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Chlorinated phenols are toxic compounds and often released into natural environments from improper disposal or non-point sources. One important factor in assessing the environmental fate and transport is the rates of microbial degradation in soils and natural waters. In this study, eight chlorophenols (4-chlorophenol (4-CP), 2,4-dichlorophenol (2,4-DCP), 3,4-dichlorophenol (3,4-DCP), 3,5-dichlorophenol (3,5-DCP), 2,3,5-trichlorophenol (2,3,5-TCP), 2,4,5-trichlorophenol (2,4,5-TCP), 3,4,5-trichlorophenol (3,4,5-TCP), and 2,4,6-trichlorophenol (2,4,6-TCP)) were chosen as the model compounds because they represent the major degradation products for the anaerobic metabolic products of pentachlorophenol (PCP). Aerobic degradation rates of all eight compounds were determined under controlled laboratory conditions.

A mixed bacterial culture was seeded from a municipal wastewater treatment plant and were grown with acetate as a primary substrate and the eight chlorophenols

as secondary substrates. Rates of degradation were measured in a batch reactor with an initial concentration of acetate of 1000 mg/l and of the individual chlorophenol of 0.1 mg/l.

Rate of acetate and chlorophenols were modelled using a Monod model. The maximum rate of acetate utilization was zero-order in relation to acetate concentration and first-order with respect to organism concentration. The rate of chlorophenol degradation were zero-order in relation to the chlorophenol concentration. The relative rates of degradation were in general as follows: monochlorophenol > dichlorophenols > trichlorophenols.

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INTRODUCTION

Chlorinated phenols and their derivatives are produced at a rate of thousands of tons annually (Reineke et. al., 1984). The most common compound, pentachlorophenol (PCP) has an annual world-wide production of over 50,000 tons (Crosby, 1981).

PCP exhibits strong toxicity because of its ability to decouple oxidative phosphorylation (Weinbach, 1957). Due to its long term toxicity, PCP is used widely as a wood preservative and also as a fungicide, herbicide and molliscide (Crosby, 1981), and a bactericide in a variety of materials including paints, adhesives, textile and paper products, leather, and cooling tower waters (Guthrie et al., 1984).

Chlorinated phenols and their derivatives are also used extensively as precursors in the production of pesticide formulation especially 2,4-D (2,4-dichlorophenoxyacetic acid) and 2,4,5-T (2,4,5-trichlorophenoxyacetic acid). Similar to other halogenated aromatics, chlorinated phenols are persistent in the environment and accumulate in sediment and organisms. Since chlorinated phenols are commonly discharged from industries using chlorination as part of their process chemistry, relatively high residues have been reported for effluent from pulp and paper mills, wood preservation plants, and certain chemical industries (Jolley, R.L., et al., 1978).

There is a total of 19 possible chlorinated phenols and all are commercially available. Monochlorophenol, consisting of three isomers, is used mainly in the production of higher chlorinated phenols. For example, 2-chlorophenol can serve as an intermediate in the synthesis of the highly chlorinated phenols (Morrison and Boyd, 1973). Combined annual U.S. production of 2-chlorophenol is 18,000 metric tons, which is considerably less than the annual production of 2,4-dichlorophenol of 39,000 tons. This latter compound is used primarily in the manufacture of 2,4-D (Moore et al., 1974).

Annual production of the two main isomers of trichlorophenol and of 2,3,4,6-tetrachlorophenol is relatively low, less than 7,000 metric tons. All three compounds find use as biocides and preservatives. In addition, 2,4,5-trichlorophenol is used in the production of 2,4,5-T and related products, whereas 2,4,6-trichlorophenol is used to manufacture tetrachlorophenol.

Chlorophenols have several significant sources in addition to industrially produced chemicals. A variety of chlorophenols are produced from the microbial degradation of several pesticides including 2,4-D and 2,4,5-T (Mikesell and Boyd, 1985) and other phenoxyalkanoate herbicides, indane, pentachloronitrobenzene, and hexachlorobenzene (Ahlborg and Thunberg, 1980). In addition, the effluent from pulp mills contain chlorophenols as a by-product of the bleaching process (Lander et al., 1977). Another mechanism for release to the environment is the reaction of unsubstituted phenols within dilute aqueous solutions such as waters and wastewaters

(Carlson et al., 1975, and Paasivirta et al., 1985). The formation of 2- and 4-chlorophenol, as well as more highly chlorinated phenols such as 2,4- and 2,6-DCP and 2,4,6-TCP, has been reported under conditions similar to those employed during the disinfection of wastewater effluents (Aly, 1968; Barnhart and Campbell, 1972).

As a group, the chlorophenols are highly toxic. The toxicity of chlorinated phenols tends to increase with their degree of chlorination. Because of their low degradation rates, the more highly chlorinated phenols tend to accumulate and persist in the environment resulting in public health concerns (Liu, et al., 1982 and Steiert et al., 1987). 2,4,6-trichlorophenol is a known animal carcinogen and 4-chlorophenol is a suspected carcinogen based on mutagenicity screening tests (Boyd and Shelton, 1984). Because of the toxicity and carcinogenicity of the chlorinated phenolic compounds, it is important to know their fate in the environment and their routes of biodegradation (Haggblom, et al., 1988).

Disposal of industrial chemicals, especially toxic xenobiotic compounds, into landfills is becoming increasingly difficult. Alternative methods of disposal, such as incineration or chemical treatment, are being investigated; however, these methods are often costly, meet with public resistance, and may even create a variety of alternate disposal problems.

The use of microorganisms for biodegradation of xenobiotic waste is one promising approach currently receiving widespread attention (Steiert and Crawford, 1985). However, this approach suffers from a lack of data concerning the rates of

biodegradation as the majority of research to date has focused upon degradative pathways (Gibson, 1980).

Recently, however, interest in quantitative aspects of biodegradation has increased due to the use of environmental models with required biodegradation rates. Quantitative measurements of biodegradation have begun to appear over the past 10 years (Sujit et al., 1984, Lyman et al., 1981). The kinetics of aerobic biodegradation to chlorophenols has been reported by Beltrame et al. (1982), Banerjee et al. (1984) and Sterert et al. (1987), and a general kinetic model for biodegradation to chlorophenols was given by Banerjee et al. (1984). However, these studies did not systematically measure the kinetic data for biodegradation to those chlorophenols which are important anaerobic metabolites of pentachlorophenol (Nicholson, 1990).

The objectives of this thesis are to determine the aerobic degradation rate of eight chlorophenols resulting from the anaerobic metabolites of pentachlorophenol using mixed aerobic bacteria. The results of this investigation can be used to further understand the fate of chlorinated compounds in complex aerobic/anaerobic environments such as contaminated soils. For examples, it is well known that the less chlorinated phenols are more mobile compared to their "mother compound" PCP. Thus, a knowledge of their biodegradation is crucial to assess their risks of transport from contaminated sites (Nicholson, 1990). Since the biodegradability and other properties such as lipophilicity and mobility are related to the chlorophenol structures, these compounds may serve as model compounds to study the fate and transport of

halogenated compounds in the environment.

AEROBIC DEGRADATION OF CHLOROPHENOLS

Aerobic biodegradation of chlorophenols is a catabolic reaction that requires the participation of oxygenases enzymes that incorporate atmospheric oxygen into their substrates (Steiert and Crawford, 1985). Dagley (1978) suggested that the benzene nucleus is very stable; for fission of the ring, it has to be dihydroxylated first by an oxygenase (or oxygenases) such that the two hydroxyl groups are attached either ortho or para position to one another on the ring. Ring fission then occurs through another oxygenase-catalyzed reaction involving the insertion of dioxygen into the aromatic nucleus.

The hydroxylation of chlorophenols is quite different from nucleophilic aromatic substitution in which halogen atom(s) on the ring are replaced by some nucleophilic reagents like OH^-, OR^-, NH_3 and CN^-. It is evident that chlorine atom(s) on an aromatic ring hinders the hydroxylation and deactivate the benzene nucleus to electrophilic attack by dioxygen because of withdrawal of electrons from the ring (Morrison and Boyd, 1973). The deactivity of chlorine atoms on the ring is of additivity; therefore, the more highly chlorinated phenols have more resistance to aerobic biodegradation and usually persist in aerobic environments.

Only a few mechanisms of aerobic microbial dechlorination of aromatic rings

are clear. Marks et al. (1984) and Muller et al. (1984) reported that 4-chlorobenzoate was dechlorinated to yield 4-hydroxybenzoate and both agreed that in the dechlorination the chlorine atom was replaced by a hydroxyl group which was derived from a water molecule instead of molecular oxygen. Reductive dehalogenation of chlorinated phenols (chlorine atoms being replaced by hydrogens) appears to occur extensively under anaerobic conditions. Nicholson (1990) showed a series of less chlorinated phenols (from tetra- to monochlorophenols) from anaerobic biodegradation of PCP.

The complete aerobic catabolic pathways for some mono- and dichlorophenols have been determined by Knacknuss (1984), and the known biochemical details are shown in Figures 1 and 2. During the aerobic catabolism of less chlorinated phenols such as some mono- and dichlorophenols mentioned above, chlorophenol-degrading bacteria usually open the dihydroxylated aromatic ring (using a dioxygenase) before they remove the chlorines. With the more highly chlorinated phenols (such as PCP), at least some of the chlorine atoms must be removed prior to ring cleavage since chlorine-mediated ring deactivation probably would be sufficient to prevent the ring-opening reactions. However, the biochemical details for the more highly chlorinated phenols are under investigation. It seems that for the trichlorinated compounds the aerobic dechlorination at least partially precedes ring cleavage (Steiert and Crawford, 1985).

There is a characteristic for the degradation of chlorinated phenoxyalkanoate

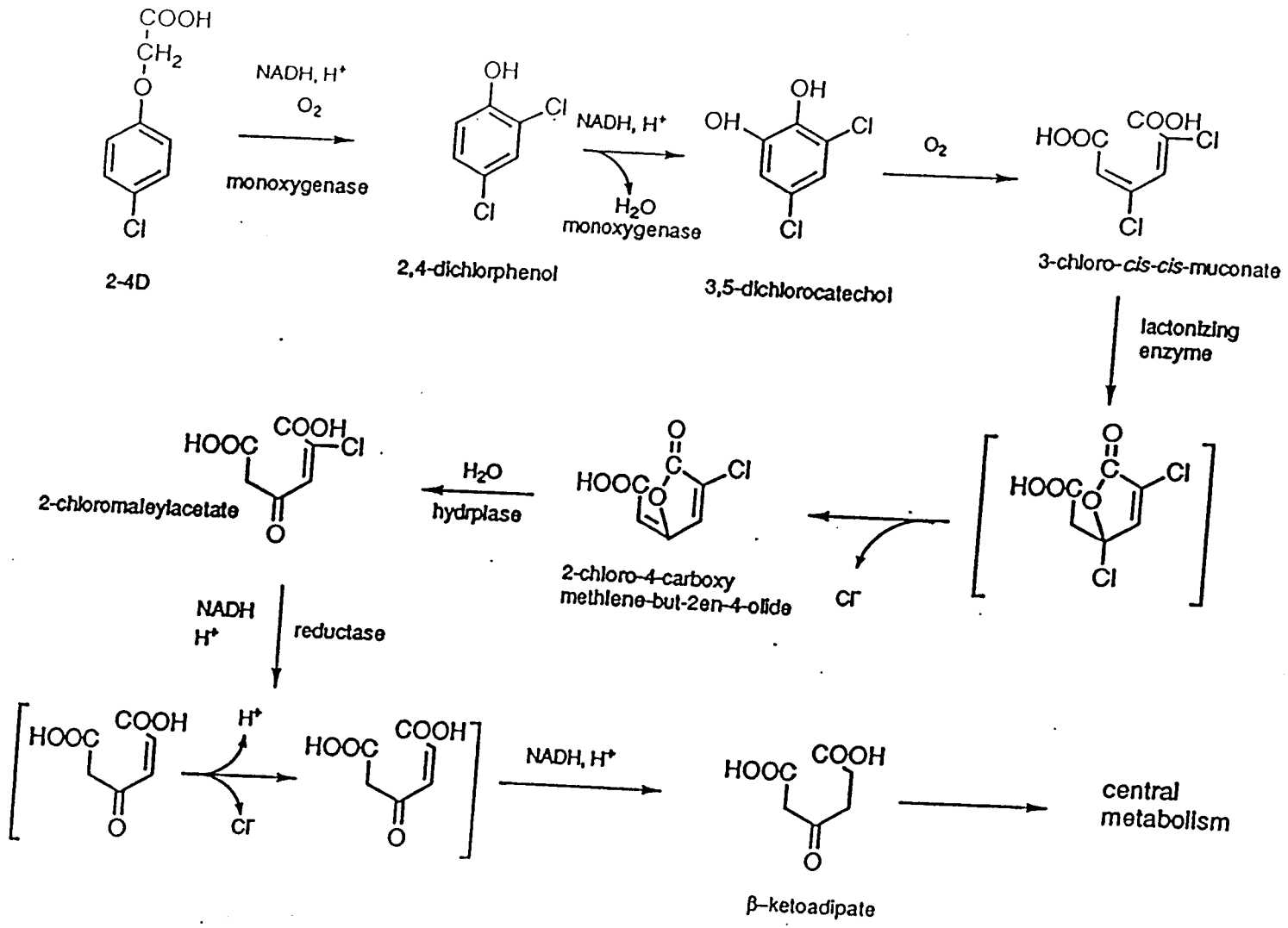


Fig. 1 The pathway for degradation of 2,4-dichlorophenoxyacetic acid (2,4-D)

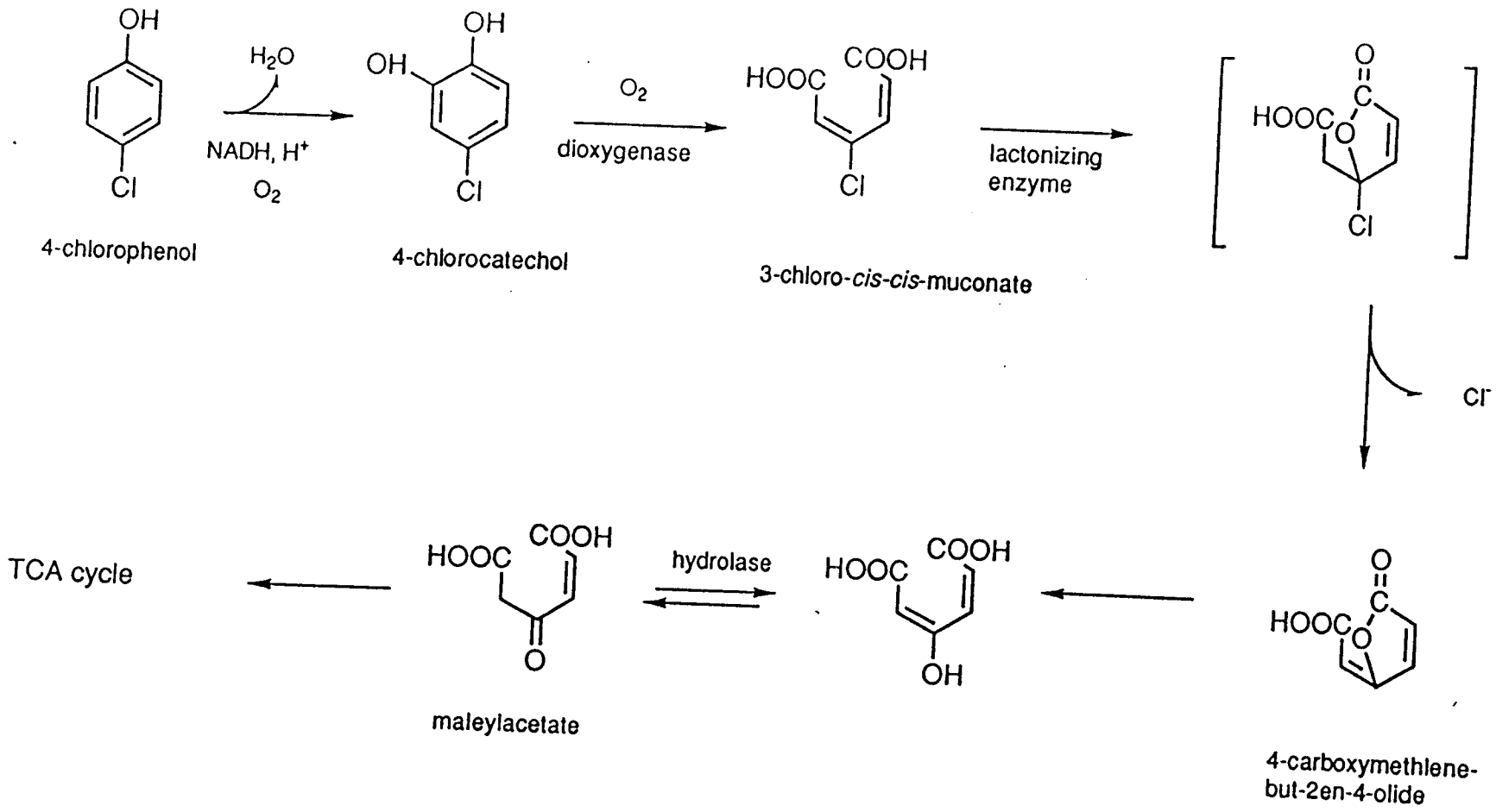


Fig. 2 Pathway for the degradation of 4-chlorophenol.

herbicides in that cleavage of the ether linkage between the phenolic and fatty acid moieties occurs before both dechlorination and ring cleavage. As a result, the corresponding free phenols are yielded. For example, 2,4-D, 4-chloro-2-methyl-, and 2- and 4-chlorophenoxyacetates are converted enzymatically to products with chromatographic characteristics of 2,4-DCP, 4-chloro-2-methyphenol, and 2- and 4-chlorophenol respectively (Loos et al., 1967a).

Both aerobic and anaerobic sludge have been used for biodegradation of PCP and other chlorinated phenols (Nicholson, 1990, Boyd and Shelton, 1984, Guthrie, et al., 1984, Salkinoja-Salonen, et al., 1984 and Moos, et al., 1983). Boyd and Shelton (1984) investigated the anaerobic degradation of mono- and dichlorophenols using adapted and unadapted sewage sludge.

They found that with unadapted anaerobic organisms, the degradation rate of monochlorophenol was dependent on the relative substitution position of chlorine to phenolic hydroxyl and increased in the order ortho > meta > para. Dichlorophenols with a chlorine ortho to the hydroxyl were degraded in an unacclimated system with the ortho chlorine being removed first and the remaining chlorine removed later. Dichlorophenols with no ortho chlorine (i.e. 3,4- and 3,5-dichlorophenol) persisted in this system. Once sludge was adapted to 2-chlorophenol, 2- and 4-chlorophenol, and 2,4-dichlorophenol, but not 3-chlorophenol were degraded. The 3- and 4-chlorophenol, 3,4- and 3,5-dichlorophenol, but not 2-chlorophenol were degraded with sludge adapted to 3-chlorophenol. Sludge acclimated to 4-chlorophenol could degrade all

monochlorophenols in addition to 2,4- and 3,4-dichlorophenols.

It is evident that substitution patterns of chlorine atoms on the phenol ring appears to be important regulators of dechlorination and mineralization of the chlorophenol compounds. Steiert et al. (1987) found that chlorophenols with chlorines substituted in positions 2 and 6 were completely dechlorinated and mineralized. Those phenols that did not contain chlorine in both position 2 and 6 were not readily mineralized. This observation coincides with those of Liu et al.(1982). Though the toxicity of chlorinated phenols increases with the degree of chlorination mentioned as before, phenols chlorinated in positions 2 and 6 were least toxic.

METHODS AND MATERIALS

Aerobically digested sewage sludge was collected from the primary anaerobic digester at the Corvallis Wastewater Reclamation Plant. The digester was operated at a temperature of 25°C and has a solid retention time of about 30 days. The predominant source to the plant is domestic sewage from the City of Corvallis.

The sludge collected from the plant was transported to the laboratory in a 20-liter carboy and transferred to a 8-liter "mother reactor" described below. The mother reactor was set in a constant temperature chamber at 31°C. The sludge was fed a "nutrient solution", which was a modified recipe described by Owen et al. (1978) at a rate of 2 liter/day. The solution contains inorganic nutrients and vitamins as follows in

mg/l: $(NH_4)_2HPO_4$ 27.09; $CaCl_2.2H_2O$ 225.45; NH_4Cl, 359.10; $MgCl_2.6H_2O$, 1620; KCl, 1170.45; $MnCl_2.4H_2O$, 17.96; $CoCl_2.H_2O$, 27; H_3BO_3, 5.13; $CuCl_2.2H_2O$, 2.43; $Na_2MoO_4.2H_2O$, 2.30; $ZnCl_2$, 1.89; biotin, 0.018; folic acid, 0.018; pyridoxin hydrochloride, 0.09; riboflavin, 0.045; thiamin, 0.045; pantolthenic acid, 0.045; B_{12}, 0.0009; P-aminobenzoic acid 0.045; and thioctic acid 0.045.

Acetate at an influent COD of 500 mg/l was used as a primary carbon source as well as an energy source to support the growth of bacteria and the metabolization of chlorophenols. PCP was added to the feed solution in a concentration of 1 mg/l to acclimate the organisms until an apparent degradation of PCP was observed. The acclimation to PCP required about four weeks. Once the organisms were adapted to PCP, eight chlorinated phenols, 4-chlorophenol (4-CP), 2,4-dichlorophenol (2,4-DCP), 3,4-dichlorophenol (3,4DCP), 3,5-dichlorophenol (3,5-DCP), 2,3,5-trichlorophenol (2,3,5-TCP), 2,4,5-trichlorophenol (2,4,5-TCP), 3,4,5-trichlorophenol (3,4,5-TCP), and 2,4,6-trichlorophenol (2,4,6-TCP) were added to the feed solution instead of PCP. These eight phenols represent the major anaerobic metabolic products of PCP (Nichlson, 1990). The concentration of the individual chlorophenol was 0.1 mg/l. The effluent from the mother reactor was periodically measured to monitor acetate concentration by ion chromatograph (IC). The sludge was acclimated until steady-state conditions were achieved.

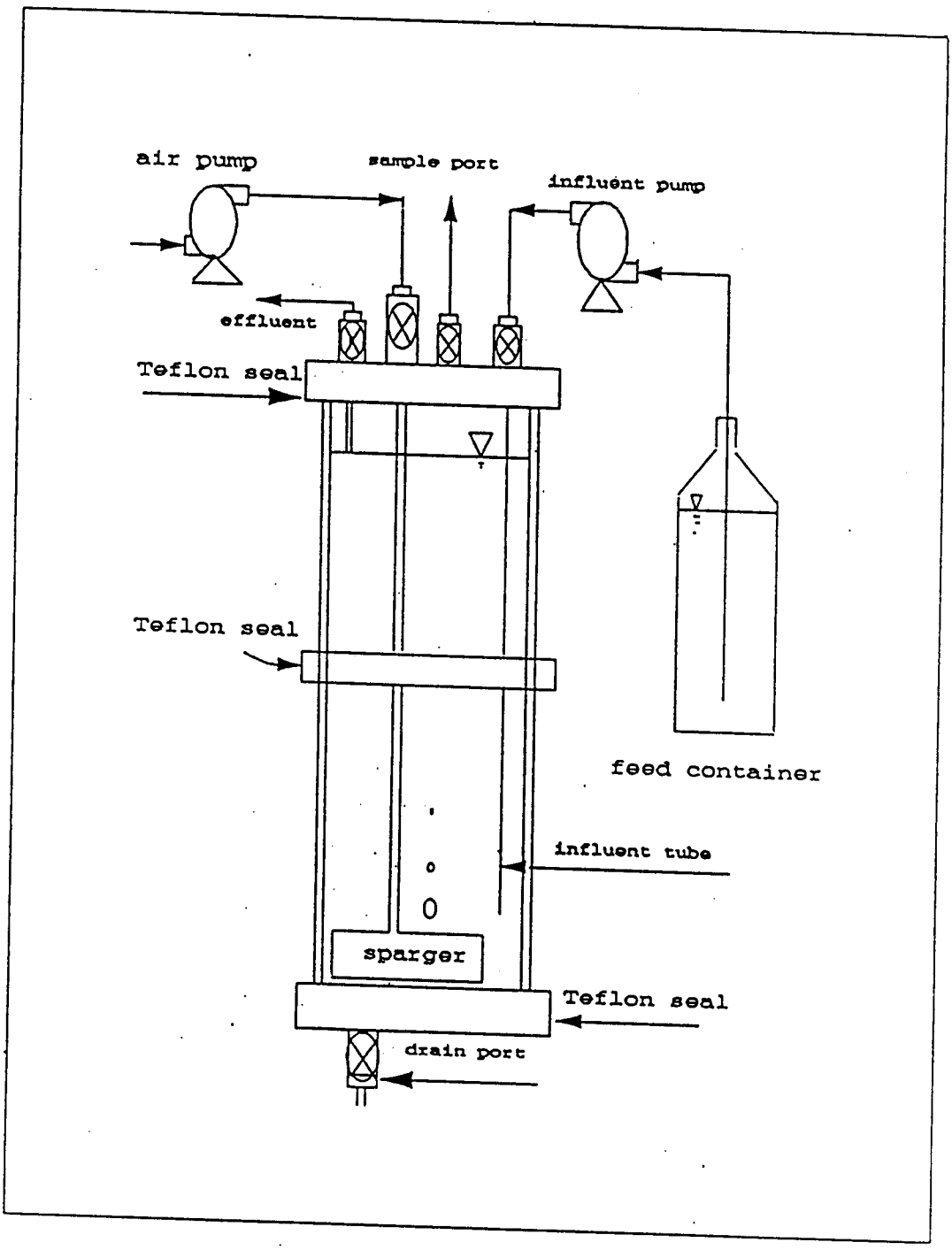
3,4,5-TCP (98%) was obtained from Ultra Scientific Co., Hope, R.I., and all other chlorophenols, 2,6-dibromophenol and 2,4,6-tribromophenol, were obtained from

Aldrich Chemical Company, Inc. Milwankie, WN, in purities of at least 98%. All chemicals were used without further purification.

Reactor System

The reactors used are shown in Fig. 3 and 4. A 8-liter "mother reactor" was used as a reservoir to provide active organisms. This reactor was composed of a cylinder with stainless steel and Teflon-lined flange fittings (Ace Glass Company, Vineland, N.J.). Brass valves set on the top served as ports for liquid sampling, liquid influent, liquid effluent and air bubbling. These materials were chosen in order to minimize sorption of chlorophenols to the reactor surfaces. The sludge was kept suspended by pumping air bubble through a Teflon tubing and a piece of sparger set at the bottom of the reactor. The reactor was set up in an environmental chamber maintained at 31°C. Feed solution was kept in a 20-liter carboy at 4°C to minimize microbial growth and transferred to the reactor through Teflon tubing by a FMI RP-G50 laboratory pump (Fluid Metering Inc. Oyster Bay, N.Y.) The flow rate was maintained at 1.3 ml/min to provide a hydraulic retention time of 3.5 days.

Kinetic analysis experiments were carried out in a 2.5-liter batch reactor which was similar to the mother reactor (Fig. 4). This reactor had a sampling port as well as air bubbling valve and was stirred by a magnetic stir bar. An electrode was set up on the top of the reactor for monitoring pH. This reactor was maintained at 31°C. Sludge was collected from the mother reactor through a drainage at the bottom and transferred



Schematic diagram of mother reactor

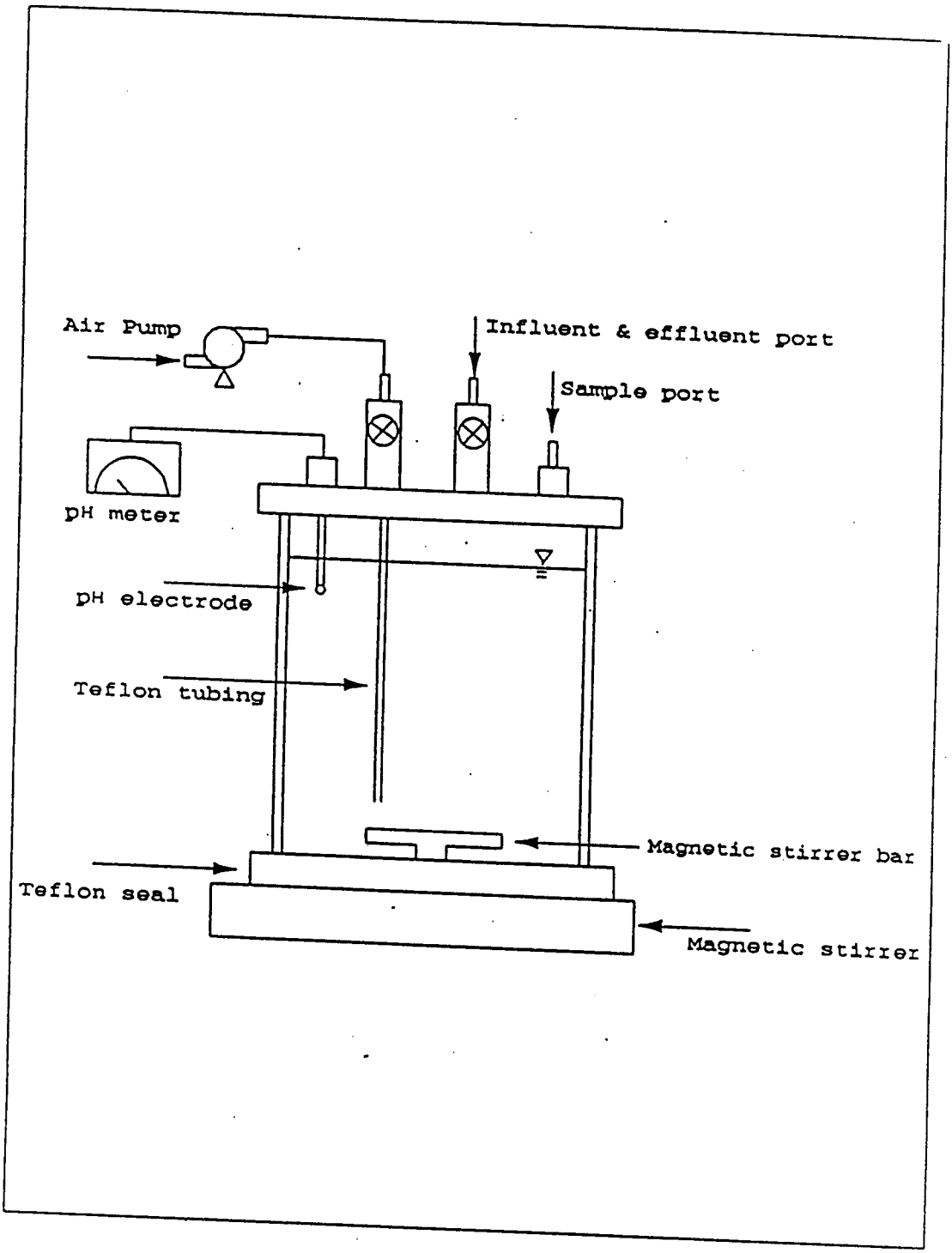


Figure 4. Schematic diagram of batch reactor

to the batch reactor. For each experiment, the batch reactor was filled with sludge from the mother reactor, an individual chlorophenol at 1 mg/l, acetic acid, and a phosphate buffer (42.5 gr. NaH_2PO_4 and 51.7 gr. Na_2HPO_4 per liter). The initial acetate concentration was 1000 mg/l for all batch experiments and the initial pH was adjusted to 6.5 with saturated sodium bicarbonate solution.

Sampling Procedure

Liquid samples (15 ml) were taken from the batch reactor and immediately filtered using a Millipore filtration apparatus and Whatman type GF/C glass microfiber filters with a 1 micron pore size. The first three ml of filtrate were discarded and the remaining was used for separate concentration analyses for both chlorophenols and acetate.

Analytical Procedures

The analysis of chlorophenols were conducted by using a modified method described by Voss et al. (1981) and NCASI (1981). The samples were first acetylated and then extracted into hexane. For each analysis, 5 ml of the filtrate, after filtration mentioned above, were immediately transferred into a 250-ml separatory funnel in which 45 ml of deionized distilled water was added. Internal standard, either 2,6-dibromophenol (25 ul) or 2,4,6-tribromophenol (50 ul) was added using a 50-ul syringe based on different expected chlorophenols. After one ml of a 0.73 gram/ml

solution of potassium carbonate and one ml of acetic anhydride were added, the funnel was shaken for exactly two minutes. The complete acetylation reaction would take at least two hours, after which exact 5 ml of hexane was added using a dedicated 5-ml volumetric pipette and again the funnel was shaken for another two minutes. Two hours were needed for the complete extraction. After the water layer was drained, the hexane layer was withdrawn using a new pasteur pipette and transferred to 2-ml amber glass vials with Telfon-lined cap, which were stored in a refrigerator until gas chromatograph analysis was conducted.

Five ml of filtrate was transferred into a 100-ml volumetric flask and diluted with a solution of 0.191 g/l $NaCO_3$ and 0.143 g/l $NaHCO_3$. The $NaCO_3$/$NaHCO_3$ solution serves as the anion eluent for ion chromatograph and minimizes "negative peaks". The prepared samples were stored in a refrigerator until analyzed using a Dionex Series 4000i liquid ion chromatograph. The ion chromatograph was operated using a temperature compensation of 1.7, output range 30 microsiemens and flow rate of 2 ml/min.

Using an autosampler, 1 ul of hexane solution was injected into a Hewlett Packard Model 5890A Gas Chromatograph equipped with a 63Ni electron capture detector (ECD) and 30 m by 0.323 mm i.d. DB-5 fused-silica capillary column (J+W Scientific, Orangeville, CA). Helium (5 psi) was used as the carrier gas and a 95% argon/5% methane mixture was used as the ECD auxiliary gas. The detector temperature was set at 320°C and injector temperature was 250°C. The samples were

run using a temperature program as follows: an initial oven temperature of 45°C was held for 2 minutes, increased by 15°C/min to 150°C, and then by 5°C/min to a final temperature of 215°C which was held for 5 minutes.

Fifty ml of sludge was taken for solid analyses (TSS or VSS) using inverted pipette by following procedure 2540 D and 2540 E, Standard Methods, 17th edition (1989).

RESULTS AND DISCUSSION

Kinetic Results for Acetate

The rate of acetate utilization can be modelled using the Monod equation (eq. 1) as:

$$u = um \times [S]/(Ks + [S]) \qquad (1)$$

The u and um are the actual and maximum growth rate of the bacteria, respectively, [S] is substrate concentration and Ks is the half-velocity coefficient. The removal rate of a compound in a batch reactor can be expressed as:

$$-[dS]/dt = um \times [S] \times [B]/Y \times (Ks + [S]) \qquad (2)$$

Here, [B] refers to biomass and Y is the yield factor, which numerically equals the difference of biomass divided by the difference of substrate during an experiment period. For short experiments, Y can be expressed as:

$$Y = ([B]_f - [B]_i)/([S]_i - [S]_f) \quad (3)$$

where the subscripts i and f substitute the initial and final time of an experiment respectively.

At high substrate concentration where [S] > Ks, eq. 2 yields eq. 4 where the degradation rate of a substrate is of first order with respect to biomass and independent of substrate concentration. The term um/Y represents the maximum rate of substrate utilization per unit mass of microorganisms (km).

$$-d[S]/dt = (um/Y) \times [B] \quad (4)$$

$$km = um/Y \quad (5)$$

$$-d[S]/dt = km \times [B] \quad (6)$$

Solving eq. 3 for [B] as a function of time, then:

$$-d[S]/dt = km \times ([B]i + Y \times [S]i) - km \times Y \times [S] \quad (7)$$

Integrating, as

$$-d[S]/dt = a - b \times [S] \quad (8)$$

where $a = km \times ([B]i + Y \times [S]i)$

$b = km \times Y$

then,

$$[S] = 1/b \times \{a - (a - b \times [S]i) \times \exp(b \times t)\} \quad (9)$$

The yield values for the experiments were calculated based upon eq. 3 and varied between 0.35 and 0.75 mg/mg with a mean value of 0.62 mg/mg (Table 1).

In the batch experiments, acetate concentrations were maintained much greater than K_S over the first 24 hours. These data were fit by eye using eq. 9 by varing the km value as shown in Appendix I. The resulting values of km are listed in Table 1.

The maximum removal of acetate occurred in the absence of chlorophenols with a rate 0.14 mg acetate/mg cell-hr. All chlorophenols resulted in decreased rates

of acetate removal from 30 to 90% for 3,5-DCP and 2,4,6-TCP, respectively. The mean reduction in acetate removal rate was 67%

Kinetic Results for Chlorophenols

It was assumed that the degradation of the chlorophenols occurred by acclimated or a separate group of organisms within the acetate population initially seeded to the reactor. As such, the rate of chlorophenol removal assuming [S] > K_s for the chlorophenols can be modelled as:

$$-d[S]/dt = km\ [B] \qquad (10)$$

where:

-d[S]/dt = rate of substrate degradation, umol/l-hr.

km = maximum substrate utilization rate, umol/g TSS-hr.

[B] = cell concentration, g/l

Because it has been assumed that the growth supported by the degradation of chlorophenols was immeasurably small, the initial cell concentration was used in eq. 10 to determine the k_m values listed in Table 2.

4-CP was rapidly degraded and all of the 4-CP was removed in eight hours. The pathway for the degradation of 4-CP could be an ortho-ring cleavage dioxygenase mechanism (Fig. 1) with catabolism starting with hydroxylation to form 4-

Table 1. Degradation of Acetate with Chlorophenols

Chlorophenols	Y (mg/mg)	k_m (mg/mg-hr)
no chlorophenol	0.48	0.138
4-CP	0.55	0.052
2,4-DCP	0.64	0.068
3,4-DCP	0.61	0.023
3,5-DCP	0.75	0.016
3,4,5-TCP	0.73	0.040
2,4,6-TCP	0.35	0.095
2,3,5-TCP	0.73	0.045
2,4,5-TCP	0.76	0.025

chlorocatechol (4-chloro-1,2-dihydroxybenzene). Intradiol ring cleavage (insertion of O_2 between carbons 1 and 2) would then yield chloromuconic acid. Lactonization of the chloromuconic acids to unstable intermediates result in spontaneous losses of the chlorines, forming butenolides. Hydrolyses then catalyze lactone cleavage with formation of maleylacetate, which is further broken down for use in central metabolism (Steiert et al., 1985).

The three dichlorophenols, 2,4-DCP, 3,4-DCP and 3,5-DCP, degraded slower than 4-CP as expected. However, the degradation was still appreciable, and their removal in 36 hour period was 60%, 91% and 23%, respectively.

Among the three dichlorophenols, 2,4-DCP has been most extensively studied in connection with work on the herbicide 2,4-D, the complete catabolic routes have been determined for several pure strains (Knackmuss, 1981). The known biochemical details are shown in Fig. 2; the degradation proceeds by removal of the sidechain forming 2,4-DCP, which is hydroxylated forming dichlorocatechol. Intradiol ring cleavage and lactonization follow, with the spontaneous loss of one chlorine atom. The remaining chlorine is removed later from chloromaleylacetic acid by a reductive mechanism yielding beta-ketoadipate which leads into central metabolic routes (Steiert et al., 1985).

Alexander and Aleem (1961) claimed complete disappearance of 2,4-DCP in 5 or 9 days in two different silt loam suspensions when the initial concentration was 50 mg/l. Aly and Faust (1964) studied 2,4-DCP oxidative degradation in samples of

Table 2. Summary of Zero Order Reaction Rates for Various Chlorophenols

Chlorophenol	km[B] (uM/l-hr)	$[B]_i$ (g/L)	km (uM/g-hr)	r^2	n
4-CP	3.80	0.391	9.72	0.988	10
2,4-DCP	0.075	0.420	0.179	0.974	10
3,4-DCP	0.193	0.807	0.240	0.957	10
3,5-DCP	0.036	0.565	0.064	0.950	10
2,4,6-TCP	0.046	0.210	0.217	0.993	10
2,3,5-TCP	0.036	0.439	0.083	0.989	10
2,4,5-TCP	0.020	0.309	0.064	0.824	8
3,4,5-TCP	0.021	0.339	0.062	0.714	8
2,4-w/ 3,4-DCP	0.148	0.137	1.080	0.951	10
3,4-w/ 2,4-DCP	0.082	0.137	0.601	0.951	10
24DCP w/o Ac	0.082	0.434	0.188	0.988	10
34DCP w/o Ac	0.082	0.276	0.298	0.926	10
246TCP w/o Ac	0.035	0.402	0.088	0.984	10

1. [B] = concentration of cells in grams per liter.

2. r2 = regression coefficient.

3. n = number of data points.

4. 2,4-&3,4-DCP means that 2,4-DCP was degraded at the presence of 3,4-DCP. 3,4-&2,4-DCP means that 3,4-DCP was degraded at the presence of 2,4-DCP.

natural lake water under laboratory conditions (pH 7, aeration, 25°C). They found that the half-life of 2,4-D in their cultures was 6 days and adaptation of the cultures was unnecessary. Chu and Kirsch (1972) showed that an unidentified bacillus soil culture adapted to pentachlorophenol was also readily able to degrade 2,4-DCP. The dichlorophenol was 67 percent oxidized by a suspension of the bacillus in 150 min. Ingols et al. (1966) also observed 2,4-DCP degradation by pentachlorophenol-adapted sludge.

In this study, 3,5-DCP degraded as slowly as trichlorophenols, which could be explained by its pathway: degradation of both 3-chlorophenol (3-CP) and 3,5-DCP would yield 3-chlorocatecol (Fig. 5) and 3,5-dichlorocatecol, respectively, as intermediates. Meta cleavage of the catecols, rather than the normal intermediate catecols, would form a highly reactive acylchloride that is a suicide inhibitor of the 2,3-dioxygenase. The formed inhibitor would cease further degradation in this pathway. However, the ortho (intradiol) cleavage pathway does not form acylchlorides.

3,4-DCP degraded faster than 3,5-DCP even though it could be subject to meta

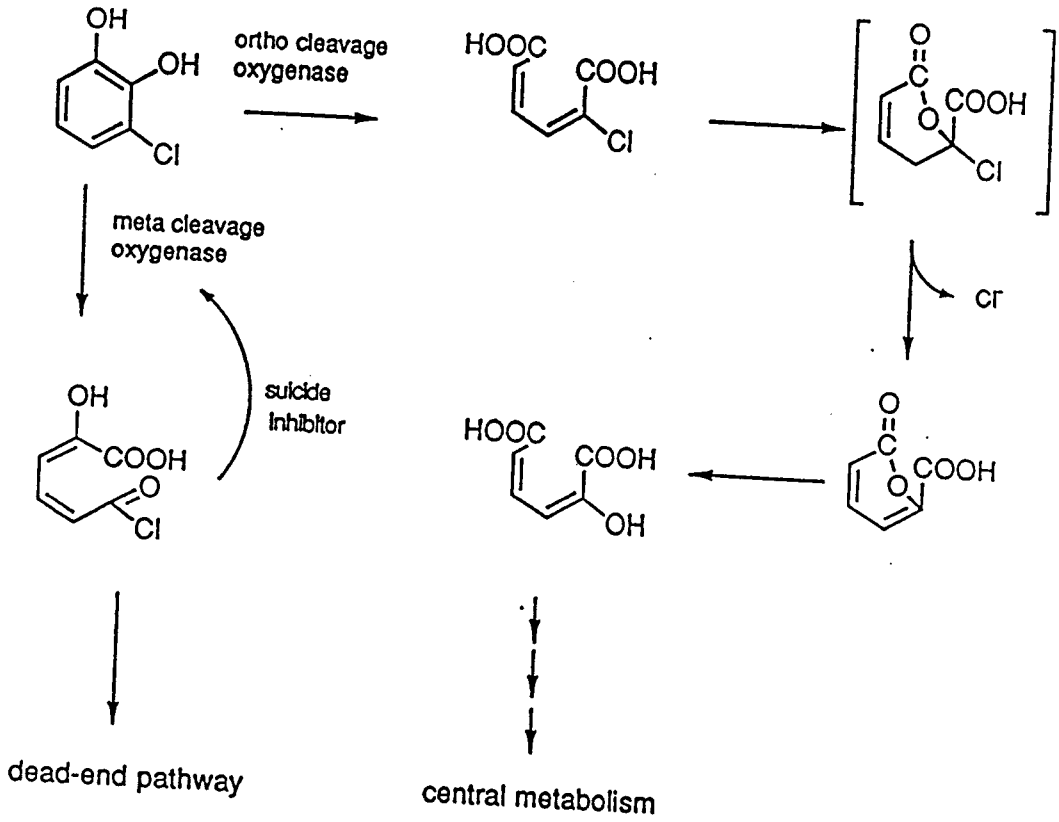


Fig. 5 Pathways for 3-chlorocatechol

cleavage. This possibly results from the fact that 3,4-DCP only has one meta chlorine atom, but 3,5-DCP has two meta chlorine atoms. Therefore, the latter would statistically have twice as many as chances to form a highly reactive acylchloride which is a suicide inhibitor as mentioned above and stop its further degradation.

All trichlorophenols, except 2,4,6-DCP, degraded slowly as expected. The slow degradation of 2,3,5-TCP, 2,4,5-TCP and 3,4,5-TCP could be explained because highly chlorinated phenols, particularly those with chlorine at the meta position on the ring, are the most resistant to degradation (Alexander and Aleem, 1961). The degradation mechanism of trichlorophenols is not as clear as less chlorinated phenols. Also, pathway of trichlorophenols would be quite different from that of dichlorophenols or chlorophenols. As expected, with trichlorinated phenols, dechlorination appears to at least partially precede ring cleavage (Steiert et al., 1985).

Among the trichlorophenols, 2,4,6-TCP seems to have been most extensively investigated. Tabak et al. (1964) reported that in flask cultures inoculated with sludge bacteria, 7 to 10 days were required to remove 90% of the 2,4,6-TCP at an initial concentrations of 300 ppm. At lower concentrations (100 ppm), respirometry experiments indicated that 70% of the 2,4,6-TCP was removed in three hours. In soil cultures 5-13 days were required for complete removal of 2,4,6-TCP. Alexander and Aleem (1961) found in their experiments that the time required for the complete disappearance of some chlorophenols, including 2,4,6-TCP from various soil samples ranged from 1 to 9 days. Ingols et al. (1966) reported complete aromatic ring

degradation of 2,4,6-TCP within 5 days by microbial action in an acclimated sludge.

The data in Table 2 show that acetate, the primary substrate, almost had no effect on the degradation of 2,4-DCP and 3,4-DCP. However, the degradation of 2,4,6-TCP was decreased in the absence of acetate. The reasons for these effects are unknown.

Competition was shown to exist between 2,4-DCP and 3,4-DCP. The presence of 3,4-DCP greatly accelerated the degradation of 2,4-DCP in that the maximum utilization rate of 2,4-DCP was increased six times with the presence of 3,4-DCP compared to that with no 3,4-DCP present. However, the maximum utilization rate of 3,4-DCP was only increased 2.5 times with the presence of 2,4-DCP compared to that with no 2,4-DCP present.

CONCLUSIONS

Based upon the results of this study, the following conclusion can be made concerning the degradation of chlorophenols by a mixed culture of aerobic bacteria:

1. Chlorophenols exhibit a strong toxic effect upon the utilization of acetate.
2. The degradation of 4-CP is rapid.
3. 3,5-DCP degrades slower than 2,4- and 3,4-DCP.
4. 2,4,6-TCP degrades much more rapidly than 2,3,5-, 2,4,5-, or 3,4,5-TCP.
5. Acetate is not required to obtain degradation of the chlorophenols which suggests

that the reaction mechanism is not simply co-metabolism.

6. The presence of 2,4-DCP and 3,4-DCP together increases the rate of metabolism for both compounds as compare to the compounds being singlely present.

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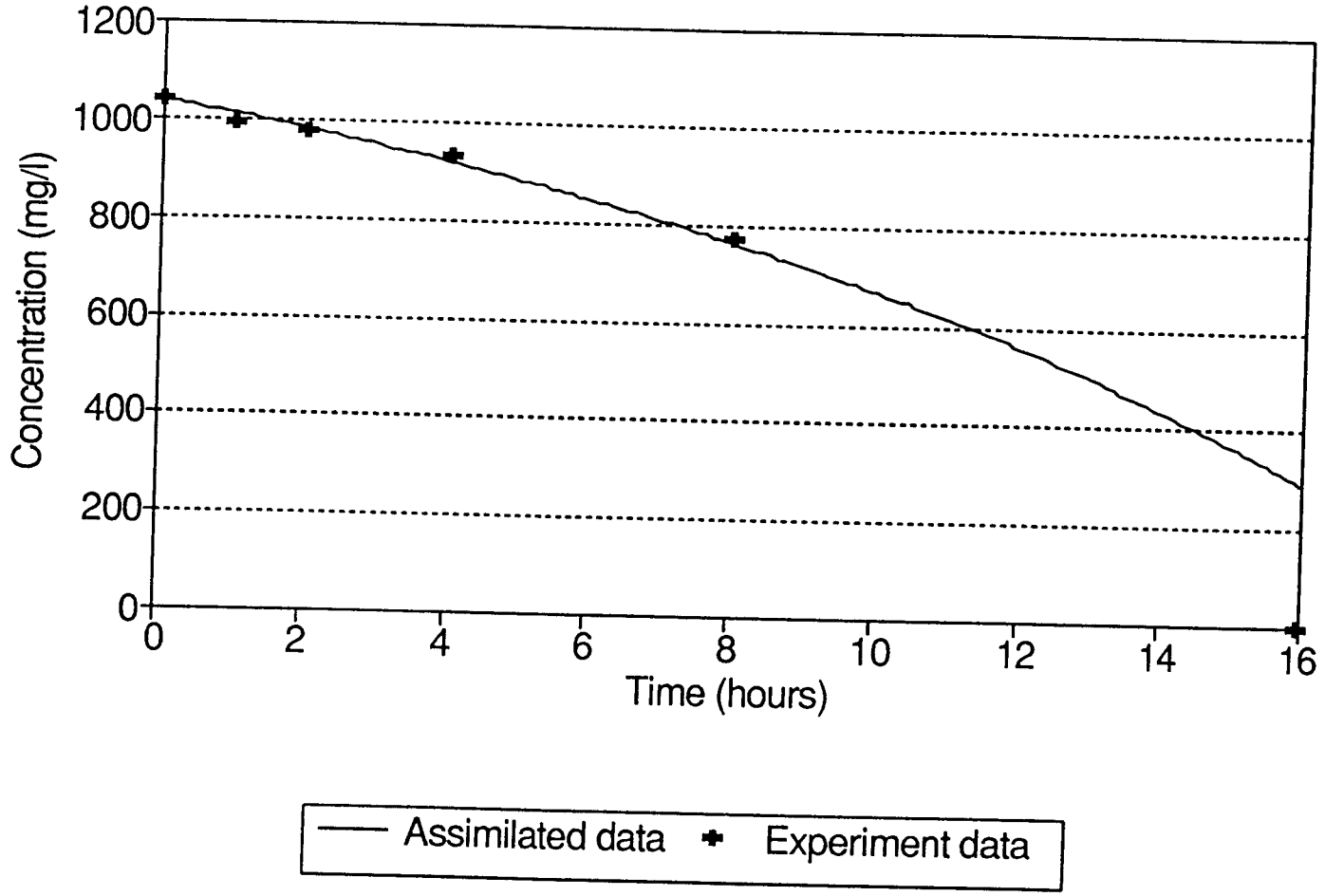
APPENDICES

Appendix A

Graphs for the Degradation of Acetate with Chlorophenols

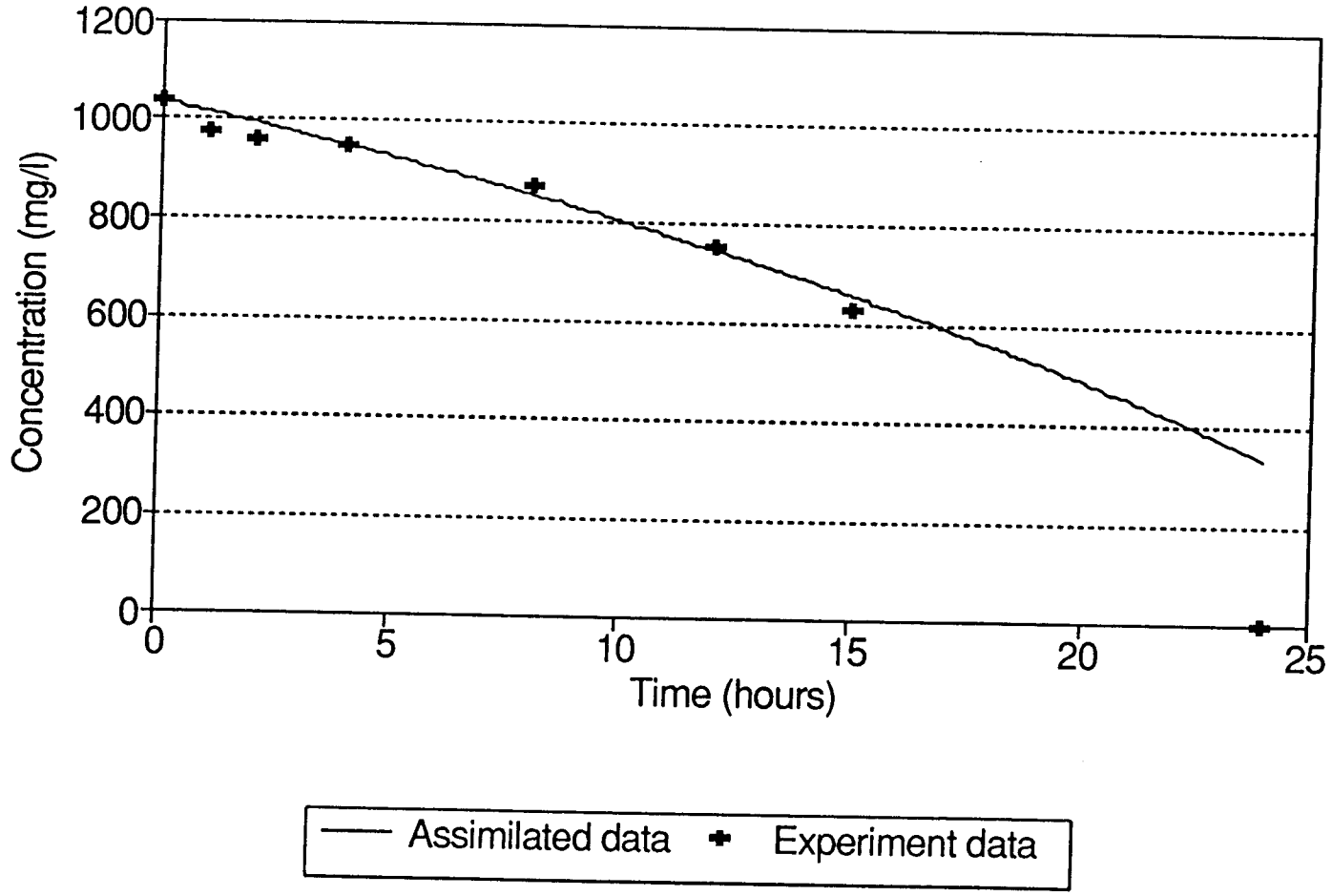
Degradation of Acetate(no chlorophenol)

(Xi=192 mg/l, Xf=687.8 mg/l, km=0.138)



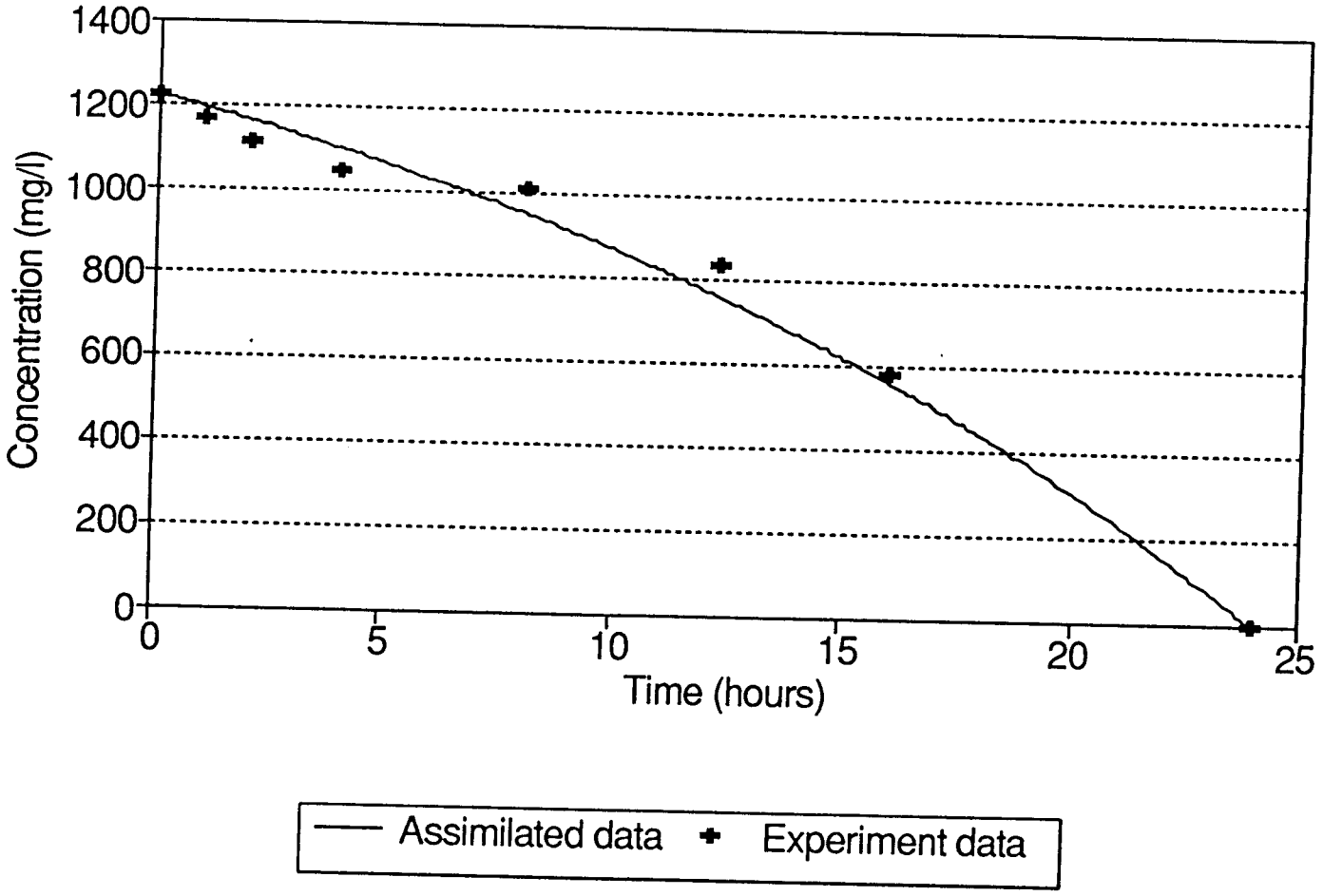
Degradation of Acetate with 4-CP

(Xi=391 mg/l, Xf=955.8 mg/l, km=0.052)



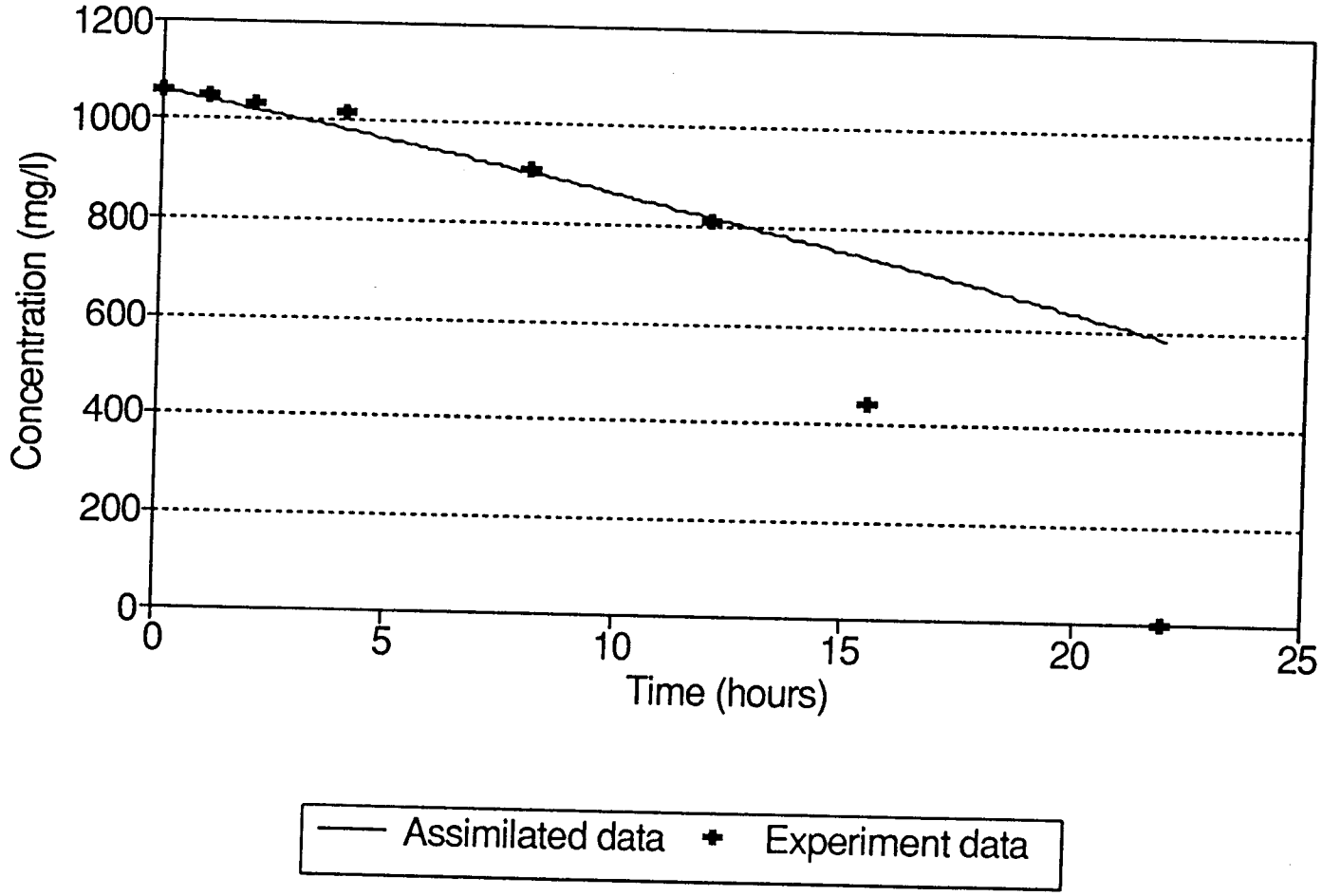
Biodegradation of Acetate with 2,4-DCP

(Xi=419.6 mg/l, Xf=1220 mg/l, km=0.068)



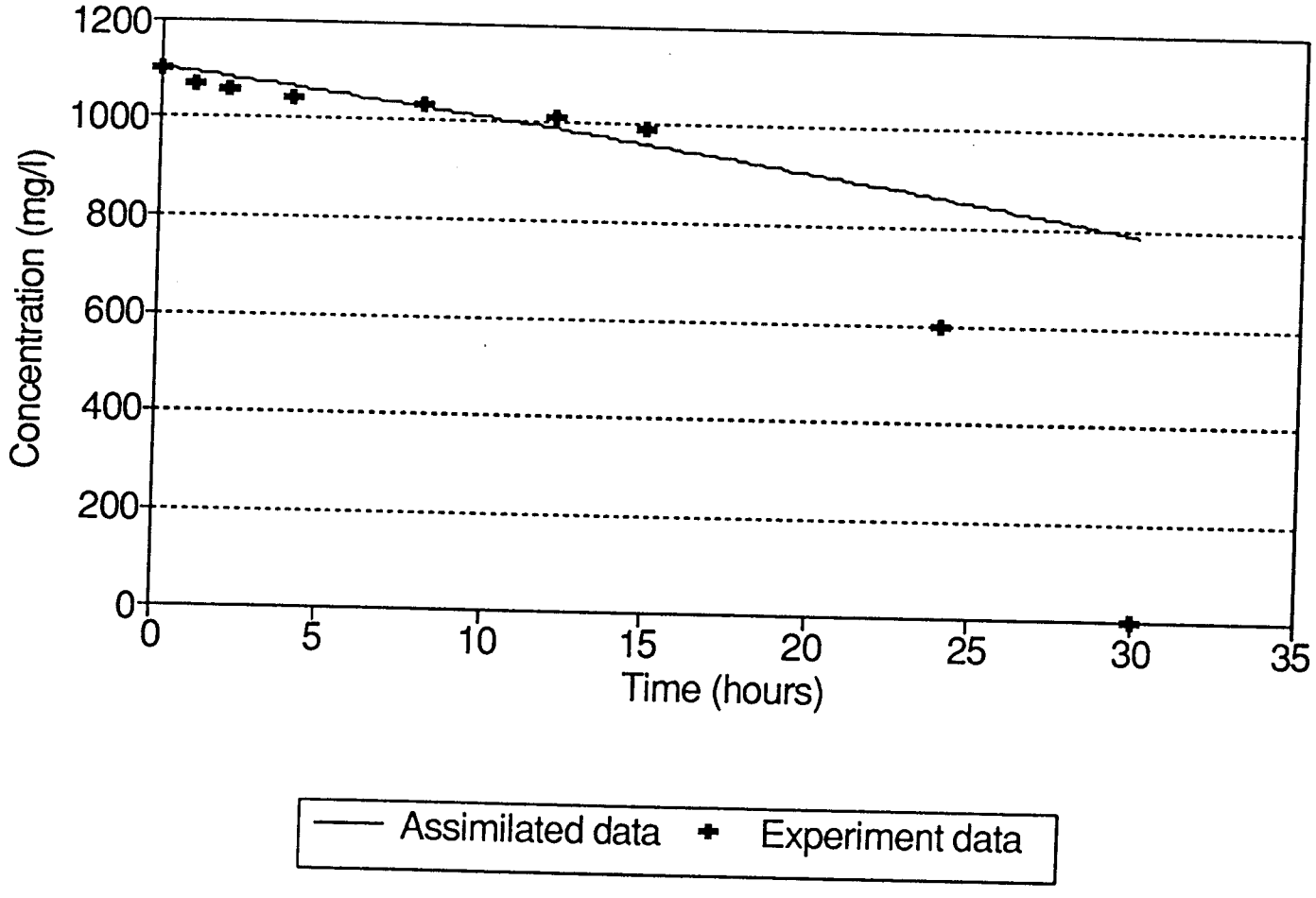
Degradation of Acetate with 3,4-DCP

(Xi=807 mg/l, Xf=1449 mg/l, km=0.023)



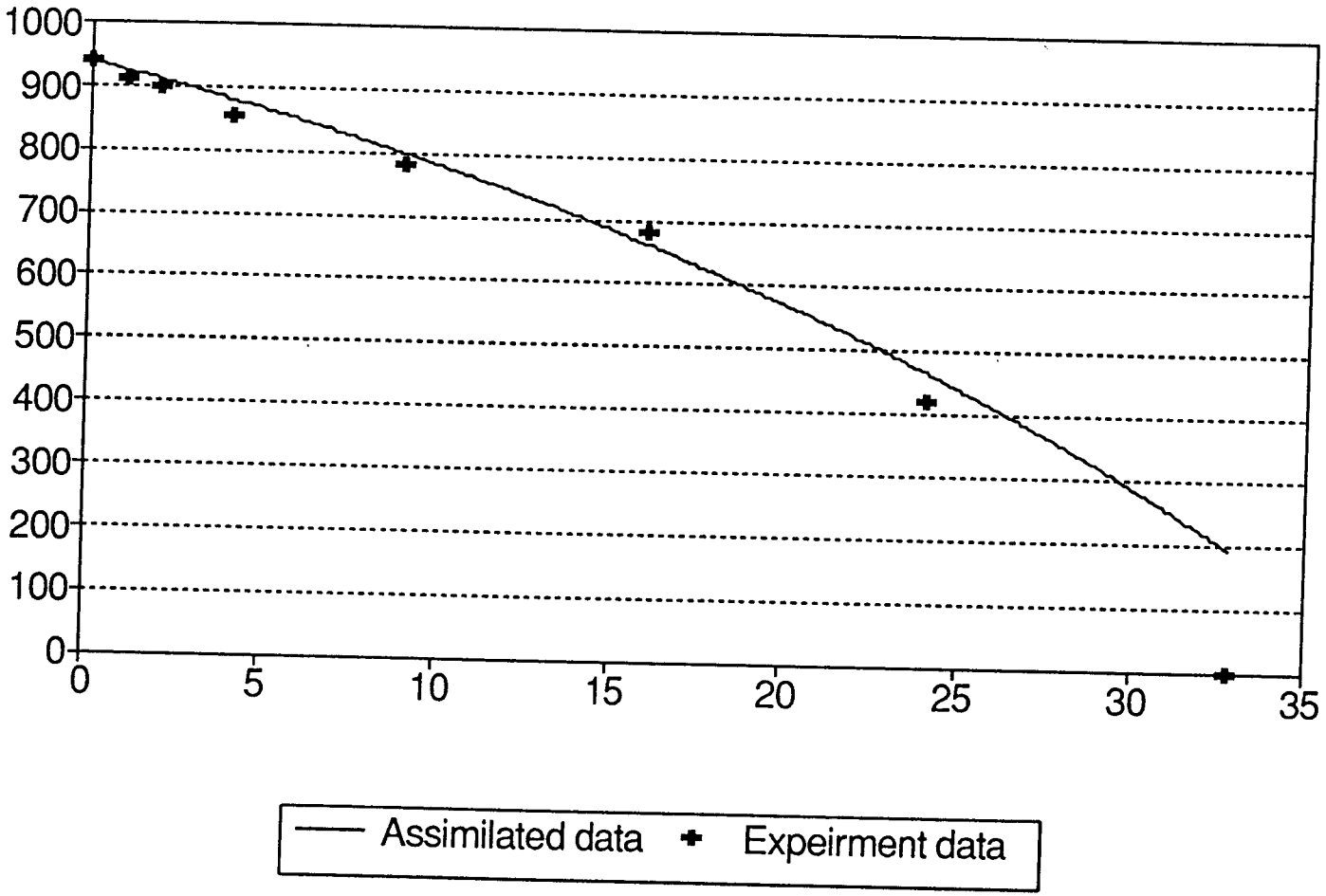
Degradation of Acetate with 3,5-TCP

(Xi=547 mg/l, Xf=1652 mg/l, km=0.016)



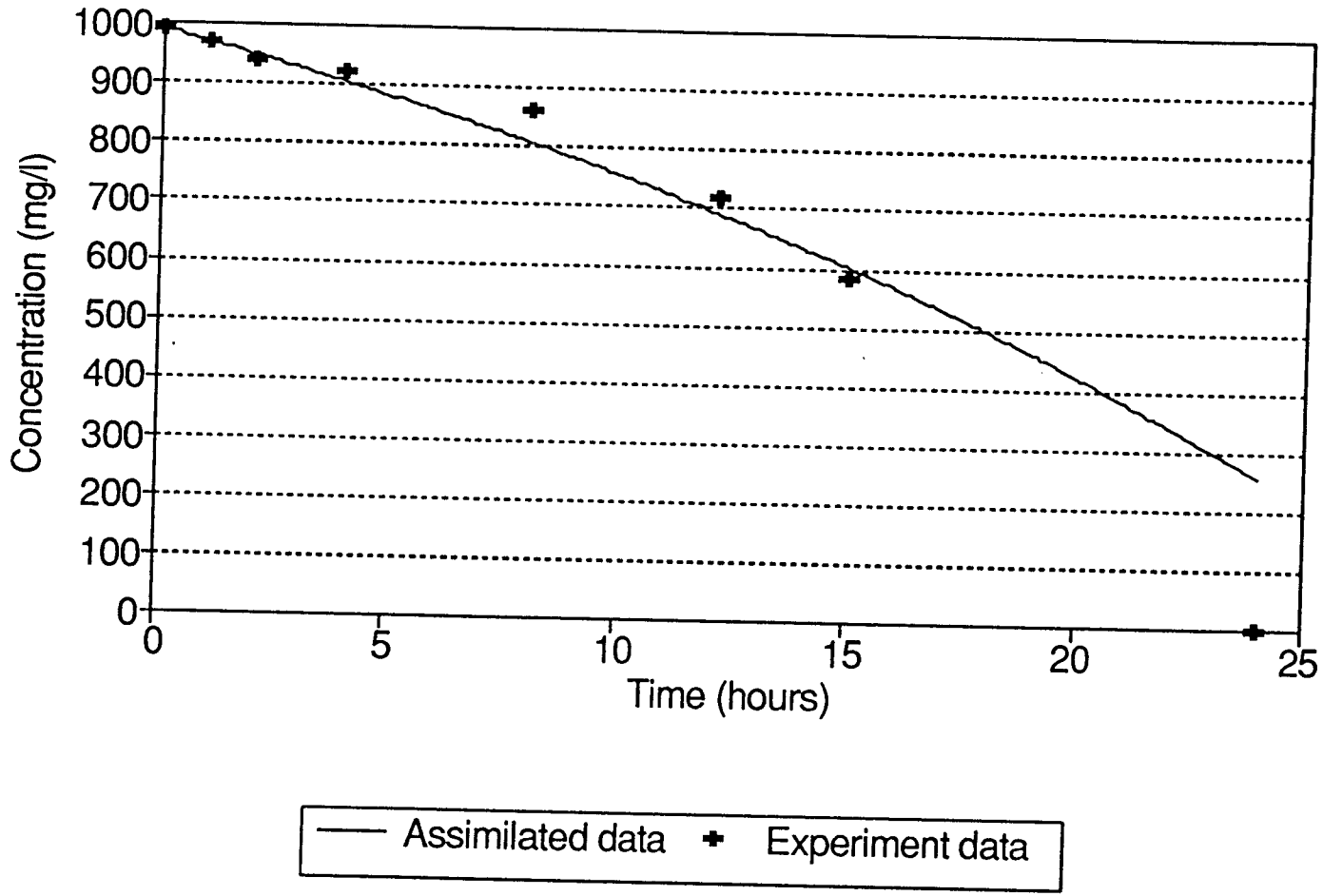
Degradation of Acetate with 3,4,5-TCP

(Xi=339 mg/l, Xf=1022 mg/l, km=0.040)



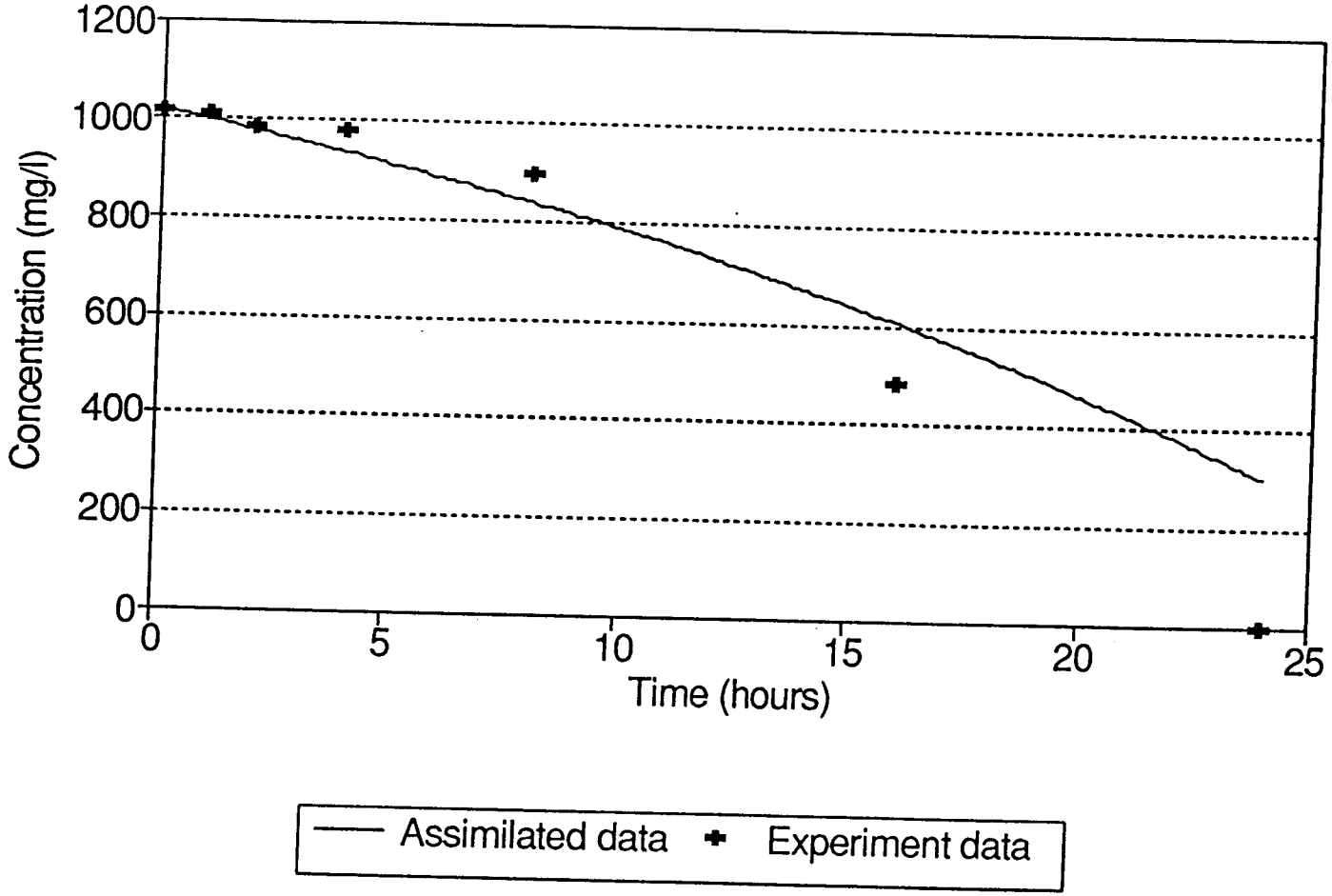
Degradation of Acetate with 2,4,6-TCP

(Xi=210.3 mg/l, Xf=555.8 mg/l, km=.095)



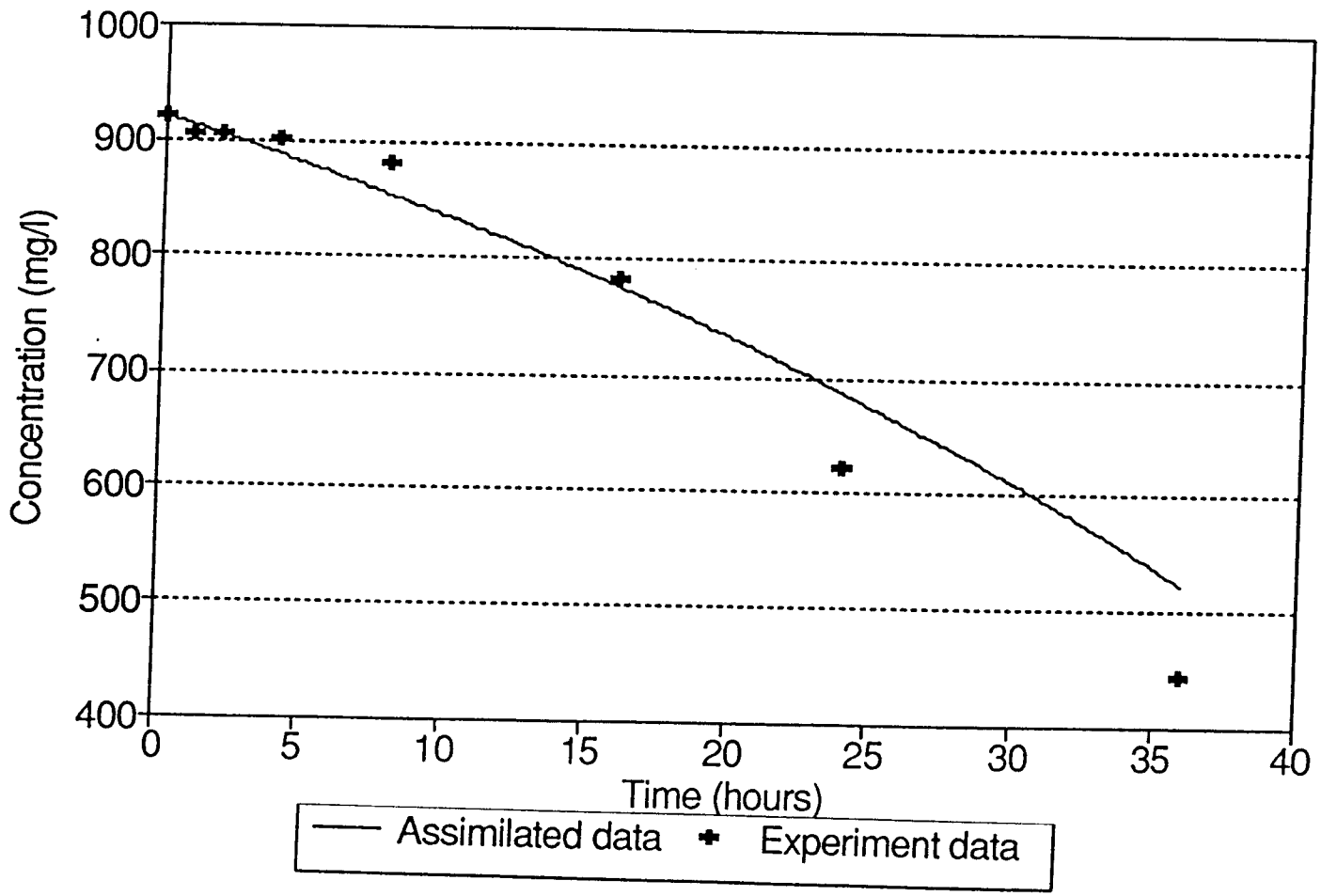
Degradation of Acetate with 2,3,5-TCP

(Xi=439 mg/l, Xf=1184 mg/l), km=0.045)



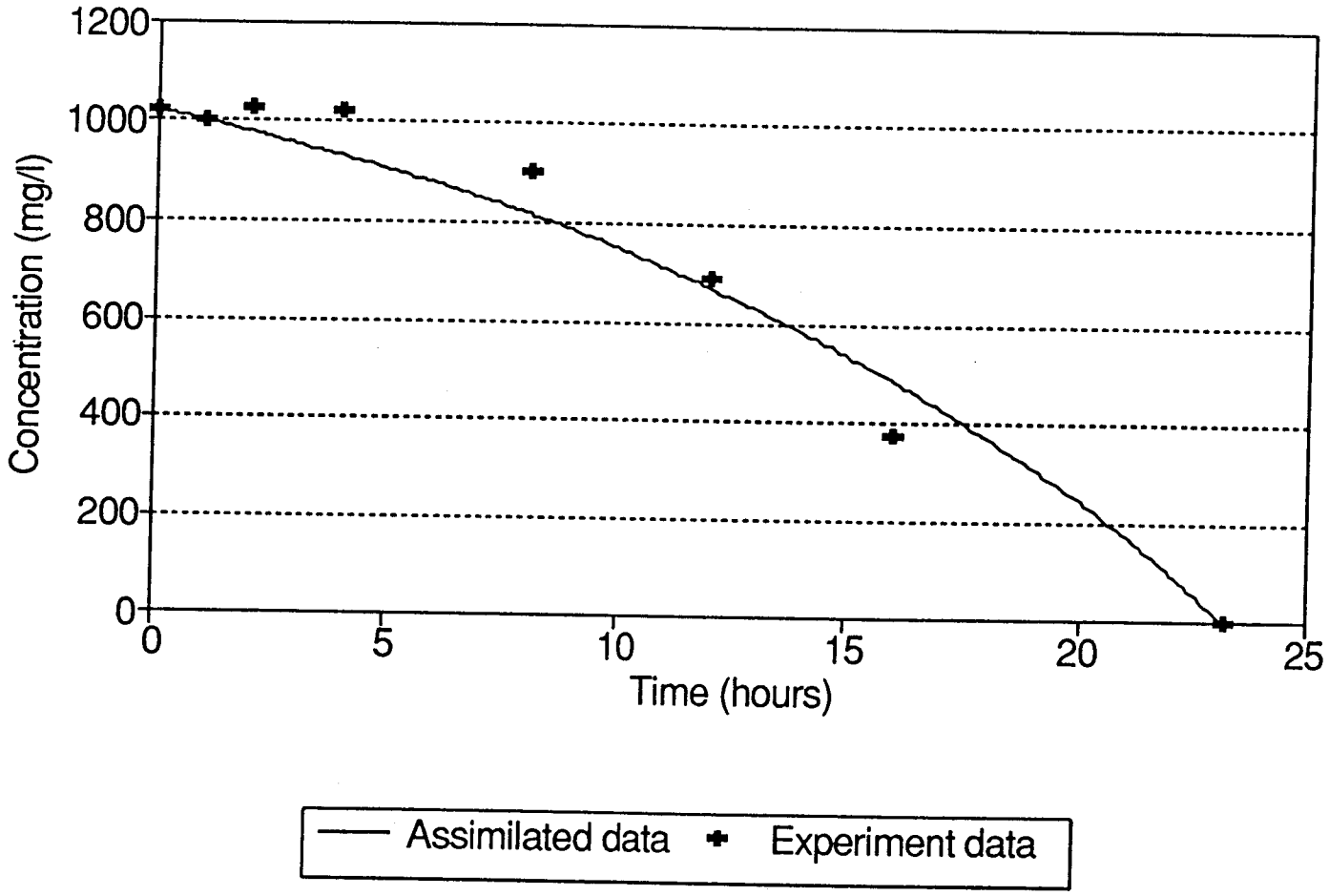
Degradation of Acetate with 2,4,5-TCP

(Xi=308.9 mg/l, Xf=806.1 mg/l, km=.025)



Degradation of Acetate with 2,4&3,4-DCP

(Xi=139 mg/l, Xf=575mg/l, km=0.144)



Appendix B

Experimental Protocol

1. Prepare a solution containing the chlorophenols of interest; analyze for chlorophenol concentrations on the gas chromatograph; calculate the volume of solution needed for the experiment.

2. Calibrate pH electrode whenever use.

3. Run acetate concentration on ion chromatograph to determine an initial COD of 500 mg/l.

4. Add glacial acetic acid and buffer to the calculated volume of chlorophenol solution to obtain an initial acetate concentration of 1000 mg/l in each batch experiment.

5. Connect the drain port of the mother reactor to the influent valve of the batch reactor using a Teflon tubing to collect 1.5 liter of "mother solution" for each batch experiment.

6. Add the prepared solution containing chlorophenol of interest, acetate and buffer to the batch reactor in which mother solution was added.

7. Place the batch reactor on a magnetic stirrer set at intensity 10 in a 31°C environmental chamber; set an electrode on the reactor to measure pH; connect air pump to the air port; start both the stirrer and pump.

8. Adjust the pH to 6.5 using saturated sodium bicarbonate solution.

9. Sample immediately once pH value is stable and at intervals appropriate for the experimental duration.

10. Record pH value for each sampling.

11. Analyze TSS for the first and last sampling of each batch experiment.

12. Withdraw 15 ml of liquid sample from the sample port using an inverted disposable pipette.

13. Filter 3 ml of sample through a pre-washed and weighted Whatman type GF/C glass microfiber filter using a Millipore filtration apparatus, discard this portion, then filter the remaining sample; place the paper in an oven for solid analysis.

14. Extract 5 ml of the filtered sample into hexane by following the Acetylation/extraction Protocol; fill 2 ml amber autosampler and storage vials with teflon caps with a new pasteur pipette.

15. Transfer 5 ml of the filtered sample into a 100-ml volumetric flask and dilute it using an anion eluent for acetate analysis on ion chromatograph.

16. Record all data in the notebook.

17. Transfer the consortia back to the mother reactor by pumping into the influent line.

18. Run chlorophenol samples on GC using the Gas Chromatograph Protocol and acetate samples on the ion chromatograph; run standard curves each time for both GC and IC analyses.

19. Save hexane extracts in a freezer in the dark; refreeze remaining acetate samples.

Appendix C

Acetylation/Extraction Protocol

1. Clean glassware by acid-washing in 50% H_2SO_4 solution, rinsing at least three times with tap water, three times with distilled water and three times with hexane (for separatory funnels).

2. Add 45 ml glass-distilled water to the separatory funnel using a 50-ml volumetric pipette.

3. Add 25 l of internal standard 1. (2,6-DBP) or 50 l of internal standard 2. (2,4,6-TBP) to the funnel using a dedicated 50- l syringe.

4. Add 5 ml of the filtered sample to the funnel using a clean and dry 5-ml volumetric pipette.

5. Add 1 ml of 0.73 gr/ml K_2CO_3 solution to the funnel and shake.

6. Add 1 ml of acetic anhydride to the funnel

7. Shake the funnel for exactly two minutes, venting every 10-20 second.

8. After two hours, add 5 ml of hexane to the funnel using a dedicated 5-ml volumetric pipette.

9. Shake the funnel for exactly 2 minutes, venting every 20-30 seconds.

10. Withdraw water from the funnel until the hexane layer is easy to withdraw without contaminating the hexane extract with water.

11. Transfer the hexane extract to a new, amber glass vial with Teflon-lined cap using a disposable pasteur pipette.

12. Always fill the vial to the same level. Store the filled vial in the refrigerator in the dark until analysis on GC.

13. Record all data in a research notebook.

Appendix D

Gas Chromatograph Protocol

1. Record all "blank", standard and sample order in the notebook. The general order is as follows: two hexane rinses as blank, standard curves, two hexane rinses, chlorophenol samples (duplicated), two hexane rinses and standard curves. For the both standard curves and samples, the order is from the most dilute to the most concentrated.

2. Load all samples into the autosampler rack based on the order mentioned above.

3. Check the gas tanks to make sure that the regulator is open and that the tank contains at least 500 psi gas. Adjust the regulator to the following pressures if necessary: helium, 60 psi, argon/methane, 40 psi.

4. Check printing paper on the integrator to make sure that there is enough paper for the run.

5. Fill the autosampler solvent rinse A and B vials with hexane.

6. Edit the method on the integrator to select the temperature program and set the number of samples to run, by keying the following sequence: edit seq. enter 3 enter (method names, e.g. "mark. met" or "computer.met") enter 1 enter (first and last sample, enter for all other questions) enter.

7. Print the operating conditions and temperature program by keying list meth enter.

8. Start the analyses by keying seq start.

9. List the method again after all samples have been run.

10. Keep the sample set as one continuous roll of paper.

11. If the "computer met" is used, follow the procedure: (1)

Turn the computer on and boot it up using the disk in the A drive. (2) Input the correct date and time. (3) Insert a blank formatted disk into drive b. (4) Copy the file SERVER.EXE from the A drive to B drive. (5) Change the directory to the B drive. (6) Type in "SERVER" to activate the datalogging program. By doing so, a post-run report at the end of each chromatographic run will be duplicated as a computer file on a floppy disk in the B drive. A separate file is generated for each report and is in ascii format. The file name for each report consists of 8 randomly generated alpha-numeric characters with an .RPT extension. Computer report filename can be matched with their corresponding chromatograms by observing the report filename printed on the integrator.

Appendix E

Preparation of Feed Media: The solution was prepared using a modified receipt given by Owen et al. (1979). The stock solution containing nutrients and vitamins was prepared as follows:

Solution	Components	Conc., g/L
S3	$(NH_4)_2HPO_4$	26.7
S4	$CaCl_2.2H_2O$	16.7
	NH_4Cl	26.6
	$MgCl_2.6H_2O$	120
	KCl	86.7
	$MnCl_2.4H_2O$	1.33
	$CoCl_2.6H_2O$	2.0
	H_3BO_3	0.38
	$CuCl_2.2H_2O$	0.18
	$Na_2MoO_4.2H_2O$	0.17
	$ZnCl_2$	0.14
S7	Biotin	0.02
	Folic acid	0.02
	Pyridoxine hydrochloride	0.1
	Riboflavin	0.05
	Thiamin	0.05
	Nicotinic acid	0.05
	Pantothenic acid	0.05
	B_{12}	0.001
	p-aminobenzoic acid	0.05
	Thioctic acid	0.05

To make 6 liters of feed solution, the following receipt was used:

16.2 ml S3

81.0 ml S4

5.4 ml S7

2.68 ml glacial acetic acid (COD = 500 mg/l)

6.0 ml of each of the eight chlorophenols (100 mg/l)

10 grams sodium bicarbonate

distilled water to make 6 liters

* 5 liters of distilled water were added first, followed by the acetic acid, S4, S3 and S7 and chlorophenol solutions. Distilled water was then added to fill 6 liters, finally the sodium bicarbonate added to adjust pH.

Appendix F

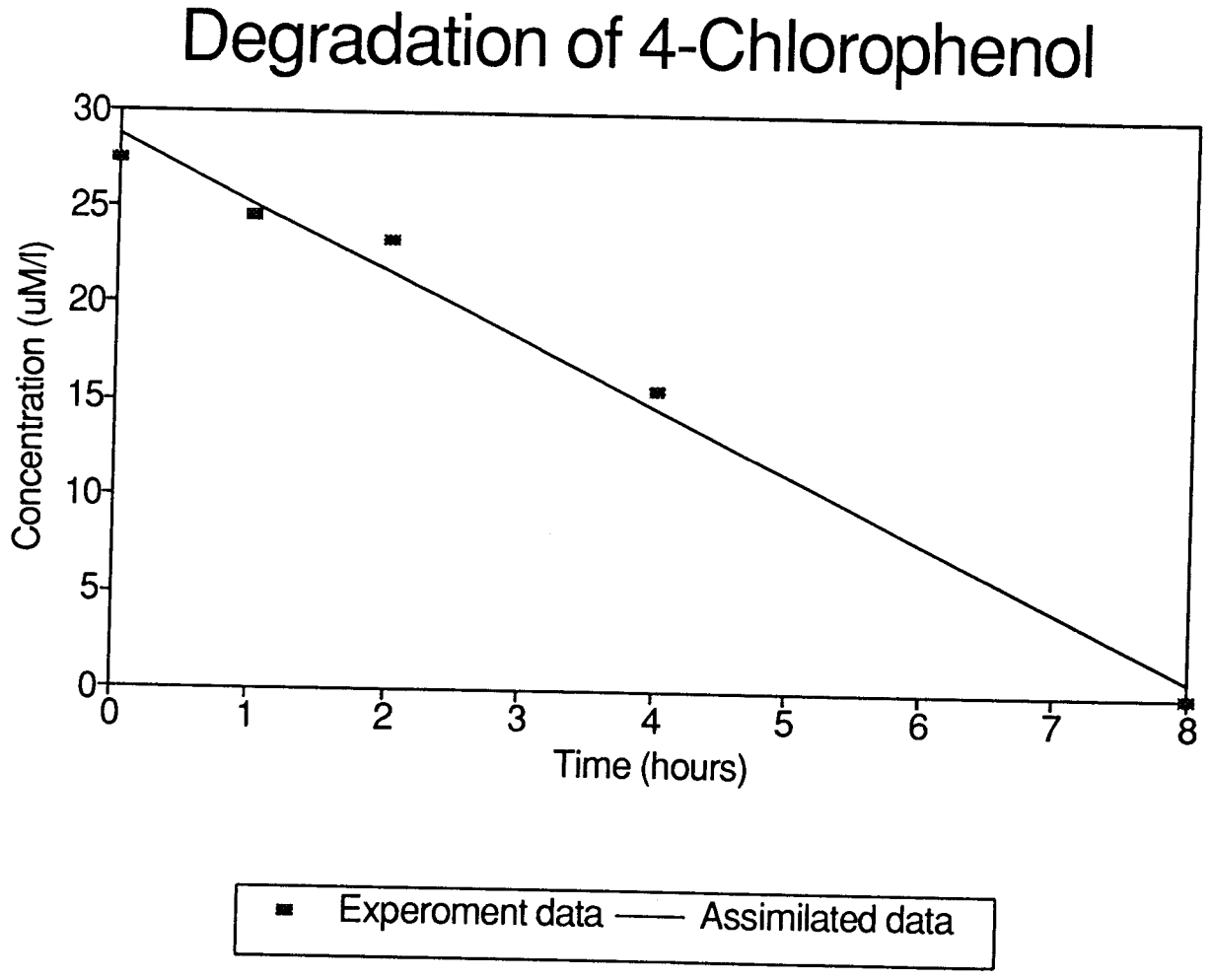
Retention Time & Relative Retention Time for Chlorophenols.

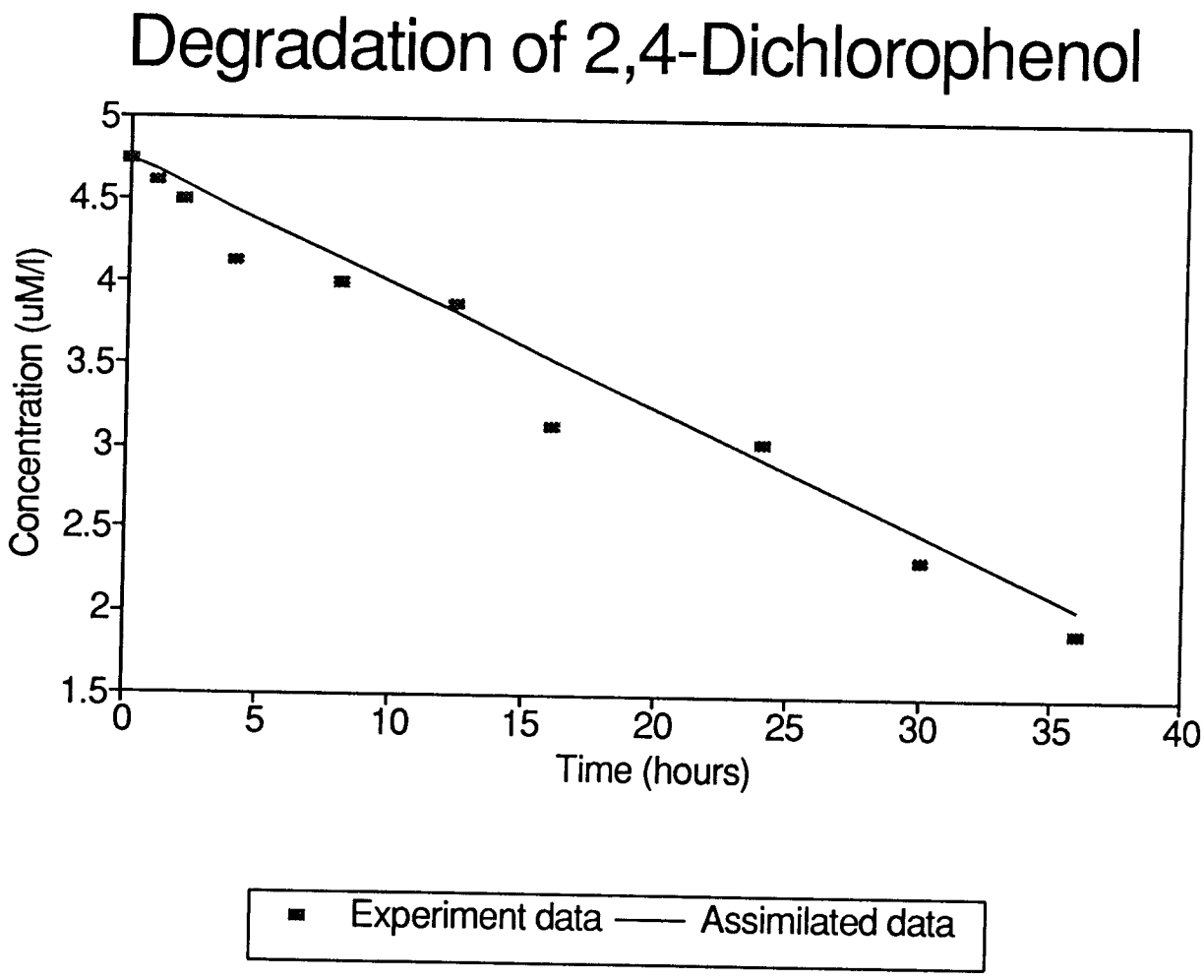
Congeners	Retention time min	ISTD	Relative retention time[1]
4-CP	12.385	2,6-DBP	0.662
2,4-DCP	15.029	2,6-DBP	0.804
3,4-DCP	16.404	2,6-DBP	0.877
3,5-DCP	15.381	2,4,6-TBP	0.625
2,4,6-TCP	17.353	2,6-DBP	0.928
3,4,5-TCP	20.200	2,6-DBP	1.080
2,4,5-TCP	19.249	2,4,6-TBP	0.782
2,3,5-TCP	19.100	2,4,6-TBP	0.776
PCP	26.069	2,6-DBP	1.394

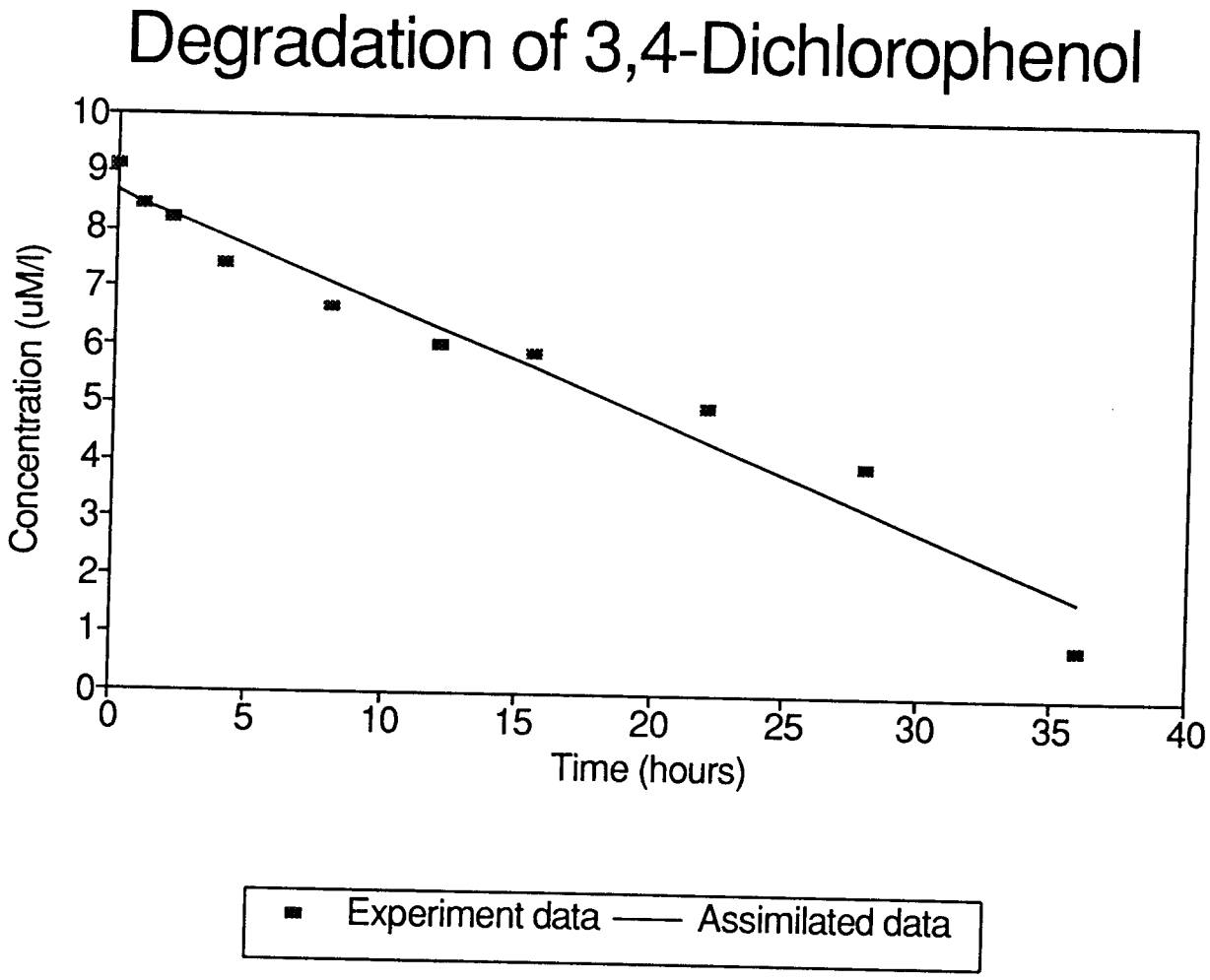
[1] Retention time divided by the retention time of internal standard (ISTD). Typical retention times for the internal standards are 18.701 min for 2,6-DBP and 24.608 min for 2,4,6-TBP.

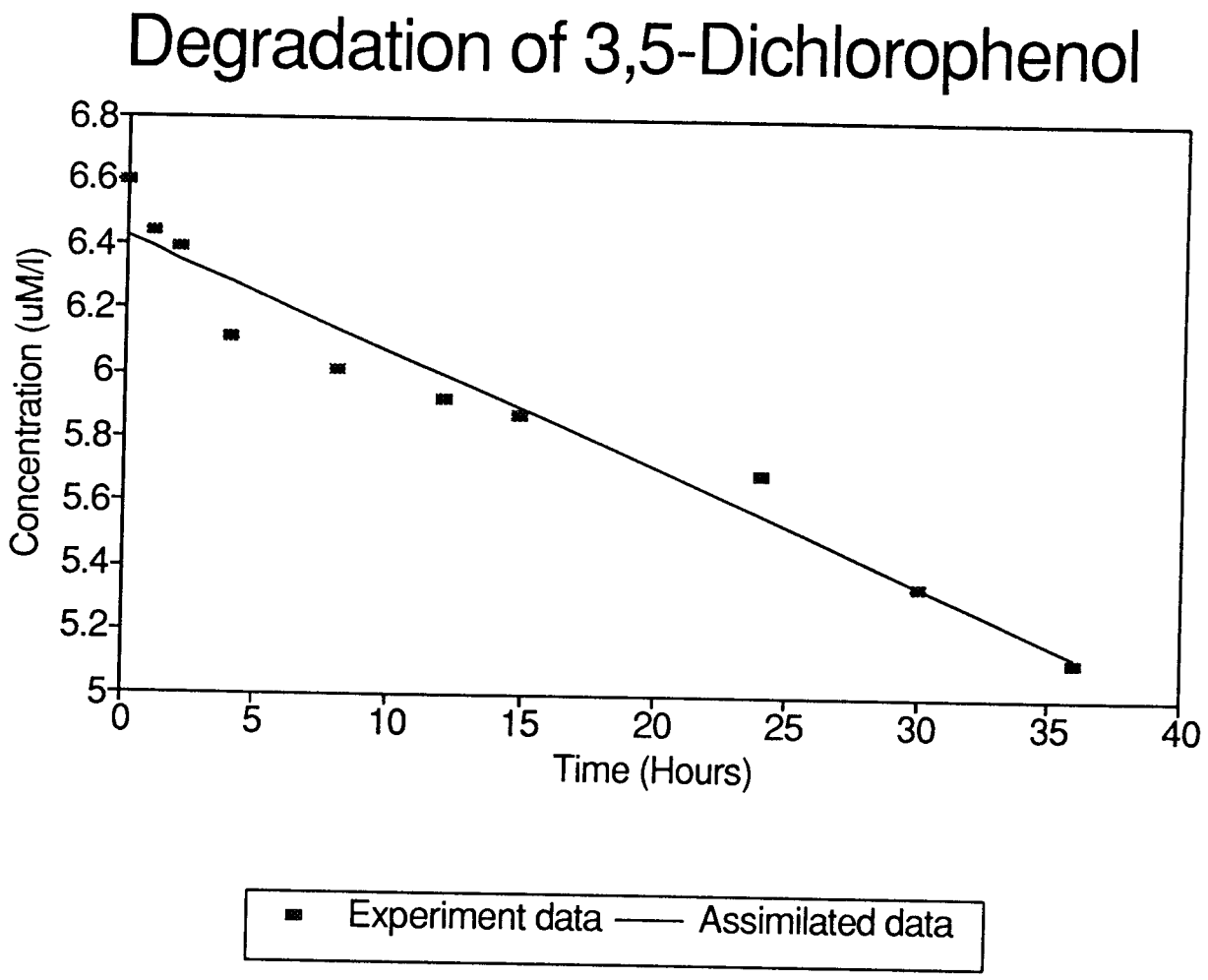
Appendix G

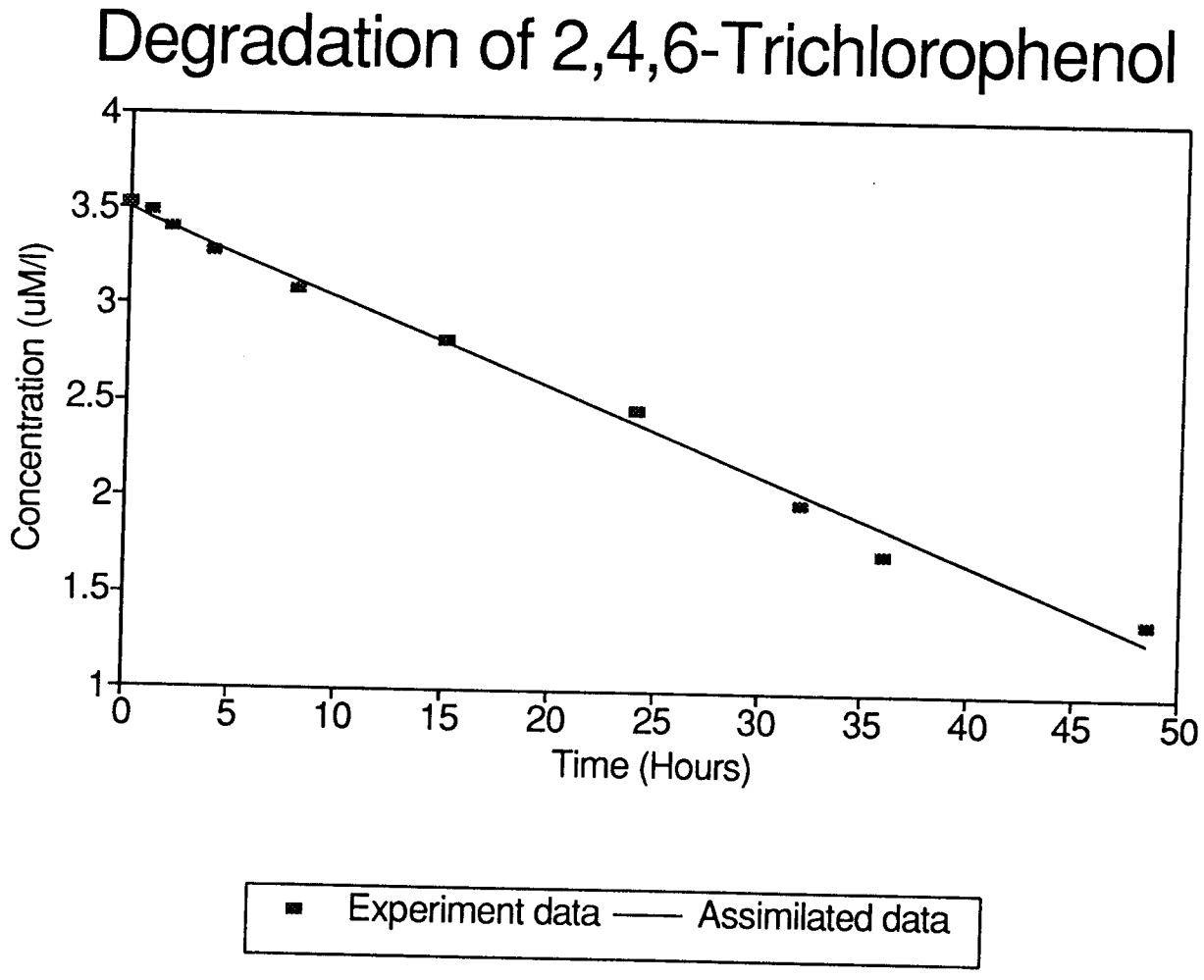
Graphs for the Degradation of Chlorophenols

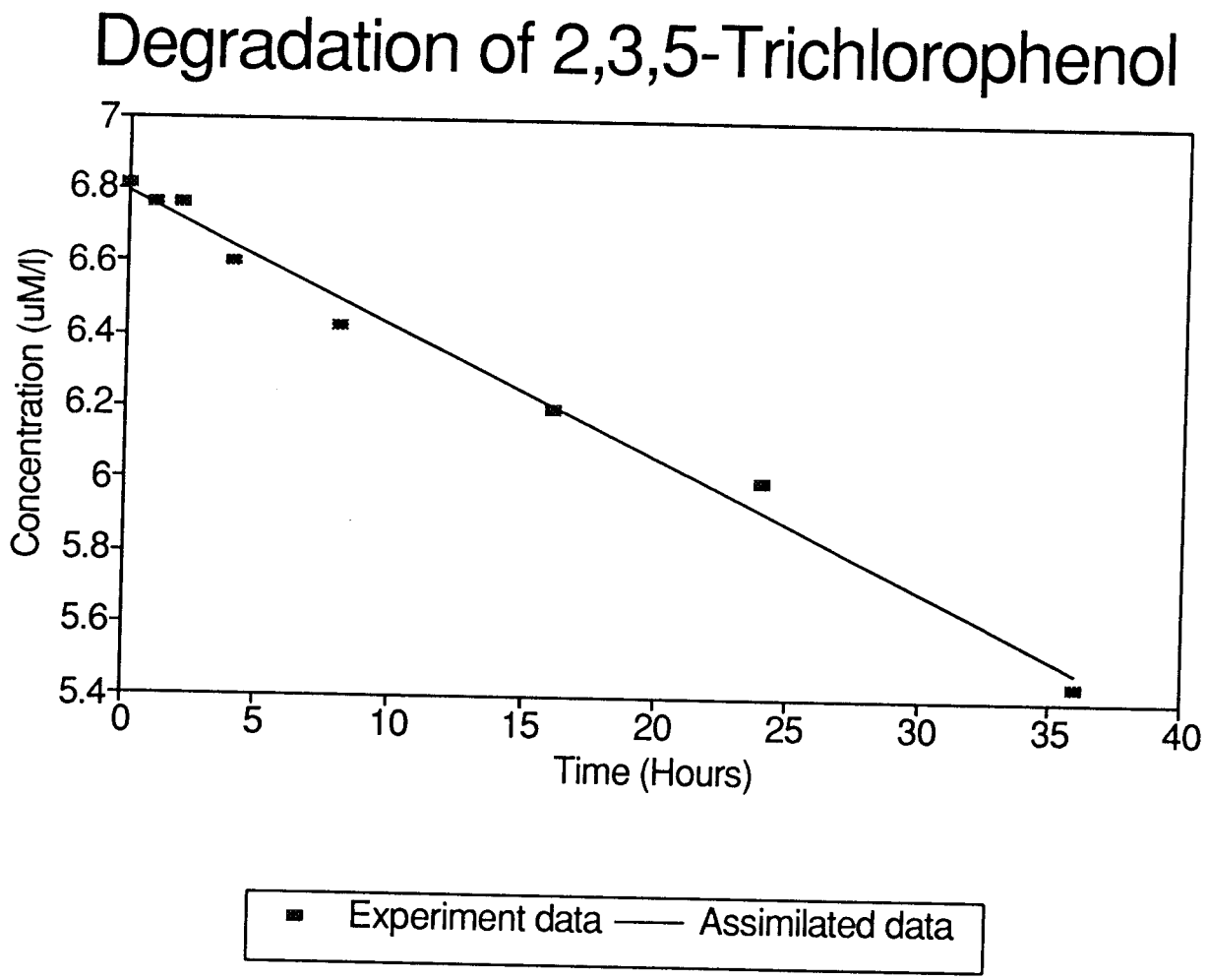

Degradation of 4-Chlorophenol
Concentration (uM/l)
30
25
20
15
10
5
0
0
1
2
3
4
5
6
7
8
Time (hours)
Experoment data
Assimilated data

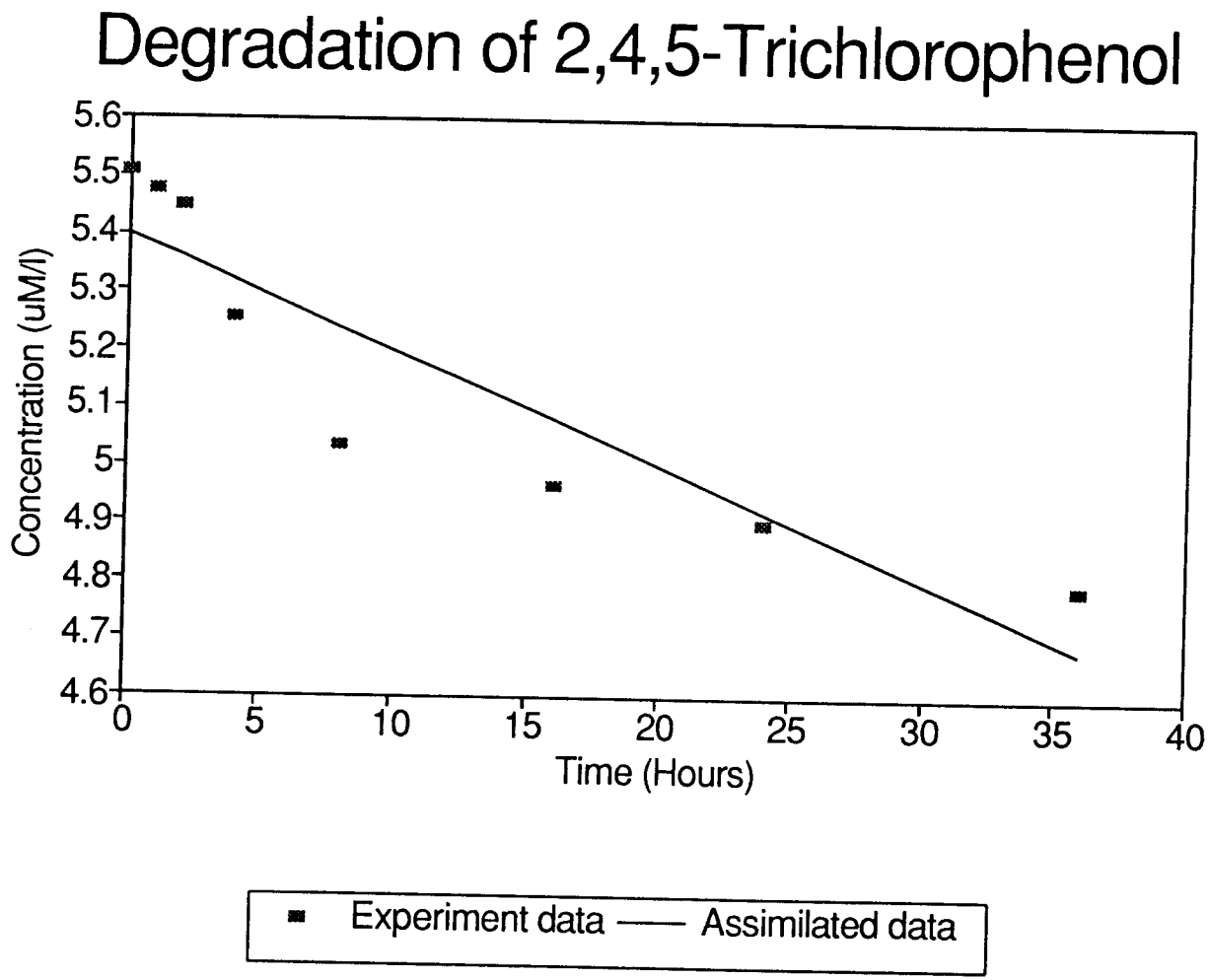

Degradation of 2,4-Dichlorophenol
Concentration (uM/l)
5
4.5
4
3.5
3
2.5
2
1.5
0
5
10
15
20
25
30
35
40
Time (hours)
Experiment data
Assimilated data

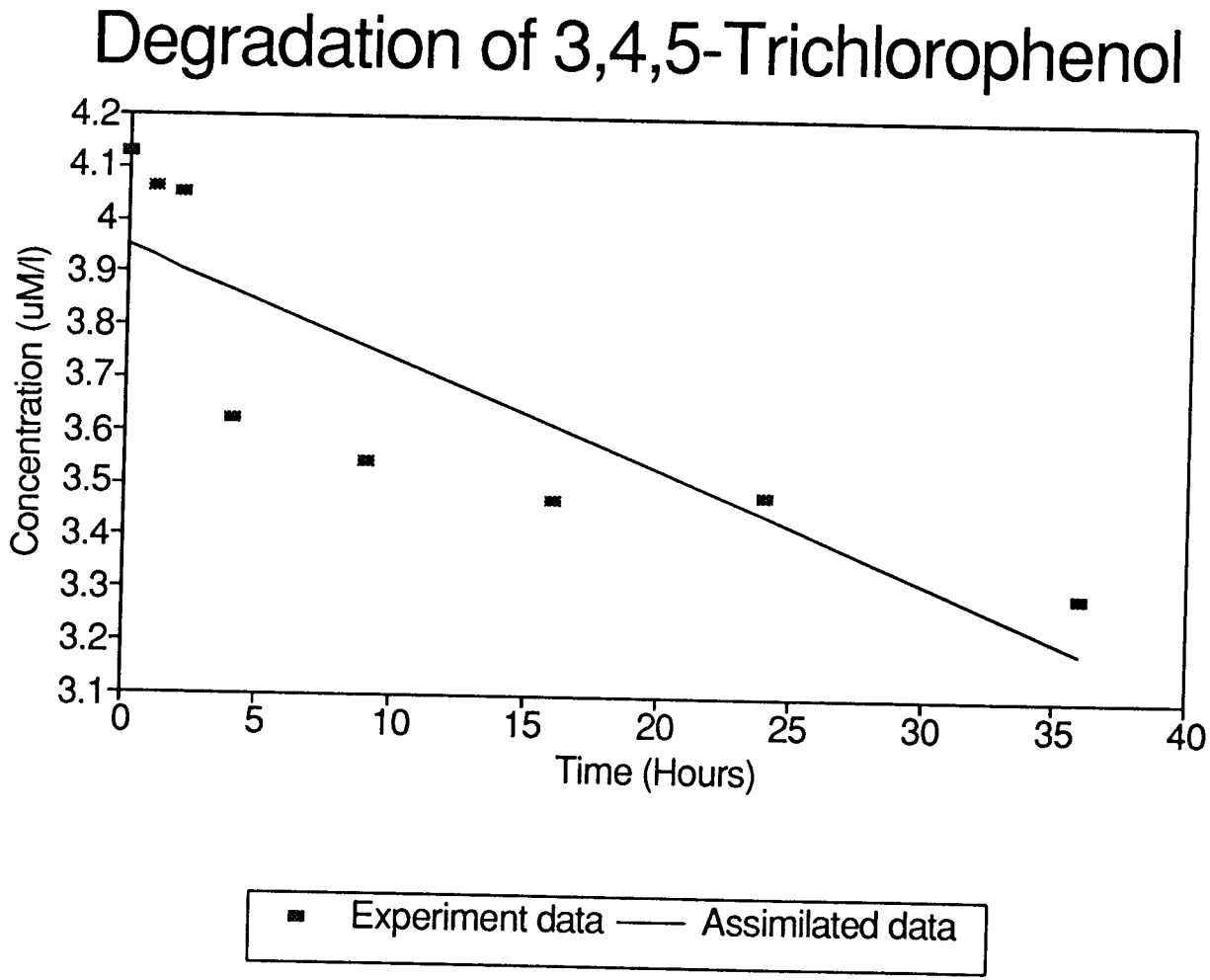

Degradation of 3,4-Dichlorophenol
Concentration (uM/l)
0
1
2
3
4
5
6
7
8
9
10
0
5
10
15
20
25
30
35
40
Time (hours)
Experiment data
Assimilated data

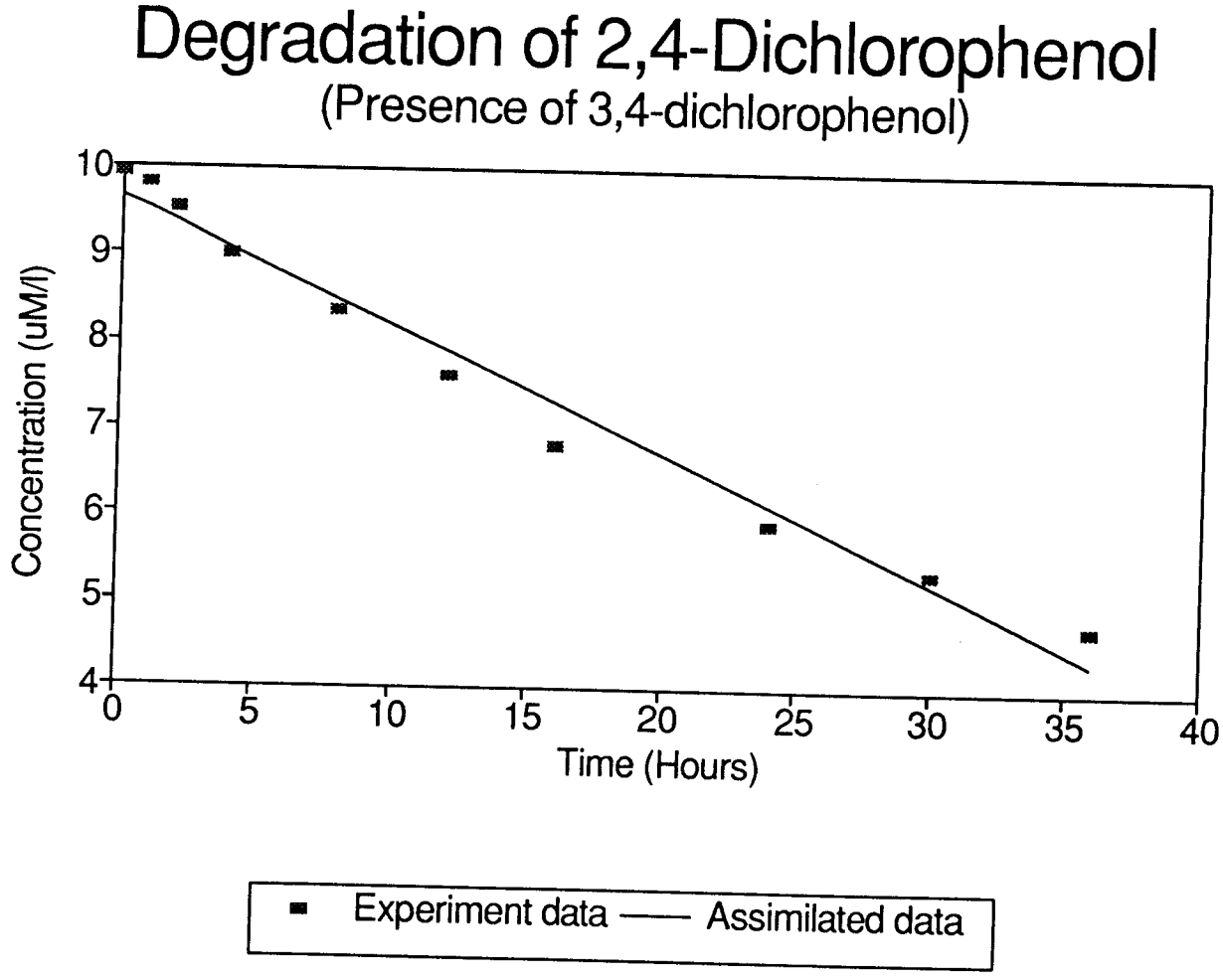

Degradation of 3,5-Dichlorophenol
Concentration (uM/l)
6.8
6.6
6.4
6.2
6
5.8
5.6
5.4
5.2
5
0
5
10
15
20
25
30
35
40
Time (Hours)
Experiment data
Assimilated data

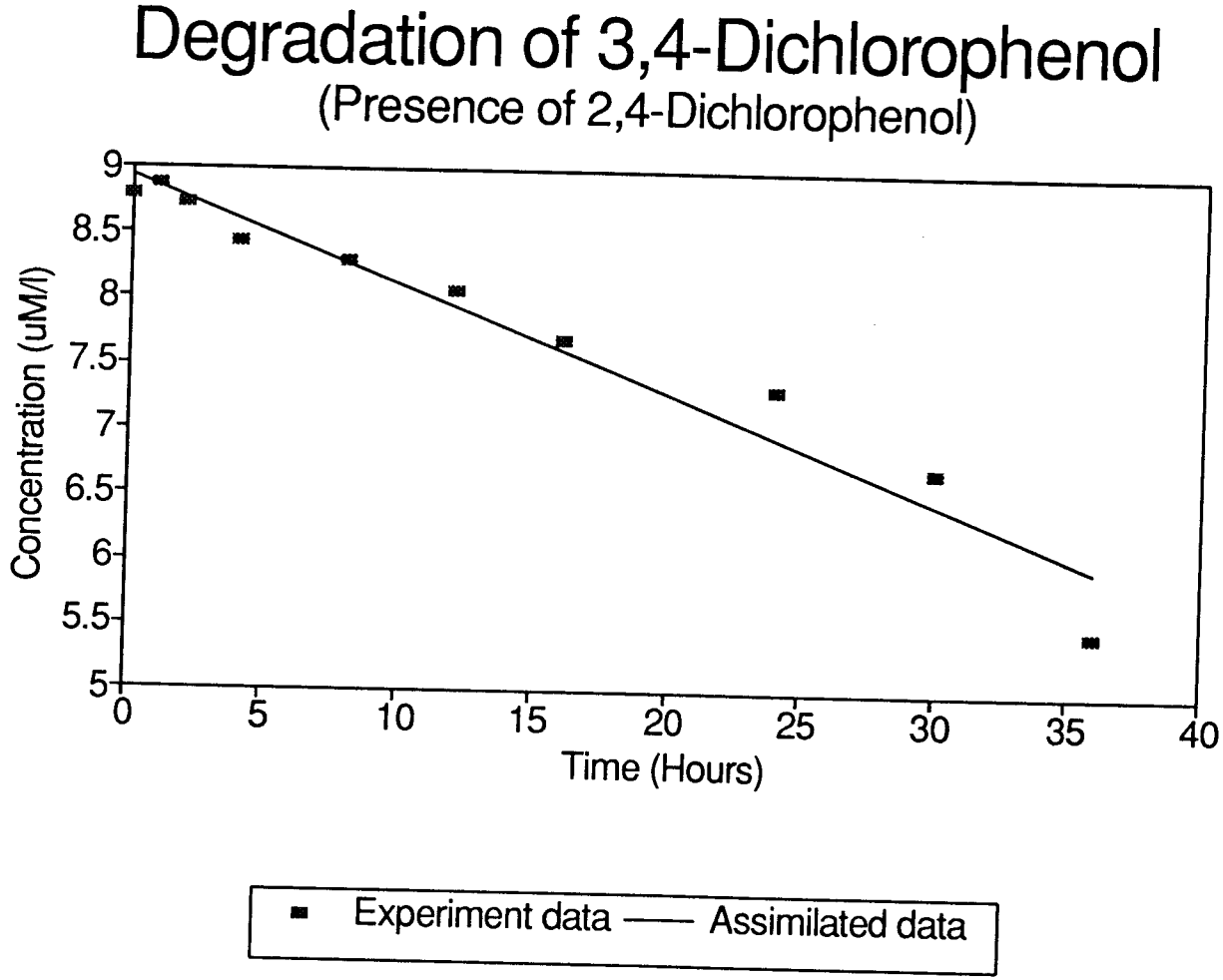

Degradation of 2,4,6-Trichlorophenol
Concentration (uM/l)
4
3.5
3
2.5
2
1.5
1
0
5
10
15
20
25
30
35
40
45
50
Time (Hours)
Experiment data
Assimilated data

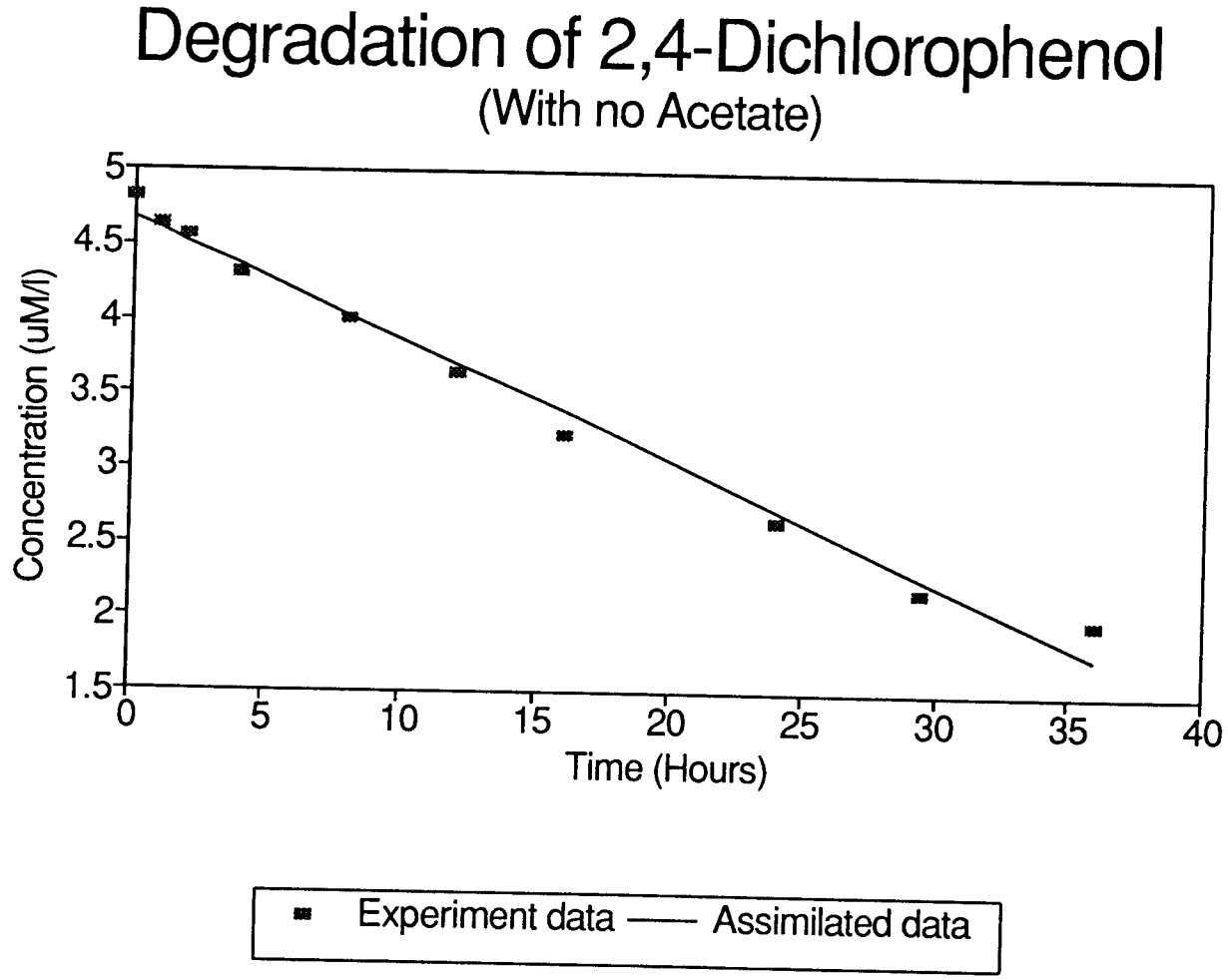

Degradation of 2,3,5-Trichlorophenol
Concentration (uM/l)
7
6.8
6.6
6.4
6.2
6
5.8
5.6
5.4
0
5
10
15
20
25
30
35
40
Time (Hours)
Experiment data
Assimilated data

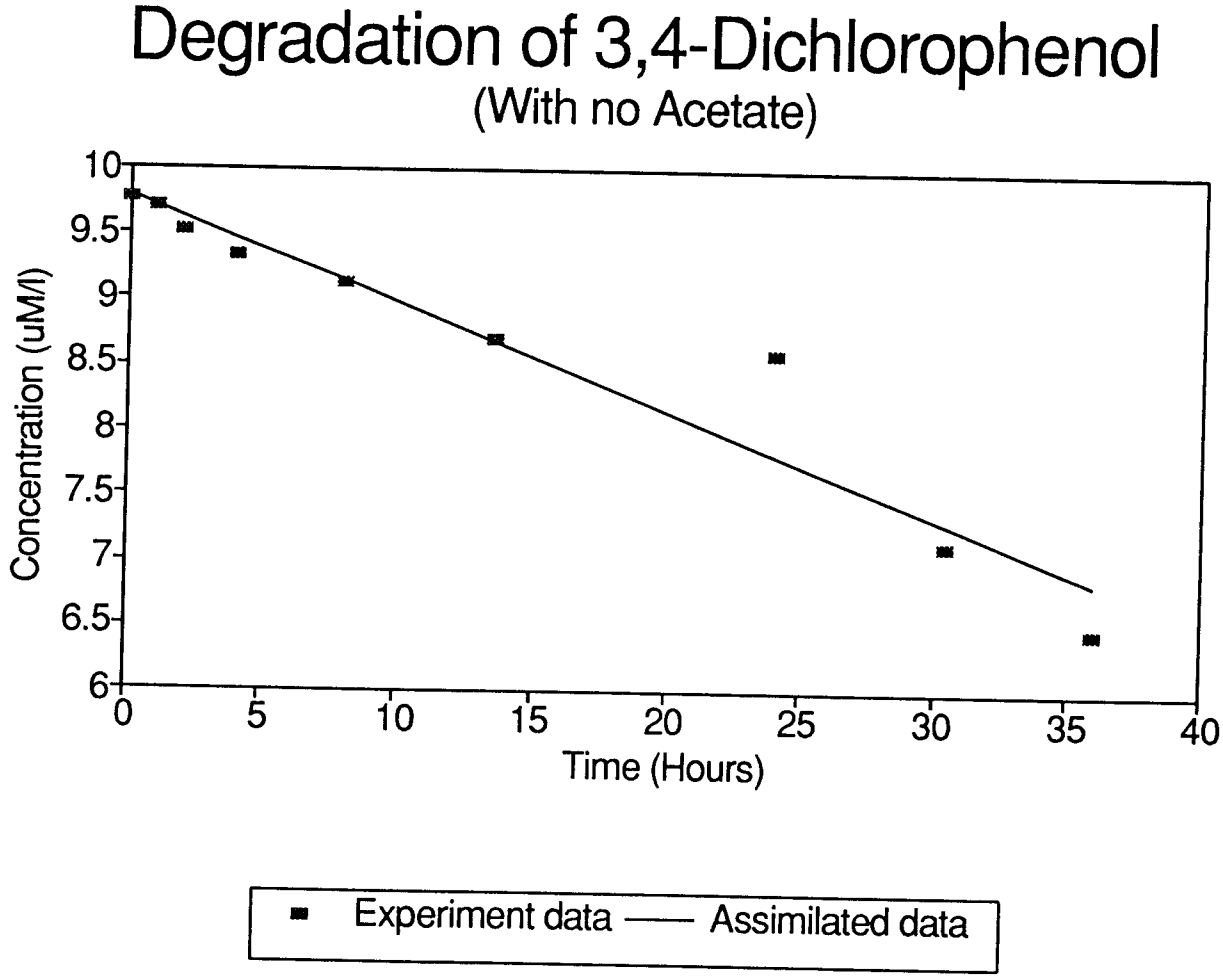

Degradation of 2,4,5-Trichlorophenol
Concentration (uM/l)
5.6
5.5
5.4
5.3
5.2
5.1
5
4.9
4.8
4.7
4.6
0
5
10
15
20
25
30
35
40
Time (Hours)
Experiment data
Assimilated data

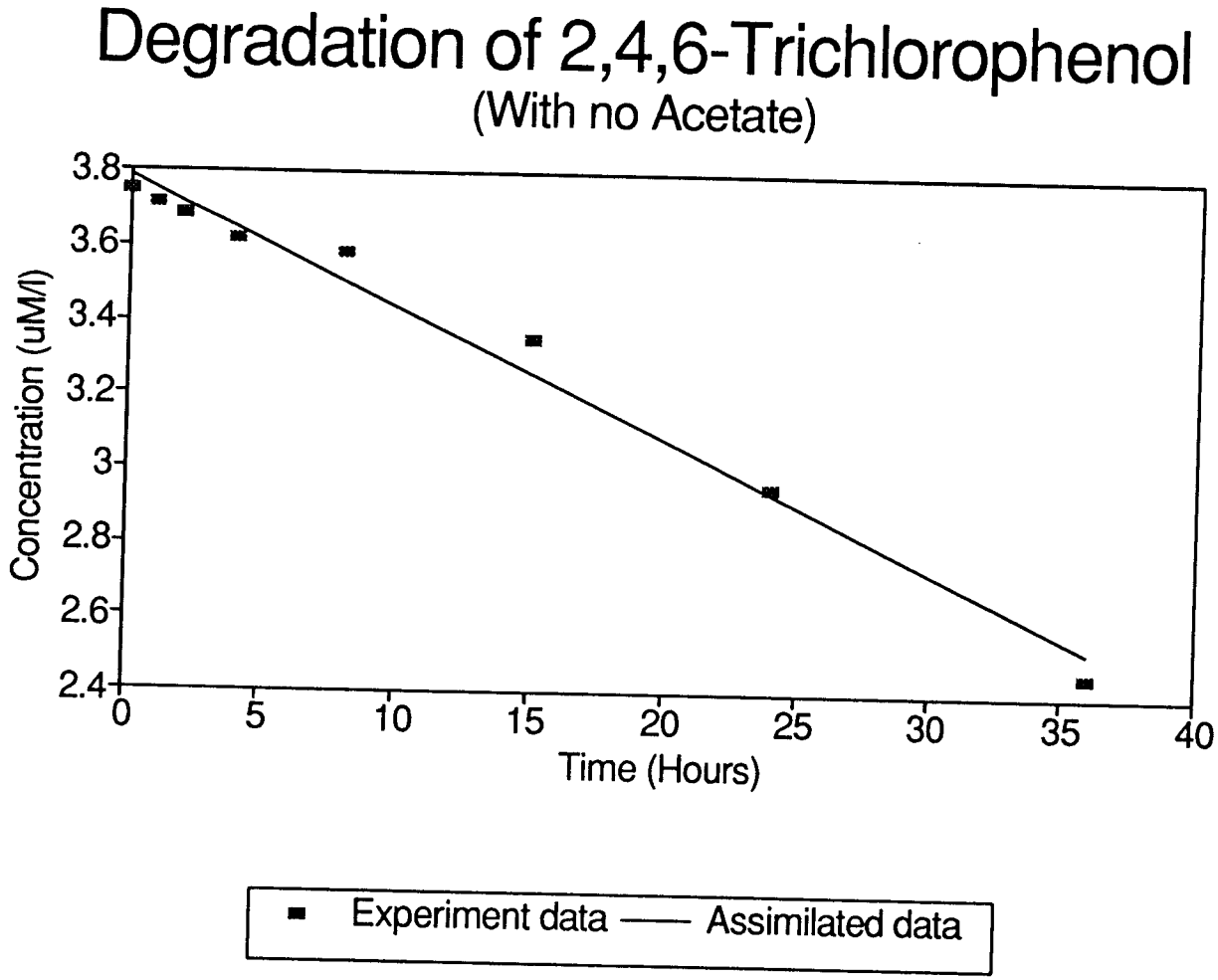

Degradation of 3,4,5-Trichlorophenol
Concentration (uM/l)
4.2
4.1
4
3.9
3.8
3.7
3.6
3.5
3.4
3.3
3.2
3.1
0
5
10
15
20
25
30
35
40
Time (Hours)
Experiment data
Assimilated data


Degradation of 2,4-Dichlorophenol
(Presence of 3,4-dichlorophenol)
Concentration (uM/l)
10
9
8
7
6
5
4
0
5
10
15
20
25
30
35
40
Time (Hours)
Experiment data
Assimilated data


Degradation of 3,4-Dichlorophenol
(Presence of 2,4-Dichlorophenol)
Concentration (uM/l)
9
8.5
8
7.5
7
6.5
6
5.5
5
0
5
10
15
20
25
30
35
40
Time (Hours)
Experiment data
Assimilated data


Degradation of 2,4-Dichlorophenol
(With no Acetate)
Concentration (uM/l)
5
4.5
4
3.5
3
2.5
2
1.5
0
5
10
15
20
25
30
35
40
Time (Hours)
Experiment data
Assimilated data


Degradation of 3,4-Dichlorophenol
(With no Acetate)
Concentration (uM/l)
10
9.5
9
8.5
8
7.5
7
6.5
6
0
5
10
15
20
25
30
35
40
Time (Hours)
Experiment data
Assimilated data


Degradation of 2,4,6-Trichlorophenol
(With no Acetate)
Concentration (uM/l)
3.8
3.6
3.4
3.2
3
2.8
2.6
2.4
0
5
10
15
20
25
30
35
40
Time (Hours)
Experiment data
Assimilated data