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THE ANAEROBIC BIODEGRADATION OF 2-CHLOROPHENOL AND 4-CHLOROPHENOL IN ATTACHED AND SUSPENDED GROWTH REACTORS

BY

TWILA GRIFFITH ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of **Master of Science**

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Twila Griffith

Twila D. Griffith
2707 - 124th Street
Edmonton, Alberta
T6J 4T2

April 25, 1994
Date

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **The Anaerobic Biodegradation of 2-Chlorophenol and 4-Chlorophenol in Attached and Suspended Growth Reactors** by **Twila D. Griffith** in partial fulfillment of the requirements for the degree of **Master of Science**.

Dr. E. Knettig (Supervisor)

Dr. D. W. Smith

Dr. N. O. Egiebor

April 28, 1994
Date

Abstract

During this study the anaerobic biodegradation of 2-chlorophenol and 4-chlorophenol by attached and suspended growth cultures was examined.

Cultures were set-up and maintained in 50 mL serum bottle reactors. These reactors, containing either attached growth or suspended growth bacterial cultures, were successfully established and acclimated to either 2-chlorophenol or 4- chlorophenol as their sole sources of substrate.

A semicontinuous fermentation study was conducted to evaluate the performance of attached growth, suspended growth and supernatant only cultures in batch assays with respect to the removal of 2- or 4-chlorophenol under conditions of steady state. Serum bottle reactors established during the acclimation study were subject to draw and feed conditions of 50 mg/L 2-chlorophenol or 4-chlorophenol substrate and a 10 day hydraulic retention time. Under these conditions the reactors were monitored for removal of the chlorophenol substrate and methane production. Reactors containing supernatant only were established during supernatant exchange to provide a control for the attached growth and suspended growth reactors.

The study found that the attached growth reactors performed superior when compared to suspended growth reactors. Attached growth reactors reported shorter acclimation times for both 2-chlorophenol and 4-chlorophenol than suspended growth reactors. The presence of resin provided a solid support material that promoted an extremely stable biomass. The attached growth reactors were much less prone to the effects of "wash-out", loading changes and substrate toxicity. Higher 2- chlorophenol and 4-chlorophenol degradation rates were achieved for attached growth reactors than suspended growth reactors.

Under pseudo steady state conditions, 2-chlorophenol and 4-chlorophenol degradation rates of 5.2 mg/L per day were achieved by the attached growth reactors.

However, over an extended period of exposure all reactors were affected by the toxicity of the monochlorophenol substrate. Anaerobic degradation of reactors containing attached or suspended growth cultures was noticeably inhibited by the continued presence of 4-chlorophenol, culminating in reactor failure.

Slight morphological differences were noted in the attached bacteria consortia. More types of rod shaped bacteria were present in reactors fed 2-chlorophenol. In comparison, more filamentous and coccoid types of bacteria were present in reactors fed 4-chlorophenol. No visual difference or increase in bacterial colonization of the resin was noted throughout the study.

The study concluded that the long term sustainability of anaerobic bioreactors with 2- or 4-chlorophenol as their sole source of substrate is in need of further investigation. Chlorophenols are highly toxic and in long term exposure may be disruptive to the bacterial enzymatic system.

Acknowledgements

I would like to thank my supervisor Dr. E. Knettig for her excellent guidance, advice and patience throughout the course of this project.

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1.0 Introduction and Research Objectives

Chlorinated phenols comprise a large group of toxic man made chemicals that are serious environmental pollutants (Steiert et al., 1985). Recent concern has been expressed on the environmental fate of these compounds due to their carcinogenic and toxic properties.

In 1992 approximately 72,000 metric tonnes of phenol were consumed in Canada, with 3,000 metric tonnes including chlorophenols such as pentachlorophenol (PCP). As of May, 1992, domestic production of chlorophenols ceased and industrial supplies of primarily pentachlorophenol and tetrachlorophenol have been imported (Fujise et al., 1993).

Chlorophenols are considered priority pollutants due to their harmful environmental effects as a consequence of their toxicity, bioaccumulation and resistance to biodegradation. Bioaccumulation and persistence of the chlorinated phenols in the aquatic environment have created concern over their discharge from bleached kraft pulp mills into the receiving waters (Kovacs et al., 1984). The toxicity of chlorinated phenols tends to increase with their degree of chlorination, and because few microbes can decompose them, the multichlorinated phenols tend to accumulate in the environment (Steiert and Crawford, 1985). Some chlorophenols are not only toxic to aquatic vertebrates and fish but are also suspected to be potential carcinogens and mutagens (Wan, 1992).

Chlorinated phenols are used as antifungal agents, wood preservatives, insecticides and are produced as degradation products of chlorophenoxy herbicides (Rochkind-Dubinsky et al., 1987). Pentachlorophenol (PCP) has been used as a wood preservative, preharvest herbicide, and a

fungicide/bactericide in cooling tower waters, adhesives, construction materials, textiles, leather, paint, paper, and oil well drilling mud (Mikesell and Boyd, 1986 and Guthrie et al., 1984). 2,4-Dichlorophenol and 4-chlorophenol are used in the production of PCP and the herbicides 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid (Kohring et al., 1989b). Chlorophenols are also intermediates in the aerobic metabolism of chlorobenzoates, chlorobenzenes and chlorinated phenoxy acids (Krumme and Boyd, 1988).

Chlorinated phenols are found in pulp mill effluents from chlorine-bleached soft wood processing. Low molecular weight chlorinated phenolic compounds formed during pulp chlorination have been identified in pulp mill bleachery effluents (Kovacs et al., 1984). Trichlorophenol is the principal chlorinated phenol in bleached sulphite discharges (CEPA, 1991).

2-Chlorophenol (orthochlorophenol) and 4-chlorophenol (parachlorophenol) are formed when gaseous chlorine is passed into molten phenol and separated by fractional distillation (Krijgsheld and van der Gen, 1986). 2-Chlorophenol is also produced as an intermediate in the synthesis of higher chlorophenols and phenolic resins. 4-Chlorophenol is produced as an intermediate in the production of higher chlorinated phenols, in the synthesis of dyes and drugs, as a denaturant for alcohol and as a selective solvent in refining of mineral oils (Krijgsheld and van der Gen, 1986).

Chlorophenols are also formed during the chlorination of effluents of various industrial waste treatment plants or in drinking water sources containing low phenol concentrations and high chlorine concentrations (Kovacs et al., 1984; Krijgsheld and van der Gen, 1986).

Chlorinated phenols are found in lake and river sediments, municipal waste systems, soils and in groundwater. They are released to the environment deliberately in pest control, by direct disposal of industrial chemicals into landfills or into waterways through wastewater disposal, as breakdown products of chlorinated phenolics or accidentally as in seepage from toxic waste disposal sites. PCP has been identified as a contaminant in the vicinity of lumber mills and paper and pulp factories. Chlorophenols also enter the environment through chemical "bleeding" from newly treated and installed poles and ties, followed by migration into the soil during rainstorms or by surface runoff and leaching processes active in the soil (Wan, 1992). In Finland, environmental soil, groundwater and fauna samples have been found to contain a variety of compounds originating from the technical-grade PCP formulation used for lumber treatment. The contaminants identified include polychlorophenoxy-phenols, polychlorophenoxy anisoles, polychlorodibenzodioxins, and furans, polychlorinated phenols, catechols and guaiacols (Mikesell and Boyd, 1986).

High water solubility and widespread use of chlorinated phenolic compounds have resulted in concern over their potential contamination of potable ground water supplies (Krumme and Boyd, 1988). If aerobic degradation is slow, these compounds pass into the anaerobic zone above the groundwater zones (Kohring et al., 1989b). If not degraded, these compounds will contaminate aquifers. Aromatic hydrocarbons, phenols and cresols are among the most prevalent aromatic contaminants in groundwater (Lovley and Lonergan, 1990). Taste and odour-causing compounds in surface water and potable water have also been associated with chlorinated phenols (Kovacs et al., 1984).

Disposal of pollutants, including chlorinated phenols, has become very difficult from both a practical and political standpoint. There is a definite need for new disposal methods. One approach currently being investigated, bioremediation, involves the use of microorganisms for biodegradation and biotransformation of these wastes. Other approaches include volatilization, photodegradation, chemical oxidation, and sorption. However, biodegradation by microorganisms is considered to be one of the major mechanisms by which chlorophenols and other pollutants can be successfully broken down into simpler compounds that are less harmful to the environment.

Bioremediation has been shown to be favorable in the cleanup of soils contaminated with high concentrations of chlorophenols. Concentrations of 1002 mg PCP/kg of soil, contaminated during wood treatment, have been successfully reduced to less than 5 mg PCP/kg of soil (Seech, 1993).

There have been many studies directed towards the anaerobic degradation of multichlorinated phenols. The scope of this research was to examine specific aspects of 2-chlorophenol and 4-chlorophenol with respect to optimizing the design of facilities required to treat chlorophenolic wastes.

1.1 Research Objectives

The overall objective of this project was to provide a rational base for optimizing design and performance of anaerobic biological process for chlorophenol wastewaters. Specific objectives of the project were:

a) to investigate the acclimation of anaerobic microbial cultures with different types of growth;

b) to compare the performance of semi-continuous reactors (draw and feed) with contain attached and suspended growth cultures;

c) to determine degradation rates for 2- and 4-chlorophenol substrates; and

d) to examine bacterial colonization of a solid support media.

2.0 Literature Review

The aim of this literature review is to provide background information on the physical properties of chlorophenols, toxicity of chlorophenols, chlorophenol biodegradation and solid support media as a biological support.

2.1 Physical Properties of Chlorophenols

Chlorophenols are members of the arene family of organic compounds. Benzene is the common name given to this family which exhibits the general formula ArH and is symbolized by the aromatic ring.

The aromatic ring is made up of a six-member carbon ring which contains three double bonds between alternate carbon atoms, linked in a cyclic hexagonal form. Carbon atoms in these ring compounds have a single covalent bond available.

The simplest aromatic ring is the benzene ring (C_6H_6) and is known as the parent compound of the aromatic series (Sawyer and McCarty, 1978). Phenol (C_6H_5OH) is the monohydroxy derivative of benzene.

Chlorophenols are comprised of a single aromatic ring with one hydroxy radical attached in the first position and a single chlorine atom attached in one or more of five possible positions.

Positions of the chlorine atoms are defined with respect to the point of substitution (Figure 2.1). If substitution occurs on carbon atoms adjacent to the hydroxy radical the term ortho (o-) or position 2 or 6 is applied. If substitution occurs on carbon atoms once removed from the hydroxy radical the term meta (m-) or position 3 or 5 is applied, and substitution on carbon atoms opposite the hydroxy radical the term para (p-) or position 4 is used.

The prefixes mono, di, tri, tetra and penta are used to indicate the number of chlorine substitutions. Monochlorophenols have a single chlorine atom substituted for a hydrogen while pentachlorophenol has all five positions (2 to 6 inclusive) substituted by chlorine atoms.

Most pure monochlorophenol compounds are white, crystalline substances. Pure 2-chlorophenol is a colourless liquid at room temperature. Chlorophenols behave as weak acids with an increase in solubility in alkaline solutions (Krijgsheld and van der Gen, 1986). Other important physical properties of chlorophenols are presented in Table 2.1.

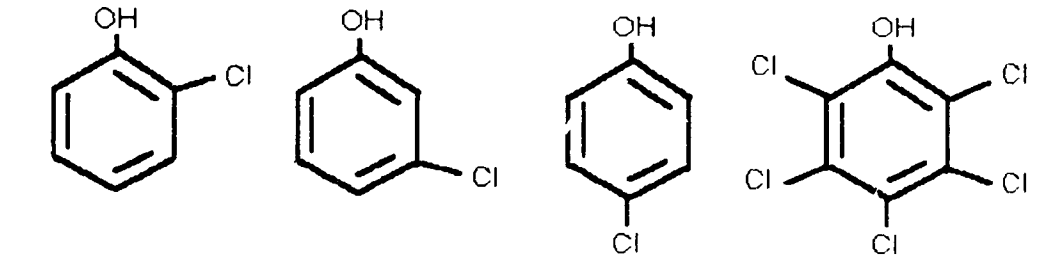


FIGURE 2.1 Molecular Structure of Chlorophenols (Adapted from Solomons, 1984).

TABLE 2.1 PHYSICAL PROPERTIES OF CHLOROPHENOLS

Parameter	2-chlorophenol	3-chlorophenol	4-chlorophenol	2,4-dichlorophenol
Molecular Mass (g/mol)	128.6	128.6	128.6	163.0
Physical State	liquid	solid	solid	solid
Colour	colourless	white	white	white
Melting Point (°C)	9.3	33	43	45
Boiling Point (°C)	175	214	217	210
Vapour Pressure	1 mm Hg @ 12.1°C	1 mm Hg @ 44.2°C	1 mm Hg @ 49.8°C	1 mm Hg @ 53°C
Water Solubility (@ 20°C, g/L H_2O)	28.5	26	27.1	4.5
Log Partition Coefficient (n-oct/water)	2.15	2.50	2.39	3.08
pK_a	8.48	9.02	9.38	7.85

Adapted from Krijgsheld and van der Gen (1986).

2.2 Toxicity

As a group, chlorophenols are highly toxic and often precursors to dioxins. Dioxins may be present as minor contaminants in some chlorophenol-based products. Technical pentachlorophenol(PCP) has been reported to typically contain 500 to 1500 mg/L of dioxin (Lamparski et al., 1980). Dioxins may also be produced when organic and chlorine-containing materials such as chlorinated phenols are combusted (Lamparski et al., 1980; Bridle et al., 1983; Boyd and Shelton, 1984). 4-chlorophenol is a suspected carcinogen and along with 2,4-dichlorophenol is highly toxic (Kohring et al., 1989b). PCP is toxic to a variety of organisms and along with other chlorophenols such as 2-chlorophenol, 2,4-dichlorophenol and 2,4,5-trichlorophenol has been placed on the US. Environmental Protection Agency (USEPA) list of 129 priority pollutants (Mikesell and Boyd, 1986; Krumme and Boyd, 1988; Eckenfelder, 1989).

Detectable levels of chlorophenols have been shown to accumulate in feral pink salmon and juvenile chinook salmon (Servizi et al., 1988). The probable sources of contamination cited were lumber mills using chlorophenol based fungicides and pulp mill effluents. Rogers et al., (1988) presented evidence of bioconcentration of chlorophenols and chloroguaiacols in feral and juvenile chinook salmon during winter conditions. These fish were exposed to effluent from sewage, pulp mills and commercial wood preservative operations.

Toxicity tests performed with chlorophenol include the LC_{50} and Microtox® EC_{50} test. The LC_{50} or lethal concentration test defines the lethal concentration of a sample in which 50% of the test animals immersed in the sample die over a specified time period. The Microtox® EC_{50} bacterial luminescence test estimates the effective concentration of a sample that causes

a 50% decrease in light output at 15°C after a specified period of time, usually 15 minutes. Results of toxicity tests performed with select chlorophenols are provided in Table 2.2.

The primary routes of chlorophenol exposure for humans and other mammals are oral intake (drinking contaminated water or eating food than contains chlorophenol residues) and skin absorption (Krijgsheld and van der Gen, 1986). Kauppinen and Lindroos (1985) measured personnel exposure to chlorophenols in 10 Finnish sawmills. High concentrations (up to 0.21 mmol/L) of chlorophenols were detected in workers that had skin contact with chlorophenols. They concluded that skin absorption appeared to be the main route of exposure. In mammals, phenolic compounds such as 2-chlorophenol, 4-chlorophenol and 2,4-dichlorophenol become largely combined with sulfate or glucuronic acid, leading to their detoxification and efficient elimination. If not transformed, these compounds may bioaccumulate and be distributed into organ and fatty tissues.

A summary of relevant toxicity data for selected chlorophenols is given in Table 2.3.

TABLE 2.2 SUMMARY OF TOXICITY TESTS FOR SELECTED CHLOROPHENOLS

Medium	Test	Animal	Toxic Level	Reference
2-Chlorophenol	LC_{50}(96 hr)	water flea and rainbow trout	2.6 mg/L	1
	LC_{50}(96 hr)	water flea	< 0.77 mg/L	2
	Microtox® EC_{50}	-	0.51-0.58 mg/L	2
	LC_{50}(96 hr)	fathead minnow	11.63 mg/L	3
	LC_{50}(96 hr)	bluegill sunfish	10.00 mg/L	3
	LC_{50}(96 hr)	goldfish	12.37 mg/L	3
	LC_{50}(96 hr)	guppies	20.17 mg/L	3
4-Chlorophenol	LC_{50}(48 hr)	golden orfe	3 mg/L	1
	96 hr EC_{50}(growth)	alga	4.8 mg/L	2
	LC_{50}(96 hr)	water flea	1.17 mg/L	2
	LC_{50}(96 hr)	water flea	4.1 mg/L	1
	Microtox® EC_{50} (5 to 30 min)	-	1.15-1.19 mg/L	2
2,4-Dichlorophenol	LC_{50}(24 hr)	trout	1.7 mg/L	1
	LC_{50}(48 hr)	water flea	1.21 mg/L	2
	Microtox® EC_{50} (5 to 30 min)	-	1.47-1.54 mg/L	2
Pentachlorophenol	LC_{50}(48 hr)	salmonids	32-130 m g/L	4

1. Krijgsheld and van der Gen, 1986.
2. Munkittrick and Power, 1991
3. Pickering and Henderson, 1966.
4. Davis and Hoos, 1974; Iwama and Greer, 1979.

TABLE 2.3 SUMMARY OF TOXICITY DATA FOR SELECTED CHLOROPHENOLS

Parameter	2-Chlorophenol	3-Chlorophenol	4-Chlorophenol	2,4-Dichlorophenol
Commercial Production	moderate (5 kT/yr)	small (<1000 kg/yr)	moderate (5 kT/yr)	large (20 kT/yr)
Persistence (half life)	low to moderate (1 - 14 days)	moderate to high (>1 month)	low to moderate (1-20 days)	low to moderate (2-15 days)
Bioaccumulation (bioconcentration factor)	low (10)	low (10)	low (10)	low (50)
Aquatic toxicity: LC_{50}, acute (96 hr)	moderate (3-14 mg/L)	moderate (3-8 mg/L)	moderate (3-9 mg/L)	moderate (2-8 mg/L)
Mammalian toxicity (oral LD_{50} rat)	moderate (670 mg/kg)	moderate 570 mg/Kg)	moderate (670 mg/kg)	moderate (580 mg/kg)
Organoleptic effects	strong	strong	strong	strong
(Taste Threshold in water, ppb)	0.1	0.1	0.1	0.3
(Threshold fish tainting, ppb)	15	60	45	0.4

Adapted from Krijgsheld and van der Gen, 1986.

2.3 Biodegradation

Successful anaerobic biodegradation of phenol and phenolic wastewaters has been reported in a number of studies (Churn, 1993).

When compared to phenol, the anaerobic biodegradation of chlorophenols is much more difficult. The carbon-halogen bonds are very resistant to metabolic attack. This resistance increases substantially if these bonds are connected to a very stable planar aromatic structure. Removal of halogen substitutents requires a high energy input. As the degree of halogenation increases, the compound becomes virtually unmetabolizable.

Positions of the chlorines on the aromatic ring also dictate how biodegradable the compound is likely to be (Rothmel et al., 1991). On aromatic rings, halogen atoms hinder ring fission of the benzene nucleus. This deactivates the benzene nucleus to electrophilic attack by dioxygen, withdrawing electrons from the ring (Steiert and Crawford, 1985).

Generally the higher the extent of chlorination, the higher the level of recalcitrance of the haloaromatic compound to microbial attack (Rothmel et al., 1991). While aromatic compounds with one or two chlorine atoms are usually biodegradable, compounds with three or more chlorine atoms generally are recalcitrant and are biodegraded in nature extremely slowly. The deactivating effect of halogen atoms increases with the number of halogen substituents, thus highly halogenated phenols can be resistant to aerobic biodegradation and often persist in aerobic environments (Steiert and Crawford, 1985).

Halogenation increases the compound's tendency to be removed from the liquid phase as indicated by an increase in the octanol/water partition coefficient (Woods, 1985) (Table 2.1). The octanol/water partition coefficient (K_{OW}) is a

constant that describes the equilibrium partitioning of a compound between equal volumes of n-octanol and water at a given temperature. The octanol/water partition coefficient increases with halogen replacement in the following order: -I>-Br>-Cl>-F (Woods, 1985).

Inhibition of chlorophenols on phenol degradation has been studied by Beltrame et al., (1984, 1988, 1989). Their studies concluded that the inhibiting action of chlorophenols increases with the number of chloro substituents. Toxicity increased from least to most as follows:

2-CP <4-CP <3-CP <2,6-DCP <2,3-DCP & 2,4-DCP & 2,5-DCP <3,4-DCP, & 3,5-DCP < 3,4,5-TCP < 2,3,4,5-TTCP < PCP .

Biodegradation of chlorophenols proceeds under both aerobic and anaerobic conditions.

2.3.1 Aerobic Biodegradation of Chlorophenols

Under aerobic conditions, degradation of chlorophenols proceeds through methylation or hydroxylation, with the latter being most common. Chlorophenols are hydroxylated to corresponding chlorocatechols or methylated to chloranisoles before ring cleavage and further degradation for use in central metabolism.

2.3.2 Anaerobic Biodegradation of Chlorophenols

If destruction of chlorophenols does not occur in aerobic environments, these compounds may eventually end up in anaerobic environments. Flooded soils, sediments, landfills, lagoons, fresh and ocean water and some groundwaters may contain anaerobic or anoxic environments.

2.3.2.a Anaerobic Metabolic Pathways

Under anaerobic conditions degradation of chloroaromatics involves complete removal of chlorine from the aromatic ring by reductive dechlorination (where chlorine is exchanged with hydrogen), before further degradation and mineralization (Gibson and Suflita, 1986) (Figure 2.2). No chlorine shift takes place (Horowitz et al., 1983; Rochkind-Dubinsky et al., 1987). This step, followed by ring cleavage (fission) has been observed for chlorinated benzoates and chlorinated phenols. Complete dehalogenation is required before a chloroaromatic can be mineralized to CH_4 and CO_2.

According to Zhang and Wiegel, (1990) the initial step in the anaerobic pathway for degradation of chlorophenols is also reductive dechlorination. The phenol ring is then carboxylated to benzoate, which undergoes ring fission and acetogenesis yielding acetate, CO_2 and H_2. These products then undergo methanogenesis to produce the end products of methane (CH_4) and carbon dioxide (CO_2). A proven pathway for the degradation of 2,4-dichlorophenol is illustrated in Figure 2.3.

Knoll and Winter (1987) have also shown benzoate to be an intermediate in the anaerobic degradation of phenol in sewage sludge. Sharak Genthner et al., (1989) have identified benzoate in several 2-chlorophenol primary cultures and in laboratory transfers of cultures which degraded 2-chlorophenol and 3-chlorophenol.

2.3.2.b Review of Anaerobic Degradation

Woods et al., (1989) examined the fate of chlorinated phenols during continuous anaerobic treatment of a complex concentrated waste water in a

continuous flow, upflow anaerobic sludge blanket reactor over a period of 7 months. Using unacclimated sewage sludge, bacteria removed ortho chlorines from 2,6-, 2,3-, and 2,4-dichlorophenol, 2,4,6-trichlorophenol, 2,3,4,5-tetrachlorophenol and PCP (Figure 2.4). With acclimation, meta chlorines were also removed from 3,5-dichlorophenol, 3,4-dichlorophenol and 3,4,5-trichlorophenol. No evidence for dechlorination of monochlorophenols, ring cleavage and complete degradation to methane and carbon dioxide, were found for any of the chlorophenols evaluated.

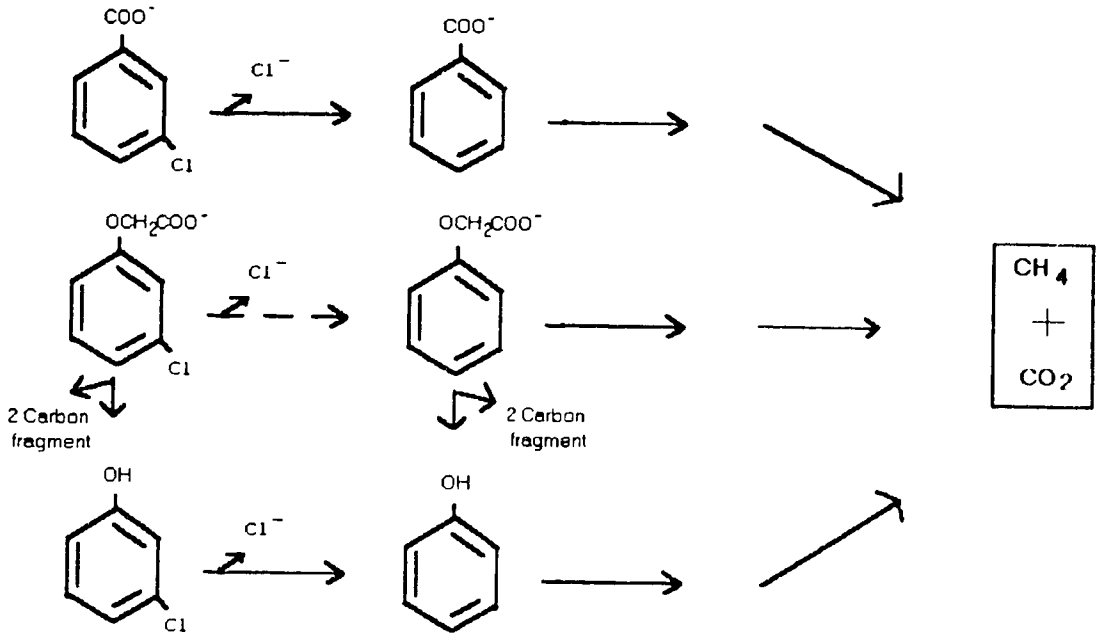


FIGURE 2.2 Proposed Pathway for Methanogenic Degradation of, from Top to Bottom, Chlorinated Benzoates, Phenoxyacetates and Phenols (Gibson and Suflita, 1986).

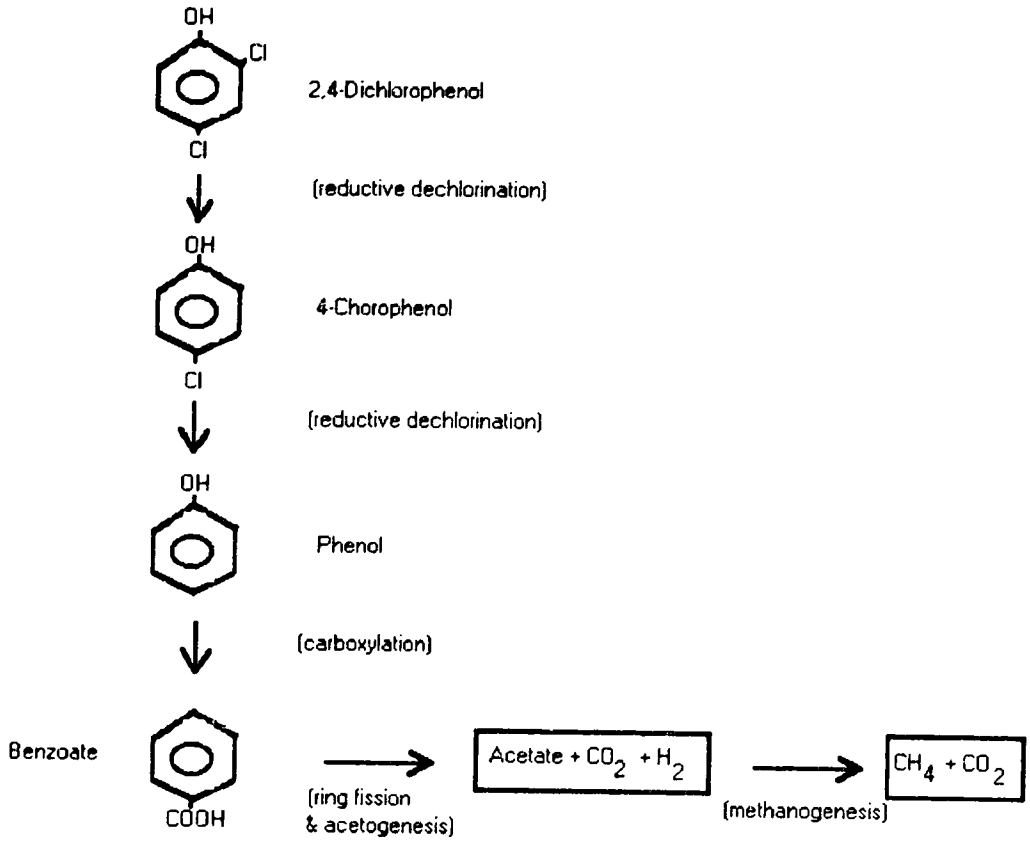


FIGURE 2.3 Biodegradation Pathway for 2,4-Dichlorophenol (Adapted from Zhang and Wiegel, 1990).

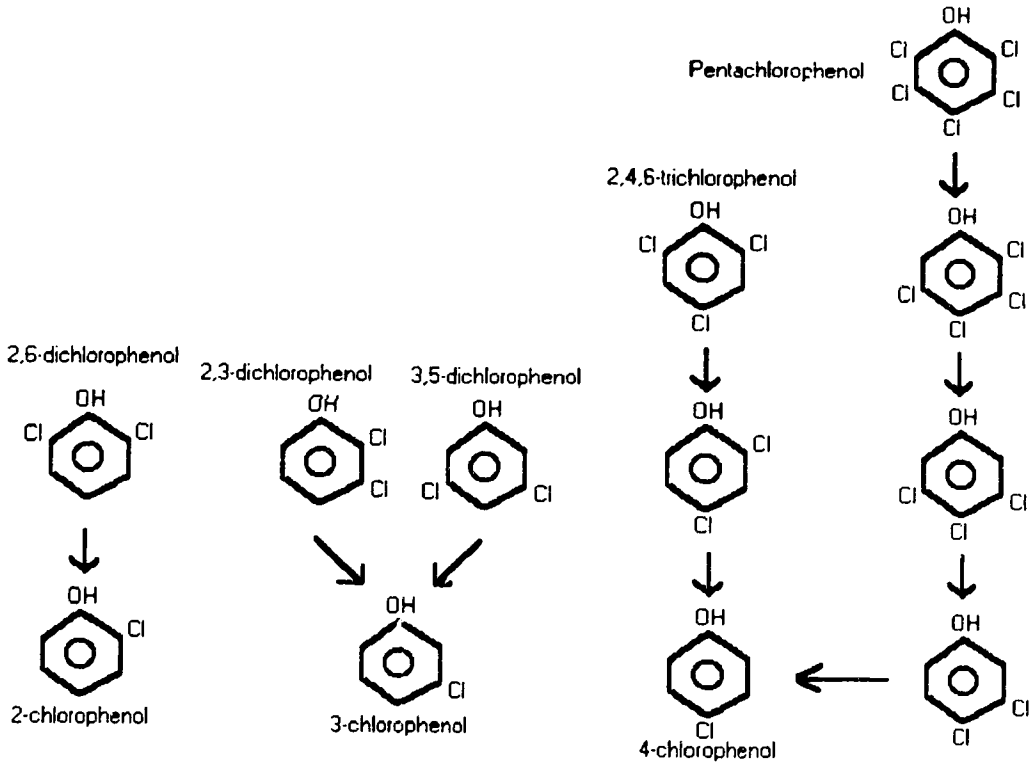


FIGURE 2.4 A Summary of Observed Dechlorination Pathways for Selected Chlorophenols (Adapted From Woods et al., 1989).

Boyd et al., (1983) examined the biodegradability of 2-, 3-, and 4-chlorophenol in fresh 10% anaerobic digester sludge. They found that 50 mg/L of 2-chlorophenol disappeared in 3 weeks and 50 mg/L of 3-chlorophenol disappeared in 7 weeks, while 50 mg/L of 4-chlorophenol was completely degraded after 16 weeks. The production of phenol corresponded with the disappearance of 2-chlorophenol. Phenol was subsequently metabolized to methane.

Woods (1985) determined the pathways of degradation for 2,4,6-trichlorophenol in a continuous flow, upflow anaerobic sludge blanket reactor, acclimated to chlorophenols for 3 months. After 6 hours, 1 mg/L of 2,4,6-trichlorophenol was transformed to 2,4-dichlorophenol. After 3 days, 4-chlorophenol was subsequently detected in the reactor while 2-chlorophenol was not found during the length of the experiment. 4-Chlorophenol was not degraded any further during the 12 day experiment. Woods concluded that 2,4,6-trichlorophenol was anaerobically degraded by reductive dehalogenation of the ortho chlorine.

Gibson and Suflita (1986) monitored the reductive biodegradation of chlorophenols, chlorobenzoates and pesticides in a methanogenic aquifer, a sulfate-reducing aquifer, pond sediment and sewage sludge. Final substrate concentrations ranged from 300 to 500 micromole/Litre (μmol/L). Substrate disappearance and initial intermediates were monitored for 5 months. In sewage sludge samples, monochlorophenols were detected as intermediates during the metabolism of 2,4- and 2,5-dichlorophenol. No degradation was observed for 4-chlorophenol in either the sewage sludge or sulfate- reducing environment. Phenol was detected as an intermediate in the degradation of 4-chlorophenol

present in both the pond sediment and methanogenic aquifer, but was not observed in either the sewage sludge or sulfate-reducing aquifer. They concluded that the removal of aryl halides and their substitution by a hydrogen atom represented the primary route of reductive dehalogenation of aromatic compounds.

Krumme and Boyd (1988) studied the reductive dechlorination of mixture of chlorophenols in upflow bioreactors using chlorophenols as sole carbon and energy source and acclimated digester sewage sludge as inoculum. Chlorophenol substrate concentrations ranged from 30 to 150 mg/L with hydraulic retention times (HRT) from 2 to 10 days. The authors noted that 100% of 3-chlorophenol was mineralized over a period of 44 days (average influent concentration of 10.2 mg/L per day and an average HRT of 6.7 days) and 99% of 4-chlorophenol was mineralized over a period of 46 days (average influent concentration of 15.5 mg/L per day and an average HRT of 7.2 days). Mineralization of chlorophenols was demonstrated by the presence of gaseous end products, CH_4 and CO_2. However, the authors recovered only one of these products, CH_4 during the mineralization of 3-chlorophenol and labeled CO_2 during the mineralization of 4-chlorophenol. The theoretical quantity of the other gaseous end product was then calculated. Krumme and Boyd concluded that under anaerobic conditions, chlorines were removed from the aromatic ring by reductive dechlorination resulting in partially or fully dehalogenated products which were then more susceptible to either aerobic or anaerobic attack.

Kohring et al., (1989b) studied the effects of temperature on the anaerobic degradation of 2,4-dichlorophenol in unacclimated pond sediment slurries. At temperatures between 5 and 40°C, 20 mg/L of 2,4-dichlorophenol was

transformed stoichiometrically to 4-chlorophenol after an initial lag period. The intermediate 4-chlorophenol was degraded after a longer lag time. Phenol was not detected as an intermediate product.

Kohring et al., (1989a) also examined the fate of 2,4-dichlorophenol and 4-chlorophenol in anaerobic freshwater sediments in the presence of added sulfate. At temperatures between 19 and 40°C, 27 mg/L of 2,4-dichlorophenol was stoichiometrically transformed to 4-chlorophenol over adaptation periods ranging from 50 to 366 days. No additional dechlorination of 4-chlorophenol was observed for 1 year. However when 32 to 37 mg/L of 4-chlorophenol plus sodium sulfate was added to non-acclimated sediments or to sediments acclimated to 4-chlorophenol, 4-chlorophenol was stoichiometrically converted to phenol at a rate up to 2 mg/L per day.

Zhang and Wiegel (1990) studied the sequential anaerobic degradation of 2,4-dichlorophenol in freshwater sediments and enrichment cultures using serum bottles incubated at 30°C. In acclimated sediments, 2,4-dichlorophenol was transformed to 4-chlorophenol at a rate of 40 mg/L per day with an adaptation time of 7 days. 4-Chlorophenol was subsequently transformed to phenol at a rate of 7 mg/L per day with an adaptation time of 37 days. Phenol was then transformed to benzoate at a rate of 82 mg/L per day with an adaptation time of 11 days. It was observed that phenol was not detected in dechlorinating cultures when concentrations of 4-chlorophenol were less than 100 mg/L. Acetate, CO_2 and H_2, and consequently methane were formed from benzoate.

Madsen and Aamand (1992) studied the transformation and toxicity of trichlorophenols by acclimated digester sludge. Sludge was acclimated to pentachlorophenol (PCP) over a 2 year period. Dechlorination was verified by

analyzing concentrations of trichlorophenol and daily measurements of methane formation. Approximately 30 mg/L of 2,4,6-trichlorophenol was transformed to 2,4-dichlorophenol in 3 days. 2,4-Dichlorophenol was subsequently transformed to 4-chlorophenol within 4 days. Disappearance of 4-chlorophenol was noted after 40 days, however phenol was not detected. All intermediates of 2,4,6-trichlorophenol were accumulated in less than stoichiometric amounts.

2.3.2.c **Effects of Electrophilic Aromatic Substitution**

Studies suggest that biodegradability of chlorinated aromatic compounds is dependent upon aromatic structure, the position of chlorine atoms on the aromatic ring and acclimation of the microbial consortia (Sharak Genthner et al., 1989 and Woods et al., 1989).

An aromatic compound is traditionally defined as "having the chemistry typified by benzene" (Solomons, 1984). The molecules of aromatic compounds are cyclic and highly stable with respect to their hypothetical resonance structure. They tend to react with electrophilic (electron-deficient) species by substitution rather than addition. A substituent group such as hydroxyl (OH), affects both reactivity and orientation in electrophilic aromatic substitutions. This group causes the aromatic ring to be more reactive than benzene and is referred to as an activating group. Substituents that tend to orient electrophilic substitution to primarily ortho and para positions on the benzene ring, are referred to as ortho-para directors. Hydroxybenzene or phenol is a very powerful activating group and ortho-para director (Solomons, 1984).

Anaerobic degradation of a substituted benzene such as phenol is reliant on the functional group and the position of the halogen on the aromatic ring.

Preferential removal of chlorines from the ortho position of chlorophenols is consistent with the ortho-para directing character of this functional group, resulting in a structure yielding the lowest overall energy (Woods, 1985).

Although these effects describe the substitution of groups on a benzene ring, the same inductive and resonance effects support the degradation pathways observed for chlorophenols and chlorobenzoates by Boyd et al., (1983), Suflita et al., (1982), and Woods (1985)

2.3.2.d Anaerobic Structural Isomer Selectivity

Boyd et al., (1983) studied the anaerobic degradation of several substituted phenols by an unacclimated mixed culture in fresh, 10% anaerobic digester sludge. Phenolic substrate concentrations and methane production were monitored for periods up to 16 weeks. Results indicated that within 3 weeks 29 mg/L of 2-chlorophenol was degraded, without a lag period. Phenol was produced, indicating reductive dechlorination and subsequently metabolized to methane. Acclimation was required before the 3- and 4-chlorophenol were degraded. After acclimation each monochlorophenol isomer was completely degraded within 16 weeks.

Boyd and Shelton (1984) investigated the anaerobic biodegradation of monochlorophenols and dichlorophenols in serum bottle reactors using fresh (unacclimated) digester sewage sludge and sludge acclimated to either 2-chlorophenol, 3-chlorophenol or 4-chlorophenol. Each monochlorophenol isomer was degraded in the unacclimated sludge reactors. Degradation of 50 mg/L of 2-chlorophenol occurred without a lag period, whereas 47 mg/L of 3-, and 4-chlorophenol required approximately 4 weeks before degradation

occurred. The authors concluded the rate of monochlorophenol degradation was dependent on the position of the chlorine isomer and increasing in the following order: ortho > meta > para.

In the unacclimated sludge, reductive dechlorination of dichlorophenol isomers resulted in initial removal of chlorine in the position ortho to the hydroxyl with the remaining chlorine removed later. Both 3,5- and 3,4-dichlorophenol were persistent during the 6 week incubation period. Sludge acclimated to 2-chlorophenol degraded 16 to 18 mg/L of 2- and 4-chlorophenol with no lag period in 3 to 4 days; 18 mg/L of 2,4-dichlorophenol but not 3-chlorophenol. Sludge acclimated to 3-chlorophenol degraded 15 to 18 mg/L of 3- and 4-chlorophenol with no lag period in less than 2 weeks, 15 to 17 mg/L of 3,4- and 3,5-dichlorophenol but not 2-chlorophenol. Sludge acclimated to 4-chlorophenol could degrade 17 to 20 mg/L of 2-, 3- and 4-chlorophenol in 32 days, and 17 to 18 mg/L of 2,4- and 3,4-dichlorophenol. Relative rates of degradation were as follows: 4-CP > 3-CP > 2-CP. The authors concluded that in activated sludge, adaptation by microorganisms for the degradation of chlorophenols was isomer specific.

Reductive dechlorination results obtained by Mikesell and Boyd (1986) indicated that pentachlorophenol (PCP) degradation occurred most rapidly in the 2-chlorophenol acclimated sludge; PCP was degraded within 3 days. PCP degradation in 3- and 4-chlorophenol acclimated sludge was considerably slower requiring 12 to 9 days, respectively, for complete disappearance. Mikesell and Boyd suggested that chlorine substituents ortho to the phenolic hydroxyl group are removed more rapidly than chlorine in the meta or para

positions. Phenol was not observed as a dechlorination product in the acclimated sludge or enrichments.

Hrudey et al., (1987a) investigated the biodegradability of 2-, 3-, and 4-chlorophenol in 50 mL batch cultures using fresh (unacclimated) digester sewage sludge as inoculum. Substrate concentration and methane production were monitored. Concentrations of 2-chlorophenol were dechlorinated to phenol, which was also degraded. During batch culture experiments, dechlorination rates varied from 2.0 mg/wk (5.7 mg/L per day) with a 2-chlorophenol concentration of 97 mg/L, to 0.5 mg/wk (1.4 mg/L per day) with a 2-chlorophenol concentration of 285 mg/L. Degradation of 3- and 4-chlorophenol was observed only after 16 to 20 weeks. After 32 weeks, initial concentrations of up to 100 mg/L 3- and 4-chlorophenol were dechlorinated to 30 mg/L.

In another study, Hrudey et. al., (1987b) observed the rate of dechlorination of the second chlorine atom from dichlorophenol substrates in 50 mL semicontinuous cultures with 50% unacclimated anaerobic digester sewage sludge. Substrate concentration and methane analyses were performed weekly for 131 days. Dechlorination of 12 mg/L of 2,6-dichlorophenol to 2-chlorophenol occurred at a rate of 1.2 mg/wk, with 2-chlorophenol subsequently degraded to phenol. Both 2-chlorophenol and phenol appeared in low concentrations but were quickly degraded. Dechlorination of 12 mg/L of 2,4-dichlorophenol to 4-chlorophenol occurred at a rate of 1.7 mg/wk. Approximately 173 mg/L of 4-chlorophenol accumulated in the serum bottle in 57 days before decreasing to constant concentration of 130 to 150 mg/L with no further degradation. Dechlorination of 12 mg/L of 2,3-dichlorophenol to 3-chlorophenol occurred at a rate of 0.7 mg/wk. During a 36 day period approximately 68 mg/L of 3-

chlorophenol accumulated in the serum bottle before decreasing to a constant concentration of 60 to 70 mg/L with no further degradation. No phenol was observed during the dechlorination of either 3- or 4-chlorophenol. After 131 days, the authors found that 19 mg of 2,6-dichlorophenol had been degraded in comparison to 7.5 mg of 2,4-dichlorophenol and 3.4 mg of 2,3-dichlorophenol. In dichlorophenols, high rates of dechlorination at the ortho position resulted in increased concentrations of 3- and 4-chlorophenol. The authors noted that this accumulation coincided with the corresponding decrease in rate of dechlorination at the ortho position. The sequence of dechlorination for the second chlorine atom was ortho > para > meta.

Sharak Genthner et al., (1989) compared the anaerobic degradation of chloroaromatic compounds in the laboratory under a variety of enrichment conditions; methanogenic, nitrate, sulfate and bromoethane sulfonic acid (BESA) amended. Anaerobic sediments (freshwater and estuarine) and site water served as inoculum. Monochlorophenols were added to final concentrations of 3 g/L, 514 mg/L, 10 mg/L and 6 mg/L to the enrichments and sampled bimonthly for a period of approximately 2 years. Acclimation time for degradation of each chlorophenol isomer varied; 1 to 2 months for 2-chlorophenol, 3 to 4 months for 3-chlorophenol and 2 to 12 months for 4-chlorophenol. The criterion for degradation was a substantial decrease ($\geq$ 25%) in the substrate concentration. After laboratory transfer, degradative activity was highly maintained with 2-chlorophenol (11 of 12 enrichments), well maintained with 3-chlorophenol (7 of 11 enrichments) and poorly maintained with 4-chlorophenol (3 of 8 enrichments). Based on the acclimation times and degradability the authors concluded that in

anaerobic sediments 2-chlorophenol would be more readily degraded than 3-chlorophenol, which would degrade more readily than 4-chlorophenol.

Reductive dechlorination of pentachlorophenol (PCP) has been demonstrated in anaerobic soils. Investigations led to the conclusion that the chlorine atoms, located ortho and para to the hydroxyl group are utilized preferentially (Ide et al., 1972).

In a study by Haggblom and Young (1990), under sulfate-reducing conditions chlorine was preferentially removed from the ortho position of 2,4-dichlorophenol and 2,6-dichlorophenol by a biomass. The biomass originated from an anaerobic reactor used to treat pulp bleaching effluent. 2,6-Dichlorophenol was sequentially dechlorinated first to 2-chlorophenol and then to phenol. 2,4-Dichlorophenol was dechlorinated to 4-chlorophenol with no further degradation. Additional research using freshwater and saline water cultures, acclimated to monochlorophenols as substrate, under sulfate-reducing conditions produced the following results: 4-CP > 3-CP > 2-CP. 4-Chlorophenol was degraded approximately three times faster than 2-chlorophenol.

Haggblom and Young (1990) stated that these results were the reverse of those found for methanogenic cultures, namely that 2-chlorophenol was rapidly degraded while degradation of 4-chlorophenol was much slower.

While examining the dechlorination sequence of 2,4-dichlorophenol Zhang and Wiegel (1990) could not detect 2-chlorophenol. Even at high concentrations of 50 mg/L 2,4-dichlorophenol, 4-chlorophenol was accumulated, but no 2-chlorophenol was encountered. According to the authors, these results were indicative of a high specificity for the removal of the chlorine in the ortho position in comparison with the para position.

Madsen and Aamand (1992) examined dechlorination of trichlorophenol isomers by a polychlorophenol mixed culture enriched from digester sewage sludge. A 97 mg/L concentration of 2,4,6-trichlorophenol was partially dechlorinated to 22 mg/L of 2,4-dichlorophenol over a 14 day period. Approximately 2 to 8 mg/L of 2,4,5-trichlorophenol required 14 days before dechlorination to 3,4-dichlorophenol, which persisted throughout the study. Approximately 2 to 4 mg/L of 3,4,5-trichlorophenol was completely dechlorinated to 3,5-dichlorophenol in 2 days, while dechlorination of 8 mg/L of 3,4,5-trichlorophenol took 14 days. Results indicated that removal of chlorine in the ortho position was rapid when compared to chlorines in either meta or para position. They concluded that the positions of the substituents influenced the rate of dechlorination.

Haggblom et al., (1993) studied the influence of alternative electron acceptors on the biodegradability of chlorinated phenols and benzoic acids. Under methanogenic conditions 13 mg/L of 2-chlorophenol was degraded in approximately 30 days while 13 mg/L of 4-chlorophenol was degraded in approximately 60 days. Cultures were refed to the original concentration once substrates were completely depleted. Phenol was detected as an intermediate during degradation of 2-chlorophenol. In acclimated cultures, under methanogenic conditions 2-chlorophenol was metabolized at 4.11 $\pm$ 0.39 mg/L per day, 4-chlorophenol was metabolized at 2.70 $\pm$ 0.13 mg/L per day and 3-chlorophenol was metabolized at 6.81 $\pm$ 0.77 mg/L per day. The 3-CP > 2-CP > 4-CP results are in contrast to the preferential removal of the ortho position of chlorophenol but are in agreement with the preferential removal of the meta position of chlorobenzoates.

2.3.2.e Acclimation Effects on Isomer Degradation

Boyd and Shelton (1984) investigated the anaerobic biodegradation of mono and dichlorophenol isomers by fresh (unacclimated) digester sewage sludge and sludge acclimated to either 2-, 3-, or 4-chlorophenol in serum bottle reactors. Substrate concentration was monitored for 6 weeks.

In unacclimated sludge, each monochlorophenol isomer was degraded. 50 mg/L of 2-chlorophenol was completely degraded within 6 weeks, without a lag period. 46 mg/L of 3-chlorophenol was completely degraded within 6 weeks, with a lag period of 4 weeks. 46 mg/L of 4-chlorophenol was completely degraded after an initial lag period of 4 weeks.

In unacclimated sludge, only dichlorophenol isomers with a chlorine in the ortho position were reductively dechlorinated without a lag period. 2,5-dichlorophenol did require a one week acclimation period before degradation began. The two dichlorophenol isomers 3,5- and 3,4-dichlorophenol, were not degraded throughout the 6 week incubation period.

Acclimated sludge showed very different degradation patterns from the fresh (unacclimated) sludge. Serum bottles containing a single dichlorophenol as the sole source of carbon were inoculated with sludge acclimated to either 2-, 3- or 4-chlorophenol. Sludge acclimation to the monochlorophenols took place over a 4 month period.

Sludge acclimated to 2-chlorophenol degraded 16 to 18 mg/L of both 2-chlorophenol and 4-chlorophenol within 3 to 4 days, but was unable to degrade 3-chlorophenol within the 30 day incubation period. Only 19 mg/L of 2,4-dichlorophenol was degraded in the sludge acclimated to 2-chlorophenol, other

dichlorophenol isomers with chlorine ortho substituents (2,3-, 2,5- and 2,6-dichlorophenol) were not removed within the 30 day incubation period.

Sludge acclimated to 3-chlorophenol cross-acclimated to 4-chlorophenol and degraded both 3-chlorophenol (14 mg/L in 7 days) and 4-chlorophenol (18 mg/L in 14 days) with no lag period. Only dichlorophenol isomers containing meta chlorine substituents or meta and para substituents were degraded. 17 to 18 mg/L of 3,4- and 3,5-dichlorophenol were degraded in 14 days. 2-chlorophenol and 2,5-dichlorophenol were not degraded within the 30 day incubation period.

Sludge acclimated to 4-chlorophenol cross-acclimated to both 2- and 3-chlorophenol. 20 mg/L of 4-chlorophenol was degraded within 8 days, 17 mg/L of 3-chlorophenol within 12 days and 18 mg/L of 2-chlorophenol within 32 days. Both 2,4- and 3,4-dichlorophenol were also degraded. No other dichlorophenol isomers were tested.

Boyd and Shelton observed that sludge acclimated to both the 2- and 3-chlorophenol cross-acclimated to 4-chlorophenol. 4-Chlorophenol acclimated sludge could successfully degrade all three monochlorophenols. However only 2,4- and 3,4-dichlorophenol were the only dichlorophenol isomers consistently degraded by acclimated sludge. According to Boyd and Shelton these results indicate the ability to remove chlorines in the ortho position was lost with acclimation. Degradation of 2,4- and 3,4-dichlorophenol suggest that with acclimation, chlorines in the para position were preferentially removed.

From experimental evidence, Boyd and Shelton suggested the involvement of specific microbial process responsible for anaerobic chlorophenol degradation by acclimated sludge and another nonspecific process (responsible for the

removal of ortho chlorine in unacclimated sludge) which disappeared with acclimation. However, the nonspecific process may be dependent on alternate carbon sources, which were present in the fresh sludge. These sources, depleted during the 4 month acclimation period, may help to explain the inability of the 2-chlorophenol acclimated sludge to remove ortho chlorines from the dichlorophenol isomers.

Woods (1985) examined the pathways of anaerobic degradation of several dichlorophenols, trichlorophenols and pentachlorophenol in a continuous flow, upflow anaerobic sludge blanket reactor. Municipal sewage sludge was acclimated to chlorophenols for 3 months. A concentration of 1 mg/L of 2,3-dichlorophenol was dechlorinated to 3-chlorophenol after 2 hours. No 2-chlorophenol was detected. Dechlorination of ortho chlorines from 2,6-, and 2,4-dichlorophenol, 2,4,6-trichlorophenol, 2,3,4,5-tetrachlorophenol and pentachlorophenol was also detected. With acclimation, meta chlorines were removed from 3,5- and 2,3-dichlorophenol. However there was no evidence of further degradation of the monochlorophenols or chlorines present in the para position to the hydroxyl group. Woods noted that with longer acclimation periods, chlorines could also be removed from the para position during continuous treatment.

Hrudey et al., (1987a) examined the feasibility of sustaining anaerobic treatment of 2-chlorophenol at high concentrations with semi-continuous (draw and feed) cultures using unacclimated digester sludge as inoculum. For 42 days prior to beginning the draw and feed experiment, 2-chlorophenol was used as the sole source of substrate for the cultures. During the experiment, rapid

dechlorination and phenol degradation were sustained with 2-chlorophenol concentrations up to 100 mg/L for a period of approximately 43 days.

2.3.2.f Inhibition of Anaerobic Biodegradation

For natural microorganisms to utilize a chlorinated compound, the organism must have an array of enzymes that convert the target compound through a series of intermediates to a product. The product can then enter the general metabolic pool (Rothmel et al., 1991). For many chlorinated compounds the enzymes are extremely inefficient since they are designed to attack a structurally analogous non-chlorinated natural compound rather than a synthetic chlorinated compound as a substrate. As a result the chlorinated compound is degraded rather slowly, which explains the relative persistence of many synthetic chlorinated compounds in nature (Rothmel et al., 1991).

Inhibition of anaerobic biodegradation processes have been attributed to several different factors. These include the position of chlorine atoms in the benzene ring (Boyd et al., 1983; Guthrie et al., 1984), interference of sulfate or other oxidized sulfur species on dehalogenation reactions (Gibson and Suflita, 1986), cessation of microbial metabolism due to toxic concentrations of fermentable substrates (Fedorak and Hrudey, 1984) and selective substrate metabolism due to competitive substrate inhibition (Folsom et al., 1990).

Preliminary studies by Gibson and Suflita (1986) on reductive dehalogenation of aromatic compounds in four anoxic habitats found sulfate inhibition of microbially catalyzed aryl dehalogenation reactions. In a sulfate-reducing environment the authors reported a phenol substrate disappearance of

99%, as compared with the substrate disappearances of 2- and 4-chlorophenol of 20% and 26% respectively.

Hrudey et al., (1987a) also investigated the inhibition of phenol degradation by monochlorophenols in 50 mL batch cultures with fresh anaerobic digester sewage sludge as inoculum. Concentrations of substrate and methane production were monitored for 46 weeks. For 97 mg/L of 2-chlorophenol, the addition of 86 mg/L phenol decreased the rate of 2-chlorophenol degradation, from 2.0 mg/wk (without added phenol) to 0.4 mg/wk (with added phenol). For 285 mg/L of 2-chlorophenol, the rate decreased from 0.5 mg/wk (without added phenol) to 0.1 mg/wk (with added phenol). Even at higher concentrations (285 mg/L), 2-chlorophenol was not toxic to methanogenesis and had degraded 145 mg/L within the 46 week incubation period. Phenol degradation was not inhibited by 2-chlorophenol concentrations less than 97 mg/L and was completely degraded in 7 weeks. Addition of 3- and 4-chlorophenol, at concentrations greater than 30 mg/L, inhibited the degradation of 84 mg/L phenol. Lag times for phenol degradation increased from less than 1 week (4-chlorophenol concentrations from 3 to 10 mg/L) to 4 weeks (256 mg/L of 4-chlorophenol). With 3-chlorophenol, phenol degradation lag times increased from 1 week (3-chlorophenol concentrations less than 10 mg/L) to greater than 33 weeks (282 mg/L of 3-chlorophenol). In comparison, 3-chlorophenol was somewhat more resistant to phenol degradation than 4-chlorophenol; 0% after 33 weeks of incubation versus 60% after the same time period, respectively.

Sharak Genthner et al., (1989) compared the anaerobic degradation of chloroaromatic compounds in the laboratory under a variety of enrichment conditions; methanogenic, nitrate, sulfate and bromoethane sulfonic acid (BESA)

amended. Degradative activity was monitored bi-monthly in enrichments containing anaerobic freshwater and estuarine sediments, and site water as inocula with chlorophenol concentrations ranging from 3 g/L to 6 mg/L. The authors found that acclimation to para-chlorinated compounds was most variable, from 2 to 12 months. Also, among chlorophenols, 4-chlorophenol degradation was observed least often (8 out of 17 enrichments).

Madsen and Aamand (1992) have proposed a general relationship between the positions of chlorine atoms in the aromatic ring and toxicity, with antibacterial activities increasing from ortho via meta to para. Toxicity of trichlorophenols by a polychlorophenol-transforming mixed culture enriched from digester sewage sludge was examined. Toxicity and compound inhibition was evaluated by production of volatile fatty acid and methane formation. Results indicated that 197 mg/L of 2,4,6-trichlorophenol was found to be the least inhibitory. At concentrations of 79 mg/L 2,4,5-trichlorophenol reduced the production of volatile fatty acids by 50% while 79 mg/L of 3,4,5-trichlorophenol inhibited acid production by greater than 60% relative to the control cultures.

Inhibition of 4-chlorophenol to degradation has also been reported in sewage sludge (Gibson and Suflita, 1986) and in the treatment of pulp bleaching effluents (Haggblom and Young, 1990).

Under certain conditions, bacteria from environments receiving several structurally related chemicals may metabolize substrates selectively due to competitive substrate inhibition. Competitive substrate inhibition occurs when one enzyme involved in multiple steps of a degradative pathway acts only on the parent compound until its concentration falls below a threshold level (Rochkind-Dubinsky et al., 1987).

Woods (1985) found that during continuous treatment of a suite of chlorophenols, chlorines were not removed from the para position to the hydroxyl during the 7 months of experimental treatment.

In comparison, Boyd and Shelton (1984) examined dechlorination of 3,4-, 2,4-dichlorophenol and 4-chlorophenol in batch experiments and found that chlorines were removed from the para position.

In a study by Armenante et al., (1992), serum bottles spiked with 2 mg/L 2,4,6-trichlorophenol were dechlorinated to 2,4-dichlorophenol, which was, in turn reduced to 4-chlorophenol. Small amounts of phenol were also produced. However, with continued spikings of 2,4,6-trichlorophenol, the anaerobic culture was unable to degrade 4-chlorophenol any further and the concentration of 4-chlorophenol increased within the serum bottles.

2.4 Solid Support Media

The advantages of using a solid support media for the attachment of microbes over a system of suspended microbes in an aqueous environment has been well documented. Solid support systems should encourage the adsorption of microbes to the surface of the support material and allow biofilm development and maintenance. Allowing unhindered biofilm development is important in anaerobic processes in order to retain the slow growing microorganisms and provide maximum bioactivity (Kindzierski, 1989). A highly irregular surface composed of pits and crevices in addition to a well-developed internal structure large enough to accommodate microorganisms, would provide the ideal material for protection of a developing biofilm while still providing the microbes easy access to food and nutrients.

Bryers (1987) reviewed the superiorities of attached growth systems over free suspended systems.

The efficiency of immobilized yeast cells compared to suspended yeast cells was studied by Doran and Bailey (1986). The immobilized cells consumed glucose twice as fast as compared to suspended yeast cells, requiring less expenditure of energy in obtaining food and nutrients in addition to reducing their specific growth rate by 45%.

Wilkie and Colleran (1984) and Wilkie et al. (1985) examined the effects of different support materials; plastic rings, coal, mussel shells and fired clay fragments in four anaerobic reactors to treat pig slurry supernatant. Using percent chemical oxygen demand (%COD) removal as a measure of the biological activity in each reactor, those reactors containing material with higher surface area (plastic rings, coal and mussel shells) performed poorly when compared to the reactor containing the material with the lowest total surface area (fired clay fragments) during the initial start-up period. The reactor containing the fired clay fragments may have developed active attached-biomass faster than the other reactors due to improved initial adsorption and attachment of bacteria (Kindzierski, 1989).

Kennedy and Droste (1985) researched the use of various support materials in the startup of an anaerobic reactor to treat bean blanching wastewater. They found that support materials with roughened surfaces such as needle-punched polyester and fired clay allowed better biofilm development than PVC or glass supported materials. The authors mentioned that bacteria, especially the methanogens, had difficulties adhering to the smooth surface of the PVC or glass.

Hrudey et al., (1987b) examined the use of activated carbon in the anaerobic degradation of 2,3-dichlorophenol. A series of 50 mL semi-continuous cultures, enriched by 50% diluted unacclimated digester sludge, were monitored for substrate concentration and methane production over a 33 week period. Cultures with 1000 mg/L of activated carbon removed 1 to 3 mg/L of 2,3-dichlorophenol continuously for 13 weeks (with and without a 2-chlorophenol supplement) at a maximum rate of 1.5 mg/wk. In comparison, 5 to 12 mg/L of 2,3-dichlorophenol was dechlorinated in an experiment without activated carbon at a rate of 0.7 mg/wk (same study). Cultures of 300 mg/L activated carbon stopped dechlorination of 2,3-dichlorophenol after the 2-chlorophenol supplements were discontinued. The intermediate 3-chlorophenol was removed at a rate of 1.5 mg/wk in cultures containing 300 mg/L activated carbon. There was also removal of 3-chlorophenol by cultures containing 1000 mg/L activated carbon, however no rate was given.

Krumme and Boyd (1988) found little methanogenic and dechlorinating activity to be associated with glass beads that were designed for attachment of the biomass in three upflow bioreactors containing sludge acclimated to monochlorophenols. The glass beads were subsequently removed from within the bioreactors without loss of biological and dechlorinating activity.

Selle Sardi et al., (1986) studied the adhesion and growth of anaerobic biofilms on ion exchange resins. The authors found that anaerobic biofilm development was favorable towards strong cation exchange resins. Preference was thought to occur due to two properties of the resin; divalent cation binding which may aid in the adsorption and firm attachment of bacteria to stable supports, and the adsorption of volatile organic acids to the resin surface.

Salkinoja-Salonen et al., (1983) examined the anaerobic biodegradation of seven chlorophenolic compounds in a fixed film reactor. Removal of pentachlorophenol (PCP) in the reactor was efficient when the PCP degrading biomass was attached to a solid support. PCP biodegradation was most active with softwood bark, less active on unglazed pottery and inactive on glass (beads or fibre), cellulosic fibre, clay and ion exchange resin (styrene divinyl benzene).

Kindzierski (1989) assessed the importance of activated carbon and non-adsorptive synthetic ion exchange resin (styrene divinyl benzene) as biological supports in serum bottle reactors. The three support materials studied were Filtrasorb® 300 activated carbon, Ambergard™ XE-352 anion exchange resin and Dowex™ MSC-1 cation exchange resin. Little or no bioactivity was associated with cultures attached to the MSC-1. Cultures attached to the XE-352 resin exhibited the highest phenol removal rates on a reactor liquid volume, support material mass and estimated spherical surface area basis using a concentration approximately 3 times smaller than the concentration of activated carbon. Cultures attached to activated carbon exhibited the highest phenol removal rates on a support material pore volume and surface area basis. After 88 days of incubation the biofilm growth of the XE-352 resin was still increasing. On the activated carbon, biofilm growth had reached full capacity and did not increase after day 75 of incubation. Kindzierski thought that this difference may be due to the fact that the resin had more available pore volume and surface area per bottle for biomass growth than the activated carbon. When comparing the bioactivity of 76% supernatant cultures, the activated carbon cultures removed phenol at a much higher rate (150 $\pm$ 39 mg/L per day) as compared to the resin cultures (64 $\pm$ 15 mg/L per day). Kindzierski attributed this difference

to detachment of the biomass, as it expanded outwards from the activated carbon surface, by liquid shearing and inter-particle abrasion. The XE-352 resin was not subject to the same phenomena as biomass growth had not reached full capacity at the time the assay was terminated.

3.0 Experimental Procedures

3.1 Anaerobic Culture Methods

A series of batch reactors were set up to study the capacity of microbial cultures to degrade 2- or 4-chlorophenol under anaerobic conditions. The primary objective of the study was to produce a series of microbial cultures acclimated to specific quantities of chlorophenols. The cultures were classified as either attached growth or suspended growth depending upon the presence of a solid support media within the reactor. The biological activity of the cultures was observed by monitoring the disappearance of 2- or 4-chlorophenol and the periodic qualification of methane in the headspace of the reactors.

3.1.1 Serum Bottle Procedure

3.1.1.1 Setting up the Reactors

Batch reactors were set up in clean, sterilized, 158 mL serum bottles (Wheaton Scientific). To produce an environment free of oxygen each individual bottle was sparged with oxygen free gas (Section 3.1.6) for approximately 3 to 5 minutes before filling. Volumetric pipets or plastic syringes fitted with disposable needles (Becton-Dickenson; B-D) were used to add resin, mineral media growth solution, inoculum and substrate to the reactors. Before transfer of media or resin, the pipet or syringe was flushed several times with oxygen free gas to minimize the potential for oxygen contamination. Aliquots of the media growth solution were dispensed through a volumetric pipet. To prevent any free oxygen from entering the system, the volumetric pipet was attached to a three-way pipet bulb connected to a line of oxygen free gas. Aliquots of the media growth

solution and sludge inocula were added to the reactor while continually sparging the serum bottle headspace. Once the desired volume was reached, cut black butyl stoppers (Wheaton Scientific) were loosely fitted on top of the serum bottles while continuously sparging the headspace with oxygen free gas for approximately 2 to 3 minutes. Serum bottles were closed with the stoppers, crimped with tear-away aluminum caps (Wheaton Scientific) and stored in the dark at 37°C.

After the serum bottles were closed, additional media growth solution and substrate was injected or removed using the syringe and needle method.

3.1.1.1.1 Attached Growth Reactors (Series A)

Attached growth batch reactors, with biomass attachment support media Ambergard™, were prepared according to the procedure described in section 3.1.1.1. Approximately 500 mg (dry weight) of wet Ambergard™ XE-352 anion exchange resin and 48.5 mL of sterile mineral media growth solution were added into each serum bottle. During sparging, reactors received approximately 1 mL of substrate to bring the concentration in each bottle to approximately 60 mg/L of either 2- or 4-chlorophenol. One serum bottle reactor did not receive any chlorophenol substrate and served as a control for the study. The sparger was removed, and the bottles were stoppered and capped. To ensure a reducing environment, 0.5 mL of sodium thiosulphate (1% v/v) was added to each bottle using a plastic disposable syringe and needle pre-rinsed several times with oxygen free gas. Serum bottles were left to equilibrate in the dark at 37 °C.

After 24 hours, the bottles were opened. While continuously sparging with oxygen free gas, a 25 mL liquid aliquot of reactor supernatant was withdrawn

and replaced with 25 mL of sludge inoculum. Sparging continued for 3 to 5 minutes after the exchange. Each attached growth serum bottle reactor contained approximately 500 mg of dry resin (added as wet) and a total of 50 mL liquid. Bottles were then stoppered, capped and stored in the dark at 37°C to equilibrate.

After 24 hours, concentrations of 2- and 4-chlorophenol substrate within the reactor supernatant were determined by gas chromatography (Section 4.1.1). To maintain a 2- or 4-chlorophenol substrate concentration of 30 mg/L, additional substrate was added up to this level in all serum bottles except the control. Repeat analyses for chlorophenol substrate concentration were performed after approximately 14 days. Substrate was added up to the predetermined concentration. Subsequent supernatant removal (70% to 88%) after acclimation was used to encourage biomass attachment to the support media (resin).

3.1.1.1.2 **Suspended Growth Reactors (Series B)**

Suspended growth reactors were prepared according to the procedure described in section 3.1.1.1 (without anion exchange resin) with the supernatant from series A. The corresponding attached growth parent reactor containing solid support media (series A) was opened and sparged with oxygen free gas. Approximately 70% (35 mL) of the supernatant was removed by plastic syringe equipped with disposable tip and replaced with 35 mL of fresh, reduced mineral media solution (Table 3.1). The supernatant was immediately injected into a corresponding empty serum bottle (series B). Sparging of the reactor bottle

headspace continued for 2 to 3 minutes. The attached growth parent reactor was then stoppered and capped.

While continually sparging the headspace of the suspended growth reactor (series B), 15 mL of mineral media was added to the reactor by graduated pipet that had been thoroughly flushed with oxygen free gas. The reactor was continually sparged with the gas for 2 to 3 minutes, then stoppered and capped.

All reactors were fed 50 mg/L of either 2- or 4-chlorophenol substrate then stored in the dark at 37°C.

3.1.1.1.3 Supernatant Only Reactors (Series E, F and G)

Supernatant only reactors were prepared according to the procedure described in section 3.1.1.2 (without anion exchange resin or inoculum) with the supernatant from series A. These reactors were set up in each phase of the semi-continuous study. Their purpose was to provide a control and a comparison of chlorophenol degradation by the same suspended microbes present in the supernatant of the attached growth reactors (series A).

3.1.1.2 Analyses of Headspace Gas for Methane

A sample of headspace gas was obtained by direct injection using a gas tight syringe (Hamilton #1801). The syringe was used to puncture the cut black butyl stopper and remove 3 microlitres (μL) of headspace gas. The gas sample was then directly injected into a gas chromatograph (GC).

Between injections, the syringe was rinsed several times with deionized, distilled water.

3.1.1.3 Extraction of Supernatant for Analyses

Serum bottles were inverted and left to settle into two distinct layers, resin (if present) and sludge at the base topped by the clear supernatant. A Hamilton syringe (Microlitre #701) was used to puncture the cut black butyl stopper and push through the sludge/resin layer to the clear supernatant. The syringe plunger was pulled back to extract 3 microlitres (μL) of supernatant, then removed from the serum bottle. The amount of liquid was discarded and the needle tip wiped clean. The entire procedure was repeated twice. After the second removal, 1.5 μL of supernatant was discarded, leaving a 1.5 μL sample remaining in the syringe. The sample was then injected into the Gas Chromatograph (GC).

The syringe was rinsed several times with deionized, distilled water between each sample injection.

3.1.1.4 Substrate Addition

Samples of reactor supernatant were analyzed for either 2-chlorophenol or 4-chlorophenol concentration as described in section 4.1.1. Volume amounts of substrate were calculated to bring reactor supernatant to the required concentration. To maintain a total reactor volume of 50 mL, a volume of supernatant equal to the volume of substrate required, was withdrawn by needle and syringe from each reactor. The required volume of chlorophenol substrate was added into the reactor bottles by direct aqueous injection.

3.1.1.5 Exchanging Caps on Reactors

The cut black butyl stoppers (Wheaton Scientific) and tear-away aluminum caps (Wheaton Scientific) were exchanged bi-weekly, between periods of supernatant removal.

Old aluminum caps were torn off and used stoppers removed from the serum bottles. Immediately after stopper removal, the reactor headspace was sparged with oxygen free gas while a new cut black butyl stopper was placed loosely on top of the serum bottle. Sparging continued for 2 to 3 minutes. Upon removing the sparger, the serum bottle reactors were quickly stoppered and capped.

3.1.1.6 Supernatant Exchange

A specific volume of supernatant was removed from a reactor containing an attached growth culture, replaced with an equal amount of mineral media solution, then placed into a 158 mL serum bottle reactor to start a new culture. These new cultures were classified as either suspended growth or supernatant only, depending upon reactor contents.

The attached growth reactor containing solid support media was opened and sparged with oxygen free gas. Various amounts of the supernatant was removed by plastic syringe equipped with disposable tip and replaced with an equal volume of fresh, reduced mineral media solution (Table 3.1). The supernatant was immediately ejected into a labeled empty serum bottle. Sparging of the attached growth reactor bottle headspace continued for 2 to 3 minutes. The reactor was then stoppered and capped.

While continually sparging the headspace of the corresponding serum bottle reactor, mineral media and/or inoculum sludge was added to the reactor by graduated pipet that had been thoroughly flushed with oxygen free gas. The reactor was continually sparged with oxygen free gas for 2 to 3 minutes, then stoppered and capped.

All reactors were fed a specific concentration of either 2- or 4-chlorophenol substrate then stored in the dark at 37°C.

3.1.1.7 Draw and Feed

The draw and feed procedure involved drawing of the supernatant for initial analysis of substrate concentration by GC and injections of an equal volume with required substrate. Based on a stock substrate solution of 2500 mg/L, the volume of 2- or 4-chlorophenol substrate required to maintain a reactor concentration of 50 mg/L was added into the mineral media injected into the reactor.

To withdraw the supernatant, a 5 mL aliquot of supernatant was removed by plastic syringe fitted with a disposable needle, once the biological solids had settled. This was replaced with 5 mL of freshly prepared and reduced mineral media growth solution (Section 3.1.3) minus the calculated volume of chlorophenol substrate required, to maintain a total exchanged volume of 5 mL. The calculated volume of either 2-chlorophenol or 4-chlorophenol substrate was then added to the reactor. All aqueous extractions and additions were performed by direct injection into closed and capped serum bottle reactors. Plastic syringes and disposable needles were rinsed thoroughly with an oxygen free gas mixture before injection (Section 3.1.1).

3.1.2 Sludge Inoculum

The sludge used as a source of inoculum for all attached growth and suspended growth reactors in this study was obtained from Parachlorophenol Series 6 sludge bank established on May 29, 1990. Sludge was originally collected from the Goldbar Wastewater Treatment Plant digester then acclimated to 4-chlorophenol (4-CP). All sludge was stored in an incubator kept at a constant temperature of 37°C and fed with 4-chlorophenol and mineral media growth solution once or twice per month.

3.1.3 Anaerobic Mineral Media Growth Solution

Anaerobic mineral media growth solution was prepared in advance, sterilized and kept refrigerated for approximately 2 weeks from date of preparation. Preparation of the mineral media solution from stock solutions is listed in Table 3.1. Chemical components of the stock solutions are listed in Table 3.2. Stock solutions were prepared in advance and stored in white plastic 1 L nalgene bottles.

TABLE 3.1 PREPARATION OF ANAEROBIC GROWTH MEDIA

Solution	Concentration (per 100 mL)
Deionized, distilled water	100 mL
Mineral Solution 1	1.0 mL
Mineral Solution 2	0.1 mL
Vitamin B Solution	0.1 mL
Phosphate Solution	1.0 mL
Resazurin Solution	1.0 mL
Sodium Bicarbonate	0.57 g

(Kindzierski, 1989)

TABLE 3.2 PREPARATION OF STOCK SOLUTIONS IN THE ANAEROBIC GROWTH MEDIA

Solution	Chemical	Concentration in Distilled Water (g/L)
Mineral 1	NaCl	50
	$CaCl_2\ 2H_2O$	10
	NH_4Cl	50
	$MgCl\ 6H_2O$	10
Mineral 2	$(NH_4)_6Mo_7O_{24}\ 4H_2O$	10
	$ZnSO_4\ 7H_2O$	0.1
	H_3BO_3	0.3
	$FeCl_2\ 4H_2O$	1.5
	$CoCl_2\ 6H_2O$	10
	$MnCl_2\ 4H_2O$	0.03
	$NiCl_2\ 6H_2O$	0.03
	$AlK(SO_4)_2\ 12H_2O$	0.1
Vitamin B	Nicotinic Acid	0.1
	Cyanocobalamine	0.1
	Thiamine	0.05
	p-Aminobenzoic Acid	0.05
	Pyridoxine	0.25
	Pantothenic Acid	0.025
Phosphate	KH_2PO_4	50
Resazurin		0.1

(Fedorak and Hrudey, 1984)

To prepare the media, all solutions listed in Table 3.1 (except the sodium bicarbonate) were added together in an erlenmeyer flask and boiled for approximately 2 minutes to drive off dissolved oxygen. Immediately after removal from the heat source, the hot solution was sparged with oxygen free gas. Sparging continued until the solution cooled to room temperature. Sodium bicarbonate was then slowly added to the cooled solution to bring the pH into the range of 6.9 to 7.1.

Once the proper pH was reached, the solution was dispensed into clean 158 mL serum bottles (Wheaton Scientific) pretreated with oxygen free gas. Sparging continued for 2 to 3 minutes. The bottles were stoppered, capped, autoclaved for 20 minutes at 121°C, cooled to room temperature and stored in a refrigerator.

Prior to use the anaerobic media solution was reduced. A solution of 2.5% w/v sodium thiosulphate was added by disposable syringe pre-flushed with oxygen free gas (1 mL sodium thiosulphate per 100 mL of media solution). The reduced anaerobic media solution was colourless. Presence of free oxygen within the bottles was indicated by resazurin (Table 3.1), a redox indicator, which turns pink in the presence of atmospheric oxygen.

Reduced media solution was kept refrigerated for 2 weeks and then discarded.

3.1.4 Substrate Preparation

Exactly 0.2500 g of 4-chlorophenol (Fisher Scientific, Fairlawn, N.J.) was weighed into a clean, sterilized, 158 mL serum bottle (Wheaton Scientific) on a covered balance. While sparging the bottle headspace with oxygen free gas,

100 mL of freshly boiled, deoxygenated water was added to the serum bottle. After sparging for 2 to 3 minutes the bottle was stoppered, capped, autoclaved at 121°C for 20 minutes, cooled to room temperature and refrigerated.

Exactly 0.2 mL of 2-chlorophenol (Fisher Scientific, Fairlawn, N.J.) was ejected from a volumetric pipet into a clean, sterilized, 158 mL serum bottle. The remaining preparation of 2-chlorophenol substrate was accomplished using the same procedures and equipment as 4-chlorophenol.

Preparation of 2500 mg/L solutions of 2,4-dichlorophenol (Aldrich Chemical Company, Inc., Milwaukee) and 3,4-dichlorophenol (Aldrich Chemical Company, Inc., Milwaukee) were accomplished using the same procedures and equipment as 4-chlorophenol.

3.1.5 Standard Preparation

2-Chlorophenol (Fisher Scientific, Fairlawn, N.J.) and 4-chlorophenol (Fisher Scientific, Fairlawn, N.J.) standards were prepared by dilution of a standard 1000 mg/L stock solution with distilled, deionized water.

Exactly 0.2500 g of 4-chlorophenol was weighed into a clean glass beaker on a covered balance then deposited into a clean, dry 250 mL volumetric flask. A 0.20 mL volume of 2-chlorophenol was transferred by graduated pipet into the same volumetric flask. Distilled, deionized water was added to the flask and made up to the proper volume. The appropriate volume of stock solution was dispensed by pipet into 50 mL volumetric flasks then diluted with distilled, deionized water to the proper volume (Table 3.3).

Standards were transferred into clean, labeled, 60 mL serum bottles (Wheaton Scientific), stoppered with grey butyl stoppers (Wheaton Scientific), capped by tear-away aluminum caps (Wheaton Scientific) and refrigerated.

Additional 100 mg/L, 50 mg/L and 10 mg/L standards of phenol (BDH Chemicals, England), 3,4-dichlorophenol (Aldrich Chemical, WS) and 3-chlorophenol (Eastman Kodiak Company, NY) were prepared by dilution of individual 100 mg/L stock solutions with distilled, deionized water.

Stock solutions were prepared by weighing exactly 0.0050 g of either phenol, 3,4-dichlorophenol or 3-chlorophenol into clean, dry, individual beakers on covered balances. The chemicals were transferred into 50 mL volumetric flasks and filled to volume with distilled, deionized water. Aliquots of 5 mL and 25 mL were taken from the stock solution, placed into 50 mL volumetric flasks and diluted to volume. Contents of volumetric flasks were transferred to labeled 60 mL serum bottles, stoppered, capped and refrigerated.

A 50 mg/L standard of 2,4-dichlorophenol (Aldrich Chemical, WS) was prepared by dilution of a 2 mL aliquot from a 2500 mg/L stock solution (substrate) into a 50 mL volumetric flask. Contents of the volumetric flask were transferred into a labeled 60 mL serum bottle, stoppered, capped and refrigerated.

A 10% and a 30% methane standard was prepared by dispensing 16 mL and 48 mL of methane gas into empty, clean, capped 158 mL serum bottles, respectively. A gas tight syringe (Hamilton# 1801) was used to inject the standard into the GC (Section 3.1.1.2).

TABLE 3.3 2-CHLOROPHENOL AND 4-CHLOROPHENOL STANDARDS

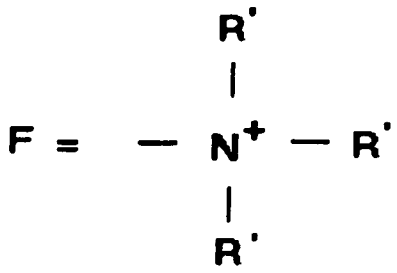
Standard (mg/L)	Volume of Stock Solution Required (mL)
10	0.5
25	1.25
50	2.5
75	3.75
100	5.0
150	7.5
200	10.0
250	12.5
300	15.0
350	17.5
400	20.0
450	22.5
500	25.0

3.1.6 Oxygen Free Gas

The oxygen free gas mixture consisted of 30% CO_2 in N_2. To remove any free oxygen the gas was run through a heated glass column containing copper shavings (Hungate, 1969). A line attached to the glass column was split into three sections. Two sections were connected to metal spargers. The third line was connected to a 158 mL serum bottle equipped with a butyl rubber cap. Flushing of needles and syringes was done by direct injection through the rubber cap.

3.1.7 Biological Solid Support Media

Ambergard™ XE-352 anion exchange resin (Rohm and Haas Co.) was used as the solid support media for attachment of biological growth. The resin is comprised of styrene-divinyl benzene (DVB) polymers, which are a combination of aromatic (benzene) and aliphatic (-CH-CH_2-) structures. XE-352 resin has a "strongly basic" Type 1 quaternary ammonium ionic group (Figure 3.1) as the dominant functional group on its surface (Kindzierski, 1989). The average size of resin particles ranges from 300 to 850 micrometre (μm). XE-352 resin pore volume and surface area characteristics are summarized in Table 3.4.



R• = Methyl ($-CH_3$) or other hydrocarbon group

FIGURE 3.1 Chemistry of Functional Group with Ion Exchange Sites for Ambergard™ XE-352 Synthetic Resins (Kindzierski, 1989).

TABLE 3.4 PORE VOLUME AND SURFACE AREA CHARACTERISTICS OF THE AMBERGARD™ XE-352 RESIN FROM MERCURY POROSIMETRY

Characteristic	XE-352 Resin
Available Cumulative Pore Volume with Mean Pore Diameters ≥ 15 μm (mL/g)	1.220
Available Cumulative Surface Area with Mean Pore Diameters ≥ 15 μm (m^2/g)	0.227
Total Cumulative Surface Area with Mean Pore Diameters ≥ 0.008 μm (m^2/g)	67.1
Estimated Spherical Surface Area (m^2/g)	0.0085
% Area Available For Bacteria	0.336

Adapted from Kindzierski (1989)

3.1.7.1 Preparation of Solid Support Media

Ambergard™ XE-352 (Cl^-) anion exchange resin (10 g/L) was used in each attached growth reactor as a solid support media for biomass attachment.

3.1.7.2 Resin Conditioning

Resin was subject to a two step conditioning process to load with Cl^- before usage as a solid support media. In step one, the resin was placed in a glass column which was screened at the lower end. Approximately 750 mL of a 10% hydrochloric acid regenerator was passed through the column. During step two, the resin was placed in a sintered glass filter funnel and hooked up to a vacuum apparatus. Approximately 4.5 L of bacteria free wash water was then passed through the resin. The resin was allowed to dry thoroughly before use. Resin conditioning was done by Sara Ebert, Department of Microbiology, University of Alberta, Edmonton, Alberta.

3.1.7.3 Determination of Moisture Content

To maintain a total reactor volume of 50 mL, the percentage of moisture required by the dry resin was calculated. Approximately 0.5037 g of resin (dry weight) was combined with 50 mL of non-sterile mineral media growth solution in a 158 mL serum bottle. The bottle was capped with a cut black butyl stopper, crimped by a tear-away aluminum cap and allowed to equilibrate in the dark at 37°C.

After 24 hours, the bottle was opened and approximately 48.5 mL of media solution was withdrawn by disposable syringe into a graduated cylinder. Moisture content of the resin was calculated as follows:

$$\text{Moisture Content (\%)} = \frac{50\ \text{mL} - 48.5\ \text{mL}}{50\ \text{mL}} = 3\ \%$$

To compensate for the amount of media adsorbed by the dry resin, approximately 1.5 mL of additional mineral media growth solution was added to reactors during initial set-up.

4.0 Analytical Methods

4.1 Analytical Determination by Gas Chromatography

4.1.1 Substrate Analysis

The concentration of 2- and 4-chlorophenol present in the batch reactors was measured by direct aqueous injection of reactor supernatant into a Gas Chromatograph (GC).

4.1.1.1 GC Conditions

The Hewlett-Packard Model 5730A Gas Chromatograph (GC) contained a 0.91 m x 2 mm ID glass column with a 1% SP1000 60/80 Carbopak B (Supelco) solid phase. The GC was equipped with a flame ionization detector (FID) and attached to a Hewlett-Packard Integrator Model 3390A. Nitrogen was used as a carrier gas at 35 mL/min. Hydrogen and air were used as the flame ionization detector gases at 35 mL/min and 240 mL/min respectively. The oven, injector and detector temperatures were set and held isothermal at 220°C, 250°C, and 250°C respectively.

4.1.1.2 Calibration

The system was calibrated using standard solutions of known concentrations (Section 3.1.5). Substrates and intermediates were identified by comparison with the retention times of the known standards. Peak areas generated from serum bottle reactor samples were compared against peak areas generated from substrate standards.

4.1.1.3 Quality Control

Quality control was maintained by producing a new calibration curve every second day (during continuous analyses) or before every analytical determination of substrate concentration (APHA, 1992). Retention times were carefully monitored during the calibration and substrate analysis. All injections were done in sets of 2 or 3 to maintain an acceptable error of determination of 5%.

4.1.2 Methane Gas Analyses

In the serum bottle reactors, methane gas was produced from mineralization of chlorophenol substrate by anaerobic microbes. Methane presence was qualitatively confirmed after stopper exchange and complete headspace flushing with oxygen free gas. Analyses were performed by extracting headspace gas from the culture bottle and injecting directly into a GC.

4.1.2.1 GC Conditions

A Hewlett-Packard Model 5730A gas chromatograph (GC) equipped with a flame ionization detector (FID) was used. The sample of headspace gas was injected onto a 0.91 m x 2 mm ID glass column containing 1% SP1000 60/80 Carbopak B (Supelco). Integration of peak areas was performed by a Hewlett-Packard Model 3390A integrator which was attached to the GC. Nitrogen was used as the carrier gas at 35 mL/min. Hydrogen and air were used as the flame ionization detector gases at 35 mL/min and 240 mL/min respectively. Temperatures were set and held isothermal at 25°C for the oven and 100°C for

the detector. The temperature control on the injector port was not turned on and operated at room temperature.

4.1.2.2 Calibration

The system was calibrated using methane standard solutions of known concentrations (Section 3.1.5). Methane gas was identified by comparison with the retention times of the known standards. The resulting peak areas were related to the injections of the headspace gas from the reactors.

4.2 Scanning Electron Microscope Photography

Scanning electron microscope (SEM) photography was used to examine the solid support media for bacteria morphology and quantity. The SEM procedure consisted of collecting samples of resin from active cultures, preparing the sample for viewing, followed by sample examination and photographing areas of interest.

A disposable needle attached to a plastic syringe was inserted into the inverted serum bottle reactor and a 2 mL sample of sludge removed. The sample, containing at least 5 granules of resin, was deposited into a solution of 2.5% glutaraldehyde in Millonig's buffer to fixate the biological growth onto the resin. The fixation process preserves the bacterial cell structure and protects against any changes that may occur during the sample preparation procedure. After 24 hours the sample was washed three times with Millonig's buffer for 10 minutes each to remove all traces of sludge. The resin sample was then postfixed with 1% Osmium Tetroxide (OsO_4) in Millonig's buffer. After one hour the sample was washed with distilled water then dehydrated in ethanol. The

dehydration began with immersion of the sample in a 50% ethanol mixture for 10 minutes followed by 70% and 90% ethanol mixtures for the same time period. Final sample dehydration took place in absolute ethanol for two, 10 minute intervals each. After dehydration the resin sample was critical point dried in a See Vac Inc. Critical Point Dryer (Florida, U.S.) with liquid CO_2, at 40°C for 10 to 15 minutes, then mounted on aluminum stubs using a fine layer of adhesive and dried overnight in a vacuum desiccator. Once dry the resin sample was sputter-coated with gold (75 to 150 nm thickness) in an Edwards Sputter Coater (Model S150B), then mounted in a Hitachi S-2500 Scanning Electron Microscope (SEM) and examined for presence and morphology of bacteria. Areas of interest on the resin samples were photographed.

Dr. M. Chen of the Medicine/Dentistry E.M. Unit, Surgical-Medical Research Institute, University of Alberta, Edmonton, Alberta developed all photographs, assisted with sample preparation, examination, photography and provided all materials or solutions needed for sample preparation.

4.3 Total Solids Determination

Each reactor was shaken well to thoroughly mix the solids and supernatant. A disposable needle attached to a plastic syringe was inserted into the reactor and a 5 mL sample extracted. The syringe was previously well flushed by an oxygen free gas mixture. Total solids were then determined by standard methods (APHA 1992, section 2540B).

4.4 Intermediate and By-Product Analyses

4.4.1 Solvent Extraction

Solvent extraction procedure was modified from the Method 625 - Base/Neutrals and Acids (USEPA, 1984) by S. Kenefick, Health Services Administration and Community Medicine, University of Alberta, Edmonton, Alberta to accommodate the limited amount of sample available.

4.4.1.1 Reactor Sampling and Preparation

A 5 mL sample of supernatant was removed by injection of a plastic syringe with disposable tip through the stopper of the serum bottle reactor. Before injection, both syringe and tip were thoroughly flushed with a mixture of oxygen free gas. The 5 mL sample was filtered through a 0.45 micrometer (μm) filter (Millipore HA) and diluted to 10 mL with deionized, distilled water.

4.4.1.2 Base/Neutral Extraction

The diluted sample was brought to pH 10 by the addition of a few drops of sodium hydroxide, pH was verified with litmus paper. Approximately 1.3 mL of double distilled methylene chloride (CH_2Cl_2) was added to the sample. This mixture was then shaken for 2 minutes with periodic venting to release built up pressure. The mixture was allowed to stand for 10 minutes to facilitate the separation of two separate phases, methylene chloride and water. Approximately 1 mL of methylene chloride was removed from the bottom of the centrifuge tube and collected into a labeled 3 mL vial. The procedure was repeated three times using 0.5 mL of CH_2Cl_2 for the second and third extraction. A total volume of 2 mL of CH_2Cl_2 was collected in the vial. Before sealing the

4.4.2.1 Operating Conditions

A 0.2 microlitre (μL) sample of each extract was analyzed using a Hewlett Packard 5890 Gas Chromatograph (GC), coupled with an HP 5970 mass-selective detector and HP 59940 Chemstation® data system. The HP5-MS column was 30 m x 0.25 mm ID with a 0.25 micrometer (μm) film thickness. The GC injection port temperature was 225°C and detector temperature 250°C. Column head pressure was 70 kPa. The oven was temperature programmed at 100°C for 2 min., 5°C per minute to 200°C, with the final temperature held for 3 minutes to obtain the best identification possible.

A computer library search was used to match the mass spectra of interest with those stored in the data library. GC/MS work was performed by S. Kenefick and T. Perley, Health Services Administration and Community Medicine, University of Alberta, Edmonton, Alberta.

4.4.3 Qualitative Analysis for Amino Acids

Exactly 0.2063 g of ninhydrin (1,2,3-indanetrione monohydrate) was weighed into a clean, dry, glass beaker under a covered balance. The ninhydrin was transferred to a 50 mL volumetric flask and made up to volume with distilled, deionized water. A 1 mL aliquot of this solution was placed into a clean, dry, test tube.

A 2 mL sample of reactor supernatant was removed by injection of a plastic syringe with disposable tip through the stopper of the serum bottle reactor. Before injection, both syringe and tip were thoroughly flushed with a mixture of oxygen free gas. The supernatant sample was filtered through a 0.45 μm filter (Millipore HA) into the test tube containing the ninhydrin solution. The test tube

mixture was placed into a hot water bath and heated to boiling for 15 to 30 seconds. The color of the solution was noted. A blue to blue-violet color given by the presence of amino acids constitutes a positive test, while all other colors are negative.

4.4.4 Derivatization and Extraction Procedure

Chlorinated phenols and their by-products were determined as their acetate derivatives by GC/MS using a modification of the method described by Coutts et al., (1980). The method was modified by S. Kenefick, Health Services Administration and Community Medicine, University of Alberta, Edmonton, Alberta to accommodate the limited amount of sample available. The analysis was used as a qualitative procedure only, no internal standards were prepared or added.

4.4.4.1 Sampling and Preparation

A 10 mL sample was removed by injection of a plastic syringe with disposable tip through the stopper of the serum bottle. Before injection, both syringe and tip were thoroughly flushed with a mixture of oxygen free gas. The 10 mL sample was filtered through a 0.45 micrometer (μm) filter (Millipore HA) into a separatory funnel.

4.4.4.2 Derivatization and Extraction

The sample in the separatory funnel was combined with 1.000 g of $NaHCO_3$, preweighed under a covered balance, and 0.5 mL of acetic anhydrite, added by glass syringe (Hamilton microlitre #750). This mixture was then

shaken with periodic venting every 5 seconds to release built-up pressure. Shaking continued until all bubbling ceased and no more CO_2 gas was evolving. Approximately 4 mL of methylene chloride was added to the separatory funnel. The mixture was then shaken for 2 minutes, with periodic venting to release built up pressure and allowed to stand for a couple of minutes to facilitate the separation of two separate phases, methylene chloride and water. Approximately 4 mL of methylene chloride was removed from the bottom of the separatory funnel and collected into a clean, dry erlenmeyer flask. The addition of methylene chloride, shaking and separation procedure was repeated three times with approximately 12 mL of sample extract collected in the flask. To remove any water, the extract was passed through a Pasteur pipet containing approximately 1 g of Na_2SO_4 immobilized between glass wool plugs, then deposited into a Kuderna Danish evaporator tube (KD tube). Concentration of the sample extract was done by passing N_2 gas over the KD tube and evaporating the sample slowly to a volume of 0.2 mL. The concentrated sample was immediately transferred to a clean, labeled 1 mL vial and capped.

4.4.4.3 Gas Chromatography/Mass Spectroscopy

Gas Chromatography/mass spectroscopy (GC/MS) was used to identify the derivatized chlorophenols and any intermediate or by-product compounds produced during microbial degradation of the chlorophenol substrate.

4.4.4.3.1 Operating Conditions

A 0.2 microlitre (μL) sample of each extract was analyzed using a Hewlett Packard 5890 Gas Chromatograph (GC), coupled with an HP 5970 mass-

selective detector and HP 59940 Chemstation® data system. The GC conditions were: 100°C for 2 min., 5°C/min to 200°C, final temperature hold for 3 min. The HP5-MS column was 30 m x 0.25 mm ID and 0.25 micrometer (μm) film thickness. Column head pressure was 70 kPa; injector temperature was 225 °C and detector temperature 250°C.

A computer library search was used to match the mass spectra of interest with those stored in the data library. GC/MS work was performed by S. Kenefick, Health Services Administration and Community Medicine, University of Alberta, Edmonton, Alberta.

4.4.5 Statistical Analysis of Data

The degradation rate was calculated by obtaining the slope of a line produced by a linear regression through selected points. Only points that occupied positions along the exponential portion of the curve were selected. The line was then plotted on a graph of time versus chlorophenol concentration.

The average (arithmetic mean) influent and effluent values were calculated through statistical analysis of influent and effluent data. Data points outside of two standard deviations (95.44% Probability) were excluded and the averages recalculated before plotted as lines on graphs of time versus chlorophenol concentration.

Regression analyses and statistical descriptions are located in Appendix V.

5.0 Semicontinuous Anaerobic Microbial Degradation of 2-Chlorophenol and 4-Chlorophenol

Batch reactors with attached growth (series A and C), suspended growth (series B) were established and acclimated to specific isomers of chlorophenol (ortho or para). Supernatant was withdrawn to encourage biomass attachment in series A. Five series (designated series B to C and series E, F and G) of microbial cultures were set-up using supernatant removed from attached growth reactors in series A. Biological activity of the cultures was observed by monitoring the disappearance of 2- or 4-chlorophenol and the periodic analysis for the presence of methane.

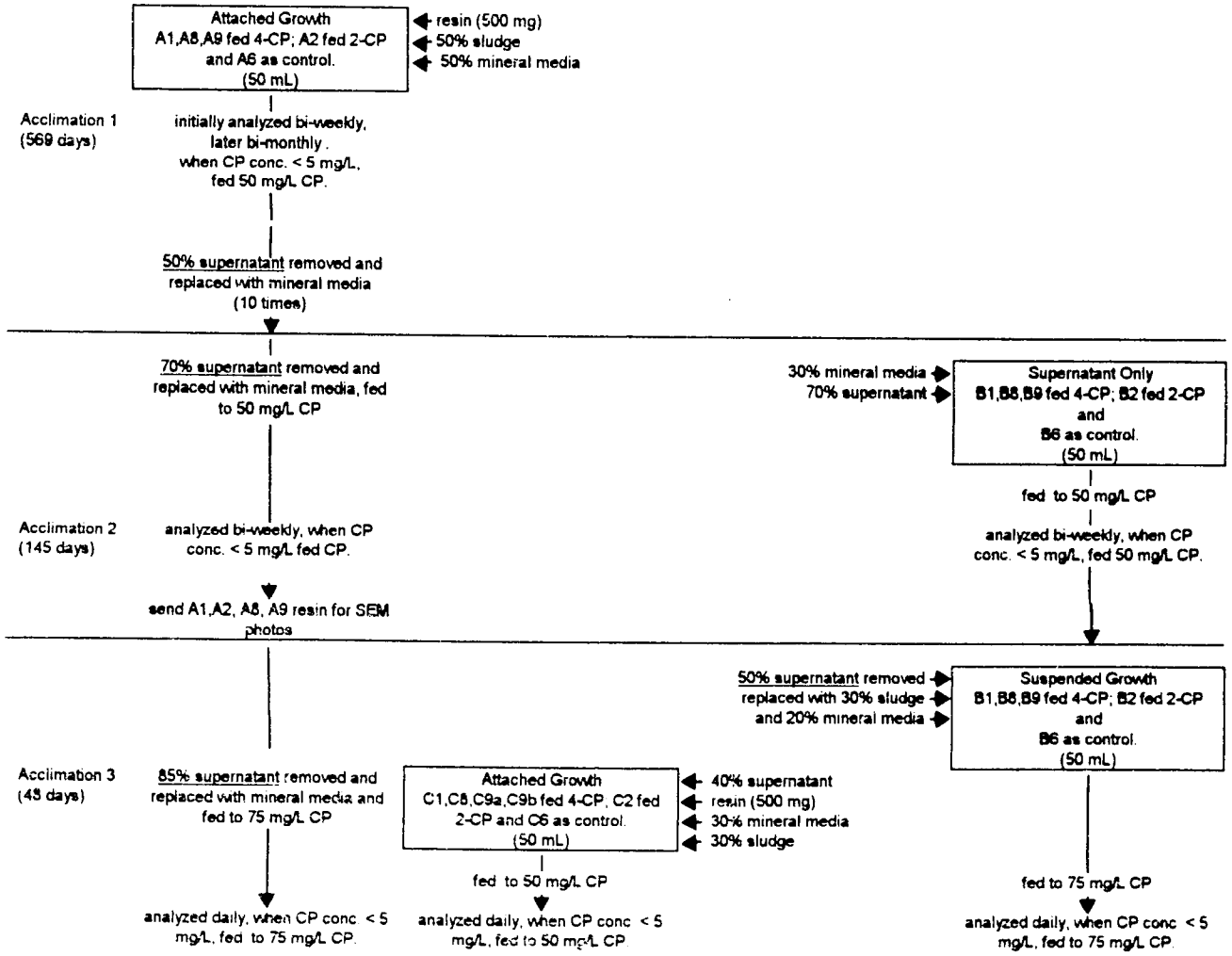
The semicontinuous (draw and feed) experimental technique (Fedorak, 1984) simulates continuous study in small volume reactors and permits the evaluation of a specific microbiological system under a controlled set of organic loading conditions such as concentration and hydraulic retention time (HRT).

5.1 Acclimation Study

The acclimation study was conducted in three phases during which a specific type of microbial (attached or suspended) growth was encouraged.

5.1.1 Procedures

Reactor set-up and the acclimation study was conducted according to the experimental summary in Figure 5.1.



note: % = % of v/v of original liquid volume in reactor (50 mL)

CP= Chlorophenol, 2-CP= 2-Chlorophenol, 4-CP= 4-Chlorophenol

FIGURE 5.1 Flow Diagram of the Acclimation Study.

5.1.1.1 Acclimation 1 (day 1 to 570)

Series A: Attached growth reactors were set-up and the supernatant periodically removed to promote microbe attachment onto a solid support media and colonization (Figure 5.1). Reactors labeled A1, A8 and A9 initially received approximately 30 mg/L of 4-chlorophenol and reactor A2 received approximately 30 mg/L of 2-chlorophenol. Reactor A6 did not receive any substrate and served as a control for the study.

Repeat GC analyses for chlorophenol concentration were initially performed approximately every 2 weeks and later performed bi-monthly. Once the concentration had declined to less than 5 mg/L, reactors were refed with either 2-chlorophenol or 4-chlorophenol up to maximum concentrations of 50 mg/L.

On 10 occasions, when chlorophenol concentrations declined to less than 5 mg/L, 50% (25 mL) of the supernatant was removed from all A series reactors, replaced with 25 mL of mineral media solution and fed up to maximum concentrations of 50 mg/L. Chlorophenol analysis, refeeding and supernatant exchange continued for approximately 81 weeks (569 days).

5.1.1.2 Acclimation 2 (day 570 to 715)

Series B: Supernatant only reactors were set up from series A supernatant (Figure 5.1). Approximately 70% (35 mL) of supernatant removed from each of the attached growth reactors (series A) was used to set up new comparable supernatant only reactors (designated series B) and supplemented by 30% (15 mL) fresh anaerobic mineral media. The supernatant only reactors were fed the same substrate as the A series, attached growth parent reactors. Four of these

reactors (B1, B2, B8 and B9) were acclimated to metabolize 50 mg/L 2-chlorophenol or 4-chlorophenol as their sole substrate source. One reactor (B6) was used as a control.

Approximately every 2 weeks reactor supernatant was analyzed for chlorophenol concentration by GC. When concentrations decreased to less than 5 mg/L, attached growth reactors (series A) received 75 mg/L either 2-chlorophenol or 4-chlorophenol while supernatant only reactors (series B) received a maximum of 50 mg/L of either 2-chlorophenol or 4-chlorophenol substrate.

Analysis for substrate and refeeding continued for approximately 21 weeks (145 days).

On day 715, samples of resin contained in attached growth reactors A1, A2, A8 and A9 were taken for SEM photographs.

5.1.1.3 **Acclimation 3 (day 715 to 763)**

Series C: Attached growth reactors were set up from series A supernatant (Figure 5.1), to provide an additional set of attached growth reactors in case of reactor failure. Reactors contained solid support media for microbe colonization.

A plastic syringe equipped with disposable tip removed 85% (42.5 mL) of the supernatant from the series A attached growth reactor. A 20 mL aliquot of the supernatant was ejected into an open reactor bottle labeled series C, containing 500 mg of dry resin (wetted). An additional syringe previously loaded with 42.5 mL of fresh, reduced anaerobic mineral media growth solution (Table 3.1) was ejected into the series A attached growth reactor. Sparging of the

reactors continued for 2 to 3 minutes. Attached growth reactors, series A, were then stoppered and capped.

While continually sparging the headspace of the series C attached growth reactor, 15 mL of sludge inoculum and 10 mL of reduced mineral media growth solution were added. Reactors were continually sparged with oxygen free gas for 2 to 3 minutes, stoppered and capped.

Attached growth reactors (C1, C2, C8, C9a and C9b) were acclimated to metabolize 50 mg/L of either 2-chlorophenol or 4-chlorophenol, the same substrate as their parent attached growth reactors (series A). One reactor (C6) was not provided with any substrate and served as a control. Reactors were then stored in the dark at 37°C .

Series B: Approximately 50% (25 mL) of supernatant from the series B reactors was exchanged for 10 mL of reduced mineral media growth solution and 15 mL of sludge inoculum. Supernatant only reactors (series B) then became suspended growth reactors. Reactors were fed 75 mg/L of either 2- or 4-chlorophenol substrate. Reactors were then stored in the dark at 37°C.

Series A: Attached growth reactors (series A) were fed 75 mg/L of either 2- or 4-chlorophenol substrate. Reactors were then stored in the dark at 37°C.

Approximately every two weeks, all reactors were analyzed for chlorophenol concentration by GC and refed when the concentration of the substrate within the supernatant decreased to less than 5 mg/L. Reactors were analyzed for chlorophenol concentration and refed over a period of approximately 7 weeks (48 days).

Table 5.1 provides a summary of the chlorophenol feeding in the acclimation study. Table 5.2 provides a summary of the components in each reactor.

TABLE 5.1 ACCLIMATION STUDY SUMMARY

Acclimation	Reactors	Culture	No. of Days From	To	Total	CP Isomer	Conc. (mg/L)
1	A2	A	1	569	569	2-CP	50
	A1,A8,A9	A				4-CP	50
	A6	A				-	-
2	A2	A	570	714	145	2-CP	75
	A1,A8,A9	A				4-CP	75
	A6	A				-	-
	B2	O				2-CP	50
	B1,B8,B9	O				4-CP	50
	B6	O				-	-
3	A2	A	715	763	48	2-CP	75
	A8,A9,C8	A				4-CP	75
	A6	A				-	-
	B2	S				2-CP	75
	B8,B9	S				4-CP	75
	B6	S				-	-
	C2	A				2-CP	50
	C1,C8,C9A, C9B	A				4-CP	50
	C6	A				-	-

Culture; A - Attached Growth, S - Suspended Growth, O - Supernatant Only.
CP - Chlorophenol; 2-CP, 2-Chlorophenol; 4-CP, 4-Chlorophenol.

TABLE 5.2 BATCH REACTOR COMPOSITION

Reactor	Series		
Composition	**A**	**B**	**C**
Solid Support Media (mg)	500	None	500
Supernatant (mL)	None	25*	20*
Mineral Media Growth Solution (mL)	25	10	15
Sludge Inoculum (mL)	25	15	15
Total Liquid Volume in Reactor (mL)	50	50	50

* Supernatant for reactors B, and C originated from reactors in series A removed at different times.

5.1.2 Results and Discussion

The purpose of the acclimation study was to set-up serum bottle reactors containing attached growth and suspended growth bacterial cultures adapted to either 2-chlorophenol or 4-chlorophenol as their sole source of substrate.

Acclimation to 2- or 4-chlorophenol substrate was accomplished through a three stage procedure (Figure 5.1 - p. 72). A long initial stage with slow degradation occurred after the first feeding, followed by an stage of increased degradation after the second feeding. The third and final stage showed that less time was required for substrate degradation with the third feeding. Performance during phase 3 for reactors A2, A8, B2 and B8 is presented in Figures 5.5 to 5.8 inclusive. With more frequent feedings the cultures exhibited a marked slowdown in the rate of degradation. During the incubation period of 20 to 50 days, the number of feedings increases with a corresponding decrease in the rate of chlorophenol substrate degradation.

Results from this study indicate that reactors with attached growth cultures acclimate to changes in substrate concentration easier than reactors with suspended growth cultures. The presence of a solid support media, such as resin, for the attachment of bacteria cultures enabled acclimation to either 2- or 4-chlorophenol to proceed over a shorter period of time than was required for acclimation of suspended growth cultures to the same substrate.

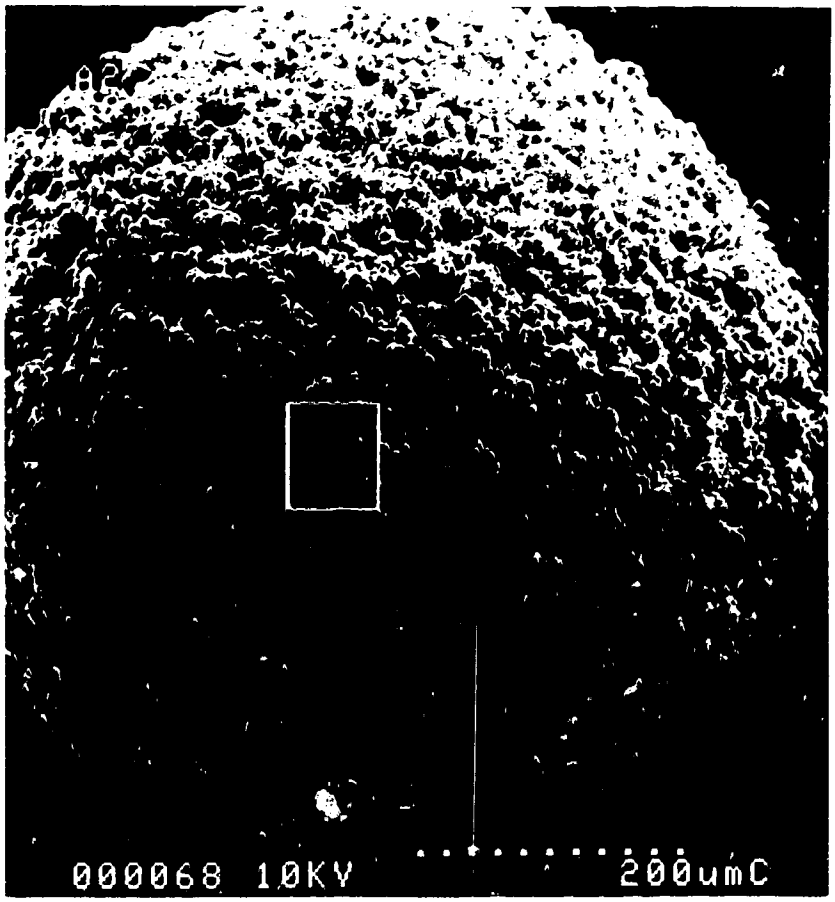


FIGURE 5.2 A Scanning Electron Microscope Photograph of a Granule of Ambergard™ XE-352 Anion Exchange Resin Used as Solid Support in Attached Growth Reactors.

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THE ANAEROBIC BIODEGRADATION OF 2-CHLOROPHENOL AND 4-CHLOROPHENOL IN ATTACHED AND SUSPENDED GROWTH REACTORS

BY

TWILA GRIFFITH 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of **Master of Science**

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Twila Griffith

Twila D. Griffith
2707 - 124th Street
Edmonton, Alberta
T6J 4T2

April 25, 1994
Date

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **The Anaerobic Biodegradation of 2-Chlorophenol and 4-Chlorophenol in Attached and Suspended Growth Reactors** by **Twila D. Griffith** in partial fulfillment of the requirements for the degree of **Master of Science**.

Dr. E. Knettig (Supervisor)

Dr. D. W. Smith

Dr. N. O. Egiebor

April 23, 1994
Date

Abstract

During this study the anaerobic biodegradation of 2-chlorophenol and 4-chlorophenol by attached and suspended growth cultures was examined.

Cultures were set-up and maintained in 50 mL serum bottle reactors. These reactors, containing either attached growth or suspended growth bacterial cultures, were successfully established and acclimated to either 2-chlorophenol or 4- chlorophenol as their sole sources of substrate.

A semicontinuous fermentation study was conducted to evaluate the performance of attached growth, suspended growth and supernatant only cultures in batch assays with respect to the removal of 2- or 4-chlorophenol under conditions of steady state. Serum bottle reactors established during the acclimation study were subject to draw and feed conditions of 50 mg/L 2-chlorophenol or 4-chlorophenol substrate and a 10 day hydraulic retention time. Under these conditions the reactors were monitored for removal of the chlorophenol substrate and methane production. Reactors containing supernatant only were established during supernatant exchange to provide a control for the attached growth and suspended growth reactors.

The study found that the attached growth reactors performed superior when compared to suspended growth reactors. Attached growth reactors reported shorter acclimation times for both 2-chlorophenol and 4-chlorophenol than suspended growth reactors. The presence of resin provided a solid support material that promoted an extremely stable biomass. The attached growth reactors were much less prone to the effects of "wash-out", loading changes and substrate toxicity. Higher 2- chlorophenol and 4-chlorophenol degradation rates were achieved for attached growth reactors than suspended growth reactors.

Under pseudo steady state conditions, 2-chlorophenol and 4-chlorophenol degradation rates of 5.2 mg/L per day were achieved by the attached growth reactors.

However, over an extended period of exposure all reactors were affected by the toxicity of the monochlorophenol substrate. Anaerobic degradation of reactors containing attached or suspended growth cultures was noticeably inhibited by the continued presence of 4-chlorophenol, culminating in reactor failure.

Slight morphological differences were noted in the attached bacteria consortia. More types of rod shaped bacteria were present in reactors fed 2-chlorophenol. In comparison, more filamentous and coccoid types of bacteria were present in reactors fed 4-chlorophenol. No visual difference or increase in bacterial colonization of the resin was noted throughout the study.

The study concluded that the long term sustainability of anaerobic bioreactors with 2- or 4-chlorophenol as their sole source of substrate is in need of further investigation. Chlorophenols are highly toxic and in long term exposure may be disruptive to the bacterial enzymatic system.

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I would like to thank my supervisor Dr. E. Knettig for her excellent guidance, advice and patience throughout the course of this project.

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1.0 Introduction and Research Objectives

Chlorinated phenols comprise a large group of toxic man made chemicals that are serious environmental pollutants (Steiert et al., 1985). Recent concern has been expressed on the environmental fate of these compounds due to their carcinogenic and toxic properties.

In 1992 approximately 72,000 metric tonnes of phenol were consumed in Canada, with 3,000 metric tonnes including chlorophenols such as pentachlorophenol (PCP). As of May, 1992, domestic production of chlorophenols ceased and industrial supplies of primarily pentachlorophenol and tetrachlorophenol have been imported (Fujise et al., 1993).

Chlorophenols are considered priority pollutants due to their harmful environmental effects as a consequence of their toxicity, bioaccumulation and resistance to biodegradation. Bioaccumulation and persistence of the chlorinated phenols in the aquatic environment have created concern over their discharge from bleached kraft pulp mills into the receiving waters (Kovacs et al., 1984). The toxicity of chlorinated phenols tends to increase with their degree of chlorination, and because few microbes can decompose them, the multichlorinated phenols tend to accumulate in the environment (Steiert and Crawford, 1985). Some chlorophenols are not only toxic to aquatic vertebrates and fish but are also suspected to be potential carcinogens and mutagens (Wan, 1992).

Chlorinated phenols are used as antifungal agents, wood preservatives, insecticides and are produced as degradation products of chlorophenoxy herbicides (Rochkind-Dubinsky et al., 1987). Pentachlorophenol (PCP) has been used as a wood preservative, preharvest herbicide, and a

fungicide/bactericide in cooling tower waters, adhesives, construction materials, textiles, leather, paint, paper, and oil well drilling mud (Mikesell and Boyd, 1986 and Guthrie et al., 1984). 2,4-Dichlorophenol and 4-chlorophenol are used in the production of PCP and the herbicides 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid (Kohring et al., 1989b). Chlorophenols are also intermediates in the aerobic metabolism of chlorobenzoates, chlorobenzenes and chlorinated phenoxy acids (Krumme and Boyd, 1988).

Chlorinated phenols are found in pulp mill effluents from chlorine-bleached soft wood processing. Low molecular weight chlorinated phenolic compounds formed during pulp chlorination have been identified in pulp mill bleachery effluents (Kovacs et al., 1984). Trichlorophenol is the principal chlorinated phenol in bleached sulphite discharges (CEPA, 1991).

2-Chlorophenol (orthochlorophenol) and 4-chlorophenol (parachlorophenol) are formed when gaseous chlorine is passed into molten phenol and separated by fractional distillation (Krijgsheld and van der Gen, 1986). 2-Chlorophenol is also produced as an intermediate in the synthesis of higher chlorophenols and phenolic resins. 4-Chlorophenol is produced as an intermediate in the production of higher chlorinated phenols, in the synthesis of dyes and drugs, as a denaturant for alcohol and as a selective solvent in refining of mineral oils (Krijgsheld and van der Gen, 1986).

Chlorophenols are also formed during the chlorination of effluents of various industrial waste treatment plants or in drinking water sources containing low phenol concentrations and high chlorine concentrations (Kovacs et al., 1984; Krijgsheld and van der Gen, 1986).

Chlorinated phenols are found in lake and river sediments, municipal waste systems, soils and in groundwater. They are released to the environment deliberately in pest control, by direct disposal of industrial chemicals into landfills or into waterways through wastewater disposal, as breakdown products of chlorinated phenolics or accidentally as in seepage from toxic waste disposal sites. PCP has been identified as a contaminant in the vicinity of lumber mills and paper and pulp factories. Chlorophenols also enter the environment through chemical "bleeding" from newly treated and installed poles and ties, followed by migration into the soil during rainstorms or by surface runoff and leaching processes active in the soil (Wan, 1992). In Finland, environmental soil, groundwater and fauna samples have been found to contain a variety of compounds originating from the technical-grade PCP formulation used for lumber treatment. The contaminants identified include polychlorophenoxy-phenols, polychlorophenoxy anisoles, polychlorodibenzodioxins, and furans, polychlorinated phenols, catechols and guaiacols (Mikesell and Boyd, 1986).

High water solubility and widespread use of chlorinated phenolic compounds have resulted in concern over their potential contamination of potable ground water supplies (Krumme and Boyd, 1988). If aerobic degradation is slow, these compounds pass into the anaerobic zone above the groundwater zones (Kohring et al., 1989b). If not degraded, these compounds will contaminate aquifers. Aromatic hydrocarbons, phenols and cresols are among the most prevalent aromatic contaminants in groundwater (Lovley and Lonergan, 1990). Taste and odour-causing compounds in surface water and potable water have also been associated with chlorinated phenols (Kovacs et al., 1984).

Disposal of pollutants, including chlorinated phenols, has become very difficult from both a practical and political standpoint. There is a definite need for new disposal methods. One approach currently being investigated, bioremediation, involves the use of microorganisms for biodegradation and biotransformation of these wastes. Other approaches include volatilization, photodegradation, chemical oxidation, and sorption. However, biodegradation by microorganisms is considered to be one of the major mechanisms by which chlorophenols and other pollutants can be successfully broken down into simpler compounds that are less harmful to the environment.

Bioremediation has been shown to be favorable in the cleanup of soils contaminated with high concentrations of chlorophenols. Concentrations of 1002 mg PCP/kg of soil, contaminated during wood treatment, have been successfully reduced to less than 5 mg PCP/kg of soil (Seech, 1993).

There have been many studies directed towards the anaerobic degradation of multichlorinated phenols. The scope of this research was to examine specific aspects of 2-chlorophenol and 4-chlorophenol with respect to optimizing the design of facilities required to treat chlorophenolic wastes.

1.1 Research Objectives

The overall objective of this project was to provide a rational base for optimizing design and performance of anaerobic biological process for chlorophenol wastewaters. Specific objectives of the project were:

a) to investigate the acclimation of anaerobic microbial cultures with different types of growth;

b) to compare the performance of semi-continuous reactors (draw and feed) with contain attached and suspended growth cultures;

c) to determine degradation rates for 2- and 4-chlorophenol substrates; and

d) to examine bacterial colonization of a solid support media.

2.0 Literature Review

The aim of this literature review is to provide background information on the physical properties of chlorophenols, toxicity of chlorophenols, chlorophenol biodegradation and solid support media as a biological support.

2.1 Physical Properties of Chlorophenols

Chlorophenols are members of the arene family of organic compounds. Benzene is the common name given to this family which exhibits the general formula ArH and is symbolized by the aromatic ring.

The aromatic ring is made up of a six-member carbon ring which contains three double bonds between alternate carbon atoms, linked in a cyclic hexagonal form. Carbon atoms in these ring compounds have a single covalent bond available.

The simplest aromatic ring is the benzene ring (C_6H_6) and is known as the parent compound of the aromatic series (Sawyer and McCarty, 1978). Phenol (C_6H_5OH) is the monohydroxy derivative of benzene.

Chlorophenols are comprised of a single aromatic ring with one hydroxy radical attached in the first position and a single chlorine atom attached in one or more of five possible positions.

Positions of the chlorine atoms are defined with respect to the point of substitution (Figure 2.1). If substitution occurs on carbon atoms adjacent to the hydroxy radical the term ortho (o-) or position 2 or 6 is applied. If substitution occurs on carbon atoms once removed from the hydroxy radical the term meta (m-) or position 3 or 5 is applied, and substitution on carbon atoms opposite the hydroxy radical the term para (p-) or position 4 is used.

The prefixes mono, di, tri, tetra and penta are used to indicate the number of chlorine substitutions. Monochlorophenols have a single chlorine atom substituted for a hydrogen while pentachlorophenol has all five positions (2 to 6 inclusive) substituted by chlorine atoms.

Most pure monochlorophenol compounds are white, crystalline substances. Pure 2-chlorophenol is a colourless liquid at room temperature. Chlorophenols behave as weak acids with an increase in solubility in alkaline solutions (Krijgsheld and van der Gen, 1986). Other important physical properties of chlorophenols are presented in Table 2.1.

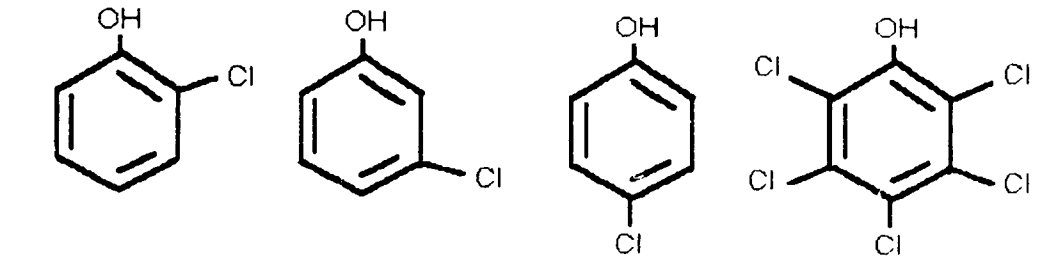


FIGURE 2.1 Molecular Structure of Chlorophenols (Adapted from Solomons, 1984).

TABLE 2.1 PHYSICAL PROPERTIES OF CHLOROPHENOLS

Parameter	2-chlorophenol	3-chlorophenol	4-chlorophenol	2,4-dichlorophenol
Molecular Mass (g/mol)	128.6	128.6	128.6	163.0
Physical State	liquid	solid	solid	solid
Colour	colourless	white	white	white
Melting Point (°C)	9.3	33	43	45
Boiling Point (°C)	175	214	217	210
Vapour Pressure	1 mm Hg @ 12.1°C	1 mm Hg @ 44.2°C	1 mm Hg @ 49.8°C	1 mm Hg @ 53°C
Water Solubility (@ 20°C, g/L H_2O)	28.5	26	27.1	4.5
Log Partition Coefficient (n-oct/water)	2.15	2.50	2.39	3.08
pK_a	8.48	9.02	9.38	7.85

Adapted from Krijgsheld and van der Gen (1986).

2.2 Toxicity

As a group, chlorophenols are highly toxic and often precursors to dioxins. Dioxins may be present as minor contaminants in some chlorophenol-based products. Technical pentachlorophenol(PCP) has been reported to typically contain 500 to 1500 mg/L of dioxin (Lamparski et al., 1980). Dioxins may also be produced when organic and chlorine-containing materials such as chlorinated phenols are combusted (Lamparski et al., 1980; Bridle et al., 1983; Boyd and Shelton, 1984). 4-chlorophenol is a suspected carcinogen and along with 2,4-dichlorophenol is highly toxic (Kohring et al., 1989b). PCP is toxic to a variety of organisms and along with other chlorophenols such as 2-chlorophenol, 2,4-dichlorophenol and 2,4,5-trichlorophenol has been placed on the US. Environmental Protection Agency (USEPA) list of 129 priority pollutants (Mikesell and Boyd, 1986; Krumme and Boyd, 1988; Eckenfelder, 1989).

Detectable levels of chlorophenols have been shown to accumulate in feral pink salmon and juvenile chinook salmon (Servizi et al., 1988). The probable sources of contamination cited were lumber mills using chlorophenol based fungicides and pulp mill effluents. Rogers et al., (1988) presented evidence of bioconcentration of chlorophenols and chloroguaiacols in feral and juvenile chinook salmon during winter conditions. These fish were exposed to effluent from sewage, pulp mills and commercial wood preservative operations.

Toxicity tests performed with chlorophenol include the LC_{50} and Microtox® EC_{50} test. The LC_{50} or lethal concentration test defines the lethal concentration of a sample in which 50% of the test animals immersed in the sample die over a specified time period. The Microtox® EC_{50} bacterial luminescence test estimates the effective concentration of a sample that causes

a 50% decrease in light output at 15°C after a specified period of time, usually 15 minutes. Results of toxicity tests performed with select chlorophenols are provided in Table 2.2.

The primary routes of chlorophenol exposure for humans and other mammals are oral intake (drinking contaminated water or eating food than contains chlorophenol residues) and skin absorption (Krijgsheld and van der Gen, 1986). Kauppinen and Lindroos (1985) measured personnel exposure to chlorophenols in 10 Finnish sawmills. High concentrations (up to 0.21 mmol/L) of chlorophenols were detected in workers that had skin contact with chlorophenols. They concluded that skin absorption appeared to be the main route of exposure. In mammals, phenolic compounds such as 2-chlorophenol, 4-chlorophenol and 2,4-dichlorophenol become largely combined with sulfate or glucuronic acid, leading to their detoxification and efficient elimination. If not transformed, these compounds may bioaccumulate and be distributed into organ and fatty tissues.

A summary of relevant toxicity data for selected chlorophenols is given in Table 2.3.

TABLE 2.2 SUMMARY OF TOXICITY TESTS FOR SELECTED CHLOROPHENOLS

Medium	Test	Animal	Toxic Level	Reference
2-Chlorophenol	LC_{50}(96 hr)	water flea and rainbow trout	2.6 mg/L	1
	LC_{50}(96 hr)	water flea	< 0.77 mg/L	2
	Microtox® EC_{50}	-	0.51-0.58 mg/L	2
	LC_{50}(96 hr)	fathead minnow	11.63 mg/L	3
	LC_{50}(96 hr)	bluegill sunfish	10.00 mg/L	3
	LC_{50}(96 hr)	goldfish	12.37 mg/L	3
	LC_{50}(96 hr)	guppies	20.17 mg/L	3
4-Chlorophenol	LC_{50}(48 hr)	golden orfe	3 mg/L	1
	96 hr EC_{50}(growth)	alga	4.8 mg/L	2
	LC_{50}(96 hr)	water flea	1.17 mg/L	2
	LC_{50}(96 hr)	water flea	4.1 mg/L	1
	Microtox® EC_{50} (5 to 30 min)	-	1.15-1.19 mg/L	2
2,4-Dichlorophenol	LC_{50}(24 hr)	trout	1.7 mg/L	1
	LC_{50}(48 hr)	water flea	1.21 mg/L	2
	Microtox® EC_{50} (5 to 30 min)	-	1.47-1.54 mg/L	2
Pentachlorophenol	LC_{50}(48 hr)	salmonids	32-130 m g/L	4

1. Krijgsheld and van der Gen, 1986.
2. Munkittrick and Power, 1991
3. Pickering and Henderson, 1966.
4. Davis and Hoos, 1974; Iwama and Greer, 1979.

TABLE 2.3 SUMMARY OF TOXICITY DATA FOR SELECTED CHLOROPHENOLS

Parameter	2-Chlorophenol	3-Chlorophenol	4-Chlorophenol	2,4-Dichlorophenol
Commercial Production	moderate (5 kT/yr)	small (<1000 kg/yr)	moderate (5 kT/yr)	large (20 kT/yr)
Persistence (half life)	low to moderate (1 - 14 days)	moderate to high (>1 month)	low to moderate (1-20 days)	low to moderate (2-15 days)
Bioaccumulation (bioconcentration factor)	low (10)	low (10)	low (10)	low (50)
Aquatic toxicity: LC_{50}, acute (96 hr)	moderate (3-14 mg/L)	moderate (3-8 mg/L)	moderate (3-9 mg/L)	moderate (2-8 mg/L)
Mammalian toxicity (oral LD_{50} rat)	moderate (670 mg/kg)	moderate 570 mg/Kg)	moderate (670 mg/kg)	moderate (580 mg/kg)
Organoleptic effects	strong	strong	strong	strong
(Taste Threshold in water, ppb)	0.1	0.1	0.1	0.3
(Threshold fish tainting, ppb)	15	60	45	0.4

Adapted from Krijgsheld and van der Gen, 1986.

2.3 Biodegradation

Successful anaerobic biodegradation of phenol and phenolic wastewaters has been reported in a number of studies (Churn, 1993).

When compared to phenol, the anaerobic biodegradation of chlorophenols is much more difficult. The carbon-halogen bonds are very resistant to metabolic attack. This resistance increases substantially if these bonds are connected to a very stable planar aromatic structure. Removal of halogen substitutents requires a high energy input. As the degree of halogenation increases, the compound becomes virtually unmetabolizable.

Positions of the chlorines on the aromatic ring also dictate how biodegradable the compound is likely to be (Rothmel et al., 1991). On aromatic rings, halogen atoms hinder ring fission of the benzene nucleus. This deactivates the benzene nucleus to electrophilic attack by dioxygen, withdrawing electrons from the ring (Steiert and Crawford, 1985).

Generally the higher the extent of chlorination, the higher the level of recalcitrance of the haloaromatic compound to microbial attack (Rothmel et al., 1991). While aromatic compounds with one or two chlorine atoms are usually biodegradable, compounds with three or more chlorine atoms generally are recalcitrant and are biodegraded in nature extremely slowly. The deactivating effect of halogen atoms increases with the number of halogen substituents, thus highly halogenated phenols can be resistant to aerobic biodegradation and often persist in aerobic environments (Steiert and Crawford, 1985).

Halogenation increases the compound's tendency to be removed from the liquid phase as indicated by an increase in the octanol/water partition coefficient (Woods, 1985) (Table 2.1). The octanol/water partition coefficient (K_{OW}) is a

constant that describes the equilibrium partitioning of a compound between equal volumes of n-octanol and water at a given temperature. The octanol/water partition coefficient increases with halogen replacement in the following order: -I>-Br>-Cl>-F (Woods, 1985).

Inhibition of chlorophenols on phenol degradation has been studied by Beltrame et al., (1984, 1988, 1989). Their studies concluded that the inhibiting action of chlorophenols increases with the number of chloro substituents. Toxicity increased from least to most as follows:

2-CP <4-CP <3-CP <2,6-DCP <2,3-DCP & 2,4-DCP & 2,5-DCP <3,4-DCP, & 3,5-DCP < 3,4,5-TCP < 2,3,4,5-TTCP < PCP .

Biodegradation of chlorophenols proceeds under both aerobic and anaerobic conditions.

2.3.1 Aerobic Biodegradation of Chlorophenols

Under aerobic conditions, degradation of chlorophenols proceeds through methylation or hydroxylation, with the latter being most common. Chlorophenols are hydroxylated to corresponding chlorocatechols or methylated to chloranisoles before ring cleavage and further degradation for use in central metabolism.

2.3.2 Anaerobic Biodegradation of Chlorophenols

If destruction of chlorophenols does not occur in aerobic environments, these compounds may eventually end up in anaerobic environments. Flooded soils, sediments, landfills, lagoons, fresh and ocean water and some groundwaters may contain anaerobic or anoxic environments.

2.3.2.a Anaerobic Metabolic Pathways

Under anaerobic conditions degradation of chloroaromatics involves complete removal of chlorine from the aromatic ring by reductive dechlorination (where chlorine is exchanged with hydrogen), before further degradation and mineralization (Gibson and Suflita, 1986) (Figure 2.2). No chlorine shift takes place (Horowitz et al., 1983; Rochkind-Dubinsky et al., 1987). This step, followed by ring cleavage (fission) has been observed for chlorinated benzoates and chlorinated phenols. Complete dehalogenation is required before a chloroaromatic can be mineralized to CH_4 and CO_2.

According to Zhang and Wiegel, (1990) the initial step in the anaerobic pathway for degradation of chlorophenols is also reductive dechlorination. The phenol ring is then carboxylated to benzoate, which undergoes ring fission and acetogenesis yielding acetate, CO_2 and H_2. These products then undergo methanogenesis to produce the end products of methane (CH_4) and carbon dioxide (CO_2). A proven pathway for the degradation of 2,4-dichlorophenol is illustrated in Figure 2.3.

Knoll and Winter (1987) have also shown benzoate to be an intermediate in the anaerobic degradation of phenol in sewage sludge. Sharak Genthner et al., (1989) have identified benzoate in several 2-chlorophenol primary cultures and in laboratory transfers of cultures which degraded 2-chlorophenol and 3-chlorophenol.

2.3.2.b Review of Anaerobic Degradation

Woods et al., (1989) examined the fate of chlorinated phenols during continuous anaerobic treatment of a complex concentrated waste water in a

continuous flow, upflow anaerobic sludge blanket reactor over a period of 7 months. Using unacclimated sewage sludge, bacteria removed ortho chlorines from 2,6-, 2,3-, and 2,4-dichlorophenol, 2,4,6-trichlorophenol, 2,3,4,5-tetrachlorophenol and PCP (Figure 2.4). With acclimation, meta chlorines were also removed from 3,5-dichlorophenol, 3,4-dichlorophenol and 3,4,5-trichlorophenol. No evidence for dechlorination of monochlorophenols, ring cleavage and complete degradation to methane and carbon dioxide, were found for any of the chlorophenols evaluated.

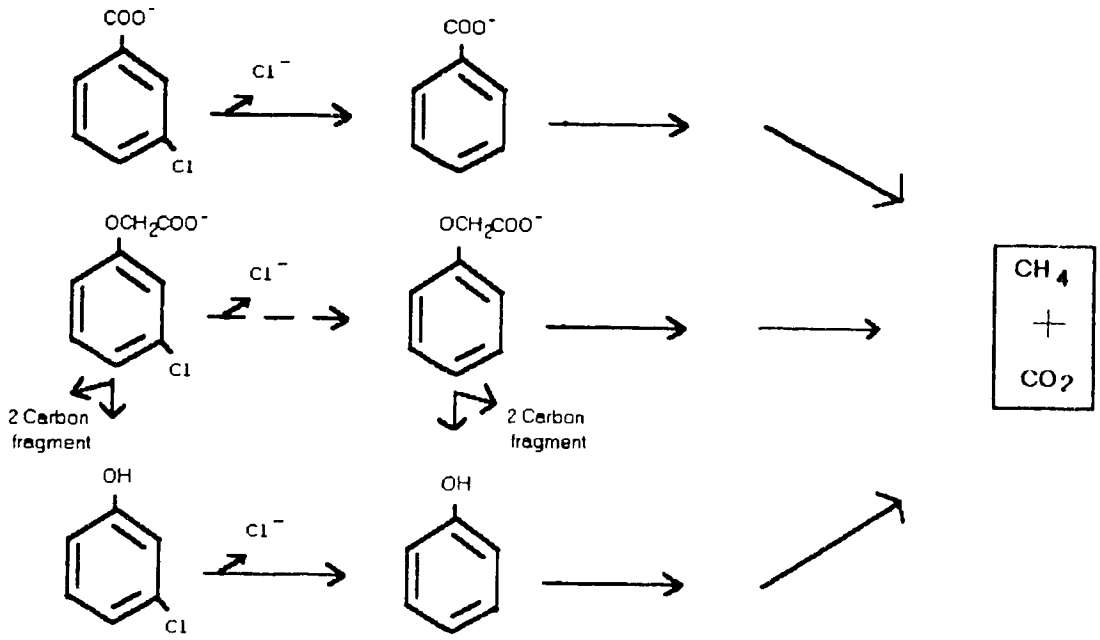


FIGURE 2.2 Proposed Pathway for Methanogenic Degradation of, from Top to Bottom, Chlorinated Benzoates, Phenoxyacetates and Phenols (Gibson and Suflita, 1986).

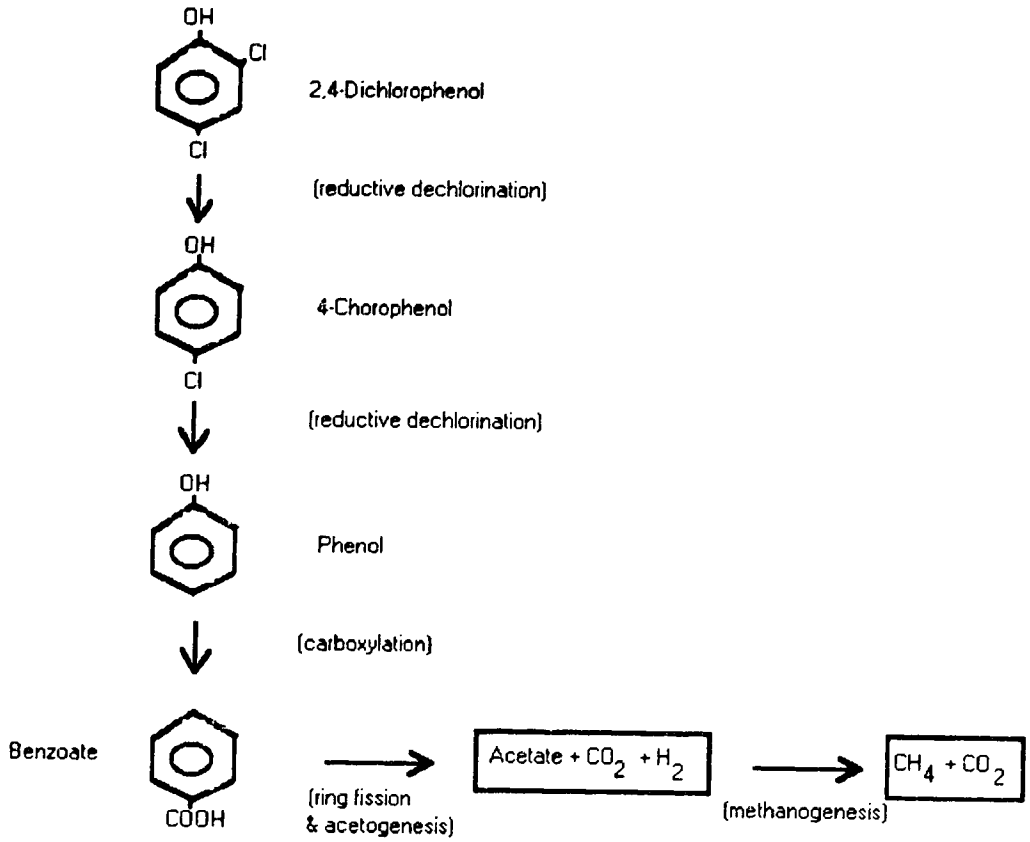


FIGURE 2.3 Biodegradation Pathway for 2,4-Dichlorophenol (Adapted from Zhang and Wiegel, 1990).

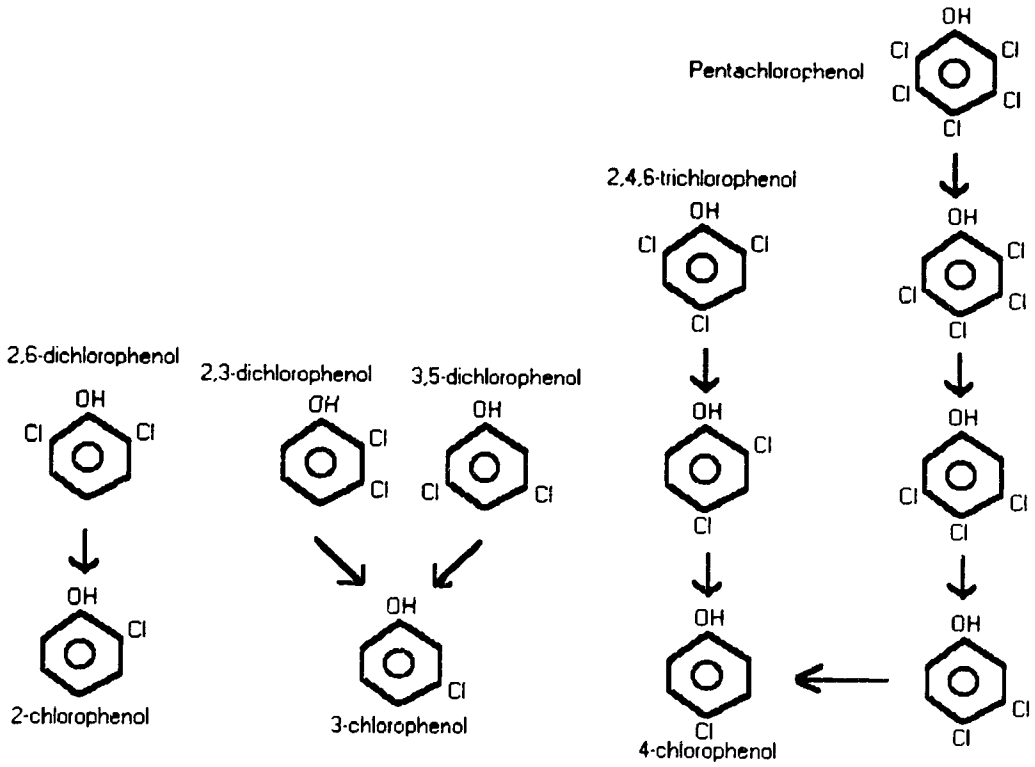


FIGURE 2.4 A Summary of Observed Dechlorination Pathways for Selected Chlorophenols (Adapted From Woods et al., 1989).

Boyd et al., (1983) examined the biodegradability of 2-, 3-, and 4-chlorophenol in fresh 10% anaerobic digester sludge. They found that 50 mg/L of 2-chlorophenol disappeared in 3 weeks and 50 mg/L of 3-chlorophenol disappeared in 7 weeks, while 50 mg/L of 4-chlorophenol was completely degraded after 16 weeks. The production of phenol corresponded with the disappearance of 2-chlorophenol. Phenol was subsequently metabolized to methane.

Woods (1985) determined the pathways of degradation for 2,4,6-trichlorophenol in a continuous flow, upflow anaerobic sludge blanket reactor, acclimated to chlorophenols for 3 months. After 6 hours, 1 mg/L of 2,4,6-trichlorophenol was transformed to 2,4-dichlorophenol. After 3 days, 4-chlorophenol was subsequently detected in the reactor while 2-chlorophenol was not found during the length of the experiment. 4-Chlorophenol was not degraded any further during the 12 day experiment. Woods concluded that 2,4,6-trichlorophenol was anaerobically degraded by reductive dehalogenation of the ortho chlorine.

Gibson and Suflita (1986) monitored the reductive biodegradation of chlorophenols, chlorobenzoates and pesticides in a methanogenic aquifer, a sulfate-reducing aquifer, pond sediment and sewage sludge. Final substrate concentrations ranged from 300 to 500 micromole/Litre (μmol/L). Substrate disappearance and initial intermediates were monitored for 5 months. In sewage sludge samples, monochlorophenols were detected as intermediates during the metabolism of 2,4- and 2,5-dichlorophenol. No degradation was observed for 4-chlorophenol in either the sewage sludge or sulfate- reducing environment. Phenol was detected as an intermediate in the degradation of 4-chlorophenol

present in both the pond sediment and methanogenic aquifer, but was not observed in either the sewage sludge or sulfate-reducing aquifer. They concluded that the removal of aryl halides and their substitution by a hydrogen atom represented the primary route of reductive dehalogenation of aromatic compounds.

Krumme and Boyd (1988) studied the reductive dechlorination of mixture of chlorophenols in upflow bioreactors using chlorophenols as sole carbon and energy source and acclimated digester sewage sludge as inoculum. Chlorophenol substrate concentrations ranged from 30 to 150 mg/L with hydraulic retention times (HRT) from 2 to 10 days. The authors noted that 100% of 3-chlorophenol was mineralized over a period of 44 days (average influent concentration of 10.2 mg/L per day and an average HRT of 6.7 days) and 99% of 4-chlorophenol was mineralized over a period of 46 days (average influent concentration of 15.5 mg/L per day and an average HRT of 7.2 days). Mineralization of chlorophenols was demonstrated by the presence of gaseous end products, CH_4 and CO_2. However, the authors recovered only one of these products, CH_4 during the mineralization of 3-chlorophenol and labeled CO_2 during the mineralization of 4-chlorophenol. The theoretical quantity of the other gaseous end product was then calculated. Krumme and Boyd concluded that under anaerobic conditions, chlorines were removed from the aromatic ring by reductive dechlorination resulting in partially or fully dehalogenated products which were then more susceptible to either aerobic or anaerobic attack.

Kohring et al., (1989b) studied the effects of temperature on the anaerobic degradation of 2,4-dichlorophenol in unacclimated pond sediment slurries. At temperatures between 5 and 40°C, 20 mg/L of 2,4-dichlorophenol was

transformed stoichiometrically to 4-chlorophenol after an initial lag period. The intermediate 4-chlorophenol was degraded after a longer lag time. Phenol was not detected as an intermediate product.

Kohring et al., (1989a) also examined the fate of 2,4-dichlorophenol and 4-chlorophenol in anaerobic freshwater sediments in the presence of added sulfate. At temperatures between 19 and 40°C, 27 mg/L of 2,4-dichlorophenol was stoichiometrically transformed to 4-chlorophenol over adaptation periods ranging from 50 to 366 days. No additional dechlorination of 4-chlorophenol was observed for 1 year. However when 32 to 37 mg/L of 4-chlorophenol plus sodium sulfate was added to non-acclimated sediments or to sediments acclimated to 4-chlorophenol, 4-chlorophenol was stoichiometrically converted to phenol at a rate up to 2 mg/L per day.

Zhang and Wiegel (1990) studied the sequential anaerobic degradation of 2,4-dichlorophenol in freshwater sediments and enrichment cultures using serum bottles incubated at 30°C. In acclimated sediments, 2,4-dichlorophenol was transformed to 4-chlorophenol at a rate of 40 mg/L per day with an adaptation time of 7 days. 4-Chlorophenol was subsequently transformed to phenol at a rate of 7 mg/L per day with an adaptation time of 37 days. Phenol was then transformed to benzoate at a rate of 82 mg/L per day with an adaptation time of 11 days. It was observed that phenol was not detected in dechlorinating cultures when concentrations of 4-chlorophenol were less than 100 mg/L. Acetate, CO_2 and H_2, and consequently methane were formed from benzoate.

Madsen and Aamand (1992) studied the transformation and toxicity of trichlorophenols by acclimated digester sludge. Sludge was acclimated to pentachlorophenol (PCP) over a 2 year period. Dechlorination was verified by

analyzing concentrations of trichlorophenol and daily measurements of methane formation. Approximately 30 mg/L of 2,4,6-trichlorophenol was transformed to 2,4-dichlorophenol in 3 days. 2,4-Dichlorophenol was subsequently transformed to 4-chlorophenol within 4 days. Disappearance of 4-chlorophenol was noted after 40 days, however phenol was not detected. All intermediates of 2,4,6-trichlorophenol were accumulated in less than stoichiometric amounts.

2.3.2.c **Effects of Electrophilic Aromatic Substitution**

Studies suggest that biodegradability of chlorinated aromatic compounds is dependent upon aromatic structure, the position of chlorine atoms on the aromatic ring and acclimation of the microbial consortia (Sharak Genthner et al., 1989 and Woods et al., 1989).

An aromatic compound is traditionally defined as "having the chemistry typified by benzene" (Solomons, 1984). The molecules of aromatic compounds are cyclic and highly stable with respect to their hypothetical resonance structure. They tend to react with electrophilic (electron-deficient) species by substitution rather than addition. A substituent group such as hydroxyl (OH), affects both reactivity and orientation in electrophilic aromatic substitutions. This group causes the aromatic ring to be more reactive than benzene and is referred to as an activating group. Substituents that tend to orient electrophilic substitution to primarily ortho and para positions on the benzene ring, are referred to as ortho-para directors. Hydroxybenzene or phenol is a very powerful activating group and ortho-para director (Solomons, 1984).

Anaerobic degradation of a substituted benzene such as phenol is reliant on the functional group and the position of the halogen on the aromatic ring.

Preferential removal of chlorines from the ortho position of chlorophenols is consistent with the ortho-para directing character of this functional group, resulting in a structure yielding the lowest overall energy (Woods, 1985).

Although these effects describe the substitution of groups on a benzene ring, the same inductive and resonance effects support the degradation pathways observed for chlorophenols and chlorobenzoates by Boyd et al., (1983), Suflita et al., (1982), and Woods (1985).

2.3.2.d Anaerobic Structural Isomer Selectivity

Boyd et al., (1983) studied the anaerobic degradation of several substituted phenols by an unacclimated mixed culture in fresh, 10% anaerobic digester sludge. Phenolic substrate concentrations and methane production were monitored for periods up to 16 weeks. Results indicated that within 3 weeks 29 mg/L of 2-chlorophenol was degraded, without a lag period. Phenol was produced, indicating reductive dechlorination and subsequently metabolized to methane. Acclimation was required before the 3- and 4-chlorophenol were degraded. After acclimation each monochlorophenol isomer was completely degraded within 16 weeks.

Boyd and Shelton (1984) investigated the anaerobic biodegradation of monochlorophenols and dichlorophenols in serum bottle reactors using fresh (unacclimated) digester sewage sludge and sludge acclimated to either 2-chlorophenol, 3-chlorophenol or 4-chlorophenol. Each monochlorophenol isomer was degraded in the unacclimated sludge reactors. Degradation of 50 mg/L of 2-chlorophenol occurred without a lag period, whereas 47 mg/L of 3-, and 4-chlorophenol required approximately 4 weeks before degradation

occurred. The authors concluded the rate of monochlorophenol degradation was dependent on the position of the chlorine isomer and increasing in the following order: ortho > meta > para.

In the unacclimated sludge, reductive dechlorination of dichlorophenol isomers resulted in initial removal of chlorine in the position ortho to the hydroxyl with the remaining chlorine removed later. Both 3,5- and 3,4-dichlorophenol were persistent during the 6 week incubation period. Sludge acclimated to 2-chlorophenol degraded 16 to 18 mg/L of 2- and 4-chlorophenol with no lag period in 3 to 4 days; 18 mg/L of 2,4-dichlorophenol but not 3-chlorophenol. Sludge acclimated to 3-chlorophenol degraded 15 to 18 mg/L of 3- and 4-chlorophenol with no lag period in less than 2 weeks, 15 to 17 mg/L of 3,4- and 3,5-dichlorophenol but not 2-chlorophenol. Sludge acclimated to 4-chlorophenol could degrade 17 to 20 mg/L of 2-, 3- and 4-chlorophenol in 32 days, and 17 to 18 mg/L of 2,4- and 3,4-dichlorophenol. Relative rates of degradation were as follows: 4-CP > 3-CP > 2-CP. The authors concluded that in activated sludge, adaptation by microorganisms for the degradation of chlorophenols was isomer specific.

Reductive dechlorination results obtained by Mikesell and Boyd (1986) indicated that pentachlorophenol (PCP) degradation occurred most rapidly in the 2-chlorophenol acclimated sludge; PCP was degraded within 3 days. PCP degradation in 3- and 4-chlorophenol acclimated sludge was considerably slower requiring 12 to 9 days, respectively, for complete disappearance. Mikesell and Boyd suggested that chlorine substituents ortho to the phenolic hydroxyl group are removed more rapidly than chlorine in the meta or para

positions. Phenol was not observed as a dechlorination product in the acclimated sludge or enrichments.

Hrudey et al., (1987a) investigated the biodegradability of 2-, 3-, and 4-chlorophenol in 50 mL batch cultures using fresh (unacclimated) digester sewage sludge as inoculum. Substrate concentration and methane production were monitored. Concentrations of 2-chlorophenol were dechlorinated to phenol, which was also degraded. During batch culture experiments, dechlorination rates varied from 2.0 mg/wk (5.7 mg/L per day) with a 2-chlorophenol concentration of 97 mg/L, to 0.5 mg/wk (1.4 mg/L per day) with a 2-chlorophenol concentration of 285 mg/L. Degradation of 3- and 4-chlorophenol was observed only after 16 to 20 weeks. After 32 weeks, initial concentrations of up to 100 mg/L 3- and 4-chlorophenol were dechlorinated to 30 mg/L.

In another study, Hrudey et. al., (1987b) observed the rate of dechlorination of the second chlorine atom from dichlorophenol substrates in 50 mL semicontinuous cultures with 50% unacclimated anaerobic digester sewage sludge. Substrate concentration and methane analyses were performed weekly for 131 days. Dechlorination of 12 mg/L of 2,6-dichlorophenol to 2-chlorophenol occurred at a rate of 1.2 mg/wk, with 2-chlorophenol subsequently degraded to phenol. Both 2-chlorophenol and phenol appeared in low concentrations but were quickly degraded. Dechlorination of 12 mg/L of 2,4-dichlorophenol to 4-chlorophenol occurred at a rate of 1.7 mg/wk. Approximately 173 mg/L of 4-chlorophenol accumulated in the serum bottle in 57 days before decreasing to constant concentration of 130 to 150 mg/L with no further degradation. Dechlorination of 12 mg/L of 2,3-dichlorophenol to 3-chlorophenol occurred at a rate of 0.7 mg/wk. Dur g a 36 day period approximately 68 mg/L of 3-

chlorophenol accumulated in the serum bottle before decreasing to a constant concentration of 60 to 70 mg/L with no further degradation. No phenol was observed during the dechlorination of either 3- or 4-chlorophenol. After 131 days, the authors found that 19 mg of 2,6-dichlorophenol had been degraded in comparison to 7.5 mg of 2,4-dichlorophenol and 3.4 mg of 2,3-dichlorophenol. In dichlorophenols, high rates of dechlorination at the ortho position resulted in increased concentrations of 3- and 4-chlorophenol. The authors noted that this accumulation coincided with the corresponding decrease in rate of dechlorination at the ortho position. The sequence of dechlorination for the second chlorine atom was ortho > para > meta.

Sharak Genthner et al., (1989) compared the anaerobic degradation of chloroaromatic compounds in the laboratory under a variety of enrichment conditions; methanogenic, nitrate, sulfate and bromoethane sulfonic acid (BESA) amended. Anaerobic sediments (freshwater and estuarine) and site water served as inoculum. Monochlorophenols were added to final concentrations of 3 g/L, 514 mg/L, 10 mg/L and 6 mg/L to the enrichments and sampled bimonthly for a period of approximately 2 years. Acclimation time for degradation of each chlorophenol isomer varied; 1 to 2 months for 2-chlorophenol, 3 to 4 months for 3-chlorophenol and 2 to 12 months for 4-chlorophenol. The criterion for degradation was a substantial decrease ($\geq$ 25%) in the substrate concentration. After laboratory transfer, degradative activity was highly maintained with 2-chlorophenol (11 of 12 enrichments), well maintained with 3-chlorophenol (7 of 11 enrichments) and poorly maintained with 4-chlorophenol (3 of 8 enrichments). Based on the acclimation times and degradability the authors concluded that in

anaerobic sediments 2-chlorophenol would be more readily degraded than 3-chlorophenol, which would degrade more readily than 4-chlorophenol.

Reductive dechlorination of pentachlorophenol (PCP) has been demonstrated in anaerobic soils. Investigations led to the conclusion that the chlorine atoms, located ortho and para to the hydroxyl group are utilized preferentially (Ide et al., 1972).

In a study by Haggblom and Young (1990), under sulfate-reducing conditions chlorine was preferentially removed from the ortho position of 2,4-dichlorophenol and 2,6-dichlorophenol by a biomass. The biomass originated from an anaerobic reactor used to treat pulp bleaching effluent. 2,6-Dichlorophenol was sequentially dechlorinated first to 2-chlorophenol and then to phenol. 2,4-Dichlorophenol was dechlorinated to 4-chlorophenol with no further degradation. Additional research using freshwater and saline water cultures, acclimated to monochlorophenols as substrate, under sulfate-reducing conditions produced the following results: 4-CP > 3-CP > 2-CP. 4-Chlorophenol was degraded approximately three times faster than 2-chlorophenol.

Haggblom and Young (1990) stated that these results were the reverse of those found for methanogenic cultures, namely that 2-chlorophenol was rapidly degraded while degradation of 4-chlorophenol was much slower.

While examining the dechlorination sequence of 2,4-dichlorophenol Zhang and Wiegel (1990) could not detect 2-chlorophenol. Even at high concentrations of 50 mg/L 2,4-dichlorophenol, 4-chlorophenol was accumulated, but no 2-chlorophenol was encountered. According to the authors, these results were indicative of a high specificity for the removal of the chlorine in the ortho position in comparison with the para position.

Madsen and Aamand (1992) examined dechlorination of trichlorophenol isomers by a polychlorophenol mixed culture enriched from digester sewage sludge. A 97 mg/L concentration of 2,4,6-trichlorophenol was partially dechlorinated to 22 mg/L of 2,4-dichlorophenol over a 14 day period. Approximately 2 to 8 mg/L of 2,4,5-trichlorophenol required 14 days before dechlorination to 3,4-dichlorophenol, which persisted throughout the study. Approximately 2 to 4 mg/L of 3,4,5-trichlorophenol was completely dechlorinated to 3,5-dichlorophenol in 2 days, while dechlorination of 8 mg/L of 3,4,5-trichlorophenol took 14 days. Results indicated that removal of chlorine in the ortho position was rapid when compared to chlorines in either meta or para position. They concluded that the positions of the substituents influenced the rate of dechlorination.

Haggblom et al., (1993) studied the influence of alternative electron acceptors on the biodegradability of chlorinated phenols and benzoic acids. Under methanogenic conditions 13 mg/L of 2-chlorophenol was degraded in approximately 30 days while 13 mg/L of 4-chlorophenol was degraded in approximately 60 days. Cultures were refed to the original concentration once substrates were completely depleted. Phenol was detected as an intermediate during degradation of 2-chlorophenol. In acclimated cultures, under methanogenic conditions 2-chlorophenol was metabolized at 4.11 $\pm$ 0.39 mg/L per day, 4-chlorophenol was metabolized at 2.70 $\pm$ 0.13 mg/L per day and 3-chlorophenol was metabolized at 6.81 $\pm$ 0.77 mg/L per day. The 3-CP > 2-CP > 4-CP results are in contrast to the preferential removal of the ortho position of chlorophenol but are in agreement with the preferential removal of the meta position of chlorobenzoates.

2.3.2.e Acclimation Effects on Isomer Degradation

Boyd and Shelton (1984) investigated the anaerobic biodegradation of mono and dichlorophenol isomers by fresh (unacclimated) digester sewage sludge and sludge acclimated to either 2-, 3-, or 4-chlorophenol in serum bottle reactors. Substrate concentration was monitored for 6 weeks.

In unacclimated sludge, each monochlorophenol isomer was degraded. 50 mg/L of 2-chlorophenol was completely degraded within 6 weeks, without a lag period. 46 mg/L of 3-chlorophenol was completely degraded within 6 weeks, with a lag period of 4 weeks. 46 mg/L of 4-chlorophenol was completeiy degraded after an initial lag period of 4 weeks.

In unacclimated sludge, only dichlorophenol isomers with a chlorine in the ortho position were reductively dechlorinated without a lag period. 2,5-dichlorophenol did require a one week acclimation period before degradation began. The two dichlorophenol isomers 3,5- and 3,4-dichlorophenol, were not degraded throughout the 6 week incubation period.

Acclimated sludge showed very different degradation patterns from the fresh (unacclimated) sludge. Serum bottles containing a single dichlorophenol as the sole source of carbon were inoculated with sludge acclimated to either 2-, 3- or 4-chlorophenol. Sludge acclimation to the monochlorophenols took place over a 4 month period.

Sludge acclimated to 2-chlorophenol degraded 16 to 18 mg/L of both 2-chlorophenol and 4-chlorophenol within 3 to 4 days, but was unable to degrade 3-chlorophenol within the 30 day incubation period. Only 19 mg/L of 2,4-dichlorophenol was degraded in the sludge acclimated to 2-chlorophenol, other

dichlorophenol isomers with chlorine ortho substituents (2,3-, 2,5- and 2,6-dichlorophenol) were not removed within the 30 day incubation period.

Sludge acclimated to 3-chlorophenol cross-acclimated to 4-chlorophenol and degraded both 3-chlorophenol (14 mg/L in 7 days) and 4-chlorophenol (18 mg/L in 14 days) with no lag period. Only dichlorophenol isomers containing meta chlorine substituents or meta and para substituents were degraded. 17 to 18 mg/L of 3,4- and 3,5-dichlorophenol were degraded in 14 days. 2-chlorophenol and 2,5-dichlorophenol were not degraded within the 30 day incubation period.

Sludge acclimated to 4-chlorophenol cross-acclimated to both 2- and 3-chlorophenol. 20 mg/L of 4-chlorophenol was degraded within 8 days, 17 mg/L of 3-chlorophenol within 12 days and 18 mg/L of 2-chlorophenol within 32 days. Both 2,4- and 3,4-dichlorophenol were also degraded. No other dichlorophenol isomers were tested.

Boyd and Shelton observed that sludge acclimated to both the 2- and 3-chlorophenol cross-acclimated to 4-chlorophenol. 4-Chlorophenol acclimated sludge could successfully degrade all three monochlorophenols. However only 2,4- and 3,4-dichlorophenol were the only dichlorophenol isomers consistently degraded by acclimated sludge. According to Boyd and Shelton these results indicate the ability to remove chlorines in the ortho position was lost with acclimation. Degradation of 2,4- and 3,4-dichlorophenol suggest that with acclimation, chlorines in the para position were preferentially removed.

From experimental evidence, Boyd and Shelton suggested the involvement of specific microbial process responsible for anaerobic chlorophenol degradation by acclimated sludge and another nonspecific process (responsible for the

removal of ortho chlorine in unacclimated sludge) which disappeared with acclimation. However, the nonspecific process may be dependent on alternate carbon sources, which were present in the fresh sludge. These sources, depleted during the 4 month acclimation period, may help to explain the inability of the 2-chlorophenol acclimated sludge to remove ortho chlorines from the dichlorophenol isomers.

Woods (1985) examined the pathways of anaerobic degradation of several dichlorophenols, trichlorophenols and pentachlorophenol in a continuous flow, upflow anaerobic sludge blanket reactor. Municipal sewage sludge was acclimated to chlorophenols for 3 months. A concentration of 1 mg/L of 2,3-dichlorophenol was dechlorinated to 3-chlorophenol after 2 hours. No 2-chlorophenol was detected. Dechlorination of ortho chlorines from 2,6-, and 2,4-dichlorophenol, 2,4,6-trichlorophenol, 2,3,4,5-tetrachlorophenol and pentachlorophenol was also detected. With acclimation, meta chlorines were removed from 3,5- and 2,3-dichlorophenol. However there was no evidence of further degradation of the monochlorophenols or chlorines present in the para position to the hydroxyl group. Woods noted that with longer acclimation periods, chlorines could also be removed from the para position during continuous treatment.

Hrudey et al., (1987a) examined the feasibility of sustaining anaerobic treatment of 2-chlorophenol at high concentrations with semi-continuous (draw and feed) cultures using unacclimated digester sludge as inoculum. For 42 days prior to beginning the draw and feed experiment, 2-chlorophenol was used as the sole source of substrate for the cultures. During the experiment, rapid

dechlorination and phenol degradation were sustained with 2-chlorophenol concentrations up to 100 mg/L for a period of approximately 43 days.

2.3.2.f Inhibition of Anaerobic Biodegradation

For natural microorganisms to utilize a chlorinated compound, the organism must have an array of enzymes that convert the target compound through a series of intermediates to a product. The product can then enter the general metabolic pool (Rothmel et al., 1991). For many chlorinated compounds the enzymes are extremely inefficient since they are designed to attack a structurally analogous non-chlorinated natural compound rather than a synthetic chlorinated compound as a substrate. As a result the chlorinated compound is degraded rather slowly, which explains the relative persistence of many synthetic chlorinated compounds in nature (Rothmel et al., 1991).

Inhibition of anaerobic biodegradation processes have been attributed to several different factors. These include the position of chlorine atoms in the benzene ring (Boyd et al., 1983; Guthrie et al., 1984), interference of sulfate or other oxidized sulfur species on dehalogenation reactions (Gibson and Suflita, 1986), cessation of microbial metabolism due to toxic concentrations of fermentable substrates (Fedorak and Hrudey, 1984) and selective substrate metabolism due to competitive substrate inhibition (Folsom et al., 1990).

Preliminary studies by Gibson and Suflita (1986) on reductive dehalogenation of aromatic compounds in four anoxic habitats found sulfate inhibition of microbially catalyzed aryl dehalogenation reactions. In a sulfate-reducing environment the authors reported a phenol substrate disappearance of

99%, as compared with the substrate disappearances of 2- and 4-chlorophenol of 20% and 26% respectively.

Hrudey et al., (1987a) also investigated the inhibition of phenol degradation by monochlorophenols in 50 mL batch cultures with fresh anaerobic digester sewage sludge as inoculum. Concentrations of substrate and methane production were monitored for 46 weeks. For 97 mg/L of 2-chlorophenol, the addition of 86 mg/L phenol decreased the rate of 2-chlorophenol degradation, from 2.0 mg/wk (without added phenol) to 0.4 mg/wk (with added phenol). For 285 mg/L of 2-chlorophenol, the rate decreased from 0.5 mg/wk (without added phenol) to 0.1 mg/wk (with added phenol). Even at higher concentrations (285 mg/L), 2-chlorophenol was not toxic to methanogenesis and had degraded 145 mg/L within the 46 week incubation period. Phenol degradation was not inhibited by 2-chlorophenol concentrations less than 97 mg/L and was completely degraded in 7 weeks. Addition of 3- and 4-chlorophenol, at concentrations greater than 30 mg/L, inhibited the degradation of 84 mg/L phenol. Lag times for phenol degradation increased from less than 1 week (4-chlorophenol concentrations from 3 to 10 mg/L) to 4 weeks (256 mg/L of 4-chlorophenol). With 3-chlorophenol, phenol degradation lag times increased from 1 week (3-chlorophenol concentrations less than 10 mg/L) to greater than 33 weeks (282 mg/L of 3-chlorophenol). In comparison, 3-chlorophenol was somewhat more resistant to phenol degradation than 4-chlorophenol; 0% after 33 weeks of incubation versus 60% after the same time period, respectively.

Sharak Genthner et al., (1989) compared the anaerobic degradation of chloroaromatic compounds in the laboratory under a variety of enrichment conditions; methanogenic, nitrate, sulfate and bromoethane sulfonic acid (BESA)

amended. Degradative activity was monitored bi-monthly in enrichments containing anaerobic freshwater and estuarine sediments, and site water as inocula with chlorophenol concentrations ranging from 3 g/L to 6 mg/L. The authors found that acclimation to para-chlorinated compounds was most variable, from 2 to 12 months. Also, among chlorophenols, 4-chlorophenol degradation was observed least often (8 out of 17 enrichments).

Madsen and Aamand (1992) have proposed a general relationship between the positions of chlorine atoms in the aromatic ring and toxicity, with antibacterial activities increasing from ortho via meta to para. Toxicity of trichlorophenols by a polychlorophenol-transforming mixed culture enriched from digester sewage sludge was examined. Toxicity and compound inhibition was evaluated by production of volatile fatty acid and methane formation. Results indicated that 197 mg/L of 2,4,6-trichlorophenol was found to be the least inhibitory. At concentrations of 79 mg/L 2,4,5-trichlorophenol reduced the production of volatile fatty acids by 50% while 79 mg/L of 3,4,5-trichlorophenol inhibited acid production by greater than 60% relative to the control cultures.

Inhibition of 4-chlorophenol to degradation has also been reported in sewage sludge (Gibson and Suflita, 1986) and in the treatment of pulp bleaching effluents (Haggblom and Young, 1990).

Under certain conditions, bacteria from environments receiving several structurally related chemicals may metabolize substrates selectively due to competitive substrate inhibition. Competitive substrate inhibition occurs when one enzyme involved in multiple steps of a degradative pathway acts only on the parent compound until its concentration falls below a threshold level (Rochkind-Dubinsky et al., 1987).

Woods (1985) found that during continuous treatment of a suite of chlorophenols, chlorines were not removed from the para position to the hydroxyl during the 7 months of experimental treatment.

In comparison, Boyd and Shelton (1984) examined dechlorination of 3,4-, 2,4-dichlorophenol and 4-chlorophenol in batch experiments and found that chlorines were removed from the para position.

In a study by Armenante et al., (1992), serum bottles spiked with 2 mg/L 2,4,6-trichlorophenol were dechlorinated to 2,4-dichlorophenol, which was, in turn reduced to 4-chlorophenol. Small amounts of phenol were also produced. However, with continued spikings of 2,4,6-trichlorophenol, the anaerobic culture was unable to degrade 4-chlorophenol any further and the concentration of 4-chlorophenol increased within the serum bottles.

2.4 Solid Support Media

The advantages of using a solid support media for the attachment of microbes over a system of suspended microbes in an aqueous environment has been well documented. Solid support systems should encourage the adsorption of microbes to the surface of the support material and allow biofilm development and maintenance. Allowing unhindered biofilm development is important in anaerobic processes in order to retain the slow growing microorganisms and provide maximum bioactivity (Kindzierski, 1989). A highly irregular surface composed of pits and crevices in addition to a well-developed internal structure large enough to accommodate microorganisms, would provide the ideal material for protection of a developing biofilm while still providing the microbes easy access to food and nutrients.

Bryers (1987) reviewed the superiorities of attached growth systems over free suspended systems.

The efficiency of immobilized yeast cells compared to suspended yeast cells was studied by Doran and Bailey (1986). The immobilized cells consumed glucose twice as fast as compared to suspended yeast cells, requiring less expenditure of energy in obtaining food and nutrients in addition to reducing their specific growth rate by 45%.

Wilkie and Colleran (1984) and Wilkie et al. (1985) examined the effects of different support materials; plastic rings, coal, mussel shells and fired clay fragments in four anaerobic reactors to treat pig slurry supernatant. Using percent chemical oxygen demand (%COD) removal as a measure of the biological activity in each reactor, those reactors containing material with higher surface area (plastic rings, coal and mussel shells) performed poorly when compared to the reactor containing the material with the lowest total surface area (fired clay fragments) during the initial start-up period. The reactor containing the fired clay fragments may have developed active attached-biomass faster than the other reactors due to improved initial adsorption and attachment of bacteria (Kindzierski, 1989).

Kennedy and Droste (1985) researched the use of various support materials in the startup of an anaerobic reactor to treat bean blanching wastewater. They found that support materials with roughened surfaces such as needle-punched polyester and fired clay allowed better biofilm development than PVC or glass supported materials. The authors mentioned that bacteria, especially the methanogens, had difficulties adhering to the smooth surface of the PVC or glass.

Hrudey et al., (1987b) examined the use of activated carbon in the anaerobic degradation of 2,3-dichlorophenol. A series of 50 mL semi-continuous cultures, enriched by 50% diluted unacclimated digester sludge, were monitored for substrate concentration and methane production over a 33 week period. Cultures with 1000 mg/L of activated carbon removed 1 to 3 mg/L of 2,3-dichlorophenol continuously for 13 weeks (with and without a 2-chlorophenol supplement) at a maximum rate of 1.5 mg/wk. In comparison, 5 to 12 mg/L of 2,3-dichlorophenol was dechlorinated in an experiment without activated carbon at a rate of 0.7 mg/wk (same study). Cultures of 300 mg/L activated carbon stopped dechlorination of 2,3-dichlorophenol after the 2-chlorophenol supplements were discontinued. The intermediate 3-chlorophenol was removed at a rate of 1.5 mg/wk in cultures containing 300 mg/L activated carbon. There was also removal of 3-chlorophenol by cultures containing 1000 mg/L activated carbon, however no rate was given.

Krumme and Boyd (1988) found little methanogenic and dechlorinating activity to be associated with glass beads that were designed for attachment of the biomass in three upflow bioreactors containing sludge acclimated to monochlorophenols. The glass beads were subsequently removed from within the bioreactors without loss of biological and dechlorinating activity.

Selle Sardi et al., (1986) studied the adhesion and growth of anaerobic biofilms on ion exchange resins. The authors found that anaerobic biofilm development was favorable towards strong cation exchange resins. Preference was thought to occur due to two properties of the resin; divalent cation binding which may aid in the adsorption and firm attachment of bacteria to stable supports, and the adsorption of volatile organic acids to the resin surface.

Salkinoja-Salonen et al., (1983) examined the anaerobic biodegradation of seven chlorophenolic compounds in a fixed film reactor. Removal of pentachlorophenol (PCP) in the reactor was efficient when the PCP degrading biomass was attached to a solid support. PCP biodegradation was most active with softwood bark, less active on unglazed pottery and inactive on glass (beads or fibre), cellulosic fibre, clay and ion exchange resin (styrene divinyl benzene).

Kindzierski (1989) assessed the importance of activated carbon and non-adsorptive synthetic ion exchange resin (styrene divinyl benzene) as biological supports in serum bottle reactors. The three support materials studied were Filtrasorb® 300 activated carbon, Ambergard™ XE-352 anion exchange resin and Dowex™ MSC-1 cation exchange resin. Little or no bioactivity was associated with cultures attached to the MSC-1. Cultures attached to the XE-352 resin exhibited the highest phenol removal rates on a reactor liquid volume, support material mass and estimated spherical surface area basis using a concentration approximately 3 times smaller than the concentration of activated carbon. Cultures attached to activated carbon exhibited the highest phenol removal rates on a support material pore volume and surface area basis. After 88 days of incubation the biofilm growth of the XE-352 resin was still increasing. On the activated carbon, biofilm growth had reached full capacity and did not increase after day 75 of incubation. Kindzierski thought that this difference may be due to the fact that the resin had more available pore volume and surface area per bottle for biomass growth than the activated carbon. When comparing the bioactivity of 76% supernatant cultures, the activated carbon cultures removed phenol at a much higher rate (150 $\pm$ 39 mg/L per day) as compared to the resin cultures (64 $\pm$ 15 mg/L per day). Kindzierski attributed this difference

to detachment of the biomass, as it expanded outwards from the activated carbon surface, by liquid shearing and inter-particle abrasion. The XE-352 resin was not subject to the same phenomena as biomass growth had not reached full capacity at the time the assay was terminated.

3.0 Experimental Procedures

3.1 Anaerobic Culture Methods

A series of batch reactors were set up to study the capacity of microbial cultures to degrade 2- or 4-chlorophenol under anaerobic conditions. The primary objective of the study was to produce a series of microbial cultures acclimated to specific quantities of chlorophenols. The cultures were classified as either attached growth or suspended growth depending upon the presence of a solid support media within the reactor. The biological activity of the cultures was observed by monitoring the disappearance of 2- or 4-chlorophenol and the periodic qualification of methane in the headspace of the reactors.

3.1.1 Serum Bottle Procedure

3.1.1.1 Setting up the Reactors

Batch reactors were set up in clean, sterilized, 158 mL serum bottles (Wheaton Scientific). To produce an environment free of oxygen each individual bottle was sparged with oxygen free gas (Section 3.1.6) for approximately 3 to 5 minutes before filling. Volumetric pipets or plastic syringes fitted with disposable needles (Becton-Dickenson; B-D) were used to add resin, mineral media growth solution, inoculum and substrate to the reactors. Before transfer of media or resin, the pipet or syringe was flushed several times with oxygen free gas to minimize the potential for oxygen contamination. Aliquots of the media growth solution were dispensed through a volumetric pipet. To prevent any free oxygen from entering the system, the volumetric pipet was attached to a three-way pipet bulb connected to a line of oxygen free gas. Aliquots of the media growth

solution and sludge inocula were added to the reactor while continually sparging the serum bottle headspace. Once the desired volume was reached, cut black butyl stoppers (Wheaton Scientific) were loosely fitted on top of the serum bottles while continuously sparging the headspace with oxygen free gas for approximately 2 to 3 minutes. Serum bottles were closed with the stoppers, crimped with tear-away aluminum caps (Wheaton Scientific) and stored in the dark at 37°C.

After the serum bottles were closed, additional media growth solution and substrate was injected or removed using the syringe and needle method.

3.1.1.1.1 **Attached Growth Reactors (Series A)**

Attached growth batch reactors, with biomass attachment support media Ambergard™, were prepared according to the procedure described in section 3.1.1.1. Approximately 500 mg (dry weight) of wet Ambergard™ XE-352 anion exchange resin and 48.5 mL of sterile mineral media growth solution were added into each serum bottle. During sparging, reactors received approximately 1 mL of substrate to bring the concentration in each bottle to approximately 60 mg/L of either 2- or 4-chlorophenol. One serum bottle reactor did not receive any chlorophenol substrate and served as a control for the study. The sparger was removed, and the bottles were stoppered and capped. To ensure a reducing environment, 0.5 mL of sodium thiosulphate (1% v/v) was added to each bottle using a plastic disposable syringe and needle pre-rinsed several times with oxygen free gas. Serum bottles were left to equilibrate in the dark at 37 °C.

After 24 hours, the bottles were opened. While continuously sparging with oxygen free gas, a 25 mL liquid aliquot of reactor supernatant was withdrawn

and replaced with 25 mL of sludge inoculum. Sparging continued for 3 to 5 minutes after the exchange. Each attached growth serum bottle reactor contained approximately 500 mg of dry resin (added as wet) and a total of 50 mL liquid. Bottles were then stoppered, capped and stored in the dark at 37°C to equilibrate.

After 24 hours, concentrations of 2- and 4-chlorophenol substrate within the reactor supernatant were determined by gas chromatography (Section 4.1.1). To maintain a 2- or 4-chlorophenol substrate concentration of 30 mg/L, additional substrate was added up to this level in all serum bottles except the control. Repeat analyses for chlorophenol substrate concentration were performed after approximately 14 days. Substrate was added up to the predetermined concentration. Subsequent supernatant removal (70% to 88%) after acclimation was used to encourage biomass attachment to the support media (resin).

3.1.1.1.2 **Suspended Growth Reactors (Series B)**

Suspended growth reactors were prepared according to the procedure described in section 3.1.1.1 (without anion exchange resin) with the supernatant from series A. The corresponding attached growth parent reactor containing solid support media (series A) was opened and sparged with oxygen free gas. Approximately 70% (35 mL) of the supernatant was removed by plastic syringe equipped with disposable tip and replaced with 35 mL of fresh, reduced mineral media solution (Table 3.1). The supernatant was immediately injected into a corresponding empty serum bottle (series B). Sparging of the reactor bottle

headspace continued for 2 to 3 minutes. The attached growth parent reactor was then stoppered and capped.

While continually sparging the headspace of the suspended growth reactor (series B), 15 mL of mineral media was added to the reactor by graduated pipet that had been thoroughly flushed with oxygen free gas. The reactor was continually sparged with the gas for 2 to 3 minutes, then stoppered and capped.

All reactors were fed 50 mg/L of either 2- or 4-chlorophenol substrate then stored in the dark at 37°C.

3.1.1.1.3 Supernatant Only Reactors (Series E, F and G)

Supernatant only reactors were prepared according to the procedure described in section 3.1.1.2 (without anion exchange resin or inoculum) with the supernatant from series A. These reactors were set up in each phase of the semi-continuous study. Their purpose was to provide a control and a comparison of chlorophenol degradation by the same suspended microbes present in the supernatant of the attached growth reactors (series A).

3.1.1.2 Analyses of Headspace Gas for Methane

A sample of headspace gas was obtained by direct injection using a gas tight syringe (Hamilton #1801). The syringe was used to puncture the cut black butyl stopper and remove 3 microlitres (μL) of headspace gas. The gas sample was then directly injected into a gas chromatograph (GC).

Between injections, the syringe was rinsed several times with deionized, distilled water.

3.1.1.3 Extraction of Supernatant for Analyses

Serum bottles were inverted and left to settle into two distinct layers, resin (if present) and sludge at the base topped by the clear supernatant. A Hamilton syringe (Microlitre #701) was used to puncture the cut black butyl stopper and push through the sludge/resin layer to the clear supernatant. The syringe plunger was pulled back to extract 3 microlitres (μL) of supernatant, then removed from the serum bottle. The amount of liquid was discarded and the needle tip wiped clean. The entire procedure was repeated twice. After the second removal, 1.5 μL of supernatant was discarded, leaving a 1.5 μL sample remaining in the syringe. The sample was then injected into the Gas Chromatograph (GC).

The syringe was rinsed several times with deionized, distilled water between each sample injection.

3.1.1.4 Substrate Addition

Samples of reactor supernatant were analyzed for either 2-chlorophenol or 4-chlorophenol concentration as described in section 4.1.1. Volume amounts of substrate were calculated to bring reactor supernatant to the required concentration. To maintain a total reactor volume of 50 mL, a volume of supernatant equal to the volume of substrate required, was withdrawn by needle and syringe from each reactor. The required volume of chlorophenol substrate was added into the reactor bottles by direct aqueous injection.

3.1.1.5 Exchanging Caps on Reactors

The cut black butyl stoppers (Wheaton Scientific) and tear-away aluminum caps (Wheaton Scientific) were exchanged bi-weekly, between periods of supernatant removal.

Old aluminum caps were torn off and used stoppers removed from the serum bottles. Immediately after stopper removal, the reactor headspace was sparged with oxygen free gas while a new cut black butyl stopper was placed loosely on top of the serum bottle. Sparging continued for 2 to 3 minutes. Upon removing the sparger, the serum bottle reactors were quickly stoppered and capped.

3.1.1.6 Supernatant Exchange

A specific volume of supernatant was removed from a reactor containing an attached growth culture, replaced with an equal amount of mineral media solution, then placed into a 158 mL serum bottle reactor to start a new culture. These new cultures were classified as either suspended growth or supernatant only, depending upon reactor contents.

The attached growth reactor containing solid support media was opened and sparged with oxygen free gas. Various amounts of the supernatant was removed by plastic syringe equipped with disposable tip and replaced with an equal volume of fresh, reduced mineral media solution (Table 3.1). The supernatant was immediately ejected into a labeled empty serum bottle. Sparging of the attached growth reactor bottle headspace continued for 2 to 3 minutes. The reactor was then stoppered and capped.

While continually sparging the headspace of the corresponding serum bottle reactor, mineral media and/or inoculum sludge was added to the reactor by graduated pipet that had been thoroughly flushed with oxygen free gas. The reactor was continually sparged with oxygen free gas for 2 to 3 minutes, then stoppered and capped.

All reactors were fed a specific concentration of either 2- or 4-chlorophenol substrate then stored in the dark at 37°C.

3.1.1.7 Draw and Feed

The draw and feed procedure involved drawing of the supernatant for initial analysis of substrate concentration by GC and injections of an equal volume with required substrate. Based on a stock substrate solution of 2500 mg/L, the volume of 2- or 4-chlorophenol substrate required to maintain a reactor concentration of 50 mg/L was added into the mineral media injected into the reactor.

To withdraw the supernatant, a 5 mL aliquot of supernatant was removed by plastic syringe fitted with a disposable needle, once the biological solids had settled. This was replaced with 5 mL of freshly prepared and reduced mineral media growth solution (Section 3.1.3) minus the calculated volume of chlorophenol substrate required, to maintain a total exchanged volume of 5 mL. The calculated volume of either 2-chlorophenol or 4-chlorophenol substrate was then added to the reactor. All aqueous extractions and additions were performed by direct injection into closed and capped serum bottle reactors. Plastic syringes and disposable needles were rinsed thoroughly with an oxygen free gas mixture before injection (Section 3.1.1).

3.1.2 Sludge Inoculum

The sludge used as a source of inoculum for all attached growth and suspended growth reactors in this study was obtained from Parachlorophenol Series 6 sludge bank established on May 29, 1990. Sludge was originally collected from the Goldbar Wastewater Treatment Plant digester then acclimated to 4-chlorophenol (4-CP). All sludge was stored in an incubator kept at a constant temperature of 37°C and fed with 4-chlorophenol and mineral media growth solution once or twice per month.

3.1.3 Anaerobic Mineral Media Growth Solution

Anaerobic mineral media growth solution was prepared in advance, sterilized and kept refrigerated for approximately 2 weeks from date of preparation. Preparation of the mineral media solution from stock solutions is listed in Table 3.1. Chemical components of the stock solutions are listed in Table 3.2. Stock solutions were prepared in advance and stored in white plastic 1 L nalgene bottles.

TABLE 3.1 PREPARATION OF ANAEROBIC GROWTH MEDIA

Solution	Concentration (per 100 mL)
Deionized, distilled water	100 mL
Mineral Solution 1	1.0 mL
Mineral Solution 2	0.1 mL
Vitamin B Solution	0.1 mL
Phosphate Solution	1.0 mL
Resazurin Solution	1.0 mL
Sodium Bicarbonate	0.57 g

(Kindzierski, 1989)

TABLE 3.2 PREPARATION OF STOCK SOLUTIONS IN THE ANAEROBIC GROWTH MEDIA

Solution	Chemical	Concentration in Distilled Water (g/L)
Mineral 1	NaCl	50
	$CaCl_2\ 2H_2O$	10
	NH_4Cl	50
	$MgCl\ 6H_2O$	10
Mineral 2	$(NH_4)_6Mo_7O_{24}\ 4H_2O$	10
	$ZnSO_4\ 7H_2O$	0.1
	H_3BO_3	0.3
	$FeCl_2\ 4H_2O$	1.5
	$CoCl_2\ 6H_2O$	10
	$MnCl_2\ 4H_2O$	0.03
	$NiCl_2\ 6H_2O$	0.03
	$AlK(SO_4)_2\ 12H_2O$	0.1
Vitamin B	Nicotinic Acid	0.1
	Cyanocobalamine	0.1
	Thiamine	0.05
	p-Aminobenzoic Acid	0.05
	Pyridoxine	0.25
	Pantothenic Acid	0.025
Phosphate	KH_2PO_4	50
Resazurin		0.1

(Fedorak and Hrudey, 1984)

To prepare the media, all solutions listed in Table 3.1 (except the sodium bicarbonate) were added together in an erlenmeyer flask and boiled for approximately 2 minutes to drive off dissolved oxygen. Immediately after removal from the heat source, the hot solution was sparged with oxygen free gas. Sparging continued until the solution cooled to room temperature. Sodium bicarbonate was then slowly added to the cooled solution to bring the pH into the range of 6.9 to 7.1.

Once the proper pH was reached, the solution was dispensed into clean 158 mL serum bottles (Wheaton Scientific) pretreated with oxygen free gas. Sparging continued for 2 to 3 minutes. The bottles were stoppered, capped, autoclaved for 20 minutes at 121°C, cooled to room temperature and stored in a refrigerator.

Prior to use the anaerobic media solution was reduced. A solution of 2.5% w/v sodium thiosulphate was added by disposable syringe pre-flushed with oxygen free gas (1 mL sodium thiosulphate per 100 mL of media solution). The reduced anaerobic media solution was colourless. Presence of free oxygen within the bottles was indicated by resazurin (Table 3.1), a redox indicator, which turns pink in the presence of atmospheric oxygen.

Reduced media solution was kept refrigerated for 2 weeks and then discarded.

3.1.4 Substrate Preparation

Exactly 0.2500 g of 4-chlorophenol (Fisher Scientific, Fairlawn, N.J.) was weighed into a clean, sterilized, 158 mL serum bottle (Wheaton Scientific) on a covered balance. While sparging the bottle headspace with oxygen free gas,

100 mL of freshly boiled, deoxygenated water was added to the serum bottle. After sparging for 2 to 3 minutes the bottle was stoppered, capped, autoclaved at 121°C for 20 minutes, cooled to room temperature and refrigerated.

Exactly 0.2 mL of 2-chlorophenol (Fisher Scientific, Fairlawn, N.J.) was ejected from a volumetric pipet into a clean, sterilized, 158 mL serum bottle. The remaining preparation of 2-chlorophenol substrate was accomplished using the same procedures and equipment as 4-chlorophenol.

Preparation of 2500 mg/L solutions of 2,4-dichlorophenol (Aldrich Chemical Company, Inc., Milwaukee) and 3,4-dichlorophenol (Aldrich Chemical Company, Inc., Milwaukee) were accomplished using the same procedures and equipment as 4-chlorophenol.

3.1.5 Standard Preparation

2-Chlorophenol (Fisher Scientific, Fairlawn, N.J.) and 4-chlorophenol (Fisher Scientific, Fairlawn, N.J.) standards were prepared by dilution of a standard 1000 mg/L stock solution with distilled, deionized water.

Exactly 0.2500 g of 4-chlorophenol was weighed into a clean glass beaker on a covered balance then deposited into a clean, dry 250 mL volumetric flask. A 0.20 mL volume of 2-chlorophenol was transferred by graduated pipet into the same volumetric flask. Distilled, deionized water was added to the flask and made up to the proper volume. The appropriate volume of stock solution was dispensed by pipet into 50 mL volumetric flasks then diluted with distilled, deionized water to the proper volume (Table 3.3).

Standards were transferred into clean, labeled, 60 mL serum bottles (Wheaton Scientific), stoppered with grey butyl stoppers (Wheaton Scientific), capped by tear-away aluminum caps (Wheaton Scientific) and refrigerated.

Additional 100 mg/L, 50 mg/L and 10 mg/L standards of phenol (BDH Chemicals, England), 3,4-dichlorophenol (Aldrich Chemical, WS) and 3-chlorophenol (Eastman Kodiak Company, NY) were prepared by dilution of individual 100 mg/L stock solutions with distilled, deionized water.

Stock solutions were prepared by weighing exactly 0.0050 g of either phenol, 3,4-dichlorophenol or 3-chlorophenol into clean, dry, individual beakers on covered balances. The chemicals were transferred into 50 mL volumetric flasks and filled to volume with distilled, deionized water. Aliquots of 5 mL and 25 mL were taken from the stock solution, placed into 50 mL volumetric flasks and diluted to volume. Contents of volumetric flasks were transferred to labeled 60 mL serum bottles, stoppered, capped and refrigerated.

A 50 mg/L standard of 2,4-dichlorophenol (Aldrich Chemical, WS) was prepared by dilution of a 2 mL aliquot from a 2500 mg/L stock solution (substrate) into a 50 mL volumetric flask. Contents of the volumetric flask were transferred into a labeled 60 mL serum bottle, stoppered, capped and refrigerated.

A 10% and a 30% methane standard was prepared by dispensing 16 mL and 48 mL of methane gas into empty, clean, capped 158 mL serum bottles, respectively. A gas tight syringe (Hamilton# 1801) was used to inject the standard into the GC (Section 3.1.1.2).

TABLE 3.3 2-CHLOROPHENOL AND 4-CHLOROPHENOL STANDARDS

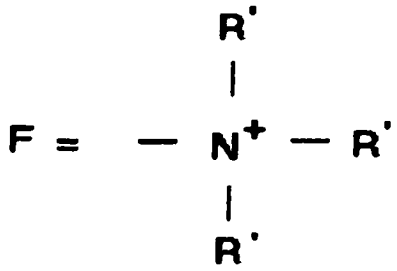
Standard (mg/L)	Volume of Stock Solution Required (mL)
10	0.5
25	1.25
50	2.5
75	3.75
100	5.0
150	7.5
200	10.0
250	12.5
300	15.0
350	17.5
400	20.0
450	22.5
500	25.0

3.1.6 Oxygen Free Gas

The oxygen free gas mixture consisted of 30% CO_2 in N_2. To remove any free oxygen the gas was run through a heated glass column containing copper shavings (Hungate, 1969). A line attached to the glass column was split into three sections. Two sections were connected to metal spargers. The third line was connected to a 158 mL serum bottle equipped with a butyl rubber cap. Flushing of needles and syringes was done by direct injection through the rubber cap.

3.1.7 Biological Solid Support Media

Ambergard™ XE-352 anion exchange resin (Rohm and Haas Co.) was used as the solid support media for attachment of biological growth. The resin is comprised of styrene-divinyl benzene (DVB) polymers, which are a combination of aromatic (benzene) and aliphatic (-CH-CH_2-) structures. XE-352 resin has a "strongly basic" Type 1 quaternary ammonium ionic group (Figure 3.1) as the dominant functional group on its surface (Kindzierski, 1989). The average size of resin particles ranges from 300 to 850 micrometre (μm). XE-352 resin pore volume and surface area characteristics are summarized in Table 3.4.



R• = Methyl (-CH_3) or other hydrocarbon group

FIGURE 3.1 Chemistry of Functional Group with Ion Exchange Sites for Ambergard™ XE-352 Synthetic Resins (Kindzierski, 1989).

TABLE 3.4 PORE VOLUME AND SURFACE AREA CHARACTERISTICS OF THE AMBERGARD™ XE-352 RESIN FROM MERCURY POROSIMETRY

Characteristic	XE-352 Resin
Available Cumulative Pore Volume with Mean Pore Diameters ≥ 15 μm (mL/g)	1.220
Available Cumulative Surface Area with Mean Pore Diameters ≥ 15 μm (m^2/g)	0.227
Total Cumulative Surface Area with Mean Pore Diameters ≥ 0.008 μm (m^2/g)	67.1
Estimated Spherical Surface Area (m^2/g)	0.0085
% Area Available For Bacteria	0.336

Adapted from Kindzierski (1989)

3.1.7.1 Preparation of Solid Support Media

Ambergard™ XE-352 (Cl^-) anion exchange resin (10 g/L) was used in each attached growth reactor as a solid support media for biomass attachment.

3.1.7.2 Resin Conditioning

Resin was subject to a two step conditioning process to load with Cl^- before usage as a solid support media. In step one, the resin was placed in a glass column which was screened at the lower end. Approximately 750 mL of a 10% hydrochloric acid regenerator was passed through the column. During step two, the resin was placed in a sintered glass filter funnel and hooked up to a vacuum apparatus. Approximately 4.5 L of bacteria free wash water was then passed through the resin. The resin was allowed to dry thoroughly before use. Resin conditioning was done by Sara Ebert, Department of Microbiology, University of Alberta, Edmonton, Alberta.

3.1.7.3 Determination of Moisture Content

To maintain a total reactor volume of 50 mL, the percentage of moisture required by the dry resin was calculated. Approximately 0.5037 g of resin (dry weight) was combined with 50 mL of non-sterile mineral media growth solution in a 158 mL serum bottle. The bottle was capped with a cut black butyl stopper, crimped by a tear-away aluminum cap and allowed to equilibrate in the dark at 37°C.

After 24 hours, the bottle was opened and approximately 48.5 mL of media solution was withdrawn by disposable syringe into a graduated cylinder. Moisture content of the resin was calculated as follows:

$$\text{Moisture Content (\%)} = \frac{50\ \text{mL} - 48.5\ \text{mL}}{50\ \text{mL}} = 3\ \%$$

To compensate for the amount of media adsorbed by the dry resin, approximately 1.5 mL of additional mineral media growth solution was added to reactors during initial set-up.

4.0 Analytical Methods

4.1 Analytical Determination by Gas Chromatography

4.1.1 Substrate Analysis

The concentration of 2- and 4-chlorophenol present in the batch reactors was measured by direct aqueous injection of reactor supernatant into a Gas Chromatograph (GC).

4.1.1.1 GC Conditions

The Hewlett-Packard Model 5730A Gas Chromatograph (GC) contained a 0.91 m x 2 mm ID glass column with a 1% SP1000 60/80 Carbopak B (Supelco) solid phase. The GC was equipped with a flame ionization detector (FID) and attached to a Hewlett-Packard Integrator Model 3390A. Nitrogen was used as a carrier gas at 35 mL/min. Hydrogen and air were used as the flame ionization detector gases at 35 mL/min and 240 mL/min respectively. The oven, injector and detector temperatures were set and held isothermal at 220°C, 250°C, and 250°C respectively.

4.1.1.2 Calibration

The system was calibrated using standard solutions of known concentrations (Section 3.1.5). Substrates and intermediates were identified by comparison with the retention times of the known standards. Peak areas generated from serum bottle reactor samples were compared against peak areas generated from substrate standards.

4.1.1.3 Quality Control

Quality control was maintained by producing a new calibration curve every second day (during continuous analyses) or before every analytical determination of substrate concentration (APHA, 1992). Retention times were carefully monitored during the calibration and substrate analysis. All injections were done in sets of 2 or 3 to maintain an acceptable error of determination of 5%.

4.1.2 Methane Gas Analyses

In the serum bottle reactors, methane gas was produced from mineralization of chlorophenol substrate by anaerobic microbes. Methane presence was qualitatively confirmed after stopper exchange and complete headspace flushing with oxygen free gas. Analyses were performed by extracting headspace gas from the culture bottle and injecting directly into a GC.

4.1.2.1 GC Conditions

A Hewlett-Packard Model 5730A gas chromatograph (GC) equipped with a flame ionization detector (FID) was used. The sample of headspace gas was injected onto a 0.91 m x 2 mm ID glass column containing 1% SP1000 60/80 Carbopak B (Supelco). Integration of peak areas was performed by a Hewlett-Packard Model 3390A integrator which was attached to the GC. Nitrogen was used as the carrier gas at 35 mL/min. Hydrogen and air were used as the flame ionization detector gases at 35 mL/min and 240 mL/min respectively. Temperatures were set and held isothermal at 25°C for the oven and 100°C for

the detector. The temperature control on the injector port was not turned on and operated at room temperature.

4.1.2.2 Calibration

The system was calibrated using methane standard solutions of known concentrations (Section 3.1.5). Methane gas was identified by comparison with the retention times of the known standards. The resulting peak areas were related to the injections of the headspace gas from the reactors.

4.2 Scanning Electron Microscope Photography

Scanning electron microscope (SEM) photography was used to examine the solid support media for bacteria morphology and quantity. The SEM procedure consisted of collecting samples of resin from active cultures, preparing the sample for viewing, followed by sample examination and photographing areas of interest.

A disposable needle attached to a plastic syringe was inserted into the inverted serum bottle reactor and a 2 mL sample of sludge removed. The sample, containing at least 5 granules of resin, was deposited into a solution of 2.5% glutaraldehyde in Millonig's buffer to fixate the biological growth onto the resin. The fixation process preserves the bacterial cell structure and protects against any changes that may occur during the sample preparation procedure. After 24 hours the sample was washed three times with Millonig's buffer for 10 minutes each to remove all traces of sludge. The resin sample was then postfixed with 1% Osmium Tetroxide (OsO_4) in Millonig's buffer. After one hour the sample was washed with distilled water then dehydrated in ethanol. The

dehydration began with immersion of the sample in a 50% ethanol mixture for 10 minutes followed by 70% and 90% ethanol mixtures for the same time period. Final sample dehydration took place in absolute ethanol for two, 10 minute intervals each. After dehydration the resin sample was critical point dried in a See Vac Inc. Critical Point Dryer (Florida, U.S.) with liquid CO_2, at 40°C for 10 to 15 minutes, then mounted on aluminum stubs using a fine layer of adhesive and dried overnight in a vacuum desiccator. Once dry the resin sample was sputter-coated with gold (75 to 150 nm thickness) in an Edwards Sputter Coater (Model S150B), then mounted in a Hitachi S-2500 Scanning Electron Microscope (SEM) and examined for presence and morphology of bacteria. Areas of interest on the resin samples were photographed.

Dr. M. Chen of the Medicine/Dentistry E.M. Unit, Surgical-Medical Research Institute, University of Alberta, Edmonton, Alberta developed all photographs, assisted with sample preparation, examination, photography and provided all materials or solutions needed for sample preparation.

4.3 Total Solids Determination

Each reactor was shaken well to thoroughly mix the solids and supernatant. A disposable needle attached to a plastic syringe was inserted into the reactor and a 5 mL sample extracted. The syringe was previously well flushed by an oxygen free gas mixture. Total solids were then determined by standard methods (APHA 1992, section 2540B).

4.4 Intermediate and By-Product Analyses

4.4.1 Solvent Extraction

Solvent extraction procedure was modified from the Method 625 - Base/Neutrals and Acids (USEPA, 1984) by S. Kenefick, Health Services Administration and Community Medicine, University of Alberta, Edmonton, Alberta to accommodate the limited amount of sample available.

4.4.1.1 Reactor Sampling and Preparation

A 5 mL sample of supernatant was removed by injection of a plastic syringe with disposable tip through the stopper of the serum bottle reactor. Before injection, both syringe and tip were thoroughly flushed with a mixture of oxygen free gas. The 5 mL sample was filtered through a 0.45 micrometer (μm) filter (Millipore HA) and diluted to 10 mL with deionized, distilled water.

4.4.1.2 Base/Neutral Extraction

The diluted sample was brought to pH 10 by the addition of a few drops of sodium hydroxide, pH was verified with litmus paper. Approximately 1.3 mL of double distilled methylene chloride (CH_2Cl_2) was added to the sample. This mixture was then shaken for 2 minutes with periodic venting to release built up pressure. The mixture was allowed to stand for 10 minutes to facilitate the separation of two separate phases, methylene chloride and water. Approximately 1 mL of methylene chloride was removed from the bottom of the centrifuge tube and collected into a labeled 3 mL vial. The procedure was repeated three times using 0.5 mL of CH_2Cl_2 for the second and third extraction. A total volume of 2 mL of CH_2Cl_2 was collected in the vial. Before sealing the

4.4.2.1 Operating Conditions

A 0.2 microlitre (μL) sample of each extract was analyzed using a Hewlett Packard 5890 Gas Chromatograph (GC), coupled with an HP 5970 mass-selective detector and HP 59940 Chemstation® data system. The HP5-MS column was 30 m x 0.25 mm ID with a 0.25 micrometer (μm) film thickness. The GC injection port temperature was 225°C and detector temperature 250°C. Column head pressure was 70 kPa. The oven was temperature programmed at 100°C for 2 min., 5°C per minute to 200°C, with the final temperature held for 3 minutes to obtain the best identification possible.

A computer library search was used to match the mass spectra of interest with those stored in the data library. GC/MS work was performed by S. Kenefick and T. Perley, Health Services Administration and Community Medicine, University of Alberta, Edmonton, Alberta.

4.4.3 Qualitative Analysis for Amino Acids

Exactly 0.2063 g of ninhydrin (1,2,3-indanetrione monohydrate) was weighed into a clean, dry, glass beaker under a covered balance. The ninhydrin was transferred to a 50 mL volumetric flask and made up to volume with distilled, deionized water. A 1 mL aliquot of this solution was placed into a clean, dry, test tube.

A 2 mL sample of reactor supernatant was removed by injection of a plastic syringe with disposable tip through the stopper of the serum bottle reactor. Before injection, both syringe and tip were thoroughly flushed with a mixture of oxygen free gas. The supernatant sample was filtered through a 0.45 μm filter (Millipore HA) into the test tube containing the ninhydrin solution. The test tube

mixture was placed into a hot water bath and heated to boiling for 15 to 30 seconds. The color of the solution was noted. A blue to blue-violet color given by the presence of amino acids constitutes a positive test, while all other colors are negative.

4.4.4 Derivatization and Extraction Procedure

Chlorinated phenols and their by-products were determined as their acetate derivatives by GC/MS using a modification of the method described by Coutts et al., (1980). The method was modified by S. Kenefick, Health Services Administration and Community Medicine, University of Alberta, Edmonton, Alberta to accommodate the limited amount of sample available. The analysis was used as a qualitative procedure only, no internal standards were prepared or added.

4.4.4.1 Sampling and Preparation

A 10 mL sample was removed by injection of a plastic syringe with disposable tip through the stopper of the serum bottle. Before injection, both syringe and tip were thoroughly flushed with a mixture of oxygen free gas. The 10 mL sample was filtered through a 0.45 micrometer (μm) filter (Millipore HA) into a separatory funnel.

4.4.4.2 Derivatization and Extraction

The sample in the separatory funnel was combined with 1.000 g of $NaHCO_3$, preweighed under a covered balance, and 0.5 mL of acetic anhydrite, added by glass syringe (Hamilton microlitre #750). This mixture was then

shaken with periodic venting every 5 seconds to release built-up pressure. Shaking continued until all bubbling ceased and no more CO_2 gas was evolving. Approximately 4 mL of methylene chloride was added to the separatory funnel. The mixture was then shaken for 2 minutes, with periodic venting to release built up pressure and allowed to stand for a couple of minutes to facilitate the separation of two separate phases, methylene chloride and water. Approximately 4 mL of methylene chloride was removed from the bottom of the separatory funnel and collected into a clean, dry erlenmeyer flask. The addition of methylene chloride, shaking and separation procedure was repeated three times with approximately 12 mL of sample extract collected in the flask. To remove any water, the extract was passed through a Pasteur pipet containing approximately 1 g of Na_2SO_4 immobilized between glass wool plugs, then deposited into a Kuderna Danish evaporator tube (KD tube). Concentration of the sample extract was done by passing N_2 gas over the KD tube and evaporating the sample slowly to a volume of 0.2 mL. The concentrated sample was immediately transferred to a clean, labeled 1 mL vial and capped.

4.4.4.3 Gas Chromatography/Mass Spectroscopy

Gas Chromatography/mass spectroscopy (GC/MS) was used to identify the derivatized chlorophenols and any intermediate or by-product compounds produced during microbial degradation of the chlorophenol substrate.

4.4.4.3.1 Operating Conditions

A 0.2 microlitre (μL) sample of each extract was analyzed using a Hewlett Packard 5890 Gas Chromatograph (GC), coupled with an HP 5970 mass-

selective detector and HP 59940 Chemstation® data system. The GC conditions were: 100°C for 2 min., 5°C/min to 200°C, final temperature hold for 3 min. The HP5-MS column was 30 m x 0.25 mm ID and 0.25 micrometer (μm) film thickness. Column head pressure was 70 kPa; injector temperature was 225 °C and detector temperature 250°C.

A computer library search was used to match the mass spectra of interest with those stored in the data library. GC/MS work was performed by S. Kenefick, Health Services Administration and Community Medicine, University of Alberta, Edmonton, Alberta.

4.4.5 Statistical Analysis of Data

The degradation rate was calculated by obtaining the slope of a line produced by a linear regression through selected points. Only points that occupied positions along the exponential portion of the curve were selected. The line was then plotted on a graph of time versus chlorophenol concentration.

The average (arithmetic mean) influent and effluent values were calculated through statistical analysis of influent and effluent data. Data points outside of two standard deviations (95.44% Probability) were excluded and the averages recalculated before plotted as lines on graphs of time versus chlorophenol concentration.

Regression analyses and statistical descriptions are located in Appendix V.

5.0 Semicontinuous Anaerobic Microbial Degradation of 2-Chlorophenol and 4-Chlorophenol

Batch reactors with attached growth (series A and C), suspended growth (series B) were established and acclimated to specific isomers of chlorophenol (ortho or para). Supernatant was withdrawn to encourage biomass attachment in series A. Five series (designated series B to C and series E, F and G) of microbial cultures were set-up using supernatant removed from attached growth reactors in series A. Biological activity of the cultures was observed by monitoring the disappearance of 2- or 4-chlorophenol and the periodic analysis for the presence of methane.

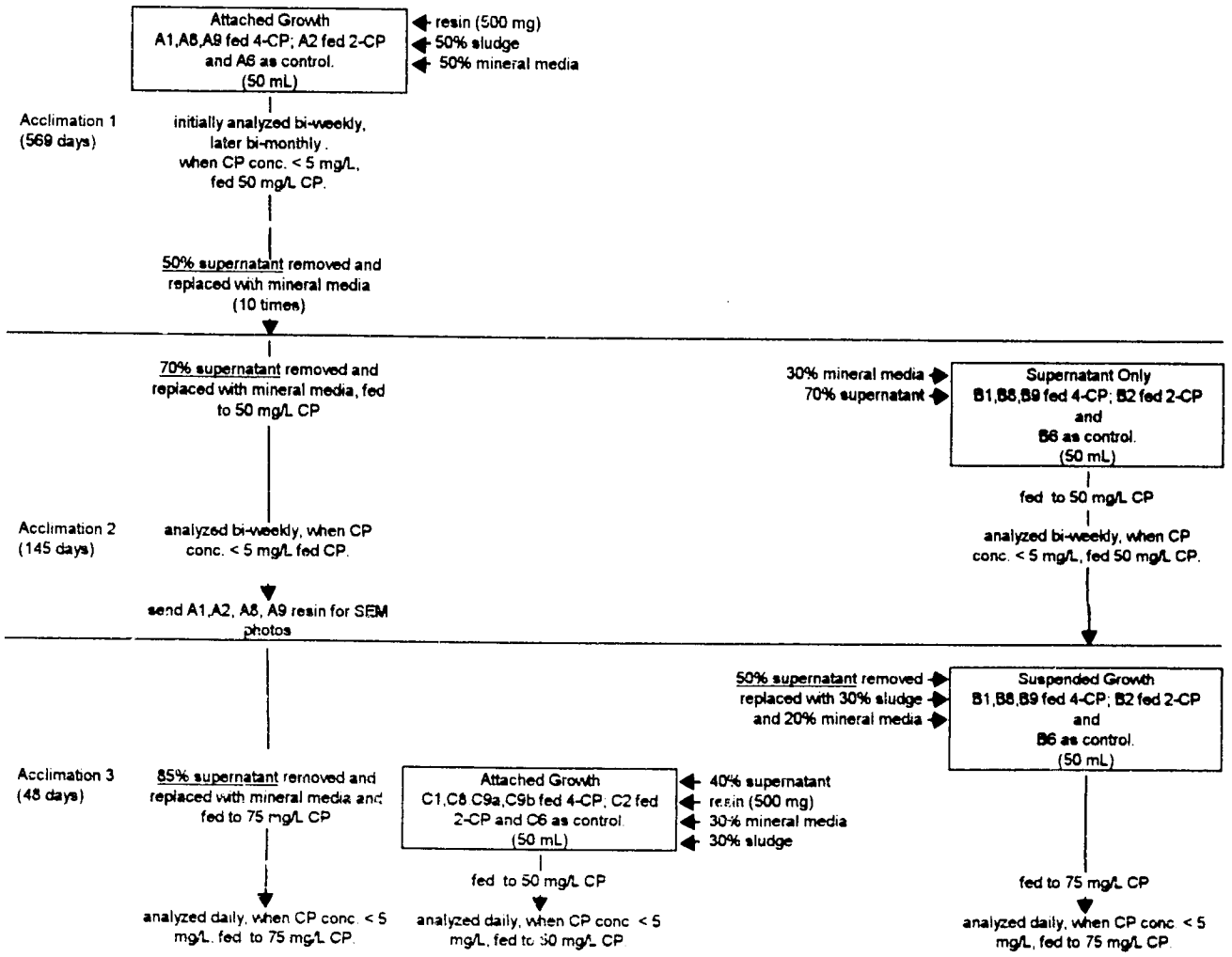
The semicontinuous (draw and feed) experimental technique (Fedorak, 1984) simulates continuous study in small volume reactors and permits the evaluation of a specific microbiological system under a controlled set of organic loading conditions such as concentration and hydraulic retention time (HRT).

5.1 Acclimation Study

The acclimation study was conducted in three phases during which a specific type of microbial (attached or suspended) growth was encouraged.

5.1.1 Procedures

Reactor set-up and the acclimation study was conducted according to the experimental summary in Figure 5.1.



note: % = % of v/v of original liquid volume in reactor (50 mL)

CP= Chlorophenol, 2-CP= 2-Chlorophenol, 4-CP= 4-Chlorophenol

FIGURE 5.1 Flow Diagram of the Acclimation Study.

5.1.1.1 Acclimation 1 (day 1 to 570)

Series A: Attached growth reactors were set-up and the supernatant periodically removed to promote microbe attachment onto a solid support media and colonization (Figure 5.1). Reactors labeled A1, A8 and A9 initially received approximately 30 mg/L of 4-chlorophenol and reactor A2 received approximately 30 mg/L of 2-chlorophenol. Reactor A6 did not receive any substrate and served as a control for the study.

Repeat GC analyses for chlorophenol concentration were initially performed approximately every 2 weeks and later performed bi-monthly. Once the concentration had declined to less than 5 mg/L, reactors were refed with either 2-chlorophenol or 4-chlorophenol up to maximum concentrations of 50 mg/L.

On 10 occasions, when chlorophenol concentrations declined to less than 5 mg/L, 50% (25 mL) of the supernatant was removed from all A series reactors, replaced with 25 mL of mineral media solution and fed up to maximum concentrations of 50 mg/L. Chlorophenol analysis, refeeding and supernatant exchange continued for approximately 81 weeks (569 days).

5.1.1.2 Acclimation 2 (day 570 to 715)

Series B: Supernatant only reactors were set up from series A supernatant (Figure 5.1). Approximately 70% (35 mL) of supernatant removed from each of the attached growth reactors (series A) was used to set up new comparable supernatant only reactors (designated series B) and supplemented by 30% (15 mL) fresh anaerobic mineral media. The supernatant only reactors were fed the same substrate as the A series, attached growth parent reactors. Four of these

reactors (B1, B2, B8 and B9) were acclimated to metabolize 50 mg/L 2-chlorophenol or 4-chlorophenol as their sole substrate source. One reactor (B6) was used as a control.

Approximately every 2 weeks reactor supernatant was analyzed for chlorophenol concentration by GC. When concentrations decreased to less than 5 mg/L, attached growth reactors (series A) received 75 mg/L either 2-chlorophenol or 4-chlorophenol while supernatant only reactors (series B) received a maximum of 50 mg/L of either 2-chlorophenol or 4-chlorophenol substrate.

Analysis for substrate and refeeding continued for approximately 21 weeks (145 days).

On day 715, samples of resin contained in attached growth reactors A1, A2, A8 and A9 were taken for SEM photographs.

5.1.1.3 **Acclimation 3 (day 715 to 763)**

Series C: Attached growth reactors were set up from series A supernatant (Figure 5.1), to provide an additional set of attached growth reactors in case of reactor failure. Reactors contained solid support media for microbe colonization.

A plastic syringe equipped with disposable tip removed 85% (42.5 mL) of the supernatant from the series A attached growth reactor. A 20 mL aliquot of the supernatant was ejected into an open reactor bottle labeled series C, containing 500 mg of dry resin (wetted). An additional syringe previously loaded with 42.5 mL of fresh, reduced anaerobic mineral media growth solution (Table 3.1) was ejected into the series A attached growth reactor. Sparging of the

reactors continued for 2 to 3 minutes. Attached growth reactors, series A, were then stoppered and capped.

While continually sparging the headspace of the series C attached growth reactor, 15 mL of sludge inoculum and 10 mL of reduced mineral media growth solution were added. Reactors were continually sparged with oxygen free gas for 2 to 3 minutes, stoppered and capped.

Attached growth reactors (C1, C2, C8, C9a and C9b) were acclimated to metabolize 50 mg/L of either 2-chlorophenol or 4-chlorophenol, the same substrate as their parent attached growth reactors (series A). One reactor (C6) was not provided with any substrate and served as a control. Reactors were then stored in the dark at 37°C .

Series B: Approximately 50% (25 mL) of supernatant from the series B reactors was exchanged for 10 mL of reduced mineral media growth solution and 15 mL of sludge inoculum. Supernatant only reactors (series B) then became suspended growth reactors. Reactors were fed 75 mg/L of either 2- or 4-chlorophenol substrate. Reactors were then stored in the dark at 37°C.

Series A: Attached growth reactors (series A) were fed 75 mg/L of either 2- or 4-chlorophenol substrate. Reactors were then stored in the dark at 37°C.

Approximately every two weeks, all reactors were analyzed for chlorophenol concentration by GC and refed when the concentration of the substrate within the supernatant decreased to less than 5 mg/L. Reactors were analyzed for chlorophenol concentration and refed over a period of approximately 7 weeks (48 days).

Table 5.1 provides a summary of the chlorophenol feeding in the acclimation study. Table 5.2 provides a summary of the components in each reactor.

TABLE 5.1 ACCLIMATION STUDY SUMMARY

Acclimation	Reactors	Culture	No. of Days From	No. of Days To	No. of Days Total	CP Isomer	Conc. (mg/L)
1	A2	A	1	569	569	2-CP	50
	A1,A8,A9	A				4-CP	50
	A6	A				-	-
2	A2	A	570	714	145	2-CP	75
	A1,A8,A9	A				4-CP	75
	A6	A				-	-
	B2	O				2-CP	50
	B1,B8,B9	O				4-CP	50
	B6	O				-	-
3	A2	A	715	763	48	2-CP	75
	A8,A9,C8	A				4-CP	75
	A6	A				-	-
	B2	S				2-CP	75
	B8,B9	S				4-CP	75
	B6	S				-	-
	C2	A				2-CP	50
	C1,C8,C9A, C9B	A				4-CP	50
	C6	A				-	-

Culture; A - Attached Growth, S - Suspended Growth, O - Supernatant Only.
CP - Chlorophenol; 2-CP, 2-Chlorophenol; 4-CP, 4-Chlorophenol.

TABLE 5.2 BATCH REACTOR COMPOSITION

Reactor	Series		
Composition	**A**	**B**	**C**
Solid Support Media (mg)	500	None	500
Supern ·t (mL)	None	25*	20*
Mineral Media Growth Solution (mL)	25	10	15
Sludge Inoculum (mL)	25	15	15
Total Liquid Volume in Reactor (mL)	50	50	50

* Supernatant for reactors B, and C originated from reactors in series A removed at different times.

5.1.2 Results and Discussion

The purpose of the acclimation study was to set-up serum bottle reactors containing attached growth and suspended growth bacterial cultures adapted to either 2-chlorophenol or 4-chlorophenol as their sole source of substrate.

Acclimation to 2- or 4-chlorophenol substrate was accomplished through a three stage procedure (Figure 5.1 - p. 72). A long initial stage with slow degradation occurred after the first feeding, followed by an stage of increased degradation after the second feeding. The third and final stage showed that less time was required for substrate degradation with the third feeding. Performance during phase 3 for reactors A2, A8, B2 and B8 is presented in Figures 5.5 to 5.8 inclusive. With more frequent feedings the cultures exhibited a marked slowdown in the rate of degradation. During the incubation period of 20 to 50 days, the number of feedings increases with a corresponding decrease in the rate of chlorophenol substrate degradation.

Results from this study indicate that reactors with attached growth cultures acclimate to changes in substrate concentration easier than reactors with suspended growth cultures. The presence of a solid support media, such as resin, for the attachment of bacteria cultures enabled acclimation to either 2- or 4-chlorophenol to proceed over a shorter period of time than was required for acclimation of suspended growth cultures to the same substrate.

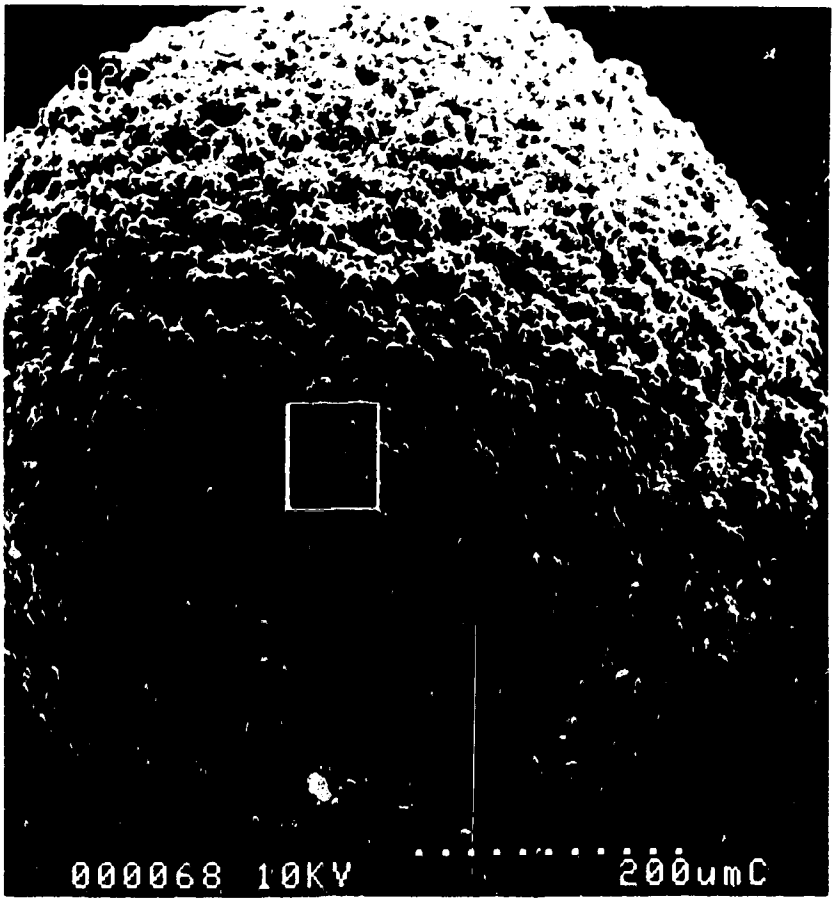


FIGURE 5.2 A Scanning Electron Microscope Photograph of a Granule of Ambergard™ XE-352 Anion Exchange Resin Used as Solid Support in Attached Growth Reactors.

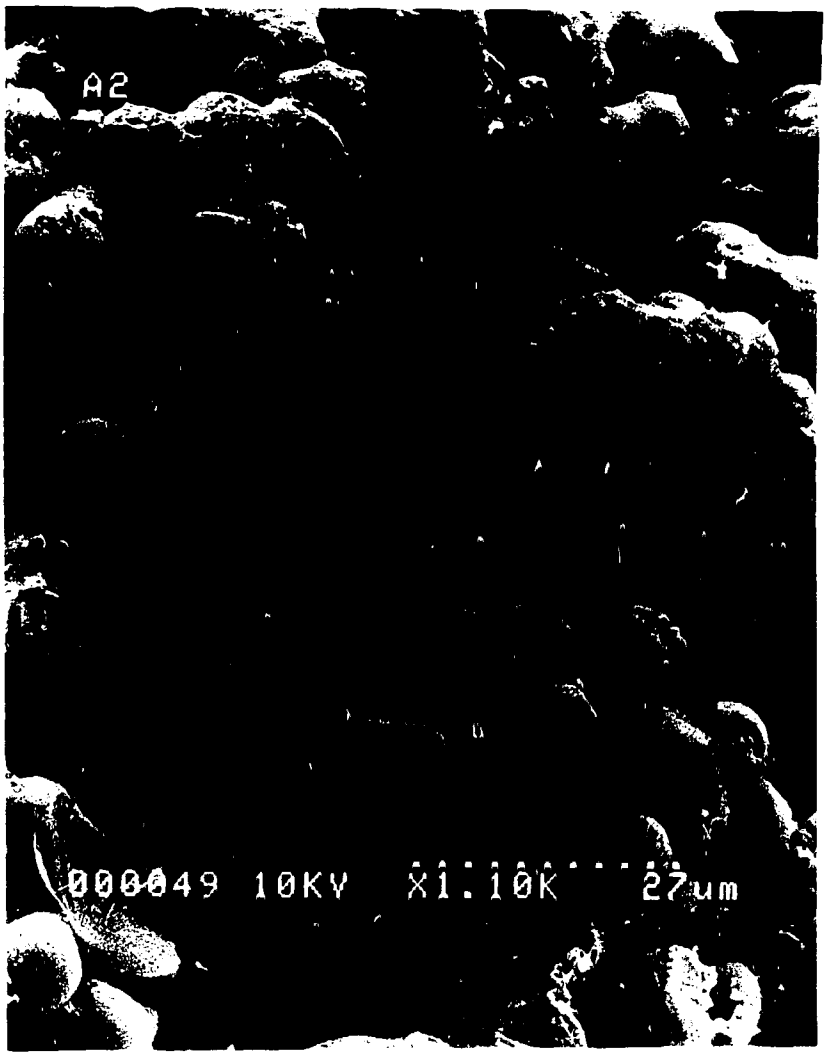


FIGURE 5.3 A Scanning Electron Microscope Photograph of Bacterial Colonization of the Resin Surface in Attached Growth Reactor A2 - Acclimated to 2-Chlorophenol.

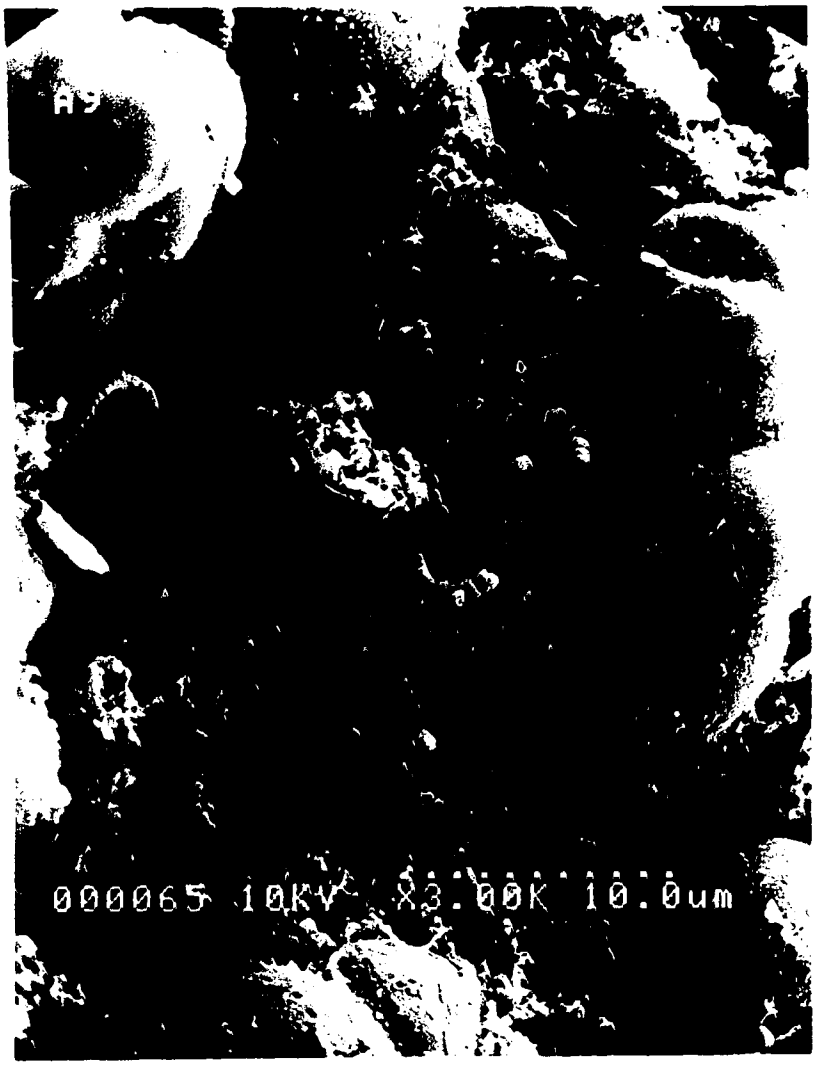


FIGURE 5.4 A Scanning Electron Microscope Photograph of Bacterial Colonization of the Resin Surface in Attached Growth Reactor A9 - Acclimated to 4-Chlorophenol.

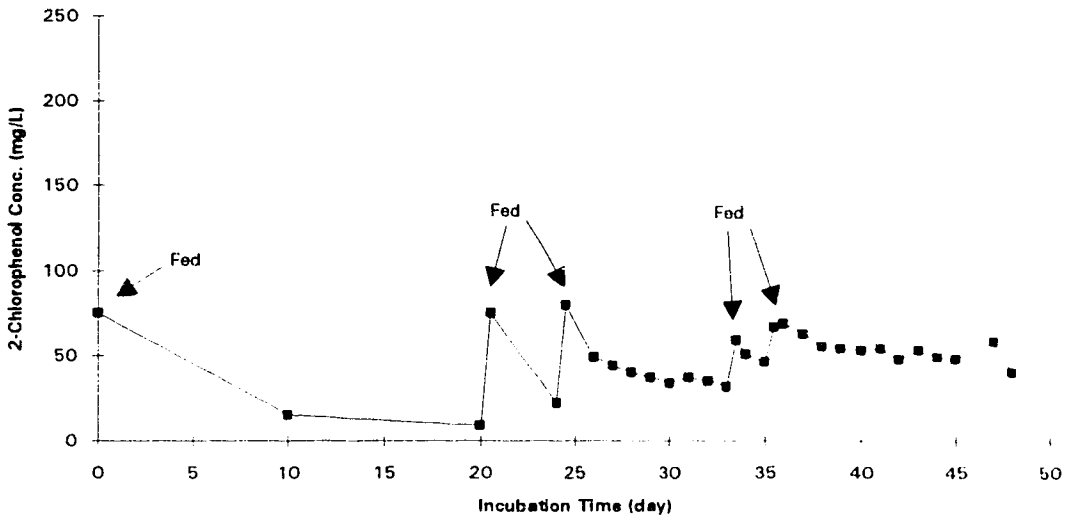


FIGURE 5.5 2-Chlorophenol Degradation in Attached Growth Reactor A2 - Acclimation 3.

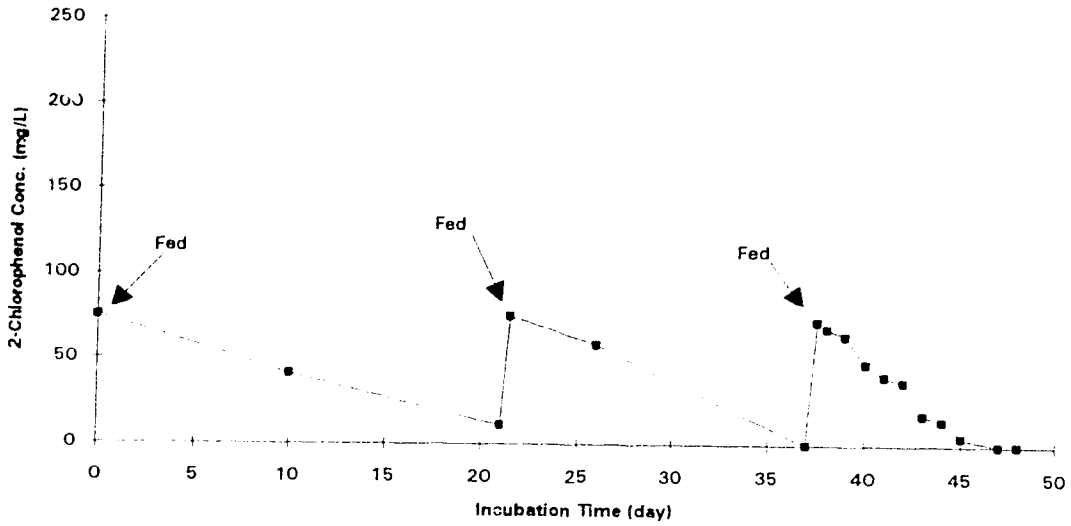


FIGURE 5.6 2-Chlorophenol Degradation in Suspended Growth Reactor B2 - Acclimation 3.

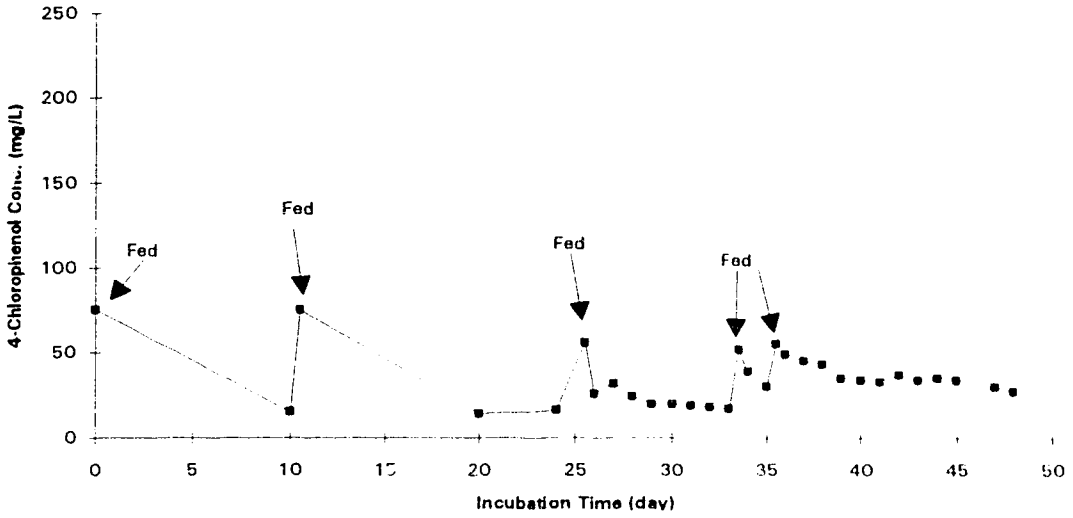


FIGURE 5.7 4-Chlorophenol Degradation in Attached Growth Reactor A8 - Acclimation 3.

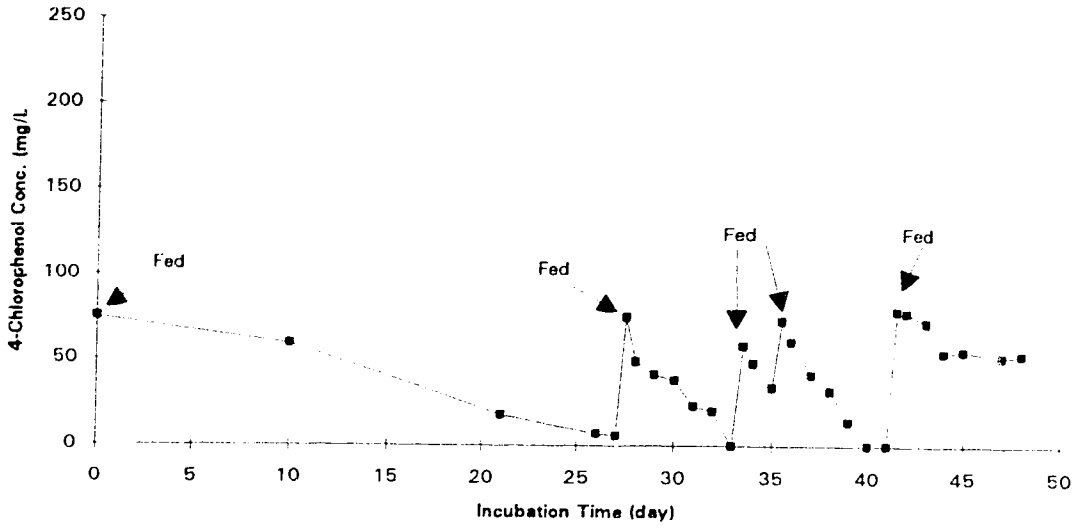


FIGURE 5.8 4-Chlorophenol Degradation in Suspended Growth Reactor B8 - Acclimation 3.

5.2 Semicontinuous Fermentation (Draw and Feed) Study

Anaerobic serum bottle reactors established during the acclimation study were subject to draw and feed conditions of 50 mg/L chlorophenol substrate and a 10 day hydraulic retention time (HRT). Under these conditions the reactors were monitored for removal of either 2-chlorophenol or 4-chlorophenol and methane production. Reactors containing supernatant only were established during supernatant exchange to provide a comparison with the attached growth and suspended growth reactors.

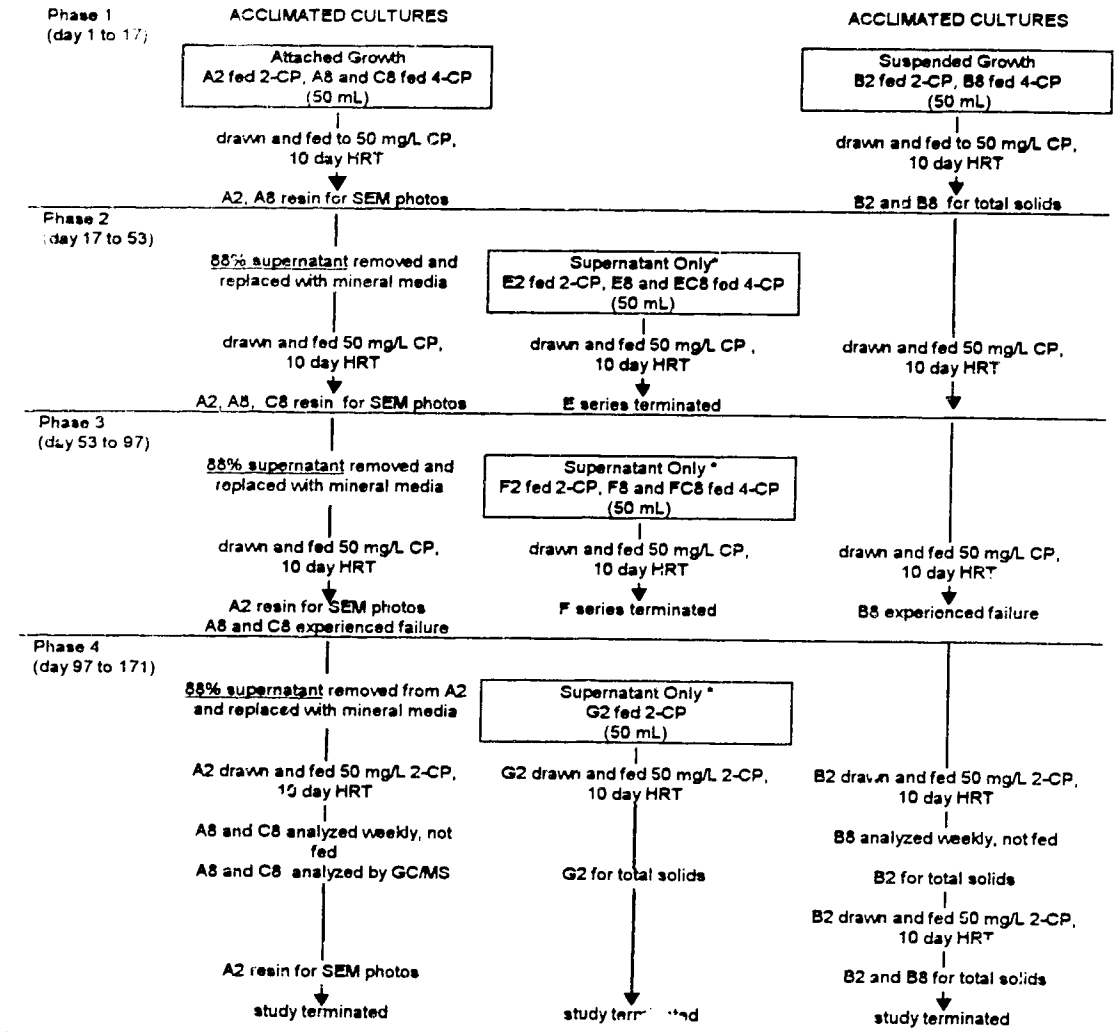
5.2.1 Procedures

The semicontinuous fermentation (draw and feed) experiment was conducted according to the experimental summary in Figure 5.9 and Table 5.3. Cultures were fed semicontinuously for 171 days before the experiment was terminated.

5.2.1.1 Phase 1 (day 1 to 17)

Series A and B: From day 1 to 16, reactors were drawn and fed daily in order to maintain a steady substrate concentration of 50 mg/L 2- or 4-chlorophenol in reactors and a HRT of 10 days.

On day 17, samples of resin contained in attached growth reactors A2 and A8 were taken for SEM photographs. Also on day 17, reactors containing suspended cultures (B2 and B8) were sampled and sent for a total solids determination.



Note: % = % of v/v of original liquid volume in reactor (50 mL).

CP= Chlorophenol; 2-CP= 2-Chlorophenol, 4-CP= 4-Chlorophenols

* Supernatant Only Reactors Series E, F, and G reactors were set up with 44 mL of supernatant removed from Series A reactors and 6 mL of fresh mineral media to constitute a total volume of 50 mL.

FIGURE 5.9 Flow Diagram of the Semicontinuous Fermentation (Draw and Feed) Study.

TABLE 5.3 SUMMARY OF THE DRAW AND FEED EXPERIMENT

Phase	Reactor	Culture	No. of Days From	To	Total	CP Isomer	Conc. (mg/L)	HRT (day)
1	A2	A	1	16	16	2-CP	50	10
	A8,C8	A				4-CP	50	10
	B2	S				2-CP	50	10
	B8	S				4-CP	50	10
2	A2	A	17	52	36	2-CP	50	10
	A8,C8	A				4-CP	50	10
	B2	S				2-CP	50	10
	B8	S				4-CP	50	10
	E2	O				2-CP	50	10
	E8,EC8	O				4-CP	50	10
3	A2	A	53	96	44	2-CP	50	10
	A8,C8	A				4-CP	50	10
	B2	S				2-CP	50	10
	B8	S				4-CP	50	10
	F2	O				2-CP	50	10
	F8,FC8	O				4-CP	50	10
4	A2	A	97	171	75	2-CP	50	10
	B2	S				4-CP	50	10
	G2	O				2-CP	50	10

Culture; A - Attached Growth, S - Suspended Growth, O - Supernatant Only.
CP - Chlorophenol; 2-CP, 2-Chlorophenol; 4-CP, 4-Chlorophenol.
HRT - Hydraulic Retention Time

5.2.1.2 **Phase 2 (day 17 to 53)**

Series E: Supernatant only reactors, designated series E, were set-up on day 17 from attached growth reactors, (Figure 5.9) according to the supernatant exchange procedure described in section 3.1.1.6. Approximately 88% (44 mL) of supernatant was removed from attached growth reactors (A2, A8 and C8) and used to set up comparable supernatant only reactors (E2, E8, and EC8). To provide a total reactor volume of 50 mL, 6 mL of freshly reduced mineral media solution growth was also added. Attached growth and suspended growth reactors were fed 50 mg/L of either 2- or 4-chlorophenol. Supernatant only reactors received 50 mg/L of the same substrate as their corresponding attached growth reactor.

From day 17 to day 52, all reactors were maintained at a substrate concentration of 50 mg/L total 2- or 4-chlorophenol concentration and a HRT of 10 days. The reactors were drawn and fed daily. Phase 2 continued for approximately 36 days.

On day 53, samples of resin from attached growth reactors A2, A8 and C8 were taken for SEM photographs. The use of the supernatant only reactors (E2, E8 and EC8) was also terminated on day 53.

5.2.1.3 **Phase 3 (day 53 to 97)**

Series F: On day 53, three supernatant only reactors, designated series F, were set-up from three attached growth reactors (Figure 5.9). Approximately 88% (44 mL) of supernatant was removed from attached growth reactors (A2, A8 and C8) and used to set up comparable supernatant only reactors (F2, F8 and FC8). To provide a total reactor volume of 50 mL, 6 mL of freshly reduced

mineral media solution growth was also added. No sludge inoculum or solid support media was placed into the serum reactor bottles.

These supernatant only reactors received the same substrate as their corresponding attached growth reactor. Reactors A2 and F2 were fed 50 mg/L 2-chlorophenol. The remaining 4 reactors (A8, F8, C8 and FC8) were fed 50 mg/L 4-chlorophenol.

Series B: Two suspended growth reactors (B2 and B8) did not exchange any supernatant for mineral media growth solution but were drawn and fed accordingly.

From day 53 to 96, reactors were drawn daily and fed upon demand to maintain a chlorophenol substrate concentration of 50 mg/L and a HRT of 10 days. Phase 3 continued for approximately 44 days.

Headspace gas was analyzed by GC for the presence of methane within each reactor. Methane presence, after stopper and anaerobic headspace gas exchange, indicates a methanogenic type of degradation.

On day 97, samples of resin from two attached growth reactors, A2 and A6, were taken and sent for SEM photographs. Draw and feed experiments with supernatant only reactors (F2, F8 and FC8) were also terminated on day 97.

5.2.1.4 **Phase 4 (day 97 to 171)**

Series G: On day 97, one supernatant only reactor designated series G, was set-up from an attached growth reactor (Figure 5.9). Approximately 88% (44 mL) of supernatant was removed from attached growth reactor A2, and used to set up a comparable supernatant only reactor G2. To provide a total reactor volume of 50 mL, 6 mL of freshly reduced mineral media solution growth was

also added. No sludge inoculum or solid support media was placed into the serum reactor bottle. Reactors A2 and G2 were drawn and fed 50 mg/L 2-chlorophenol.

Series A, B and C: Other attached growth reactors (A8 and C8) and suspended growth reactors (B2 and B8) did not participate in any exchange of supernatant.

From day 98 to 171, three reactors fed 2-chlorophenol (A2, B2 and G2) were drawn daily and fed to maintain a substrate concentration of 50 mg/L and a 10 day HRT. Weekly, reactors fed 4-chlorophenol substrate (A8, B8 and C8) were analyzed for substrate concentration by GC and fed substrate to maintain a concentration of 50 mg/L.

On day 101, two reactors (B2 and G2) were sampled for total solids determination.

On day 146, two reactors (B2 and B8) were sampled for total solids determination. A sample of resin from attached growth reactor A2, was taken and sent for SEM photographs.

On day 153, two attached growth reactors (A8 and C8) were sampled for GC/MS analysis of 4-chlorophenol degradation intermediates and by-products.

During phase 4 stoppers were changed bi-weekly on reactors A2, A8, B2, B8, C8, F2, F8, and G2. Headspace gas was analyzed by GC for the presence of methane within each reactor. Methane presence, after stopper and anaerobic headspace gas exchange, indicates a methanogenic type of degradation.

Phase 4 continued for approximately 75 days.

5.2.2 Results and Discussion

The purpose of the semicontinuous fermentation (draw and feed) study was to evaluate the performance of attached growth, suspended growth and supernatant only cultures with respect to the removal of 2- or 4-chlorophenol under conditions of steady state.

The study was conducted in 4 phases. Phase 1 allowed the acclimated cultures in reactors A2, A8, C8, B2 and B8 time to become accustom to the conditions of draw and feed, thus minimizing any stress to the active cultures. These reactors were chosen to participate in this study because of their excellent performance during the acclimation study. Results for reactor C8 were not provided as it served as a duplicate attached growth reactor. At the beginning of each phase all supernatant (88% v/v) was removed from reactor series A and C (leaving only attached bacterial growth) and replaced with fresh mineral media. Phases 2, 3, and 4 involved subjecting reactors to the conditions of draw and feed and evaluating their performance. These results are presented in Table 5.4 (p. 97).

Results obtained for this study indicated that steady state conditions were never achieved by all reactors. However, attached growth cultures were more successful at substrate degradation than either suspended growth or supernatant only cultures. Cultures fed 2-chlorophenol performed better than cultures fed 4-chlorophenol. Overall rates of dechlorination of chlorine in the ortho position were higher than chlorine located in the para position.

During the study steady state conditions were never fully achieved by both attached growth and suspended growth reactors. Achieving steady state conditio would have permitted a direct comparison of the removal efficiency.

Data collected over phases 2 to 4 was not consistent enough. These results were not expected when compared to the results of other studies.

During phases 2, 3 and 4, reactors fed 2-chlorophenol came closest to achieving steady state conditions. The performance of attached growth reactor A2 and suspended growth reactor B2 are presented in Figures 5.10 and 5.11, respectively (p. 98 to 99). Reactors fed 4-chlorophenol stopped degrading the substrate and began to fail during phase 3. Draw and feed conditions were suspended during phase 4 to help revive the cultures. The performance of attached growth reactor A8 and suspended growth B8 are presented in Figures 5.12 and 5.13, respectively (p. 100 to 101).

During phases 2, 3 and 4, attached growth reactors exhibited a better rate of 2-chlorophenol or 4-chlorophenol degradation than suspended growth reactors or reactors containing supernatant only.

Reactors fed 2-chlorophenol exhibited relatively constant rates of degradation throughout the study. Reactors fed 4-chlorophenol experienced decreasing rates of degradation as the study progressed. This pattern does agree with results of other studies.

Throughout the semicontinuous fermentation study, the cumulative chlorophenol degradation rates for the "pseudo-steady state" degradation stages were indicated by extrapolated linear regression lines. These lines are presented in Figures 5.14 to 5.16 inclusive (p. 102 to 104), Figures 5.18 to 5.23 inclusive (p. 106 to 111) and Figure 5.25 (p. 113). The slopes are described in terms of degradation rate within each individual phase in Table 5.4 (p. 97). Statistical analysis are presented in Appendix V.

The cessation of chlorophenol degradation was exhibited as a slowdown in the rates of substrate degradation, which corresponded to a change in the slope of the cumulative rate.

This change indicated that the cultures were experiencing stress, probably by continued exposure to high concentrations of chlorophenol substrate. The cultures appeared to lose the ability to degrade any additional chlorophenol substrate (the slope of the cumulative rate lies parallel to the "x" axis (Time axis)).

If the cultures were unable to degrade either 2- or 4-chlorophenol substrate for over a two month period, the reactors were considered to have "failed" and were terminated (eg. suspended growth reactor B8 was terminated during phase 4).

Supernatant only reactors, which were created as a control for the corresponding attached growth reactors, were monitored throughout each individual phase without addressing their ability to degrade the chlorophenol substrate.

Figures 5.10 to 5.13 inclusive show statistically calculated averages (Appendix V) of influent and effluent from each reactor. Thus illustrating the overall average 2- or 4-chlorophenol degradation rate, presented in Table 5.4.

In conclusion, steady state conditions were never achieved by all reactors therefore the efficiency of the conditions of draw and feed could not be fully evaluated. Attached growth cultures were more successful at substrate degradation than either suspended growth or supernatant only cultures due to the presence of solid support media. This may be attributed to the ability of the resin to support a large bacterial population capable of metabolizing

chlorophenol and prevent the effects of wash-out. Dechlorination at the ortho position appeared to be much more favorable than dechlorination at the para position. Cultures capable of 2-chlorophenol degradation exhibited the best overall performance during the study. The inhibition of 4-chlorophenol degradation, which also occurred during this study, was independent of reactor composition.

TABLE 5.4 RESULTS OF THE SEMICONTINUOUS FERMENTATION (DRAW AND FEED) STUDY

Phase/ Reactor	Degradation Rate (mg/L per day)*	Lag Period (days)	Total Fermentation Time (days)
ATTACHED GROWTH			
2-Chlorophenol			
Phase 2/A2	5.2	0	36
Phase 3/A2	5.1	5	44
Phase 4/A2	4.8	0	75
4-Chlorophenol			
Phase 2/A8	5.2	0	36
Phase 3/A8	2.2	8	24
Phase 4/A8	Reactor Failure		
SUSPENDED GROWTH			
2-Chlorophenol			
Phase 2/B2	4.9	0	36
Phase 3/B2	3.4	0	44
Phase 4/B2	3.9	0	75
4-Chlorophenol			
Phase 2/B8	1.3	3	36
Phase 3/B8	1.0	10	24
Phase 4/B8	Reactor Failure		
SUPERNATANT ONLY			
2-Chlorophenol			
Phase 2/E2	2.7	0	36
Phase 3/F2	3.1	0	44
Phase 4/G2	3.6	0	75
4-Chlorophenol			
Phase 2/E8	2.3	9	36
Phase 3/F8	Reactor Failure		

* See Appendix V for statistical calculation of degradation rate.

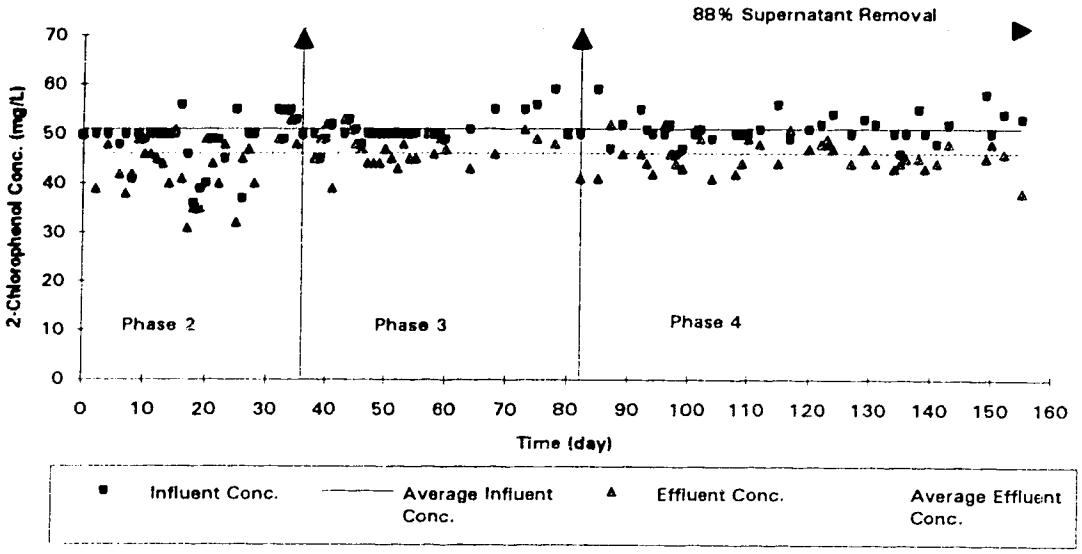


FIGURE 5.10 SEMiCONTINUOUS FERMENTATION (10 day HRT, Fed 50 mg/L 2-Chlorophenol) - Attached Growth Reactor A2.

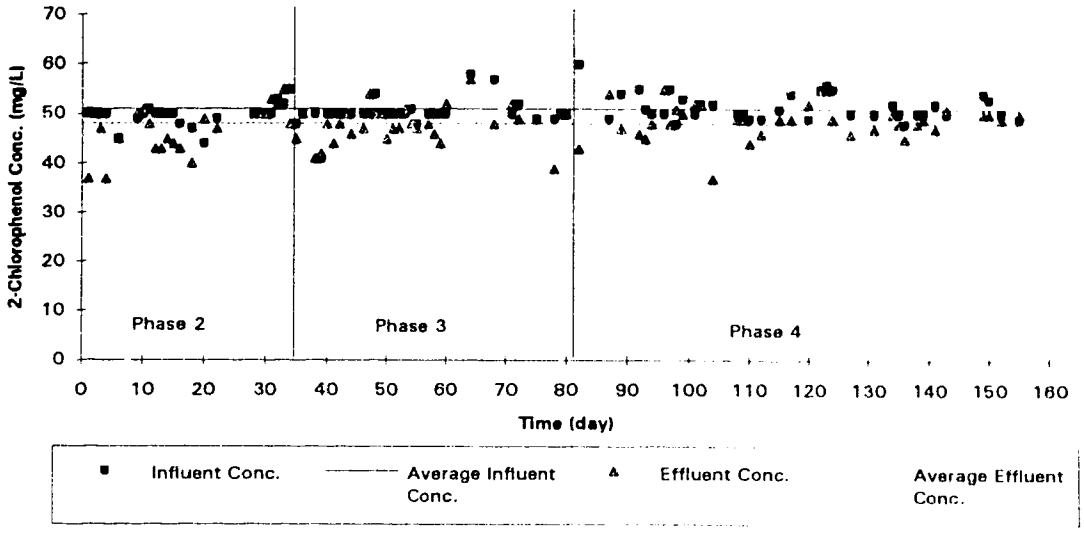


FIGURE 5.11 SEMICONTINUOUS FERMENTATION (10 day HRT, Fed 50 mg/L 2-Chlorophenol) - Suspended Growth Reactor B2

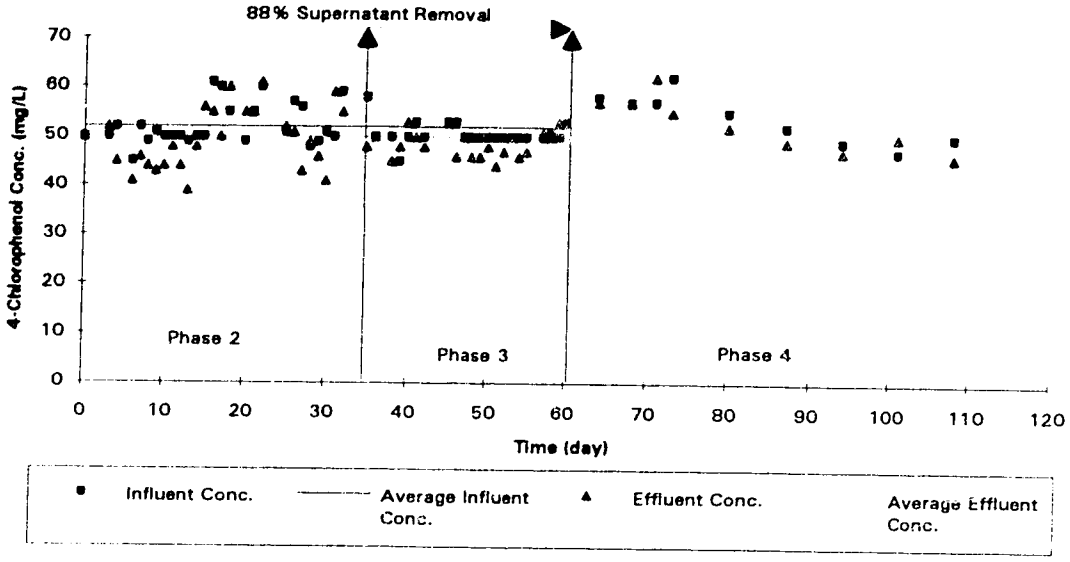


FIGURE 5.12 SEMICONTINUOUS FERMENTATION (10 day HRT, Fed 50 mg/L 4-Chlorophenol) - Attached Growth Reactor A8

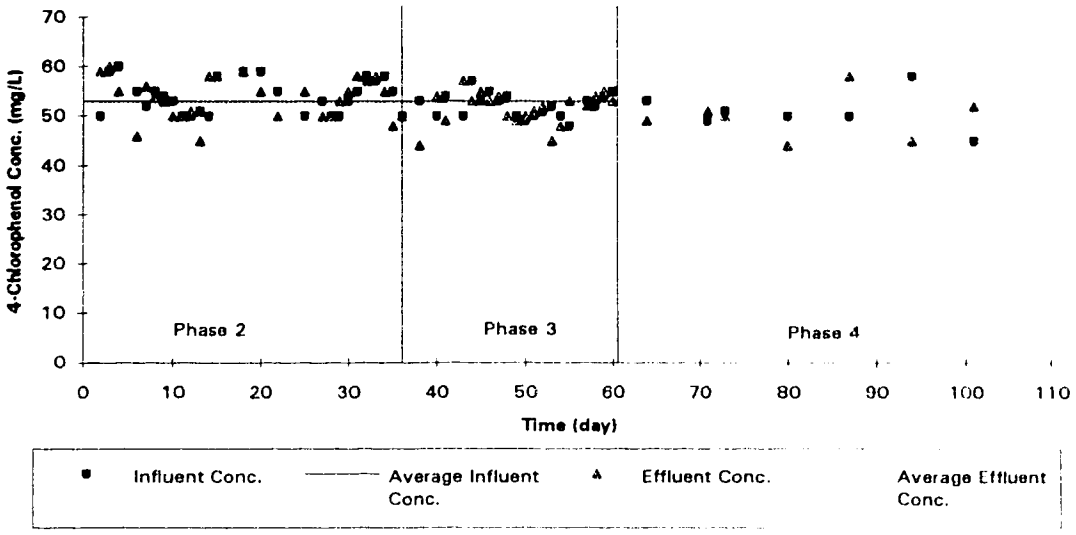


FIGURE 5.13 SEMICONTINUOUS FERMENTATION (10 day HRT, Fed 50 mg/L 4-Chlorophenol) - Suspended Growth Reactor B8

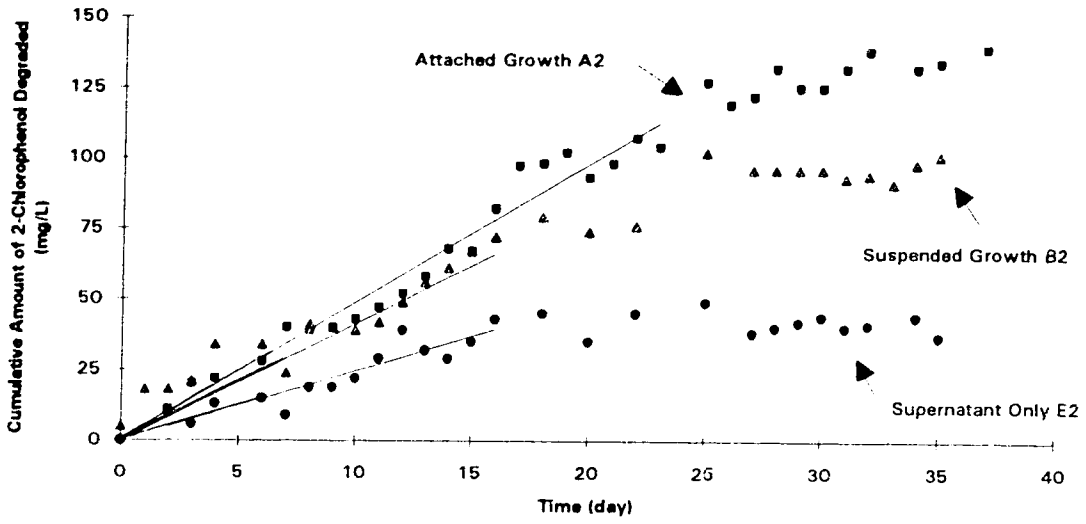


FIGURE 5.14 SEMICONTINUOUS FERMENTATION (10 day HRT, Fed 50 mg/L 2-Chlorophenol) - Cumulative Amount of 2-Chlorophenol Degraded (Phase 2, Various Reactors).

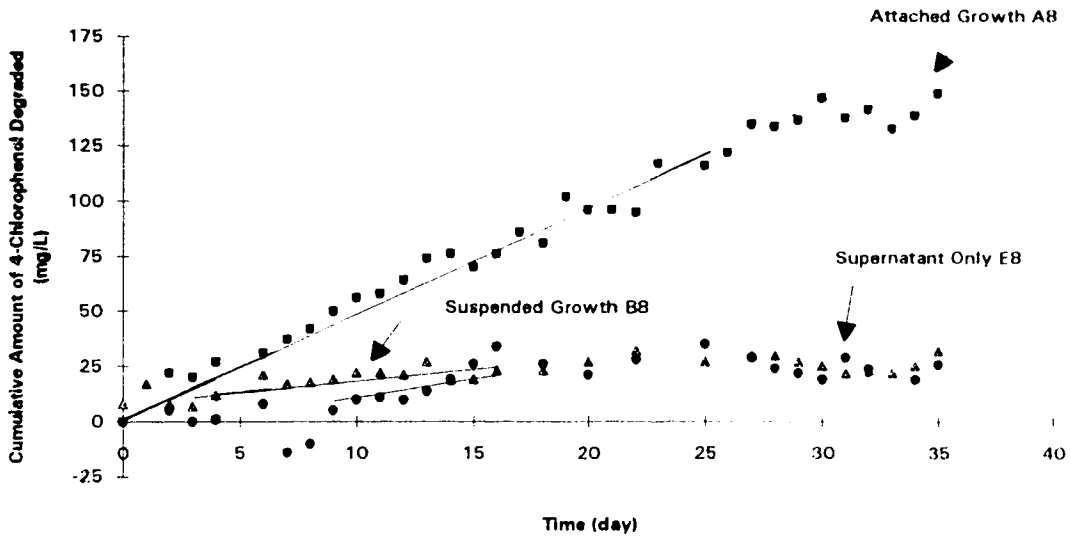


FIGURE 5.15 SEMICONTINUOUS FERMENTATION (10 day HRT, Fed 50 mg/L 4-Chlorophenol) - Cumulative Amount of 4-Chlorophenol Degraded (Phase 2, Various Reactors).

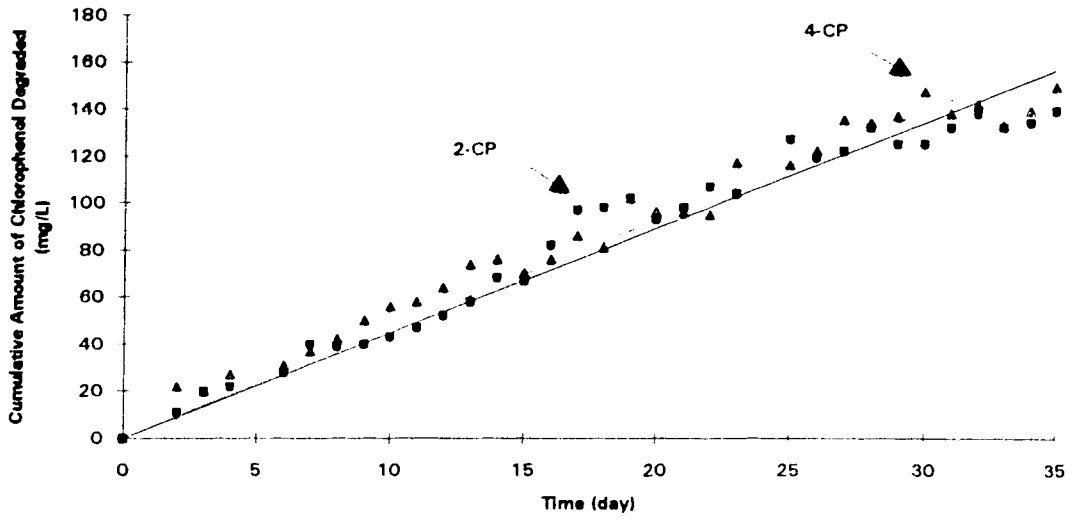


FIGURE 5.16 SEMICONTINUOUS FERMENTATION (10 day HRT, Fed 50 mg/L 2-Chlorophenol or 4-Chlorophenol) - Cumulative Amount of Chlorophenol Degraded by Attached Growth Reactors (Phase 2).

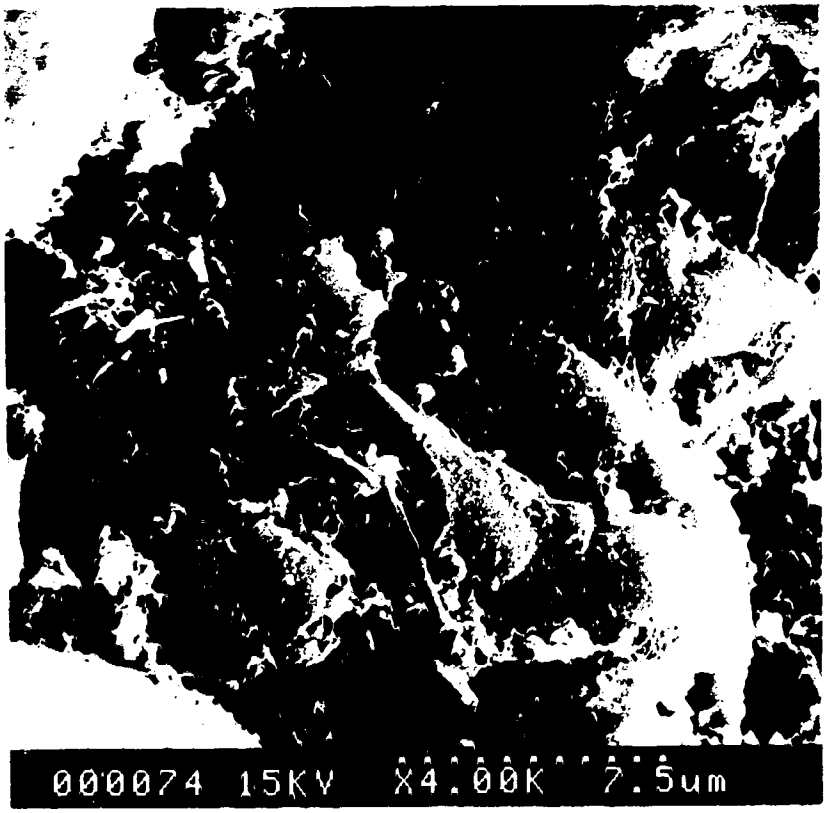


FIGURE 5.17 A Scanning Electron Microscope Photograph of Bacterial Colonization on the Resin Surface in Attached Growth Reactor A8 - Phase 2

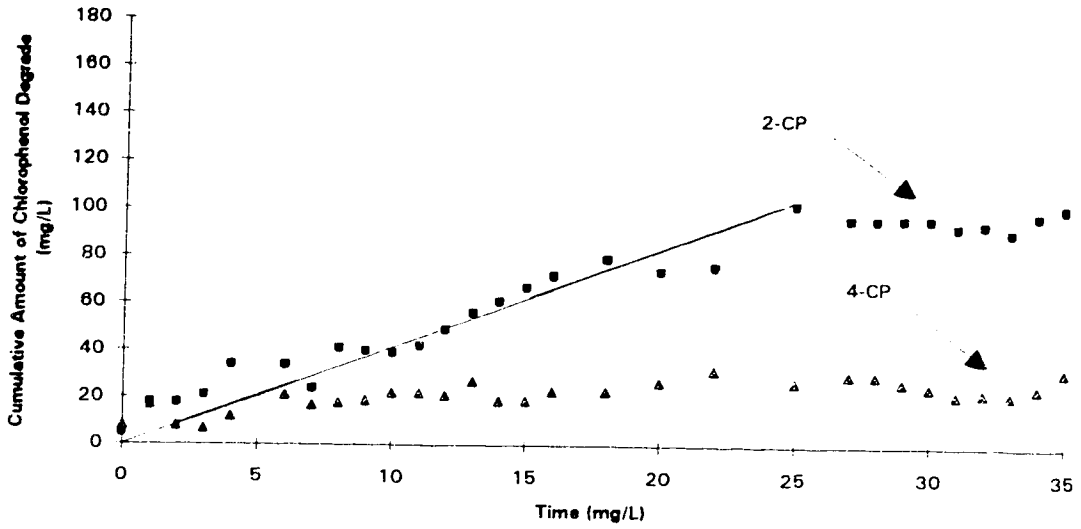


FIGURE 5.18 SEMICONTINUOUS FERMENTATION (10 day HRT, Fed 50 mg/L 2-Chlorophenol or 4-Chlorophenol) - Cumulative Amount of Chlorophenol Degraded by Suspended Growth Reactors (Phase 2).

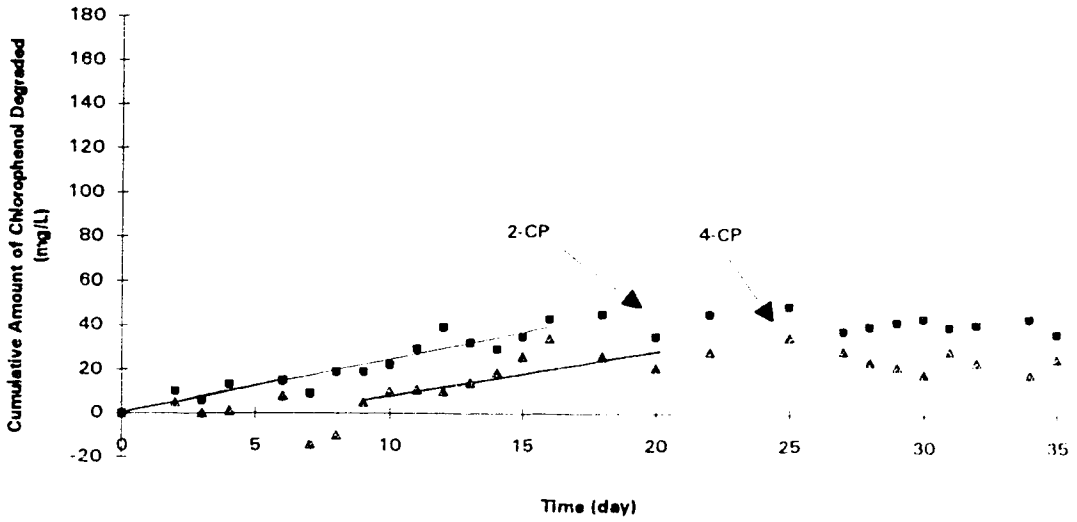


FIGURE 5.19 SEMICONTINUOUS FERMENTATION (10 day HRT, Fed 50 mg/L 2-Chlorophenol or 4-Chlorophenol) - Cumulative Amount of Chlorophenol Degraded by Supernatant Only Reactors (Phase 2).

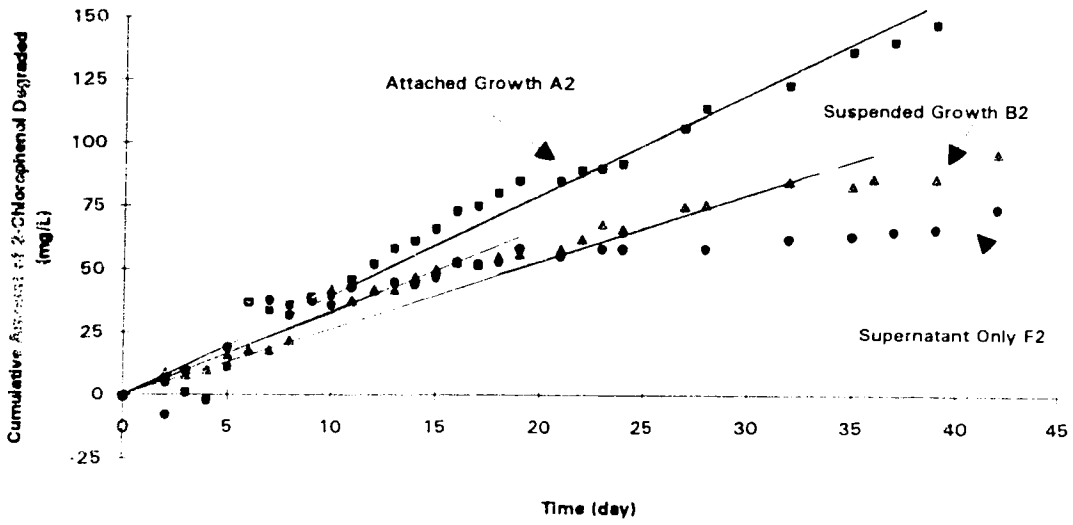


FIGURE 5.20 SEMICONTINUOUS FERMENTATION (10 day HRT, Fed 50 mg/L 2-Chlorophenol) - Cumulative Amount of 2-Chlorophenol Degraded (Phase 3, Various Reactors).

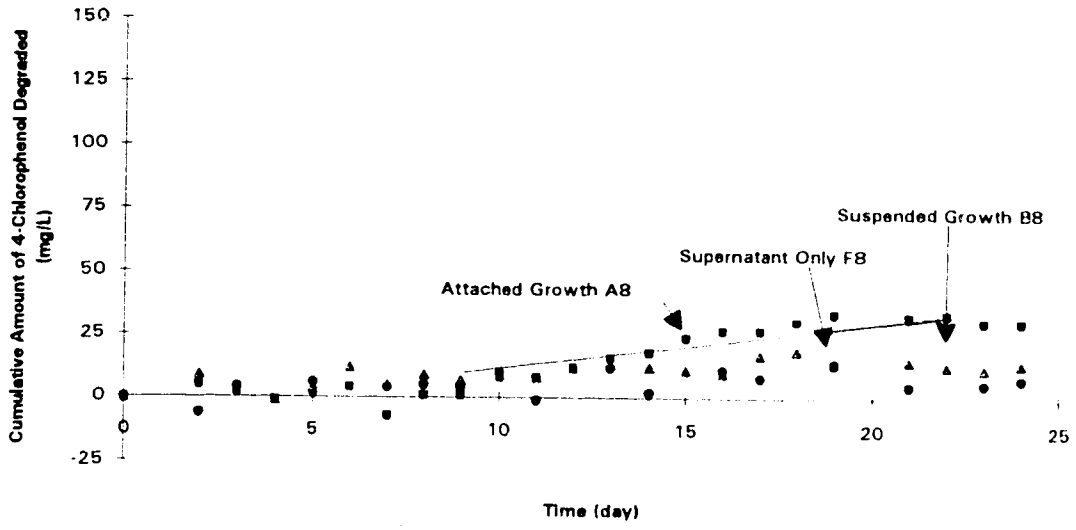


FIGURE 5.21 SEMICONTINUOUS FERMENTATION (10 day HRT, Fed 50 mg/L 4-Chlorophenol) - Cumulative Amount of 4-Chlorophenol Degraded (Phase 3, Various Reactors).

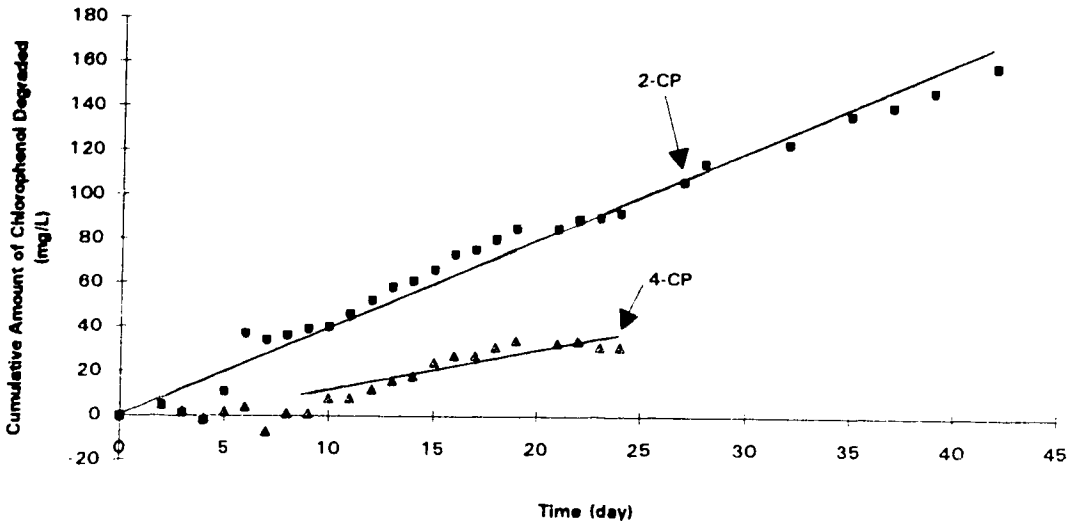


FIGURE 5.22 SEMICONTINUOUS FERMENTATION (10 day HRT, Fed 50 mg/L 2-Chlorophenol or 4-Chlorophenol) - Cumulative Amount of Chlorophenol Degraded by Attached Growth Reactors (Phase 3).

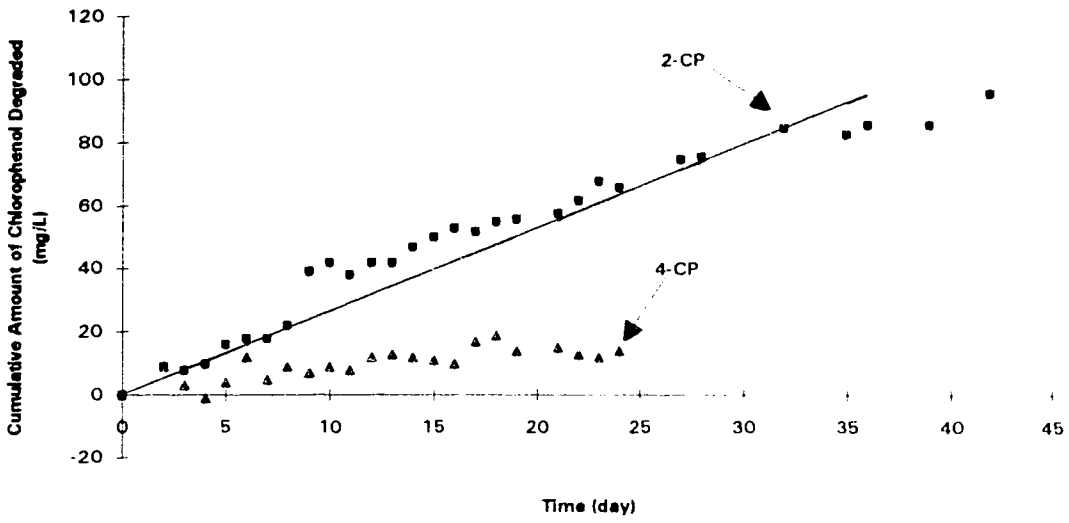


FIGURE 5.23 SEMICONTINUOUS FERMENTATION (10 day HRT, Fed 50 mg/L 2-Chlorophenol or 4-Chlorophenol) - Cumulative Amount of Chlorophenol Degraded by Suspended Growth Reactors (Phase 3).

FIGURE 5.24 A Scanning Electron Photograph of Bacterial Colonization on the Resin Surface in Attached Growth Reactor A2 - Phase 4.

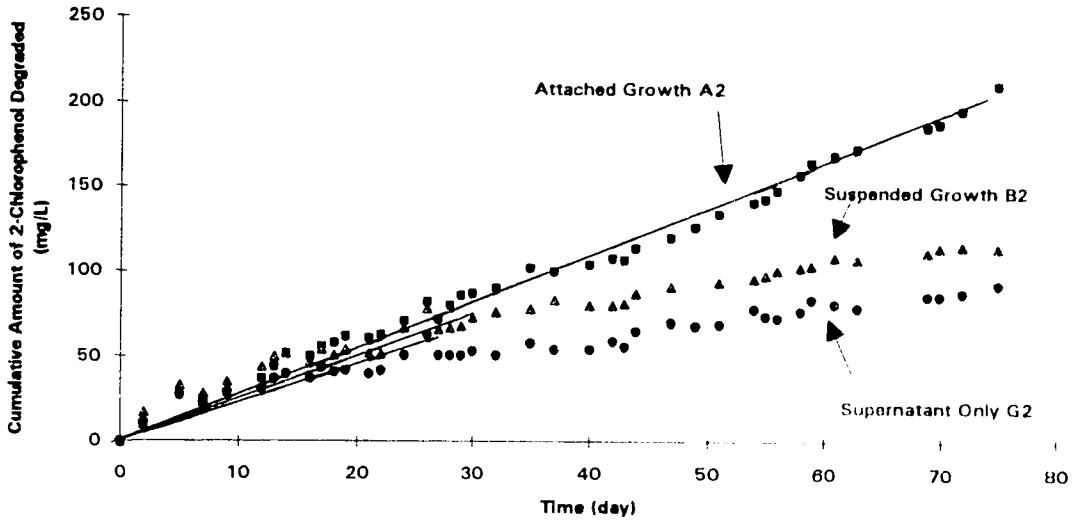


FIGURE 5.25 SEMICONTINUOUS FERMENTATION (10 day HRT, Fed 50 mg/L 2-Chlorophenol) - Cumulative Amount of 2-Chlorophenol Degraded (Phase 4, Various Reactors).

5.2.2.1 Summary of Individual Phases

Phase 2

Cumulative 2-chlorophenol degradation is presented in Figure 5.14 (p. 102). Degradation rates for attached growth, suspended growth and supernatant only reactors were 5.2 mg/L per day, 4.9 mg/L per day and 2.7 mg/L per day, respectively (Table 5.4) (p. 97).

Cumulative 4-chlorophenol degradation rates are presented in Figure 5.15 (p. 103). Degradation rates for attached growth, suspended growth and supernatant only cultures are 5.2 mg/L per day, 1.3 mg/L per day and 2.3 mg/L per day, respectively (Table 5.4) (p. 97).

Under pseudo steady state conditions in Figures 5.14 to 5.16 (p. 102 to 104) and 5.18 to 5.19 (p. 106 to 107), inclusive, degradation rates are represented by linear regression lines. These rates are expressed as the results of statistical analysis (Appendix V) in Table 5.4 (p. 97).

During phase 2, methane was qualitatively detected in reactors A2, A8, B2 but not in reactor B8. Reactor B8 performed poorly and degraded 4-chlorophenol at a rate of 1.3 mg/L per day, after a 3 day lag.

Supernatant only reactors had lower degradation rates than the other reactors; 2.7 mg/L per day of 2-chlorophenol by reactor E2 and 2.3 mg/L per day of 4-chlorophenol by reactor E8. Degradation in reactor E8 proceeded after a 9 day lag (Figure 5.19). Both reactors showed no further degradation after day 20 of phase 2. No methane was detected in either reactor during this phase.

Phase 3

Reactors wih 2-chlorophenol substrate continued to perform well during this phase. In comparison, reactors with 4-chlorophenol substrate exhibited a poor performance and experienced reactor failure after 24 days.

Cumulative degradation of 2-chlorophenol is presented in Figure 5.20 (p. 108). Degradation rates for attached growth, suspended growth and supernatant only reactors were; 5.1 mg/L per day, 3.4 mg/L per day and 3.1 mg/L per day, respectively (Table 5.4) (p. 97). Reactor A2 experienced a 5 day initial lag.

Cumulative degradation of 4-chlorophenol is presented in Figure 5.21 (p. 109). Degradation rates for attached growth, and suspended growth reactor were; 2.2 mg/L per day and 1.0 mg/L per day, respectively (Table 5.4, p. 97). No rate of degradation is included for reactor F8 due to the variability of the data. Actual degradation for reactors A8 and B8 took place from day 10 to day 24, after experiencing a initial lag period. Phase 3 ended on day 24 due to reactor failure.

Under pseudo steady state conditions in Figures 5.20 to 5.23 (p. 108 to 111), inclusive, degradation rates are represented by linear regression lines. These rates are expressed as the results of statistical analysis (Appendix V) in Table 5.4 (p. 97).

During phase 3 there was a distinct difference in the rate of chlorophenol degradation between reactors fed 2-chlorophenol and those fed 4-chlorophenol. Dechlorination at the ortho position appeared to be much more favorable than dechlorination at the para position.

A comparison of the cumulative degradation of two chlorophenol isomers by attached growth reactors is presented in Figure 5.22 (p. 110). Degradation rates were 5.1 mg/L per day for 2-chlorophenol (2-CP) and 2.2 mg/L per day for 4-chlorophenol (4-CP) in reactors A2 and A8, respectively (Table 5.4, p. 97).

A comparison of the cumulative degradation of two chlorophenol isomers by suspended growth reactors is presented in Figure 5.23 (p. 111). Degradation rates were 3.4 mg/L per day for 2-chlorophenol (2-CP) and 1.0 mg/L per day for 4-chlorophenol (4-CP) in reactors B2 and B8, respectively (Table 5.4, p. 97).

Methane was qualitatively detected during phase 3 in attached growth reactors A2 and A8, in suspended growth reactor B2. Methane was not detected in the suspended growth reactor B8 nor any of the reactors containing supernatant only.

Phase 4

By phase 4 only reactors A2, B2 and G2, fed 2-chlorophenol, were still involved in the draw and feed study. Rates of 2-chlorophenol degradation by reactors A2 and B2 compared favorably with rates from previous phases (Table 5.4, p. 97). Bacteria were still actively metabolizing 2-chlorophenol. This was especially evident in attached growth reactor A2 where bacteria were numerous (Figure 5.24, p. 112). Cumulative degradation of 2-chlorophenol is presented in Figure 5.25 (p. 113). Degradation rates of attached growth, suspended growth and supernatant only reactors were; 4.8 mg/L per day, 3.9 mg/L per day and 3.6 mg/L per day, respectively (Table 5.4, p. 97).

Under "pseudo" steady state conditions in Figure 5.25, degradation rates are represented by linear regression lines. These rates are expressed as the results of statistical analysis (Appendix V) in Table 5.4.

During phase 4, methane was detected in all three reactors fed 2-chlorophenol (A2, B2 and G2) and in attached growth reactor A8, fed 4-chlorophenol. Methane was not detected in suspended growth reactor B8, fed 4-chlorophenol, until near the end of phase 4 (Appendix I).

During phase 4, unknown peaks were chromatographed during a qualitative methane analysis in attached growth reactors A8 and C8. In order to identify the source of these peaks, samples of supernatant from each reactor were obtained and subsequently treated with a base/neutral and acid extraction procedure. Extracts were analyzed by GC/MS for the presence of any intermediates. Results were inconclusive as samples chromatographed poorly and the separation of peaks for spectrum identification was not possible. No further analysis was done.

Unknown peaks were also chromatographed during the GC analysis of the concentration of 2-chlorophenol in suspended growth reactor B2. The retention times of these peaks were similar to the known retention times of a standard phenol solution. No further tests were conducted to accurately identify the compound as phenol. However, these results do agree with the studies of Boyd et al., (1983), Hrudey et al., (1987a), Hrudey et al., (1987b), Haggblom and Young (1990) and Haggblom et al., (1993); where the appearance of phenol was noted during the dechlorination of 2-chlorophenol.

Total solids were determined for suspended growth reactors B2 and B8 during phases 2 and phase 4 (Appendix II). The amount of solids determined for each reactor remained relatively constant during phases 2 and 4. These results may be reflected in the total amount of chlorophenol consumed and the overall performance of the reactors during the study. Reactor B8, which performed poorly throughout the study, may have been stressed and started to experience reactor failure during phase 2.

6.0 Isomer Selectivity and Toxicity Study

The selectivity of microbial cultures for either of two monochlorophenol isomers (2- or 4-) was studied. The purpose of Experiment 1 was to observe whether cultures acclimated to one specific isomer were capable of degrading substrate of the other isomer.

In addition, an inhibition/toxicity study was conducted as Experiment 2 to assess the effect of increasing the chlorophenol concentration (2-chlorophenol and/or 4-chlorophenol).

6.1 Procedures

6.1.1 Experiment 1 (day 1 to 42)

Experiment 1 studied the degradation of 4-chlorophenol by 2-chlorophenol acclimated cultures (Table 6.1). In this study, three reactors contained cultures that were acclimated to 2-chlorophenol in the previous draw and feed study. These consisted of an attached growth reactor A2, a suspended growth reactor B2 and a supernatant only reactor G2. Three other attached growth reactors (C8, C9a and C9b) contained cultures that were acclimated to 4-chlorophenol over approximately 217 days.

On day 1, a sample of resin from attached growth reactor A2, was taken and sent for SEM photographs.

From day 1 to day 20, reactors with cultures acclimated to 2-chlorophenol (A2, B2 and G2) were fed 4-chlorophenol as the sole source of substrate. All reactors (A2, B2, G2, C8, C9a and C9b) were fed and drawn daily to maintain a constant 4-chlorophenol concentration of 50 mg/L with a HRT of 10 days.

Around day 21, degradation of chlorophenol ceased and the reactors began to exhibit signs of failure. To help revive the cultures, draw and feed conditions were suspended. Substrate concentration was maintained at 50 mg/L with 5 mL of supernatant drawn and exchanged once a week to supply fresh mineral media.

6.1.2 Experiment 2 (day 43 to 196)

Experiment 2 studied the effect of different ratios of 2-chlorophenol and 4-chlorophenol substrate on the cultures and toxicity caused by increasing the substrate concentration. Reactor A2 was fed 4-chlorophenol only, reactor C9a was fed twice as much 2- as 4-chlorophenol (2-CP:4-CP = 2:1) and reactor C9b was fed equal amounts of 2- and 4-chlorophenol (2-CP:4-CP = 1:1).

From day 44 to day 89, reactors A2, C9a and C9b were analyzed weekly by GC. Total substrate concentrations were increased by approximately 25 mg/L per week.

From day 90 to day 196, reactors A2, C9a and C9b were analyzed every two weeks by GC. Total substrate concentrations were increased by approximately 20 mg/L per week. Reactor A2 was fed a total of 548 mg/L 4-chlorophenol. Reactor C9a was fed a total of 595 mg/L combined 2- and 4-chlorophenol (2-CP:4-CP = 2:1). Reactor C9b was fed a total of 484 mg/L combined 2- and 4-chlorophenol (2-CP:4-CP = 1:1).

The experiment was terminated after 196 days as 2- and/or 4-chlorophenol degradation within the cultures had ceased. Table 6.1 provides a summary of the isomer selectivity and toxicity experiment.

TABLE 6.1. ISOMER SELECTIVITY AND TOXICITY STUDY SUMMARY

Experiment Details	Reactor	Culture	Original CP Isomer	Total Days	Acclimate CP Isomer	CP (mg/L per week)	Final CP Conc. (mg/L)
Experiment 1							
Day 1 to 20: CP isomer acclimation under draw & feed conditions of 50 mg/L and 10 day HRT.	A2	A	2-CP	42	4-CP		50
	B2	S	2-CP	42	4-CP		50
	G2	O	2-CP	42	4-CP		50
Day 21 to 42: Feed 50 mg/L 2-CP or 4-CP weekly and draw bi-weekly using a 70 day HRT.	C8,C9a, C9b	A	4-CP	42	4-CP		50
Experiment 2							
Day 43 to 89: Weekly 2-CP and 4-CP conc. analyses. Increased CP conc. approximately 25 mg/L per week.	A2	A	2-CP	47	4-CP	24	243
	C9a	A	4-CP	47	2-CP:4-CP = 2:1	24	250
	C9b	A	4-CP	47	2-CP:4-CP = 1:1	23	200
Day 90 to 196: Bi-weekly 2-CP and 4-CP conc. analyses and increased CP conc. approximately 20 mg/L per week.	A2	A	2-CP	107	4-CP	19	548
	C9a	A	4-CP	107	2-CP:4-CP = 2:1	21	595
	C9b	A	4-CP	107	2-CP:4-CP = 1:1	16	484

Culture; A - Attached Growth, S - Suspended Growth, O - Supernatant Only.
CP - Chlorophenol; 2-CP - 2-Chlorophenol; 4-CP - 4-Chlorophenol.
HRT - Hydraulic Retention Time.

6.2 Results and Discussion

Results of Experiment 1 and 2 are presented in Table 6.2.

Results from Experiment 1 indicate that cultures acclimated to 2-chlorophenol could be re-acclimated to 4-chlorophenol. A 10 day hydraulic retention time (HRT) was maintained by all reactors. Methane gas was present in all reactors during the initial days of Experiment 1. However near the end of Experiment 1, suspended growth reactor B2 and attached growth reactor C8 began to fail and were removed from the study.

Results from Experiment 2 were not conclusive due to poor reactor performance but did indicate that cultures acclimated to 4-chlorophenol degraded 4-chlorophenol instead of 2-chlorophenol, when offered both. There were no intermediates found during the degradation of either 2- or 4-chlorophenol. Overall degradation rates were low and seemed to correlate with the concentration of the individual chlorophenolic substrate. The resulting degradation rates from Experiment 2 are presented in Table 6.2.

Average degradation rate values were calculated by summing the daily differences between the amount of chlorophenol isomer fed to each reactor and the amount of chlorophenol isomer remaining in each reactor, over a 145 day period. An average of the summation differences was obtained and the standard deviation calculated.

During Experiment 2, unknown peaks were chromatographed during an analysis of reactor supernatant for chlorophenol concentration. Retention times of the peaks corresponded to the known retention time of a phenol standard. To identify the source of this peak, samples of supernatant from each reactor were obtained and subsequently treated by derivatization and extraction (Section

4.4.4). Extracts were analyzed by GC/MS for the presence of any intermediates. There was nothing found except a quantity of 2,4-dichlorophenol, present as a contaminant within the 2-chlorophenol feed solution. A qualitative test for amino acids was also performed. Nothing was found except an amino acid that was present in the mineral media solution. These results are presented in Appendices IV and III, respectively.

In conclusion, attached growth and suspended growth cultures, originally acclimated to 2-chlorophenol, can be re-acclimated to 4-chlorophenol without a lag period. When presented with both ortho and para isomers, cultures acclimated to 4-chlorophenol appeared to degrade 4-chlorophenol rather than 2-chlorophenol. No intermediates were detected during the study. Toxicity results could not be confirmed due to poor reactor performance. Dechlorination could not be confirmed as there was no proof of methane production.

TABLE 6.2 RESULTS OF THE ISOMER SELECTIVITY AND TOXICITY STUDY FOR EXPERIMENT 2

Reactor	Acclimate CP Isomer	CP	Average Conc. Degraded (mg/L per day)*	Standard Deviation (mg/L per day)	Max. Conc. (mg/L)**
A2	4-CP	2-CP	0	0.2	25
		4-CP	2.4	1.8	440
C9a	2-CP:4-CP = 2:1	2-CP	1.1	1.5	326
		4-CP	0.7	1.2	183
C9b	2-CP:4-CP = 1:1	2-CP	0.6	1.3	187
		4-CP	0.8	1.6	194

CP - Chlorophenol; 2-CP - 2-Chlorophenol; 4-CP - 4-Chlorophenol

* Conc. Degraded is the Average Difference Between Feed Concentration (Influent) and Reactor Concentration (Effluent) Per Day.

** Max. Conc. is the Maximum Concentration the Reactor was Fed Before the Experiment was Terminated.

7.0 Summary and Conclusions

Anaerobic biodegradation of 2-chlorophenol and 4-chlorophenol was investigated in attached growth, suspended growth and supernatant only type reactors during semicontinuous (draw & feed) conditions.

Prior to beginning of each phase the attached growth reactors, which contained resin as growth support media, had 88% supernatant removed to retain maximum colonization of the attached growth biomass. The suspended growth reactors did not have any supernatant removed (except during the semicontinuous draw & feed procedure) since their initial set-up. The supernatant only reactors were created from the supernatant removed from attached growth reactors at the beginning of each new phase. They served as a control reactor for individual corresponding attached growth reactor during each phase. Since they contained no resin or sludge, they were therefore free of any attached biomass.

The attached growth reactors performed superior when compared to suspended growth reactors. The presence of resin provided a support material with available pore volume for bacterial attachment and colonization. Attached growth reactors illustrated; (a) shorter acclimation times for both 2-chlorophenol and 4-chlorophenol substrate, (b) a more stable bacterial consortium (biomass) and were less prone to the effects of "wash-out", loading changes, loss of enzymes, or toxicity of prolonged high substrate concentration and (c) higher degradation rates for either 2- or 4-chlorophenol than corresponding suspended growth reactors.

Under "pseudo" steady state conditions achieved during individual phases of the semicontinuous fermentation study (using a 10 day hydraulic retention

time (HRT)), the highest 2-chlorophenol or 4-chlorophenol degradation rates obtained was 5.2 mg/L per day. These rates were similar to the highest degradation rates reported by other researchers in batch studies. Hrudey et al., (1987a) reported 5.7 mg/L per day for 2-chlorophenol, Zhang and Weigel (1990) reported 7 mg/L per day for 4-chlorophenol, and Haggblom et al., (1993) reported a range of 3.7 to 4.5 mg/L per day for 2-chlorophenol. The semicontinous (draw and feed) study showed that attaining much higher degradation rates for chlorophenols is very unlikely. Over an extended period of exposure, all reactors were affected to some degree by the toxicity of either the 2-chlorophenol or 4-chlorophenol substrate.

The suspended growth reactors experienced instability or signs of toxicity build-up, possibly due to a loss or "wash-out" of some important enzyme, much earlier than attached growth reactors. This effect was more pronounced and the suspended growth reactors were less likely to recover when compared to the attached growth reactors (Figures 5.14, 5.15, 5.20 and 5.21). The cessation of chlorophenol degradation was the first indication that these reactors were experiencing stress.

Throughout the entire study, dechlorination at the ortho position (2-chlorophenol) appeared to be much more favorable than dechlorination at the para (4-chlorophenol) position. Also instability and subsequent reactor failure was considerably more visible in reactors degrading 4-chlorophenol than in reactors degrading 2-chlorophenol substrate. Reactor failure was independent of reactor type. This was in agreement with other investigators. When compared with chlorine attached in either ortho or meta positions on the phenol molecule, dechlorination of the para position has been shown to be more

difficult. Many researchers found 4-chlorophenol the most difficult monochlorophenol to degrade, and rates of degradation varied (Sharak Genthner et al., 1989; Madsen and Aamand, 1992; Armenante et al., 1992). An investigation by Hrudey et al., (1987a) indicated that specific monochlorophenols, particularly 3-chlorophenol and 4-chlorophenol, at concentrations greater than 30 mg/L inhibited the degradation of other available substrate such as phenol at concentrations of 97 to 285 mg/L. Under these conditions the degradation rates of the monochlorophenols were also greatly decreased.

The supernatant only reactors performed at about half the degradation rate of the attached growth reactors. These reactors contained only a limited quantity of suspended microbial growths. However their results also help confirm the superiority of attached growth biomass.

There were slight morphological differences in attached bacterial consortia with different chlorophenol substrate isomers. Upon examination of the SEM photos, it appears that more rod shape bacteria were present in the reactors fed 2-chlorophenol substrate and more filamentous and coccoid types of bacteria were present in reactors fed 4-chlorophenol substrate. However, there was no visual difference in bacterial colonization of resin or increase in attached biomass from the time of acclimation to the end of the semicontinuous fermentation study.

In a brief attempt to study chlorophenol isomer selectivity and the toxicity of increasing chlorophenolic substrate, bacterial cultures acclimated to 2-chlorophenol were fed 4-chlorophenol. Other reactors were fed both isomers in different proportions. Concentrations of substrate increased about 5 fold before

reactors failed and 10 fold before the experiment was terminated. However, due to extreme variability in the data, the final results were inconclusive. No intermediates were detected.

In conclusion, this study indicated that the long term sustainability of anaerobic bioreactors with chlorophenol as their sole source of substrate, is in need of further investigation. Chlorophenols are highly toxic and in long term exposure may disrupt the enzymatic system required for life sustaining activities of bacteria.

8.0 Recommendations for Future Work

Future studies should include a detailed examination of the long term effects of chlorophenol degradation by microbial cultures in an attached growth system.

The studies should investigate the following: (i) toxicity of various concentrations of individual chlorophenol, using Microtox® or digester sludge acclimated to phenol to evaluate the levels of toxicity as a function of the changing conditions, (ii) the effect of one of the proposed intermediates such as phenol or benzoates, in very low concentrations (less than 5 mg/L), as a life supporting supplement, and (iii) a semicontinuous fermentation study with various hydraulic retention times.

References

American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Water and Wastewater,* 18th ed., Washington, D.C.

Armenante, P.M., D. Kafkewitz, G. Lewandowski and C.M. Kung. 1992. Integrated Anaerobic-Aerobic Process for the Biodegradation of Chlorinated Aromatic Compounds. Environmental Progress. 11(2): 113-122.

Banerjee, S., P.H. Howard, A.M. Rosenberg, A.E. Dombrowski, H. Sikka and D.L. Tullis. 1984. Development of a General Kinetic Model for Biodegradation and Its Application to Chlorophenols and Related Compounds. Environ. Sci. Technol. 18(6): 416-422.

Beltrame, P., P.L. Beltrame and P. Carniti. 1984. Inhibiting Action of Chloro- and Nitro-Phenols in Biodegradation of Phenol: A Structure-Toxicity Relationship. Chemosphere. 13(1): 3-9.

Beltrame, P., P.L. Beltrame, P. Carniti, D. Guardione and C. Lanzetta. 1988. Inhibiting Action of Chlorophenols on Biodegradation of Phenol and Its Correlation with Structural Properties of Inhibitors. Biotech. and Bioeng. 31: 821-828.

Beltrame, P., P.L. Beltrame and P. Carniti. 1989. Lipophilic and Electrophilic Factors in the Inhibiting Action of Substituted Phenols on the Biodegradation of Phenol. Chemosphere. 19(10/11): 1623-1627.

Berry, D.F., A.J. Francis and J.M. Bollag. 1987. Microbial Metabolism of Homocyclic and Hetrocyclic Aromatic Compounds under Anaerobic Conditions. Microbiol Rev. 51(1): 43-59.

Box, G.E.P., W.G. Hunter and J.S. Hunter. 1978. *Statistics for Experimenters.* John Wiley & Sons. New York. pp: 653.

Boyd, S.A., D.R. Shelton, D. Berry and J.M. Tiedje. 1983. Anaerobic Biodegradation of Phenolic Compounds in Digested Sludge. Appl. Environ. Microbiol. 46 (1): 50-54.

Boyd, S.A. and D.R. Shelton. 1984. Anaerobic Biodegradation of Chlorophenols in Fresh and Acclimated Sludge. Appl. Environ. Microbiol. 47(2): 272-277.

Bridle, T.R., H.W. Campbell and A.K. Sachdev. 1983. Thermal Destruction of Chlorinated Residues. *Proc. 38th Indus. Waste Conf.* Purdue University, Ann Arbor Science Publishers, Ann Arbor, Mich. pp. 299-309.

Bryers, J.D. 1987. Biologically Active Surfaces: Processes Governing the Formation and Persistence of Biofilms. Biotechnol. Prog. 3(2): 57-68.

Chen, M.H. 1992. Workshop for Biological Scanning Electron Microscopy. Medicine/Dentistry E.M. Unit, Surgical-Medical Research Institute, University of Alberta, Edmonton, Alberta. pp: 79.

Churn, C. 1993. Chemical and Allied Products, Industrial Wastes. In: Water Environment Research Literature Review. Water Environ. Fed. 65(4): 386-398.

Coutts, R.T., E.E. Haresheimer and F.M. Pasutto. 1980. Application of Direct Aqueous Acetylation Technique to the Gas Chromatographic Quantitation of Nitrophenols and 1-Naphthol in Environmental Water Samples. Journal of Chromatography. 195: 105-112.

Davis, J.C. and A.W. Hoos. 1974. Use of Sodium Pentachlorophenate and Dehydroabietic Acid as Reference Toxicants for Salmonid Bioassays. Fisheries and Marine Service. Technical Report No. 464. pp: 26.

Doran, P.M. and J.E. Bailey. 1986. Effects of Immobilization on Growth, Fermentation Properties, and Macromolecular Composition of *Saccharomyces cerevisiae* Attached to Gelatin. Biotechnol. Bioeng. 28: 73-87.

Eckenfelder, W. W. Jr. 1989. *Industrial Water Pollution Control*, 2nd Edition. McGraw-Hill, New York, N.Y. pp: 400.

Evans, W.C. 1977. Biochemistry of the Bacterial Catabolism of Aromatic Compounds in Anaerobic Environments. Nature. 270: 17-22.

Environment Canada. 1991. Canadian Environmental Protection Act, Priority Substances List Assessment Report No. 2: Effluents from Pulp Mills Using Bleaching, En40-215/2E, ISBN 0-662-18734-2. 60 pp.

Fedorak, P.M. 1984 Anaerobic Biological Treatment of Phenolic Wastewaters. Ph.D. Thesis. University of Alberta, Edmonton. 298 pp.

Fedorak, P.M. and S.E. Hrudey. 1984. The Effects of Phenol and Some Alkyl Phenolics on Batch Anaerobic Methanogenesis. Water Res. 18(3): 361-367.

Folsom, B.R., P.J. Chapman and P.H. Pritchard. 1990. Phenol and Trichloroethylene Degradation by *Pseudomonas cepacia* G4: Kinetics and Interactions Between Substrates. Appl. Environ. Microbiol. 56(5): 1279-1285.

Fujise, L., W. Cox and M. Tashiro. 1993. Phenol, April 1993. In: *Chemical Economics Handbook*. SRI International. 686.5000-686.5002.

Gibson, S.A. and J.M. Suflita. 1986. Extrapolation of Biodegradation Results to Groundwater Aquifers: Reductive Dehalogenation of Aromatic Compounds. Appl. Environ. Microbiol. 52(4): 681-688.

Guthrie, M.A., E.J. Kirsch, R.F. Wukasch and C.P.L. Grady Jr. 1984. Pentachlorophenol Biodegradation -II. Anaerobic. Water Res. 18(4): 451-461.

Haggblom, M.M. and L.Y. Young. 1990. Chlorophenol Degradation Coupled to Sulfate Reduction. Appl. Environ. Microbiol. 56(11): 3255-3260.

Haggblom, M.M., M.D. Rivera and L.Y. Young. 1993. Influence of Alternative Electron Acceptors on the Anaerobic Biodegradability of Chlorinated Phenols and Benzoic Acids. Appl. Environ. Microbiol. 59(4): 1162-1167.

Hansson, G. 1982. End Product Inhibition in Methane Fermentations. Process Biochem. 17(6):45-49.

Horowitz, A., J.M. Sulfita and J.M. Tiedje. 1983. Reductive Dehalogenations of Halobenzoates by Anaerobic Lake Sediment Microorganisms. Appl. Environ. Microbiol. 45(5): 1459-1465.

Hrudey, S.E., E. Knettig, S.A. Daignault and P.M. Fedorak. 1987a. Anaerobic Biodegradation of Monochlorophenols. Environ. Technol. Lett. 8: 65-76.

Hrudey, S.E., E. Knettig, P.M. Fedorak, and S.A. Daignault. 1987b. Anaerobic Semi-Continuous Culture Biodegradation of Dichlorophenols Containing an Ortho Chlorine. Water Poll. Res. J. Canada. 22(3): 427-436.

Hungate, R.E. 1969. A Roll Tube Methods for Cultivation of Strict Anaerobes. In: *Methods in Microbiology*. Ed: J.R. Norris and D.W. Ribbons. Academic Press, New York. Vol. 3B, pp: 117-132.

Ide, A., Y. Niki, F. Sakamoto, I. Watanabe, and H. Watanabe. 1972. Decomposition of Pentachlorophenol in Paddy Soil. Agric. Biol. Chem. 36: 1937-1944.

Iwama, G.K. and G.L. Greer. 1979. Toxicity of Pentachlorophenate to Juvenile chinook Salmon Under Conditions of High Loading Density and Continuous-flow Exposure. Bull. Environm. Contam. Toxicol. 23: 711-716.

Kauppinen, T and L. Lindroos. 1985. Chlorophenol Exposure in Sawmills. Am. Ind. Hyg. Assoc. J. 46: 34-38.

Kennedy, K.J., and R.L. Droste. 1985. Startup of Anaerobic Downflow Stationary Fixed Film (DSFF) Reactors. Biotechnol. Bioeng. 27: 1152-1165.

Kindzierski, W.B. 1989. Enhanced Anaerobic Biological Treatment of Phenolic Wastewaters. PhD Thesis. University of Alberta, Edmonton, Alberta. 383 pp.

Knoll, G. and J. Winter. 1987. Anaerobic Degradtion of Phenol in Sewage Sludge. Appl. Microbiol. Biotechnol. 25: 384-391.

Kohring, G.W., X. Zhang and J. Wiegel. 1989a. Anaerobic Dechlorination of 2,4-Dichlorophenol in Freshwater Sediments in the Presence of Sulfate. Appl. Environ. Microbiol. 55(1): 2735-2737.

Kohring, G.W., J.E. Rogers and J. Wiegel. 1989b. Anaerobic Biodegradation of 2,4-Dichlorophenol in Freshwater Lake Sediments at Different Temperatures. Appl. Environ. Microbiol. 55(2): 348-353.

Kovacs, T.G., R.H. Voss and A. Wong. 1984. Chlorinated Phenolics of Bleached Kraft Mill Origin. An Olfactory Evaluation. Water Res. 18(7): 911-916.

Krijgsheld, K.R. and A. van der Gen. 1986. Assessment of the Impact of the Emission of Certain Organochlorine Compounds on the Aquatic Environment - I. Monochlorophenols and 2,4-Dichlorophenol. Chemosphere 15(7): 825-860.

Krumme M.L. and S.A. Boyd. 1988. Reductive Dechloration of Chlorinated Phenols in Anaerobic Upflow Bioreactors. Water Res. 22(2): 171-177.

Lamparski, L.L., R.H. Stehl and R.L. Johnston. 1980. Photolysis of Pentachlorophenol-Treated Wood. Chlorinated Dibenzo-*p*-dioxin Formation. Environ. Sci. Technol. 14(2): 196-200.

Lovley, D.R. and D.J. Lonergan. 1990. Anaerobic Oxidation of Toluene, Phenol and p-Cresol by the Dissimilatory Iron-Reducing Organism, GS-15. Appl. Environ. Microbiol. 56(6): 1858-1864.

Madsen, T. and J. Aamand. 1992. Anaerobic Transformation and Toxicity of Trichlorophenols in a Stable Enrichment Culture. Appl. Environ. Microbiol. 58(2): 557-561.

Mikesell, M.D. and S. A. Boyd. 1986. Complete Reductive Dechloration and Mineralization of Pentachlorophenol by Anaerobic Microorganisms. Appl. Environ. Microbiol. 52(4): 861-865.

Munkittrick, K.R. and E.A. Power. 1991. The Relative Sensivity of Microtox, Daphnid, Rainbow Trout, and Flathead Minnow Acute Lethality Tests. Environ. Toxicol. Water Qual. 6: 35-62.

Philbrook, D.M. and C.P.L. Grady, Jr. 1985. Evaluation of Biodegradation Kinetics For Priority Pollutants. *Proc. 40th Indus. Waste Conf.* Purdue University, Ann Arbor Science Publishers, Ann Arbor, Mich. pp. 795-804.

Pickering, Q.H. and C. Henderson. 1966. Acute Toxicity of Some Important Petrochemicals to Fish. J. Water Poll. Control Fed. 38(9): 1419-1429.

Rochkind-Dubinsky, M.L., G.S. Sayler and J.W. Blackburn. 1987. *Microbiological Decomposition of Chlorinated Aromatic Compounds*, Vol.18. Marcel Dekker, Inc. New York. pp: 317.

Rogers, I.H., J.A. Servizi and C.D. Levings. 1988. Bioconcentration of Chlorophenols by Juvenile Chinook Salmon (*Oncorhynchus tshawytscha*) Overwintering in the Upper Fraser River: Field and Laboratory Tests. Water Poll. Research J. Canada. 23(1): 100-113.

Rothmel, S., J.M. Haugland, R. Sangodkar, K.L. Coco and R.S. Chakrabarty. 1991. Microbial Degradation of Synthetic Chlorinated Compounds: Evolutionary Implications. In: *Biodeterioration and Biodegradation 8.* Ed: H.W. Rossmore. Elsevier Science Publishers Ltd., New York. pp: 276-291.

Sahm, H. and M. Brunner and S.M. Schoberth. 1986. Anaerobic Degradation of Halogenated Aromatic Compounds. Microb. Ecol. 12: 147-153.

Salkinoja-Salonen, M.S., R. Hakulinen, R. Valo and J. Apajalahti. 1983. Biodegradation of Recalcitrant Organochlorine Compounds in Fixed Film Reactors. Water Sci. Tech. 15: 309-319.

Sawyer, C.N. and P.L. McCarty. 1978. *Chemistry of Environmental Engineering*, 3rd Ed. McGraw-Hill Book Company, New York. pp: 94-163.

Seech, A. 1993. PCP\PAH Soil Cleanup Using Bioremediation. Hazardous Materials Management. June: 16.

Selle Sardi, R.F.G., W. Bulani, W.L. Cairns, and N. Kosaric. 1986. Adhesion and Growth of Anaerobic Biofilms on Ion Exchange Resins. Water Poll. Res. J. Canada. 21(4): 486-495.

Servizi, J.A., R.W. Gordon and J.H. Carey. 1988. Bioconcentration of Chlorophenols by Early Life Stages of Fraser River Pink and Chinook Salmon (*Oncorhynchus gorbuscha, O. tshawytscha*). Water Poll. Research J. Canada. 23(1): 88-99.

Sharak Genthner, B.R., W.A. Price III and P.H. Pritchard. 1989. Anaerobic Degradation of Chloroaromatic Compounds in Aquatic Sediments under a Variety of Enrichments Conditions. Appl. Environ. Microbiol. 55(6): 1466-1471.

Shelton, D.R. and J.M. Tiedje. 1984. General Method for Determining Anaerobic Biodegradation Potential. Appl. Environ. Microbiol. 47(4): 850-857.

Solomons, T.W.G. 1984. *Organic Chemistry*, 3rd. Ed. John Wiley & Sons, Inc. New York. 1103 pp.

Speece, R.E. 1983. Anaerobic Biotechnology for Industrial Wastewater Treatment. Environ. Sci. Technol. 17 (9): 416A-427A.

Steiert J.G. and R.L. Crawford. 1985. Microbial Degradation of Chlorinated Phenols. Trends in Biotech. 3(2): 300-305.

Suflita, J.M., A. Horowitz, D.R. Shelton and J.M. Tiedje. 1982. Dehalogenation: A Novel Pathway for the Anaerobic Biodegradation of Haloaromatic Compounds. Science 218: 1115-1116.

Suflita, J.M., J.A. Robinson and J.M. Tiedje. 1983. Kinetics of Microbiol Dehalogenation of Haloaromatic Substrates in Methanogenic Environments. Appl. Environ. Microbiol. 45(5): 1466-1473.

Tiedje, J.M., S.A. Boyd and B.Z. Fathepure. 1987. Anaerobic Degradation of Chlorinated Aromatic Hydrocarbons. In: *Developments in Industrial Microbiology*. Ed: G. Pierce. Journal of Industrial Microbiology Suppl. No. 1. Volume 27: 117-127.

United States Environmental Protection Agency (USEPA), Federal Register. 1984. Guidelines Establishing Test Procedures for the Analysis of Pollutants Under the Clean Water Act; Final Rule and Interim Final Rule and Proposed Rule. Volume 49, No. 209. pp: 153-174.

VanDemark, P.J. and Batzing, B.L. 1987. *The Microbes: An Introduction to Their Nature and Importance*. Benjamin/Cummings Publishing Company, Inc. Menlo Park, California. 991 pp.

Valo, R.J., M.M. Haggblom and M.S. Salkinoja-Salonen. 1990. Bioremediation of Chlorophenol Containing Simulated Ground Water by Immobilized Bacteria. Water Res. 24(2): 253-258.

Valo, R.J., V. Kitunen, M.S. Salkinoja-Salonen and S. Raisanen. 1985. Chlorinated Phenols and their Derivatives in Soil and Ground Water Around Wood Preserving Facilities in Finland. Water Sci. Tech. 17: 1381-1384.

Voss, R.H. 1987. Trace Organic Contaminants in Pulp and Paper Mill Effluent and Their Environmental Effects. Canadian Paper Research Institute of Canada Miscellaneous Report (MR) No. 112.

Wan, M.T. 1992. Utility and Railway Right-of-Way Contaminants in British Columbia: Chlorophenols. J. Environ. Qual. 21(2): 225-231.

Wang, Y.T., H.D. Gabbard and P.C. Pai. 1991. Inhibition of Acetate Methanogenesis by Phenols. J. Environ. Eng. 117(4): 487-500.

Wilkie, A. and E. Colleran. 1984. Start-up of Anaerobic Filters Containing Different Support Materials Using Pig Slurry Supernatant. Biotechnol. Lett. 6(11): 735-740.

Wilkie, A., G. Faherty and E. Colleran. 1985. The Effect of Varying the Support Matrix on the Anaerobic Digestion of Pig Slurry in the Upflow Anaerobic Filter Design. Proceedings of the "International Conference on Biomass-1985". pp: 531-535.

Woods, S.L. 1985. The Fate of Chlorinated Hydroxylated and Methoxylated Benzenes in Anaerobic Wastewater Treatment. PhD Thesis. University of Washington, Seattle, W.A. 299 pp.

Woods, S.L., J.F. Ferguson and M.M. Benjamin. 1989. Characterization of Chlorophenol and Chloromethoxybenzene Biodegradation during Anaerobic Treatment. Environ. Sci. Technol. 23(1): 62-68.

Young, L.Y. 1984. Anaerobic Degradation of Aromatic Compounds. In: *Microbial Degradation of Organic Compounds*. Ed: D.T. Gibson. Marcel Dekker, Inc. New York, N.Y. pp: 487-523.

Young, L.Y. and M.D. Rivera. 1985. Methanogenic Degradation of Four Phenolic Compounds. Water Res. 19(10): 1325-1332.

Zhang, X. and J. Wiegel. 1990. Sequential Anaerobic Degradation of 2,4-Dichlorophenol in Freshwater Sediments. Appl. Environ. Microbiol. 56(4): 119-127.

Appendix I
Qualitative Methane Detection

TABLE I: Qualitative Methane Detection From Mineralization of Chlorophenols

Reactor/ Substrate	Phase	Date (d/m/yr)	Description of Procedure	Reactor Headspace Composition
A2/2-CP	2	5/7/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	2	17/7/92	GC analyses for CH_4	CH_4 detected
	2	19/7/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	2	1/8/92	GC analyses for CH_4	CH_4 detected
	3	10/8/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	3	24/8/92	GC analyses for CH_4	CH_4 detected
	3	4/9/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	3	11/9/92	GC analyses for CH_4	CH_4 detected
	4	23/9/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	4	30/9/92	GC analyses for CH_4	CH_4 detected
	4	2/10/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	4	7/10/92	GC analyses for CH_4	CH_4 detected
	4	11/11/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	4	25/11/92	GC analyses for CH_4	CH_4 detected
A8/4-CP	2	5/7/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	2	17/7/92	GC analyses for CH_4	CH_4 detected
	2	19/7/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	2	1/8/92	GC analyses for CH_4	CH_4 detected
	3	10/8/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	3	24/8/92	GC analyses for CH_4	CH_4 detected
	3	4/9/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	3	11/9/92	GC analyses for CH_4	CH_4 detected
	4	4/10/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	4	7/10/92	GC analyses for CH_4	CH_4 detected
	4	11/11/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	4	25/11/92	GC analyses for CH_4	CH_4 detected

TABLE I: Qualitative Methane Detection From Mineralization of Chlorophenols

Reactor/ Substrate	Phase	Date (d/m/yr)	Description of Procedure	Reactor Headspace Composition
B2/2-CP	2	11/7/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	2	17/7/92	GC analyses for CH_4	CH_4 detected
	2	5/8/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	3	24/8/92	GC analyses for CH_4	CH_4 detected
	3	4/9/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	3	11/9/92	GC analyses for CH_4	CH_4 detected
	4	2/10/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	4	7/10/92	GC analyses for CH_4	CH_4 detected
	4	15/10/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	4	6/11/92	GC analyses for CH_4	CH_4 detected
	4	11/11/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	4	25/11/92	GC analyses for CH_4	CH_4 detected
B8/4-CP	2	11/7/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	2	17/7/92	GC analyses for CH_4	CH_4 not detected
	3	4/9/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	3	11/9/92	GC analyses for CH_4	CH_4 not detected
	4	4/10/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	4	7/10/92	GC analyses for CH_4	CH_4 not detected
	4	11/11/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	4	25/11/92	GC analyses for CH_4	CH_4 detected
C8/4-CP	2	5/7/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	2	17/7/92	GC analyses for CH_4	CH_4 detected
	3	10/8/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	3	24/8/92	GC analyses for CH_4	CH_4 detected
	3	4/9/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	3	11/9/92	GC analyses for CH_4	CH_4 detected
	4	4/10/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	4	7/10/92	GC analyses for CH_4	CH_4 detected

TABLE I: Qualitative Methane Detection from Mineralization of Chlorophenols

Reactor/ Substrate	Phase	Date (d/m/yr)	Description of Procedure	Reactor Headspace Composition
C8/4-CP	4	11/11/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	4	25/11/92	GC analyses for CH_4	CH_4 detected
E2/2-CP	2	15/7/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	2	17/7/92	GC analyses for CH_4	CH_4 not detected
E8/4-CP	2	15/7/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	2	17/7/92	GC analyses for CH_4	CH_4 not detected
EC8/4-CP	2	15/7/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	2	17/7/92	GC analyses for CH_4	CH_4 not detected
F2/2-CP	3	24/8/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	3	24/8/92	GC analyses for CH_4	CH_4 not detected
	3	4/9/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	3	11/9/92	GC analyses for CH_4	CH_4 not detected
F8/4-CP	3	24/8/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	3	24/8/92	GC analyses for CH_4	CH_4 not detected
	3	4/9/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	3	11/9/92	GC analyses for CH_4	CH_4 detected
FC8/4-CP	3	24/8/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	3	24/8/92	GC analyses for CH_4	CH_4 not detected
	3	4/9/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	3	11/9/92	GC analyses for CH_4	CH_4 not detected
G2/2-CP	4	23/9/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	4	30/9/92	GC analyses for CH_4	CH_4 detected
	4	2/10/92	Stopper exchanged, oxygen free gas flush	N_2 and CO_2
	4	7/10/92	GC analyses for CH_4	CH_4 not detected

CP - Chlorophenol; 2-CP, 2-Chlorophenol; 4-CP, 4-Chlorophenol.

Appendix II
Total Solids Determination

TABLE II: Total Solids Determination

Reactor/ Substrate	Phase	Date (d/m/yr)	Solids Wt. (mg)	Volume (mL)	Biomass (mg/L)	Comments
B2/2-CP	2	5/7/92	12.0	5	2400	
	4	23/9/92	11.3	5	2260	
	4	10/11/92	11.5	5	2290	
B8/4-CP	2	5/7/92	14.9	5	2980	
	4	10/11/92	11.4	5	2284	Reactor Failure
G2/2-CP	4	23/9/92	0.15	5	30	

CP - Chlorophenol; 2-CP, 2-Chlorophenol; 4-CP, 4-Chlorophenol.

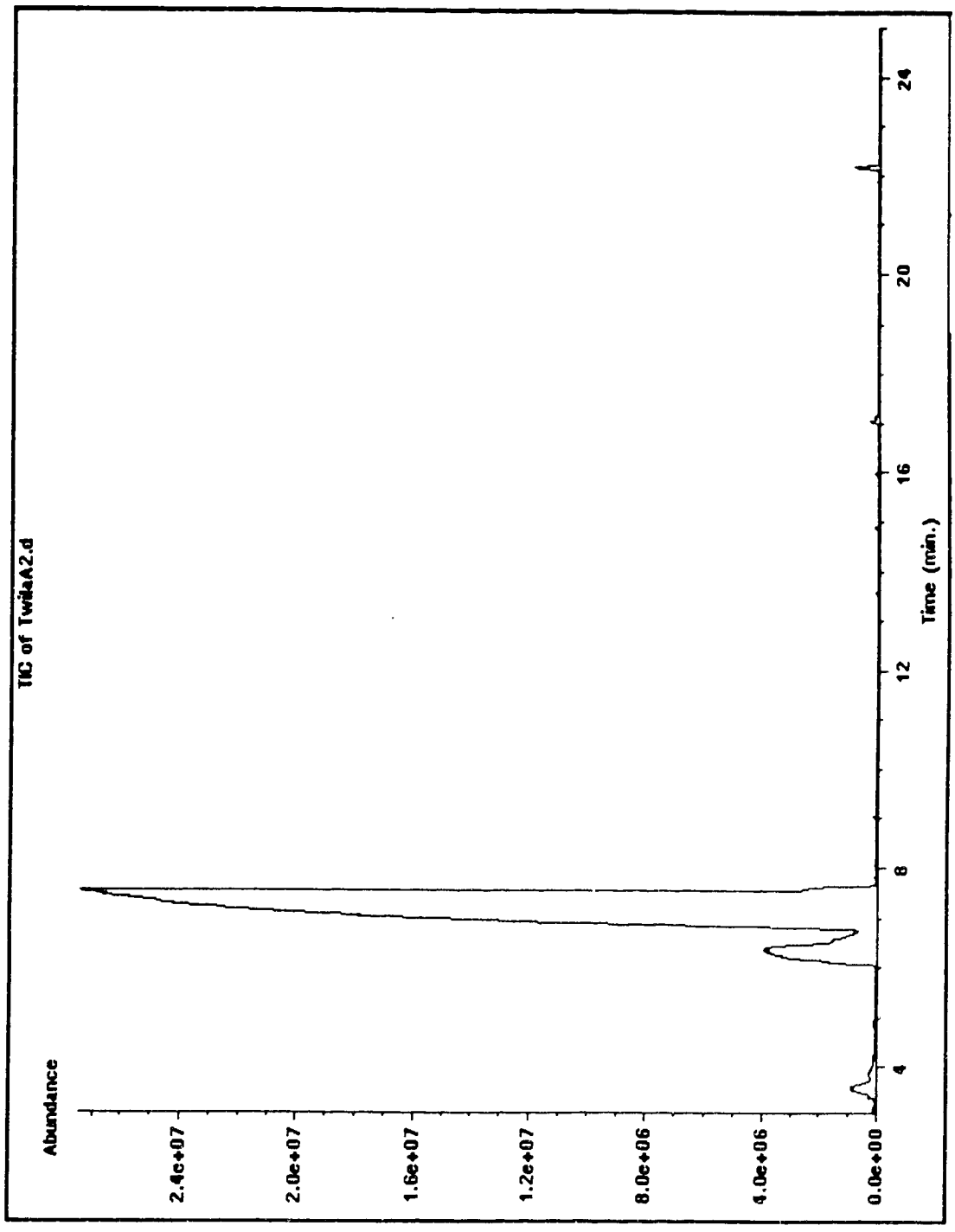
Appendix III
Qualitative Amino Acid Determination

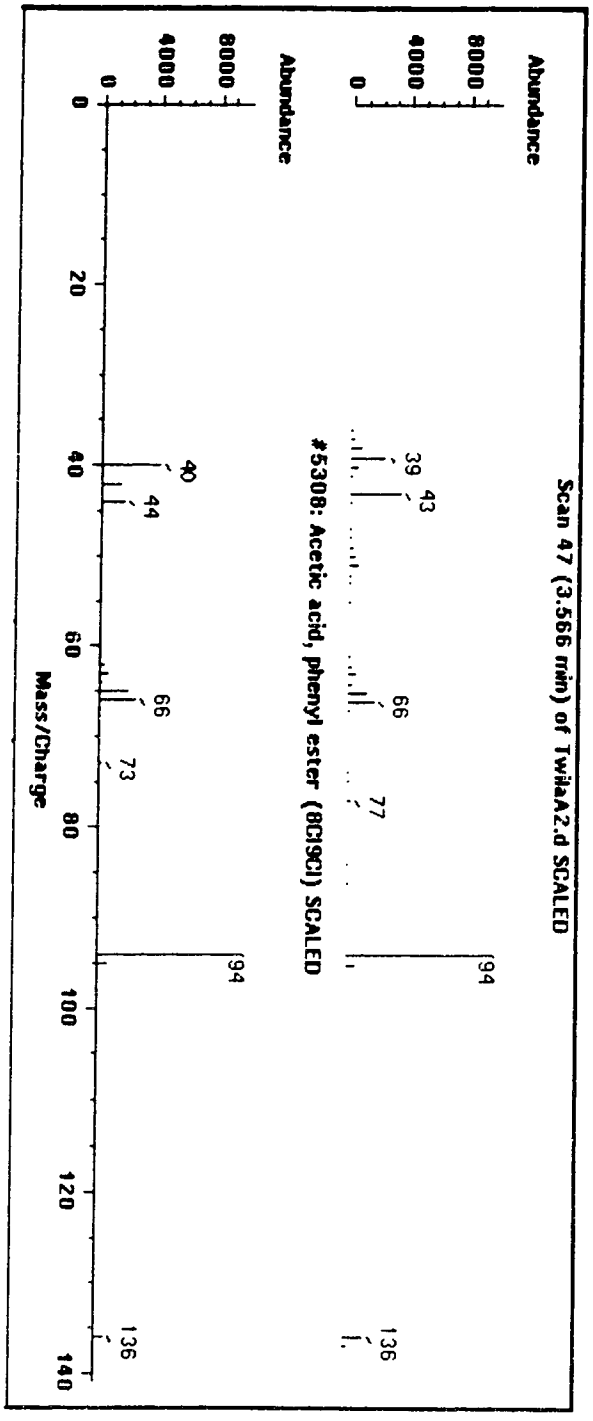
TABLE III: Qualitative Amino Acid Determination

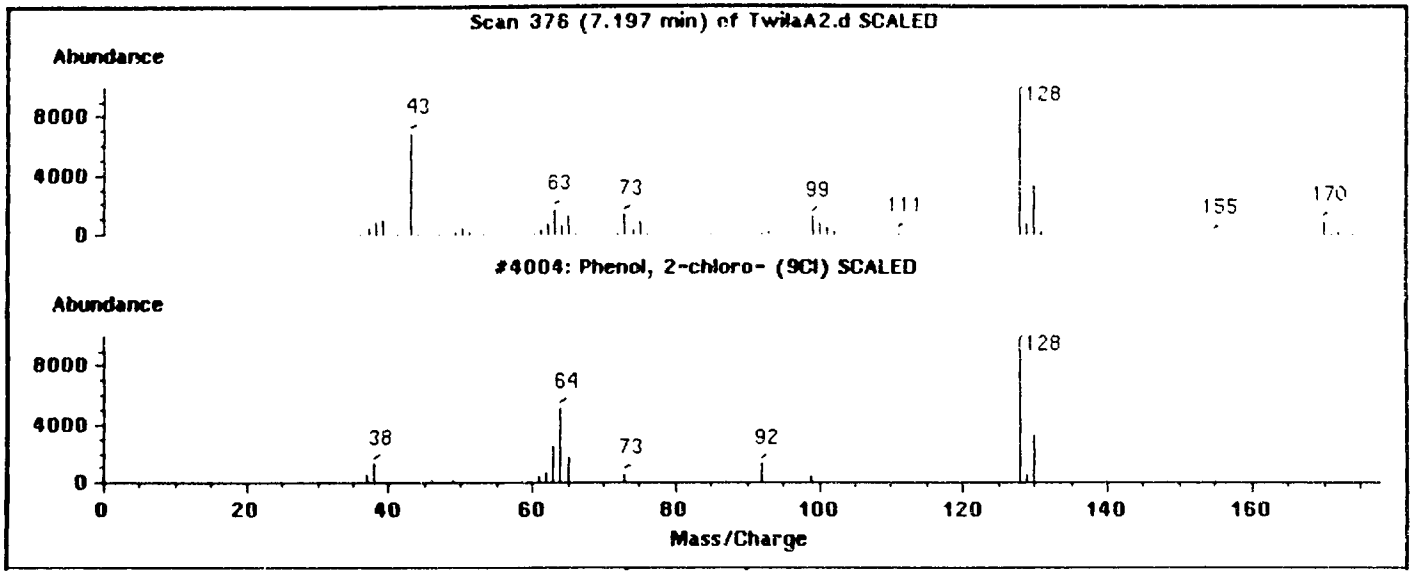
Reactor	Color After Addition of Ninhydrin	Color After Heating	Amino Acids	Comments
A2	pale pink	red-brown	A	
C9a	clear	red-brown	A	
C9b	clear	red-brown	A	
Mineral Media Solution	red-orange	dark purple to black	P	Contains p-Aminobenzoic Acid

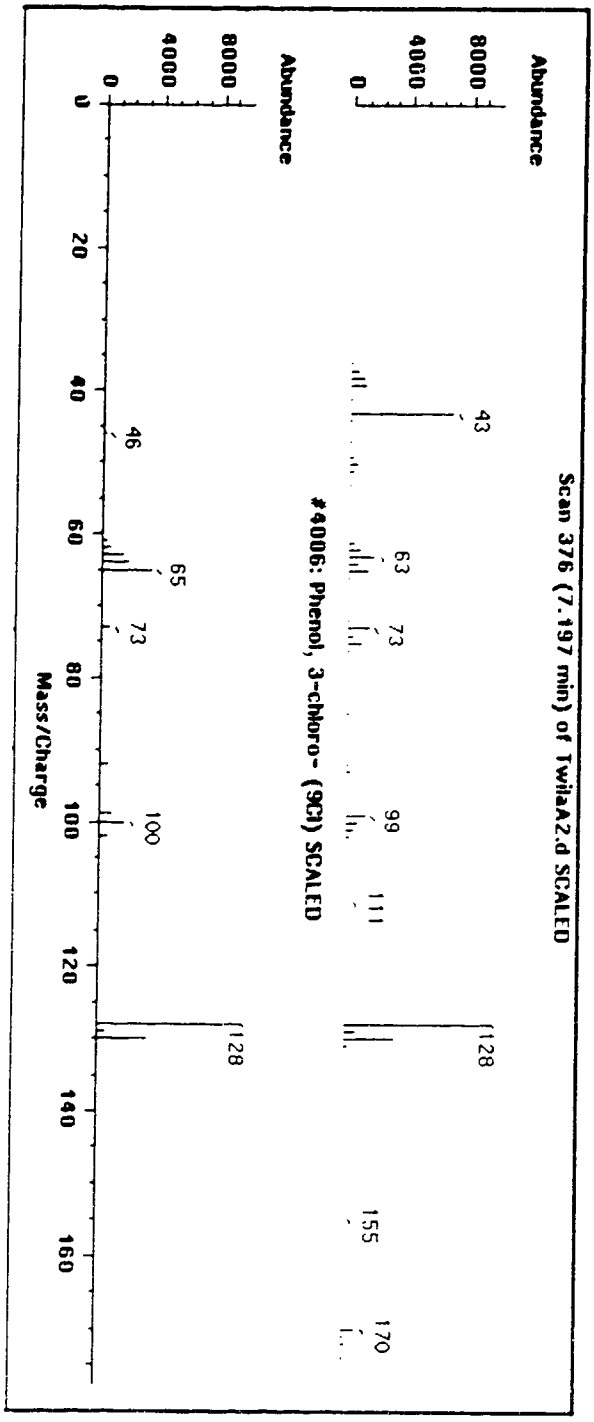
P - Present; A- Absent

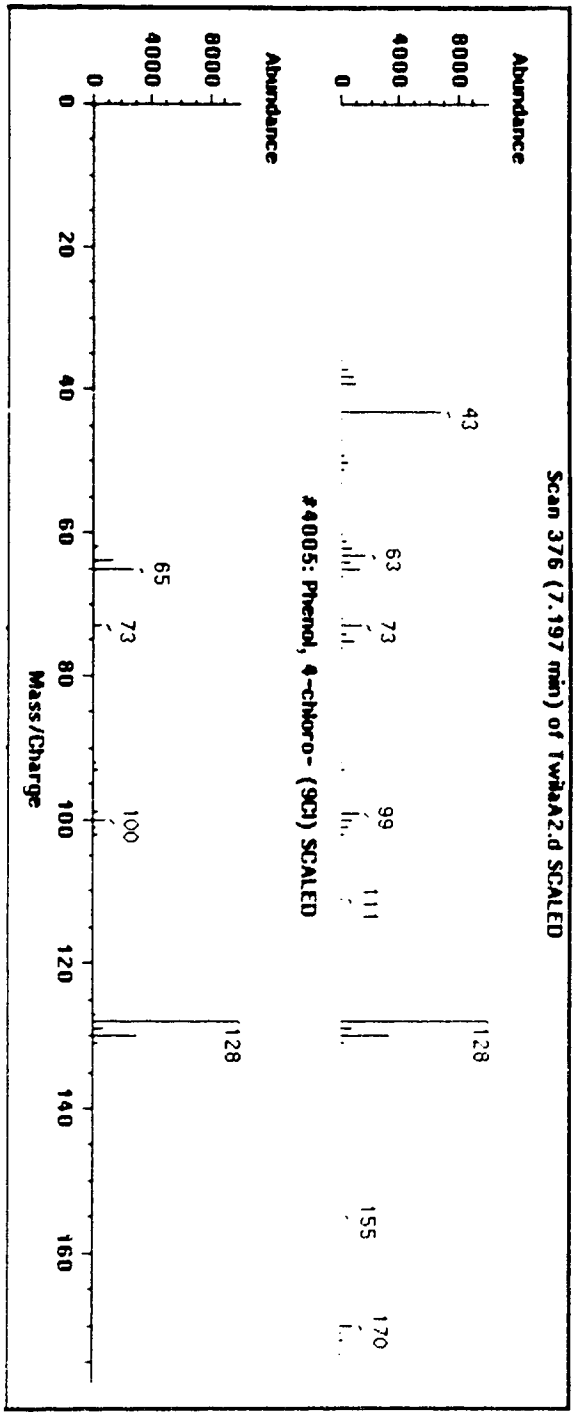
Appendix IV
Analyses of Derivatized Chlorophenols and By-Products

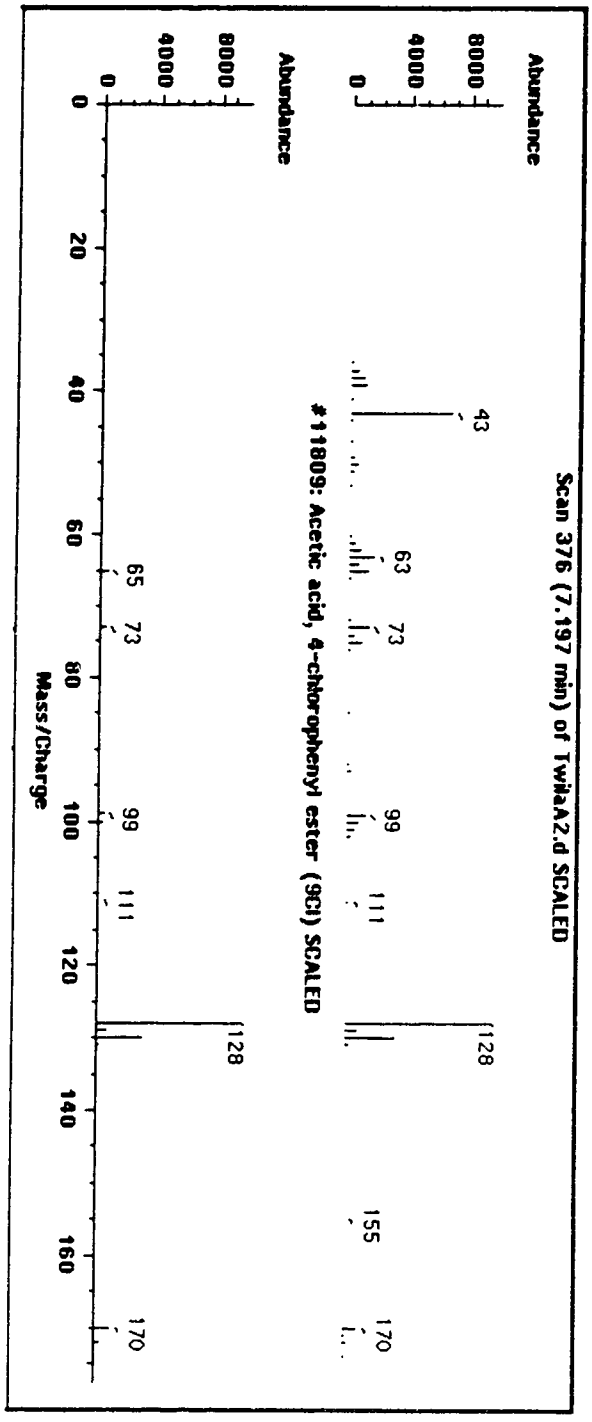

TIC of TwikaA2.d
Abundance
2.4e+07
2.0e+07
1.6e+07
1.2e+07
8.0e+06
4.0e+06
0.0e+00
4
8
12
16
20
24
Time (min.)

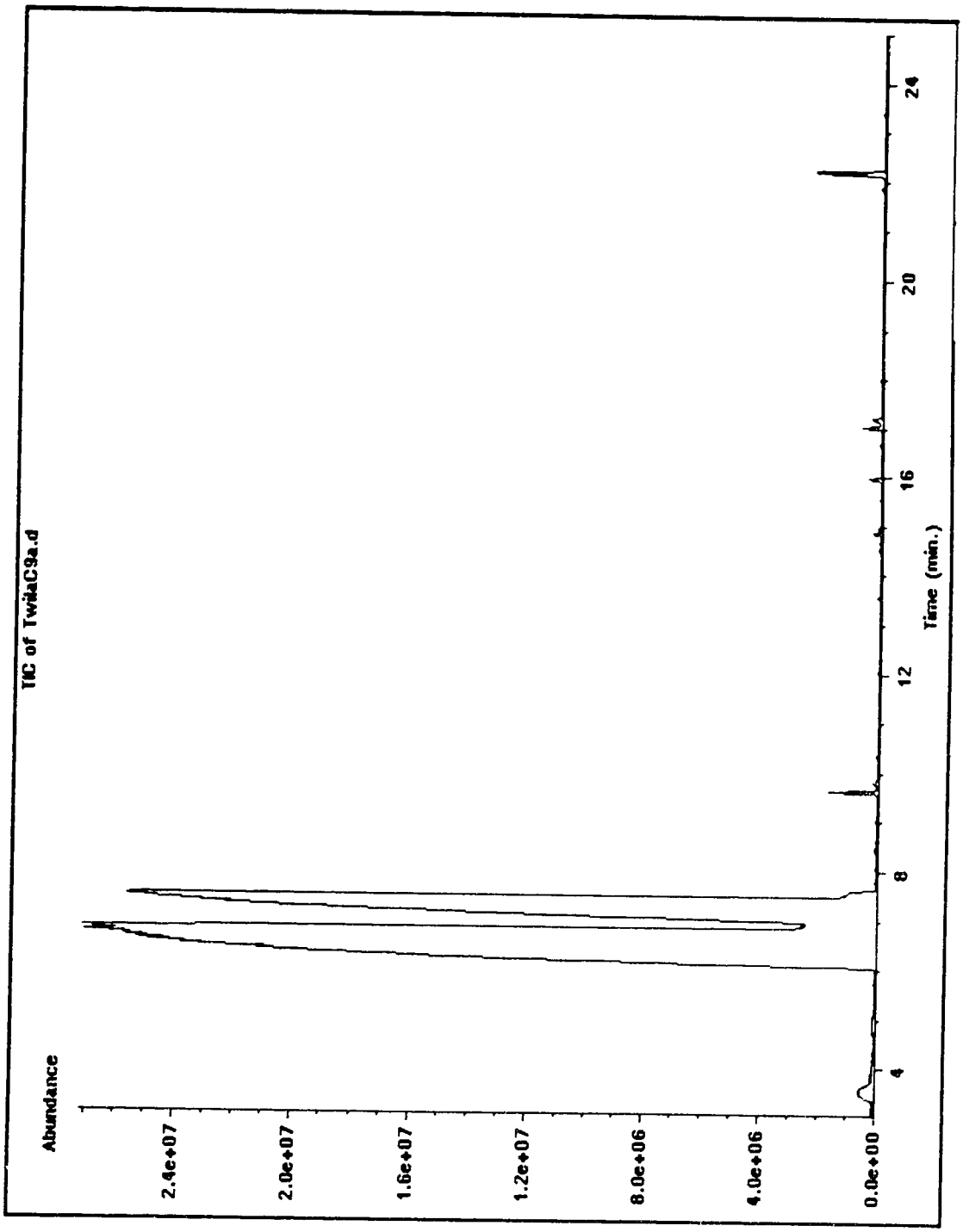

Scan 47 (3.566 min) of TwilaA2.d SCALED
Abundance
8000
4000
0
39
43
66
77
94
136
#5308: Acetic acid, phenyl ester (8CI,9CI) SCALED
Abundance
8000
4000
0
40
44
66
73
94
136
0
20
40
60
80
100
120
140
Mass/Charge

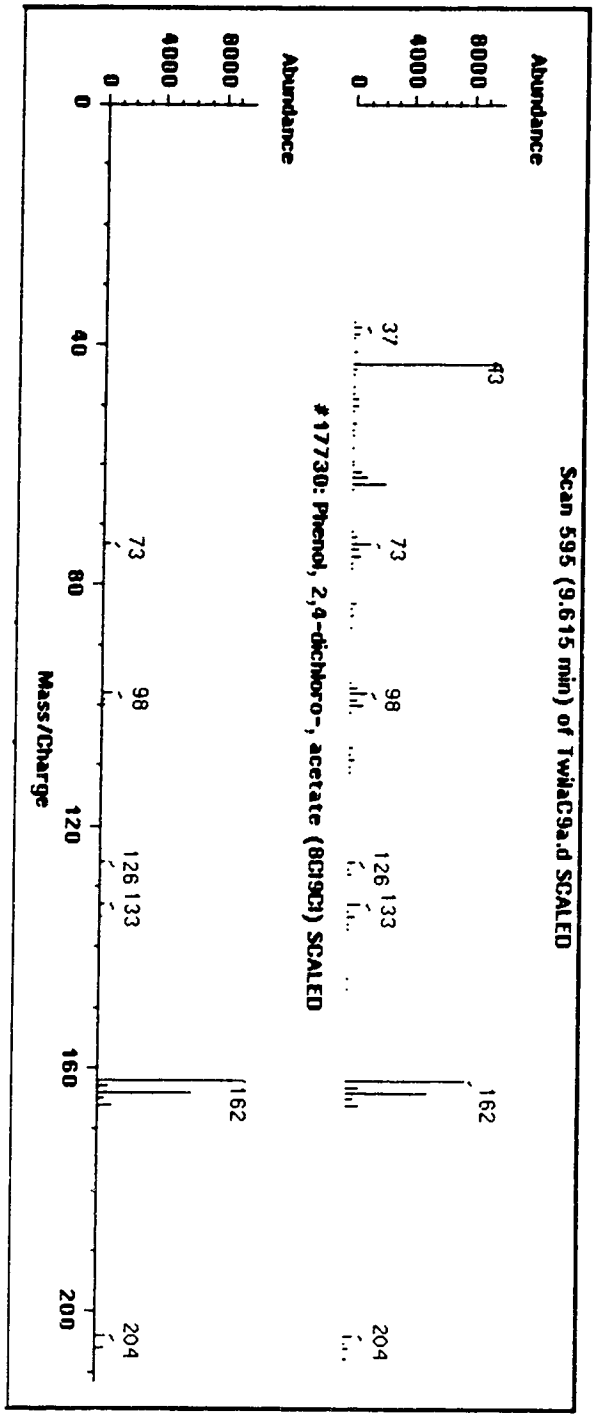

Scan 376 (7.197 min) of TwilaA2.d SCALED
Abundance
8000
4000
0
43
63
73
99
111
128
155
170
#4004: Phenol, 2-chloro- (9CI) SCALED
Abundance
8000
4000
0
38
64
73
92
128
0
20
40
60
80
100
120
140
160
Mass/Charge

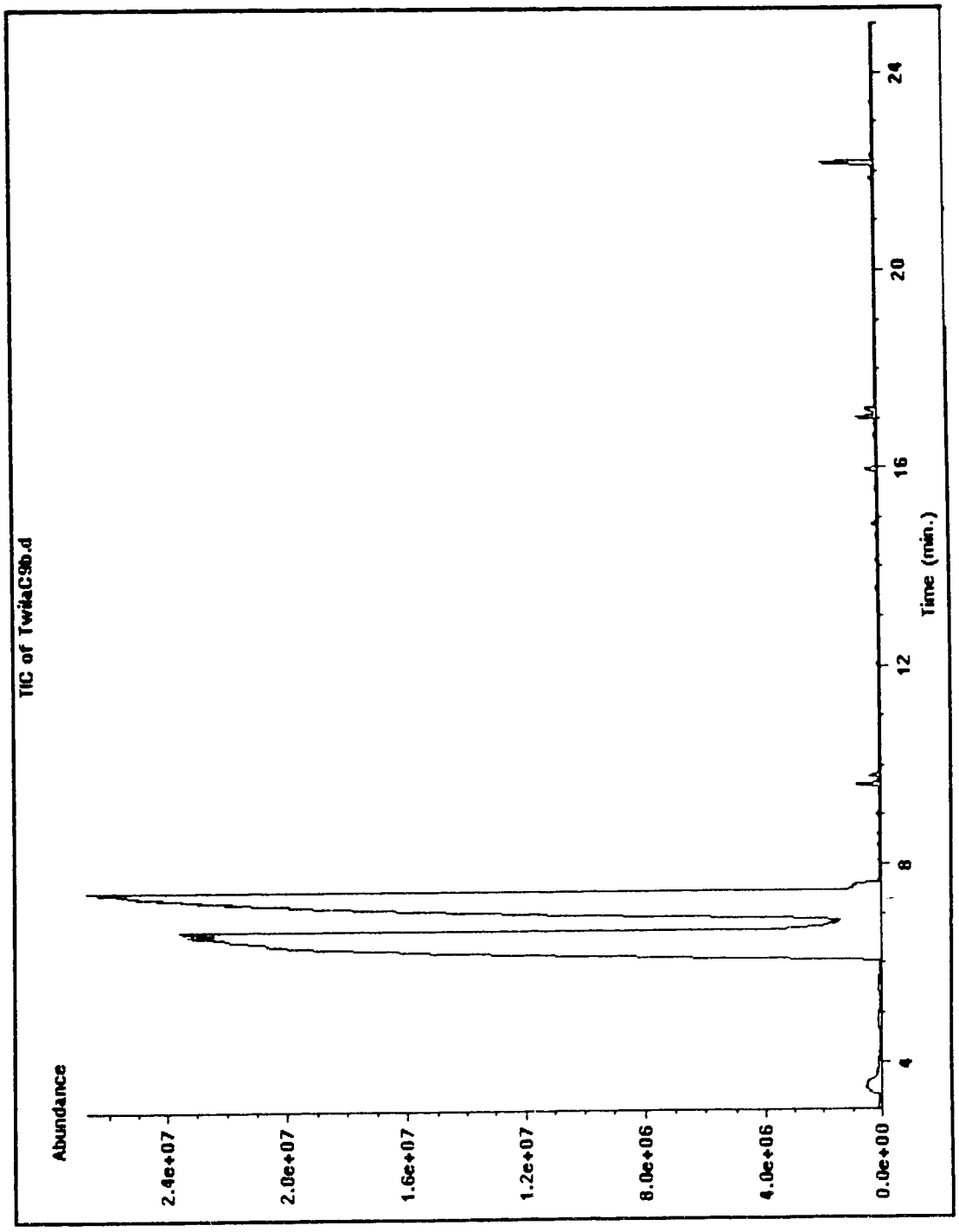

Scan 376 (7.197 min) of TwilaA2.d SCALED
Abundance
8000
4000
0
43
63
73
99
111
128
155
170
#4006: Phenol, 3-chloro- (9CI) SCALED
Abundance
8000
4000
0
46
65
73
100
128
0
20
40
60
80
100
120
140
160
Mass/Charge

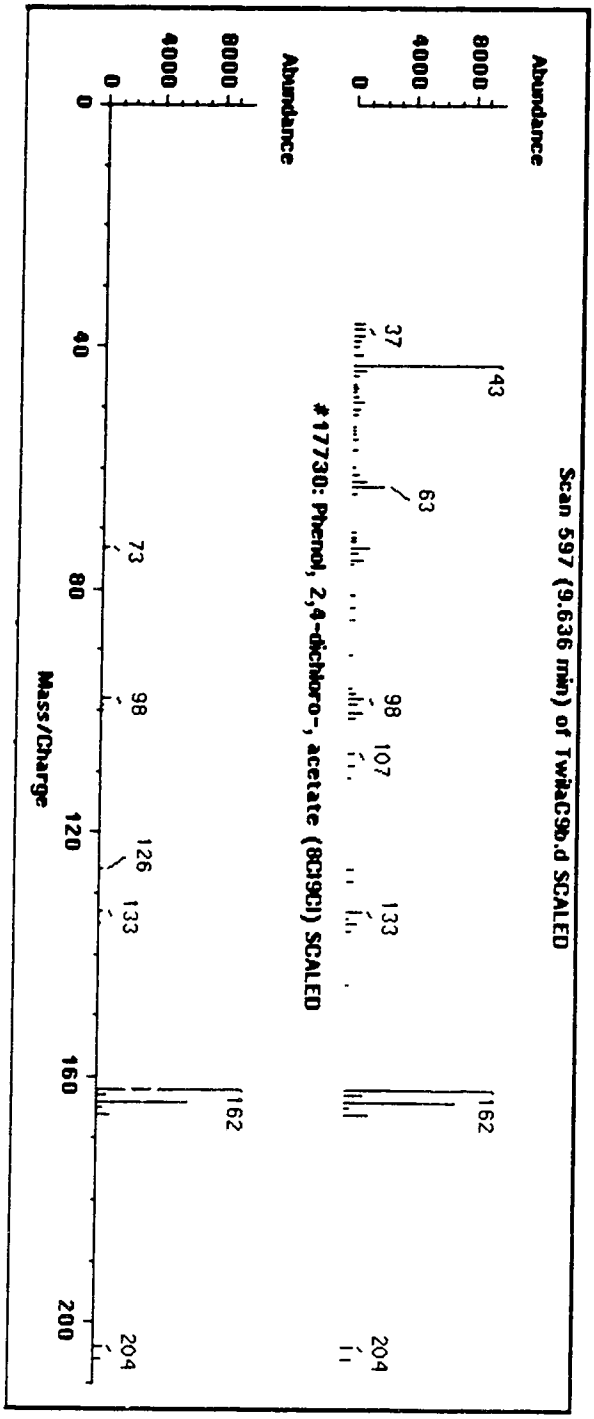

Scan 376 (7.197 min) of TwibaA2.d SCALED
Abundance
8000
4000
0
43
63
73
99
111
128
155
170
#4005: Phenol, 4-chloro- (9CI) SCALED
Abundance
8000
4000
0
65
73
100
128
0
20
40
60
80
100
120
140
160
Mass/Charge

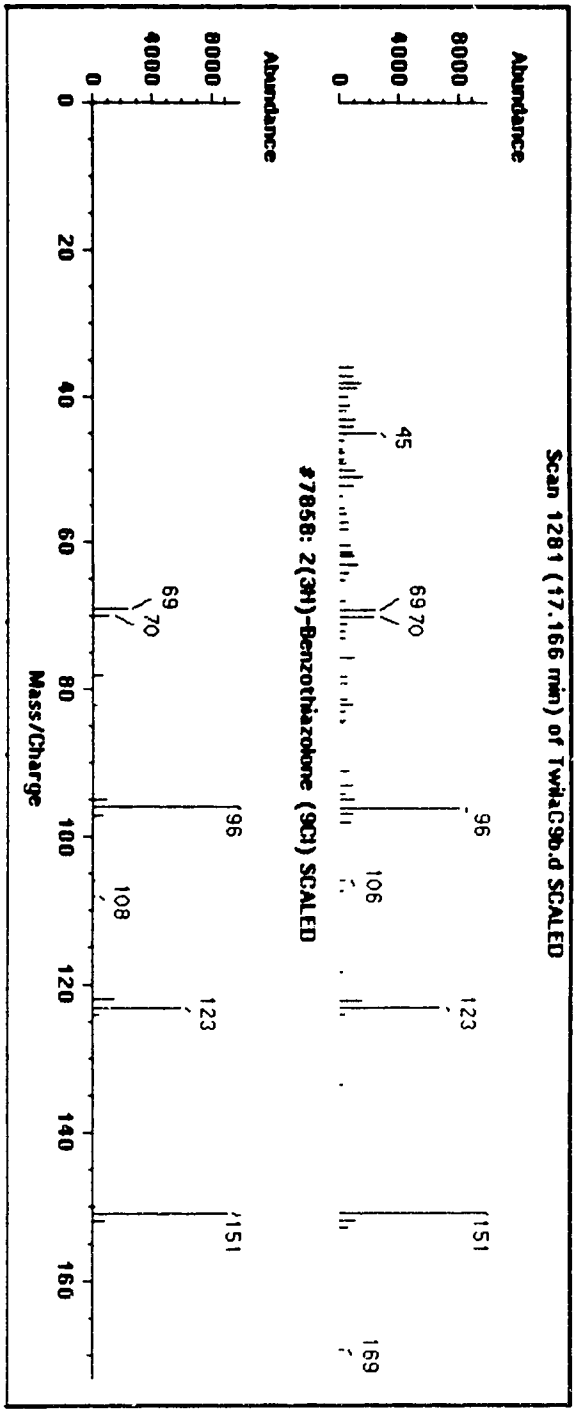

Scan 376 (7.197 min) of TwikaA2.d SCALED
Abundance
8000
4000
0
43
63
73
99
111
128
155
170
#11809: Acetic acid, 4-chlorophenyl ester (9CI) SCALED
Abundance
8000
4000
0
65
73
99
111
128
170
0
20
40
60
80
100
120
140
160
Mass/Charge

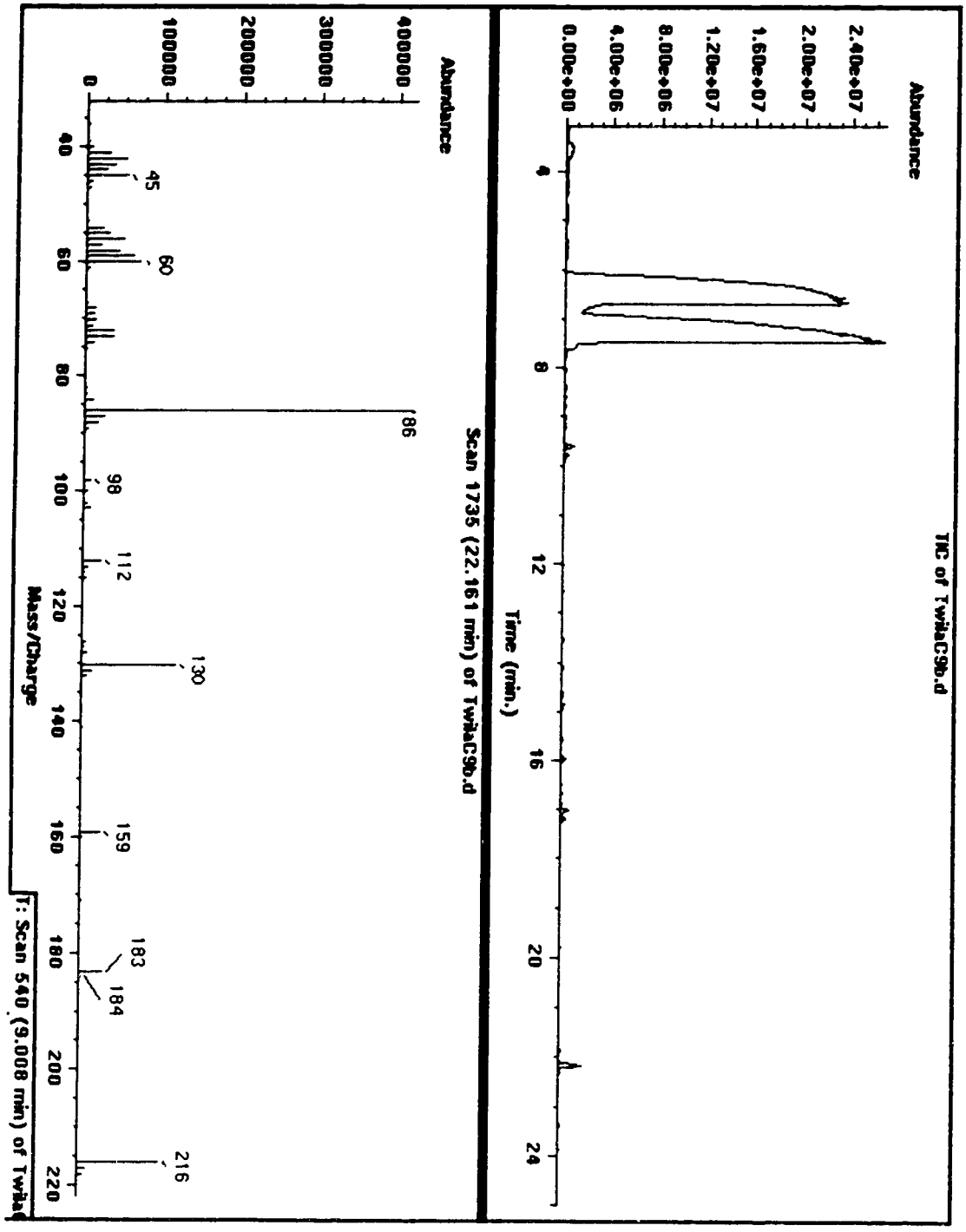

TIC of TwikaC9a.d
Abundance
2.4e+07
2.0e+07
1.6e+07
1.2e+07
8.0e+06
4.0e+06
0.0e+00
4
8
12
16
20
24
Time (min.)

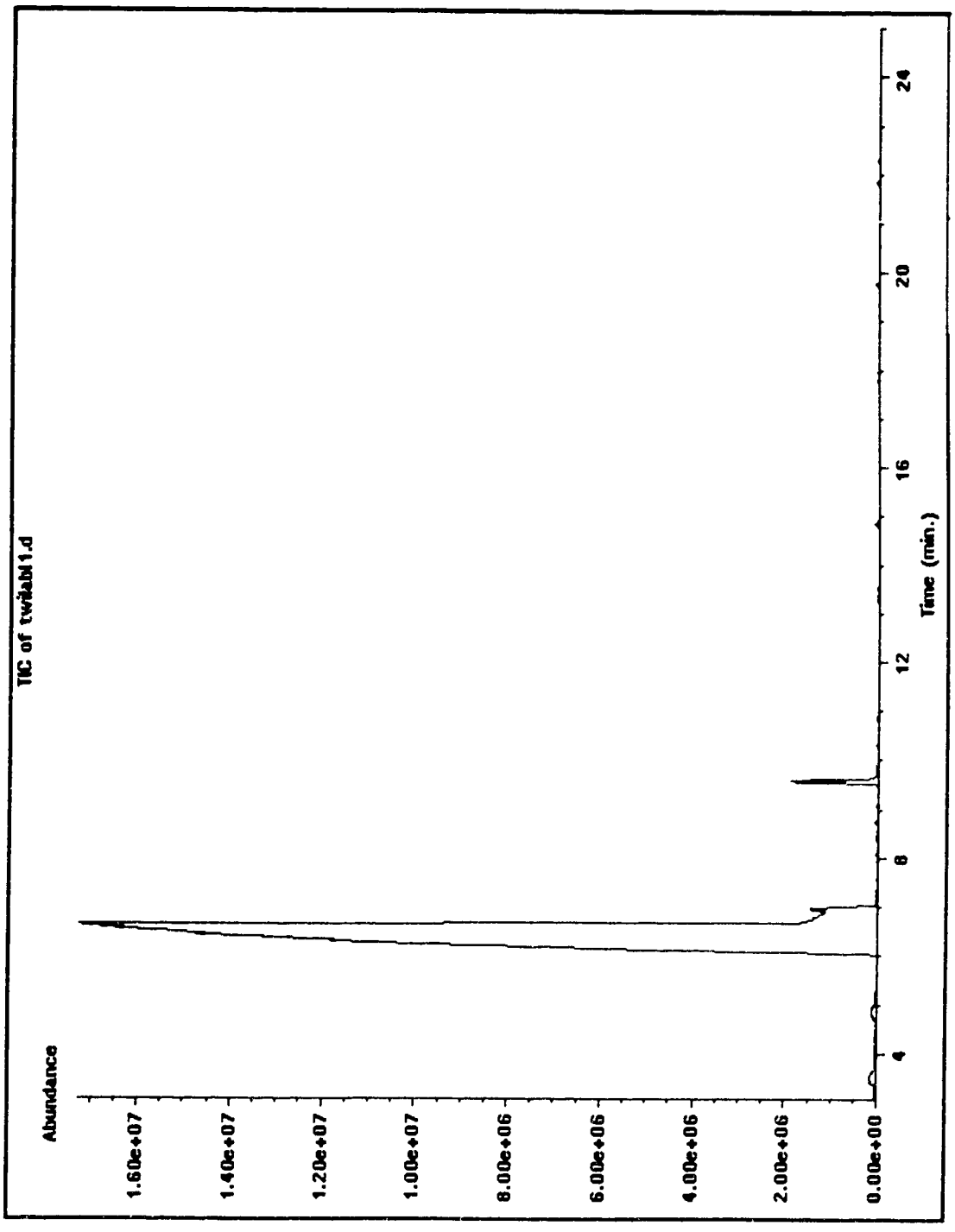

Scan 595 (9.615 min) of TwilaC9a.d SCALED
Abundance
8000
4000
0
43
37
73
98
126
133
162
204
#17730: Phenol, 2,4-dichloro-, acetate (8CI,9CI) SCALED
Abundance
8000
4000
0
162
73
98
126
133
204
0
40
80
120
160
200
Mass/Charge

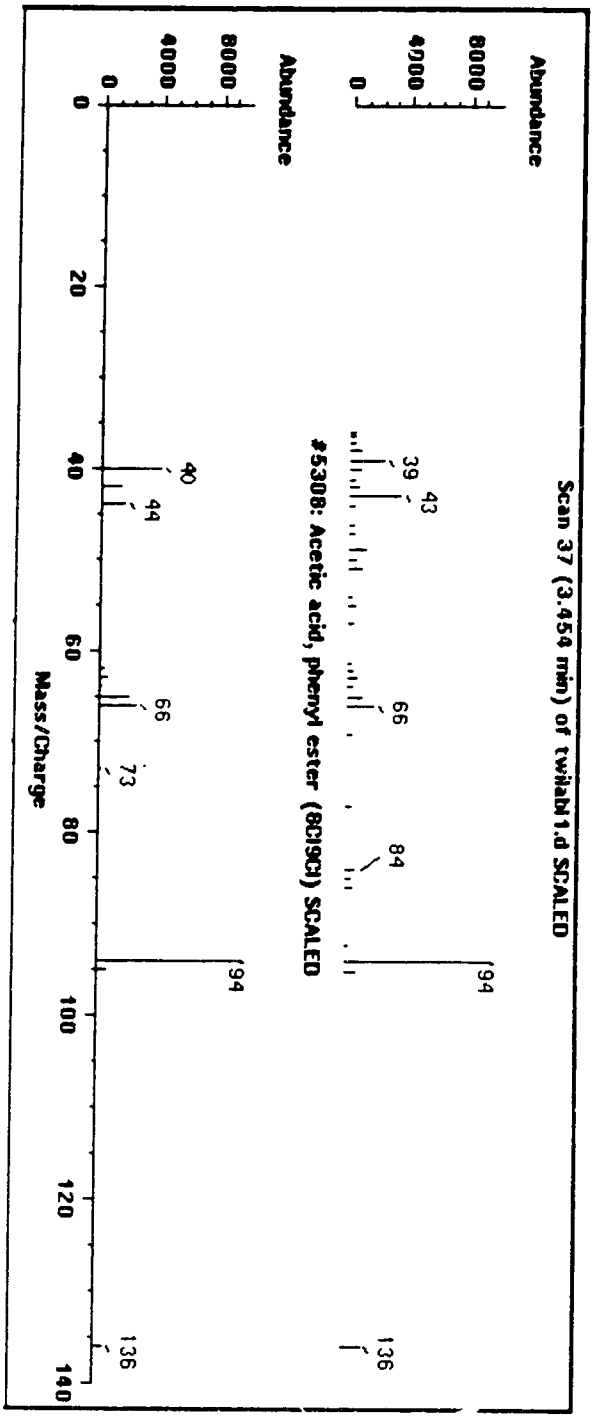

TIC of TwikaC9b.d
Abundance
2.4e+07
2.0e+07
1.6e+07
1.2e+07
8.0e+06
4.0e+06
0.0e+00
4
8
12
16
20
24
Time (min.)

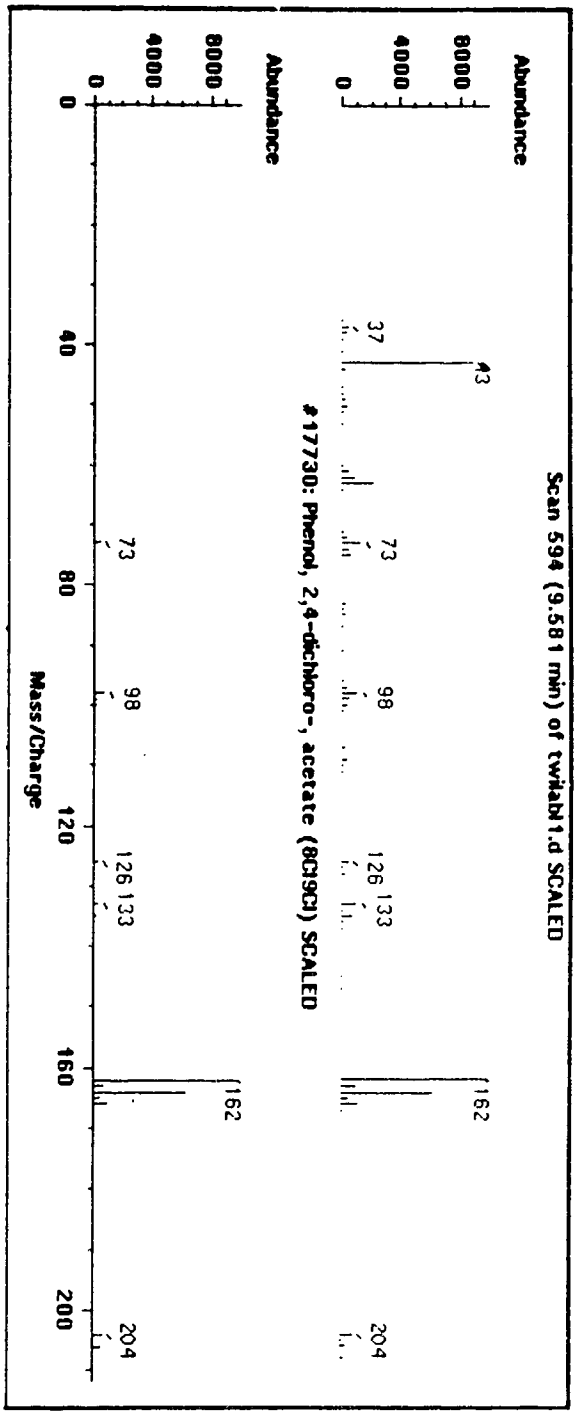

Scan 597 (9.636 min) of TwiltaC9b.d SCALED
Abundance
8000
4000
0
37
43
63
98
107
133
162
204
#17730: Phenol, 2,4-dichloro-, acetate (8CI,9CI) SCALED
Abundance
8000
4000
0
73
98
126
133
162
204
0
40
80
120
160
200
Mass/Charge

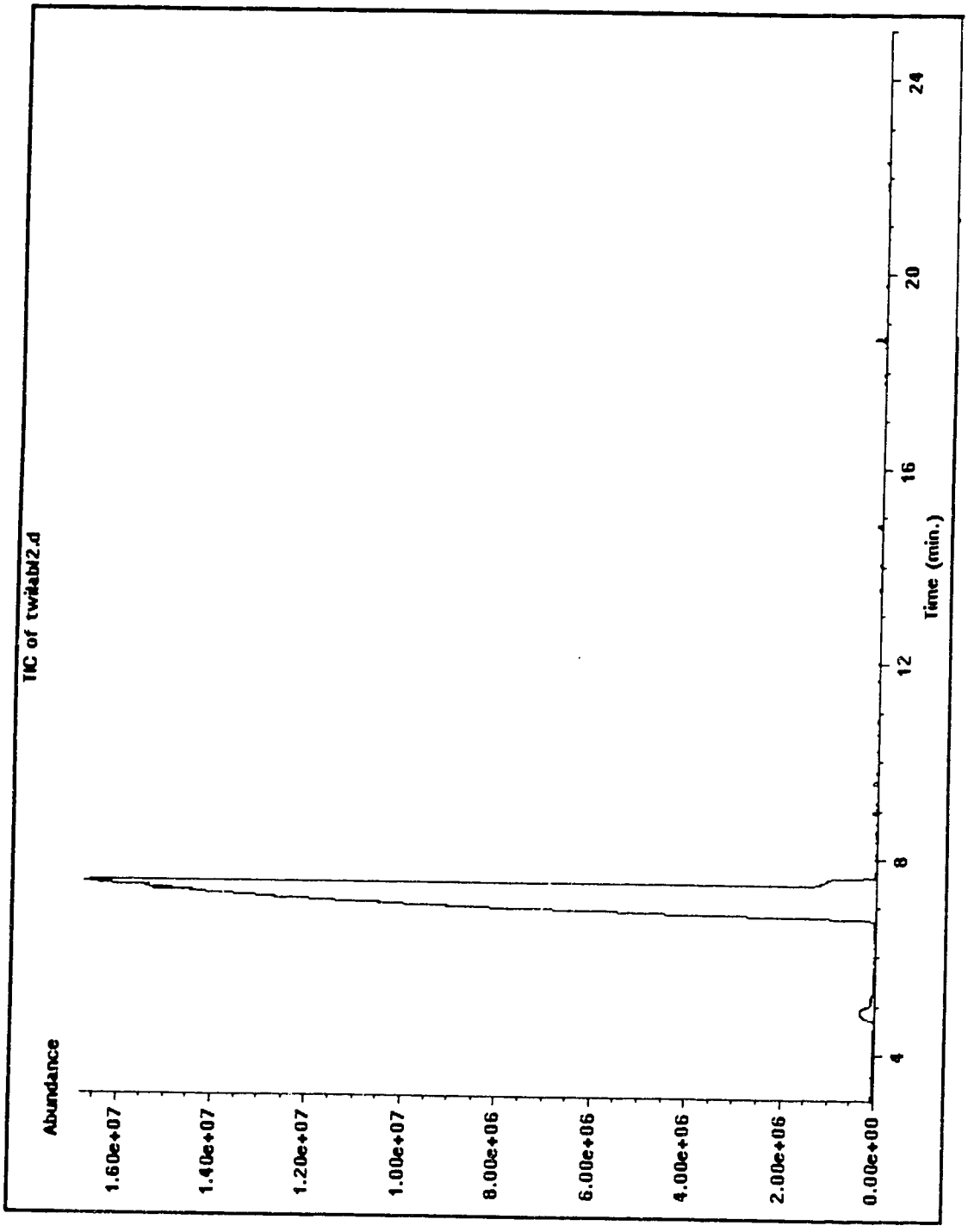

Scan 1281 (17.166 min) of TwiaC9b.d SCALED
Abundance
8000
4000
0
45
69 70
96
106
123
151
169
#7858: 2(3H)-Benzothiazolone (9CI) SCALED
Abundance
8000
4000
0
69
70
96
108
123
151
0
20
40
60
80
100
120
140
160
Mass/Charge


TIC of TwikaC9b.d
Abundance
Time (min.)
Scan 1735 (22.161 min) of TwikaC9b.d
Abundance
Mass/Charge
86
45
60
98
112
130
159
183
184
216
T: Scan 540 (9.008 min) of Twika


TIC of twiabl1.d
Abundance
1.60e+07
1.40e+07
1.20e+07
1.00e+07
8.00e+06
6.00e+06
4.00e+06
2.00e+06
0.00e+00
4
8
12
16
20
24
Time (min.)


Scan 37 (3.454 min) of twiab11.d SCALED
Abundance
8000
4000
0
39
43
66
84
94
136
#5308: Acetic acid, phenyl ester (8CI,9CI) SCALED
Abundance
8000
4000
0
40
44
66
73
94
136
0
20
40
60
80
100
120
140
Mass/Charge


Scan 594 (9.581 min) of twiab11.d SCALED
Abundance
8000
4000
0
43
37
73
98
126
133
162
204
#17730: Phenol, 2,4-dichloro-, acetate (8CI,9CI) SCALED
Abundance
8000
4000
0
73
98
126
133
162
204
0
40
80
120
160
200
Mass/Charge


TIC of twiabl2.d
Abundance
1.60e+07
1.40e+07
1.20e+07
1.00e+07
8.00e+06
6.00e+06
4.00e+06
2.00e+06
0.00e+00
4
8
12
16
20
24
Time (min.)

Appendix V
Statistical Analyses

TABLE V.A: Regression Analysis for Figure 5.14

Regression Statistics			
	A2	**B2**	**E2**
Multiple R	0.99	0.95	0.94
R Squared	0.97	0.89	0.89
Adjusted R Square	0.93	0.84	0.81
Standard Error	6.08	8.22	4.33
Observations	23	20	15
Slope	**5.18**	**4.94**	**2.71**
Analysis of Variance			
Degrees of Freedom			
Regression	1	1	1
Residual	22	19	14
Total	23	20	15
Sum of Squares			
Regression	2,791.89	10,797.23	2,028.89
Residual	812.55	1,283.57	262.45
Total	28,004.43	12,080.80	2,291.33
Mean Square			
Regression	27,191.89	1,097.23	2,028.89
Residual	36.93	67.56	18.75
F			
Regression	736.23	159.83	108.23
Significance F			
Regression	7.81E-18	2.17E-10	1.14E-07
Coefficients X 1	4.89	4.12	2.45
Intercept	0	0	0
Standard Error	0.09	0.14	0.11
t Statistic	56.46	28.82	21.73
P-Value	3.57E-26	9.20E-18	9.42E-13
Lower 95%	4.71	3.82	2.21
Upper 95%	5.07	4.42	2.70

TABLE V.B: Regression Analysis for Figure 5.15

Regression Statistics			
	A8	**B8**	**E8**
Multiple R	0.90	0.86	0.84
R Squared	0.97	0.74	0.71
Adjusted R Square	0.94	0.72	0.68
Standard Error	6.52	3.99	5.63
Observations	29	17	12
Slope	**5.17**	**1.27**	**2.29**
Analysis of Variance			
Degrees of Freedom			
Regression	1	1	1
Residual	28	15	10
Total	29	16	11
Sum of Squares			
Regression	4,584.38	672.74	783.50
Residual	1,188.86	239.14	317.42
Total	46,973.24	911.88	1,100.92
Mean Square			
Regression	45,784.38	672.74	783.50
Residual	42.46	15.94	31.74
F			
Regression	1,078.31	42.20	24.68
Significance F			
Regression	2.65E-23	1.01E-05	5.63E-04
Coefficients	4.83	7.59	1.69
Intercept	0	1.06	-6.20
Standard Error	0.07	2.05	0.34
t Statistic	73.44	0.16	4.97
P-Value	1.69E-34	7.38E-06	4.23E-04
Lower 95%	4.70	0.71	0.93
Upper 95%	4.97	1.41	2.45

TABLE V.C: Regression Analysis for Figure 5.20

Regression Statistics			
	A2	**B2**	**F2**
Multiple R	0.99	0.95	0.94
R Squared	0.98	0.90	0.88
Adjusted R Square	0.94	0.87	0.81
Standard Error	7.07	8.34	6.79
Observations	30	30	16
Slope	**5.13**	**3.42**	**3.12**
Analysis of Variance			
Degrees of Freedom			
Regression	1	1	1
Residual	29	29	15
Total	30	30	16
Sum of Squares			
Regression	58,426.22	18,645.02	5,147.03
Residual	1,449.15	2,018.34	690.97
Total	59,875.37	20,663.37	5,838.00
Mean Square			
Regression	58,426.22	18,645.02	5,147.03
Residual	49.97	69.60	46.06
F			
Regression	1,169.21	267.90	111.73
Significance F			
Regression	2.21E-24	7.22E-16	4.68E-08
Coefficients	3.96	2.65	3.30
Intercept	0	0.00	0.00
Standard Error	0.06	0.07	0.14
t Statistic	63.61	35.96	23.18
P-Value	1.46E-33	3.15E-26	9.74E-14
Lower 95%	3.83	2.50	3.00
Upper 95%	4.06	2.80	3.60

TABLE V.D: Regression Analysis for Figure 5.21

Regression Statistics			
	A8	**B8**	**F8**
Multiple R	0.92	0.82	0.89
R Squared	0.85	0.68	0.79
Adjusted R Square	0.84	0.64	0.69
Standard Error	4.85	2.29	2.85
Observations	16	10	4
Slope	**2.22**	**1.03**	**3.51**
Analysis of Variance			
Degrees of Freedom			
Regression	1	1	1
Residual	14	8	2
Total	15	9	3
Sum of Squares			
Regression	1,828.07	87.58	62.48
Residual	328.87	42.02	16.27
Total	2,156.94	129.60	78.75
Mean Square			
Regression	1,828.07	87.58	62.48
Residual	23.49	5.25	8.13
F			
Regression	77.82	16.67	7.68
Significance F			
Regression	4.31E-07	3.52E-03	1.09E-01
Coefficients	1.78	1.03	2.19
Intercept	-6.25	-2.11	-27.42
Standard Error	0.20	0.25	0.79
t Statistic	8.82	4.08	2.77
P-Value	2.53E-07	2.75E-03	6.95E-02
Lower 95%	1.35	0.45	-1.21
Upper 95%	2.22	1.61	5.60

TABLE V.E: Regression Analysis for Figure 5.25

Regression Statistics			
	A2	**B2**	**G2**
Multiple R	0.99	0.85	0.88
R Squared	0.99	0.73	0.77
Adjusted R Square	0.96	0.69	0.71
Standard Error	6.43	10.92	7.22
Observations	41	24	17
Slope	**4.81**	**3.9**	**3.63**
Analysis of Variance			
Degrees of Freedom			
Regression	1	1	1
Residual	40	23	16
Total	41	24	17
Sum of Squares			
Regression	118,586.74	7,396.89	2,783.52
Residual	1,679.75	2,740.74	834.95
Total	120,266.49	10,137.63	3,618.47
Mean Square			
Regression	118,586.74	7,396.89	2,783.52
Residual	41.99	119.16	52.18
F			
Regression	2,823.92	62.07	53.34
Significance F			
Regression	5.30E+38	7.60E-08	2.60E-06
Coefficients	2.72	2.50	2.26
Intercept	0.00	0.00	0.00
Standard Error	0.02	0.10	0.10
t Statistic	110.71	25.64	21.71
P-Value	2.06E-52	5.96E-19	7.78E-14
Lower 95%	2.67	2.30	2.04
Upper 95%	2.77	2.70	2.48

TABLE V.F: Statistical Analysis for Figure 5.10
Attached Growth Reactor A2

Statistical Parameter	Influent (Original)	Effluent (Original)	Influent (Modified)	Effluent (Modified)
Mean	50.94	46.13	**50.8**	**46.24**
Standard Error	0.43	0.54	0.33	0.4
Median	50	46	50	46
Mode	50	44	50	44
Standard Deviation	4.04	5.06	2.92	3.6
Variance	16.35	25.57	8.52	12.94
Kurtosis	3.49	4.65	2.07	-0.19
Skewness	-0.08	-0.82	0.37	-0.05
Range	27	38	18	17
Minimum	35	24	41	38
Maximum	62	62	59	55
Sum	4,534	4,106	4064	3699
Count	89	89	80	80
95.44% Probability				
Std Dev x 2	8.09	10.11		
Range - Low	42.86	36.02		
Range - High	59.03	56.25		
99.74% Probability				
Std Dev x 3	12.13	15.17		
Range - Low	-38.81	-30.96		
Range - High	63.07	61.31		

TABLE V.G: Statistical Analysis for Figure 5.11
Suspended Growth Reactor B2

Statistical Parameter	Influent (Original)	Effluent (Original)	Influent (Modified)	Effluent (Modified)
Mean	50.26	47.11	**50.62**	**47.93**
Standard Error	0.49	0.58	0.29	0.43
Median	50	48	50	48
Mode	50	50	50	50
Standard Deviation	4.91	5.78	2.69	4.01
Variance	24.15	33.37	7.26	16.09
Kurtosis	8.74	3.75	3.27	0.8
Skewness	-1.64	-1.41	0.44	-0.58
Range	37	37	19	20
Minimum	27	24	41	37
Maximum	64	61	60	57
Sum	4976	4664	4505	4266
Count	99	99	89	89
95.44% Probability				
Std Dev x 2	9.83	11.55	5.39	8.02
Range - Low	40.43	35.56	45.23	39.91
Range - High	60.09	58.66	56.01	55.95
99.74% Probability				
Std Dev x 3	14.74	17.33	8.08	12.03
Range - Low	35.52	29.78	42.53	35.9
Range - High	65.01	64.44	58.7	59.96

TABLE V.H: Statistical Analysis for Figure 5.12
Attached Growth Reactor A8

Statistical Parameter	Influent (Original)	Effluent (Original)	Influent (Modified)	Effluent (Modified)
Mean	52.71	49.41	**51.81**	**49.52**
Standard Error	0.64	0.81	0.52	0.72
Median	50	49.5	50	49.5
Mode	50	50	50	50
Standard Deviation	4.84	6.19	3.76	5.17
Variance	23.47	38.28	14.16	26.76
Kurtosis	0.73	1.63	0.68	-0.22
Skewness	1.22	-0.26	1.1	0.37
Range	21	36	16	22
Minimum	45	28	45	39
Maximum	66	64	61	61
Sum	3057	2866	2694	2575
Count	58	58	52	52
95.44% Probability				
Std Dev x 2	9.69	12.37	7.53	10.35
Range - Low	43.02	37.04	44.28	39.17
Range - High	62.4	61.79	59.33	59.87
99.74% Probability				
Std Dev x 3	14.53	18.56	11.29	15.52
Range - Low	38.17	30.85	40.52	34
Range - High	67.24	67.98	63.1	65.04

TABLE V.I: Statistical Analysis for Figure 5.13
Suspended Growth Reactor B8

Statistical Parameter	Influent (Original)	Effluent (Original)	Influent (Modified)	Effluent (Modified)
Mean	53.02	52.13	**53.23**	**52.74**
Standard Error	0.5	0.65	0.46	0.57
Median	53	53	53	53
Mode	50	50	50	50
Standard Deviation	3.6	4.6	3.17	3.94
Variance	12.96	21.96	10.05	15.5
Kurtosis	0	-0.02	-0.76	-0.44
Skewness	0.37	-0.59	0.48	-0.25
Range	18	19	12	16
Minimum	44	41	48	44
Maximum	62	60	60	60
Sum	2757	2711	2502	2479
Count	52	52	47	47
95.44% Probability				
Std Dev x 2	7.2	9.37	6.34	7.87
Range - Low	45.82	42.76	46.89	44.87
Range - High	60.22	61.51	59.58	60.62
99.74% Probability				
Std Dev x 3	10.8	14.06	9.51	11.81
Range - Low	42.22	38.08	43.72	40.93
Range - High	63.82	66.19	62.75	64.56