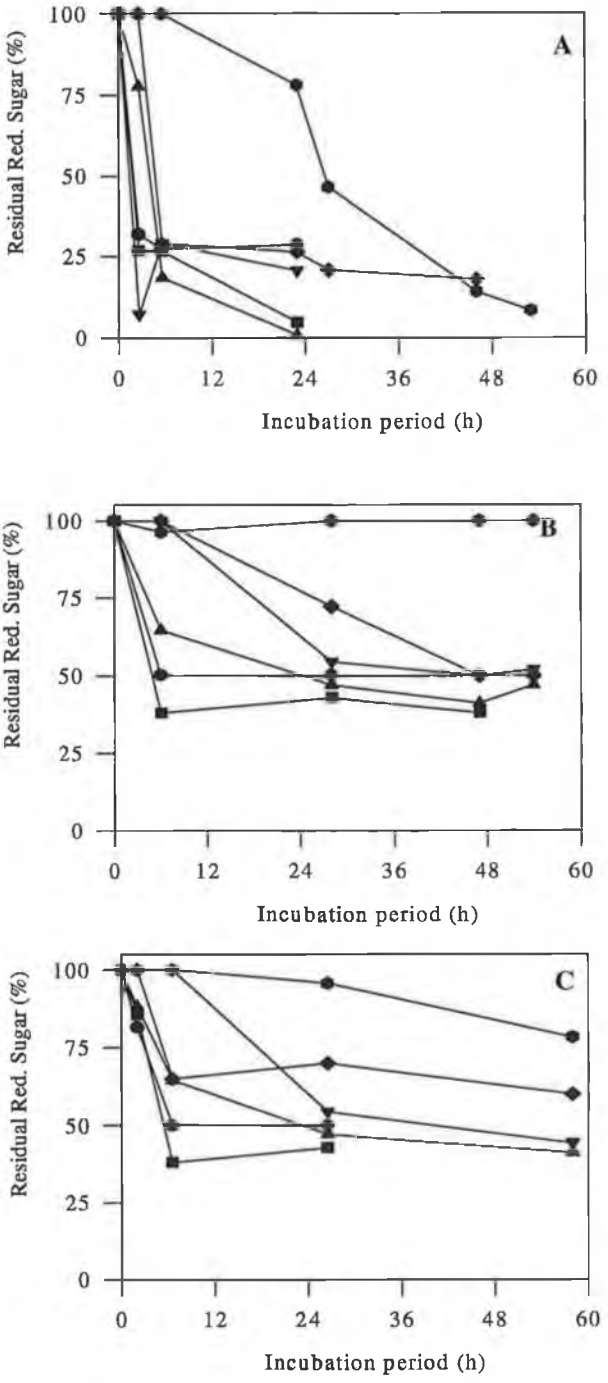


Fig. 13 Reducing sugar removal in the presence of various concentrations of 4-cp (A), 3-cp (B) and 2-cp (C) by unwashed HAB.

Legends: $50mgl^{-1}$, ●, $100mgl^{-1}$, ■, $200mgl^{-1}$, ▲, $300mgl^{-1}$, ▼, $400mgl^{-1}$, ◆, $500mgl^{-1}$,

3. 1. 3 The influence of the presence of chlorine and its orientation on the aromatic ring on substrate removal.

The relative ease of removal of phenol and the mono-chlorophenol isomers is clearly illustrated in Fig. 14. Phenol was most readily removed by the mixed microbial culture. Substitution of the aromatic ring with a chlorine atom resulted in a phenolic compound that was less readily degraded. The position of the chlorine atom on the aromatic ring was found to greatly influence removal of the mono-chlorophenols. 4-chlorophenol was the most readily removed of the isomers, followed by 3-chlorophenol and finally 2-chlorophenol. The ease of removal pattern for the mixed microbial population was as follows: phenol > 4-chlorophenol > 3-chlorophenol > 2-chlorophenol.

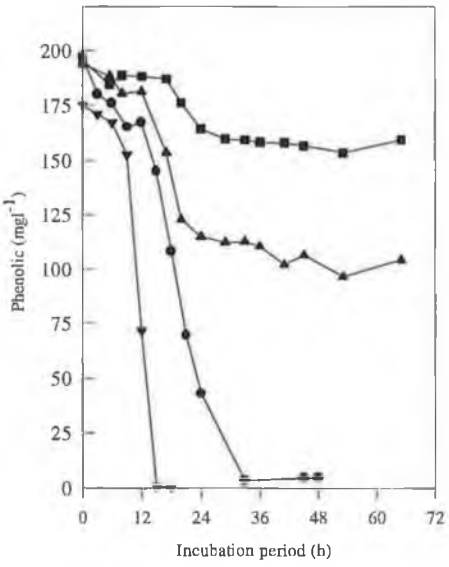


Fig. 14 The influence of the presence and position of a chlorine atom of the relative ease of removal of phenolic compounds (200 mgl^{-1}) by washed HAB. Legend: ▼ , Phenol; ●, 4-cp; ▲, 3-cp; ■, 2-cp.

3. 2 The influence of environmental conditions on substrate removal by HAB.

The effects of varying environmental conditions on substrate removal by the mixed microbial culture were investigated. A number of parameters were considered including the presence of nutrients, the exposure history of the micro-organisms, as well as incubation temperature and pH. In all cases, an initial phenolic concentration of 200mgl^{-1} was used.

3. 2. 1 The effect of the presence of glucose and fructose on phenol and mono- chlorophenol removal.

As a result of the inhibition observed with the unwashed population (Section 3. 1. 1 and 3. 1. 2) and also the detection of considerable levels of reducing sugars in the unwashed formulation (3. 1), it was of interest to examine the effects of sugars on phenolic removal. During preliminary studies, which examined the effects of a range of sugars (glucose, fructose, galactose, sucrose) on phenolic removal, two contrasting effects were noted depending on the particular sugar present. Two sugars which represented the differing effects were selected for further study; glucose to which the micro-organisms had been previously exposed and fructose to which no acclimation had taken place. The effects of a range of sugar concentrations (0.01, 0.05, 0.1, 0.2, 0.5 and 1%) on phenolic removal by the washed inoculum were examined. The flasks were incubated at 30°C and 150rpm, with an initial medium pH of 7.0.

3. 2. 1. 1 Phenol and mono-chlorophenol removal in the presence of glucose.

As in previous studies (Section 3. 1), a lag period preceded phenol and mono-chlorophenol removal by the mixed microbial population and variations in pH from the initial pH of 7.0 were noted during the incubations. The pH decreases were found to increase with increasing concentrations of glucose. At glucose concentrations up to 0.5% minor pH drops, of no greater than 0.8 unit, were observed. The pH decreases attained in the presence of 1% glucose were however more substantial and influenced substrate utilisation. The minimum pH obtained coincided with the exhaustion of glucose.

Complete phenol removal was achieved with glucose concentrations up to 0.5% (Fig. 15). The rate of phenol removal decreased with increasing glucose concentrations. In the presence of 0.01% glucose, a specific removal rate of $0.22mgl^{-1}h^{-1}mg^{-1}$ was achieved, which was comparable to the rate of $0.26mgl^{-1}h^{-1}mg^{-1}$ achieved by the washed culture (Section 3. 1. 1). With increasing glucose concentrations the removal rate decreased from $0.22mgl^{-1}h^{-1}mg^{-1}$, to a minimum of $0.03mgl^{-1}h^{-1}mg^{-1}$, in the presence of 0.5% glucose. In the presence of glucose at a concentration of 1%, glucose metabolism resulted in a large pH decrease, of 2.3 units, and phenol removal was incomplete. Glucose removal was preceded by a lag period. The duration of this period did not appear to be influenced by the glucose concentration. A sequential pattern of substrate removal from the phenol/glucose mixtures was noted, with glucose removal preceding phenol removal. Growth of the micro-organisms was preceded by a lag period, which was of equal length with that preceding glucose removal. Biomass levels increased as the substrates disappeared. Growth became more substantial with increasing glucose concentrations.

Complete removal of 4-chlorophenol was only achieved in the presence of glucose concentrations of 0.01 and 0.05%, at similar rates of 0.08 and $0.09mgl^{-1}h^{-1}mg^{-1}$, respectively (Fig. 16). Comparable removal, at a rate of $0.086mgl^{-1}h^{-1}mg^{-1}$, was achieved by the washed culture (Section 3. 1. 2). Above glucose concentrations of 0.05%, 4-chlorophenol removal decreased with increasing glucose concentrations. 4-chlorophenol removal was particularly poor in the presence of 1% glucose, where only 13.5% removal was achieved and a significant pH drop down to pH3.75 was observed. As in the presence of phenol (Fig. 15), glucose removal was preceded by a lag period. The duration of this period did not appear to be influenced by the glucose concentration. Glucose removal from the 4-chlorophenol/glucose mixtures was slow compared to its removal in the presence of phenol. For example, complete glucose (0.5%) utilisation in the presence of 4-chlorophenol was achieved in 30 hours, compared to only 13 hours in the presence of phenol. Simultaneous substrate removal from

the 4-chlorophenol/glucose mixtures occurred. Removal of the mono-chlorophenol accompanied glucose removal and ceased upon exhaustion of the readily metabolised sugar. Growth of the micro-organisms was preceded by a lag period, which was of equal length with that preceding glucose removal. Biomass levels increased as the substrates disappeared. Growth became more substantial with increasing glucose concentrations and was similar to the growth observed on phenol/glucose mixtures (Fig. 15).

3-chlorophenol removal varied little in the presence of glucose concentrations up to 0.5%, with between 40 and 50% removal achieved in all cases (Fig. 17). The time required to achieve this removal however, increased with increasing glucose concentrations, from 18 to 38 hours in the presence of 0.01 and 0.5% glucose, respectively. Within this concentration range, complete glucose utilisation was achieved. The effect of 3-chlorophenol on glucose removal was more marked than that observed in the presence of phenol and 4-chlorophenol. An additional 15 hours approx. were required for complete glucose (0.5%) removal in the presence of 3-chlorophenol, compared to in the presence of 4-chlorophenol. As before, glucose removal appeared to accompany mono-chlorophenol removal and the depletion of the sugar marked the cessation of 3-chlorophenol removal. In the presence of 1% glucose, a minimum pH of 4.2 was obtained. In this case, utilisation of both substrates was impeded, with only 21% 3-chlorophenol removal and 51% glucose removal achieved.

The percentages removal of 2-chlorophenol were similar, in the presence of all concentrations of glucose examined (Fig. 18). Glucose removal was preceded by a lag period and accompanied removal of the mono-chlorophenol isomer. The time required for complete glucose removal was further extended in the presence of 2-chlorophenol. Glucose (0.5%) removal was achieved within 48 hours compared to 45, 30 and 13 hours in the presence of 3-, 4-chlorophenol and phenol, respectively. This reflected the increased toxicity of 2-chlorophenol, relative to the other phenolics examined, which had been observed earlier (Section 3. 1. 3).

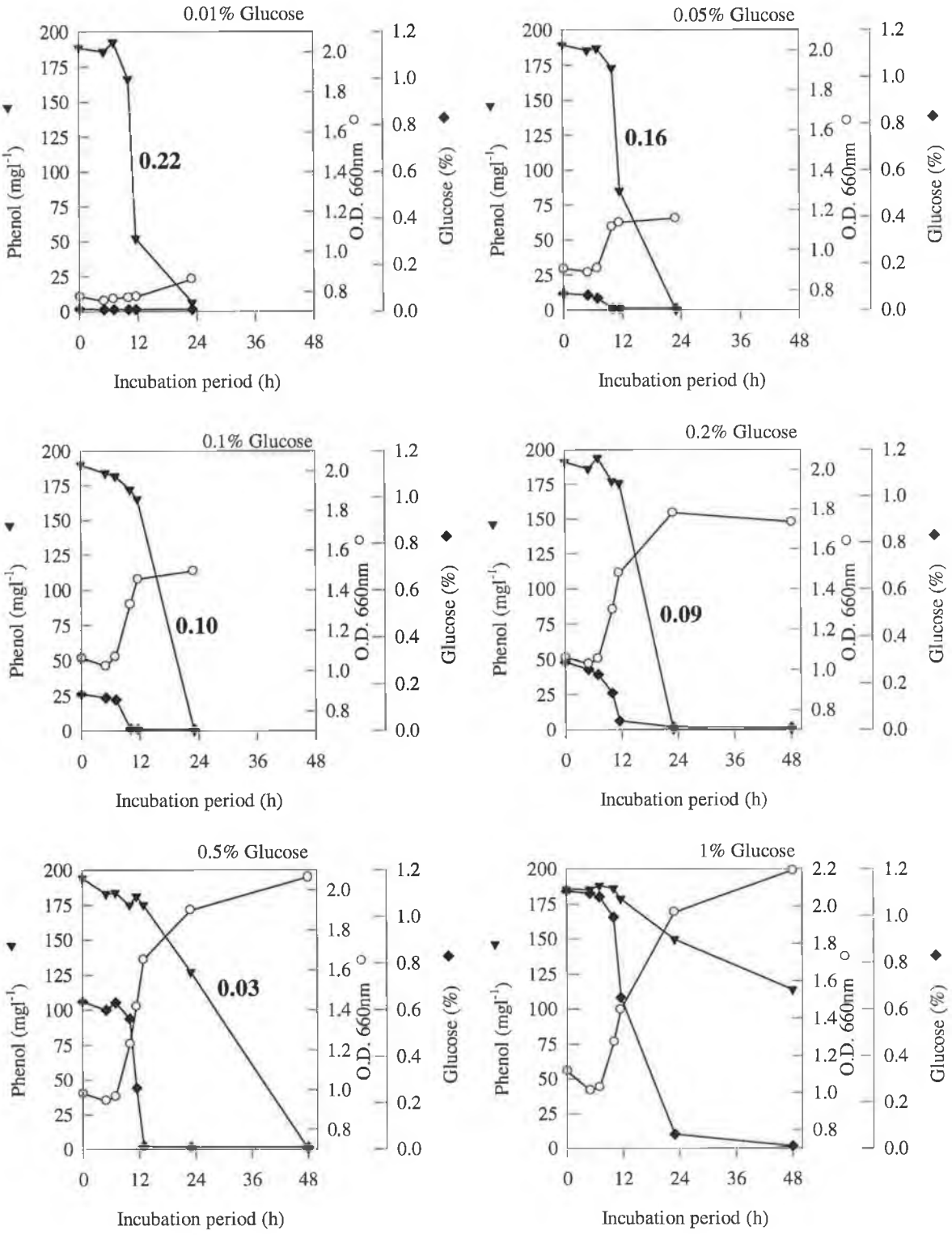


Fig. 15 The removal of phenol (200 mgl^{-1}) by washed HAB in the presence of various concentrations of glucose (0.01 - 1%). Specific phenol removal rates ($mgl^{-1}h^{-1}mg^{-1}$) are given in bold on the graphs.

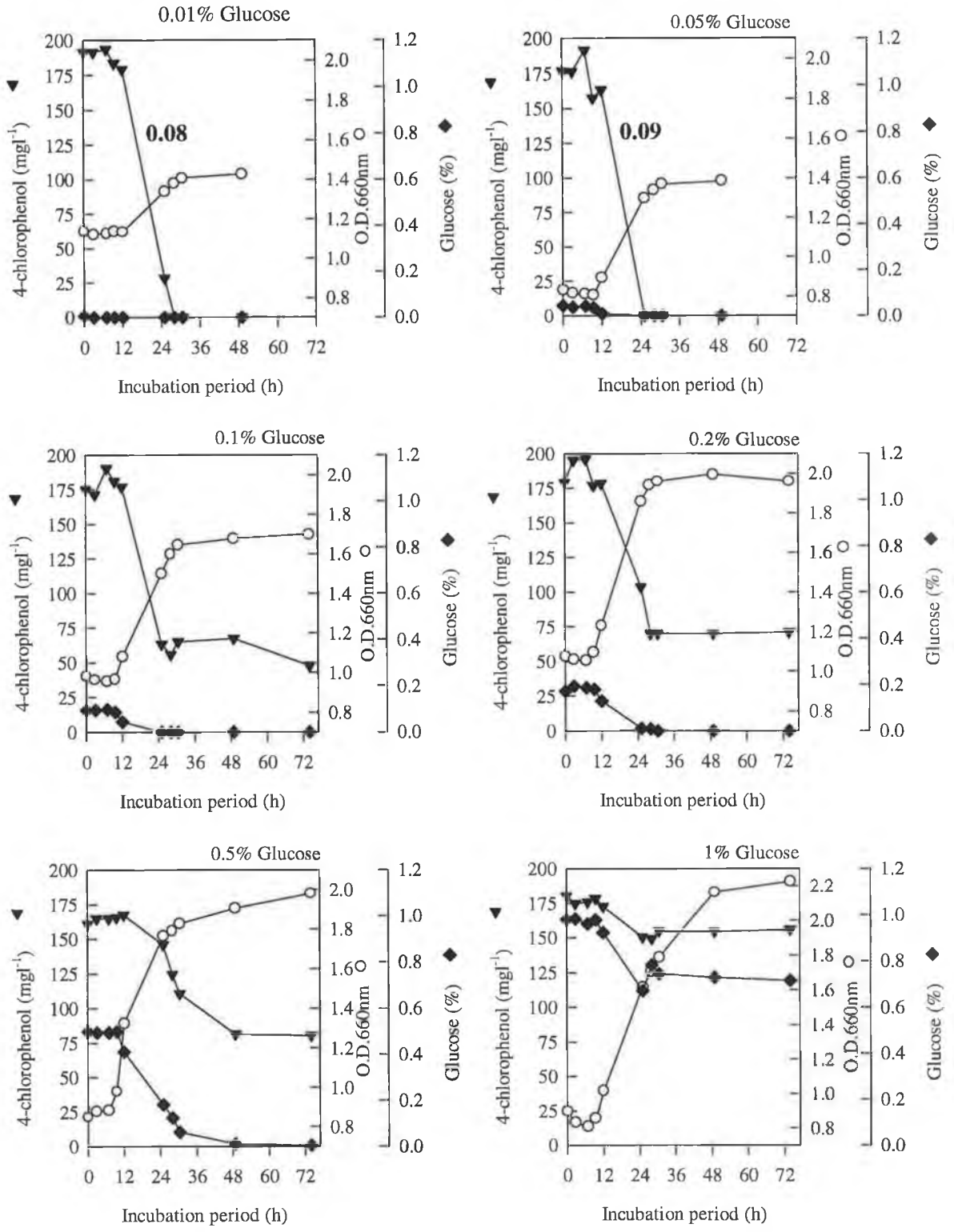


Fig. 16 The removal of 4-chlorophenol ($200mgl^{-1}$) by washed HAB in the presence of various concentrations of glucose (0.01 - 1%). Specific 4-chlorophenol removal rates ($mgl^{-1}h^{-1}mg^{-1}$) are given in bold on the graphs.

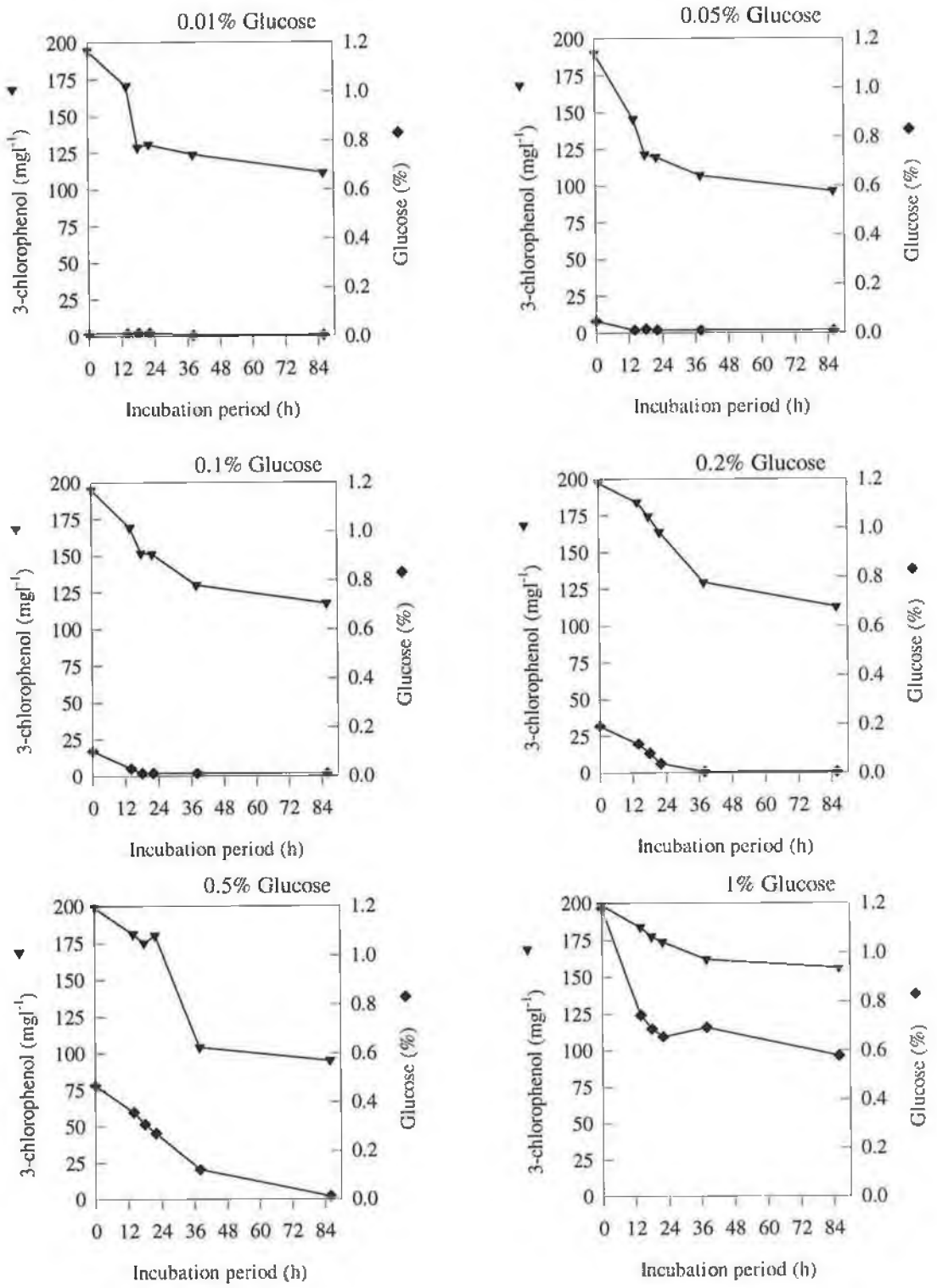


Fig. 17 The removal of 3-chlorophenol ($200mgl^{-1}$) by washed HAB in the presence of various concentrations of glucose (0.01 - 1%).

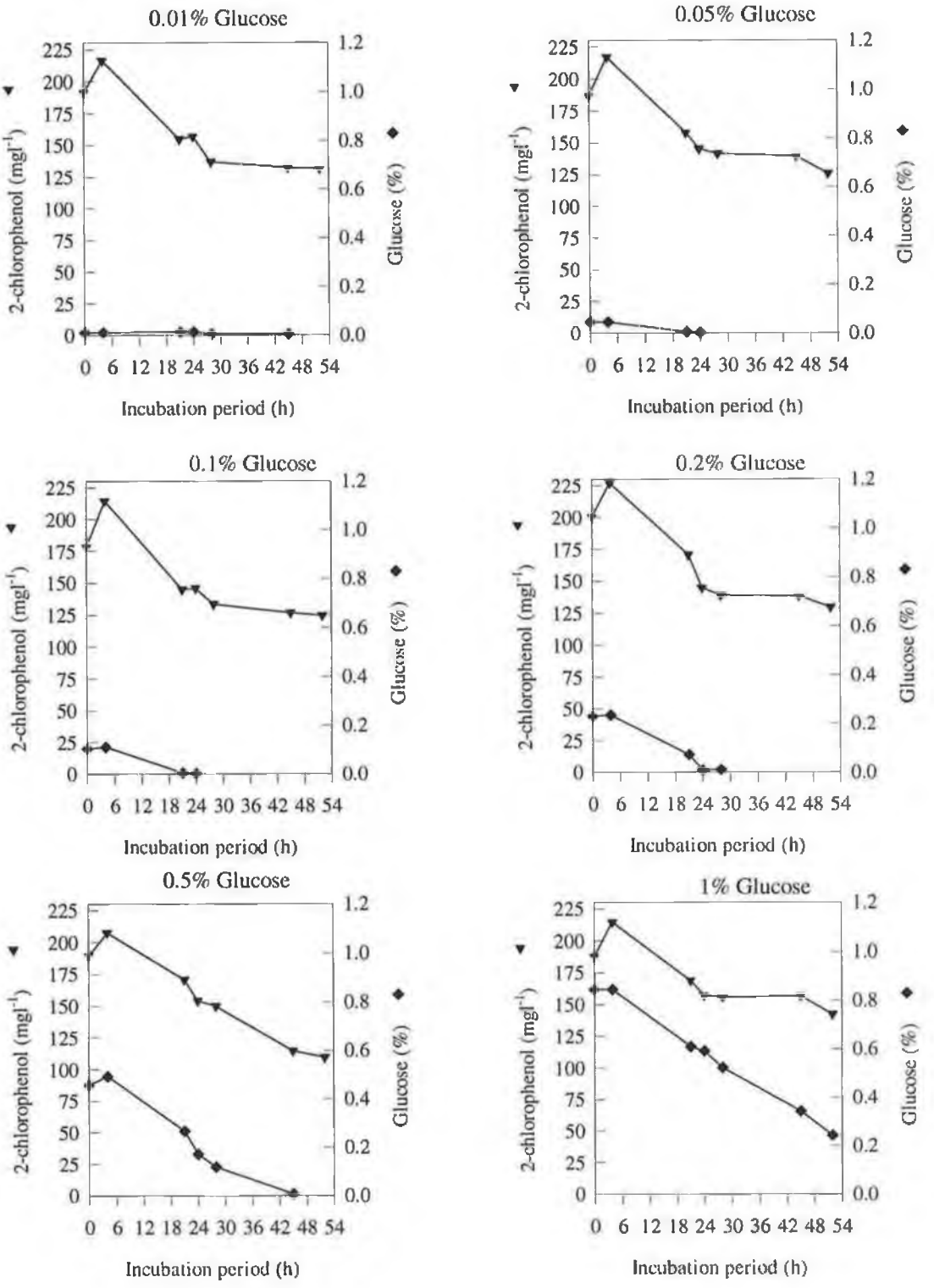


Fig. 18 The removal of 2-chlorophenol (200mgl^{-1}) by washed HAB in the presence of various concentrations of glucose (0.01 - 1%).

3. 2. 1. 2 Phenol and mono-chlorophenol removal in the presence of fructose.

Phenolic removal was preceded by a lag period, which was not influenced by increasing fructose concentrations. A lag period also preceded fructose removal. The length of this lag period was relatively long when compared to that which preceded phenolic removal, resulting in preferential utilisation of the phenolic from the substrate mixtures. The time required for complete fructose removal was longer than that required for glucose removal. During the study of the removal of phenolic/fructose mixtures, pH decreases were noted and were found to increase with increasing fructose concentration. The magnitude of these decreases, which never exceeded 1.5 pH unit, were not as substantial as those observed with glucose (Section 3. 2. 1. 1).

At all concentrations of fructose used, complete phenol removal was achieved by the mixed microbial culture (Fig. 19). The rates of phenol removal varied little within the fructose concentration range of 0.01 to 0.5%. These rates were similar to those achieved in the presence of 0.01% glucose (Section 3. 2. 1. 1) and by the washed inoculum (Section 3. 1. 1). In the presence of 1% fructose, rapid phenol removal was achieved in the initial 12 hours of the incubation, during which time no fructose removal was detected. With the onset of fructose removal however, a marked reduction in the phenol removal rate can be seen and a relatively slow overall phenol removal rate resulted. In all cases, complete fructose removal was achieved and was preceded by a lag period. In contrast to substrate removal from the phenol/glucose mixtures (Section 3. 2. 1. 1), preferential utilisation of phenol from the phenol/fructose mixtures was observed. A lag period prior to growth of the micro-organisms was noted and biomass levels increased as the substrates disappeared. Net growth increased with increasing fructose concentrations.

As with phenol, complete 4-chlorophenol removal was achieved in the presence

of all fructose concentrations used (Fig. 20). In all cases, similar rates of 4-chlorophenol removal were achieved. As with phenol, these rates were similar to those achieved in the presence of 0.01% glucose (Section 3. 2. 1. 1) and by the washed inoculum (Section 3. 1. 2). The substrate removal pattern differed to the concurrent removal observed with 4-chlorophenol/glucose mixtures. The lag period preceding fructose was longer than that which preceded glucose removal and 4-chlorophenol was removed preferentially from the 4-chlorophenol/fructose mixtures. In contrast to glucose, the time required for complete fructose removal in the presence of 4-chlorophenol was similar to that observed with phenol. With phenolic/fructose mixtures however, the concentration of phenolic present was greatly reduced prior to the onset of sugar removal and therefore the toxic effects of the phenolic on the sugar removal rate would be reduced. Biomass increased with substrate disappearance and was more substantial with increasing sugar concentrations.

Enhanced removal of 3-chlorophenol by the mixed microbial population was observed in the presence of increasing concentrations of the sugar (Fig. 21). Close to 90% removal was achieved in the presence of 0.1% fructose or higher, compared to only 41% in the presence of 0.01% fructose. In all cases, complete fructose removal was achieved and was preceded by a lag period. In contrast to glucose, the rate of fructose removal did not appear to be greatly influenced by the toxicity of the particular phenolic present and the time required for fructose removal in the presence of 3-chlorophenol was similar to that seen with 4-chlorophenol and phenol. As before, preferential utilisation of the phenolic from the phenolic/fructose mixtures was noted. The removal of 2-chlorophenol by the mixed microbial culture was also enhanced in the presence of fructose (Fig. 22). This improvement was not as marked as that seen with 3-chlorophenol. A percentage 2-chlorophenol removal of approx. 50% was achieved in the presence of 0.2% fructose or higher, compared to only 20% in the presence of 0.01% fructose. As before, fructose removal was preceded by a lag period and its removal did not appear to be influenced by the toxicity of the phenolic. Preferential utilisation of 2-chlorophenol from the 2-chlorophenol/fructose

mixtures was observed. A period of concurrent removal of the substrates was observed however, following the onset of fructose removal. Little 2-chlorophenol removal was observed following exhaustion of the sugar.

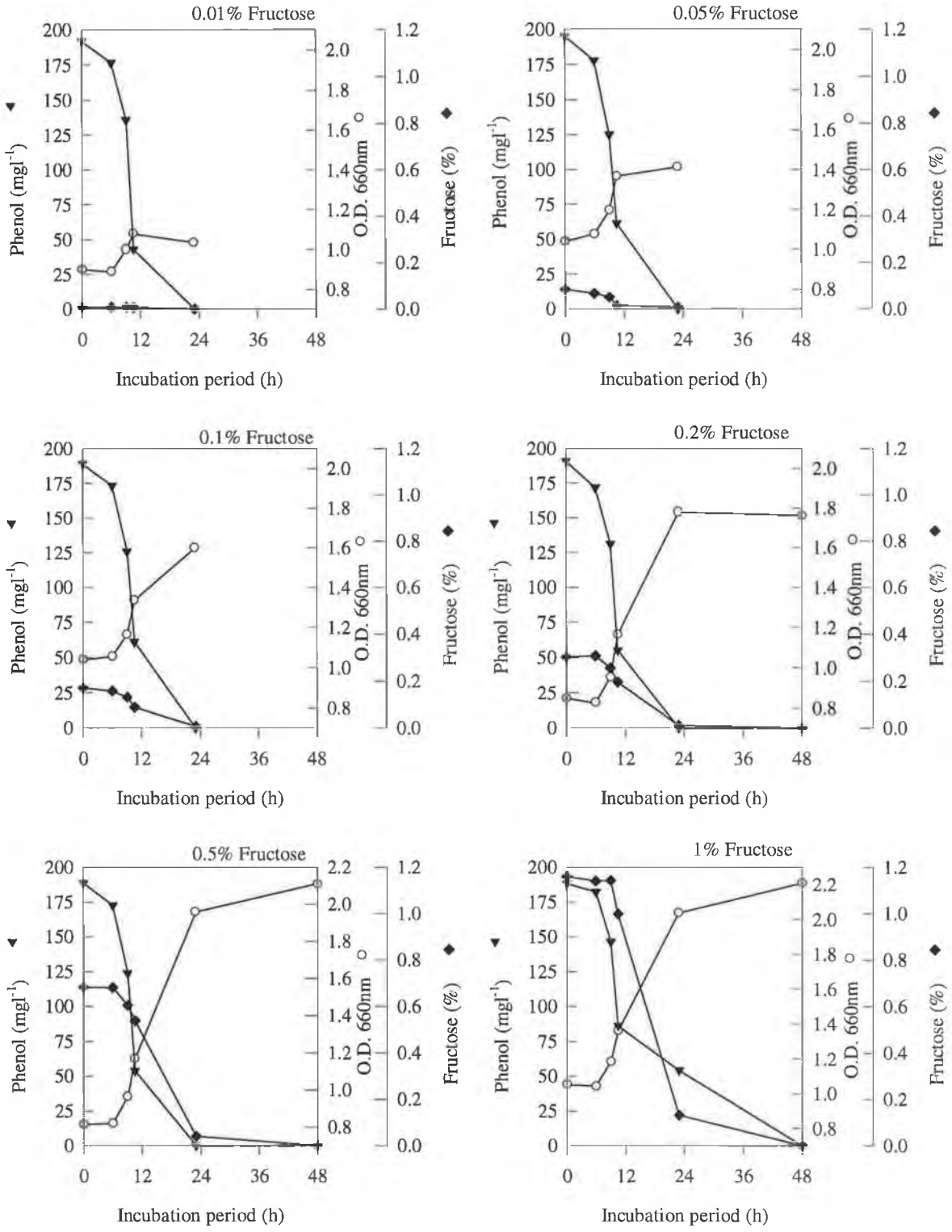


Fig. 19 The removal of phenol (200mgl^{-1}) by washed HAB in the presence of various concentrations of fructose (0.01-1%).

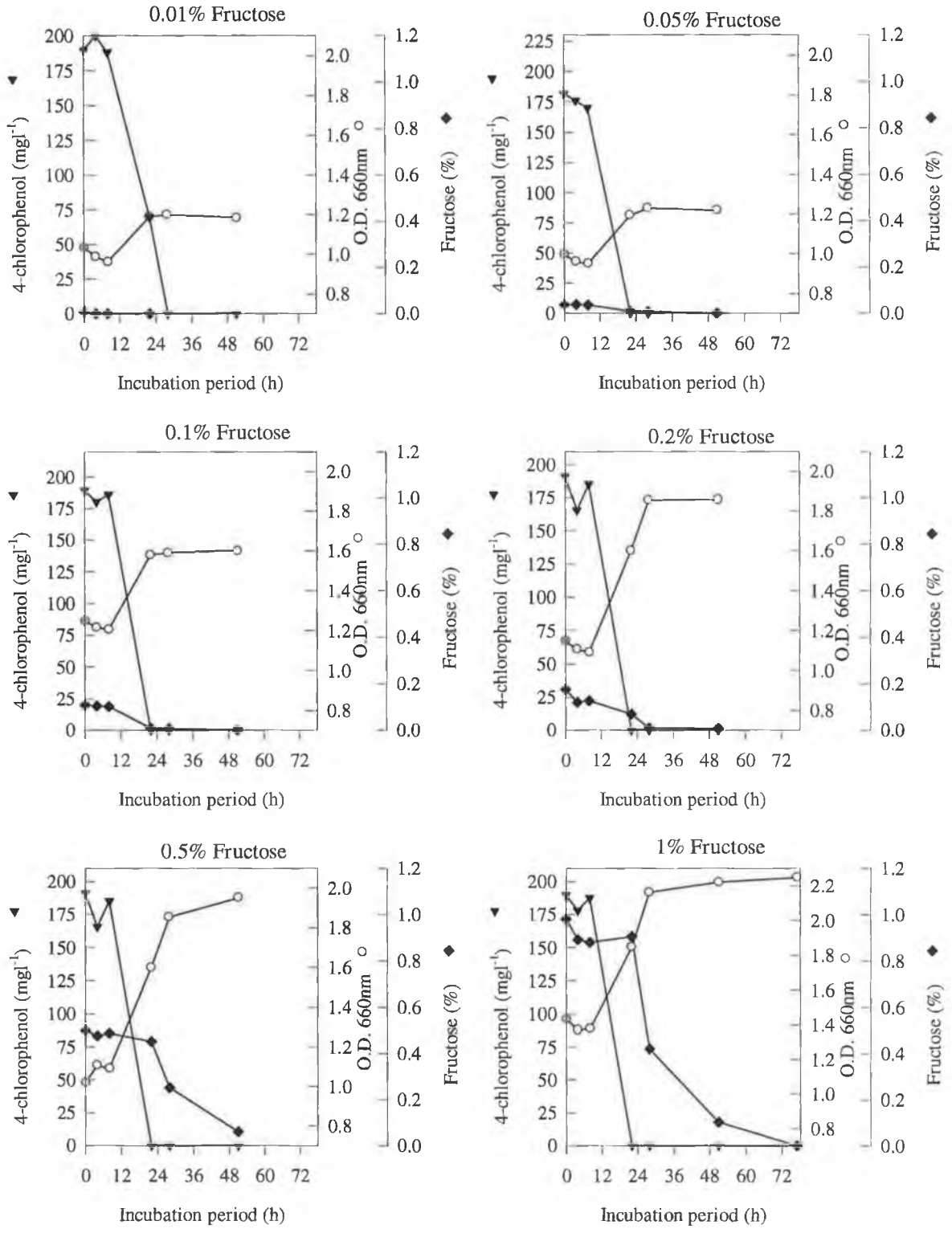


Fig. 20 The removal of 4-chlorophenol ($200mgl^{-1}$) by washed HAB in the presence of various concentrations of fructose (0.01-1%).

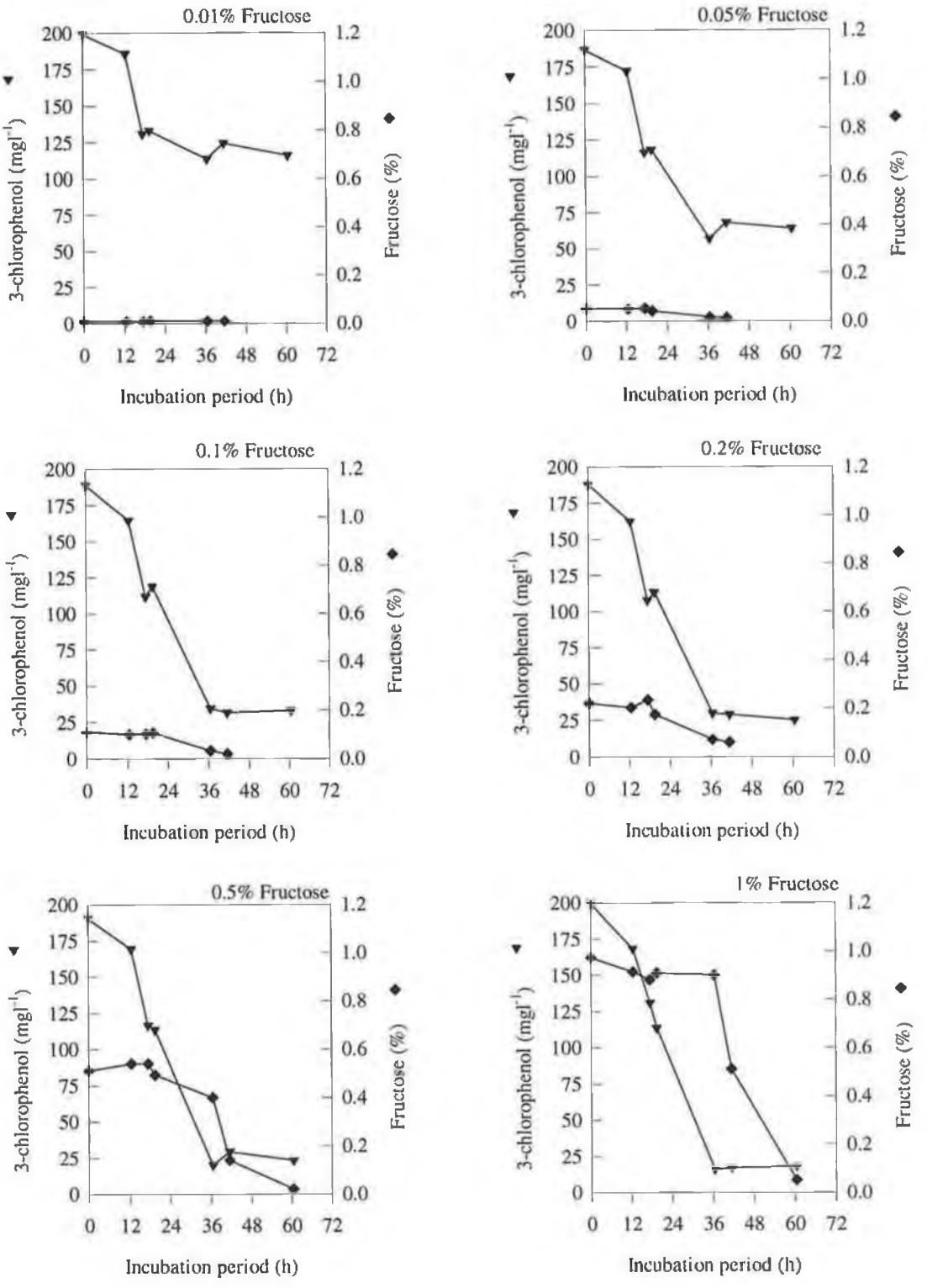


Fig. 21 The removal of 3-chlorophenol ($200mgl^{-1}$) by washed HAB in the presence of various concentrations of fructose (0.01-1%).

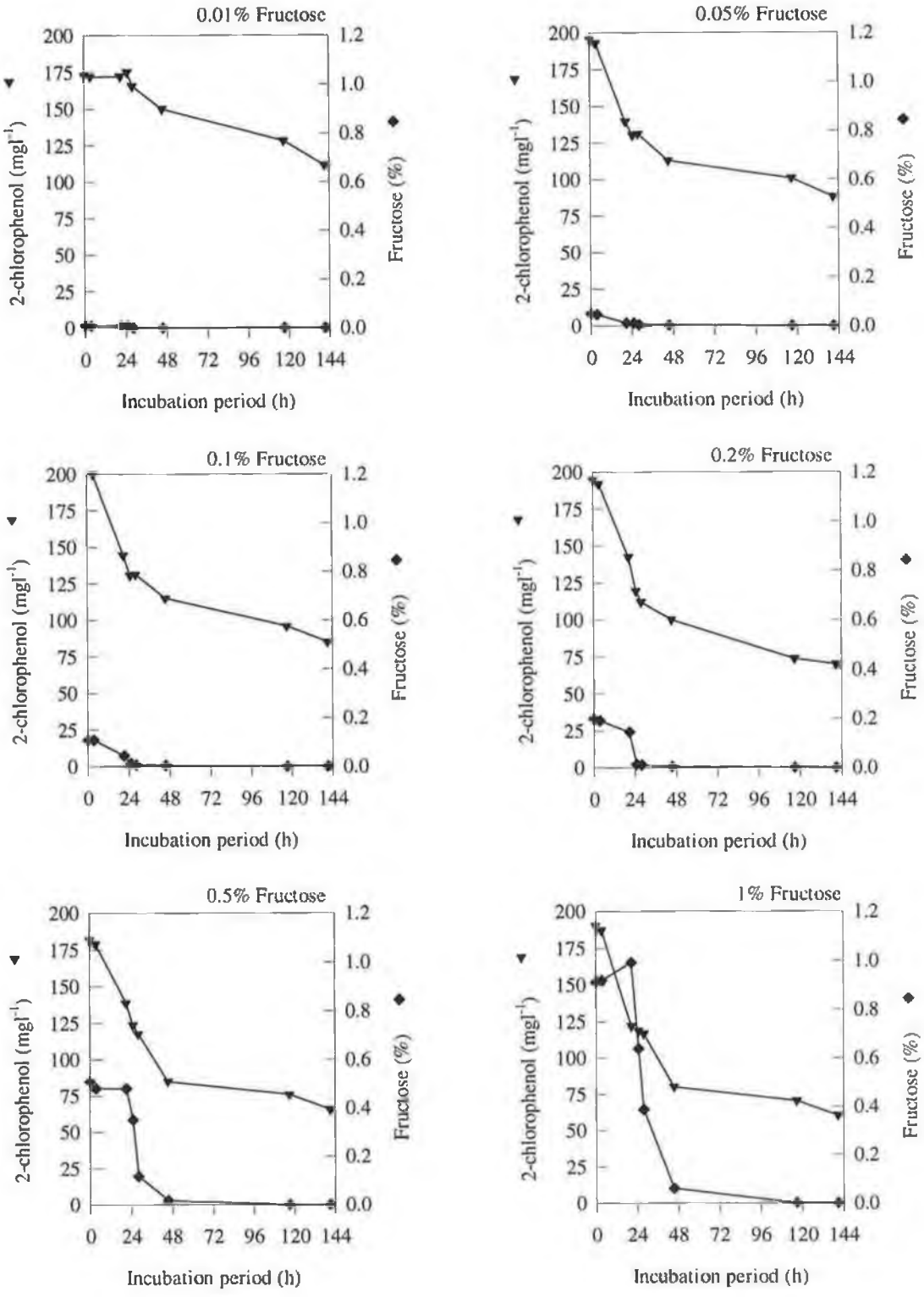


Fig. 22 The removal of 2-chlorophenol ($200mgl^{-1}$) by washed HAB in the presence of various concentrations of fructose (0.01-1%).

3. 2. 2 The influence of incubation temperature on substrate removal.

The removal of phenol and the mono-chlorophenol isomers ($200mgl^{-1}$), by both the washed and unwashed cultures, was examined at incubation temperatures of 20, 30 and 45°C. Controls were also incubated at the various temperatures, in order to monitor abiotic removal.

3. 2. 2. 1 The effect of temperature on phenol removal.

At all the selected incubation temperatures, complete phenol removal was achieved by both the washed and unwashed inocula (Fig. 23). Removal by both populations was optimal at 30°C. In all cases, phenol removal was preceded by a lag period. The length of the lag periods were similar at incubation temperatures of 20 and 30°C for the washed and unwashed cultures, but were substantially longer at 45°C. While the lengths of the lags were similar at 20 and 30°C, the rates of phenol removal at the lower temperature were decreased considerably (Table 4). At 30°C, phenol removal rates of 0.28 and $0.14mgl^{-1}h^{-1}mg^{-1}$ were achieved by the washed and unwashed cultures, respectively. These rates were comparable to those seen earlier in similar studies (Section 3. 1. 1). Rates of phenol removal of only 0.11 and $0.06mgl^{-1}h^{-1}mg^{-1}$ were achieved by the washed and unwashed cultures incubated at 20°C.

Removal of the additional carbon present in the unwashed formulation was found to be influenced by incubation temperature (Fig. 23B, C, D). As before at 30°C (Section 3. 1. 1), sequential removal of the substrates was observed. No lag period prior to sugar removal was observed and complete sugar removal preceded the onset of phenol removal. A similar pattern was observed at 45°C however, sugar removal was more rapid (Table 4). In contrast, sugar removal was preceded by a lag period during incubation at 20°C and concurrent removal of the substrates was observed.

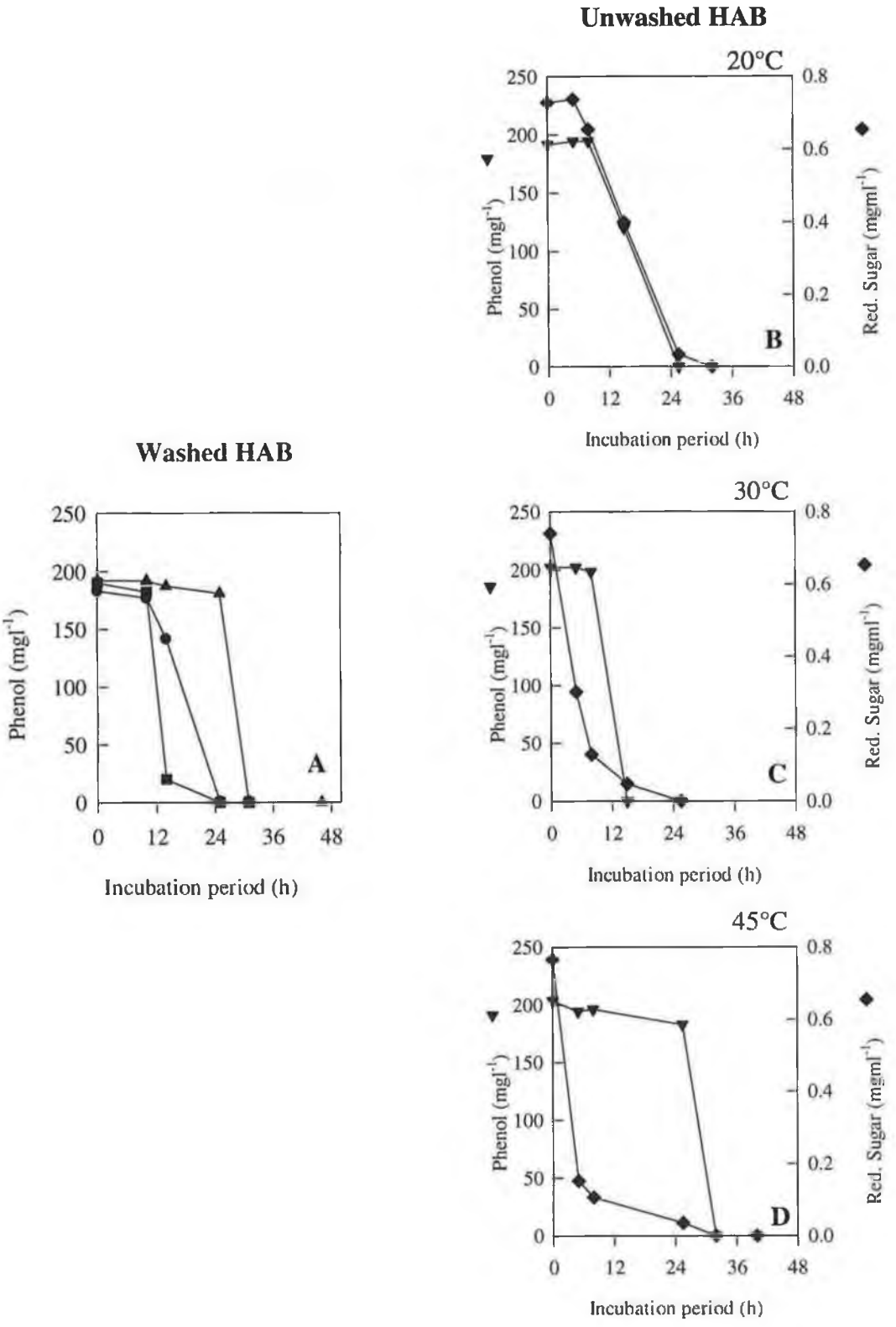


Fig. 23 Substrate removal during growth on phenol by washed (A) and unwashed (B, C, D) HAB incubated at various temperatures.

Legend to Graph A: 20°C, ●, 30°C, ■, 45°C, ▲.

Table 4: The effect of incubation temperature on substrate removal during growth of washed and unwashed HAB on phenol.

	WASHED HAB		UNWASHED HAB			
Temp	**Phenol Removal**		**Phenol Removal**		**Sugar Removal**	
(°C)	($mgl^{-1}h^{-1}$)	($mgl^{-1}h^{-1}mg^{-1}$)	($mgl^{-1}h^{-1}$)	($mgl^{-1}h^{-1}mg^{-1}$)	($mgl^{-1}h^{-1}$)	($mgl^{-1}h^{-1}mg^{-1}$)
20	12.06	0.11	11.11	0.06	34	0.17
30	31.6	0.28	28.38	0.14	76.5	0.45
45	30.18	0.28	28.15	0.15	122.6	0.77

3. 2. 2. 2 The effect of temperature on mono-chlorophenol removal.

No mono-chlorophenol removal was observed at 45°C (Figs. 24, 25, 26). The additional carbon present in the unwashed formulation was removed however, at this elevated temperature. At the lower incubation temperatures, ease of mono-chlorophenol removal followed the order: 4- > 3- > 2-chlorophenol. Where removal occurred, it was accompanied by colour development. As before, during 4-chlorophenol removal by the mixed culture, an intense yellow colour developed in the culture fluids but did not persist. With both 3- and 2-chlorophenol, black compounds appeared and persisted in the culture fluids.

Complete 4-chlorophenol removal by the washed culture was achieved at incubation temperatures of 20 and 30°C (Fig. 24A). At both temperatures, removal was preceded by a lag period of similar duration. As with phenol, optimum removal was achieved at 30°C. Similar to before at this temperature, 4-chlorophenol was removed at a rate of $0.09mgl^{-1}h^{-1}mg^{-1}$ (Section 3. 1. 2). At 20°C however, a lower removal rate, of $0.05mgl^{-1}h^{-1}mg^{-1}$, was achieved.

As before (Section 3. 1. 2), 4-chlorophenol removal was adversely affected by the presence of additional carbon in the unwashed formulation, with only 64.6% removal achieved at 30°C (Fig. 24B). Sugar removal was not preceded by a lag period and a period of concurrent removal of the substrates was observed. However, at an incubation temperature of 20°C, the inhibition observed at 30°C was alleviated and complete 4-chlorophenol removal was achieved (Fig. 24B). The sugar removal pattern was different to that observed at 30°C. A lag period now preceded removal of the additional carbon. A longer period of concurrent removal of the substrates was noted and appeared to facilitate enhanced 4-chlorophenol transformation.

The removal of 3- and 2-chlorophenol by the washed culture was relatively poor, with only 30 and 25.4% removal achieved at 30°C, respectively (Figs. 25A, 26A). Similar removal was achieved at 20°C. The substrate removal pattern observed during growth of the unwashed culture at 30°C had been noted previously (Section 3. 1. 2). No lag period preceded sugar removal and concurrent removal of the substrates was evident. Little mono-chlorophenol removal was achieved following exhaustion of the additional carbon. As with 4-chlorophenol, a contrasting substrate removal pattern was observed at 20°C which appeared to facilitate enhanced phenolic removal. A lag period prior to utilisation of both substrates was noted resulting in an extended period of concurrent removal. Percentages removal of 3 and 2-chlorophenol, of 50 and 36.2% respectively, were achieved at the lower incubation temperature.

Ease of removal by both washed and unwashed HAB followed the order: 4- > 3- > 2-chlorophenol. Removal by the washed culture was optimal at 30°C. In contrast, phenolic removal in the presence of additional carbon was optimal at 20°C. The role of the pattern of sugar removal appeared significant. Its influence has been previously noted, where slow fructose removal facilitated enhanced phenolic removal (Section 3. 2. 1. 2).

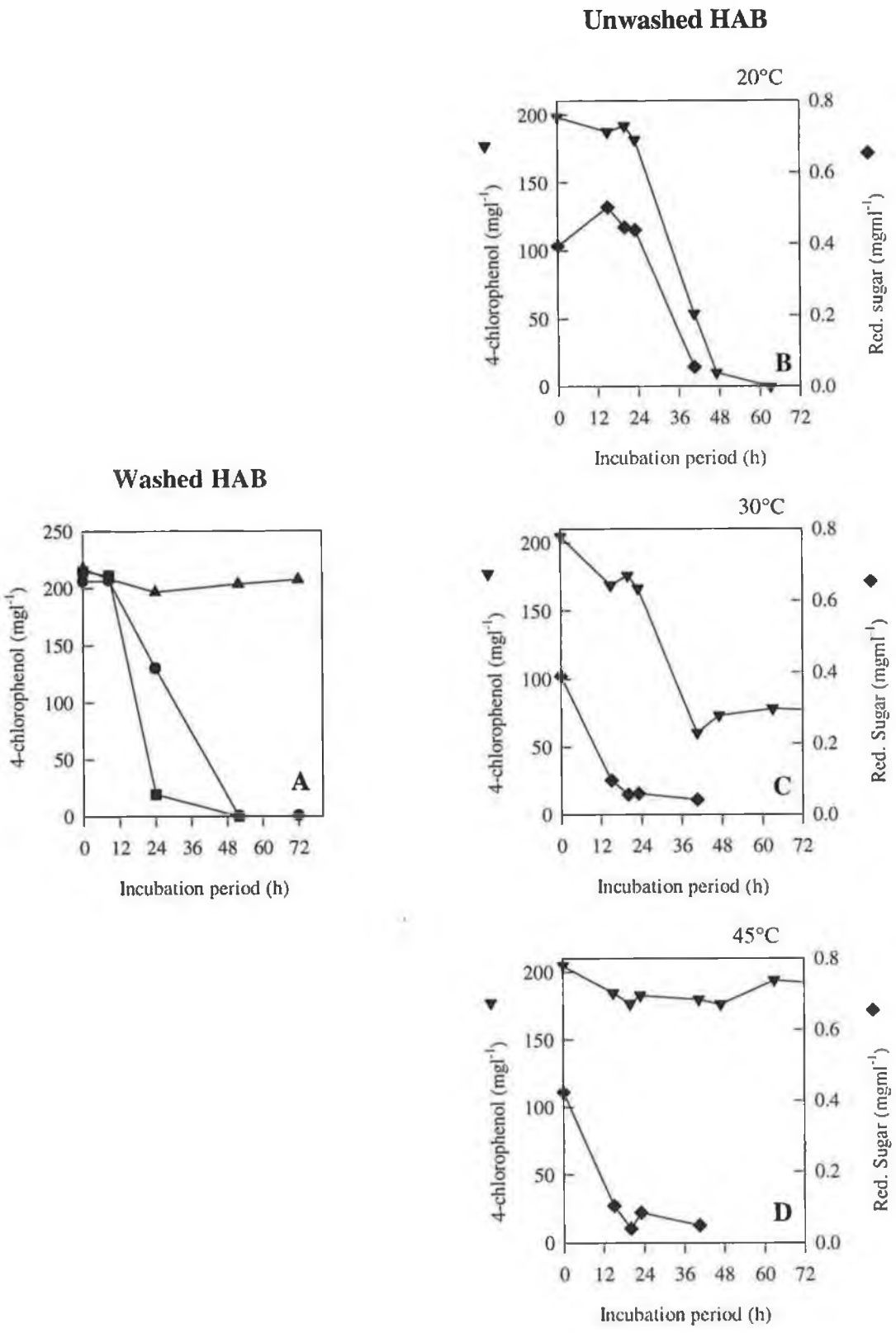


Fig. 24 Substrate removal during growth on 4-chlorophenol by washed (A) and unwashed (B, C, D) HAB incubated at various temperatures.

Legend to Graph A: 20°C, ●, 30°C, ■, 45°C, ▲.

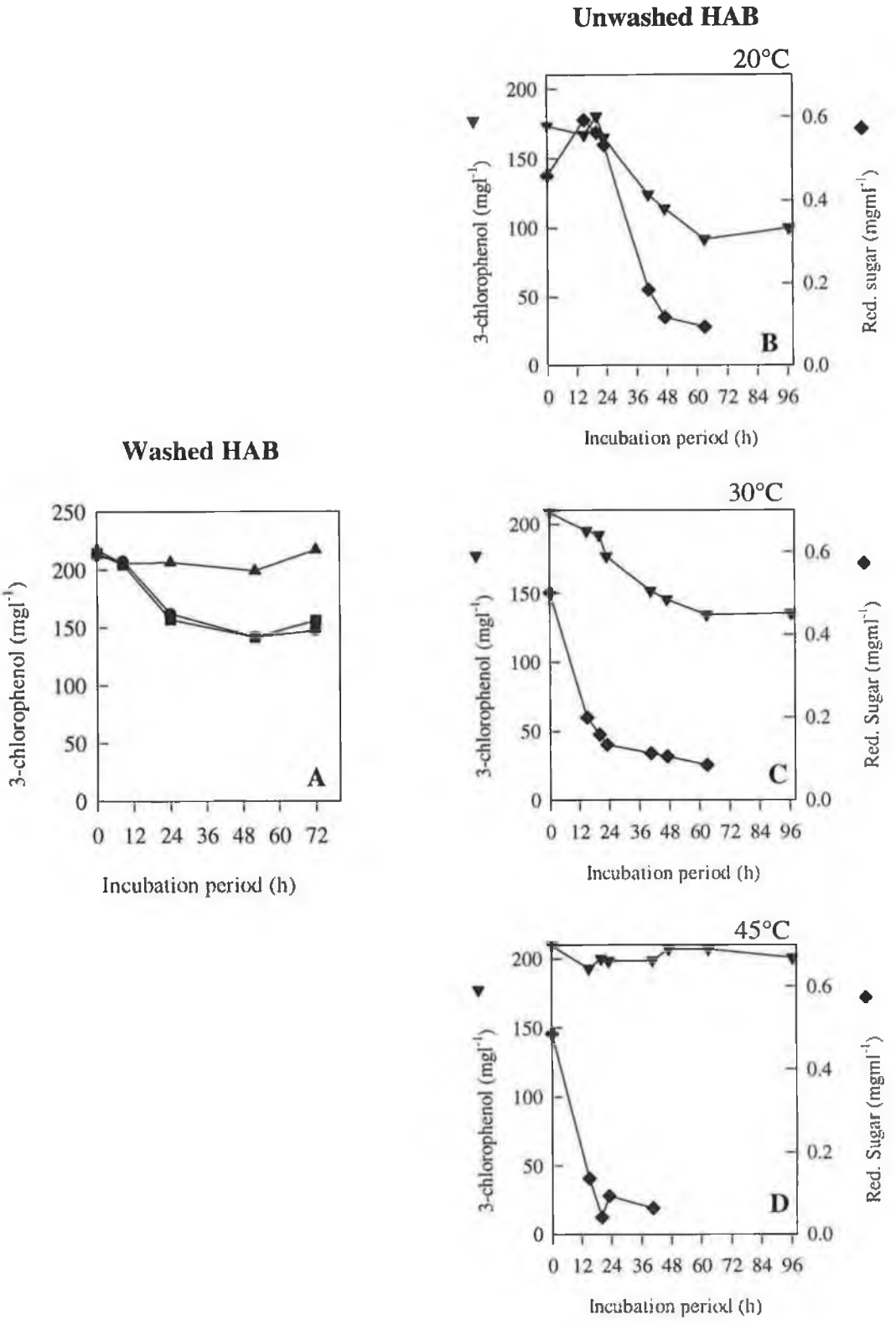


Fig. 25 Substrate removal during growth on 3-chlorophenol by washed (A) and unwashed (B, C, D) HAB incubated at various temperatures.

Legend to Graph A: 20°C, ●, 30°C, ■, 45°C, ▲.

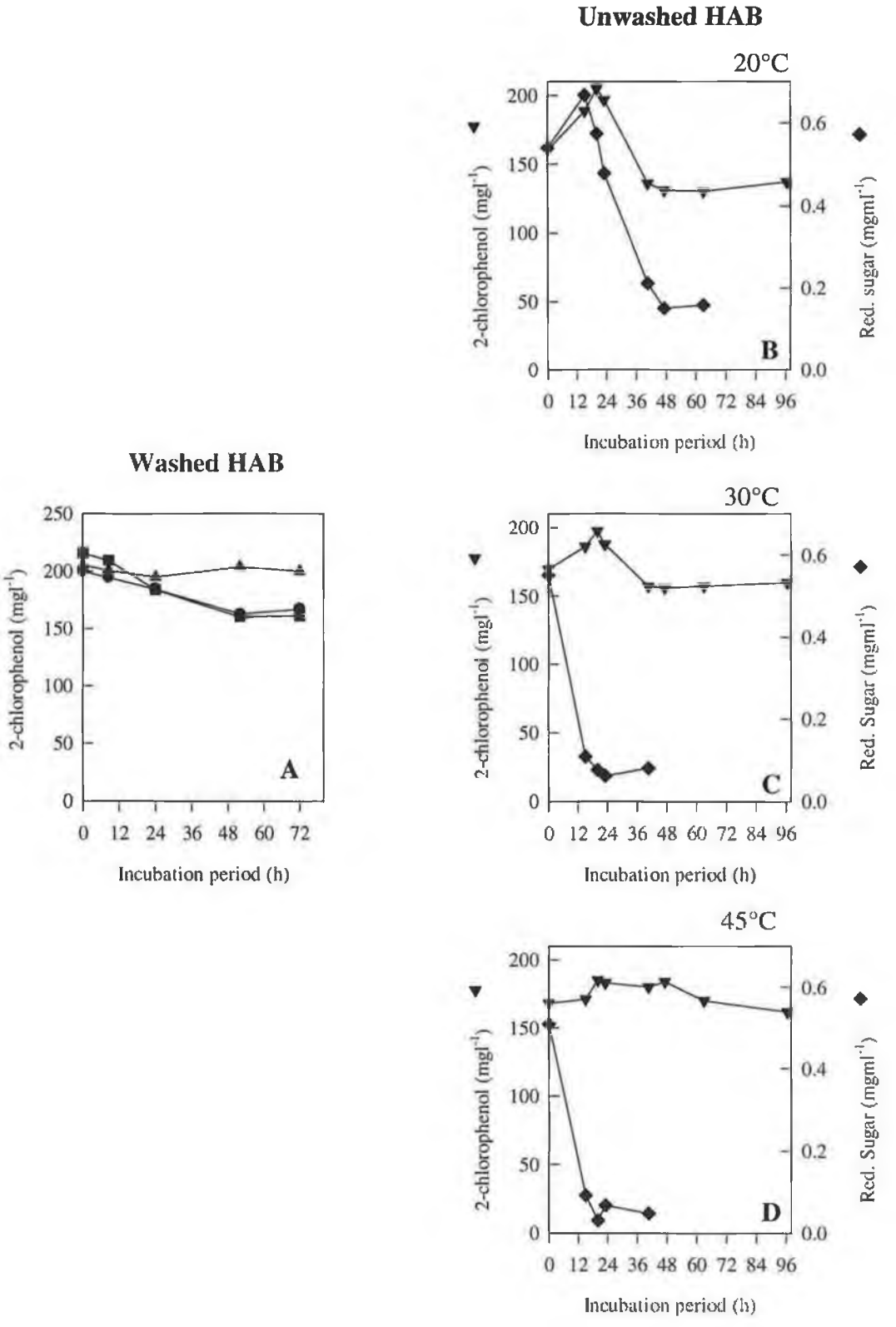


Fig. 26 Substrate removal during growth on 2-chlorophenol by washed (A) and unwashed (B, C, D) HAB incubated at various temperatures.

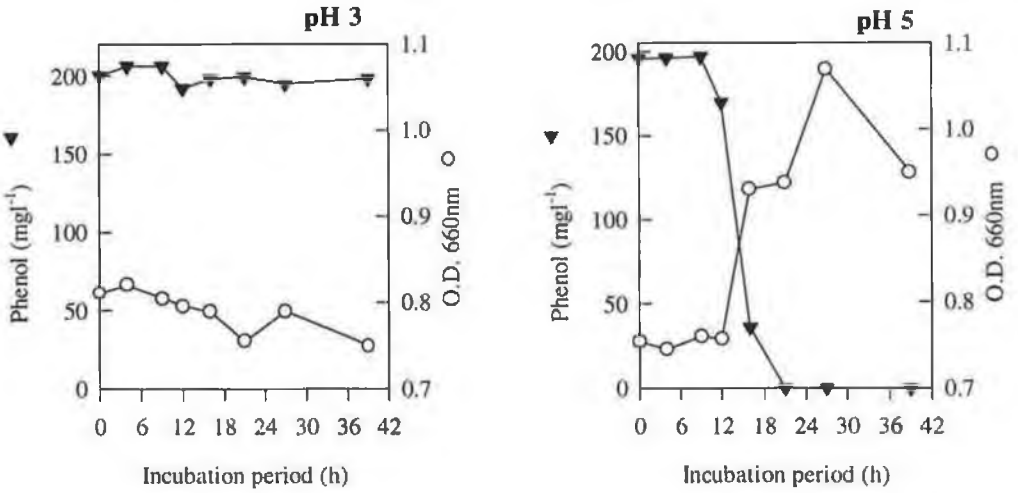
Legend to Graph A: 20°C, ●, 30°C, ■, 45°C, ▲.

3. 2. 3 The influence of medium pH on substrate removal.

Variations in pH during the removal of phenol and mono-chlorophenol by the mixed microbial population, under the variety of conditions used, were usually minimal. The prevailing pH rarely deviated substantially from the initial pH of 7.0. In the presence of 1% glucose however, large pH drops were noted. It was therefore of interest to determine the effect of various pH's, in the range pH 3-8, on phenolic removal by the mixed cultures. The use of four buffers (Universal buffer; Citric acid - Na_2HPO_4; KH_2PO_4 - NaOH; Na_2HPO_4 - NaH_2PO_4) was investigated. The effect of various concentrations (0.01, 0.05, 0.1, 0.5, 1M) of these buffers on phenolic removal at pH 7.0 was studied. The presence of the buffers exerted inhibitory effects on phenolic removal. It was subsequently decided to study the effect of medium pH, without using buffers (Greer *et al*, 1990). The pH of the *Pseudomonas* Minimal Medium was adjusted using 10M NaOH or 10M HCl, prior to autoclaving. The pH drift in the unbufferred flasks was monitored during the removal of phenol and mono-chlorophenols by the washed and unwashed inocula and only slight pH deviations, no greater than 0.5 unit, were noted.

3. 2. 3. 1 Effect of medium pH on phenol removal.

No phenol removal was achieved by washed or unwashed HAB at pH 3 (Figs. 27, 28). Removal of the additional carbon in the unwashed formulation however, was achieved at this low pH. Utilisation of this sugar was preceded by a lag period and was reflected in a certain amount of growth. With the washed culture, the rates of phenol degradation and the length of the lag periods did not vary within the range of pH 5-8. Comparable phenol removal, at a rate of 0.26 $mgl^{-1}h^{-1}mg^{-1}$, has been previously observed with washed HAB at pH 7 (Section 3. 1. 1). Similarly, little difference in phenol removal by the unwashed population was observed within the range of pH 5-8. These rates were similar to those achieved by the unwashed culture at pH7 in earlier studies (Section 3. 1. 1) and also as before, were lower than those achieved by the washed culture. The rapid sugar removal which preceded phenol removal had also been observed previously.



pH	Phenol Removal ($mgl^{-1}h^{-1}mg^{-1}$)
3	-
5	0.22
7	0.26
8	0.24

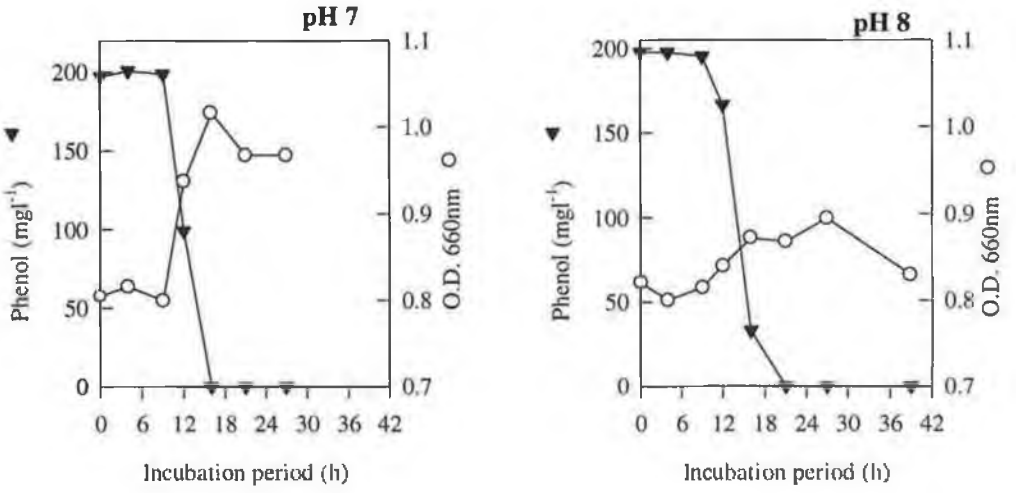
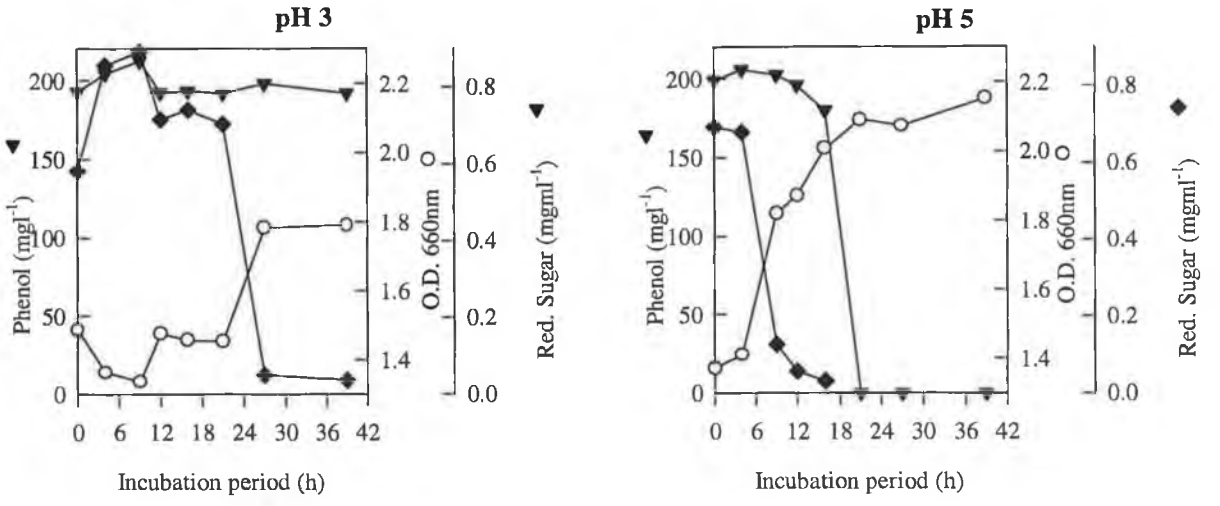


Fig. 27 The effect of pH on growth and phenol ($200mgl^{-1}$) removal by washed HAB.



pH	Phenol Removal ($mgl^{-1}h^{-1}mg^{-1}$)
3	-
5	0.11
7	0.10
8	0.10

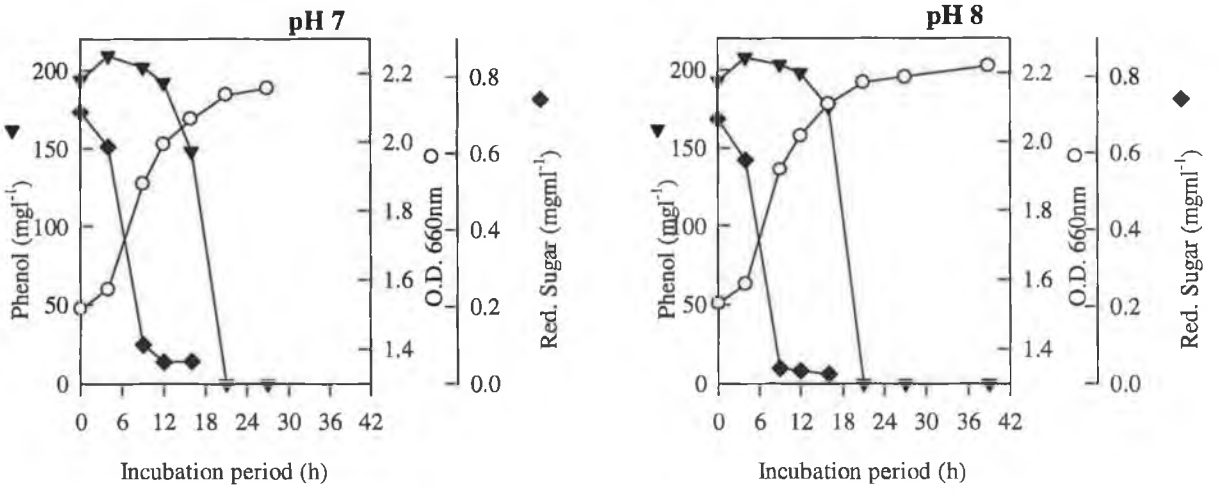


Fig. 28 The effect of pH on growth and phenol ($200mgl^{-1}$) removal by unwashed HAB.

3. 2. 3. 2 Effect of medium pH on mono-chlorophenol removal.

Mono-chlorophenol removal was significantly influenced by pH. No mono-chlorophenol removal by the mixed microbial populations was observed at pH 3. As before at pH 7 (Section 3. 1. 2), complete removal of 4-chlorophenol was achieved by the washed culture and removal in the presence of additional carbon was relatively poor (Table 5). Large decreases in 4-chlorophenol removal by the washed and unwashed cultures, down to 26.9 and 23.4% respectively, were observed at pH 8. Inhibition was even more considerable at pH 5.

Removal of 3-chlorophenol was incomplete within the pH range examined. As before at pH 7 (Section 3. 1. 2), approximately 30% removal was achieved by the washed and unwashed cultures (Table 5). Slight improvements in removal were noted at pH 8. With the unwashed culture, 3-chlorophenol removal was optimum at pH 5. As before at pH 7 (Section 3. 1. 2), 2-chlorophenol removal by both inocula was poor. Slight improvements were noted during incubation of the unwashed culture at pH 5. Optimum 2-chlorophenol was achieved by the washed population at pH 8.

Table 5: Effect of medium pH on percentage removal of mono-chlorophenols by washed (W) and unwashed (UW) HAB.

	4-cp		**3-cp**		**2-cp**	
pH	W	UW	W	UW	W	UW
3	No Removal		No Removal		No Removal	
5	<1	6.4	20.63	59.09	9.6	13.97
7	100	64.5	27.23	33.24	11.3	4.4
8	26.9	23.4	53.63	44.49	32.64	3.86

Sugar removal by the unwashed culture did not appear to be influenced by pH's of between 5 and 8 (Fig. 29). At pH3 however, marked reductions in sugar removal were observed. Complete inhibition of removal was noted in the presence of the more toxic 3- and 2-chlorophenol.

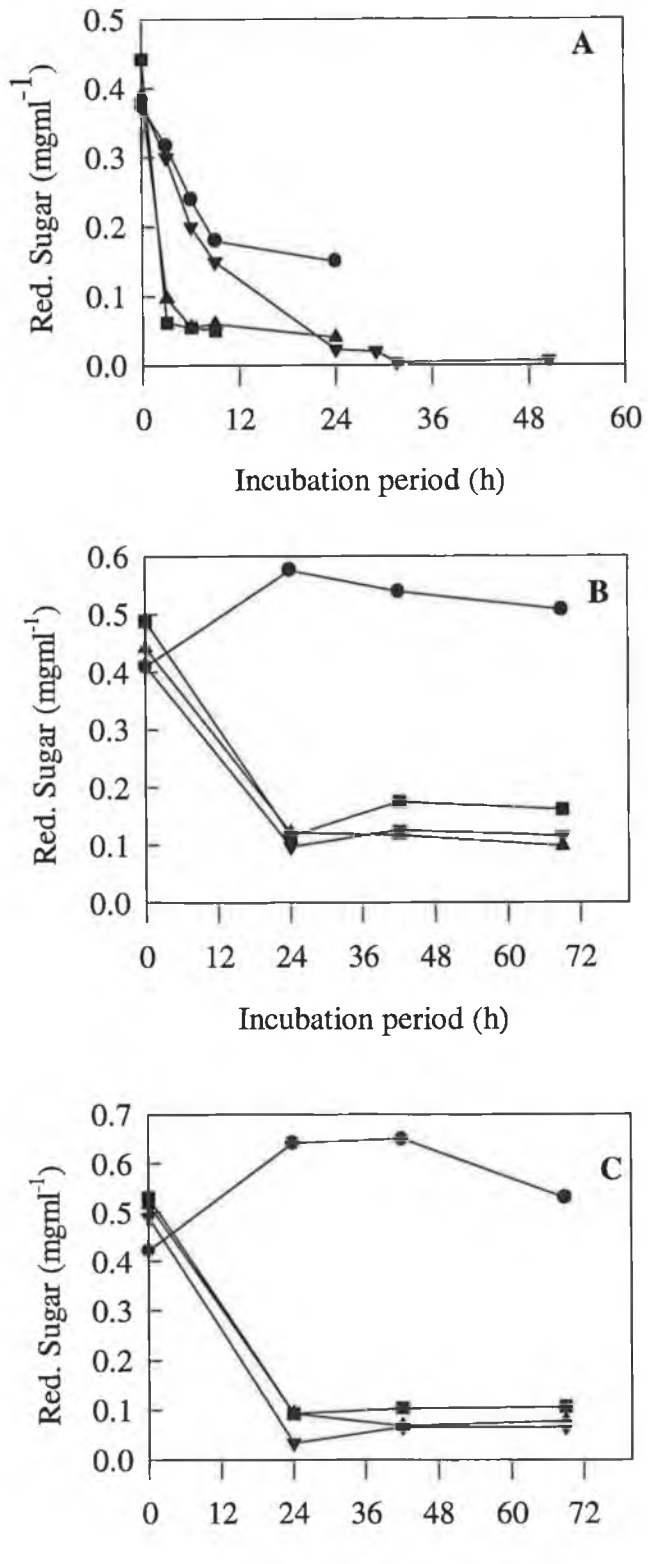


Fig. 29 The effect of pH on sugar removal by unwashed HAB in the presence of 4-chlorophenol (A), 3-chlorophenol (B) and 2-chlorophenol (C).

Legends: pH 3, ●, pH 5, ■, pH 7, ▲, pH 8, ▼.

3. 2. 4 The influence of acclimation on phenol and mono-chlorophenol removal.

HAB bacteria were grown on the substrate ($200mgl^{-1}$) until its exhaustion. The bacteria were then considered acclimated to the substrate and were used to inoculate fresh media containing a phenolic compound. The biomass was only acclimated to substrates that were fully degraded i.e. phenol and 4-chlorophenol.

3. 2. 4. 1 Phenol degradation by phenol-acclimated cells.

The washed and unwashed form of the bioaugmentation product were considered to be acclimated to phenol, following growth on the substrate for 12-15 hours. Both forms were then inoculated into fresh minimal media containing a range of phenol concentrations. Phenol removal by these acclimated cells was monitored. Removal by the acclimated cells was compared with earlier results using unacclimated HAB (Section 3. 1. 1) and displayed in Table 6.

As before (Section 3. 1. 1), no removal was observed at phenol concentrations of 1500 and $2000mgl^{-1}$ (Table 6). While phenol removal in the absence of acclimation was always preceded by a lag period, which varied with initial phenol concentration, no such delay was observed following acclimation to phenol.

Specific rates of phenol removal, at concentrations up to $800mgl^{-1}$, by the washed acclimated cells were similar to the rates achieved by thier unacclimated equivalent. At higher phenol concentrations, substrate inhibition became significant to the unacclimated cells, as rates of removal decreased with further increases in phenol concentration. Following acclimation, higher phenol concentrations, greater than $1000mgl^{-1}$, were necessary to cause this rate decrease.

Due to the presence of additional carbon, phenol removal rates achieved with unwashed cells (without acclimation) were lower than those achieved by the washed bacteria (Section 3. 1. 2). During the acclimation process, the additional carbons, normally associated with the unwashed population, were exhausted and phenol removal by both acclimated cultures was comparable (Table 6). As with the washed cultures, a substantial drop in the removal rate was observed at phenol concentrations above $1000mgl^{-1}$, rather than $800mgl^{-1}$,with the unacclimated population.

Table 6: The removal of various concentrations of phenol (200 - $2000mgl^{-1}$) by phenol-acclimated cells. Rate * - Specific removal rate in $mgl^{-1}h^{-1}mg^{-1}$.

	WASHED HAB				UNWASHED HAB			
Acclimation	-		+		-		+	
Phenol (mgl^{-1})	**Lag** (h)	**Rate ***	**Lag** (h)	**Rate ***	**Lag** (h)	**Rate ***	**Lag** (h)	**Rate ***
200	9	0.26	0	0.27	12	0.08	0	0.22
500	9	0.29	0	0.27	15	0.09	0	0.21
800	30	0.25	0	0.20	46	0.10	0	0.19
1000	52	0.12	0	0.22	56	0.07	0	0.23
1300	74	0.10	0	0.06	78	0.06	0	0.14
1500	No Removal		No Removal		No Removal		No Removal	
2000	-		No Removal		-		No Removal	

3. 2. 4. 2 Mono-chlorophenol removal by phenol-acclimated cells.

Washed HAB biomass was acclimated to phenol ($200mgl^{-1}$) and then presented with varying concentrations of the mono-chlorophenol isomers. Previously (Section 3. 1. 2), 4-chlorophenol was successfully removed by the washed culture, at concentrations up to and including $200mgl^{-1}$. Following acclimation to

phenol however, complete 4-chlorophenol removal at concentrations up to 400mgl^{-1} was achieved by the mixed microbial population (Table 7). At a concentration of 500mgl^{-1}, only 10.9% removal was achieved by the acclimated cells.

In contrast, acclimation of the cells to phenol did not enhance the removal of either 2- or 3-chlorophenol. Similar percentages removal of these mono-chlorophenol isomers were achieved by the both the acclimated and un-acclimated populations.

Table 7: The removal of various concentrations of the mono-chlorophenol isomers (200 - 2000mgl^{-1}) by phenol-acclimated cells.

	% Removal					
Acclimation	-	+	-	+	-	+
Conc. (mgl^{-1})	*4-cp*		*3-cp*		*2-cp*	
50	100	100	45.9	73.7	48.8	28.3
100	100	100	49.7	54.6	45.1	16.1
200	100	100	42.0	43.7	43.4	15.2
300	66.7	100	32.3	47.2	28.4	23.4
400	36.7	100	28.7	36.9	11.7	26.0
500	<1	10.9	<1	27.2	<1	24.3

3. 2. 4. 3 4-chlorophenol (cp) removal by 4-cp-acclimated biomass.

Complete 4-chlorophenol (200mgl^{-1}) removal was achieved by the washed culture (Section 3. 1. 3). Removal of 4-chlorophenol by unacclimated washed HAB was at a rate of 12mgl^{-1} h^{-1} and was preceded by a lag period of 12 hours. The biomass was acclimated 4-chlorophenol, at a concentration of 200mgl^{-1}.

Following acclimation, no lag period was evident prior to 4-chlorophenol removal and an improved rate of removal, of 16mgl^{-1} h^{-1}, was achieved (Fig. 30).

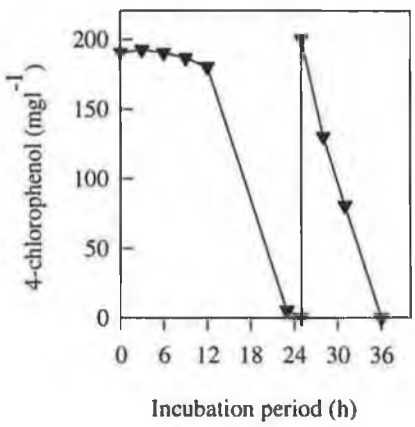


Fig. 30 The removal of primary (by unacclimated cells) and secondary (by 4-cp-acclimated cells) 4-chlorophenol quantities by the mixed microbial population.

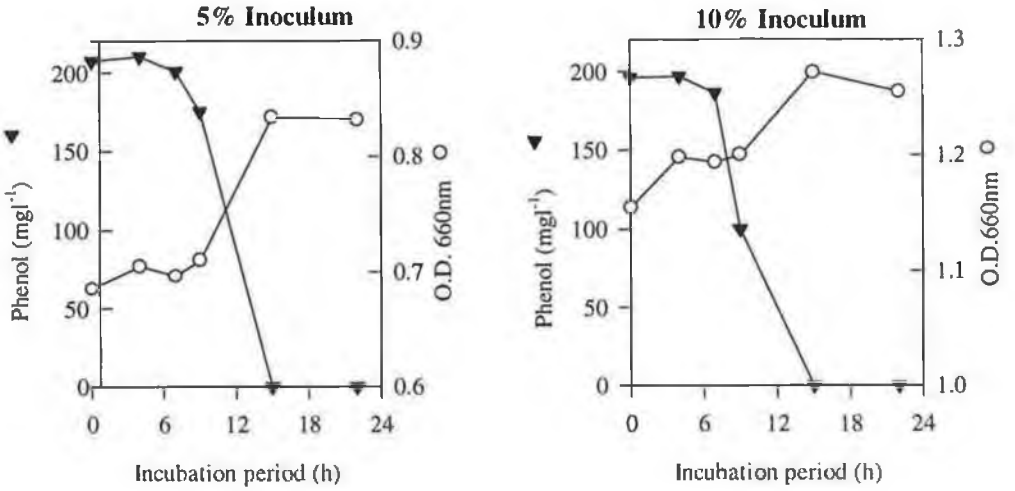
3. 2. 5 The effect of inoculum size on phenol and mono-chlorophenol removal.

The effect of varying the inoculum size, of both washed and unwashed HAB, on the removal of phenol and mono-chlorophenol was determined. The recommended level of inoculum was 10% (Goulding *et al*, 1988). Phenolic removal by the mixed microbial population at this inoculum size has been studied (Section 3.1). In order to examine the effects of varying biomass levels, a range of inoculum sizes above and below this level were selected. Phenol and mono-chlorophenol ($200mgl^{-1}$) removal, by both washed and unwashed HAB, at a variety of inoculum sizes (5, 10, 15, 20%) was monitored.

3. 2. 5. 1 Effect of inoculum size on phenol removal.

As can be seen in Fig. 31, the greater the inoculum size of washed HAB the faster the rate of removal, ranging from $25mgl^{-1}$ h^{-1} with a 5%inoculum to $72mgl^{-1}$ h^{-1} with the highest level of inoculum. The lag periods preceding removal did not change substantially with the different inoculum sizes. At an inoculum size of 10%, phenol removal was similar to that observed previously (Section 3. 1. 1).

The pattern was reversed however, with the unwashed HAB, where the additional carbon exerted a retarding effect on phenol removal (Fig. 32). Due to the mode of preparation of unwashed HAB, increasing the inoculum size also increased the concentration of additional carbon present in the medium, and therefore higher inoculum sizes resulted in greater retardation of phenol removal. With increasing inoculum sizes and consequently increasing additional carbon concentrations, the lag periods preceding phenol removal lengthened. A diauxic pattern of dual substrate removal was observed, with complete exhaustion of the secondary carbon occurring prior to the onset of phenol removal. The rate of phenol removal did not vary greatly with inoculum sizes of 5, 10 and 15%. A greater than 50% decrease in this rate was noted however, with the highest inoculum size.



Inoculum Size(%)	Phenol Removal ($mgl^{-1}h^{-1}$)
5	25.12
10	37.21
15	65.35
20	72.0

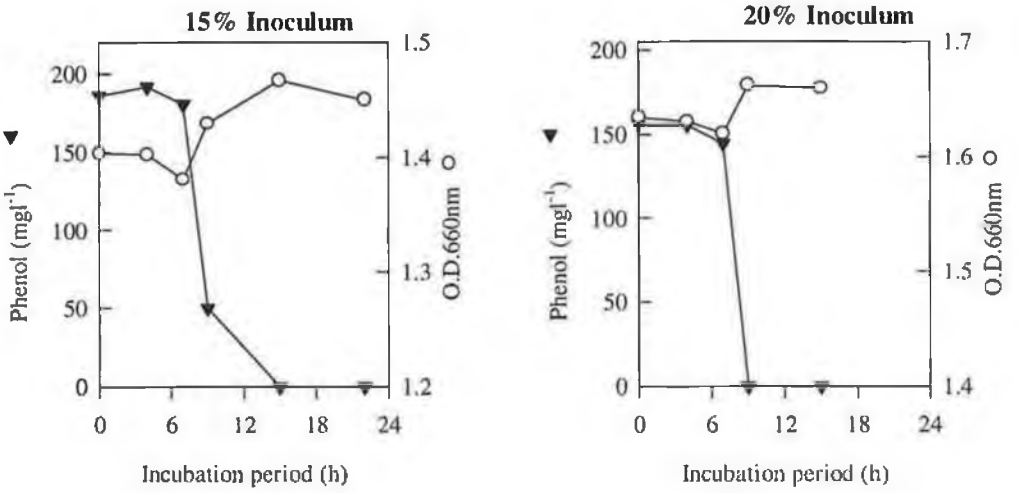
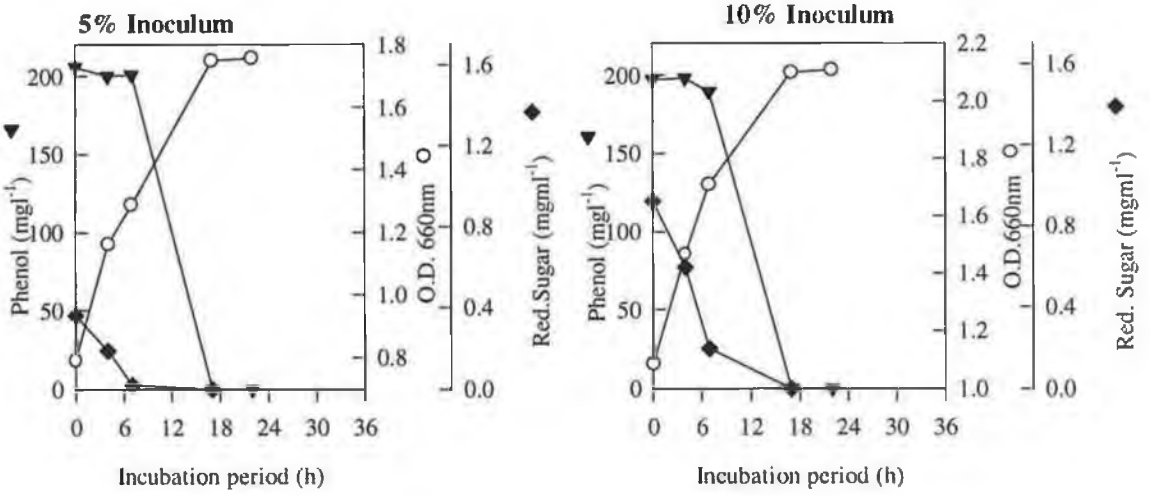


Fig. 31 The effect of inoculum size on phenol removal by washed HAB.



Inoculum Size(%)	Phenol Removal ($mgl^{-1}h^{-1}$)
5	20.6
10	18.9
15	16.0
20	8.9

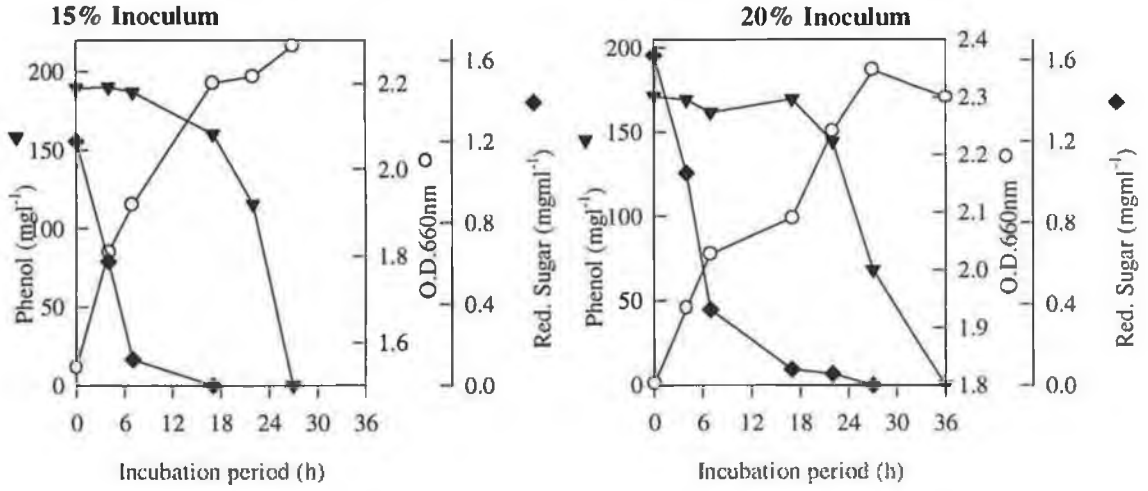


Fig. 32 The effect of inoculum size on phenol removal by unwashed HAB.

3. 2. 5. 2 Effect of inoculum size on mono-chlorophenol removal.

The percentages removal of the mono-chlorophenols were similar to before, when using a 10% inoculum size, and also the ease of removal pattern followed the order: 4- > 3- > 2-chlorophenol (Section 3. 1). As with phenol, the presence of a greater load of washed biomass produced improved mono-chlorophenol removal (Table 8). Complete 4-chlorophenol removal by the washed culture was achieved at all inoculum sizes used, with increasing inoculum sizes leading to more rapid removal. At the lowest inoculum size, complete 4-chlorophenol removal was achieved within 55 hours. Less than 24 hours were necessary for removal using an inoculum size of 20%. The removal of both 3- and 2-chlorophenol was also enhanced at the higher inoculum sizes but complete removal was never achieved.

As before, the inhibitory effect of the additional carbon was noted. Only partial 4-chlorophenol removal was achieved by the unwashed inoculum at levels of 10% or higher. At an inoculum size of 5% however, where additional carbon levels were minimal, complete 4-chlorophenol was achieved by the unwashed culture in 24 hours. The percentages removal of 3-chlorophenol by the unwashed population was optimum at the highest inoculum size, with removal improving with increasing inoculum size. Optimum 2-chlorophenol removal by the unwashed culture was also obtained when using a 20% inoculum size.

Table 8: Effect of inoculum size on mono-chlorophenol ($200mgl^{-1}$) removal by washed (W) and unwashed (UW) HAB.

	% Removal					
	4-cp		*3-cp*		*2-cp*	
Inoculum (%)	W	UW	W	UW	W	UW
5	$100_{(55h)}$	$100_{(24h)}$	24.9	19.9	6.9	2.7
10	$100_{(45h)}$	68.4	38.9	23.4	3.6	6.0
15	$100_{(24h)}$	61.8	41.5	30.2	14.7	4.2

3. 3 The use of 4-chlorophenol as a model substrate to illustrate how environmental conditions can be manipulated in order to facilitate substrate removal.

Environmental conditions had been found to significantly influence substrate removal by the mixed microbial population (Section 3. 2). Whether a given environmental condition exerted positive or negative effects on phenolic removal, was very often linked to the mode of utilisation of any additional carbon present. For example, the inhibitory effects of additional carbon in the unwashed formulation observed at 30°C, were alleviated by incubation at 20°C where sugar removal was relatively slow (Section 3. 2. 2. 2). Also, the presence of glucose, but not fructose, inhibited phenolic removal (Section 3. 2. 1). The removal patterns of the sugars were different however, with fructose removal being slower. Further investigations were necessary however, in order to substantiate these observations.

The degradative capabilities of the mixed culture have been shown to be influenced by the toxicity of the phenolic substrate (Section 3. 1), as well as the prevailing environmental conditions (Section 3. 2). Phenol proved to be the least toxic of the substrates and its removal was achieved under a broad range of environmental conditions. 3- and 2-chlorophenol were particularly toxic to the system and complete removal was never achieved. With 4-chlorophenol however, complete removal was achieved but only under limited conditions. It was therefore decided to attempt to optimise substrate removal by manipulation of environmental condition,, using 4-chlorophenol as a model toxic substrate as its removal was most influenced by environmental conditions.

3. 3. 1 The influence of glucose supplementation.

It had been shown that 4-chlorophenol ($200mgl^{-1}$) removal was inhibited in the presence of a range of concentrations of glucose (Section 3. 2. 1. 1). 4-chlorophenol

removal proceeded while the sugar was present and ceased upon exhaustion of this additional carbon. It was therefore decided to investigate the effect, if any, of further glucose additions on 4-chlorophenol transformation by the mixed microbial population. Flasks which contained 4-chlorophenol ($200mgl^{-1}$) and a range of glucose concentrations were inoculated with the washed inoculum and substrate removal was monitored. Glucose levels of 0.1 and 0.5% were selected, as removal of 4-chlorophenol was incomplete at these concentrations and the pH effects were minimal. Following the disappearance of the sugar, the medium was supplemented with additional glucose, such that the glucose concentration was returned to its original level.

Glucose supplementation did not significantly influence 4-chlorophenol transformation (Table 9). Percentages removal of 66.8 and 52.4 were achieved, in the presence of glucose at concentrations of 0.1 and 0.5%, respectively. Similar rates were seen previously in Section 3. 2. 1. 1. Comparable 4-chlorophenol transformation was achieved with glucose supplementation. The role of pH may be significant. Minor decreases in pH values, no greater than 0.9unit, were observed during substrate removal from the primary 4-chlorophenol/glucose mixtures (or in the absence of supplementation). Similar decreases have been previously noted in the presence of such glucose concentrations (Section 3. 2. 1. 1). The utilisation of the secondary quantity of glucose, by the mixed microbial population, resulted in further pH decreases in the culture fluid to below pH 5. 4-chlorophenol removal has been found to be inhibited at pH's of 5.0 and less (Section 3. 2. 3. 2).

Table 9: The influence of glucose supplementation on the percentage 4-chlorophenol(cp) ($200mgl^{-1}$) removal, in the absence of pH control.

	Without Supplementation		**With Supplementation**	
Glucose (%)	*% 4-cp Removal*	*Min. pH*	*% 4-cp Removal*	*Min. pH*
0.1	66.8	6.54	65.3	4.95
0.5	52.4	6.15	50.6	3.67

3. 3. 2 The influence of a combination of glucose supplementation and pH control.

Large variations in pH were observed in previous glucose supplementation experiments (Section 3. 3. 1. 1). Further studies were therefore necessary to determine the influence of glucose supplementation under controlled pH conditions. As before, concentrations of glucose of 0.1 and 0.5% were used and following exhaustion of the sugar, supplementation returned the glucose concentration to its original level. Manual pH adjustment was used in preference to buffering which had been shown to adversely effect phenolic removal (Section 3. 2. 3). For control purposes, pH adjustments were carried out in all flasks, both supplemented and unsupplemented.

Glucose supplementation, when used in conjunction with pH control, was found to facilitate further 4-chlorophenol transformation and resulted in the complete removal of 4-chlorophenol (Fig. 33). In the presence of 0.1% glucose, only 65% removal of 4-chlorophenol was achieved in the unsupplemented flasks. Further transformation of the 4-chlorophenol was observed however, upon the addition of supplementary glucose. Similar observations were made in the presence of 0.5% glucose. In both cases, concurrent removal of the substrates was observed, with little 4-chlorophenol removal occurring following exhaustion of the primary quantity of sugar. Glucose supplementation resulted in an extended period of concurrent substrate removal and thereby appeared to facilitate enhanced 4-chlorophenol transformation. The rate of utilisation of the secondary glucose was more rapid.

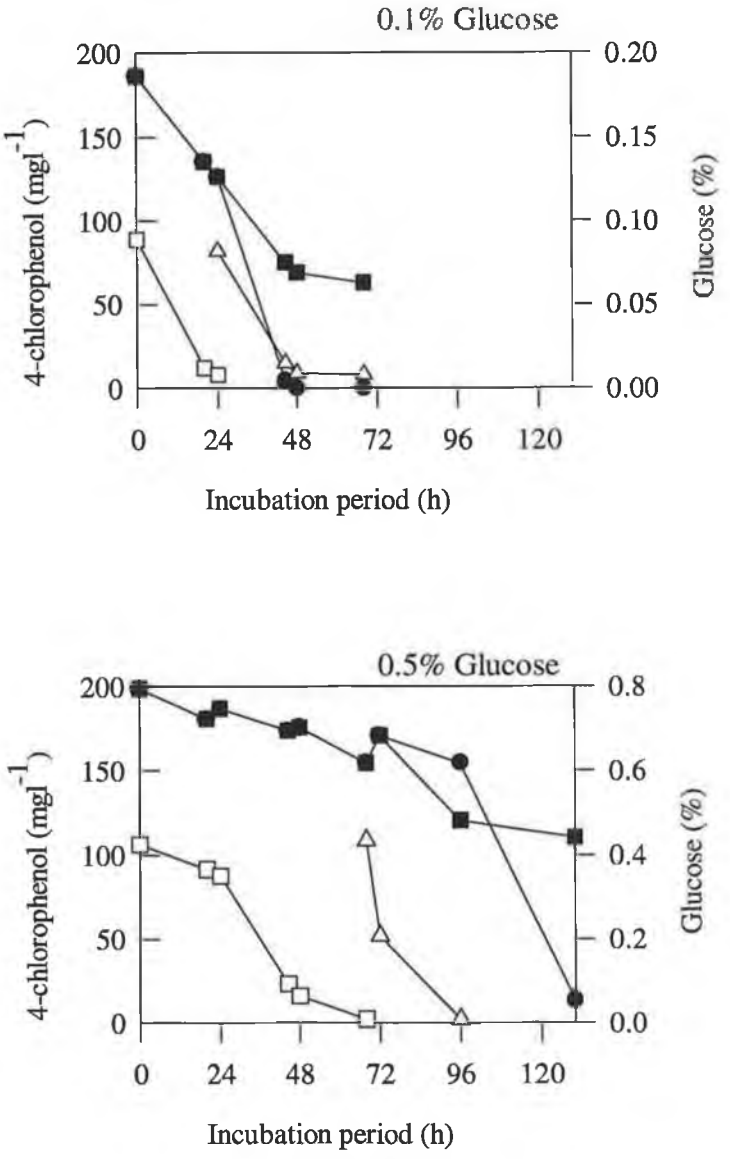


Fig. 33 The influence of glucose supplementation on 4-chlorophenol removal by the mixed microbial population under controlled pH conditions.

Legends: 4-chlorophenol removal in unsupplemented flasks, ■, supplemented flasks, ●. Primary glucose, , supplementary glucose, Δ.

3. 3. 3 The influence of incubation temperature.

Having noted the alleviation of inhibition observed with the unwashed inoculum by incubation at 20°C (Section 3. 2. 2. 2), it was of interest to determine the change in glucose removal pattern, if any, that would result from incubation at 20°C and the resulting effect on 4-chlorophenol removal. Therefore the removal of 4-chlorophenol ($200mgl^{-1}$) in the presence of a range of glucose concentrations (0.01, 0.05, 0.1, 0.2, 0.5 and 1%) was examined at the lower incubation temperature.

It was found that the inhibition observed during 4-chlorophenol removal in the presence of glucose incubated at 30°C (Section 3. 2. 1. 1) was indeed alleviated by incubation at 20°C, and complete 4-chlorophenol removal was achieved at glucose concentrations up to 0.5% (Fig. 34). In all cases, there was a lag period preceding 4-chlorophenol and glucose removal by the mixed microbial culture. The lengths of these lag periods were substantially longer than those observed at 30°C (Section 3. 2. 1. 1). The onset of glucose removal preceded that of 4-chlorophenol. A period of concurrent removal of the substrates was observed. Growth of the micro-organisms was preceded by a lag period, which was of equal length with that preceding glucose removal. Biomass levels were observed to increase as substrate concentration decreased. A pH drop did occur during the incubation (Table 10) with the minimum pH obtained coinciding with the exhaustion of glucose. This pH drop was minor, except at a glucose concentration of 1% where a minimum pH of 3.8 was reached. 4-chlorophenol removal by the mixed microbial population has been shown to be inhibited at such pH's (Section 3. 2. 3. 2). The inhibition of 4-chlorophenol removal in the presence of 1% glucose is therefore most likely a pH effect.

The rates of 4-chlorophenol removal in the presence of glucose at concentrations of 0.05% or higher were relatively slow, when compared to that achieved with 0.01% glucose (Table 10). While the presence of glucose concentrations of 0.05% or greater did result in retarded 4-chlorophenol removal, this effect was not

concentration dependent. The rates of glucose removal, which in general were slower than those achieved at 30°C, increased with increases in glucose concentration. These rates were substantially higher than the rates of 4-chlorophenol removal.

Table 10: Rates of substrate removal from 4-chlorophenol(cp)(200mgl^{-1})/glucose mixtures by the mixed microbial population incubated at 20°C.

Glucose	**4-cp Removal**		**Glucose Removal**		**ΔpH**
(%)	($mgl^{-1}h^{-1}$)	($mgl^{-1}h^{-1}mg^{-1}$)	($mgl^{-1}h^{-1}$)	($mgl^{-1}h^{-1}mg^{-1}$)	
0.01	6.90	0.057	-	-	0.16
0.05	3.96	0.031	38	0.26	0.21
0.1	4.16	0.030	82.1	0.50	0.24
0.2	3.81	0.025	161	0.98	0.33
0.5	4.61	0.026	188.8	1.13	0.64
1.0	Incomplete Removal		224.5	1.37	3.18

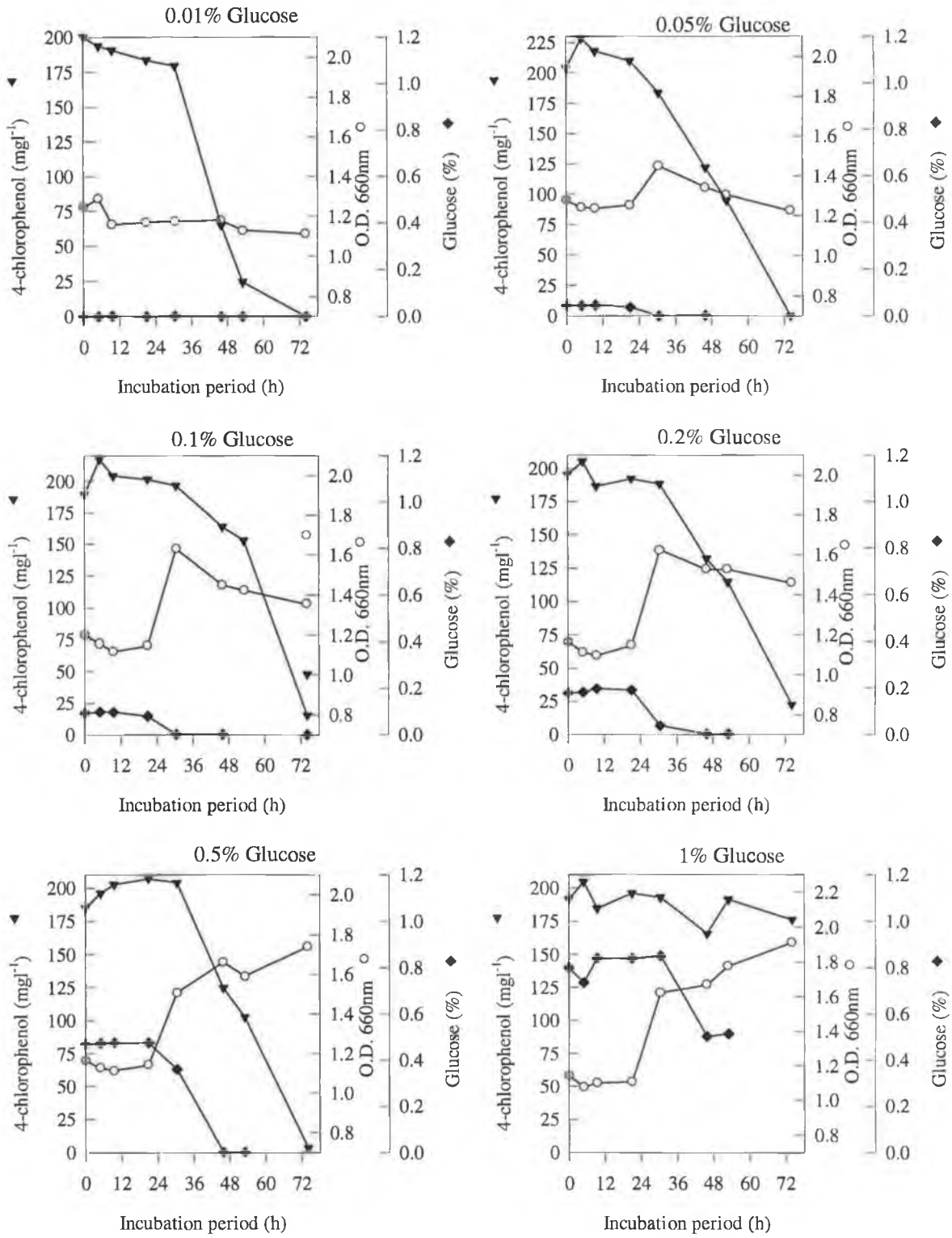


Fig. 34 The removal of 4-chlorophenol (200mgl^{-1}) in the presence of various concentrations of glucose (0.01-1%) by washed HAB incubated at 20°C.

3. 3. 4 The effect of acclimation to fructose on removal of 4-chlorophenol fructose mixtures.

Due to the manner in which the mixed microbial population had been initially produced, the organisms were acclimated to glucose, but not to fructose. This acclimation appeared to influence the removal pattern of the sugar (Section 3. 2. 1). Fructose utilisation by the mixed culture had been observed to be relatively slow compared to glucose and was usually preceded by a relatively long lag period. The removal pattern of the sugar had been suggested to influence its effect on phenolic removal by the mixed microbial population (Section 3. 2. 1). It was therefore of interest to determine if acclimation of the culture to fructose would alter substrate removal patterns and consequently alter the effect of the sugar on phenolic removal. The mixed microbial population was acclimated to fructose (0.1%) (Section 2. 2. 3) and the removal of 4-chlorophenol ($200mgl^{-1}$) in the presence of a variety of fructose concentrations (0.01 to 1%) by the acclimated culture was studied.

The removal of 4-chlorophenol by the acclimated cells was inhibited by concentrations of fructose up to 0.5% (Fig. 35). Unlike before, little 4-chlorophenol removal was observed in the presence of fructose at concentrations of 0.01 and 0.05% by the fructose-acclimated culture. 4-chlorophenol removal proceeded in the presence of fructose concentrations of 0.1% and greater, but ceased upon exhaustion of the sugar. Complete 4-chlorophenol removal, at a rate of $0.022mgl^{-1}h^{-1}mg^{-1}$, was achieved however, in the presence of sufficient quantities of fructose i.e. where a concentration of 1% was used.

In contrast to earlier studies (Section 3. 2. 1. 2), no lag period prior to fructose removal was observed (Table 11). The pattern of removal of the substrates was now concurrent and very similar to that previously obtained in the presence of glucose (Section 3. 2. 1. 1). Preferential 4-chlorophenol removal had been observed during the removal of 4-chlorophenol/fructose mixtures by the unacclimated culture

(Section 3. 2. 1. 2). The fructose removal rates were found to increase with increases in its concentration and was consistently higher than the rates achieved by the unacclimated population (Table 11).

Decreases in the pH of the culture fluid, from the initial pH of 7.0, were observed. These pH variations were minor, even in the presence of 1% fructose, less than 1.2 unit, and therefore previous inhibitory pH effects observed in the presence of high glucose concentrations (Section 3. 2. 1. 1) did not apply. Where removal occurred, increases in biomass levels were observed and accompanied substrate disappearance. Growth was more substantial at higher concentrations of fructose.

Table 11: Fructose removal from 4-chlorophenol(200mgl^{-1})/fructose mixtures by fructose- acclimated and unacclimated cells.

	Without Acclimation			**With Acclimation**		
Fructose	**Lag**	**Fructose Removal**		**Lag**	**Fructose Removal**	
(%)	(h)	($mgl^{-1}h^{-1}$)	($mgl^{-1}h^{-1}mg^{-1}$)	(h)	($mgl^{-1}h^{-1}$)	($mgl^{-1}h^{-1}mg^{-1}$)
0.01	-	-	-	-	-	-
0.05	9	19.3	0.15	0	68.6	0.56
0.1	9	67.9	0.42	0	131.4	0.96
0.2	9	58.5	0..32	0	148.0	1.0
0.5	22	204.7	1.11	0	208.5	1.06
1.0	22	276.2	1.48	0	315.9	1.52

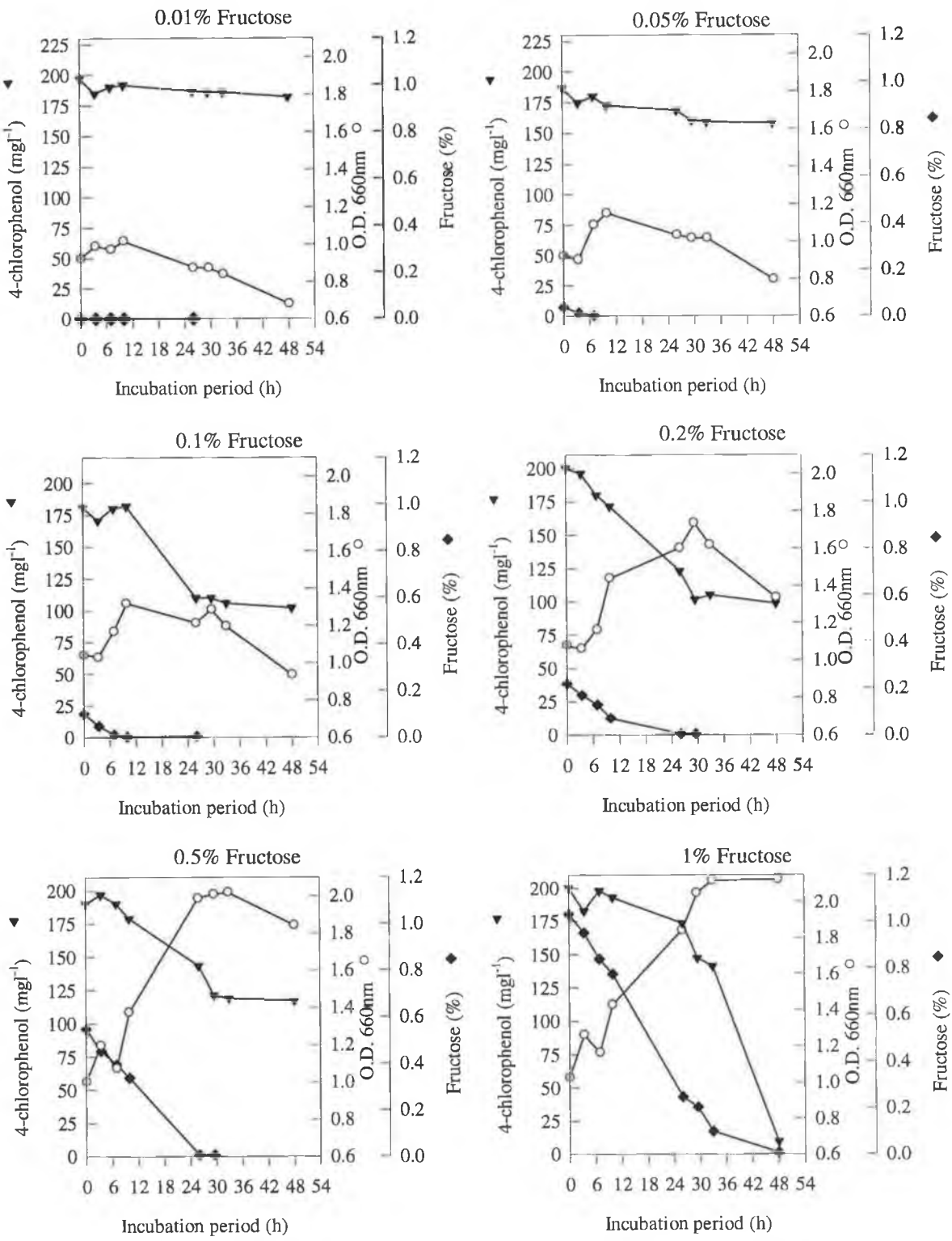


Fig. 35 The effects of the presence of fructose (0.01-1%) on 4-chlorophenol ($200mgl^{-1}$) removal by HAB, following acclimation to 0.1% fructose.

3. 4 A study of the microbiology of the system.

The bioaugmentation product, Biolyte HAB, was designed to degrade a range of substituted aromatic ocmpounds. It consisted of a minimum of eleven strains of bacteria including members of the genus *Pseudomonas* and *Actinomycetes* along with a brown rot fungus, *Trichoderma harzanium*. The micro-organisms were freeze-dried onto a cereal base. The formulation also contained surfactants, cryo-protectants and nutrients.

Phenol removal by the washed mixed culture was achieved (3. 1). The removal of 4-chlorophenol was also achieved but was found to be influenced by the prevailing environmental conditions which could be modified in order to facilitate removal (3. 3). The removal of both 3- and 2-chlorophenol was constantly poor and in general, environmental conditions exerted little influence. It was decided to determine if these results were due to the microbiology of the washed system. Flasks containing $200mgl^{-1}$ mono-chlorophenol, were inoculated with the mixed culture. Initial and final (after 24 hours) samples were taken and plated onto nutrient agar, malt extract/chloramphenicol agar and chlorophenol agar. The results are summarised in Fig. 36.

In all cases, growth of several colony types on nutrient agar was evident at both time points. The fungus was detected in the initial sample. Following growth of the mixed culture for 24 hours on both 4- and 3-chlorophenol, no fungus was detected. Fungal growth was detected however, following exposure to 2-chlorophenol. Bacterial growth on the malt extract agar was not detected due to the presence of chloramphenicol. In all cases, the growth observed on the chlorophenol agar was indistinct and it was difficult to observe individual colonies with the naked eye. The development of a yellow colour in the 4-chlorophenol agar was noted following approx. 24 hours incubation. This colour however, did not persist in the medium. Colour development was also noted on 3- and 2-chlorophenol agar plates. In this case, the colour of the agar did not change but the colonies appeared to contain a brown/black compound.

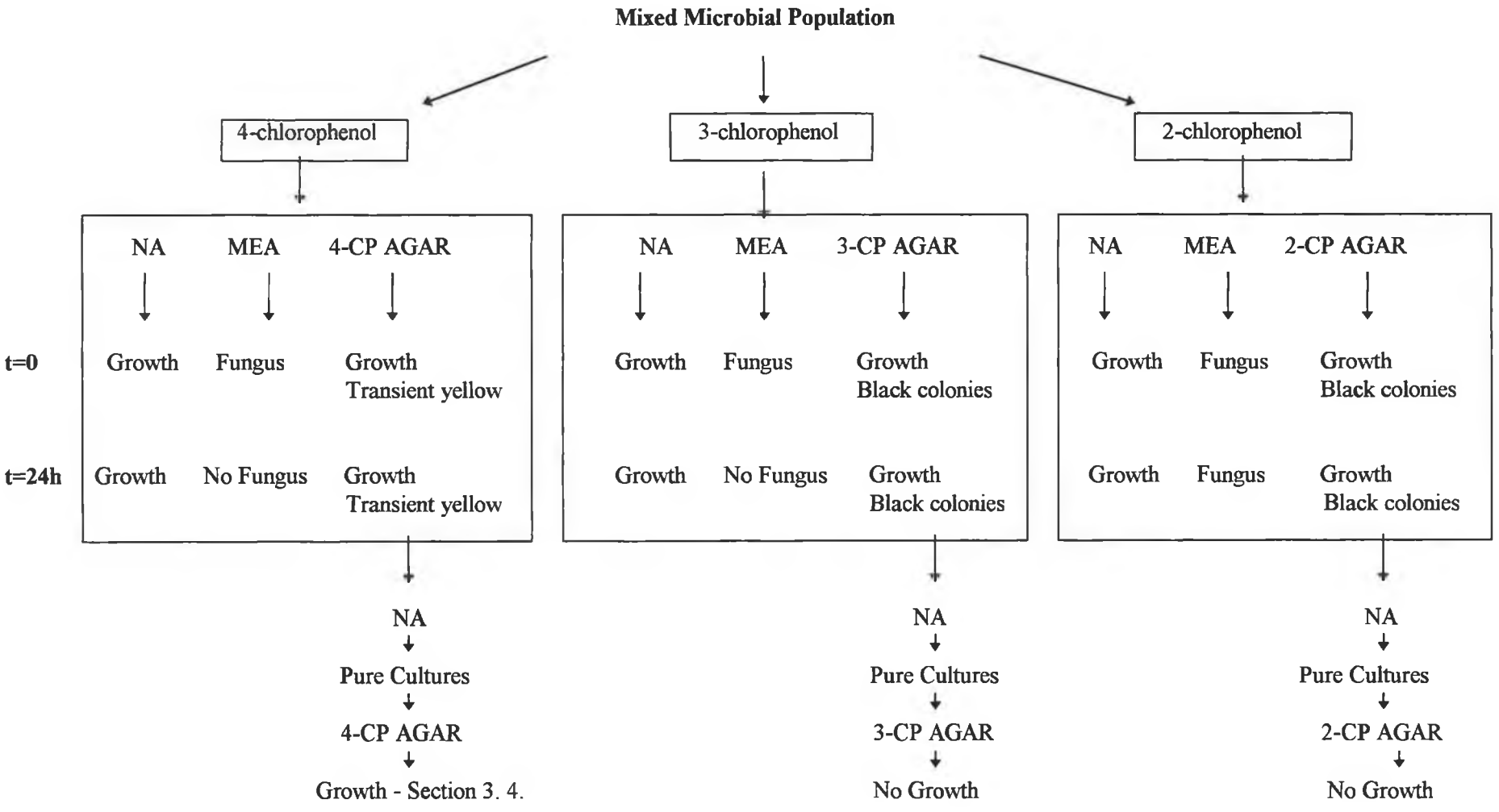


Fig. 36 The study of the microbiology of the system.

Attempts were made to isolate pure cultures from the chlorophenol agar. Streaks were taken from the chlorophenol agar plates and transferred onto nutrient agar. Several colony types were evident on the nutrient agar plates and were purified. The pure cultures were then transferred back onto chlorophenol plates. These pure cultures failed to grow on both 3- and 2-chlorophenol agar. Growth of pure cultures was obtained on 4-chlorophenol agar. These isolates were identified and characterised (Section 3. 4. 2). The fungus was isolated from the malt extract/chloramphenicol agar and characterised also (Section 3. 4. 1).

3. 4. 1 Characterisation of the role of the fungus.

A number of observations had been made which deemed it necessary to examine the contribution of the fungus to the degradative abilities of the mixed microbial population. The absence of degradative activity at low pH's, which often favour the predominance of fungi, suggested that the fungus may not play a substantial role in the degradation of the substrates examined (Section 3. 2. 3). It was therefore necessary to examine more closely the role played by this fungus in degradation by the mixed microbial population.

3. 4. 1. 1 Survival of the fungus in the mixed microbial population.

A variety of flasks were set up containing minimal medium, a phenolic substrate and HAB. It was found, following growth of the washed and unwashed mixed microbial populations on various concentrations of mono-chlorophenol isomers, that the fungus survived in the presence of 2-chlorophenol at concentrations of no more than $300mgl^{-1}$ (Table 12). No fungus was detected following exposure of the cultures to 3-chlorophenol at all concentrations examined. The fungus was again absent following exposure to 100 mgl^{-1} 4-chlorophenol or higher. Fungus was however,

detected following 24 hours incubation in the presence of 50mgl^{-1} 4-chlorophenol, but was no longer present after 48 hours. Similar results were obtained earlier (Fig. 36).

Table 12: Fungal detection following 24h incubation of the washed (W) and unwashed (UW) mixed microbial population on various concentrations of mono-chorophenol isomers. + fungus, - no fungus.

	4-chlorophenol		**3-chlorophenol**		**2-chlorophenol**	
Conc. (mgl^{-1})	W	UW	W	UW	W	UW
50	+	+	-	-	+	+
100	-	-	-	-	+	+
200	-	-	-	-	+	+
300	-	-	-	-	+	+
400	-	-	-	-	-	-
500	-	-	-	-	-	-

3. 4. 1. 2 Growth of the fungus alone on phenolic compounds.

The fungus was isolated from the mixed microbial population on malt extract agar which contained chloramphenicol. Fungal plugs were subsequently transferred to minimal media agar plates which contained various substrates and were incubated at 25°C. The fungus had demonstrated an ability to survive in the presence of 2-chlorophenol and therefore this phenolic was of particular interest. A range of 2-chlorophenol concentrations were examined and also a number of 2-chlorophenol/sugar mixtures.

Growth of the fungus was achieved on phenol and 2-chlorophenol only (Table 13). The amount of growth on 2-chlorophenol was influenced by concentration. Maximum growth was obtained with $100mgl^{-1}$, followed by $200mgl^{-1}$, with no growth observed on $400mgl^{-1}$ 2-chlorophenol. The fungus also grew on 2-chlorophenol/sugar mixtures, but to a lesser extent. The type of sugar present influenced growth, with glucose being the sugar that stimulated the most growth on a 2-chlorophenol/sugar mixture. No fungal growth was detected on the 3- or 4-chlorophenol plates.

Table 13: Fungal growth on solid minimal media containing a variety of single and mixed carbon sources. Phenolic and sugar concentration of $200mgl^{-1}$ and 0.2%, respectively unless otherwise stated. - no growth, + ~ 0.5cm radius of fungal growth.

Carbon Source(s)	Fungal Growth
Phenol	++++
4-chlorophenol	-
3-chlorophenol	-
2-chlorophenol ($100mgl^{-1}$)	++++
2-chlorophenol ($200mgl^{-1}$)	+++
2-chlorophenol ($400mgl^{-1}$)	-
2-chlorophenol/glucose	++
2-chlorophenol/fructose	+

The fungus was found to be capable of growth on both phenol and 2-chlorophenol, but not on 3- and 4-chlorophenol. The consistent absence of the fungus following exposure of the mixed microbial population to both 3- and 4-chlorophenol under a variety of nutrient conditions and its failure to grow on these phenolic substrates, suggested that it was unlikely to play a significant role in the degradation of these mono-chlorophenol isomers. The suggestion that the absence of removal of these

compounds at low pH's rules out significant fungal involvement in the degradative activity of the mixed culture is further substantiated.

3. 4. 1. 3 The influence of the fungal inoculum size on the ability of HAB to degrade 2-chlorophenol.

The fungus was capable of growth on both phenol and 2-chlorophenol (Section 3. 4. 1. 2). While phenol degradation was achieved, 2-chlorophenol removal by the fungal-containing mixed culture was always poor. Given the survival of the fungus when the mixed culture was exposed to 2-chlorophenol and also the ability of the fungus to grow on 2-chlorophenol, both alone or in the presence of additional carbon sources, the question arises as to why this phenolic proved to be most toxic to the mixed microbial population (Section 3. 1). A possible reason may be that the fungal population is not sufficiently large. It was therefore decided to supplement the mixed culture with fungus and subsequently examine its ability to remove 2-chlorophenol.

Flasks containing 2-chlorophenol ($200mgl^{-1}$) were inoculated with the mixed culture and a various numbers of fungal plugs (0 to 18) taken from malt extract agar plates. The results are shown in Table 14. Only slight increases in the percentage removal of 2-chlorophenol were achieved following supplementation of the mixed culture with fungal plugs.

Table 14: The influence of fungal supplementation on 2-chlorophenol($200mgl^{-1}$) removal by the mixed microbial population.

No. of Fungal Plugs	% 2-chlorophenol Removal
0	30.59
6	32.72
12	39.76
18	41.06

3. 4. 2 Characterisation of *Pseudomonas* species from the bioaugmentation product.

Two bacterial species, designated 7-cp and A(a), were isolated from the mixed microbial population (Section 3. 4). The cultures were obtained following enrichment of the mixed culture on 4-chlorophenol ($200mgl^{-1}$). The enrichment was carried out in liquid medium (PMM) containing 4-chlorophenol, which was incubated at 30°C for 35 hours (until the carbon source was depleted). The bacteria were therefore isolated on the basis of their ability to grow/survive in the presence of 4-chlorophenol as the sole carbon source.

3. 4. 2. 1 The identification of the isolated bacterial species.

The cell characteristics and the Gram reaction of both organisms were noted together with their response to a wide range of biochemical tests. As can be seen in Tables 15 and 16, the cell characteristics and biochemical properties of the isolates were identical. Both were found to be Gram negative, non-spore forming rods. They were oxidative organisms and were both oxidase and catalase positive. The response of the organisms to the other biochemical tests was negative.

Table 15: Cell characteristics of the isolates.

	7-cp	A(a)
Gram +/-	-	-
Shape	Rod	Rod
Spores+/-	-	-
Motility	+	+

Table 16: Biochemical properties of the isolates.

A - Hugh and Leifson, Oxidative (Ox) and Fermentative (Fer); **B** - Oxidase; **C** - Catalase; **D** - DNase; **E** - Gelatinase; **F** - Casein hydrolysis; **G** - Tween 80 hydrolysis; **+** Positive result; **-** Negative result.

	A	B	C	D	E	F	G
7-cp	Ox	+	+	-	-	-	-
A(a)	Ox	+	+	-	-	-	-

The ability of the organisms to grow on nutrient agar at a range of temperatures (4 - 42°C) was investigated (Table 17). All the organisms were capable of growth at temperatures of between 20 and 37°C, but not at 4 or 42°C.

Table 17: Growth of the isolates at various temperatures. Growth,+, No growth, -.

Temperature °C	**4**	**20**	**25**	**30**	**37**	**42**
7-cp	-	+	+	+	+	-
A(a)	-	+	+	+	+	-

The organisms were tested for their ability to utilise a range of carbohydrates as sole carbon sources (Table 18). Of the fifty four carbohydrates tested, 7-cp could use twenty three. The range of carbon sources used by A(a) was relatively limited, with only ten carbohydrates utilised.

Table 18: Carbohydrate utilisation (CH50) by the isolates.

Substrate	7-cp	A(a)	Substrate	7-cp	A(a)
N-acetyl glucosamine	+	-	2-ketogluconate	+	+
Adipate	-	-	5-ketogluconate	-	-
Adonitol	-	-	Lactose	-	-
Amygdalin	-	-	D-Lyxose	-	-
D-Arabinose	-	-	Malate	-	-
L-Arabinose	+	-	Maltose	+	-
D-Arabitol	-	-	Mannitol	+	+
L-Arabitol	-	-	Mannose	-	-
Arbutine	+	-	Melezitose	-	-
Caprate	+	+	Melibiose	-	-
Cellobiose	+	-	α-Methyl-D-Glucoside	-	-
Citrate	+	+	α-Methyl-D-Mannoside	-	-
Dulcitol	+	-	ß-Methyl-Xyloside	-	-
Erythritol	-	-	Phenylacetate	+	+
Esculine	+	-	Raffinose	-	-
Fructose	+	+	Rhamnose	+	-
D-Fucose	-	-	Ribose	+	-
L-Fucose	+	-	Saccharose	-	-
Galactose	+	-	Salicin	-	-
β-Gentiobiose	-	-	Sorbitol	+	-

Gluconate	+	+	L-Sorbose	-	-
D-Glucose	+	+	D-Tagatose	-	-
L-Glucose	+	+	Trehalose	+	-
Glycerol	+	+	D-Turanose	-	-
Glycogen	-	-	Xylitol	-	-
Inositol	-	-	D-Xylose	-	-
Inulin	-	-	L-Xylose	-	-

The isolates were both identified as *Pseudomonas putida* species using the API identification software, together with ancillary tests which were used in consultation with Bergey's Manual of Systematic Bacteriology (1984).

3. 4. 2. 2 Growth of the *Pseudomonas* species on various aromatics.

The ability of *P. putida* 7-cp and A(a) to grow at the expense of a range of aromatic substrates was investigated. The organisms were grown in liquid culture on different concentrations of benzoic acid, *m*-toluic acid, naphthalene, phenol, mono-chlorophenol and toluene in *Pseudomonas* minimal medium at 30°C, pH 7.0 and 150 rpm.

3. 4. 2. 2. 1 Growth on phenol and mono-chlorophenol isomers.

Cultures were set up in *Pseudomonas* minimal medium incorporating phenol or mono-chlorophenol, at a concentration of 200mgl^{-1}, as the sole carbon source. The extent of removal was monitored and the results are summarised below (Table 19).

Table 19: The removal of phenol and mono-chlorophenol isomers by *P. putida* 7-cp and A(a).

	% Removal	
Substrate	***P. putida* 7-cp**	***P. putida* A(a)**
Phenol	95	92
2-chlorophenol	10	14
3-chlorophenol	16	80
4-chlorophenol	26	92

Ease of removal of the substrates by both *Pseudomonas* spp. followed the order phenol > 4-chlorophenol > 3-chlorophenol > 2-chlorophenol (Table 19). This pattern was equivalent to that obtained with the mixed microbial population from which the bacteria were isolated (Section 3. 1).

Phenol removal by both isolates was accompanied by growth and a minor pH drop. While 4-chlorophenol removal by *Pseudomonas* 7-cp was incomplete, some growth was detected and a yellow compound became visible in the medium. This was also the case during the complete removal of 4-chlorophenol by *Pseudomonas* A(a). Greater 3-chlorophenol removal was achieved by *P. putida* A(a) than by strain 7-cp. During 3-chlorophenol removal, by both *Pseudomonas* spp., large increases in optical density values were attained. These increases were attributed to the development of black compounds in the culture fluids, indicative of the activity of a *meta*-cleavage pathway. With both isolates, 2-chlorophenol removal was poor. Microbial growth and colour development were not evident.

3. 4. 2. 2. 2 Growth on benzoic acid, *m*-toluic acid and naphthalene.

The extent of growth on the aromatic substrates was investigated by following the $O.D._{660nm}$ of the cultures until growth at 30°C had reached stationary phase or decline. The pH variation during growth was also noted. The duration of the lag period and the maximum $O.D._{660nm}$ attained were recorded and the specific growth rate calculated (Section 2. 2. 13) for each organism and substrate (Tables 20 and 21).

Table 20: Growth of *P. putida* 7-cp on benzoic acid, *m*-toluic acid and naphthalene.

Substrate	*Conc.(mM)*	*Lag(h)*	*Max* OD_{660}	μ (h^{-1})
Benzoic acid	5	0	0.837	0.087
	10	0	1.288	0.096
	20	0	1.285	0.110
***m*-Toluic acid**	5	0	0.284	0.010
	10	24	0.516	0.028
	20	4	0.557	0.044
Naphthalene	5	0	0.131	0.02
	10	0	0.160	0.015
	20	0	0.220	0.02

Both organisms grew best on benzoic acid followed by *m*-toluic acid. Growth of *P. putida* 7-cp on naphthalene was relatively poor and no growth of *P. putida* A(a) on

naphthalene was observed. Where growth occurred, the biomass produced increased with increasing concentrations of substrate as indicated by the maximum optical density values attained in each case. Growth was accompanied by pH deviations from an initial pH of 7.0. In most cases, there was no lag period preceding growth. Where the lag period increased, it did so with increasing substrate concentration. In general, the growth rate also increased with increasing substrate concentrations. *P. putida* 7-cp achieved the fastest growth rate following growth on benzoic acid. *P. putida* A(a) achieved similar maximum growth rates on both benzoic acid and *m*-toluic acid.

Table 21: Growth of *P. putida* A(a) on benzoic acid, *m*-toluic acid and naphthalene.

Substrate	*Conc.(mM)*	*Lag(h)*	*Max* OD_{660}	μ (h^{-1})
Benzoic acid	5	0	0.743	0.04
	10	0	1.168	0.06
	20	0	1.507	0.09
***m*-Toluic acid**	5	0	0.372	0.01
	10	0	0.577	0.02
	20	24	0.864	0.10
Naphthalene	5	-	-	-
	10	-	-	-
	20	-	-	-

3. 4. 3 Catechol Dioxygenase Activity.

The observation of the accumulation of coloured compounds following growth of the *Pseudomonas* spp. on the mono-chlorophenols and also of their preferences for benzoic acid and *m*-toluic acid, can be indicative of the activity of *meta*-cleavage pathways. Similar indications of *meta*-activity were evident during growth of the mixed microbial population on mono-chlorophenol isomers. It was therefore of interest to determine the levels of catechol dioxygenase activity in the biomass and attempt to illustrate *meta*-activity. Cells of the mixed cultures were harvested following growth on the mono-chlorophenol isomer ($200mgl^{-1}$) for 48hours and used for enzyme analysis. The results are illustrated below (Fig. 37). Dominant activity of the *meta* enzyme, catechol 2, 3-dioxygenase can be observed during growth on all three mono-chlorophenol isomers. This activity was highest during growth on 4-chlorophenol, followed by 3- and then 2-chlorophenol. In all cases, very little *ortho* activity was detected.

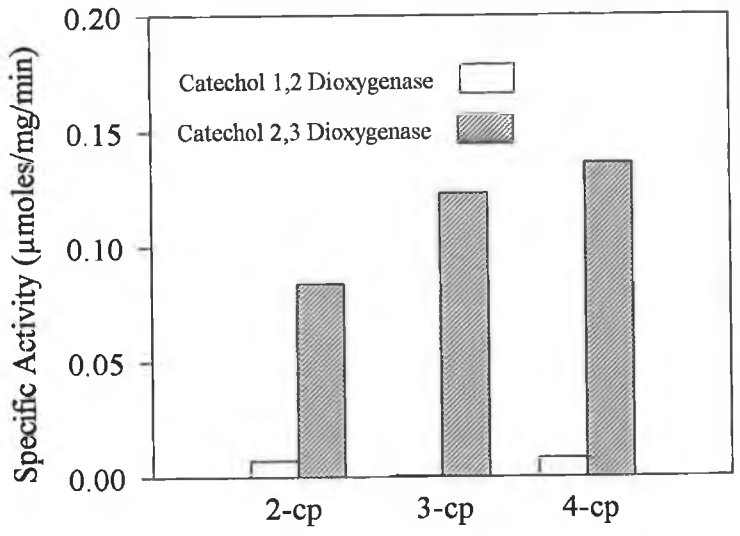


Fig. 37 Catechol dioxygenase activity during growth of HAB on mono-chlorophenols.

4. Discussion.

While phenol is toxic to most micro-organisms, complete removal was achieved by washed and unwashed HAB, at concentrations up to 1300mgl^{-1}. This compares well to studies with other bioaugmentation products, Phenobac and Polybac, where removal was achieved at concentrations of 1000 and 600mgl^{-1}, respectively (Lallai and Mura, 1989). Removal of phenol concentrations greater than 1500mgl^{-1} is uncommon, however a mutant strain of *Pseudomonas pictorum* has been reported to remove phenol, at concentrations up to 2gl^{-1} (Chitra *et al*, 1995). A study on the removal of phenol (100mgl^{-1}) by Hydrobac, BI-CHEM DC 1006/7 and Liquid Live Micro-organisms, three bioaugmentation products, reported removal rates of 25, 52 and 9mgl^{-1}h^{-1} respectively, following prior acclimation to phenol (Lewandowski *et al*, 1986). Phenol removal rates achieved in this study, where no acclimation occurred, thus compare favourably. In this study, the rates of removal decreased at phenol concentrations above 500mgl^{-1}. This observation, together with the fact that the lag periods preceding phenol removal increased with increasing phenol concentrations (Chitra *et al*, 1995, Lee *et al*, 1992) can be accounted for by the phenomenon of substrate inhibition of microbial growth at high phenol concentrations (Hill and Robinson, 1975, Shishodo and Toda, 1996). The growth of microbial cultures on toxic compounds is generally characterised by substrate inhibition kinetics, where the specific growth rate (μ) appears to be controlled by two competing substrate effects. The specific growth rate tends to increase as the substrate (S) is increased (Monod type relationship), but also tends to decrease due to the inhibitory effect of S as its concentration is increased (Kumaran and Paruchuri, 1997). There is, therefore a joint dependence of μ on S as substrate and S as inhibitor. The Haldane equation was devised to model such experimental data. It predicts that as S increases, μ rises to a peak and finally decreases as the inhibitory term dominates (Gaudy *et al*, 1986). A number of authors have described the biotransformation of phenols using Haldane inhibition kinetics (Jones *et al*, 1973, Kumaran and Paruchuri, 1997, Hill and Robinson, 1975, Yoong *et al*, 1997). During growth of the mixed culture on phenol, pH decreases

were observed and can be attributed to the production of organic acids from the intermediates which are formed in the degradation of phenol (Bayly and Dagley, 1969). While the actual pH drop obtained in this study was small, the pattern of pH change was similar to that obtained with other bioaugmentation products, where the minimum pH reached coincided with substrate exhaustion (Lallai and Mura, 1989). Studies on the degradation of phenol by a *Pseudomonas* sp. produced a similar relationship (Bettman and Rehm, 1984). During the application of the product, the simple act of measuring the pH of the liquor may give useful indications as to the understanding of the stage of the purification process, in terms of substrate disappearance.

Relatively speaking, the mono-chlorophenols were less readily degraded than phenol. Substitution of an aromatic ring with such an electron-withdrawing group which diminishes enzymatic transformation and deactivates electrophilic substitution, has been implicated as a reason for the persistence of many xenobiotics (Atlas and Bartha, 1992a, Fewson, 1988). The ease of removal pattern obtained in this study i.e. phenol > 4- > 3- > 2-chlorophenol, clearly demonstrates the effect of the presence of a chlorine atom and its orientation on the aromatic nucleus, and has been previously reported (Schöllhorn *et al*, 1994). This is in contrast however, to the outcome of a study by Beltrame *et al* (1988) which suggested that *ortho* substitution reduces the inhibiting action of chlorophenols. In this study, 4-chlorophenol was the only mono-chlorophenol isomer that was completely mineralised by the mixed microbial population. Very few strains have been reported to be able to use more than one mono-chlorophenol isomer (Häggblom, 1990). For example, the constructed strain *Alcaligenes* sp. A7-2, one of the most active chlorophenol-degrading isolates (Schwien and Schmidt, 1982) was found to be capable of growth on 2- and 4-chlorophenol, but not on 3-chlorophenol; this compound was cometabolised by strain A7-2 cells pre-grown or growing on phenol (Menke and Rehm, 1992). *Pseudomonas pickettii* strain LD1 appears to be somewhat unique among the chlorophenol-degrading bacteria described in the literature. It has been reported to be the only strain capable of using all mono-chlorophenol isomers (Fava *et al*,

1995).

Concentrations of 4-chlorophenol above 200mgl^{-1} were not successfully removed by the mixed microbial culture. This concurs with the literature where reports on mono-chlorophenol removal at concentrations greater than 200mgl^{-1} are rare. Concentrations of 4-chlorophenol of 160mgl^{-1} were removed by immobilised *Alcaligenes* strain A7-2 (Balfanz and Rehm, 1991). Two Gram-positive coryneform bacteria degraded 4-chlorophenol at a concentration of 100mgl^{-1} (Kramer and Kory, 1992). The mutant strain of *Pseudomonas pictorum* however, could degrade up to 300mgl^{-1} 4-chlorophenol (Chitra *et al*, 1995). As with phenol, increasing 4-chlorophenol concentrations caused extended lag periods (Menke and Rehm, 1992).

2-chlorophenol proved to be particularly toxic to this bioaugmentation product. Reports are available on the biodegradation of 2-chlorophenol in aerobic systems. *Pseudomonas pictorum* can remove approximately 50 percent 2-chlorophenol at concentrations of 300mgl^{-1} (Chitra *et al*, 1995). *Pseudomonas pickettii* strain LD1 can totally degrade 200mgl^{-1} 2-chlorophenol (Fava *et al*, 1995). Immobilised activated sludge micro-organisms were not inhibited by 1000mgl^{-1} 2-chlorophenol however, lower concentrations were fatal to the free micro-organisms (Sofer *et al*, 1990). Degradation of 2-chlorophenol under anaerobic conditions has also been observed. Anaerobic sludge completely degraded 50mgl^{-1} 2-chlorophenol (Basu *et al*, 1996). Cole *et al* (1994) and Zhang *et al* (1995) have reported the isolation of pure cultures capable of anaerobic 2-chlorophenol degradation.

The *meta* cleavage pathway of mono-chlorophenol degradation usually leads to the formation of dead end products and so *ortho* cleavage is often the preferred route following growth on chlorinated substrates (Williams and Murray, 1974). Oxidation of 4-chlorophenol results in the formation of 4-chlorocatechol which when cleaved by catechol 2, 3-dioxygenase yields 5-chloro-2-hydromuconic semialdehyde, a dead-end product which accumulates in the medium resulting in

an intense yellow colour (Knackmuss, 1981). The *meta* cleavage products of chlorocatechols have been reported to be lethal or unable to support the growth of micro-organisms (Bartels *et al*, 1984). In this study, both enzyme activity studies and the appearance of yellow compounds in the culture fluid, were indicative of the activity of a *meta*-cleavage pathway during growth of the mixed microbial culture on 4-chlorophenol. The yellow colour however, did not persist in the medium and complete 4-chlorophenol degradation, with stoichiometric chloride release, has been demonstrated in the laboratory. A newly isolated strain, *Comamonas testosteroni* CPW301, which can fully degrade 4-chlorophenol via a *meta* cleavage pathway has recently been reported (Bae *et al*, 1996). In this report, 5-chloro-2-hydroxymuconic semialdehyde was released as a temporary metabolic intermediate during 4-chlorophenol breakdown and slowly disappeared following the complete depletion of 4-chlorophenol.

Black compounds formed in the medium following initial degradation of both 2- and 3-chlorophenol. These may have contributed to the rise in $O.D._{660nm}$ observed. Chlorophenols can be fortuitously metabolised by the catabolic enzymes of various pathways which have broad substrate specificity (Knackmuss and Hellwig, 1978; Spain *et al*, 1989). The oxidation of both 2- and 3-chlorophenol results in the formation of 3-chlorocatechol which is a suicide substrate for catechol 2, 3-dioxygenase resulting in the formation of an acylchloride. This is an acylating agent which irreversibly inactivates catechol 2, 3-dioxygenase resulting in the accumulation of chlorocatechol and its black auto-oxidation products (Bartels *et al*, 1984).

Inhibition of the removal of phenol and the mono-chlorophenols was observed with the unwashed bioaugmentation product. The presence of a readily degradable carbon source in the product formulation was a conceivable cause of this inhibition, shifting the selection pressure away from degradation of phenolics (Møller *et al*, 1995). With phenol, degradation rates were decreased in the presence of other metabolisable substrates, but total degradation was achieved. Percentage removals of the mono-chlorophenols by the unwashed culture

however, were significantly reduced compared to the washed culture. Carbon source dependent repression in *Pseudomonas* species has previously been reported to affect the catabolism of aromatic carbon sources, such as toluene (Holtel *et al*, 1994) and styrene (O'Connor *et al*, 1995). The suppression of the removal of phenolics in the presence of readily metabolisable carbon substrates, is also well documented (Goulding *et al*, 1988, Swindoll *et al*, 1988, Chitra *et al*, 1995).

An increasing interest in mixed substrate utilisation by microbes has evolved, in recognition of the suggested importance of this process in nature and in wastewater purification systems. A discussion on strategies of mixed substrate utilisation and their significance in competition amongst microbes in natural environments, along with relevant control mechanisms, has been published by Harder and Dijkhuizen (1982). They have purported that utilisation of a particular substrate from a mixture can be controlled on at least three levels, namely the regulation of substrate uptake, of enzyme synthesis and of enzyme activity. A number of dual substrate removal patterns have been reported including diauxie, which is attributed to catabolite repression (Kim and Maier, 1986), simultaneous utilisation (Rozich and Colvin, 1986) and cometabolism (Review: Horvath, 1972). As defined by Dalton and Stirling (1982) cometabolism is the transformation of a nongrowth substrate in the "obligate presence of a growth substrate or another transformable compound".

The inevitability that toxic, or inhibitory, compounds will be found in mixtures with non-toxic or 'conventional' wastes is a matter of great practical importance to the biological destruction of toxic organic chemicals in waste treatment facilities, polluted groundwaters and hazardous waste sites. The determination of the type of interaction between toxic and non-toxic substrates, with their contrasting growth kinetics, has been the focus of a number of studies. A variety of toxic/non-toxic substrate mixtures have been used, with pure cultures and also with both defined and undefined heterogeneous cultures. The presence of additional readily metabolised carbon sources may be beneficial or detrimental to

the degradation of a target hazardous compound.

In this study, the presence of glucose exerted inhibitory effects on the removal of both phenol and mono-chlorophenols by the mixed microbial population. The specific rate of phenol removal decreased with increasing glucose concentrations. Both rates of removal and percentages removal of the mono-chlorophenol isomers were reduced in the presence of glucose. The necessity of the use of specific (per unit biomass) rather than overall removal rates has been stressed by Wang *et al* (1996) who criticised a number of authors for reaching so-called inaccurate conclusions regarding mixed substrate degradation on the basis of overall removal rates. The suppression of the removal of phenolics in the presence of readily metabolisable carbon substrates is well documented. Reduced phenol removal rates in the presence of various concentrations of glucose has previously been reported for heterogeneous populations (Rozich and Colvin, 1986). The authors suggested that the cells switched preference from the phenolic substrate to glucose, as the glucose concentration was increased. It has long been recognised that cells will tend to preferentially utilise those compounds that can realise the most rapid growth. A phenol-acclimated population of *Arthrobacter* sp. could suppress the phenol degradation pathway when presented with glucose, in order to quicken biomass acclimatisation to the carbon source that could permit more rapid growth (Kar *et al*, 1996). Such preferential utilisation of more easily degradable carbon sources has also been observed by Swindoll *et al* (1988) during mineralisation of *p*-nitrophenol, phenol and toluene by microbial communities, and by Goulding *et al* (1988) during growth of a mixed microbial culture on 3-chlorobenzoic acid. The presence of glucose exerted repressive effects on phenol removal by *Pseudomonas pictorum*, with the percentage phenol removal decreasing with increases in glucose concentration above 0.05% (Chitra *et al*, 1995). The failure of a bioaugmentation product to enhance bioremediation of oil polluted soil was attributed to the presence of a readily degradable carbon source in the formulation, which shifted the selection pressure away from degradation of the pollutant (Møller *et al*, 1995).

Several reasons, other than altered selection pressures, have been suggested in order to explain the above phenomena. For example, glucose may inhibit phenol transportation into the cell, which would effect the intracellular phenol concentration and in turn, reduce phenol degradation. Glucose may also inhibit enzymes that participate in the phenol degradation pathway. A report detailing the effect, on phenol removal, of glucose addition at different stages of microbial growth illustrated that early glucose addition had the most marked inhibitory effect (Kar *et al*, 1996). Inhibition of phenol metabolising enzymes or of phenol transport, may therefore fail to explain the negative effects observed, as glucose addition at any stage of growth would have inhibited phenol degradation.

In general, the mixed substrate removal patterns obtained with phenolic/glucose mixtures in this study were of rapid glucose utilisation and relatively slow phenolic removal. While the onset of glucose utilisation occurred prior to that of the phenolic, a period of concurrent removal of the substrates was noted with the mono-chlorophenols. Glucose exhaustion was often accompanied by a cessation in mono-chlorophenol removal by the mixed microbial culture. A dual substrate removal pattern of rapid uptake of glucose followed by a relatively slow utilisation of phenol has been reported by Rozich and Colvin (1986) when using mixed substrate (phenol/glucose) acclimated populations.

While phenol removal was reduced in the presence of glucose, the converse was not true. The rates of removal of glucose, when present alone or as part of a phenol/glucose mixture, were similar. The rate of glucose consumption by a glucose acclimatised *Arthrobacter* species was not affected in the presence of phenol, and was comparable to that realised by the phenol acclimatised culture (Kar *et al*, 1996).

When using a mono-chlorophenol/glucose mixture however, both substrate utilisation rates were reduced. In a study by Papanastasiou and Maier (1982) 2, 4-D-acclimated cultures were grown on a 2, 4-D/glucose mixture and were found to utilise the substrates concurrently. Utilisation of one substrate was

found to be inhibited by the other and vice versa. The inhibitory effect on 2, 4-D removal was however, masked by the fact that greater quantities of active biomass were produced in the presence of glucose. Mutual inhibition during the removal of two substrates may suggest that the substrates are involved in a cross-inhibitory pattern. A pattern of uncompetitive cross-inhibition was previously observed during growth of *Pseudomonas putida* ATCC 17514 on a phenol/glucose mixture (Wang *et al*, 1996). The following suggestions regarding the exact nature of this interaction, were made in the afore mentioned paper. Glucose and phenol are degraded by different enzymes. Glucose may however, have an affinity for the phenol-degrading enzyme. The binding of glucose to this enzyme reduces its affinity for phenol and therefore the phenol removal rate is reduced. Similarly, phenol has some affinity for the glucose-degrading enzyme and consequently a lower rate of glucose utilisation results. An alternative explanation for the dual inhibition may stem from the study of the ease of utilisation of the mono-chlorophenols, which was found to be related to the orientation of the chlorine atom on the aromatic ring (Section 3. 1). It was found that 4-chlorophenol was most readily removed, followed by 3-chlorophenol, with 2-chlorophenol being the most toxic. Beltrame *et al* (1988) has reported that the toxicity of chlorinated phenolics is related not only to the number of chlorine atoms present, but also to their site of attachment to the benzene ring. Given the mode of preparation of this bioaugmentation product, the presence of micro-organisms which are capable of glucose utilisation, but sensitive to concentrations of mono-chlorophenols is quite likely. The rate of glucose utilisation by such microbes would undoubtedly be reduced in such a hostile environment. The extent of retardation of glucose utilisation rate, in the presence of mono-chlorophenols, was found to be dependent on the particular mono-chlorophenol. The more toxic the mono-chlorophenol, the slower the removal of glucose from the mono-chlorophenol/glucose mixture. This offers further evidence of the relative toxicity of these pollutants to this mixed microbial culture.

In contrast to glucose, fructose did not exert any inhibitory effects on phenolic

removal by the mixed microbial population. In fact, the removal of both 2- and 3-chlorophenol was enhanced in the presence of fructose. Different effects, depending on the nutrient used, have been previously noted. Rates of phenol and *p*-nitrophenol mineralisation in samples of lake water were enhanced in the presence of inorganic nutrients, arginine and yeast extract but reduced in the presence of glucose (Rubin and Alexander, 1983).

The role of the metabolic pathways of the two sugars merits some discussion. Degradation of glucose by pseudomonads, major constituents of the HAB population, is via the Entner-Doudoroff pathway and results in the production of glyceraldehyde-3-phosphate and pyruvate. Both a direct oxidative pathway (extracellular) and a phosphorylative pathway (intracellular) are available for conversion of glucose to 6-phosphogluconate, a key intermediate in the process (Lessie and Phibbs, 1984).

Fructose metabolism by pseudomonads has been distinguished from that of other carbohydrates. While carbohydrate uptake is normally via active transport, PEP-dependent phosphotransferase activity has been demonstrated in pseudomonads grown on fructose, pointing to the transport of this sugar into the cell by vectorial phosphorylation. The sugar accumulates in the cell as fructose-1-phosphate, which is converted to fructose 1,6-diphosphate, then fructose-6-phosphate before subsequent metabolism via the Entner-Doudoroff pathway (Lessie and Phibbs, 1984).

While a number of subtle differences in the individual pathways are evident however, the central degradation pathways of the sugars are similar. The contrasting efficiencies in phenolic removal by the HAB population, depending on the specific sugar present, is unlikely therefore, to be attributed to individual features of the sugar metabolism.

The pattern of dual substrate removal in the presence of fructose was different to that observed in the presence of glucose as the additional carbon source.

Preferential utilisation of the phenolic from the phenolic/fructose mixtures was observed. The lag periods preceding fructose removal were substantially longer than those which preceded glucose utilisation by the mixed microbial population. Due to the manner in which the bioaugmentation product had been initially produced, the micro-organisms were acclimated to glucose but not to fructose. In a report by Rozich and Colvin (1986), the substrate removal pattern exhibited by cells of a heterogeneous population was significantly influenced by the acclimation characteristics of the culture.

The importance of substrate removal pattern may imply that cometabolism plays a role in the removal of the aromatic substrates. Cometabolic transformation of chlorophenols, where micro-organisms depend upon the concurrent or previous utilisation of a growth or energy substrate, has been described by many researchers (Dapaah and Hill, 1992, Hill *et al*, 1996, Janke *et al*, 1988, Liu *et al*, 1991, Saez and Rittman, 1991). In the absence of growth substrates, biomass oxidation may generate the reducing power required to facilitate substrate transformation (Chang *et* al, 1993). The rate of cometabolic transformation of 4-chlorophenol has been linked with biomass-decay rates (Saez and Rittman, 1991). In this study, the observation of the accumulation of dead-end products during the breakdown of the mono-chlorophenol isomers, resulting from the activity of a *meta*-cleavage pathway (Knackmuss and Hellwig, 1978), suggested that these substrates were only partially degraded. The micro-organisms were therefore unable to derive energy from the substrate to support growth and the definition of cometabolism applied (Horvath, 1972). The breakdown of both 3- and 2-chlorophenol led to the accumulation of black compounds in the culture fluids resulting from the auto-oxidation of chlorocatechols, which are the dead end products of an unproductive *meta*-cleavage pathway. With 4-chlorophenol, a yellow intermediate, 5-chloro-2-hydroxymuconic semialdehyde, which is indicative of *meta* activity appeared in the culture fluid. This compound however, slowly disappeared towards the end of the incubation. Stoichiometric chloride release during growth of HAB bacteria on 4-chlorophenol, has been demonstrated in the laboratory, and therefore complete 4-chlorophenol

degradation was assumed. During cometabolic transformation, one or more products are formed and tend to accumulate in pure cultures. In mixed cultures, these products may serve as growth or energy substrates for non-cometabolising populations (Criddle, 1993) and thereby facilitate the complete degradation of the substrate. Complete degradation of 4-chlorophenol by *Comamonas testosteroni* CPW301, via a *meta* pathway, has been reported (Bae *et al*, 1996). Janke *et al* (1988) have studied the cometabolic turnover of mono-chloroaromatics in *Rhodococcus* sp. An 117 and An 213. They reported an enhancement in 4-chlorophenol removal, by *Rhodococcus* sp. An 117, in the presence of glucose and fructose. This stimulating effect was said to be primarily due to the provision of the cells with energy and/or reducing power resulting from metabolism of the additional carbon source. Compounds which did not support growth of the organism (gluconate and 1-0-methyl-α-D-glucopyranoside) had no stimulating effect.

In this study, the manner in which additional carbon was utilised appeared to influence chlorophenol removal and very often a pattern of concurrent utilisation of the substrates was observed. In general, removal of the mono-chlorophenol accompanied sugar removal and ceased upon exhaustion of the more readily metabolised substrate. Kinetic models have been developed which quantify cometabolism (Criddle, 1993). Such models couple transformation of the non-growth substrate to the utilisation rate of the growth or energy substrate. The author suggests that the maximum rate of non-growth substrate utilisation depends on two factors: (1) the utilisation rate of the growth/energy substrate and (2) the maximum rate at which the cells can transform the non-growth substrate in the absence of growth/energy substrate. This model captures many features of cometabolism, including loss of activity in resting cells caused by transformation of non-growth substrate, and an increase in the rate and extent of cometabolism in the presence of growth/energy substrates. In this study, increases in the rate and extent of both 2- and 3-chlorophenol transformation in the presence of fructose were observed. The presence of the sugar throughout the period of transformation, as a result of its relatively slow utilisation

(compared to glucose), facilitated the enhanced transformation. With glucose however, mono-chlorophenol transformation was not enhanced. The rate of glucose utilisation increased with increases in its concentration. With the increasingly rapid glucose utilisation, transformation of the non-growth substrate was further reduced. Simultaneous removal of the chlorophenol in the presence of glucose was observed and no further phenolic transformation was detected following its disappearance. Glucose supplementation leading to an extended period of concurrent substrate removal however, was found to facilitate further 4-chlorophenol transformation. Chang *et al* (1993) have successfully applied the above model to describe cometabolism of *p*-xylene by a *Pseudomonas* species. They demonstrated the transformation of *p*-xylene in the presence of growth substrates such as benzene or toluene. Similar to the results obtained here, transformation of *p*-xylene slowed significantly when the growth substrate disappeared, but increased when a growth substrate was again added.

Throughout this study, substrate removal patterns were found to be indicative of the manner in which additional carbon influenced phenolic removal by the mixed microbial population. Contrasting effects were obtained in the presence of glucose, to which the micro-organisms had been previously exposed and in the presence of fructose, to which no acclimation had taken place. In a report by Rozich and Colvin (1986), the substrate removal pattern exhibited by cells of a heterogeneous population was significantly influenced by the acclimation characteristics of the culture. The role of acclimation was also found to be particularly relevant in this study. Acclimation of HAB to fructose resulted in altered substrate removal patterns and changed the effect of fructose on 4-chlorophenol removal. 4-chlorophenol removal in the absence of fructose was no longer achieved. Increasing concentrations of fructose facilitated transformation of the phenolic but, similar to removal in the presence of glucose, depletion of the sugar marked the cessation of phenolic removal. In the presence of sufficient quantities of fructose, complete 4-chlorophenol transformation was achieved. In a study by Basu and Oleszkiewicz (1995), the removal of 2-chlorophenol was achieved by activated sludge. This removal was enhanced in the presence of

dextrose. Following growth of the sludge on dextrose alone however, 2-chlorophenol removal was no longer achieved.

Microbial degradation is the main route of disappearance of chlorinated aromatic compounds. The micro-organisms responsible for such transformations are subject to a range of tolerances to ecologically important factors, such as temperature and pH, which affect their growth and activity. The effects of these parameters must be taken into consideration when assessing the environmental significance of laboratory results. If a particular environment contains several species able to bring about a particular transformation, the tolerance range is often broader than that of a single species, encompassing the tolerances of all the indigenous populations (Alexander, 1994a). The use of mixed cultures for degradation in less than stable environments therefore, merits considerable attention.

The prevailing temperature is of paramount importance and has been cited as a possible reason for the failure of inocula to function as required (Goldstein *et al*, 1985). While it is often not economically feasible to modify or control the prevailing temperature, it is important to understand its importance in order to predict the likely persistence of organic molecules in differing environments. The activity of the washed mixed microbial population was optimal at 30°C. This temperature optimum for phenol removal by the product may be a reflection of its microbial composition and the predominant role of the bacterial species. The formulation contains several species of *Pseudomonas* which function optimally at 30°C (Stanier *et al*, 1966). However, when a lower temperature was imposed upon the process, complete removal of phenol and 4-chlorophenol was achieved albeit with reduced efficiency. Temperature control during wastewater treatment however, is minimal and therefore ambient temperatures prevail, and rates of removal in an applied situation can be expected to be slower. Temperature decreases from 30 to 4°C have been found to decrease rates of mineralisation of benzoate and sphagnum by a peatland microbial community (Williams and Crawford, 1983). To a degree, the changes in rate of degradation associated with

seasons of the year are a consequence of the concomitant changes in temperature (Alexander, 1994a). The growth of micro-organisms involved in 2,4-D degradation in the Avon River in Western Australia, was inhibited during winter at water temperatures of 15°C or lower (Nesbitt and Watson, 1980). The impact of temperature on the duration of the acclimation period has previously been observed by Atlas and Bartha (1972), where there was a longer interval before the onset of rapid oil degradation at lower than at higher temperatures.

The temperature optimum for the unwashed population was 20°C. This inoculum contains levels of additional carbon sources. The rate of utilisation of added carbon has previously been found to influence its effect on phenolic removal by the mixed microbial population. A reduced incubation temperature was found to decrease the rate of utilisation of the additional carbon, which in turn appeared to facilitate enhanced phenolic removal. The inhibitory effects of glucose on 4-chlorophenol removal at 30°C were also found to be alleviated by incubation at 20°C, where glucose utilisation was slower.

No chlorophenol removal was observed at 45°C. Phenol removal however, does take place at this elevated temperature. Phenolic compounds, due to their hydrophobic nature, exert their effect by inserting into the cell membrane thereby making it more fluid. Membrane integrity is disturbed and non-specific efflux of cellular material results (Heipieper *et al*, 1991, Sikkema *et al*, 1995). A high correlation between the toxicity and hydrophobicity of different phenolic compounds has been deduced (Beltrame *et al*, 1988). It is well known that a raised growth temperature also increases the fluidity of membranous lipids. Using a mechanism known as homeoviscosic adaptation, micro-organisms react to externally dictated changes in their environment by modifying their membranes to keep them in the same fluidity condition and thereby prevent aspecific permeabilisation. The HAB population was capable of such adaptation to the combined effects of phenol and the elevated temperature. However, it is probable that the presence of the more toxic mono-chlorophenols, in conjunction with the elevated temperature, proved much too 'stressful' for the microbes leading to

cell death and the consequent lack of pollutant removal. During a study of chlorophenol degradation in fluidised bed reactors, a temperature decrease of ten degrees resulted in a 7-fold reduction in the chlorophenol degradation rate (Melin *et al*, 1998). The fact that both chlorophenols and temperature illicit membrane fluidity responses was suggested as an explanation for the observed effects, as well as the possible effect of temperature on chlorophenol transport into the cells.

While mono-chlorophenol removal by the mixed microbial population was very sensitive to pH, phenol removal proceeded unimpeded between pH 5 and 8. In this range, the pH had virtually no effect on the lag period and degradation rate. Similar results were found during the degradation of phenol by *Pseudomonas putida* EKII (Hinteregger *et al*, 1992). In contrast, Bettman and Rehm (1984) found markedly prolonged lag periods with increasing pH in growth experiments on phenol using immobilised cells of *P. putida* P8. The role of pH in treatment processes is an important one (George and Gaudy, 1973). These authors reported that changes in pH of no more than one unit from the neutral range can be tolerated without possible disruption of the biochemical efficiency of substrate removal, by a heterogeneous microbial population. They noted that the primary response mechanism to pH reduction was ecological, involving a shift in predominating species from bacterial to fungal. Low pH values are known to favour the predominance of fungi. The absence of chlorophenol degradative activity, in this study, at low pH values therefore suggests that the species of fungus, present in the product, does not play a role in mono-chlorophenol removal. Minor pH increases have been reported to extend the lag period during phenol degradation by a *Pseudomonas* sp. (Bettman and Rehm, 1984). This phenomenon was attributed to the time taken to adapt to the higher pH environment. The significance of environmental pH on the degradation of 2,4-D by *Pseudomonas cepacia* BRI6001 has been studied. Degradation was optimal in the acidic pH range from 5.1 to 6.7 (Greer *et al*, 1990). Ambient pH has a particularly important role in nature. In a study by Robertson and Alexander (1992), the effect of pH on microbial mineralisation of organic chemicals was

suggested to be a reflection of the role of pH in determining the ionic species of phosphate in solution. This has particular implications in natural environments, where the availability of phosphate is known to be a factor limiting microbial growth and therefore the biodegradation of synthetic organic compounds. The bioremediation of such contaminated sites frequently necessitates phosphate supplementation to ensure rapid biodegradation of pollutants. More effective bioremediation may stem from a deeper understanding of factors, including pH, influencing the availability to micro-organisms of such added phosphate.

The mineralisation of many organic compounds is often preceded by an acclimation period. Minimisation of this period, during which degradation is not detected, is desirable both in natural environments, in order to eliminate the risk of dissemination of the pollutant, and in treatment systems where maximal removal rates are always demanded. Several mechanisms have been suggested to account for this period including enzyme induction, genetic changes and the multiplication of specialised micro-organisms (Buitrón and Capdeville, 1995). Nutrient limitation may also affect the length of the adaptation period (Lewis *et al*, 1986), as well as the concentration of the test substrate or the presence of other substrates (Wiggins and Alexander, 1988a).

Enhancement of the biodegradation activity, of activated sludge, due to acclimation to the substrate has been reported (Buitrón and Capdeville, 1995). Degradation times of both phenol and 4-chlorophenol decreased as a result of controlled acclimation cycles in a Sequencing Batch Reactor. Similarly, river populations preexposed to *p*-nitrophenol degraded subsequent nitrophenol additions much faster than the control population (Spain *et al*, 1980). Anaerobic biodegradation of chlorophenols was also enhanced following acclimation (Boyd and Shelton, 1984). It is generally assumed that biodegradation is detected immediately following the second introduction of a chemical, because the organisms responsible for the transformation became numerous as they grew on the organic chemical following its first introduction (Alexander, 1994c). Van der Wijngaard *et al* (1993) have reported that selection pressure can increase the

cellular concentration of key enzymes in bacteria capable of degrading chlorinated compounds, and that this, in turn, results in an enhanced affinity of the bacteria for the xenobiotics.

Acclimation of a microbial community to one substrate frequently results in the simultaneous acclimation to some, but not all, structurally related molecules. Because individual species often act on several structurally similar substrates, the species favoured by the first addition may then quickly destroy the analogues (Alexander, 1994c). In this study, acclimation to phenol resulted in enhanced removal of the structurally related compound, 4-chlorophenol. Exposure to phenol has resulted in adaptation of a microbial community to the structurally related compounds *m*-cresol, *m*-aminophenol and *p*-chlorophenol (Shimp and Pfaender, 1987). Both common pathway elements and similar cell membrane transport systems were suggested as possible explanations for the ability of phenol to affect the biodegradation of related phenolic compounds. Haller (1978) observed that acclimation of sewage microflora to 3-chlorobenzoate or 4-chlorophenol reduced the lag period preceding mineralisation of other mono-substituted aromatic hydrocarbons. The presence of nutrient broth had no noticeable effect on the acclimation of activated sludge to 2, 4 dichlorophenoxyacetic acid (Kim and Maier, 1986). The specific compound to which the biomass is acclimated can influence subsequent removal patterns. While fresh anaerobic sludge utilised all mono-chlorophenol isomers, sludge acclimated to 2-chlorophenol cross-acclimated to 4-chlorophenol, but did not utilise 3-chlorophenol (Boyd and Shelton, 1984).

Inoculum density has a major impact on both the efficiency and cost of bioaugmentation, and has also been cited as a factor limiting the success of inoculation for biodegradation (Ramadan *et al*, 1990). In this study, increasing the size of the washed inoculum led to more rapid removal of the substrates that permitted growth and had little impact on the removal of the more toxic substrates, 3- and 2-chlorophenol. The role of inoculum size in the determination of the efficiency of 2, 4-D degradation has been reported. Comeau *et al* (1993)

noted a reduction in the time for complete 2,4-D degradation of 0.5 day per log increase in cell density. Growth lag times prior to the onset of 2, 4-D degradation, and the total time required for degradation were linearly related to the starting population density (Greer *et al*, 1990). A strain of *Pseudomonas cepacia* was capable of mineralisation of *p*-nitrophenol when added to nonsterile lake water at relatively high cell densities, but not at low ones (Ramadan *et al*, 1990).

Two organisms which survived in the presence of 4-chlorophenol were characterised. They were 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. The many biochemical properties of the *Pseudomonas* spp. were typical for their species as outlined in Bergey's Manual of Systematic Bacteriology (1984). The nutritional versatility, which is typical of *Pseudomonas* sp. (Palleroni, 1986), was evident in their ability to use a large number of the carbohydrates tested for growth. They were both identified as *Pseudomonas putida* species using the API identification system.

The characterisation of mixed microbial populations typically involves a logical reductionist progression from original sample, to laboratory incubation, to enrichment cultures, to the isolation of pure cultures and to the elucidation of cellular and subcellular processes. The complexity of the system under scrutiny diminishes at each step in the progression. This system of analysis forms the basis of much of the information available on ecological, physiological, biochemical, genetic and molecular aspects of microbial reactions. Such reductionist methods may however, diminish the ecological relevance of the resultant information (Madsen, 1998). The study of the *Pseudomonas* species isolated in this study can therefore only serve to provide some insight into the function of this community and does not attempt to fully encompass the complex and dynamic nature of heterogeneous populations.

In order to overcome the inadequacies of traditional methods, particularly isolate-based methods, a community-level approach to characterise and classify heterotrophic microbial populations has been developed (Garland and Mills, 1991). The authors utilised a technology developed by BIOLOG, Inc. in which carbon source utilisation was used as a basis for strain identification of bacterial isolates. This technology produced ecologically relevant classifications of heterotrophic microbial communities based on sole-carbon-source utilisation. Application of this method to the analysis of microbial communities in wastewater treatment systems has been reported (Victorio *et al*, 1996). Microbial biomass from three different treatment systems displayed different composite metabolic patterns, indicative of unique indigenous populations. By incorporation of certain carbon compounds into the test, the microbial consortia displaying the greater response to these compounds was identified and as such, the biodegradation potential associated with the treatment systems was determined.

The ability of the isolates to grow at the expense of a range of aromatic compounds was examined in the basal salts medium of Goulding *et al* (1988), which provided a source of nitrogen, phosphate and trace salts, incorporating the aromatic compound as the sole source of carbon for energy and growth. The solubility of the aromatic compounds in the aqueous medium can be important for their metabolism. Phenol, benzoate and *m*-toluate dissolved readily in the medium. Naphthalene however, did not dissolve completely. A number of bacteria have been found to be capable of metabolising aromatic compounds with low water solubilities, using various mechanisms. Some bacteria may facilitate the uptake of poorly soluble compounds by producing emulsifiers or by the possession of a hydrophobic cell surface. It is also possible that the organisms grow only at the expense of the compound in solution and that the rate of dissolution of such compounds might govern the rate of biodegradation (Stucki and Alexander, 1987).

The two *Pseudomonas* isolates grew on phenol at a concentration of $200mgl^{-1}$.

The enzymes for phenol degradation are induced by phenol (Feist and Hegeman, 1969). Phenol also induces catechol 2, 3-dioxygenase and the other enzymes of the *meta*-cleavage pathway.

Benzoate has been shown to support the growth of a variety of micro-organisms (Reiner, 1971). Both *Pseudomonas* sp. grew well on benzoate. The enzymes for benzoate degradation are chromosomally-encoded and are ubiquitous in *Pseudomonas* sp. (Harayama and Rekik, 1990). Benzoate can however, also be attacked by the broad spectrum toluate dioxygenase and other TOL-plasmid encoded enzymes. The degree of growth of both organisms was greater on higher concentrations of benzoate.

m-toluate is an intermediate in the degradation of *m*-xylene by the TOL plasmid encoded pathway (Davey and Gibson, 1974). Both organisms were capable of growth on *m*-toluate and as with the other substrates the degree of growth, as indicated by the specific growth rate and maximum O.D attained, increased on the higher *m*-toluate concentrations. The growth observed was less than that observed on benzoate. Methyl-substituted aromatic compounds induce the enzymes of the *meta*-cleavage pathway.

A number of plasmids carrying naphthalene catabolic genes have been demonstrated in *Pseudomonas* spp.. In this study however, growth of the *Pseudomonas* isolates on naphthalene was poor.

P. putida A(a) was capable of growth on 4-chlorophenol and to a lesser extent on 3-chlorophenol. This ability may be linked to its ability to metabolise both *m*-toluate and phenol, as a number of studies have demonstrated the broad substrate specificity of the enzymes of the TOL plasmid-encoded pathway and also of phenol mono-oxygenase enzymes. Toluene mono-oxygenase, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase have all been shown to act on chlorinated substrates (Abril *et al*, 1989). Degradation of chlorinated phenols by a toluene enriched mixed microbial culture has also been demonstrated (Ryding

et al, 1994). Furthermore, the oxidation of phenols by *Pseudomonas putida* was closely correlated with the induction and activity of the toluene dioxygenase system (Spain and Gibson, 1988). The authors also noted that the reaction products were always 1, 2-dihydroxy compounds and that the reactions were always fastest with phenols containing a single chloro, methyl or nitro group in the *meta* position, thus indicating the influence of steric effects on enzyme activity. Knackmuss and Hellwig (1978) suggested that the same phenol hydroxylase was induced by 4-chlorophenol and phenol, since no significant differences in the relative turnover rates were observed for the isomeric mono-chlorophenols. The broad specificity of phenol hydroxylase towards methyl- and chloro-substituted substrates has also been demonstrated during studies of the degradation of phenols by *Pseudomonas putida* EKII (Hinteregger *et al*, 1992).

The ease of removal of the phenolic compounds by the mixed microbial population i.e. phenol > 4- > 3- > 2-chlorophenol, was reflected in the microbial composition of the system. The ability of the two *Pseudomonas* sp. to grow in the presence of phenolic compounds was similar to that of the mixed culture. The predominant activity of the *meta*-cleavage pathway during growth of the mixed culture on the mono-chlorophenol isomers, was also evident in the pure cultures, where mono-chlorophenol removal was accompanied by colour production. Although the mixed culture was said to be designed for the degradation of substituted aromatic compounds, its ability to degrade both 3- and 2-chlorophenol was relatively poor. This lack of activity may be the result of a number of factors. Environmental conditions, which play a central role in the performance of any microbiological process, may have prevented the growth of an active population. The influence of such factors is however, contingent on the presence of micro-organisms which carry the relevant degradative capability. In this study, while some growth of the mixed culture on these substrates was evident, it was not possible to isolate pure bacterial cultures with similar capabilities. Even if capable micro-organisms were present, the toxicity generated through the accumulation of metabolites of the active *meta* pathway, such as chlorocatechols or chlorosubstituted ring fission products, as well as the

inherent toxicity of haloaromatics, may have severely suppressed the growth of haloaromatic-utilising micro-organisms, that may have pre-existed as marginal members of the microbial community. A defined mixed culture, capable of degradation of a synthetic sewage containing alkanols, acetone and phenols was only capable of partial chlorophenol transformation via an unproductive *meta* pathway (Schmidt *et al*, 1983). Addition of the chlorocatechol-dissimilating *Pseudomonas* sp. B13 however, resulted in prevention of the *meta* pathway and the establishment of a chlorocatechol-assimilating sequence and thereby facilitated complete chlorophenol degradation and detoxification. Such an approach may prove useful with this bioaugmentation product in order to facilitate successful chlorophenol mineralisation.

While no bacteria capable of growth on 2-chlorophenol were successfully isolated, the isolated fungus was found to grow on 2-chlorophenol, at concentrations up $200mgl^{-1}$, both as a single carbon source and in the presence of sugars. The lack of 2-chlorophenol removal by the mixed culture suggested that the unproductive bacterial population dominated and that the fungus did not play an active role. This was further substantiated by the observation that only bacterial growth was detected when the mixed culture was plated on 2-chlorophenol agar. Evidence for fungistatic activity has been demonstrated (Atlas and Bartha, 1992b). In soil, inhibitory effects on germination of fungal spores were widespread. Such effects were associated with microbial activity as they are eliminated by sterilisation. Such interactions can play a role in the control of plant pathogens. Addition of some *Bacillus* and *Streptomyces* strains to soil or seeds has been shown to control plant disease caused by the fungus *Rhizoctonia solani*. The poor fungal activity may also be a result of the prevailing environmental conditions such as shear force. *Phanerochaete chrysosporium*, a white rot fungus, has been shown to effectively degrade 2-chlorophenol (Lewandowski *et al*, 1990). This organism produced a non-specific ligninase system which enabled it to degrade a wide variety of compounds. Investigators have demonstrated the detrimental effect of agitation on enzyme induction. The degradation of 2-chlorophenol by *Phanerochaete chrysosporium* has been shown

to more efficient in reactor systems in which the fungus was immobilised (Wang and Ruckenstein, 1994, Lewandowski *et al*, 1990). It was suggested that the fungus needed to be attached to a surface to produce the ligninolytic system responsible for the degradation of toxic compounds. Both white and brown rot fungi have been implicated to play roles in the degradation of aromatic compounds. The brown rot fungi however, appear to be less versatile and reports detailing their degradative capabilities are less numerous.

5. Conclusions.

(i) The mixture of micro-organisms was capable of the degradation of phenol, up to a concentration of 1300mgl^{-1}.

(ii) The mixture was also capable of complete 4-chlorophenol removal, at concentrations up to 200mgl^{-1}, but not 3- or 2-chlorophenol.

(iii) The overall degradative capability of the mixed culture was via a *meta* cleavage pathway. This activity facilitated both phenol and 4-chlorophenol removal, but led to the likely accumulation of dead end products during growth on 3- and 2-chlorophenol.

(iv) The response of the mixed microbial population to the presence of sugar was governed by acclimation. Previous exposure of the cells to glucose led to rapid removal of this sugar and phenolic removal, by the mixed culture, was inhibited by glucose. In contrast, no inhibitory effects were observed in the presence of fructose. The cells had not been acclimated to fructose and preferential phenolic removal from the phenolic/fructose mixtures was observed.

(v) Rapid glucose removal led to inhibitory effects on phenolic removal. These inhibitory effects were alleviated however, when glucose utilisation was retarded i.e at a reduced incubation temperature, or where a continuous presence of the sugar was maintained under controlled pH conditions.

(vi) Further evidence of the central role of the sugar removal pattern was noted following acclimation of the cells to fructose. Fructose removal by the acclimated cells was rapid and phenolic removal from phenolic/fructose mixtures was inhibited.

(vii) The mixed microbial population contained one fungal species. This fungus was capable of growth on 2-chlorophenol, but was unable to compete successfully with the bacterial members of the mixture and therefore its degradative capabilities were not expressed.

(viii) Two *Pseudomonas putida* spp. capable of growth on 4-chlorophenol were isolated and characterised. They were found to be capable of growth on a range of aromatic compounds which are usually degraded via a *meta* pathway and therefore may reflect the dominance of *meta* activity observed with the mixed culture. Similar to the mixed culture, their ability to grow on phenolic compounds followed the order: phenol > 4- > 3- > 2-chlorophenol.

(ix) The removal of 3- and 2-chlorophenol was not achieved by the mixed microbial population. Attempts to isolate bacteria capable of growth on these substrates were unsuccessful. It was concluded that bacteria capable of growth on these substrates were unlikely to be present.

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7. Appendices.

Appendix 1.

Standard curves for the mono-chlorophenol isomers using the colorimetric assay.

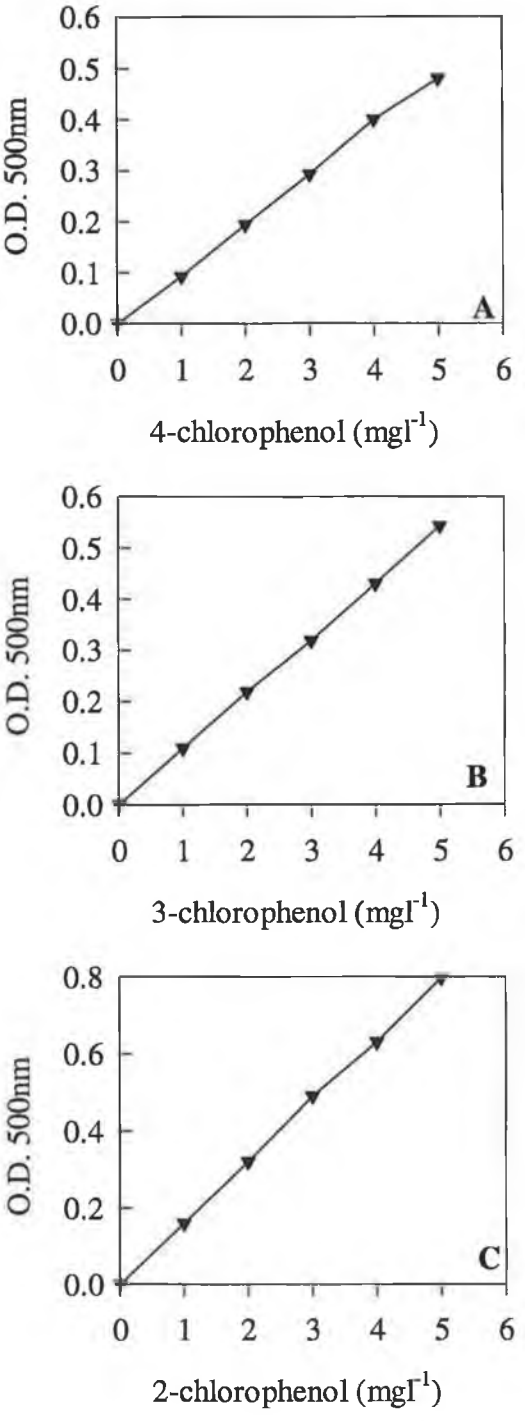

Fig 1. 1 Standard curves for 4- (A), 3- (B) and 2-chlorophenol (C) using the colorimetric assay.

Appendix 2.

Calibration curve relating optical density units to mg dry weight cells per ml.

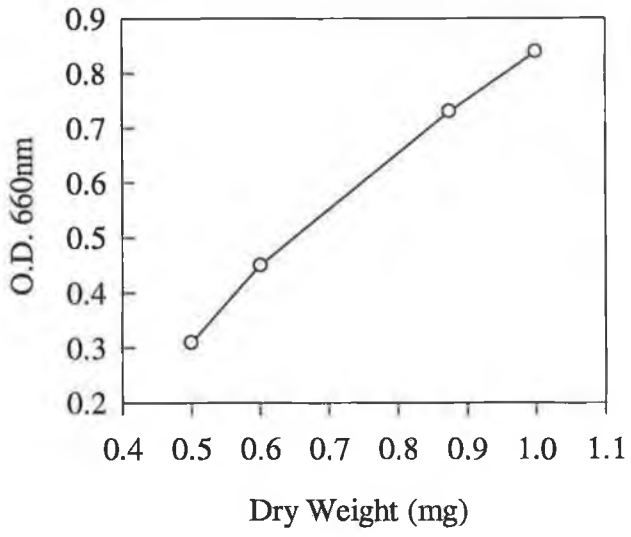

Fig 2. 1 Plot of O.D. 660nm vs. Dry Weight biomass.