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Degradation of mono-chlorophenols by a mixed microbial community via a *meta*- cleavage pathway

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Abstract

A mixed microbial community, specially designed to degrade a wide range of substituted aromatic compounds, was examined for its ability to degrade mono-chlorophenols as sole carbon source in aerobic batch cultures. The mixed culture degraded 2-, 3-, and 4 -chlorophenol (1.56 mM) via a *meta*- cleavage pathway. During the degradation of 2- and 3-chlorophenol by the mixed culture, 3-chlorocatechol production was observed. Further metabolism was toxic to cells as it led to inactivation of the catechol 2,3-dioxygenase enzyme upon *meta*- cleavage of 3-chlorocatechol resulting in incomplete degradation. Inactivation of the *meta*- cleavage enzyme led to an accumulation of brown coloured polymers, which interfered with the measurement of cell growth using optical denstiy. Degradation of 4-chlorophenol by the mixed culture led to an accumulation of 5-chloro-2-hydroxymuconic semialdehyde, the *meta*-cleavage product of 4-chlorocatechol. The accumulation of this compound did not interfere with the measurement of cell growth using optical density. 5-chloro-2-hydroxymuconic semialdehyde was further metabolized by the mixed culture with a stoichiometric release of chloride, indicating complete degradation of 4-chlorophenol by the mixed culture via a *meta*- cleavage pathway.

Introduction

The usage of chlorinated aromatic compounds, such as chlorophenols, in agricultural or industrial processes, has led to their accumulation in the environment. Despite the recalcitrant nature of mono-chlorophenols, specialized strains of micro-organisms have been shown to be capable of their degradation (Knackmuss and Hellwig, 1978; Balfanz and Rehm, 1991; Fava *et al.*, 1995). For complete degradation of chlorinated aromatic compounds to occur, two steps are necessary, cleavage of the aromatic ring and the removal of the chlorine atom (Häggblom, 1990). The initial step in the aerobic degradation of mono-chlorophenols is their transformation to chlorocatechols. 2- and 3-chlorophenol metabolism results in the production of 3-chlorocatechol, while 4-chlorophenol is transformed to 4-chlorocatechol (Fig. 1). 4-chlorocatechol production has also been observed during 3-chlorophenol degradation (Häggblom, 1990). Chlorocatechols are central metabolites in the aerobic degradation of a wide range of chlorinated aromatic compounds. Following transformation of the chlorophenols to chlorocatechols, ring cleavage by dioxygenases may proceed.

Cleavage of the aromatic ring may occur using either the *ortho*- or the *meta*- pathway. Aromatic compounds found naturally in the environment, such as phenol and benzene, are typically broken down via the *meta*- cleavage pathway. While methyl-substituted aromatic compounds are also successfully degraded via the *meta*- cleavage pathway, chlorinated aromatic compounds are generally broken down via the *ortho*-pathway. Two types of *ortho*- pathway exist - *ortho*-type I pathway, involved in the degradation of unchlorinated aromatics, via catechol, and a modified *ortho*- type II pathway, specific to the degradation of chlorinated aromatics, via chlorocatechol (Dorn

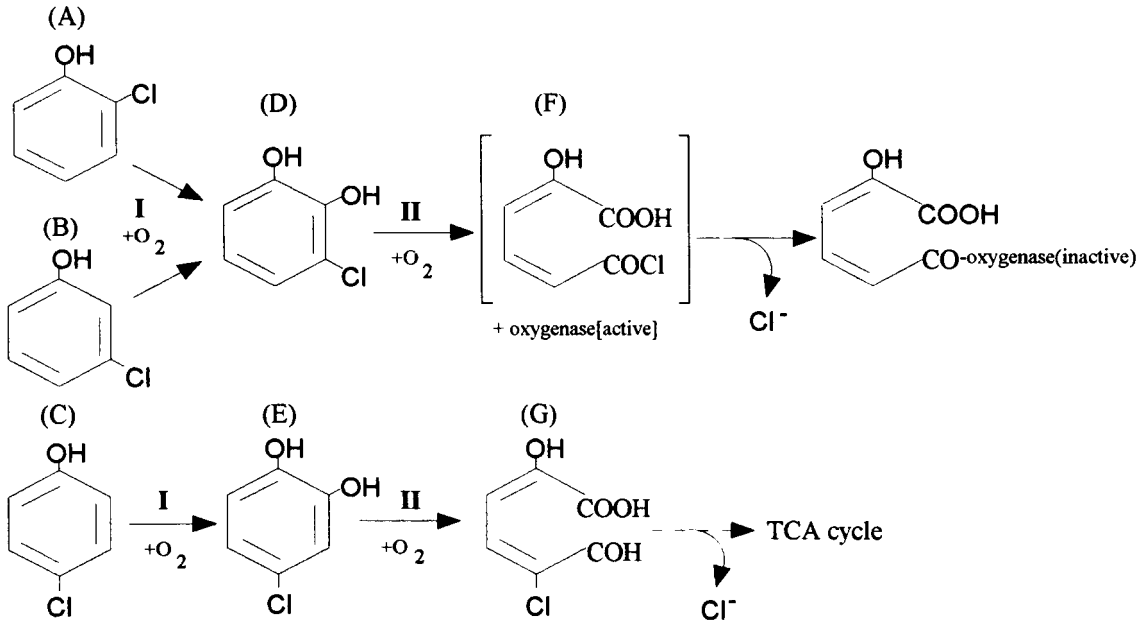


Figure 1. Degradation of mono-chlorophenols via the *meta*- cleavage pathway. A, 2-chlorophenol, B, 3-chlorophenol, C, 4-chlorophenol, D, 3-chlorocatechol, E, 4-chlorocatechol, F, 5-chloroformyl-2-hydroxy-penta-2,4-dienoic acid, G, 5-chloro-2-hydroxymuconic semialdehyde, **I**, phenol hydroxylase, **II**, catechol 2,3-dioxygenase.

and Knackmuss, 1978). *Ortho*- cleavage (Type II) of chlorinated aromatic compounds, catalyzed by the enzyme catechol 1,2-dioxygenase, is generally necessary for complete degradation, with a resulting release of the chloride atom. *Meta*- cleavage by catechol 2,3-dioxygenase is known to generally result in incomplete metabolism due to the production of dead-end or suicide-metabolites (Fig. 1) (Schmidt *et al.*, 1983). Successful degradation of chloro-aromatics via 3-chlorocatechol using the *meta*- cleavage pathway has been demonstrated (Mars *et al.*, 1997) but is extremely rare. Generally *meta*- cleavage of 3-chlorocatechol results in the inactivation of catechol 2,3-dioxygenase either by 3-chlorocatechol itself, acting as a chelating compound (Klecka and Gibson, 1981), or by the production of a highly reactive acylchloride, the product of the cleavage of 3-chlorocatechol, which binds irreversibly to the *meta*- cleavage enzyme (Bartels *et al.*, 1984). The *meta*- cleavage of 4-chlorocatechol results in the production of a chlorinated aliphatic compound, 5-chloro-2-hydroxymuconic semialdehyde (Fig. 1) (Weiser *et al.*, 1994) which has been widely reported as being a dead-end metabolite (Reineke *et al.*, 1982; Westmeier and Rehm, 1987). However, recent reports have shown that further metabolism of 5-chloro-2-hydroxymuconic semialdehyde may occur, resulting in complete degradation of 4-chlorophenol via a *meta*- cleavage pathway (Sung Bae *et al.*, 1996; Hollender *et al.*, 1997). This paper reports our findings on the ability of a mixed microbial community to aerobically degrade 2-, 3- and 4-chlorophenol as sole carbon source. The mixed culture used was a commercially produced wastewater bioaugmentation product, specially formulated to degrade a wide range of substituted aromatic compounds. Degradation of the mono-chlorophenols by the mixed microbial community was found to be via the *meta*- cleavage pathway, and the effect of this pathway on the degradative system was investigated.

Materials and Methods

Chemicals

2-chlorophenol, 3-chlorophenol and 4-chlorophenol, used in the degradation studies, as well as minimal medium components, were obtained from Sigma-Aldrich Chemical Co. (Dorset, UK). 3-chlorocatechol and 4-chlorocatechol were obtained from Helix Biotech Corporation (Richmond, British Columbia, Canada).

Mixed Culture

The mixed culture used was a commercially produced wastewater treatment bioaugmentation product comprising of bacteria belonging to the genera *Pseudomonas* and *Actinomycetes*, together with a species of white rot fungus, *Trichoderma harzanium*. Surfactants, cryo-protectants and growth media were also present along with the sterile cereal base.

Culture Conditions

Chlorophenol biodegradation experiments were performed in 250ml conical flasks containing 100mls of minimal medium and 1.56 mM chlorophenol or 0.69 mM 4-chlorocatechol as sole carbon source. The minimal medium used, adapted from Goulding *et al*. (1988), contained (g/L): K_2HPO_4, 4.36; NaH_2PO_4, 3.45; $(NH_4)_2SO_4$, 1.26; $MgSO_4.6H_2O$, 0.912; Trace salts solution 1ml/l. The trace salts solution contained (g/100ml): $CaCl_2.2H_2O$, 4.77; $FeSO_4.7H_2O$, 0.37; $CoCl_2.6H_2O$, 0.37; $MnCl_2$, 0.10; $Na_2MoO_4.2H_2O$, 0.02. The pH of the medium was adjusted to pH 7.0 and autoclaved at 121 °C for 15 mins. Chlorophenols and chlorocatechols were added to flasks following sterilization of the media.

The inoculum, supplied in the form of a buff/brown freeze-dried granular powder, was rehydrated in sterile dilution buffer (10g in 90ml), shaken for 1 hr at 30°C and allowed to settle for 5 min. Dilution buffer was made up by addition of 1.25ml 0.1M K_2HPO_4, pH 7.2 and 1ml 10% v/v Triton X-100 to 1 litre distilled H_2O. The supernatant fluid (10ml) was centrifuged at 5,000 rpm for 10 min, the pellet was then washed twice with 10 ml 0.01M sodium phosphate buffer, pH 7.0, removing any additional growth substrates contained in the mixed culture, and used to inoculate chlorophenol or chlorocatechol flasks (10% v/v).

The flasks were incubated in an orbital shaker at 150 rpm at 30 °C. Uninoculated control flasks were incubated in parallel. No drop in 3- and 4-chlorophenol concentrations were found in the control flasks over the course of the biodegradation experiments, while the average loss in 2-chlorophenol concentrations due to evaporation was found to be 4.65 μM/hr. All values were corrected to account for evaporation losses.

Measurement of Cell Growth

Growth of the mixed culture on the monochlorophenols was followed by measurement of the optical density at 660nm and by performing cell counts on Oxoid plate count agar using the pour plate method. The plates were incubated at 30 °C for 24 hours.

Analytical Methods

pH was measured using an Orion pH electrode (model 9157BN). Chloride release was followed with an Orion chloride specific electrode (model 9417). Chloride concentrations were calculated with reference to a standard curve constructed with NaCl standards. Samples and standards were diluted with 2% ionic strength adjusting solution (5M $NaNO_3$).

All optical density measurements were carried out using an ATI Unicam spectrophotometer while UV spectra were recorded using a Shimadzu UV Visable recording spectrophotometer (Model UV-160A).

Measurement of Chlorophenol Concentrations

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

Measurement of Chlorocatechol Concentrations

Chlorocatechol concentrations were quantified by the method of Arnow (1937). Samples were centrifuged at 5,000 rpm for 10 mins to remove cells. 1 ml of standard/sample was placed in a test-tube (1/10 dilution sample used). Standards were prepared in the range of 0–0.020 mg/ml chlorocatechol. To each test-tube 1 ml 0.5 N HCl was added. Tubes were mixed well and to this 1 ml nitrite-molybdate reagent was added resulting in a yellow colour. Nitrite-molybdate reagent

was prepared by dissolving sodium nitrite and sodium molybdate in water to a concentration of 1g/10 ml. After mixing, 1 ml of 1 N NaOH was added resulting in a red colour. To this 1 ml distilled water was added. Following mixing, the absorbance was read at 510 nm and concentrations were calculated from the standard curve.

Identification of Metabolites

Identification of chlorocatechols, was carried out using HPLC. Samples were centrifuged to remove cells, the resulting supernatant (5 ml) was acidified to pH 2.0 with 2M HCl, and extracted with ethyl acetate, (2 ml) in 3 successive extractions. The resulting organic phase was assayed immediately or stored at 4 °C for 2–3 days. A sample was loaded into a Reodyne injection loop and a Shimadzu LC-9A solvent delivery unit automatically injected 20 μl of sample onto the Novapak C18 column (Waters, 4 μm particle size, 3.9 $\times$ 150 mm stainless steel column). The mobile phase used was 0.075 M acetic acid/Acetonitrile (70 : 30) at a flow rate of 0.8 ml/min. Detection of chlorocatechols was at 270 nm using a Shimadzu SPD-6AV UV/Vis detector. An Axiomm Chromatography data acquisition package was used to monitor and process the data.

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

Enzyme Assays

Cells, grown up on 1.56 mM mono-chlorophenol, were harvested by centrifugation (5,000 rpm, 10 mins), and washed twice in 0.033 M Tris-HCl buffer (pH 7.6). The cells were broken by sonication and centrifuged at 20,000 rpm at 0–4 °C for 20 mins. The cell free extract was kept on ice and assayed as soon as possible for catechol dioxygenase activity.

Catechol 1,2-dioxygenase (Type I) activity was measured by following the formation of *cis,cis*-muconic acid, the *ortho*- cleavage product of catechol. The following reagents were added to a quartz cuvette: 2 ml 50 mM Tris-HCl buffer (pH 8.0); 0.7 ml distilled water; 0.1 ml 100 mM 2-mercaptoethanol; 0.1ml cell-free extract. The contents of the cuvette were mixed by inversion and 0.1ml catechol (1 mM) was then added and the contents mixed again. *Cis,cis*-muconic acid formation was followed by an increase in the absorbance at 260nm over a period of 5 mins.

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

Catechol 2,3-dioxygenase activity was measured by following the formation of 2-hydroxymuconic semialdehyde, the *meta*- cleavage product of catechol, or the formation of 5-chloro-2-hydroxymuconic semialdehyde, the *meta*- cleavage product of 4-chlorocatechol. The following reagents were added to a plastic cuvette: 2 ml 50 mM Tris-HCl buffer (pH 7.5); 0.6 ml distilled water; 0.2 ml cell free extract. The contents were mixed by inversion and 0.2 ml catechol or 4-chlorocatechol (100 mM) was added and mixed with the contents. The production of 2-hydroxymuconic semialdehyde was followed by increase in absorbance at 375 nm over a period of 5 mins, while the production of 5-chloro-2-hydroxymuconic semialdehyde was followed by the increase in absorbance at 380 nm over the same period.

Specific activities were calculated by using the following extinction coefficients for each reaction product: catechol at 260 nm = 16,800 l/mol/cm; 3-chlorocatechol at 260 nm = 17,100 l/mol/cm, 4-chlorocatechol at 380 nm = 42,600 l/mol/cm (Schmidt, 1987) and catechol at 375 nm = 36,000 l/mol/cm (Sung Bae *et al.,* 1996). The specific enzyme activity is expressed as μmoles of product formed per min per mg of protein. The protein concentrations in cell free extracts were determined by the method of Lowry *et al.,* (1951).

Results/Discussion

Degradation of 2- and 3-Chlorophenol by the Mixed Culture

Degradation of 2- and 3-chlorophenol (1.56 mM) was incomplete, as indicated by the non-stoichiometric releases of chloride. 2-chlorophenol degradation appeared to cease after 48 hours. Despite 64.8% of 2-chlorophenol being removed, only 30.2% of the total chloride was released (Fig. 2). Metabolism of 3-chlorophenol by the mixed culture continued resulting in 100% removal after 238 hours. However, only 38.0% chloride was released, indicating incomplete

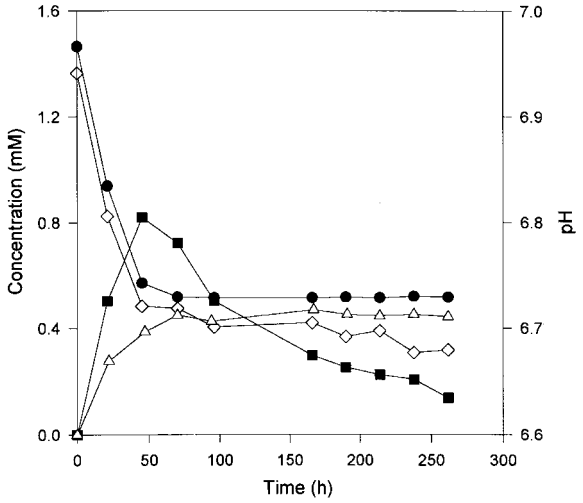


Figure 2. Degradation of 2-chlorophenol by the mixed culture. Symbols ● 2-chlorophenol, ■ 3-chlorocatechol, △ differential chloride concentration & ◊ pH.

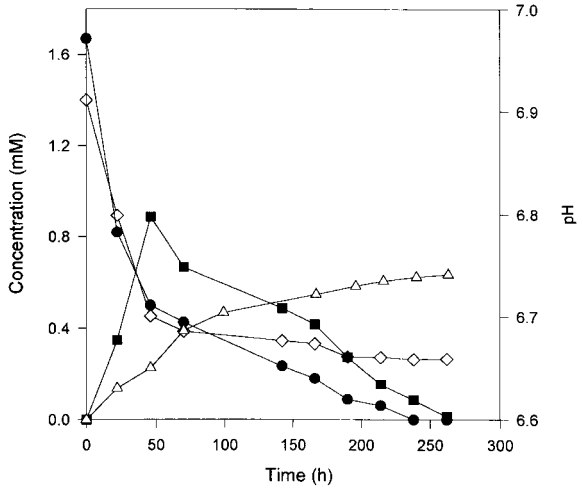


Figure 3. Degradation of 3-chlorophenol by the mixed culture. Symbols ● 3-chlorophenol, ■ 3-chlorocatechol, △ differential chloride concentration & ◊ pH.

degradation (Fig. 3). The pattern of chloride release for both 2- and 3-chlorophenol mirrored the pH drop observed for both chlorophenols. The initial chloride release was accompanied by a corresponding drop in pH which reached a plateau as the chloride release also reached completion.

2- and 3-chlorophenol metabolism caused an accumulation of a degradative intermediate in the culture medium. Using HPLC analysis, the intermediate was identified as 3-chlorocatechol. As 2- and 3-chlorophenol metabolism continued, 3-chlorocatechol concentrations as measured by Arnow's colorimetric assay and HPLC reached a peak at 48 hours and began to drop, corresponding with the development of a brown colour which remained in the medium. The production of brown colours following the degradation of chloroaromatics via 3-chlorocatechol, using the *meta*- cleavage pathway, has been widely reported in the literature (Haller and Finn, 1979; Knackmuss, 1982; Adams *et al.*, 1992). Colouration of the medium is due to the build up of 3-chlorocatechol, which polymerises due to autooxidation, causing the brown colouration in the medium. Chlorocatechol accumulation results from the negative effect 3-chlorocatechol has on the *meta*- cleavage enzyme, catechol 2,3-dioxygenase. Two mechanisms have been proposed by which 3-chlorocatechol may interfere with the activity of catechol 2,3-dioxygenase. 3-chlorocatechol may act as a chelating agent, binding to the iron core of catechol 2,3-dioxygenase, resulting in reversible inactivation (Klecka and Gibson, 1981) causing a large build up of chlorocatechols. Alternatively, *meta*-cleavage of 3-chlorocatechol can lead to the production of a highly reactive acyl halide, 5-chloroformyl-2-hydroxy-penta-2,4-dienoic acid (Bartels *et al.*, 1984) which has been proposed to act as a suicide compound, binding irreversibly to catechol 2,3-dioxygenase with a subsequent release of chloride and a destruction of metabolic activity (Fig. 1). As some chloride release was observed during the degradation of 2- and 3-chlorophenol by the mixed culture, it is assumed that the build up of 3-chlorocatechol is due to the latter mechanism. The polymerized 3-chlorocatechol produced during degradation of 2- and 3-chlorophenol by the mixed culture resulted in a drop in chlorocatechol concentrations as measured by Arnow's colorimetric assay or HPLC. The drop in chlorocatechol concentrations was not due to further microbial degradation as was confirmed by control experiments containing 3-chlorocatechol in the absence of any microbial inoculum, incubated in parallel to chlorophenol degradation studies (Fig. 4). 3-chlorocatechol concentrations decreased at a similar rate to that observed during 2- and 3-chlorophenol degradation. As in chlorophenol degradation studies, decreases in 3-chlorocatechol concentrations corresponded with the development of a brown colour in the medium. The development of the brown coloured polymers corresponded with an increase in the optical density at 660 nm (Fig. 4). The development of pigment production resulting from polymerisation of catechols has been followed by measurement of the optical density at 600 nm following removal of cells (Haller and Finn 1979), while

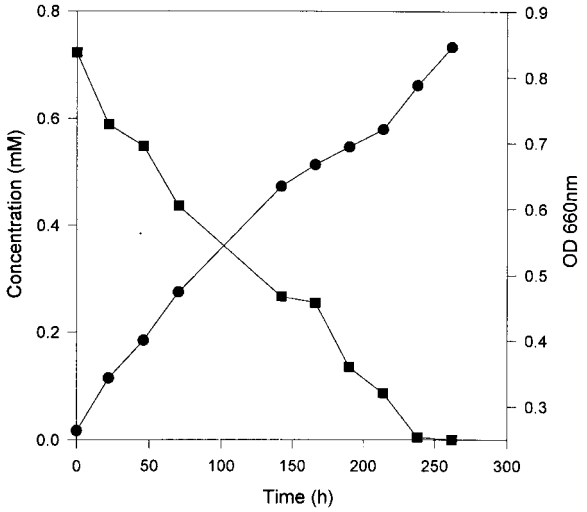
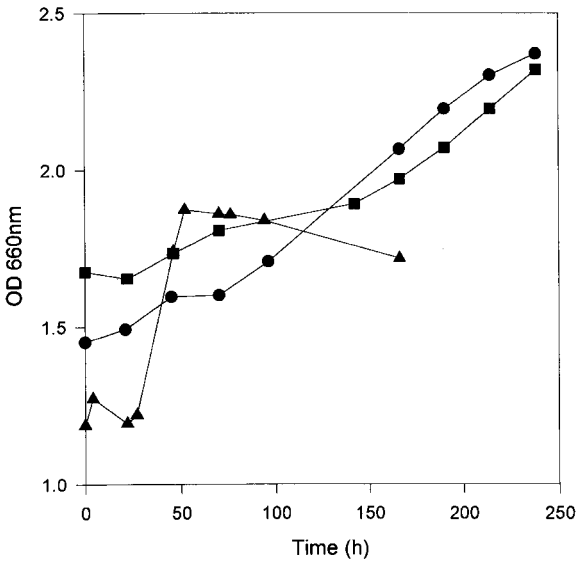


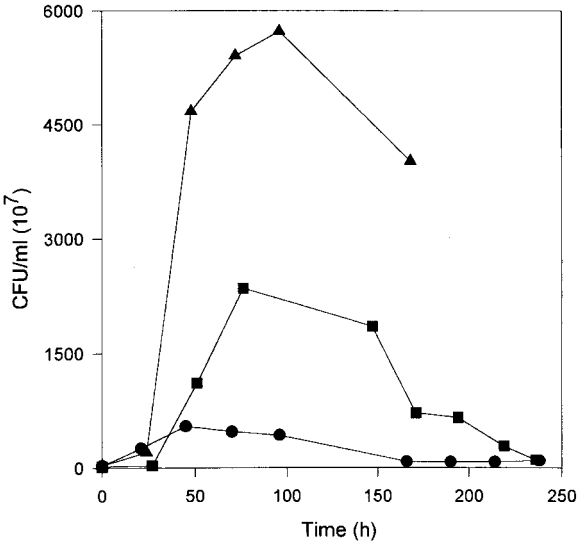
Figure 4. Incubation of 3-chlorocatechol in the absence of the mixed culture. Symbols ■ 3-chlorocatechol concentration & ● OD 660 nm.

an increase in the optical density at 600 nm due to the development of extracellular pigment production following degradation of 3-chlorobenzoate has been described in the literature (Fava *et al.*, 1993). It has been suggested that polymerization might not be due to autooxidation alone, but as a result of the activity of peroxidase and polyphenol oxidase enzymes, resulting in the darkening of the medium (Fava *et al.*, 1993). However polymerisation occurred in 3-chlorocatechol flasks in the absence of any microbial enzymes, indicating polymerisation resulted from abiotic processes.

The growth of the mixed culture on 2- and 3-chlorophenol was monitored by measurement of the optical density at 660nm and by carrying out plate counts. However while the optical density continued to rise during the degradation of 2- and 3-chlorophenol, suggesting continued cell growth (Fig. 5a), viable cell counts show that cell numbers reach a maximum after approximately 48 hours during growth on 2-chlorophenol and 72 hours on 3-chlorophenol following which viable cell numbers begin to decrease (Fig. 5b). Although the measurement of OD 660 nm suggest similar amounts of cell growth, cell numbers show significantly more growth on 3-chlorophenol than on 2-chlorophenol. The accumulation of brown coloured chlorocatechol polymers in the medium during the degradation of 2- and 3-chlorophenol appeared to interfere with the optical density around 660 nm. Arnesdorf and Focht (1994) describe how optical density could not be used as a measure of cell growth resulting from *meta*- cleavage of chloroaromatics via



(a)



(b)

Figure 5. Growth of the mixed culture on 2-chlorophenol, 3-chlorophenol and 4-chlorophenol, as measured by (a) O.D 660 nm and (b) viable cell count (CFU) per ml. Symbols: ● 2-chlorophenol, ■ 3-chlorophenol & ♦ 4-chlorophenol.

3-chlorocatechol, as cultures with very low viable-cell counts during degradation of 2- and 3-chlorobiphenyl routinely gave high optical density readings. This study further illustrates that optical density should not be used as an indication of cell growth in the presence of coloured polymers.

Experiments carried out on the degradation of 2- and 3-chlorophenol by the mixed culture had sug-

gested that ring cleavage had been via the *meta*-cleavage pathway. In order to confirm this, the key enzymes involved in catalyzing ring fission (catechol 1,2-dioxygenase, Types I and II, and catechol 2,3-dioxygenase) were assayed (Table 1). Enzyme assays confirmed that degradation was via the *meta*- cleavage pathway. Low levels of catechol 2,3-dioxygenase activities were detected during 2- and 3-chlorophenol degradation as activity is irreversibly inactivated by 3-chlorocatechol (Bartels *et al.*, 1984). No catechol 1,2-dioxygenase activity was detected during chlorophenol metabolism by the mixed culture. The low levels of catechol 2,3-dioxygenase detected, decreased quickly on accumulation of 3-chlorocatechol after 48 hours, demonstrating the toxicity of 3-chlorocatechol metabolism to the *meta*- cleavage enzyme. Even after the *meta*- cleavage capability of the mixed culture was destroyed by the accumulation of 3-chlorocatechol, no catechol 1,2-dioxygenase activity was detected, indicating the absence of any *ortho*-cleavage capability in the mixed culture.

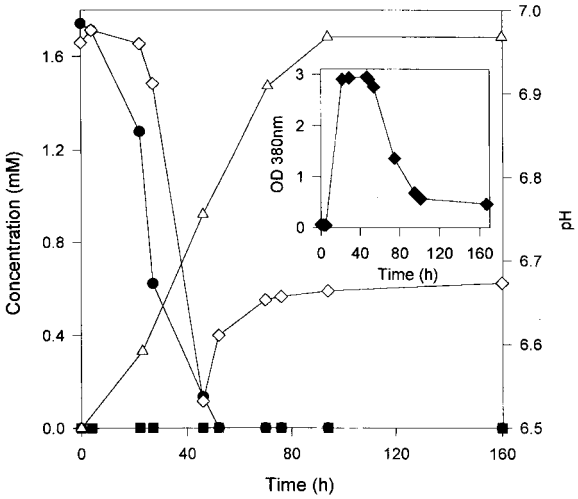


Figure 6. Degradation of 4-chlorophenol by the mixed culture. Symbols ● 4-chlorophenol, ■ 4-chlorocatechol, △ differential chloride concentration & ◊ pH. The insert shows the development of 5-chloro-2-hydroxymuconic semialdehyde as measured by O.D 380 nm, ♦.

Degradation of 4-chlorophenol by the Mixed Culture

Incubation of the mixed culture with 4-chlorophenol (1.56 mM) resulted in complete removal of the chlorophenol within 48 hours with a stoichiometric release of chloride within 100 hours (Fig. 6). The degradation of 4-chlorophenol resulted in a greater drop in pH than was observed during the degradation of 2- and 3-chlorophenol. This drop in pH resulted not only from the greater release of chloride, but due to the production of organic acids which are described as being intermediates of the degradation of 4-chlorophenol by the *meta*- cleavage pathway (Arnesdorf and Focht, 1995). However unlike the drop in pH following 2- and 3-chlorophenol degradation which reached a plateau, the pH began to rise again after 48 hours of 4-chlorophenol degradation. This rise in pH followed the further metabolism of the acidic metabolites produced and reached a plateau only after the complete metabolism of 4-chlorophenol.

No 4-chlorocatechol accumulation was detected during the degradation of 4-chlorophenol. However the ability of the mixed culture to degrade 4-chlorocatechol as an intermediate of 4-chlorophenol degradation was demonstrated by incubation of 4-chlorocatechol with the mixed culture. Growth of the mixed culture on 4-chlorocatechol (0.69 mM) resulted in complete removal within 120 hours and a stoichiometric release of chloride within 160 hours (Fig. 7). During the degradation of 4-chlorophenol and 4-chlorocatechol, a yellow colour accumulated in the medium. However, unlike the brown colour produced during 2- and 3-chlorophenol degradation, this yellow colour did not remain in the medium. During the degradation of 4-chlorophenol, the yellow colour became visible within 16 hours, developed to form a straw yellow colour and finally disappeared after 48 hours, suggesting further metabolism of the yellow intermediate. The accumulated intermediate had all the characteristics of 5-chloro-2-hydroxymuconic semialdehyde, the *meta*- cleavage product of 4-chlorocatechol, with an absorption maximum of 379nm at pH 7.0 and 334nm at pH 2.0, with the yellow colour disappearing reversibly upon acidification, as described by Wieser *et al.*, (1994). Incubation of 5-chloro-2-hydroxymuconic semialdehyde with the mixed culture resulted in further metabolism as demonstrated by the disappearance of the yellow colour, the drop in 5-chloro-2-hydroxymuconic semialdehyde concentrations as indicated by OD 380 nm measurements and the stoichiometric release of chloride (Fig. 6).

The *meta*- cleavage product of 4-chlorocatechol had been widely reported as being a dead-end metabolite (Reineke *et al.*, 1982; Balfanz and Rehm, 1991; Weiser *et al.*, 1994). However complete degradation of 4-chloroaromatics via a *meta*- cleavage pathway has been reported in the literature. Arnesdorf and

Table 1. Ring cleavage activities during the degradation of mono-chlorophenols by the mixed microbial community

Enzyme Assayed and assay substrate	Specific activity (μ moles/min/mg) after growth on:								
	2-chlorophenol			3-chlorophenol			4-chlorophenol		
	T = 24	T = 48	T = 72	T = 24	T = 48	T = 72	T = 24	T = 48	T = 72
Catechol 2,3-dioxygenase									
Catechol	0.003	0.001	<0.001	0.004	0.001	<0.001	0.096	0.042	0.001
4-chlorocatechol	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.007	0.005	<0.001
Catechol 1,2-dioxygenase									
Catechol	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
3-chlorocatechol	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

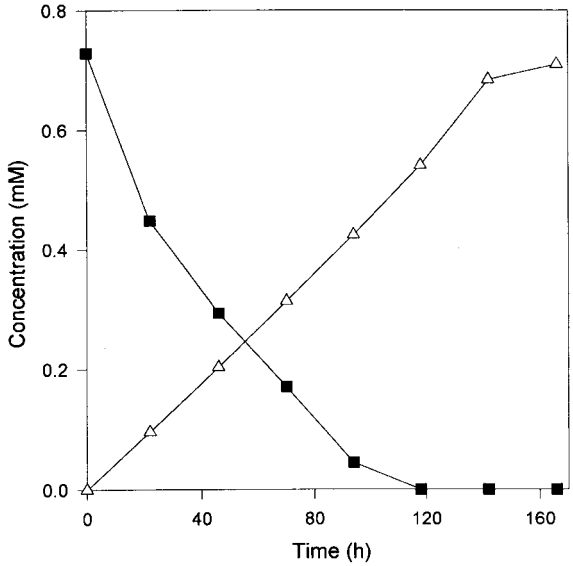


Figure 7. Degradation of 4-chlorocatechol by the mixed culture. Symbols ■ 4-chlorocatechol concentration & ◊ differential chloride concentration.

Focht (1994) described how 4-chlorobiphenyl was degraded via 4-chlorocatechol to completion using the *meta*- cleavage pathway by a *Pseudomonas cepacia* P166. The mechanism by which 4-chloroaromatics are degraded via 4-chlorocatechol by a *meta*- cleavage pathway is further described by Arnesdorf and Focht (1995). 5-chloro-2-hydroxymuconic semialdehyde is further metabolised leading to the production of pyruvic acid and chloroacetic acid. Chloroacetic acid may then be dehalogenated to form glycolate, which may be utilized along with pyruvic acid in the TCA cycle (McCullar *et al*., 1994). Hollender *et al.*, (1997) reported the successful degradation of 1.8 mM 4-chlorophenol via the *meta*- cleavage pathway by *Comamonas testosteroni* JH5, while Sung Bae *et al.*, (1996) described the complete degradation of up to 0.6 mM 4-chlorophenol by *Comamonas testosteroni* CPW301 using a *meta*- cleavage pathway. Earlier work carried out in this laboratory involved a study of the microbiology of the mixed microbial community when cultivated on 2-, 3- and 4-chlorophenol (O' Sullivan, 1998). Interestingly no organisms were found to be capable of growth on 1.56 mM 2- or 3-chlorophenol as pure cultures, while 2 *Pseudomonas* species were the only organisms in the mixed culture capable of growth on 1.56 mM 4-chlorophenol. Degradation of 4-chlorophenol by these isolated Gram negative bacteria took place via the *meta*- cleavage pathway.

The production of the yellow coloured intermediate did not interfere with optical density readings at 660 nm and hence absorbance readings could be used as an indication of growth on 4-chlorophenol. (Fig. 5a & b). The growth of the mixed culture as measured by turbidity readings and plate counts continued to rise during 4-chlorophenol degradation. Cell numbers only began to show a significant decrease as the complete metabolism of 4-chlorophenol neared completion after approximately 100 hours. Viable cell counts show a greater increase in cell numbers during the degradation of 4-chlorophenol, which is as expected due to its complete mineralization by the mixed culture, followed by 3-chlorophenol and 2-chlorophenol (Fig. 5b) indicating the relative toxicity's of the mono-chlorophenols to the mixed culture.

As was shown for 2- and 3-chlorophenol, degradation of 4-chlorophenol by the mixed culture was via the *meta*- cleavage pathway. Catechol 2,3-dioxygenase activity on 4-chlorophenol and 4-chlorocatechol was detected following growth on 4-chlorophenol. Higher levels of catechol 2,3-

dioxygenase activity were detected following growth on 4-chlorophenol than were observed during 2- and 3-chlorophenol degradation by the mixed culture (Table 1). Again, no catechol 1,2-dioxygenase activity, Types I or II, was detected during 4-chlorophenol metabolism. This result, the detection of *meta*- activity and no *ortho*- activity, was similar to that obtained when the degradation of 4-chlorophenol by *Comamonas testosteroni* CPW301 (Sung Bae *et al.*, 1996) and by *Comamonas testosteroni* JH5 (Hollender *et al.*, 1997) was investigated. The levels of catechol 2,3-dioxygenase activity detected during the degradation of 4-chlorophenol disappeared only after 4-chlorophenol was completely degraded by the mixed culture.

Conclusions

The mixed culture, a commercially produced wastewater bioaugmentation product, degraded the mono-chlorophenols via a *meta*- cleavage pathway. *Meta*- cleavage of 2- and 3-chlorophenol resulted in a dead-end pathway leading to incomplete degradation demonstrating the requirement for *ortho*- cleavage for successful degradation which could by assured by incorporating suitable microbes in the product. However, degradation via the *meta*- cleavage pathway led to complete degradation of 4-chlorophenol with a stoichiometric release of chloride.

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