REDUCTIVE DECHLORINATION OF CHLORINATED PHENOLS IN ANAEROBIC UPFLOW BIOREACTORS

By

Mary Luise Krumme

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Crop and Soil Science

1986

ABSTRACT

REDUCTIVE DECHLORINATION OF CHLORINATED PHENOLS IN ANAEROBIC UPFLOW BIOREACTORS

By

Mary Luise Krumme

The reductive dechlorination of chlorinated aromatic compounds was studied in anaerobic upflow bioreactors with chlorinated phenols as the sole carbon and energy source. Dechlorinating activity was maintained in three separate anaerobic bioreactors for 350, 400 and 190 days. Mineralization of approximately 40% of added chlorophenols to CH_4 and CO_2 was demonstrated. Substrate loading rates of up to 20 mg l^{-1} d^{-1} at a hydraulic retention time of two or four days were achieved at a substrate conversion efficiency of greater that 90% as determined by measurement of substrate concentration in the effluent. The ability to degrade a mixture of all three monochlorophenols and 3,4,5-trichlorophenol was demonstrated. The majority of the active biomass was located at the bottom of the bioreactor in the form of a sludge blanket. Three microorganisms which dominated the biomass were identified by morphology as a putative anaerobic phenol-oxidizing bacterium, a *Methanosarcina sp.* and a *Methanothrix sp.*

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The Environmental Protection Agency estimated that the United States generates more than 70 billion tons of hazardous waste annually (Thomas, 1984). Because of the vast quantity of waste produced, there is an increasing need for establishing large-scale hazardous waste disposal methods. Biological treatment can eliminate some hazardous compounds, such as chlorinated aromatic compounds, by aerobic or anaerobic degradation to yield inocuous compounds. Some halogenated aromatic compounds, such as the chlorophenols, can be degraded and mineralized in both aerobic and anaerobic systems; however, anaerobic biological treatment can offer many advantages such as lower operational costs and the possibility of novel reactions. Nevertheless, anaerobic biodegradation treatment systems for halogenated aromatics need further development in order to more fully understand the microbial processes involved and to make the processes feasible for large-scale treatment of toxic waste.

Chlorophenols are widely used industrial chemicals. Pentachlorophenol (PCP) is registered as an insecticide, fungicide, herbicide, algicide, disinfectant, and an ingredient in antifouling paint (Crosby, 1981). In 1981, the U.S. consumption of PCP was 23,000,000 kg/year (Arsenault, 1976). Approximately 80% of the PCP manufactured was used for commercial wood treatment (Crosby, 1981). Chlorophenols are also precursors for other industrially important herbicides such as 2,4-D and 2,4,5-T (Environmental Protection Agency, 1980). In addition to the deliberate manufacturing of chlorophenols, these chemicals are by-products from other processes such as the chlorination of water for disinfection (Ahlborg and Thunberg, 1980; Environmental Protection Agency, 1980) and the bleaching of paper pulp (Lindberg and Lund, 1980; Salkinoja-Salonen *et al.*, 1981).

Because of their widespread usage, chlorophenols are found throughout the environment. The chlorophenols, and in particular 2,4-dichlorophenol, 2,4,6-trichlorophenol and PCP have been placed on the Environmental Protection Agency list of 129 Priority Pollutants (Keith and Telliard, 1979). Many different types of environmental samples have been found contaminated with chlorophenols including soil, river, lake and sea water, sediment, and snow (Salkinoja-Salonen *et al.* 1981; Salkinoja-Salonen *et al.*, 1984; Paasivirta *et al.*, 1985; Valo *et al.*, 1985; Xie *et al.*, 1986). Chlorophenol contamination has been found not only near point pollution sources such as the Finnish saw mills treating wood with PCP (Valo *et al.*, 1984; Valo *et al.*, 1985), but contamination has also been found in otherwise pristine environments (Paasivirta *et al.*, 1985). Even man is contaminated with chlorophenols; PCP and other chlorophenols have been found in greater than 90% of human urine samples from occupationally unexposed groups (Edgerton *et al.*, 1981).

Chlorophenols, especially PCP, are toxic compounds. Their toxicity has made them both useful as lethal substances and harmful as environmental pollutants. PCP is toxic to nearly all forms of life including man, animals and plants (Crosby, 1981); PCP acts as an inhibitor of oxidative phosphorylation (Weinbach *et al.*, 1965; Ahlborg and Thunberg, 1980). Numerous fish kills have been reported from PCP amended paddy soils which prompted the Japanese to limit usage of PCP as a herbicide (Crosby, 1981). Methanogens, in particular, are sensitive to PCP; thus the presence of PCP in waste effluent can cause process upsets in anaerobic treatment facilities. PCP has been found to completely inhibit methanogenesis from other carbon sources at concentrations as low as 1 mg/l (Guthrie *et al.*, 1984; Godsy *et al.*, 1986).

The biodegradation of chlorophenols has been demonstrated in laboratory studies, environmental fate studies and industrial waste treatment processes. Most of the information regarding the biodegradation potential of chlorophenols and other chlorinated aromatic compounds comes from aerobic studies in which oxygen functions as the terminal electron acceptor. In general, ring cleavage precedes dehalogenation and susceptibility to biodegradation decreases with increasing halogenation (Tabak *et al.*, 1964; Dorn and Knackmuss, 1978; Dorn and Knackmuss, 1978a; Knackmuss, 1981; Kobayashi and Rittman, 1982; Ghosal *et al.*, 1985).

In contrast to aerobic systems, biodegradation of halogenated aromatic compounds generally procedes by an initial dehalogenating step resulting in partially or fully dehalogenated products (Tiedje *et al.*, 1986). In both cases, the resulting aromatic nucleus is more susceptible to either aerobic or anaerobic microbial attack.

Dehalogenation under anaerobic conditions was first observed for chlorines on aliphatic carbons such as DDT [1,1,1-trichloro-2,2,-bis (p-chlorophenyl) ethane] and lindane (γ-hexachlorocyclohexane) (McRae *et al.*, 1967; McRae *et al.*, 1969). Numerous abiotic and biotic systems have been reported which were able to remove chlorines from non-aromatic carbons under anaerobic conditions (Barker *et al.*, 1965; Miskus *et al.*, 1965; Guenzi and Beard, 1967; McRae *et al.*, 1967; Wedemeyer, 1967; McRae *et al.*, 1969; Zoro *et al.*, 1974; Jagnow *et al.*, 1977). Since these early reports, numerous studies have reported on reductive dehalogenation from aliphatic carbons including compounds such as the halogenated hydrocarbons (Bouwer *et al.*, 1981; Bouwer and McCarty, 1983; Kleopfer *et al.*, 1985; Vogel and McCarty, 1985).

One of the earliest observations of dehalogenation from an aromatic nucleus comes from field studies with PCP (Ide *et al.*, 1972; Kuwatsuka and Igarashi, 1975; Murthy *et al.*, 1978). Because PCP was used extensively as an herbicide in rice paddy fields, many investigations were conducted on flooded soils where conditions were either oxygen limited or anaerobic. Ide *et al.* (1972) found that reductive dechlorination occurred more readily for chlorines on the ortho or para position resulting in the accumulation of meta-substituted chlorophenols. Kuwatsuka and Igarashi (1975), and Murthy *et al.*(1978) also examined the fate of PCP in anaerobic soil and found reductive dechlorination at all positions on the aromatic ring. Pentachloroanisole, the methylated derivative of PCP, was also found as a product. There was no evidence for the complete mineralization of PCP in these studies.

Since these early reports, reductive dechlorination has also been observed for the pesticides diuron (3,4-dichlorophenyl-1,1-dimethylurea), (Attaway *et al.*, 1982; Attaway *et al.*, 1982a; Stepp *et* *al.*, 1985), chlornitrofen (4-nitrophenyl-2,4,6-trichlorophenyl ether), (Yamada and Suzuki, 1983), thiobencarb (S-4-chlorobenzyl-N,N-diethyl thiocarbamate), (Moon and Kuwatsuka, 1984), and techlofthalam (N-(2,3-dichlorophenol)-3,4,5,6-tetrachlorophthalamic acid), (Kirkpatrick *et al.*, 1981). In pond sediment previously treated with diuron, dechlorination of the para-chlorine was observed resulting in the formation of monuron (3-(4-chlorophenyl)-1,1-dimethylurea) which was not further metabolized (Attaway *et al.*, 1982; Attaway *et al.*, 1982a). In further experiments, compounds with chlorines in the meta and para position [3,4-dichlorophenylurea, linuron (3,4-dichloro-phenyl-1-methyl-1-methoxy urea), and isopropyl-3,4-dichlorocarbanilate] were also dehalogenated at the para-chloro position in anaerobic pond sediment (Stepp *et al.*, 1985). A loss of chlorine from the para position has also been demonstrated for the pesticides thiobencarb and chlornitrofen in flooded paddy soils (Yamada and Suzuki, 1983; Moon and Kuwatsuka, 1984). Similarily, reductive dechlorination of an aryl chlorine was demonstrated for the pesticides techlofthalam in flooded paddy soil (Kirkpatrick *et al.*, 1981).

Reductive dehalogenation has been more thoroughly investigated for the halogenated benzoates (Suflita *et al.*, 1982; Horowitz *et al.*, 1983). Substrates were examined in sediment and in an enriched bacterial consortium derived from anaerobic sewage sludge grown on meta-chlorobenzoate. A variety of halogens were removed from the benzoate ring including iodo-, bromo-, fluoro- and chloro-. Bromo- and iodo- were dehalogenated with shorter lag times than either the fluoro- or chloro-. The meta-halogens were more susceptible to attack than either the ortho- or para-halogens. No dehalogenation was observed for either ortho- or para-chlorobenzoate even after one year.

Evidence that the mechanism involved in dehalogenation is biological includes: 1) dehalogenation has not been observed in sterile sediments, 2) there are long reproducible lag times before the onset of dehalogenation, and once acclimated to the substrate these lag times are diminished, 3) dehalogenating activity is lost above 39°C and, 4) activity is substrate specific as evidenced by specific cross-acclimation patterns (Horowitz *et al.*, 1983).

In addition, a unique dechlorinating bacterium has been isolated in pure culture from meta-chlorobenzoate enrichments (Shelton and Tiedje, 1984). The organism is a Gram negative straight rod with an unusual collar surrounding the cell. It uses both thiosulfate and sulfite but not sulfate as electron acceptors (Linkfield, 1986). The only substrates demonstrated to support growth of the organism were pyruvate and rumen fluid (Shelton and Tiedje, 1984; Linkfield, 1986). The organism dechlorinates meta-chlorobenzoate to stoichiometric amounts of benzoate but does not dechlorinate at other positions on the ring or other chloroaromatic compounds.

In addition to the chlorobenzoates, reductive dechlorination of chlorophenols has been investigated in anaerobic sewage sludge. Initial investigations demonstrated the removal of chlorines from monochlorophenols in 10% anaerobic sewage sludge (Boyd *et al.*, 1983). Removal of the chlorine preceded cleavage and subsequent mineralization of the aromatic nucleus as demonstrated by the transient accumulation of phenol. The rate of chlorophenol degradation depended on the position of the chlorine on the ring. Ortho-chlorophenol degraded faster than meta-chlorophenol, and para-chlorophenol persisted over the eight week study.

In further studies, all three monochlorophenols were dehalogenated in whole, fresh, anaerobic sewage sludge (Boyd and Shelton, 1984). Mineralization of para-chlorophenol, ortho-chlorophenol, and 2,4-dichlorophenol to CH_4 and CO_2 was confirmed by radiotracer studies using ^{14}C-labeled substrate. The fate of dichlorophenols was also examined in whole, fresh sludge. Reductive dechlorination ortho to the hydroxy moeity was observed for each dichlorophenol with an ortho-chlorine including 2,3-dichlorophenol, 2,6-dichlorophenol, 2,4-dichlorophenol, and 2,5-dichlorophenol. The chlorophenols examined without ortho-chlorines, 3,4-dichlorophenol and 3,5-dichlorophenol, persisted during the study.

The anaerobic sewage sludge could be slowly acclimated to chlorophenols by weekly feedings of substrate resulting in more rapid degradation rates. Distinct patterns of acclimation were observed for sludge acclimated to either ortho-chlorophenol, meta-chlorophenol or para-chlorophenol. Sludge acclimated to ortho-chlorophenol degraded both ortho-chlorophenol and para-chlorophenol without a lag but did not degrade meta-chlorophenol. The dichlorophenols that were dehalogenated in fresh sludge, (2,3-dichlorophenol, 2,6-dichlorophenol, and 2,5-dichlorophenol), were not degraded in the ortho-chlorophenol acclimated sludge.

Sludge acclimated to meta-chlorophenol degraded both meta-chlorophenol and para-chlorophenol without a lag but did not degrade ortho-chlorophenol. The dichlorophenols with meta- and para-chlorines that persisted in fresh sludge, (3,4-dichlorophenol and 3,5-dichlorophenol) were degraded in the meta-chlorophenol acclimated sludge.

Sludge acclimated to para-chlorophenol was able to degrade all three chlorophenols at rates para > meta > ortho. The para acclimated sludge was also able to degrade both 3,4-dichlorophenol and 2,4-dichlorophenol suggesting that dechlorination could occur at all positions on the aromatic ring.

Additional experiments with anaerobic sewage sludge from three different Michigan communities confirmed the ability of the sludge to degrade all three monochlorophenols at rates of ortho > meta > para (Mikesell and Boyd, 1985). Additionally, dechlorination was observed for 2,4,6-trichlorophenol, 2,4-D, 2,4,5-T, and pentachlorophenol. The ortho-chlorines were rapidly removed from these substrates with subsequent removal of some of the para- and meta-chlorines. Pentachlorophenol was dehalogenated in the ortho and para positions resulting in the accumulation of 3,5-dichlorophenol which was not further dehalogenated in the 70 day experiment.

The initial steps in the degradation of 2,4-D were cleavage of the ether linkage and removal of the ortho-chlorines. The resultant product, para-chlorophenol, was slowly degraded. The initial steps in the degradation of 2,4,5-T were also cleavage of the ether linkage and removal of the ortho-chlorine which resulted in 3,4-dichlorophenol. Further removal of the meta-chlorine resulted in the accumulation of para-chlorophenol which was not further degraded in the study. This is in contrast to earlier studies with 2,4,5-T in which only the para-chlorine was removed resulting in the accumulation of 2,5-D (Suflita *et al.*, 1984). This para-dechlorinating activity, however, was selected for by growth on meta-chlorobenzoate.

Further experiments were conducted with pentachlorophenol in anaerobic sewage sludge acclimated to all three monochlorophenols (Mikesell and Boyd, 1986). Again, distinct patterns of acclimation were observed. In general, pentachlorophenol was dechlorinated at both ortho positions in sludge acclimated to ortho-chlorophenol. In para-chlorophenol acclimated sludge, pentachlorophenol was dechlorinated at both the para and ortho positions, and meta-chlorophenol acclimated sludge dechlorinated pentachlorophenol at the meta-positions. The products, tri- and tetra-chlorophenols tended to accumulate in the acclimated sludge.

Complete reductive dechlorination and partial mineralization of the aromatic nucleus, however, was observed when all three acclimated sludges were mixed together or when sludge was acclimated simultaneously to all three monochlorophenols. The dechlorinated products, meta-chlorophenol, 3,5-dichlorophenol, and 3,4,5-trichlorophenol accumulated at background levels in the sludge upon repeated pentachlorophenol additions but were completely dehalogenated when pentachlorophenol additions ceased. Mineralization of the pentachlorophenol carbon to CH_4 and CO_2 was also observed in the sludge mixture. Approximately 66% of the added carbon could be accounted for as CH_4 and CO_2.

Under methanogenic conditions, once the chlorophenols are completely dehalogenated, the resultant aromatic nucleus can be mineralized to CH_4 and CO_2. Several researchers have reported on the mineralization of phenol by microbial consortia from habitats including anaerobic sewage sludge and rumen fluid (Tarvin and Buswell, 1934; Healy and Young, 1978; Holladay *et al.*, 1978; Balba *et al.*, 1979; Boyd and Shelton, 1984; Fedorak and Hrudey, 1984; Barik *et al.*, 1985; Young and Rivera, 1985; Godsy *et al.*, 1986). The mineralization of phenol has been described by the stoichiometry of the following equation (Healy and Young, 1978):

$$C_6H_5OH + 4\ H_2O \ \text{------->}\ 3.5\ CH_4 + 2.5\ CO_2$$

Several investigators have proposed a pathway for anaerobic phenol metabolism based on the detection of intermediates from phenol in mixed culture experiments (Bakker, 1977; Evans, 1977; Balba *et al.*, 1979; Young and Rivera, 1985). The initial steps in the pathway are the reduction of phenol to cyclohexanone followed by ring cleavage to produce caproate or adipate (Figure 1). The fatty acids are then further oxidized to produce methanogenic precursors (Balba and Evans, 1977).

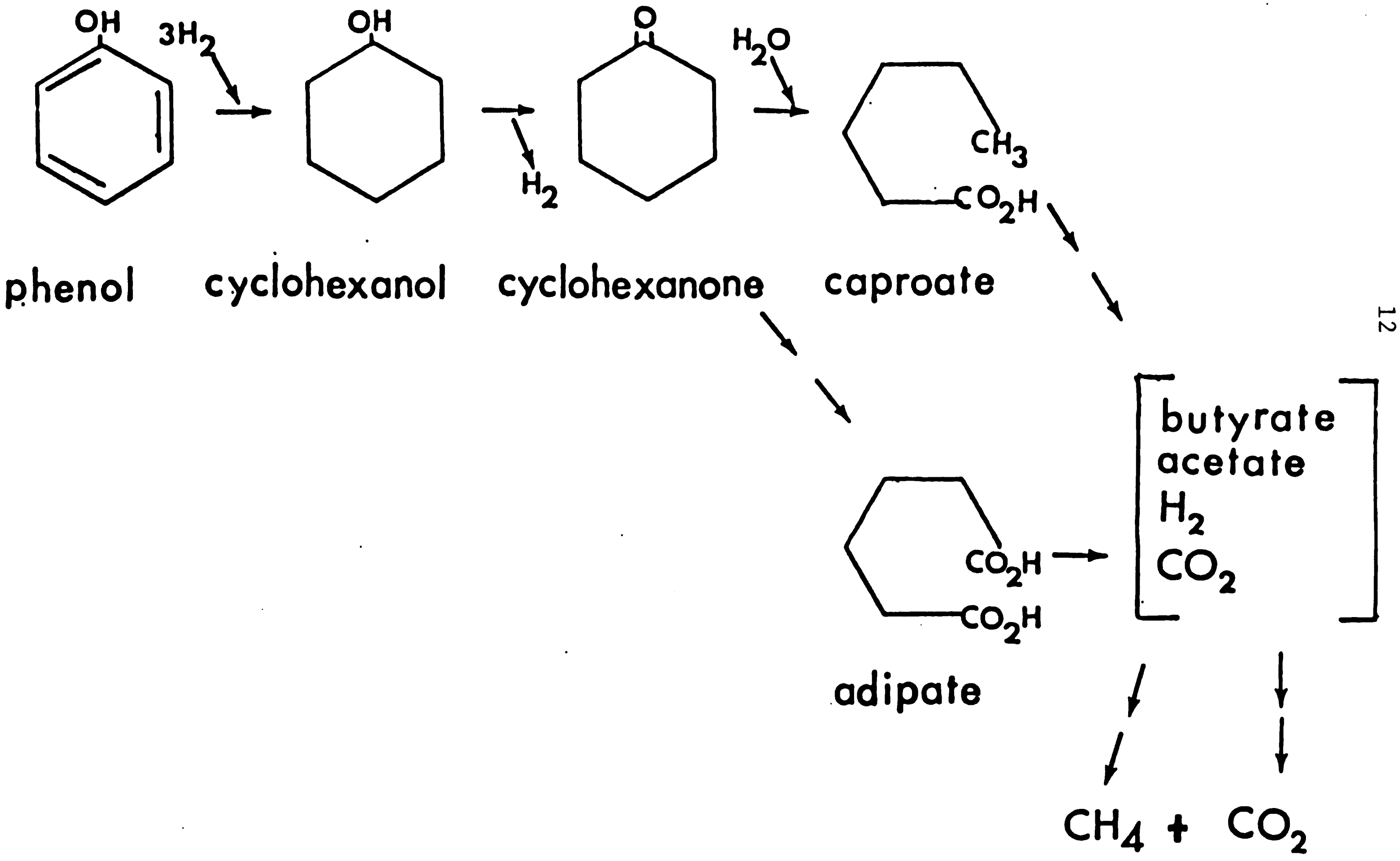
The phenol pathway has not been demonstrated in pure culture experiments because until recently pure cultures of anaerobic phenol-oxidizing bacteria were not available. The free energy change ($\Delta G^{o'}$) for phenol fermentation reactions are positive under standard conditions. When hydrogen is maintained at low partial pressures through the presence of a hydrogen-consuming microorganism, the free energy change for phenol oxidation becomes negative. Thus, anaerobic phenol-oxidizing bacterium can only be isolated on phenol in the presence of a hydrogen-consuming microorganism.

Recently, two phenol-degrading organisms have been isolated in co-culture with *Wollinella succinogenes*, a hydrogen-utilizing

Figure 1. Proposed pathway for the anaerobic metabolism of phenol.

ANAEROBIC METABOLISM OF PHENOL

Figure 1.



bacterium (Barik *et al.*, 1985). Both organisms were able to utilize benzoate, phenylacetate, phenol and hydrocinnamate as their sole carbon source. Other non-aromatic substrates were also able to support growth. An additional organism has been isolated in co-culture with *Desulfovibrio sp.* which is able to grow on benzoate or phenol (Krumme and Boyd, 1986).

To exploit the enzymatic activities involved in the anaerobic dechlorination and mineralization of chlorophenols, a continual, large-scale treatment system needs development. Anaerobic biological reactors have been widely employed for the continual treatment of waste. The advantages of anaerobic reactors versus aerobic reactors are less energy consumption, less sludge production and the production of a useful product, methane (Olthof and Oleszkiewicz, 1982; Klass, 1984).

Recently, anaerobic attached film expanded bed reactors have been introduced for waste treatment. In this reactor design, the waste stream enters the bottom of the reactor and passes by the attached microbial biomass where it can be converted to CH_4 and CO_2. Methane gas and effluent are collected at the top. The advantages of an anaerobic attached film expanded bed reactor over conventional treament are shorter retention time, increased hydraulic load, the ability to treat low strength wastes, and an increased tolerance to toxic shock (Switzenbaum and Jewell, 1980; Jewell, 1982; Binot *et al.*, 1983; van den Berg, 1984). A variety of wastes, including waste from sugar refineries and agriculture have been degraded in anaerobic upflow bioreactors (Olthof and Oleszkiewicz, 1982; Speece, 1983).

The biodegradation of chlorophenols has also been examined in anaerobic upflow bioreactors. The design scale of the bioreactor varies from small laboratory reactors to large industrial scale reactors. In all of the bioreactor studies, chlorophenols were added to the bioreactor along with other readily utilizable carbon sources.

In one study, an anaerobic reactor was fed a mixture of glucose, methanol and acetate to stimulate methanogenesis along with varying concentrations and types of chlorophenols (Woods, 1985). The chlorophenols, di-, tri-, tetra-, and penta-chlorophenol were fed to the bioreactor at influent concentrations varying from 0.1 mg/l to 27 mg/l at a hydraulic retention time (HRT) of 0.5 days. Toxicity to the dechlorinating and methanogenic activity was observed at the higher chlorophenol input concentration. As the chlorophenol concentration in the feed increased from 2.4 mg/l to 8.0 mg/l, dechlorinating activity decreased.

Rapid dechlorination was observed for chlorines ortho to the hydroxy moeity. Some meta-chlorines were removed only after four months of acclimation to the chlorophenols. Chlorines in the para position were not removed and no ring cleavage was observed. Rates of dechlorination increased with increasing chlorination of the ring. Thus, the fastest rate of dechlorination was observed for pentachlorophenol.

Several different types of microorganisms were enumerated from the bioreactor including aerobes, facultative anaerobes, methanogens and yeast (Hakulinen *et al.*, 1985; Woods, 1985). The isolated facultative anaerobes were able to grow on 2,4,6-trichlorophenol under aerobic but not under anaerobic conditions. When two of the isolates were mixed together, identified as *Klebsiella oxytoca* and *Pseudomonas aeruginosa*, anaerobic degradation of 2,4,6-trichlorophenol was observed. No degradation or mineralization products from 2,4,6-trichlorophenol were reported.

The significance of these microorganisms in chlorophenol biodegradation was not clear. The *Klebsiella oxytoca*, along with the other enumerated microbes, decreased in number in the bioreactor with increasing concentration of chlorophenol in the feed. The *Pseudomonas aeruginosa* remained constant. Yeasts, also isolated from the bioreactor, increased in number with increasing chlorophenol concentration in the feed but the increased number of yeasts had no effect on the rate of chlorophenol degradation.

The biodegradation of chlorophenols from pulp waste effluent was examined in a two-stage reactor system consisting of an anaerobic upflow reactor and an aerobic trickling filter (Hakulinen and Salkinoja-Salonen, 1982; Salkinoja-Salonen *et al.*, 1983; Salkinoja-Salonen *et al.*, 1984). The extent of chlorophenol biodegradation due to each system was not determined. The bioreactor system was effective at removing much of the biological oxygen demand, chemical oxygen demand and toxicity from the pulp waste.

Chlorophenols, typically found in pulp waste effluent, were also removed by the reactor system. The removal efficiency depended on the loading rate into the bioreactor and the HRT. The efficiency of chlorophenol removal was 93-95% with a six hour HRT and a waste stream containing 0.6-2.2 mg/l chlorophenols. The efficiency dropped to approximately 50% when the HRT decreased to three hours and the waste stream contained 1.5-2.0 mg/l chlorophenols. When the influent and effluent waste streams from the reactor system were analyzed for individual chlorophenols, the authors found that both pentachlorophenol and 2,4,6-trichlorophenol were the most resistant to biodegradation and tended to be the first chlorophenols to appear when the chlorophenol removal efficiency dropped. Mineralization of 60 to 80% of pentachlorophenol to CO_2 was demonstrated using ^{14}C-pentachlorophenol. Methane was not reported as a product.

In the present study, we have examined the dechlorination of chlorinated aromatic compounds in anaerobic upflow bioreactors. Chlorophenols have been used in these studies as model chlorinated aromatic compounds because they are a serious environmental pollution problem and represent one class of compounds that are reductively dechlorinated under anaerobic conditions.

The dechlorination of chlorophenols in anaerobic upflow bioreactors was studied for several reasons. First, anaerobic upflow bioreactors are currently used for large-scale waste treatment processes. The dechlorination of chlorophenols in the same type of bioreactor would be the first step towards utilizing these unique activities for large-scale treatment.

In addition to the potential for waste treatment, anaerobic upflow bioreactors can also be used in the laboratory as a tool for the enrichment of unique activities, such as dehalogenation, by enriching for stable bacterial communities. The bioreactor enrichment technique may be the only feasible method for studying these types of activities when conventional techniques, such as the isolation of individual community members, are difficult or impossible.

Bioreactors can also provide a means for acclimating microbial communities to degrade new substrates. New substrates can be slowly but continually introduced into the bioreactors to slowly adapt the microbes to new substrates. This may be an especially useful technique when the substrates of interest, such as the chlorophenols, are toxic to the microorganisms.

Specific research objectives were set in order to determine the feasibility of degrading chlorophenols in anaerobic upflow bioreactors. These were: 1) determine whether dechlorinating activity can be maintained in anaerobic upflow bioreactors with chlorophenols as the sole carbon and energy source, 2) determine if mineralization of the chlorophenols occurs in the bioreactors, 3) examine the bioreactor for location of biomass and dominant morphology, 4) determine the substrate range, and 5) determine the substrate loading capacity.

MATERIAL AND METHODS

Bioreactor Methods

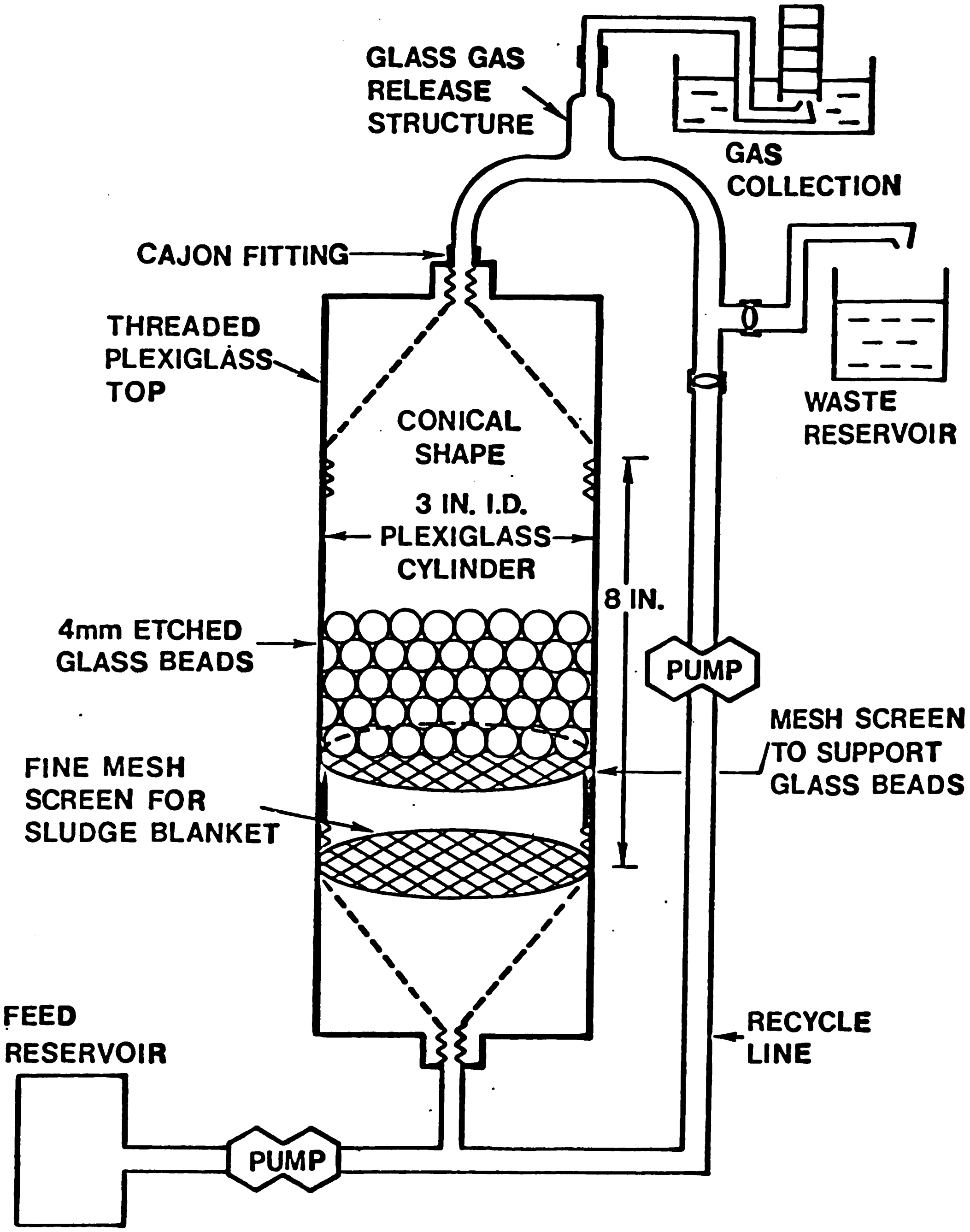
Bioreactor Design. Hybrid anaerobic upflow bioreactors were used for the continual treatment of a synthetic chlorophenol waste stream. A diagram of the reactor is shown in Figure 2. The cylindrical plexiglass reactor was approximately one liter in volume with a height of 12 inches and an inner diameter of three inches. The ends of the reactor were conical shaped and threaded for easy removal. Cajon fittings were used to connect either 1/4 inch I.D. glass or stainless steel tubing to the cylinder. Sampling ports sealed with butyl rubber stoppers were located throughout the length of the bioreactor.

Two areas inside the bioreactor were designed for the containment of the biomass. A fine mesh screen located at the bottom of the reactor was designed for the accumulation of a sludge blanket. The center of the bioreactor, designed for the attachment of the biomass, contained approximately 250 mls of 4 mm etched glass beads held in place by an additional screen.

Gaseous end products were separated from liquid effluent in a glass gas release structure attached to the top of the cylindrical bioreactor. Gas was collected in an inverted graduated cylinder

Figure 2. Diagram of hybrid anaerobic upflow bioreactor.

Figure 2.


GLASS GAS RELEASE STRUCTURE
GAS COLLECTION
CAJON FITTING
THREADED PLEXIGLASS TOP
WASTE RESERVOIR
CONICAL SHAPE
3 IN. I.D. PLEXIGLASS CYLINDER
8 IN.
4mm ETCHED GLASS BEADS
PUMP
MESH SCREEN TO SUPPORT GLASS BEADS
FINE MESH SCREEN FOR SLUDGE BLANKET
FEED RESERVOIR
RECYCLE LINE
PUMP

placed in an acid-brine solution (2% HCl, 10% NaCl). The top of the graduated cylinder was fitted with a butyl rubber stopper for taking quantitative gaseous measurements. The glass gas release structure also contained a port for the collection of effluent in a one liter Erlenmeyer flask.

The feed reservoir consisted of a one liter Erlenmeyer flask fitted with a rubber stopper. The feed flask was completely sealed by the rubber stopper and held in place by plastic tape. Tygon and stainless steel tubing connected the bottom of the feed flask through the stopper to a piston-action pump (Fluid Meter, Inc. RP6 pump equipped with a 1/4" ceramic piston pump head). Additional tygon and stainless steel tubing connected the pump to the bottom of the reactor.

A slow continual stream of oxygen-free nitrogen was supplied to the feed flask headspace via the rubber stopper. The oxygen-free nitrogen gas was provided by a tank connected in series to a two-stage regulator (Airco 9600), a low pressure regulator (Matheson Model 70) and an adjustable relief valve (Union Carbide Corp. RV5570). The pressure release valve was set at 1-2 psi to prevent excess pressure build-up in the feed flask. The feed flask, containing anaerobic media, was replaced as needed by continually flushing the flask with oxygen-free nitrogen.

Heat distribution inside the bioreactor was provided by eight aluminum strips (7.5" x 3/4" x 1/4") attached to the outside of the column. Column heat was obtained by means of a heat resistor tape wrapped around the outside of the aluminum strips and attached to a variable transformer. The temperature was controlled either by the setting of the transformer or by connecting the transformer to a temperature controller (YSI Model 64RC, Yellow Springs Instrument Co., Inc.) equipped with a YSI Series 600 thermistor attached to the outside of the column. Temperature was monitored by a thermometer placed inside the bioreactor through a top well sealed by a Cajon fitting. The entire bioreactor was placed inside a wooden box packed with styrofoam for insulation.

Inoculation Procedure. Strict anaerobic techniques were used throughout the inoculation procedure. The column was initially flushed with oxygen-free nitrogen for one hour. Flushing probes extended approximately half-way down into the column to insure adequate flushing. While flushing, reduced anaerobic mineral media, (RAMM), was added to the column using a specifically designed inoculation flask. Ports were sealed and the column was allowed to stand overnight before slowly draining the column while flushing. The column was inoculated using the inoculation flask and the ports were sealed.

The inoculation flask consisted of a two liter Erlenmeyer flask sealed by a rubber stopper. A five-inch, 11-gauge stainless steel needle was attached to tygon tubing which went through the rubber stopper and extended into the bottom on the flask. An additional stainless steel needle pierced the rubber stopper and extended into the headspace of the flask. The flask was filled with either RAMM or inoculum while continually flushing with oxygen-free nitrogen. To inoculate the column, oxygen-free nitrogen was connected to the needle that pierced the stopper. Excess pressure caused the liquid in the flask to escape through the additional needle which was placed inside the column through a top port.

Source of Inoculum. All three bioreactors were inoculated with either acclimated anaerobic sewage sludge or sewage sludge enrichment cultures that were obtained from the acclimated anaerobic sewage sludge. Anaerobic sewage sludge, obtained from a primary anaerobic sewage sludge digestor, (Jackson, MI), was acclimated by weekly feedings (20 ug/ml) of chlorophenols. The anaerobic sludge had been acclimated to the chlorophenols for approximately two years prior to use in the columns. Anaerobic enrichments were obtained by periodic transfers (25%) of acclimated sludge into RAMM. Enrichments were maintained by feeding approximately 10-20 ug/ml chlorophenols as the sole carbon and energy source every two days. Both acclimated sludge and enrichment culture were periodically monitored for chlorophenol content to prevent a toxic build-up of the substrate.

Bioreactors I and III were initially inoculated with an enrichment that had been degrading meta-chlorophenol for approximately one year. The enrichment was obtained by two transfers of 50% and 25% from acclimated sludge. The bioreactors were inoculated by completely filling the bioreactor with the enrichment culture.

Bioreactor II was initially inoculated with an equal mixture of sludge that had been acclimated to either ortho-, meta-, or para-chlorophenol. The bioreactor was seeded with a 1:1 mixture of the sludge and RAMM. When activity in Bioreactor II diminished at day 200 (see Figure 6), the bioreactor was reseeded with 100 ml (10%) of a 1:1:1 mixture of sludge acclimated to either ortho-, meta- or para-chlorophenol.

Feed Media. Feed media for all bioreactor experiments was reduced anaerobic mineral media (RAMM). The mineral salts media consisted of (per liter): 0.27 g of KH_2PO_4, 0.35 g of K_2HPO_4, 0.53 g of NH_4Cl, 0.10 g of $MgCl_2 \cdot 6H_2O$, 79 mg of $CaCl_2 \cdot 2H_2O$, 20 mg of $FeCl_2 \cdot 4H_2O$, 1.0 ml of a trace metals solution and 1.0 ml of a vitamin solution. The trace metals solution consisted of (per liter): 0.5 g of $MnCl_2 \cdot 4H_2O$, 0.05 g of H_3BO_3, 0.05 g of $ZnCl_2$, 0.03 g of $CuCl_2$, 0.01 g of $Na_2MoO_4 \cdot 2H_2O$, 0.50 g of $CoCl_2 \cdot 6H_2O$, 0.05 g of $NiCl_2 \cdot 6H_2O$, and 0.05 g of Na_2SeO_3. The vitamin solution consisted of (per liter): 0.02 g of biotin, 0.02 g of folic acid, 0.06 g of lipoic acid, 0.05 g of thiamin, 0.05 g of riboflavin, 0.05 g nicotinic acid, 0.10 g of pyridoxal-HCl, 0.05 g of pantothenic acid, 0.05 g of cyanocobalamin, and 0.05 g p-aminobenzoic acid. Media, prepared as needed in one liter Erlenmeyer flasks, was boiled for five minutes to remove oxygen and then cooled to approximately 35°C while being sparged with a 20% CO_2/80% N_2 oxygen-free gas mixture. After cooling, 2.4 g $NaHCO_3$ and 0.24 g Na_2S was added to the media and the flask was tightly stoppered. Chlorophenol substrates were added to the media just prior to use from anaerobic stock solutions of 6 mg chlorophenol/ml H_2O.

Effluent was collected and measured daily to determine the amount of feed media which entered the bioreactor. Daily samples of the effluent were frozen for future high pressure liquid chromatographic (HPLC) determination of the chlorophenol concentration.

Analytical Methods

Methane. Methane was collected as a gaseous product in the inverted cylinder placed in the acid-brine solution. Total volume of gas collected was measured daily and samples of the gas were taken periodically for methane quantification. Methane was quantified by injecting 0.2 ml of the gas into a Carle gas chromatograph equipped with a poropack Q column and a microthermistor detector.

Dissolved methane in the effluent was extracted according to the procedure developed by Robinson *et. al* (1981) for extracting dissolved H_2. Samples of the effluent were taken after separation of gaseous products in the glass gas release structure (Fig. 2), and before exposure to the atmosphere, by piercing the tygon tubing with a 22-gauge needle and slowly withdrawing a sample into a glass 50 ml syringe fitted with a one way stopcock. Methane was extracted from the effluent and then analyzed by the Carle gas chromatograph as previously described. The following equation was used to determine the amount of methane dissolved in the effluent: $N = X[1+\alpha(A/B)]/A$ where N= moles of CH_4 ml^{-1} of extracted effluent, A= volume (ml) of aqueous phase in the 50 ml syringe, B= volume (ml) of the gas phase of the syringe, and X= moles of CH_4 in the gas phase of the syringe at equilibrium. The value X is calculated by multiplying the number of moles of CH_4 ml^{-1} of gas in the gas bubble, as determined by gas chromatography, by the volume of the gas bubble. A Bunsen absorption coefficient, α, of 0.03046 was used.

Sep-Pak Extraction. Effluent samples containing 3,4,5-trichlorophenol were concentrated using Sep-Pak C18 cartridges

(Millipore) before HPLC analysis. An effluent sample of 50 ml was concentrated in the cartridge and eluted with 2.0 ml of methanol. Recovery efficiency of the trichlorophenol was determined by spiking known quantities of trichlorophenol in spent effluent and concentrating by Sep-Pak extraction. Effluent spiked with a trichlorophenol concentration of 0.1-2.0 ug/ml was concentrated 25 times at a recovery of 80%.

Analysis of Chlorophenols. Effluent samples were thawed and filtered through 0.45 uM HA Millipore filters. Monochlorophenols were routinely measured with a Waters HPLC consisting of a model 6000A pump and a model 441 *UV* absorbance detector set at 280 nm. The sample injection valve (Rheodyne 7125) was fitted with a 20-ul loop. The analytical column was a Waters Radial PAK C18 cartridge held in a RCM-100 Radial Compression Module. Peak areas were measured with a Hewlett Packard 3390A integrator. The trichlorophenols and pentachlorophenol were measured with a Waters HPLC consisting of a model 6000A pump, a model 45 pump, and a model 720 systems controller, coupled with a model 480 Lamba Max variable wavelength *UV* absorbance detector. The wavelength was set at 300.5 nm for pentachlorophenol and 280.0 nm for trichlorophenols. The analytical column was a uBondapak C18 column (Waters Assoc.).

Mobile phase for both HPLC systems consisted of a 5% aqueous acetic acid:acetonitrile mixture with a flow rate of 2.0 ml/min. The mobile phase was adjusted to give a retention time of approximately 3.5 min for the monochlorophenols (1:1 mixture); 5 min for trichlorophenols (4:6 mixture) and 7 min for pentachlorophenol (3:7

mixture). Mixtures of the monochlorophenols were separated by using a mobile phase mixture of 8:2 resulting in retention times of 5-7 min.

Experimental Procedures

General. All experiments were conducted utilizing one of three bioreactors. Preliminary experiments were conducted with Bioreactor I which was initially seeded with an enrichment culture that had been degrading meta-chlorophenol. Mineralization studies were also performed with Bioreactor I. After nearly one year of operation (350 days), the bioreactor was terminated and studies were performed utilizing the bioreactor contents.

Bioreactor II, in operation for 400 days, was initialy seeded with acclimated sludge that had been degrading all three monochlorophenols. After an initial start-up period, two mineralization studies were performed utilizing radiolabeled substrate. Bioreactor II was used for three additional experiments involving the fate of higher chlorinated phenols; 2,4,6-trichlorophenol; 3,4,5-trichlorophenol and pentachlorophenol.

Bioreactor III was originally seeded from a meta-chlorophenol degrading enrichment. After an initial start-up period, four experiments were run sequentially to determine the effect of loading rate and (HRT) on the substrate conversion efficiency. The experiments were terminated after 190 days and the bioreactor contents were further characterized.

Mineralization Studies. The mineralization of meta-chlorophenol was examined in Bioreactor I by quantitative recovery of the mineralization product, CH_4. The study took place during days 159 through 202 of operation with an average substrate loading rate of 10.2 mg l^{-1} d^{-1} and an average HRT of 6.7 days. The substrate conversion efficiency was 100%. Both gaseous and dissolved CH_4 were recovered and quantified. The total amount of theoretical CH_4 obtained from the total amount of added substrate chlorophenol during the 46 day period was determined based on the stoichiometry of the following equation:

$C_6H_5OH + 4\ H_2O$ --------> $3.5\ CH_4 + 2.5\ CO_2$ (Healy and Young, 1978)

The mineralization of chlorophenol in Bioreactor II was examined in two separate experiments by determining the fate of ^{14}C-para-chlorophenol. The first study took place from day 112 to 157 of operation with an average total substrate loading rate of 21.2 mg l^{-1} d^{-1} and an average HRT of 8.3 days. The average substrate conversion efficiency was 99%.

Uniformly ring-labeled ^{14}C-para-chlorophenol (obtained from Pathfinders with a specific activity of 11.61 mCi/mmol and purity of 98%) was added as a spike to the feed media. The spiked media contained 57.8 uCi of ^{14}C para-chlorophenol and 150 mg/l of unlabeled substrate (a 1:4:1 mixture of ortho-:meta-:para- chlorophenol) and was pumped into the bioreactor over a 48 hour period.

Labeled material was recovered in a series of traps as depicted in Figure 3a. Effluent and any gaseous products were initially collected in a stoppered one liter Erlenmeyer flask. Before daily collection and measurement of the effluent, the contents of the flask were acidified with HCl and flushed with nitrogen gas by piercing the rubber stopper with a needle. Exhausted gas passed through a series

Figure 3. Experimental design for trapping rabiolabeled material.

Figure 3a.

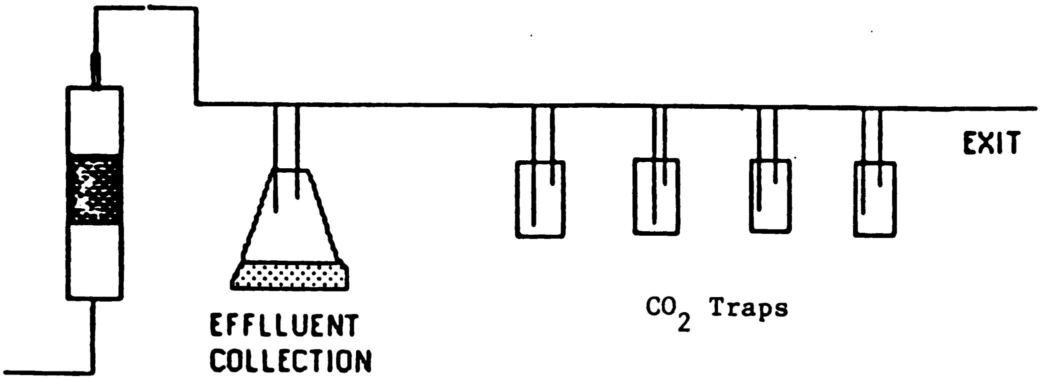
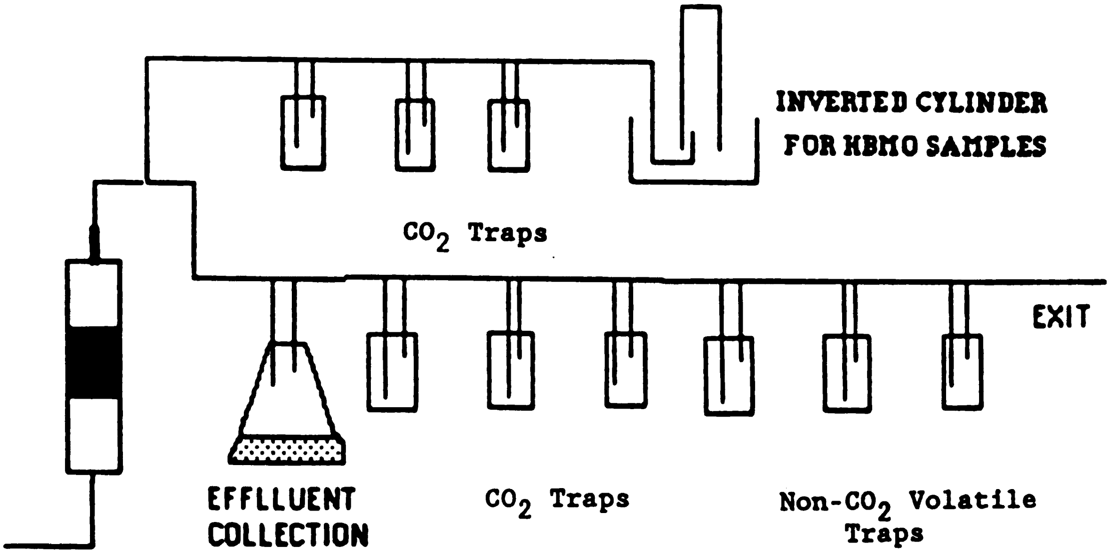


Figure 3b.



of four traps (scintillation vials containing 10 ml of a CO_2-trapping cocktail) to trap any CO_2 before venting to the atmosphere. Traps were changed daily and radioactivity was measured. Duplicate 1.0 ml samples of the effluent were added to universal cocktail (Scintiverse II, Fisher Scientific) and counted. All samples were counted in a Beckman Model LS8100 Scintillation Counter. Samples of effluent were also collected for HPLC analysis of the chlorophenol content.

The second study took place from day 157 through 202 of operation with an average substrate loading rate of 15.5 mg l^{-1} d^{-1} and average HRT of 7.2 days. The substrate conversion efficiency was 99%.

Para-chlorophenol was added as a spike to 300 ml of feed media containing 53.4 uCi of [U]-^{14}C-para-chlorophenol and 150 mg/l of total chlorophenol (1:4:1 mixture of ortho-:meta-:para-), and was delivered to the bioreactor over a 72 hour period.

Labeled material was recovered in a series of traps as depicted in Figure 3b. Gaseous products were separated from the effluent by means of the glass gas release structure. Gaseous products passed through a series of CO_2 traps before collection of non-CO_2 gases in the inverted cylinder. Samples of the gas collected in the inverted cylinder were periodically placed in 3 ml Venoject vials (Terumo Medical Co.) for later analysis. Radioactivity was measured in the gaseous samples stored in Venoject vials by oxidizing the gas to CO_2 in a Harvey Biological Material Oxidizer Model OX200 and collecting the resultant CO_2 in scintillation fluid.

The effluent, containing any dissolved ^{14}C, was collected in a sealed Erlenmeyer flask. The flask was connected to a series of CO_2

traps followed by an additional series of traps (scintillation vials containing 20 ml of: 0.375 g 2,5-diphenyloxazole, 0.100 g dimethyl POPOP in 1.0 l of toluene). Before daily collection of the effluent, tubing to the bioreactor was clamped off, the effluent was acidified with HCl and flushed with nitrogen gas. The effluent was then collected, the volume was measured and duplicate 1.0 ml samples were placed in scintillation fluid and counted. Effluent samples were also taken for high pressure liquid chromatographic determination of the chlorophenol concentration.

Turnover Time and Sorption Studies. Bioreactor I was employed to determine the turnover time and recovery of a non-mineralized compound in the bioreactor during days 334 to 341 of operation. The average HRT was 4.1 days. Ortho-chlorophenol was used as the non-mineralized compound due to its similarity to the mineralized substrate, meta-chlorophenol. Ortho-chlorophenol was added to the bioreactor by injecting the substrate (3.0 ml of a 6.0 mg chlorophenol/ml H_2O stock for a total of 18.0 mg) through tygon tubing that connected the pump to the bioreactor. Effluent samples were monitored daily for the presence of ortho-chlorophenol by HPLC.

Trichlorophenols and Pentachlorophenol Studies. Three experiments were run with Bioreactor II to determine the fate of higher chlorinated phenols in the bioreactor. The substrates used were 2,4,6-trichlorophenol, 3,4,5-trichlorophenol and pentachlorophenol. During these experiments, influent loading rates of the monochlorophenol substrates were decreased. Between experiments, the loading rates of the monochorophenols were increased to approximately 20 mg total substrate l^{-1} d^{-1}.

The higher chlorinated phenol substrates were added to the feed media just prior to use from stock solutions. Effluent was monitored for monochlorophenol, the higher chlorinated substrate and any possible chlorinated products. For the experiment with the substrate 3,4,5-trichlorophenol, effluent samples were first concentrated by Sep-Pak extraction (see analytical methods).

Substrate Loading Rate and Hydraulic Retention Time Studies. Bioreactor III was used to determine the capacity of the bioreactors to degrade chlorophenols at different loading rates and HRT's. Substrate loading rates at a constant HRT varied by changing the concentration of chlorophenol in the feed media. HRT varied by changing the volume of feed media pumped into the bioreactor. The chlorophenol removal efficiency was calculated from the average influent substrate loading rate and the average effluent substrate concentration as determined by HPLC.

Tear Down Procedures. After 350 days of operation, Bioreactor I was disassembled and the contents were examined. Strict anaerobic techniques were employed thoughout the procedure by continually flushing the bioreactor with oxygen-free nitrogen. The bioreactor was divided into ten non-equal portions as shown in Figure 21. Portions of each section were collected for protein analysis, chlorophenol analysis, methane productivity and microscopic observations.

Samples for protein determination (5.0 or 10.0 ml) were centrifuged at 10,000 rpm for 15 min, resuspended in phosphate buffer, and acidified overnight with concentrated HCl. Samples containing glass beads were vortexed and washed with phosphate buffer to remove the biomass from the beads. After acidification, the samples were neutralized with NaOH and the protein was solubilized with 2N NaOH. Samples were centrifuged at 1200 x g for 15 min to remove any remaining particulate matter and then assayed according to the procedure of Lowry *et al.* (1951).

The bioreactor contents were also analyzed for the presence of chlorophenol. A 10.0 ml aliquot from each section was extracted with 5.0 ml acetonitrile, filtered through 0.45 uM Millipore HVLP filters and analyzed by HPLC as previously described.

Methane production from each column section was also determined. A 25 ml portion of each section was placed into a 71 ml serum bottle while continually flushing the bioreactor and the serum bottle with oxygen-free nitrogen. Substrate chlorophenol was added to each bottle to give a final concentration of 48 ug/ml before sealing the bottle with a butyl rubber stopper and incubating at 37^{o}C in the dark. Methane production was monitored periodically as previously described.

After 190 days of operation, Bioreactor III was disassembled and the contents were examined. Strict anaerobic techniques were employed throughout the procedure. The bioreactor was divided into five non-equal portions as shown in Figure 22 and analyzed for protein, dechlorinating activity, methane production and microbial morphology. Protein was determined as previously described. Duplicate samples were employed in the analysis.

Dechlorinating activity and methane production were determined simultaneously, in triplicate. Methane production was determined as previously described for Bioreactor I. Periodic samples, (1.0 ml), of the aqueous phase were taken and frozen. The samples were thawed, extracted with 0.5 ml acetonitrile, filtered though HVLP filters and assayed by HPLC as previously described.

A Zeiss Photo-1 photomicroscope was utilized for microscopic observations and photomicrographs. The bioreactor material was embedded on a slide in 1.5% agarose for the photomicrographs.

RESULTS

General. One of the primary objectives of the bioreactor experiments was to determine whether dechlorinating activity could be maintained in the bioreactors for long periods of time with chlorophenols as the sole carbon and energy source. An additional primary objective was to determine feasible substrate loading rates and hydraulic retention times. Three bioreactors were employed for these general studies. Bioreactor I and III were fed only meta-chlorophenol as the sole carbon source while Bioreactor II was fed a mixture of ortho-, meta-, and para-chlorophenol.

Bioreactor I was a preliminary, long term experiment designed to answer the two primary objectives. Figure 4 describes the influent and effluent substrate loading rates for the entire operation of Bioreactor I including the start-up period. The hydraulic retention time (HRT) varied throughout the length of operation as depicted in Figure 5.

After inoculation of the bioreactor at day 0, start-up conditions were maintained for approximately 100 days (Figure 4). During the initial 40 days of operation influent feed media was applied to the one liter bioreactor at a rate of 50 ml/day. The concentration of chlorophenol in the feed media was 60 mg/l to give a loading rate of approximately 3 mg l^{-1} d^{-1}.

Figure 4. Influent and effluent substrate loading rate for Bioreactor I.

Figure 4.

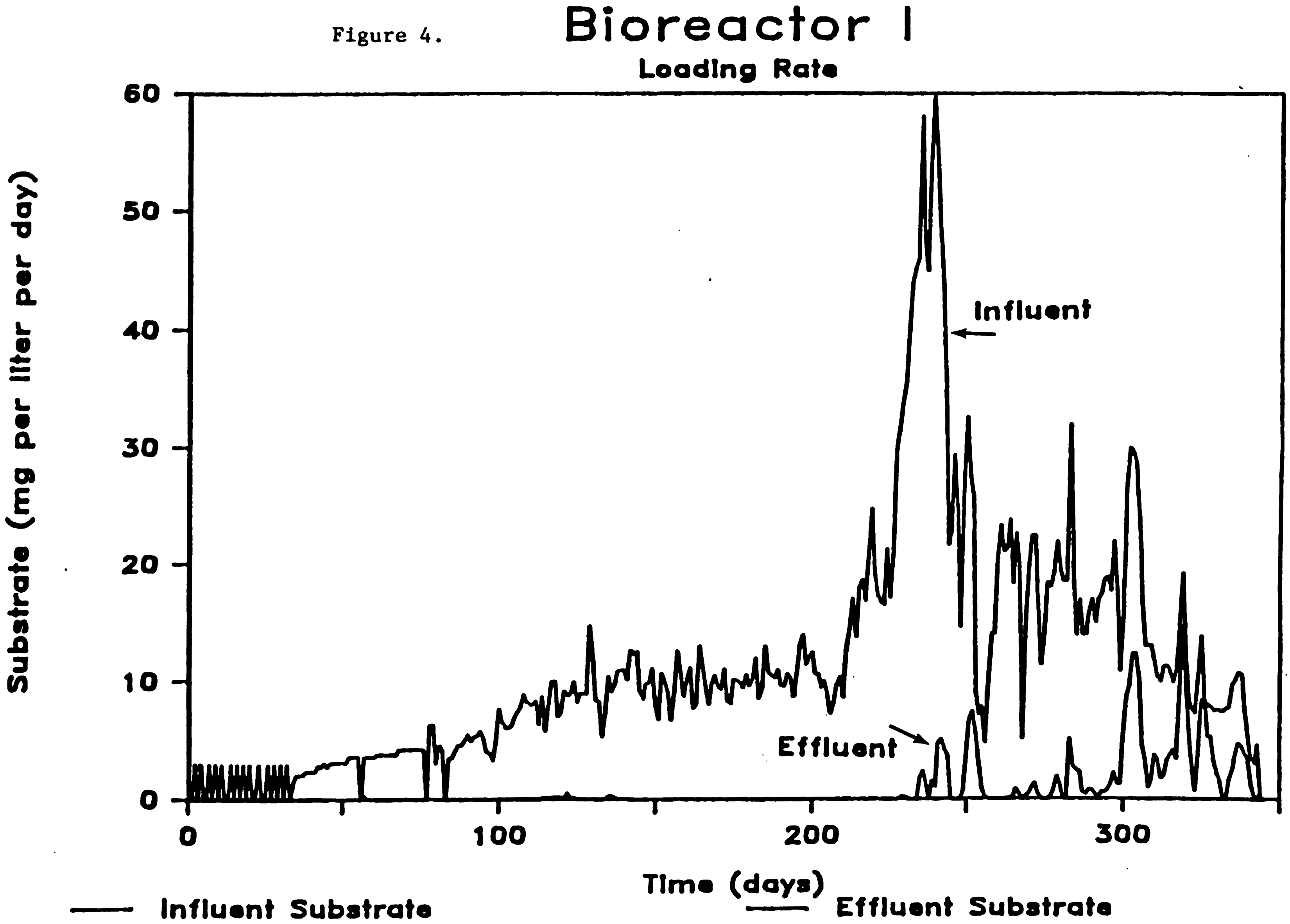
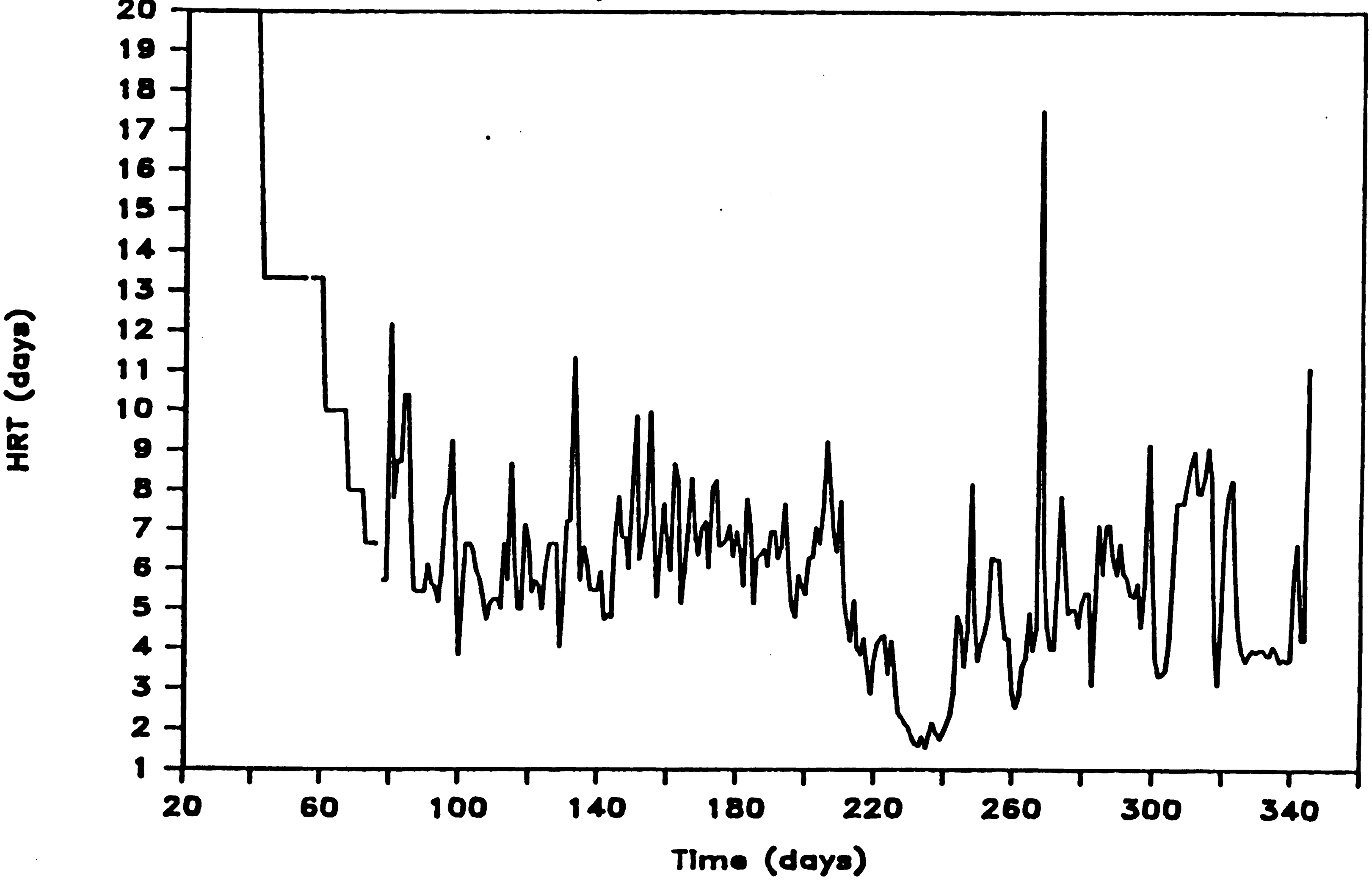

Bioreactor I
Loading Rate
Influent
Effluent
Substrate (mg per liter per day)
60
50
40
30
20
10
0
0
100
200
300
Time (days)
Influent Substrate
Effluent Substrate

Figure 5. Hydraulic retention time for Bioreactor I.

Figure 5.


Bioreactor I
Hydraulic Retention Time
HRT (days)
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
Time (days)
20
60
100
140
180
220
260
300
340

After day 100, feed media was applied to the bioreactor continually (Figure 4). From day 100 to day 165, the concentration of chlorophenol in the feed media was increased as the HRT was gradually decreased until the loading rate was approximately 10 mg l^{-1} d^{-1} at an approximate HRT of 6.5 days. Steady-state conditions were employed from day 165 to day 210 for the mineralization study described below. After day 210, the concentration of chlorophenols was again gradually increased as the HRT decreased in order to achieve the highest possible loading rates at greater than 90% conversion efficiency of the chlorophenol. Loading rates of up to 50-60 mg l^{-1} d^{-1} were achieved at hydraulic retention times of approximately two days. After day 240, significant amounts of chlorophenol were detected in the effluent. To achieve a high chlorophenol conversion efficiency, both the influent chlorophenol concentration was decreased and HRT was increased which resulted in a lowered chlorophenol loading rate. The bioreactor experiment was terminated at day 345.

Bioreactor II was fed a mixture of 1:1:1 ortho-:meta-:para-chlorophenol during day 1 to day 21, a mixture of 1:2.5:2.5 ortho-:meta-:para- during days 22 to 82 of operation and 1:4:1 ortho-:meta-:para- during the remainder of the experiment. The ratio of the monochlorophenols were varied when a higher concentration of one of the monochlorophenols was detected in the effluent. Thus, the bioreactor became acclimated to degrading approximately four times more meta-chlorophenol than ortho- or para-chlorophenol. Figure 6 decribes the overall loading rate of total influent and effluent substrate while Figure 7 decribes the HRT.

Figure 6. **Influent and effluent substrate loading rate for Bioreactor II.**

Figure 6.

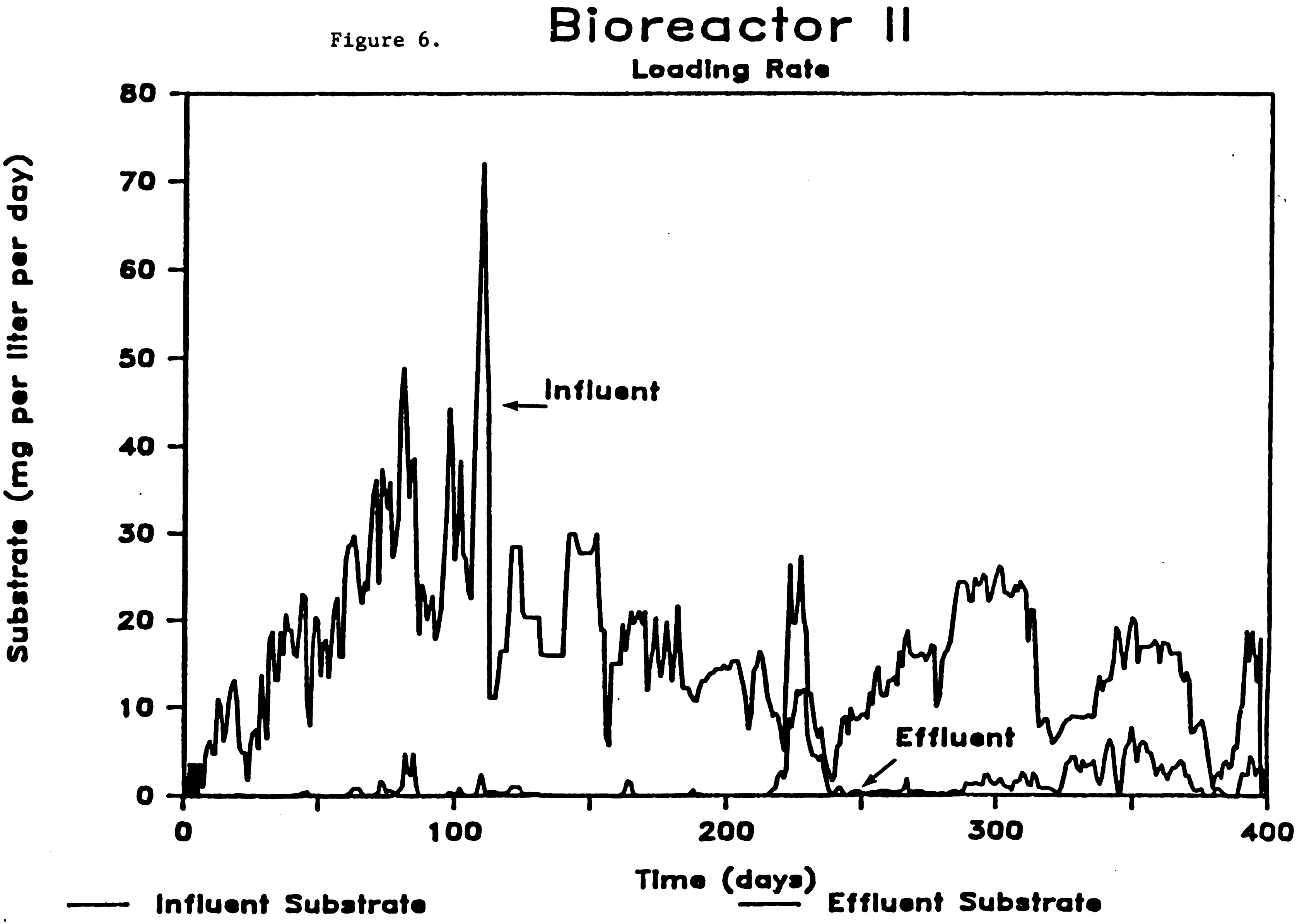
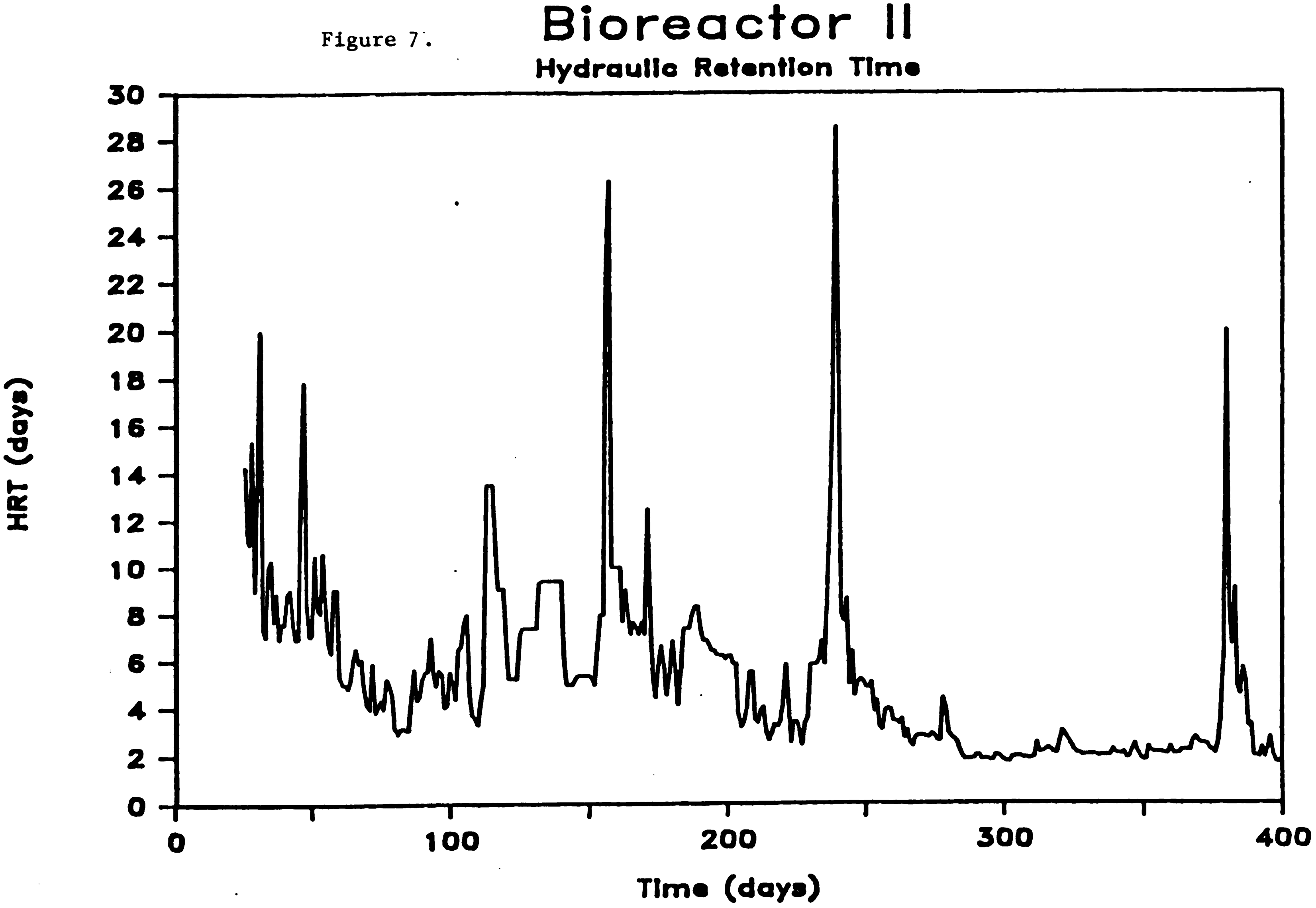

Bioreactor II
Loading Rate
Substrate (mg per liter per day)
80
70
60
50
40
30
20
10
0
0
100
200
300
400
Time (days)
Influent
Effluent
Influent Substrate
Effluent Substrate

Figure 7. Hydraulic retention time for Bioreactor II.

Figure 7.


Bioreactor II
Hydraulic Retention Time
HRT (days)
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
0
100
200
300
400
Time (days)

The start-up period was shortened considerably from the first bioreactor experiment (Bioreactor I, Figure 4). Loading rates of 10 mg l^{-1} d^{-1} were achieved after 30 days of operation (Figure 6). Both the influent chlorophenol concentration was increased and HRT was decreased from day 30 to 112. Loading rates of up to 70 mg l^{-1} d^{-1} were attained at nearly 100% conversion efficiency. From day 112 to 202, loading rates were decreased to perform mineralization studies. From day 202 to 400, loading rates varied during which time several other experiments involving the addition of higher chlorinated phenols were performed. The experimental bioreactor was terminated after 400 days of operation.

Experiments with Bioreactor III was additional evidence for the long term dechlorinating and degradative ability of the bioreactors. Bioreactor III was fed only meta-chlorophenol during its 190 days of operation. The overall influent and effluent loading rates and the HRT are shown in Figure 8 and 9. Start-up of the bioreactor was achieved after 10 days of operation, in contrast to Bioreactors I and II which lasted 100 and 30 days, respectively. The bioreactor loading rate was maintained at 20-30 mg l^{-1} d^{-1} at a HRT of two to four days through day 40 to 190 of operation with an average conversion efficiency of 90%. The bioreactor was terminated at day 190.

<u>Mineralization Studies</u>. The mineralization of substrate chlorophenol was examined in Bioreactor I and II utilizing two separate analytical methods described previously. Bioreactor I was employed for the first study where the mineralization product, CH_4, from meta-chlorophenol was measured. Chlorophenol was the sole

Figure 8. Influent and effluent substrate loading rate for Bioreactor III.

Figure 8.

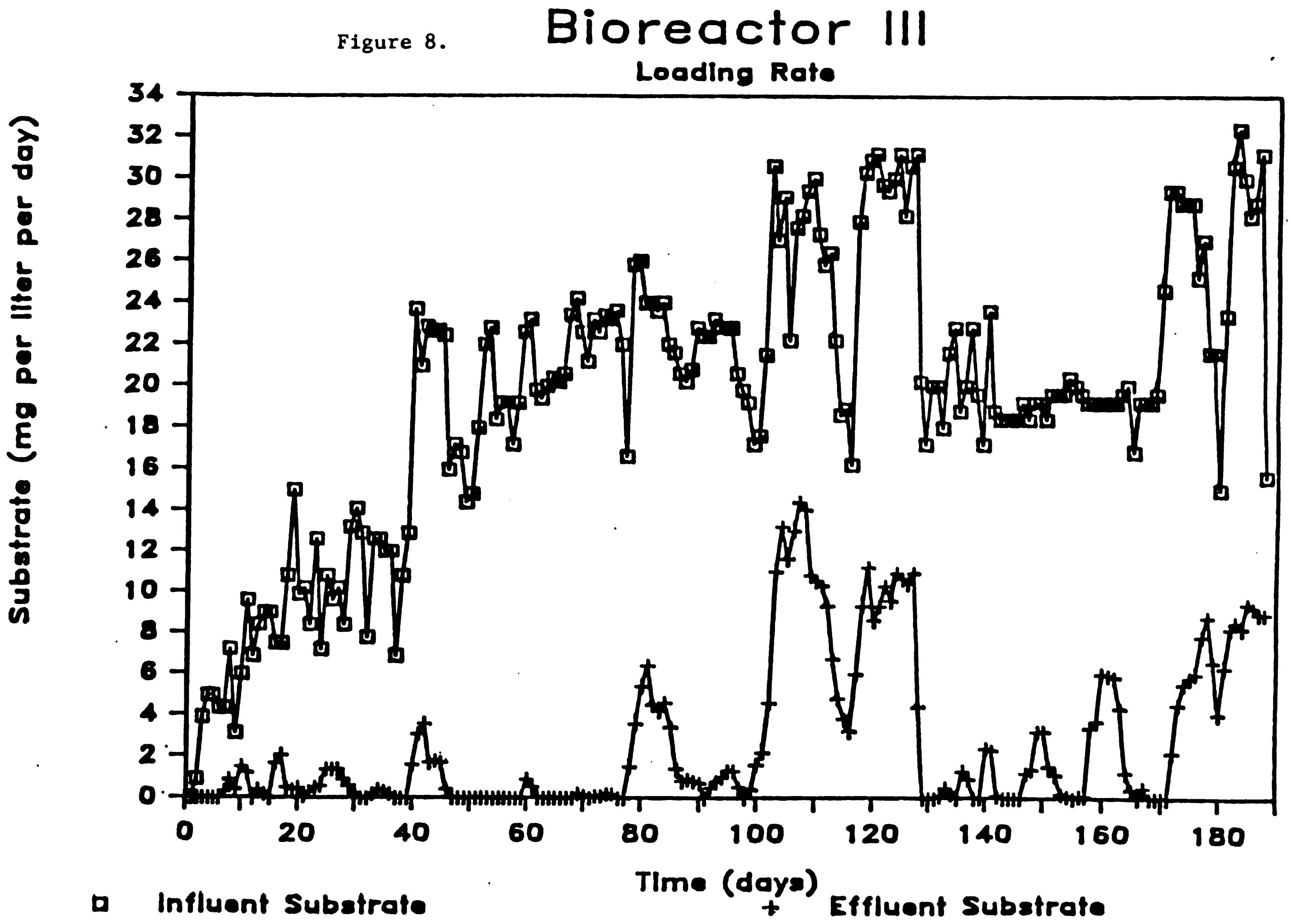
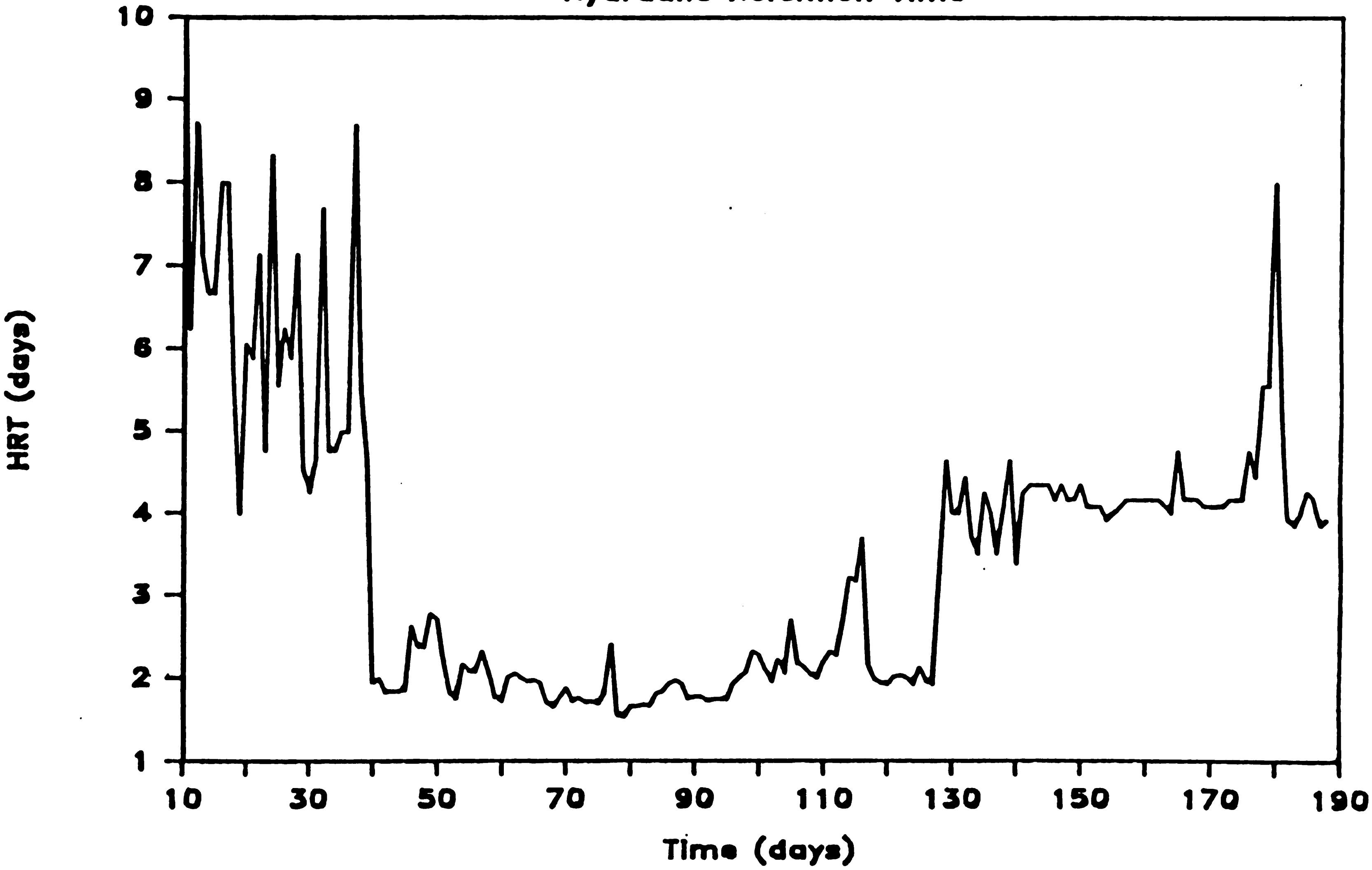

Bioreactor III
Loading Rate
Substrate (mg per liter per day)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
0
20
40
60
80
100
120
140
160
180
Time (days)
□ Influent Substrate
+ Effluent Substrate

Figure 9. Hydraulic retention time for Bioreactor III.

Figure 9. **Bioreactor III**

Hydraulic Retention Time



carbon source and thus the sole source of CH_4. Radiotracer methods were employed using Bioreactor II in two additional mineralization experiments where $^{14}CO_2$ from ^{14}C-para-chlorophenol was measured.

In the Bioreactor I mineralization study, the total amount of CH_4 collected over the 46 day period accounted for 21.7% of the total chlorophenol substrate added during this period. Methane recovery was divided between gaseous CH_4 and aqueous CH_4; 9.0% of the total carbon was detected as a gaseous product and 12.7% of the total carbon was extracted from the aqueous phase. Based on the stoichiometry of 3.5 CH_4:2.5 CO_2, another 15.6% of the total added carbon could be accounted for as CO_2 for a total carbon recovery of 37.3% (Table 1).

The results for the two mineralization studies with Bioreactor II are summarized in Table 2. The experiments differed from each other in the average substrate loading rate, substrate conversion efficiency, HRT, and the experimental methods employed to trap radiolabeled material.

In the first radiolabeled mineralization experiment, labeled material was recovered both as para-chlorophenol in the effluent, 17.6%, and as CO_2, 18.8%. The large amount of label in the effluent was most likely due to the low substrate conversion efficiency during the early part of the experiment. Based on the theoretical gaseous stoichiometry, 26.3% of the total added label could be accounted for as unrecovered CH_4 for a total gaseous recovery of 45.1%.

In the second radiolabeled mineralization experiment, the distribution of total label was as follows: 6.1% was found in the effluent as para-chlorophenol, 19.4% was collected as CO_2 and 4.1% as

Table 1. Mineralization of m-chlorophenol to gaseous end products in Bioreactor I.

	Gaseous End Product Yield (Percent of Theoretical)
Methane	21.7 %
Carbon Dioxide*	15.6 %
Total	37.3 %

*CO_2 was calculated from the methane.

Table 2. Fate of ^{14}C-para-chlorophenol in Bioreactor II.

(Percent of Added Label)

Fraction	Experiment 1	Experiment 2
Effluent (para-chlorophenol)	17.6 %	6.1 %
CO_2	18.8 %	19.4 %
Non-CO_2 Volatile	------	4.1 %
Calculated CH_4	26.3 %	27.2 %
Total Gaseous	45.1 %	46.6 %

non-CO_2 volatiles. The non-CO_2 volatile material probably represented a portion of the labeled CH_4 with the remainder of the labeled CH_4 lost. Based on the gaseous stoichiometry, 27.2% of the label could be accounted for as CH_4 for a total gas yield of 45.8%. The results are summarized in Table 2.

Sorption. Sorption of the chlorophenols in the bioreactor was minimal. Approximately 75% of a non-mineralized compound (ortho-chlorophenol) added to the bioreactor which degraded meta-chlorophenol was recovered in the effluent (Figure 10). The ortho-chlorophenol rapidly eluted from the bioreactor which had a HRT of approximately four days. The bioreactor contents were also examined for residual chlorophenol after termination of the bioreactor. No meta-chlorophenol was detected in any of the bioreactor contents including the biomass at the bottom of the bioreactor and the glass beads.

Substrate Loading Rate and Hydraulic Retention Time. The capacity of the bioreactors to degrade chlorophenol at various substrate loading rates and HRT was determined. Experiments were initiated with Bioreactor III after an initial start-up period. The overall loading rates throughout all of the experiments with Bireactor III is shown in Figure 8. The capacity of the bioreactors to degrade chlorophenols was examined by determining the efficiency of chlorophenol removal at various substrate loading rates and HRT. The results are summarized in Table 3.

In the first experiment, chlorophenol removal efficiency was determined at an average substrate loading rate of 21.1 mg l^{-1} d^{-1} and a HRT of 1.9 days (Figure 11). The experiment took place over a

Figure 10. Turnover time of a non-mineralized substrate, orthochlorophenol, in Bioreactor I.

Figure 10.

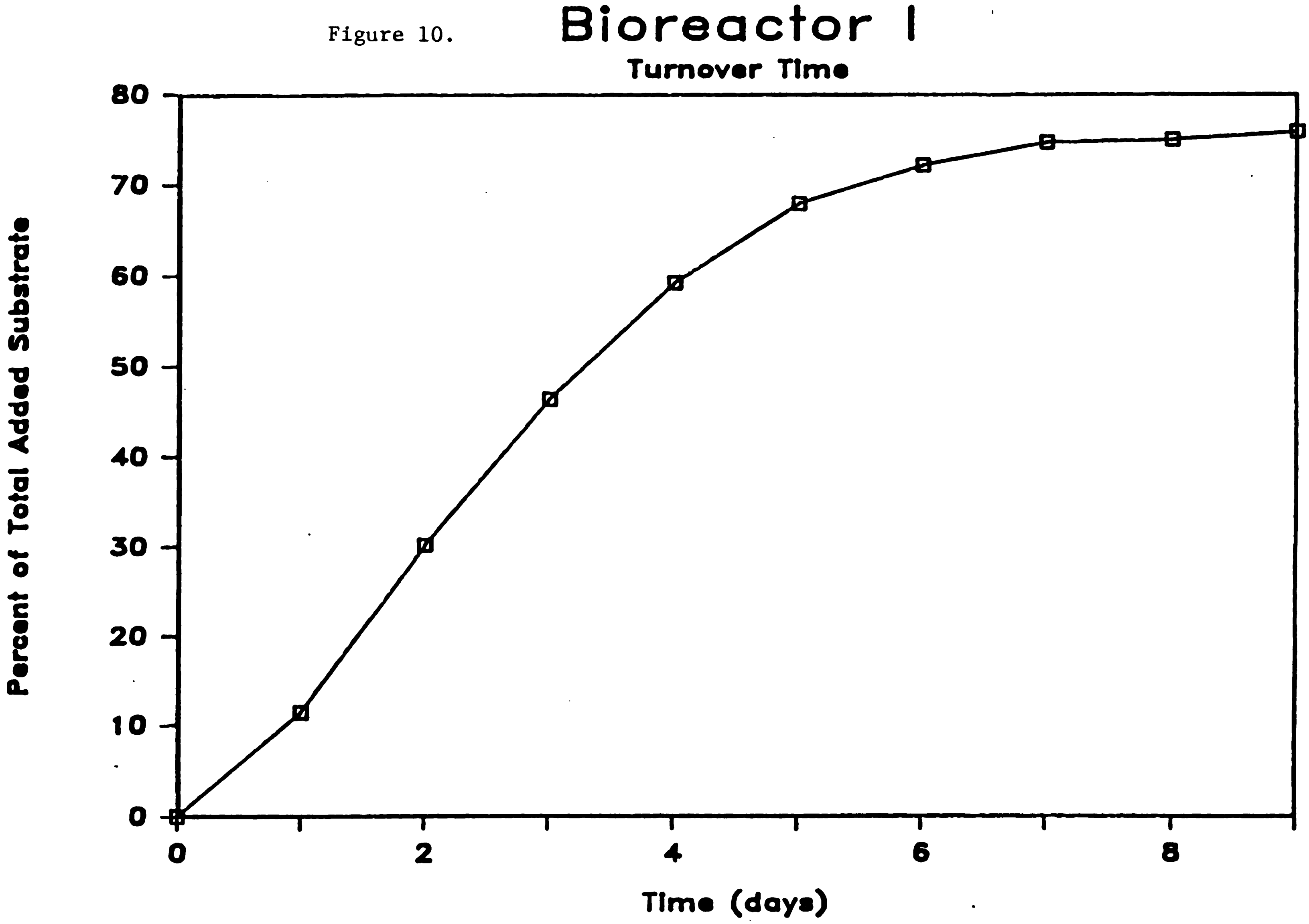
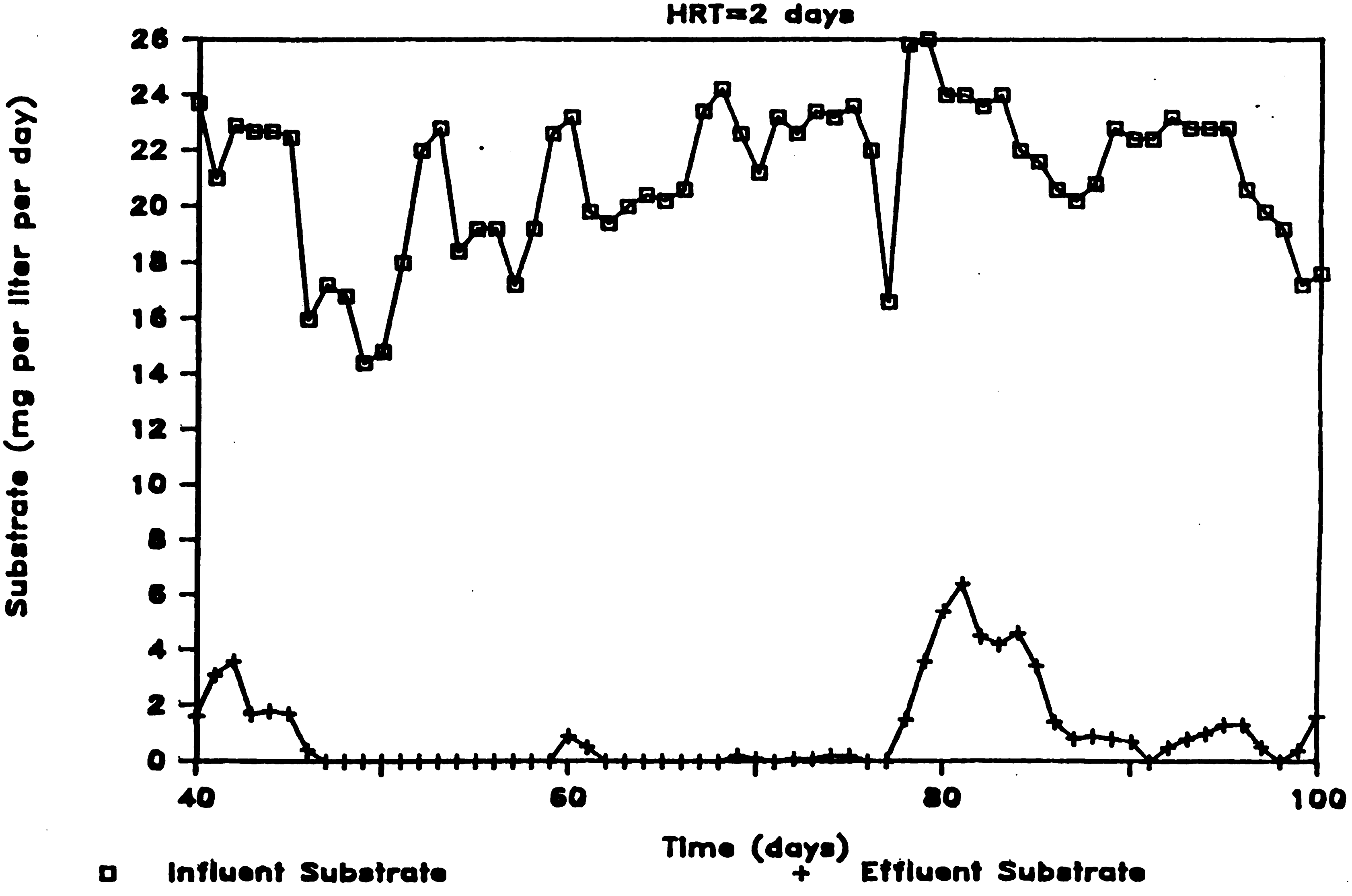

Bioreactor I
Turnover Time
Percent of Total Added Substrate
0
10
20
30
40
50
60
70
80
0
2
4
6
8
Time (days)

Table 3. Chlorophenol removal efficiency at different substrate loading rates and hydraulic retention times in Bioreactor III.

Expt. #	Substrate Loading Rate (mg 1-1 d-1)	Effluent (mg 1-1 d-1)	HRT (days)	Removal Efficiency (Percent)
1	21.1	1.01	1.9	95.2
2	27.1	9.28	2.3	65.8
3	19.4	1.25	4.1	93.6
4	27.1	6.46	4.6	76.2

Figure 11. Influent and effluent substrate loading rate for Bioreactor III during days 40 through 100.

Figure 11. Bioreactor III Loading Rate



61 day period from day 40 to 100. The influent substrate concentration in the feed was constant at 40 mg/l. The chlorophenol removal efficiency over the experimental period was 95%.

In the second experiment, the chlorophenol removal efficiency was determined at a substrate loading rate of 27.1 mg l^{-1} d^{-1} and a HRT of 2.3 days (Figure 12). Influent substrate concentration in the feed was increased to 60 mg/l. The chlorophenol removal efficiency over the 28 day period from day 100 to 127 was calculated at 66%.

Due to the low chlorophenol removal efficiency at 27 mg l^{-1} d^{-1} and HRT of two days, the HRT was increased to approximately four days to determine the effect of HRT on chlorophenol removal. The third experiment, from day 128 to 169, was performed for 42 days (Figure 13). The influent substrate concentration in the feed was 80 mg/l at an average substrate loading rate of 19.4 mg l^{-1} d^{-1} and an HRT of 4.1 days. The chlorophenol removal efficiency was 94%.

The substrate concentration in the feed was increased to 120 mg/l in the fourth experiment for an average substrate loading rate of 27.1 mg l^{-1} d^{-1} (Figure 14). The HRT remained at approximately four days (4.6) throughout the 20 day experiment from day 171 to day 187. The chlorophenol removal efficiency was 76%.

Trichlorophenols and Pentachlorophenol. Three experiments were performed utilizing Bioreactor II to determine the capacity of the bioreactors to dechlorinate and degrade higher chlorinated compounds. The bioreactor employed for these studies was able to degrade all three monochlorophenols at a 1:4:1 ratio of ortho-:meta-:para-.

The first experiment with higher chlorinated phenols was performed using the trichlorohphenol, 2,4,6-trichlorophenol. The

Figure 12. Influent and effluent substrate loading rate for Bioreactor III during days 100 through 127.

Figure 12.

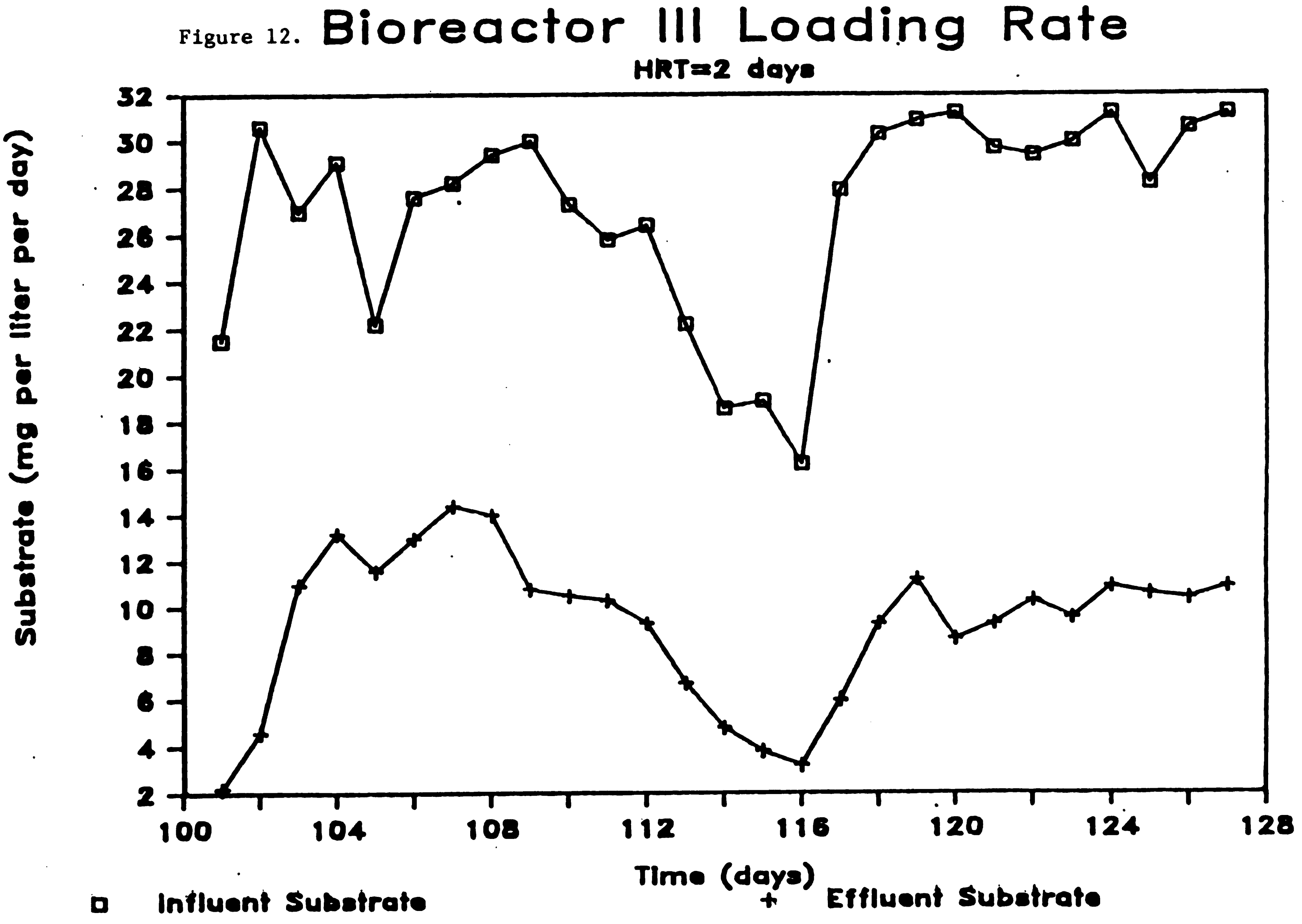

Bioreactor III Loading Rate
HRT=2 days
Substrate (mg per liter per day)
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
100
104
108
112
116
120
124
128
Time (days)
□ Influent Substrate
+ Effluent Substrate

Figure 13. Influent and effluent substrate loading rate for Bioreactor III during days 128 through 169.

Figure 13. Bioreactor III Loading Rate

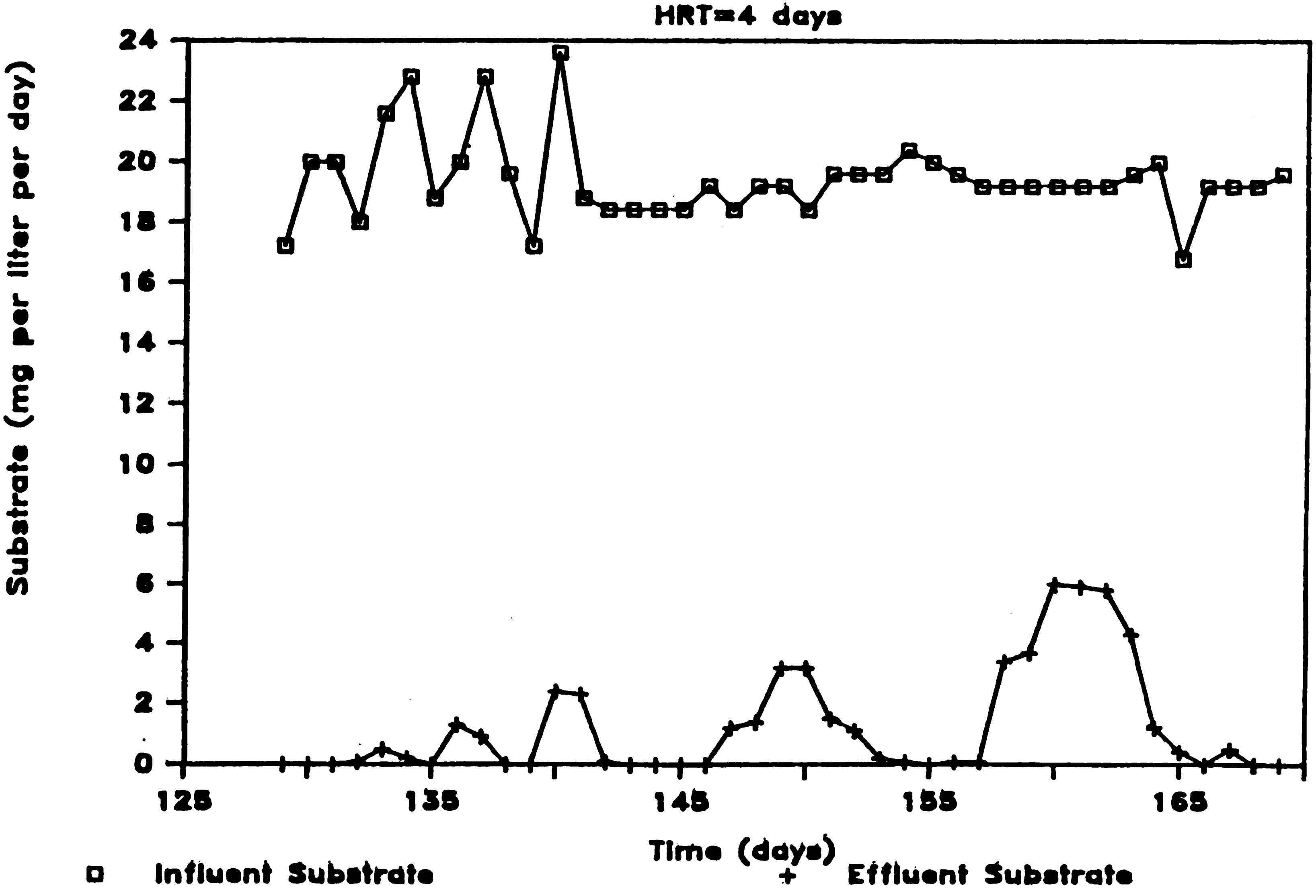
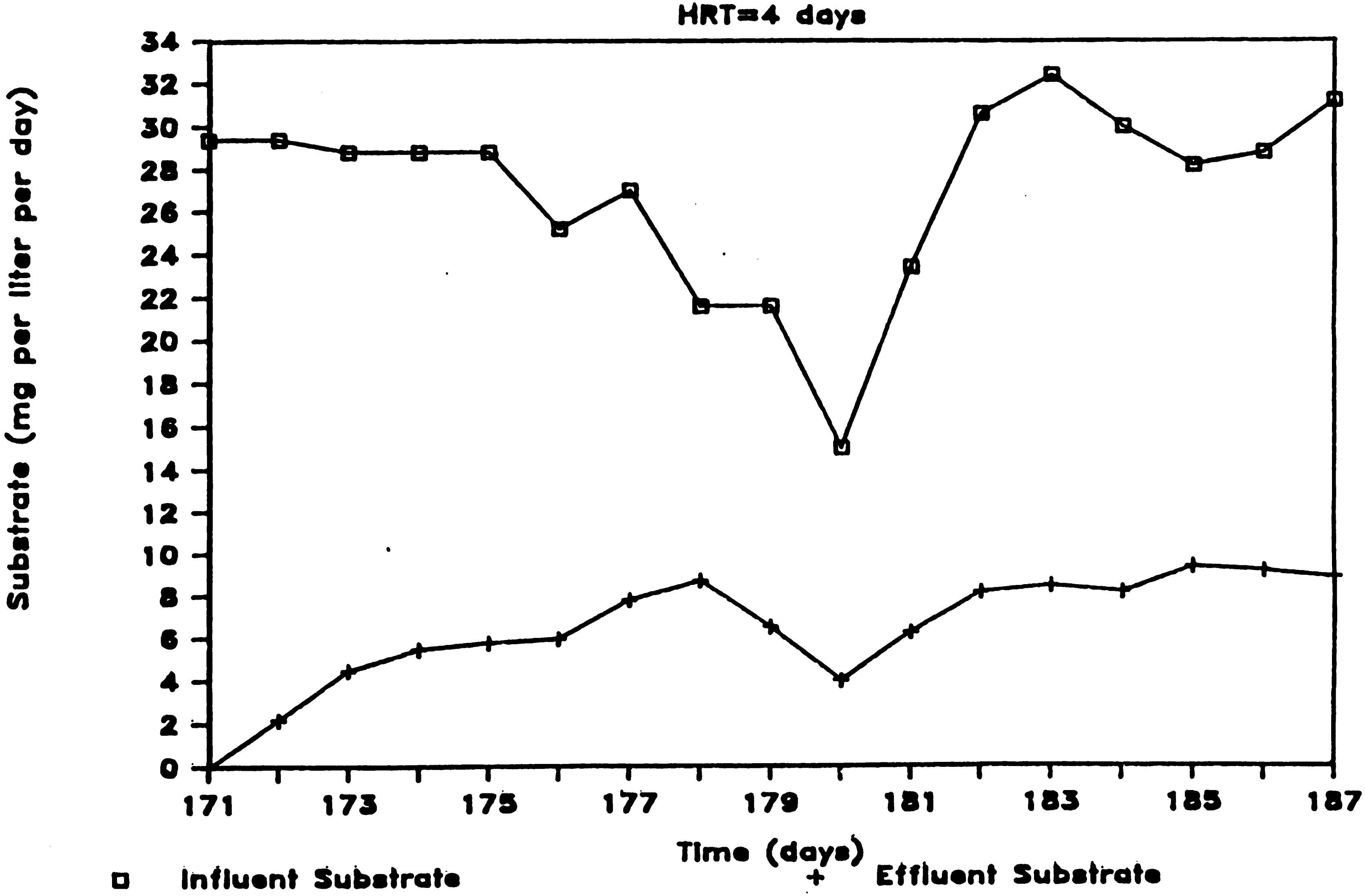


Figure 14. Influent and effluent substrate loading rate for Bioreactor III during days 171 through 187.

Figure 14. Bioreactor III Loading Rate



trichlorophenol was added to the bioreactor during days 213-222 and detected in the effluent through day 229 of operation. The average influent total substrate loading rate of the monochlorophenols was 15.1 mg l^{-1} d^{-1} at an average HRT of 3.6 days. The average substrate conversion efficiency of the monochlorophenols was 84%. The average loading rate of the 2,4,6-trichlorophenol was 6.28 mg l^{-1} d^{-1} during days 213-222 (Figure 15). The total cumulative amount of trichlorophenol added to the bioreactor is shown in Figure 16 along with the cumulative amount of trichlorophenol detected in the effluent. Approximately 75% of the total added trichlorophenol was detected in the effluent indicating that dehalogenation and degradation was minimal in the bioreactor. Dehalogenated products were not detected in the effluent. Any monochlorophenols that may have been produced from the trichlorophenol would not have been detected in the effluent because the bioreactor was also being fed monochlorophenols as a substrate.

In the second experiment with higher chlorinated phenols, the substrate 3,4,5-trichlorophenol was added to the bioreactor during days 315 to 329 of operation and detected in the effluent through day 354. The average influent total substrate loading rate of the monochlorophenols was 11.8 mg l^{-1} d^{-1} at an average HRT of 2.2 days. The average substrate conversion efficiency of the monochlorophenols was 74%. The average loading rate of the 3,4,5-trichlorophenol was 2.04 mg l^{-1} d^{-1} (Figure 17). The total cumulative amount of trichlorophenol added to the bioreactors and detected in the effluent is shown in Figure 18. In contrast to the first experiment, only 35% of the added trichlorophenol substrate was detected in the effluent

Figure 15. Influent and effluent substrate loading rate for 2,4,6-trichlorophenol.

Figure 15. 2,4,6—Trichlorophenol

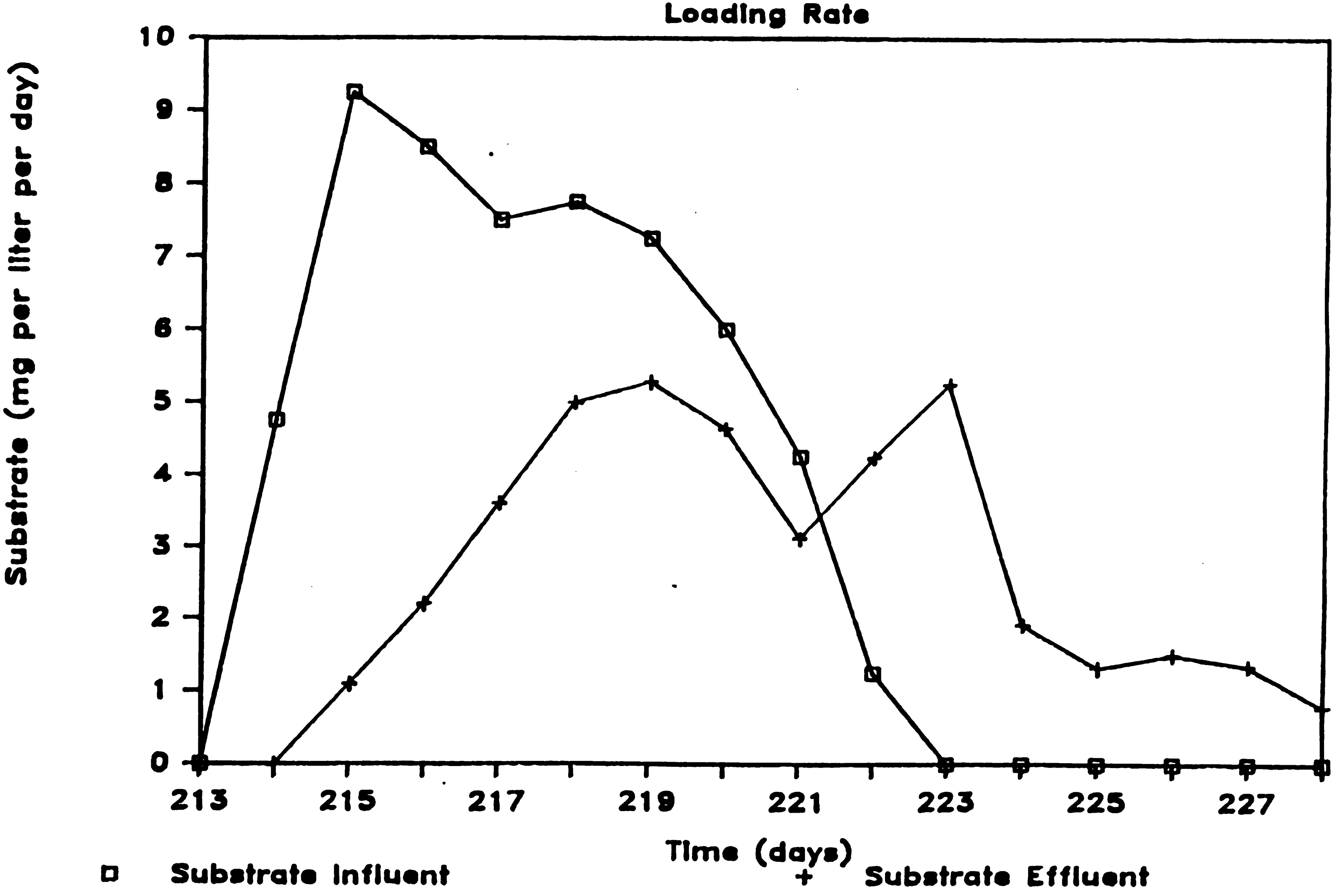


Figure 16. Cumulative substrate in the influent and effluent for 2,4,6-trichlorophenol.

Figure 16. 2,4,6–Trichlorophenol

Cumulative Substrate

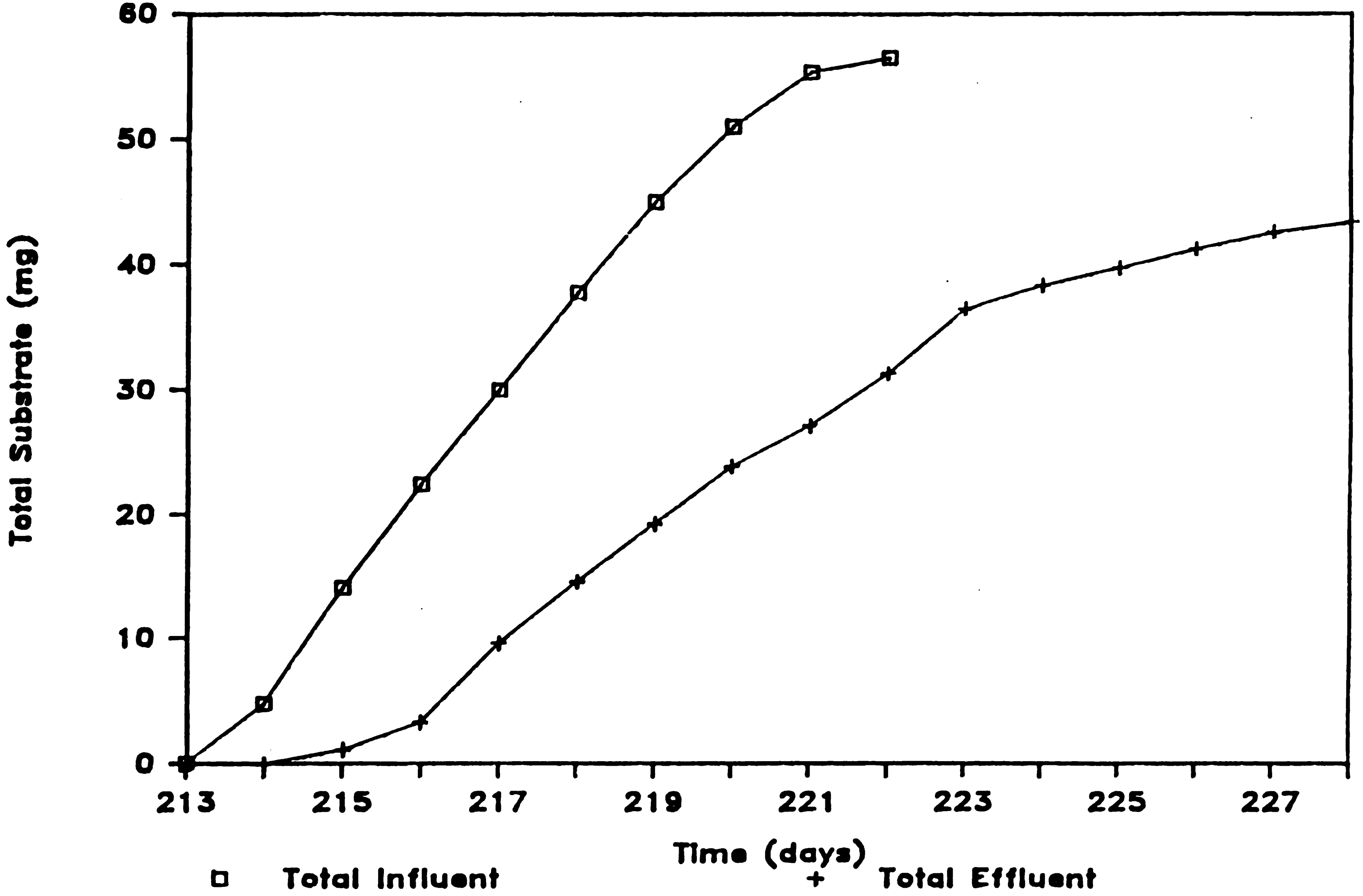


Figure 17. Influent and effluent substrate loading rate for 3,4,5-trichlorophenol.

Figure 17.

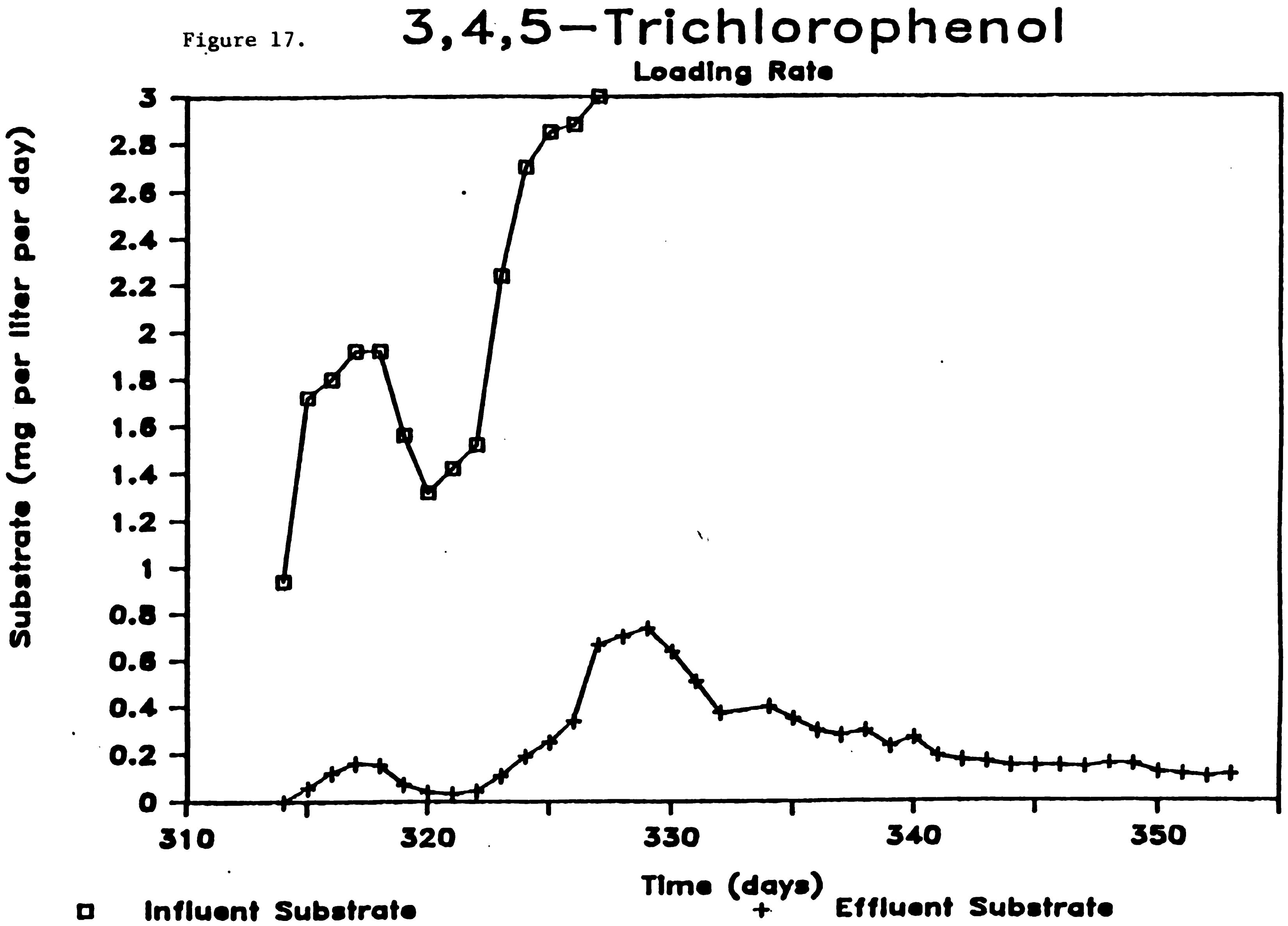
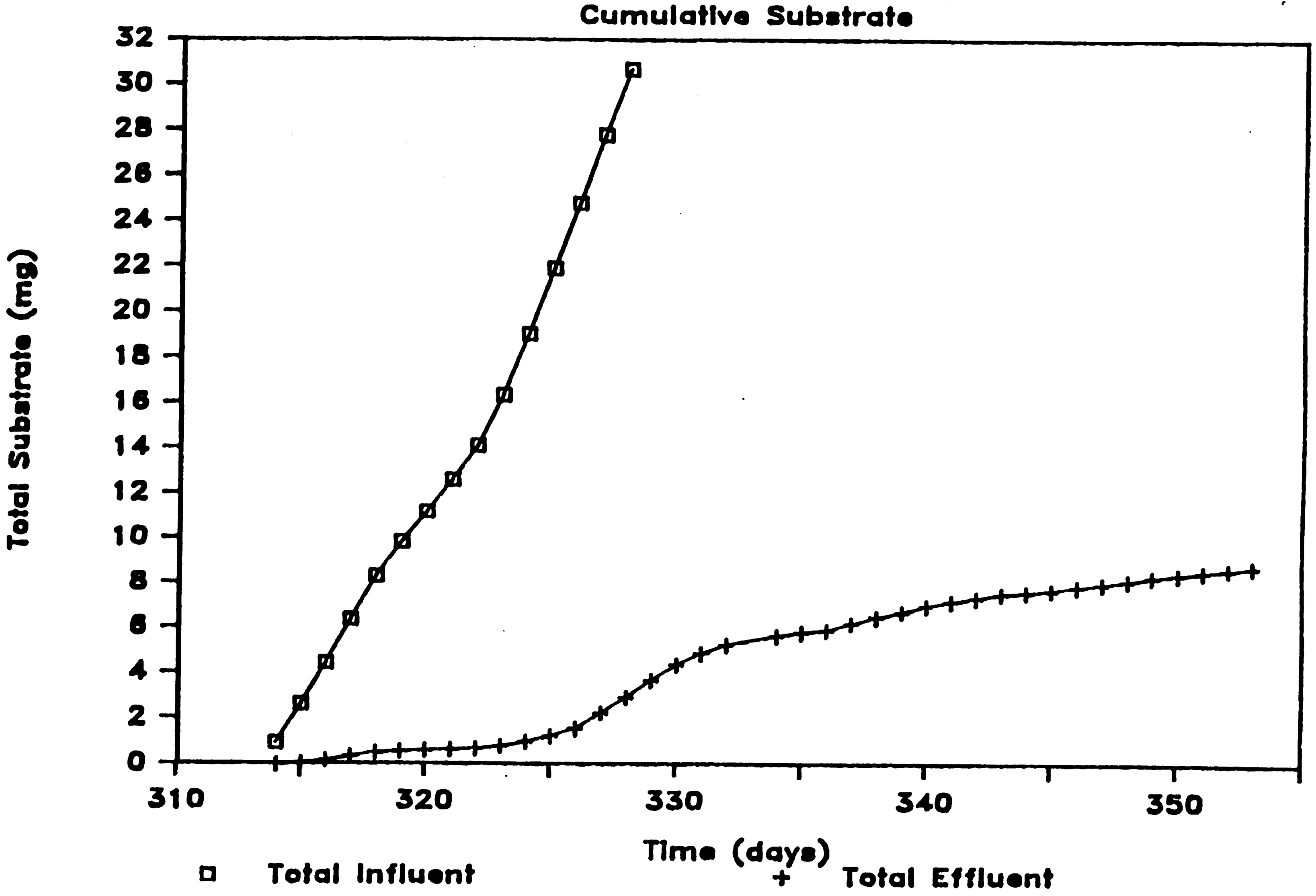

3,4,5—Trichlorophenol
Loading Rate
Substrate (mg per liter per day)
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
310
320
330
340
350
Time (days)
□ Influent Substrate
+ Effluent Substrate

Figure 18. Cumulative substrate in the influent and effluent for 3,4,5-trichlorophenol.

Figure 18.


3,4,5—Trichlorophenol
Cumulative Substrate
Total Substrate (mg)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
310
320
330
340
350
Time (days)
□ Total Influent
+ Total Effluent

indicating that degradation of the trichlorophenol occurred. Again, no dehalogenated products were detected in the effluent.

The third experiment was performed utilizing pentachlorophenol as the higher chlorinated substrate. The average influent total substrate loading rate of the monochlorophenols was 7.55 mg l^{-1} d^{-1}. The average substrate conversion efficiency of the monochlorophenols was 86%. The average loading rate of the pentachlorophenol was 1.61 mg l^{-1} d^{-1} during days 371 through 389 (Figure 19). The total cumulative amount of pentachlorophenol added to the bioreactor and detected in the effluent is depicted in Figure 20. Approximately 75% of the added pentachlorophenol could be detected in the effluent indicating that degradation was minimal. No lesser chlorinated products were observed in the effluent.

Two different HRT's were used to determine the effect on the degradation of pentachlorophenol. During days 371 to 378, a HRT of 2.6 days was employed and during days 379 to 400 a HRT of 5.1 days was employed. The change in HRT had no apparent effect on the degradation of pentachlorophenol.

Biomass Location. Bioreactors I and III, with meta-chlorophenol as the sole carbon source, were examined for the location of biomass. These experiments were performed at the termination of Bioreactor I and III after 350 and 190 days of operation, respectively.

The location of the biomass, as protein, in Bioreactor I is shown in Figure 21. The bioreactor was divided into ten non-equal portions. Section 1 and 2, at the top of the bioreactor contained very little protein indicating that washout of the cells was minimal. Sections 3-5, which contained the glass beads for the attachment of

Figure 19. Influent and effluent substrate loading rate for pentachlorophenol.

Figure 19.

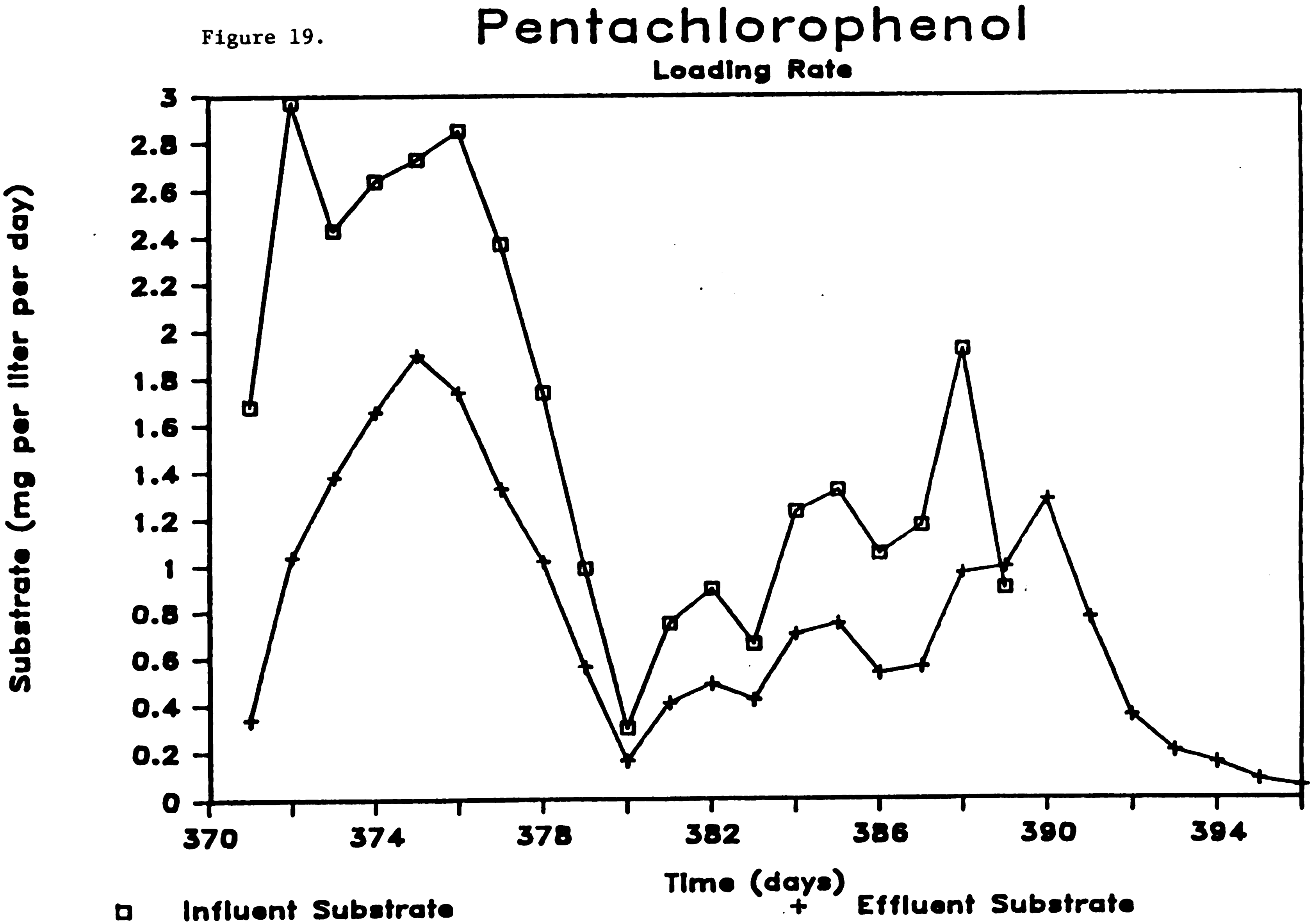

Pentachlorophenol
Loading Rate
Substrate (mg per liter per day)
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
370
374
378
382
386
390
394
Time (days)
□ Influent Substrate
+ Effluent Substrate

Figure 20. Cumulative substrate in the influent and effluent for pentachlorophenol.

Figure 20.

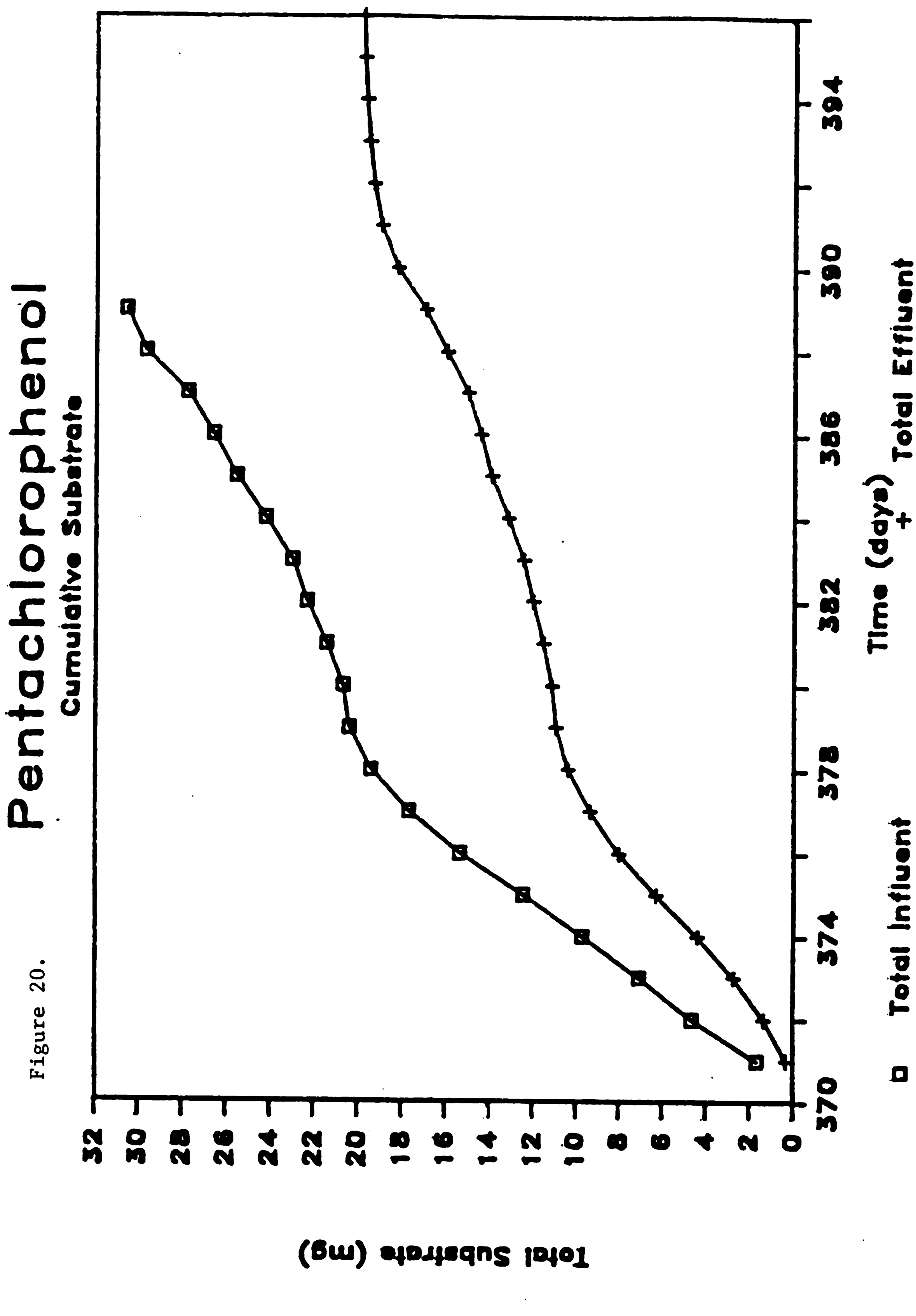
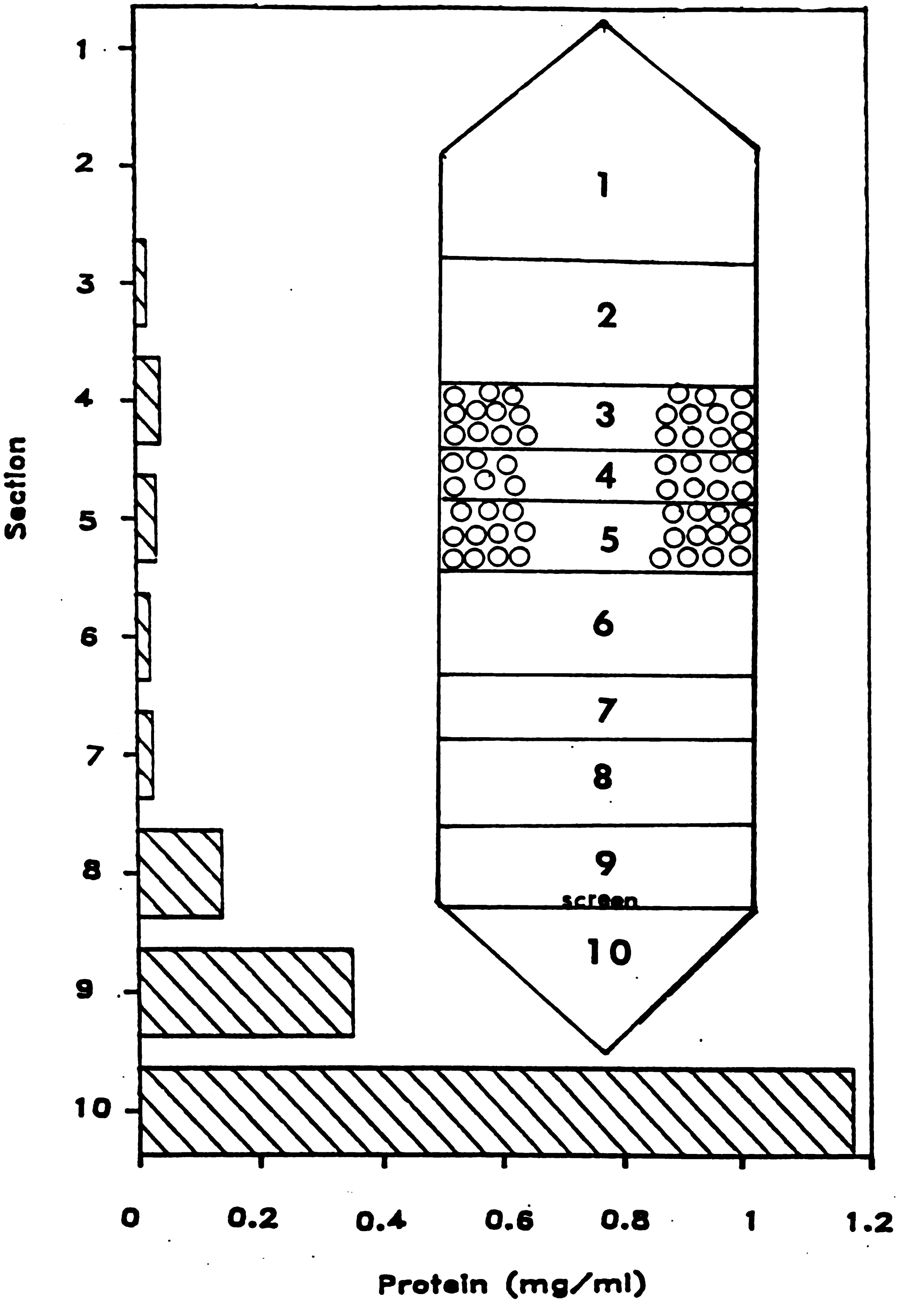

Pentachlorophenol
Cumulative Substrate
Total Substrate (mg)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
370
374
378
382
386
390
394
Time (days)
□ Total Influent
+ Total Effluent

Figure 21. Location of the biomass in Bioreactor I.

Bioreactor I

Figure 21.



the biomass, also contained very little protein, but at slightly higher levels than section 1 and 2. Attachment of the biomass to the glass beads was minimal. Sections 6-10 contained increasing amounts of biomass with the most significant amount of biomass located in section 9 and 10 near the screen designed for the accumulation of a sludge blanket. The distribution of protein was similar in Bioreactor III. (Figure 22). The majority of the protein was located at the bottom of the bioreactor near the screen.

Methane production and a chlorophenol degradation assay were used to determine the location of active biomass in both bioreactors. Although each section of the bioreactor produced small amounts of methane, the sections near the sludge blanket, sections 9 and 10 for Bioreactor I and sections 4 and 5 for Bioreactor III produced significantly more methane (Figures 23 and 24).

A chlorophenol degradation assay was also used to determine the dehalogenation activity in Bioreactor III (Figure 25). Chlorophenol was rapidly degraded in the bottom section of the bioreactor in both sections 4 and 5. No dissappearance of added chlorophenol over a 20 day period was observed for sections of the bioreactor above the sludge blanket, including the sections containing the glass beads.

Dominant Morphology. The dominant morphology in the bioreactor was determined by microscopic examination of the bioreactor contents. Although not the sole organisms present, three organisms dominated the bioreactor contents. These were:

1. a putative phenol-degrader
2. a *Methanosarcina sp.*
3. a *Methanothrix sp.*

Figure 22. Location of the biomass in Bioreactor III.

Bioreactor III

Figure 22.

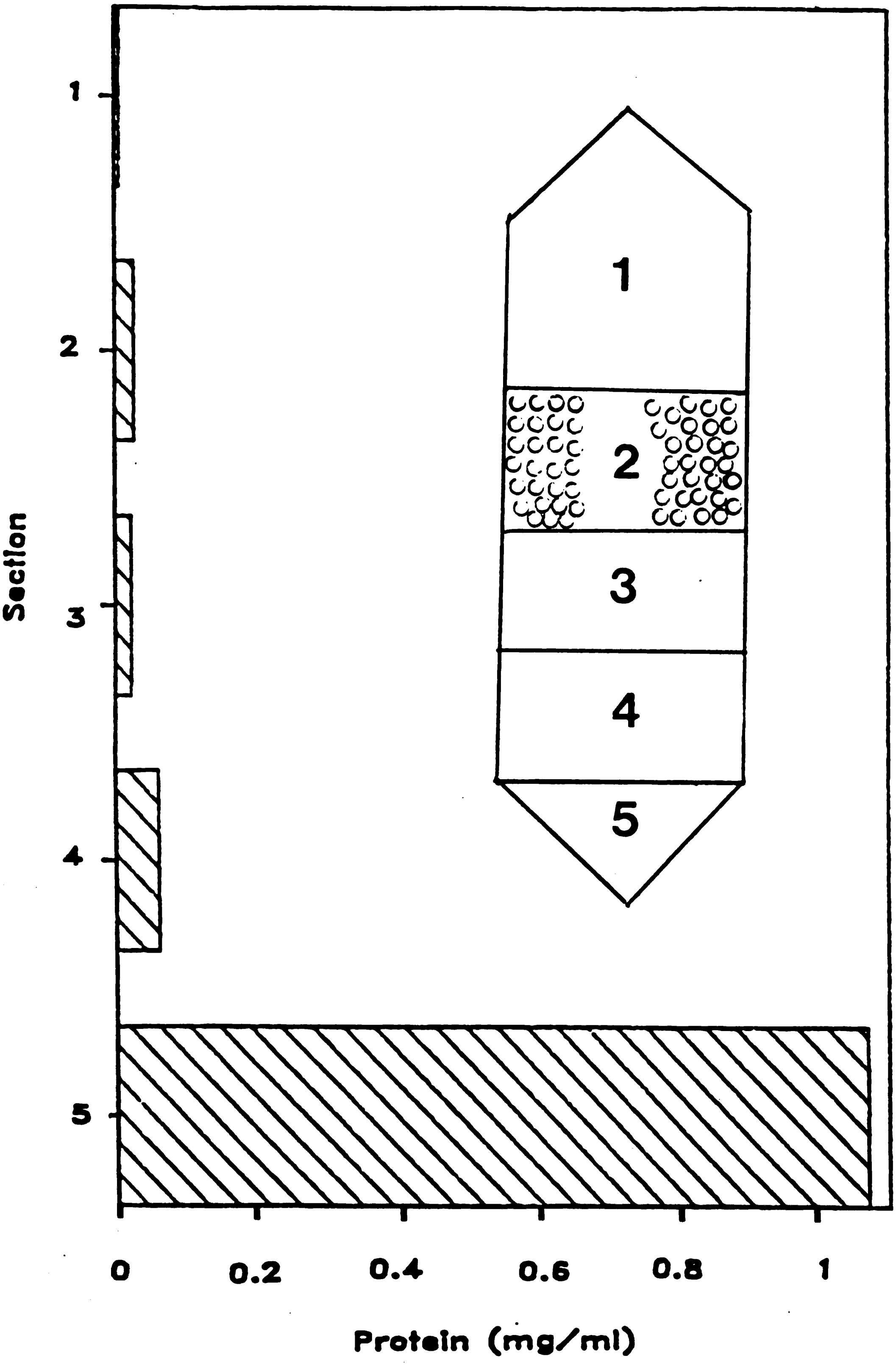


Figure 23. Methanogenic activity per column section in Bioreactor I.

Figure 23.

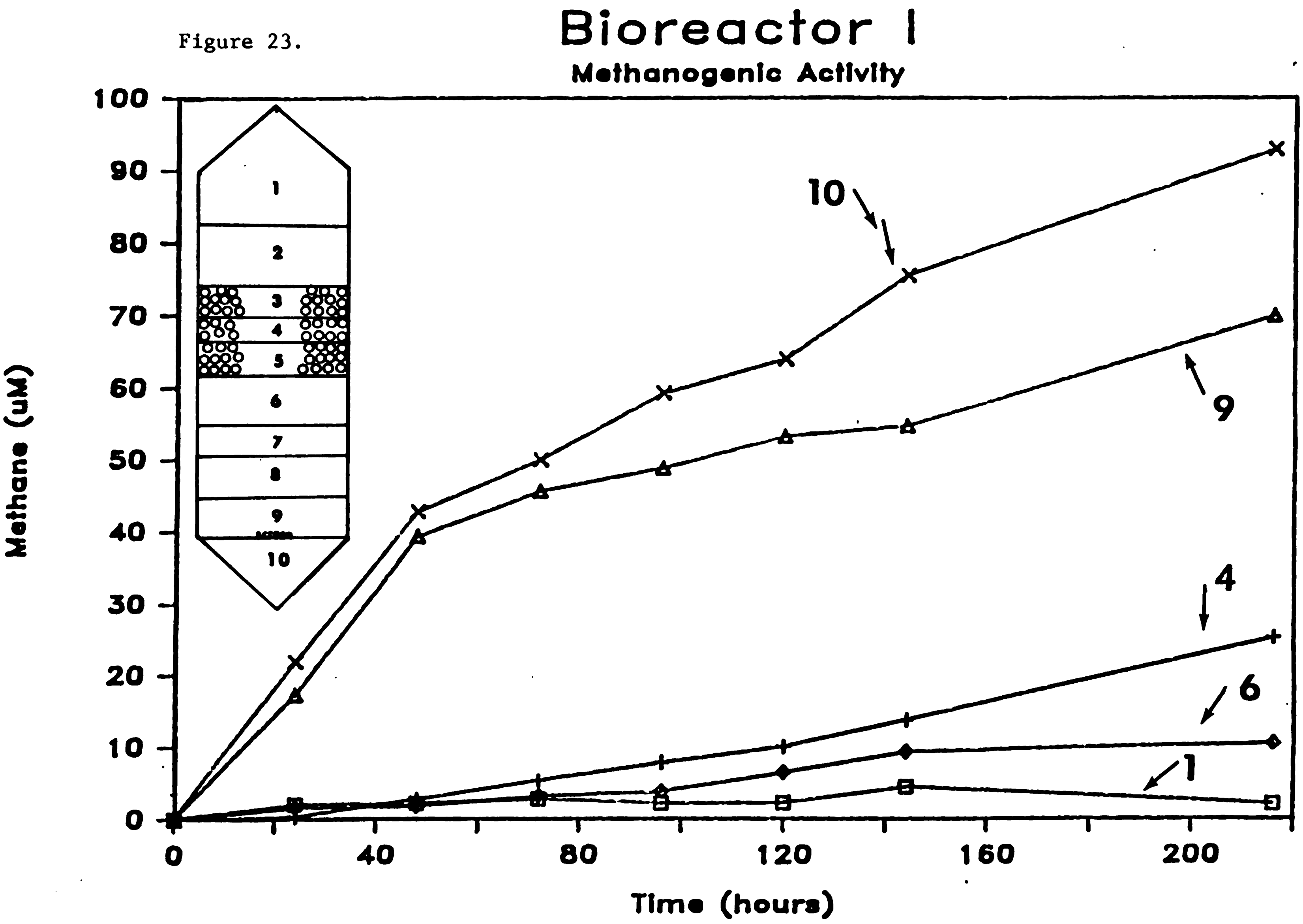

Bioreactor I
Methanogenic Activity
Methane (uM)
Time (hours)
0
10
20
30
40
50
60
70
80
90
100
0
40
80
120
160
200
10
9
4
6
1
1
2
3
4
5
6
7
8
9
10

Figure 24. Methanogenic activity per column section in Bioreactor III.

Figure 24.

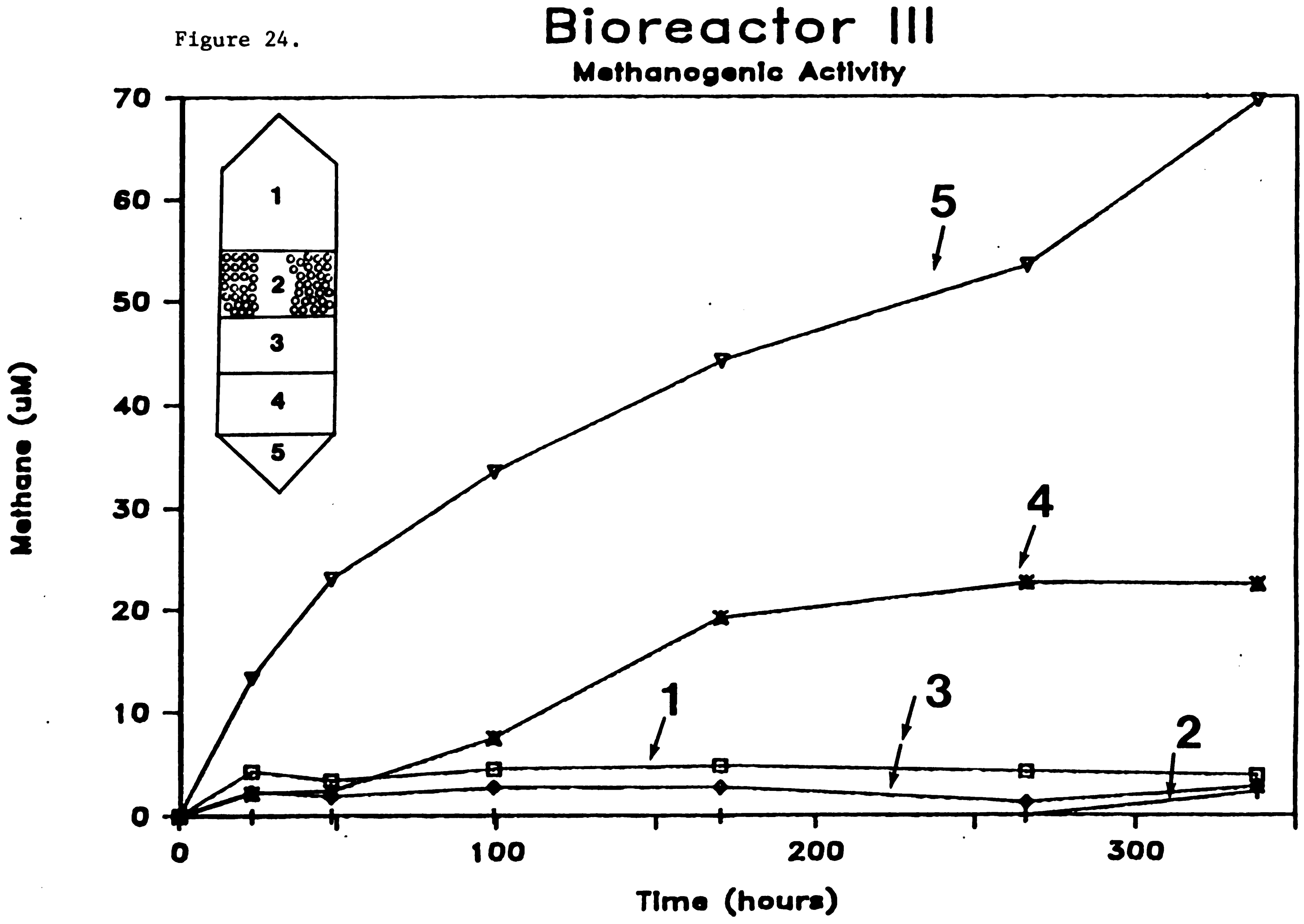
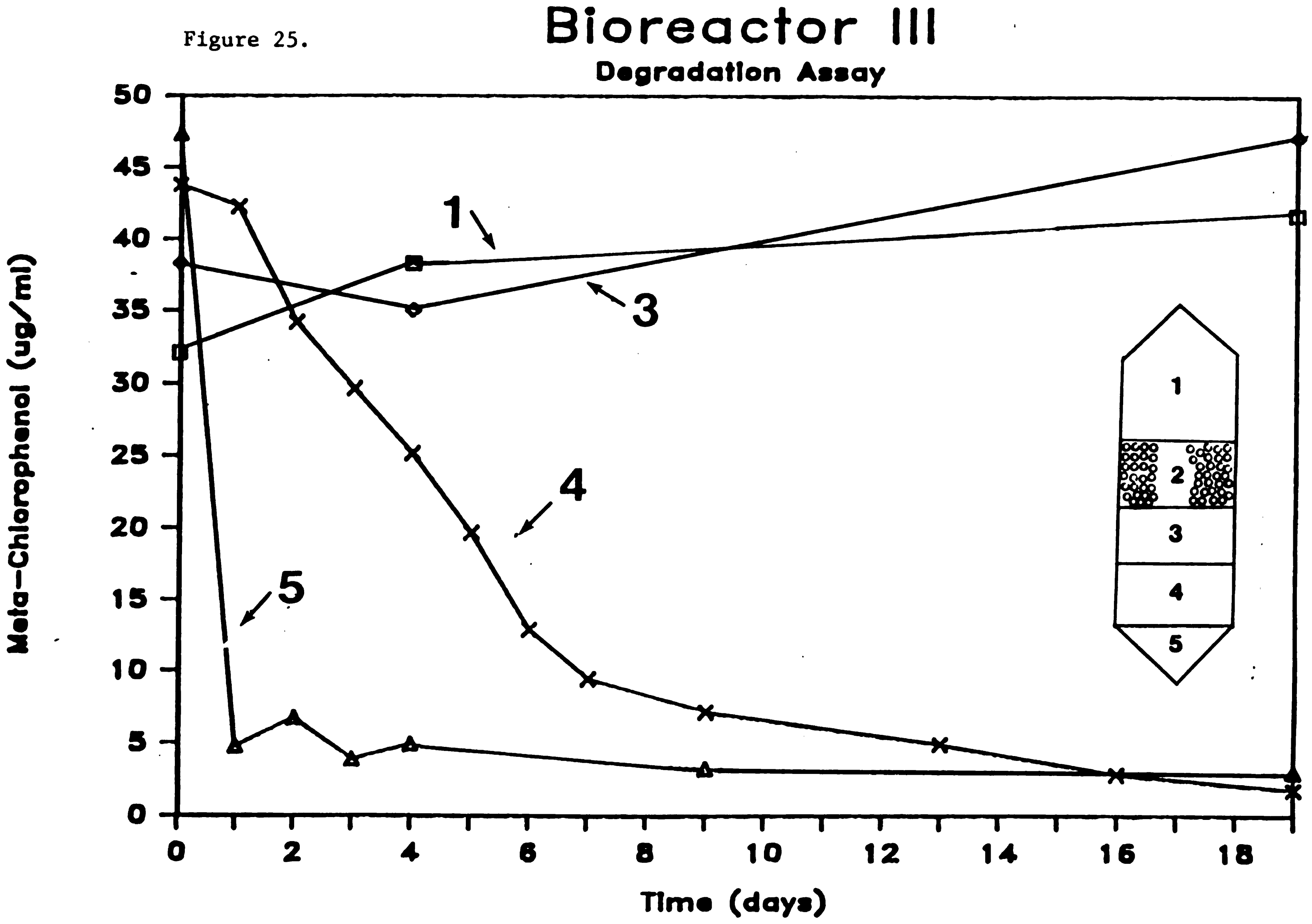

Bioreactor III
Methanogenic Activity
Methane (uM)
Time (hours)
0
10
20
30
40
50
60
70
0
100
200
300
1
2
3
4
5
1
2
3
4
5

Figure 25. Degradative activity per column section in Bioreactor III.

Figure 25.


Bioreactor III
Degradation Assay
Meta-Chlorophenol (ug/ml)
50
45
40
35
30
25
20
15
10
5
0
Time (days)
0
2
4
6
8
10
12
14
16
18
1
3
4
5
1
2
3
4
5

The morphology of the first organism, a putative phenol-degrader (Figure 26), was similar to that observed in other phenol-degrading enrichment cultures and has been isolated in co-culture from the phenol enrichments (Krumme and Boyd, 1986). The second microorganism was similar in morphology to *Methanosarcina sp.* (Figure 27), and the third organism was similar in morphology to *Methanothrix sp.* (Figure 28). Although present in chlorophenol enrichment cultures, these three organisms did not dominate the enrichment cultures to the extent that they dominated in the bioreactor. Numerical quantification was not possible due to the presence of particulate matter in both enrichment culture and bioreactor contents which obscured observations.

Figure 26. Photomicrograph of bioreactor biomass depicting morphology similar to a putative phenol-oxidizing microorganism (4000X).

Figure 27. Photomicrograph of bioreactor biomass depicting morphology similar to *Methanosarcina sp.* (4000X).

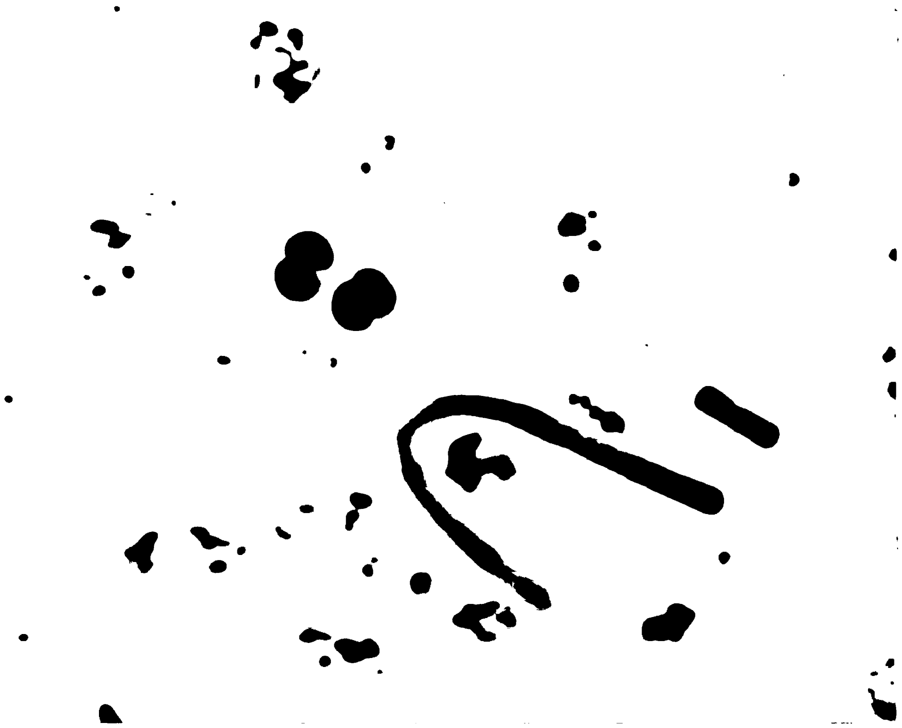


Figure 28. Photomicrograph of bioreactor biomass depicting morphology similar to *Methanothrix sp*. (4000X).

DISCUSSION

We have demonstrated with three separate bioreactors, that reductive dechlorinating activity can be maintained in anaerobic upflow bioreactors. The anaerobic conditions necessary for reductive dehalogenation were maintained with chlorophenols as the sole carbon and energy source. The bioreactors operated for 350, 400 and 190 days suggesting that reductive dechlorinating activity can be maintained indefinitely given the proper conditions.

In addition to reductive dechlorination, mineralization of the chlorophenols to CH_4 and CO_2 was demonstrated in the bioreactors. Approximately 40% of the added carbon was accounted for as gaseous end products. Gaseous products, CH_4 and CO_2, may have accounted for the remaining (60%) of the carbon but was not recovered due to the difficulty in recovering and detecting small quantities of gaseous products from large effluent volumes over long time periods in an open flow-through system. Mineralization of the chlorophenol to CH_4 and CO_2 was additional evidence that strict anaerobic conditions were maintained in the bioreactors.

In the first two bioreactor experiments, we attempted to determine a range of feasible substrate loading rates and HRT's while maintaining a substrate conversion efficiency of greater than 90%. We were concerned with both washout of the biomass from short HRT's and toxicity of the chlorophenol substrate from high loading rates. Short-term evidence, with Bioreactor I, suggested that a substrate loading rate of 60 mg l^{-1} d^{-1} at a HRT of two days was possible. During long-term mineralization studies, we demonstrated that substrate loading rates of 10 mg l^{-1} d^{-1} at a HRT of four days could be maintained at 100% substrate conversion efficiency for nearly 100 days. With Bioreactor II, short-term evidence also suggested that substrate loading rates of 70 mg l^{-1} d^{-1} at a HRT of two days and a substrate conversion efficiency of 100% was feasible. These loading rates were achieved using a mixture of all three monochlorophenols. Again, during the mineralization studies we demonstrated that loading rates of approximately 18 mg l^{-1} d^{-1} at a HRT of approximately eight days and a substrate conversion efficiency of approximately 100% could be maintained for over 100 days.

In order to more fully define maximum substrate loading rates at a specific HRT, experiments were performed with Bioreactor III, which utilized only meta-chlorophenol as the sole carbon source. Loading rates of 20 mg l^{-1} d^{-1} at HRT's of two and four days were achieved at substrate conversion efficiencies of greater than 90%. However, when the loading rate was increased to 30 mg l^{-1} d^{-1} at both retention times, the substrate conversion efficiency decreased to approximately 60%. It appeared that the biomass was adapted to degrade only 20 mg l^{-1} d^{-1} because the decrease in substrate conversion efficiency was equal to the difference between 20 and 30 mg l^{-1} d^{-1}. Toxicity of the substrate at the higher loading rate did not appear to effect the biomass since it retained the ability to degrade the lower substrate loading rate of 20 mg l^{-1} d^{-1}. In future experiments it may be possible to achieve higher substrate loading rates in bioreactors by either allowing a sufficient period of time for the biomass to adapt to a higher substrate loading rate or by some other means of increasing the amount of active biomass in the bioreactor.

We have demonstrated the ability to dehalogenate and degrade a mixture of all three monochlorophenols in anaerobic upflow bioreactors. Dehalogenating activity was maintained on a 1:4:1 mixture of ortho-:meta-:para-chlorophenol for 400 days. These results suggest that anaerobic upflow bioreactors may be capable of treating more complex waste streams containing higher chlorinated phenols since dechlorination occurs at all positions on the aromatic ring. Batch culture experiments with acclimated anaerobic sewage sludge have demonstrated the ability to dechlorinate higher chlorinated phenols such as the dichlorophenols and pentachlorophenol. (Boyd and Shelton, 1984; Mikesell and Boyd, 1985). Additional experiments with anaerobic sewage sludge acclimated to all three monochlorophenols have demonstrated the ability to dechlorinate and partially mineralize pentachlorophenol (Mikesell and Boyd, 1986). Thus, the potential for dechlorinating higher chlorinated phenols exists.

We have demonstrated the partial dissappearance of one higher chlorinated phenol, 3,4,5-trichlorophenol, in the bioreactor capable of dechlorinating all three monochlorophenols. The lack of dechlorination in the bioreactors of the other higher chlorinated phenols we tried, (i.e., 2,4,6-trichlorophenol and pentachlorophenol), may be the result of improper experimental conditions such as the loading rate or the HRT.

Anaerobic upflow bioreactors can be used as a tool in studying unique microorganisms and their activities. Bioreactors are an especially important tool when the microbial activity of interest is the result of interactions between members of microbial communities. When the microbes responsible for a unique activity are difficult to identify in a microbial community and isolate by conventional methods, such as those involved with the dechlorination and degradation of chlorinated phenols, bioreactor studies may be the only feasible method of studying these unique microbial activities.

Anaerobic upflow bioreactors can be an alternative to conventional enrichment culture techniques where periodically portions of the enrichments are transferred into fresh media. The undesirable microbes and metabolic end-products are eliminated and the microbes of interest are selected. Conventional enrichment culture techniques may be lengthy due to slow growth of the organisms after transferring portions of the enrichment. Enrichment of the desirable microbial community can be achieved faster in anaerobic upflow bioreactors for two reasons. First, the majority of the microbial biomass remains in the bioreactor, usually in the form of a sludge blanket or attached to a solid support material. Second, enrichment is continual; substrate is continually added to the bioreactor while end-products are continually removed. An upflow bioreactor also provides a feasible method of enrichment when the substrate of interest is toxic to the microbes, as is the case with chlorophenols, because low concentrations of the substrate can be continually applied to the bioreactor.

Enrichment of a microbial community occurred with the bioreactors fed only chlorophenols as a carbon source. Although a dechlorinating microorganism could not be identified, three other microorganisms dominated the biomass. These were 1) a putative phenol-degrader, 2) a *Methanosarcina sp.* and 3) a *Methanothrix sp.*

APPENDIX

Isolation of Anaerobic Phenol and Benzoate-Oxidizing Bacteria

Introduction

Phenol and phenolic compounds are widely used industrial chemicals. They are also toxic compounds and have been placed on the Environmental Protection Agency list of 129 Priority Pollutants (Keith and Telliard, 1979). The anaerobic biodegradation of phenol and related compounds has been extensively studied with mixed cultures of bacteria in waste treatment processes and other environments (Tarvin and Buswell, 1934; Healy and Young, 1978; Holladay *et al.*, 1978; Balba *et al.*, 1979; Boyd and Shelton, 1984; Fedorak and Hrudey, 1984; Young and Rivera, 1985; Godsy *et al*, 1986). However, there has been only one previous report of anaerobic phenol-oxidizing bacteria isolated in pure culture (Barik *et al*, 1985). The microorganisms, in co-culture with the hydrogen-consumer *Wolinella succinogenes*, were able to anaerobically oxidize phenol and other aromatic compounds.

Anaerobic phenol-oxidizing bacteria are presumed to be obligate syntrophs with hydrogen-consuming microorganisms when grown on phenol, because the free energy change, $\Delta G^{o'}$, for phenol fermentation is negative unless low partial pressures of hydrogen are maintained. Thus, isolation of phenol-oxidizing bacteria on phenol must be carried out in the presence of hydrogen-consuming microorganisms.

In the present study we describe the isolation of two bacteria in co-culture with the hydrogen consumer *Desulfovibrio G11* from phenol-degrading enrichment cultures.

Material and Methods

Methanogenic Phenol Enrichment. A stable methanogenic phenol-degrading enrichment was established from anaerobic sewage sludge with phenol as the sole carbon and energy source. The enrichment was established over a two to three year period by periodically transferring 25-50% of the enrichment into fresh medium (RAMM, as previously described). Approximately 100-200 ug/ml phenol was added to the enrichment every two days.

Sulfidogenic Enrichments. Sulfidogenic enrichments were established from the methanogenic phenol enrichment by transferring 25% of the methanogenic enrichment into media (RAMM) containing 2.5 mM $SO_4^=$ and either 2.5 mM phenol or 10 mM benzoate. The enrichment was also inoculated with 25% of a turbid culture of *Desulfovibrio G11*. The sulfidogenic enrichment was allowed to acclimate for approximately one month prior to use.

Isolation. Serial dilutions of the sulfidogenic enrichments were inoculated into anaerobic roll tubes containing 5.0 ml RAMM, 5% clarified rumen fluid, 2% agar, 2.5 mM $SO_4^=$, and either 2.5 mM phenol or 10 mM benzoate. The sulfidogen, 0.5 ml of a turbid culture, was also added to each tube to insure an adequate lawn of hydrogen consumers. Colonies were picked from the roll tubes after 8-12 weeks by a bent Pasteur pipette and transferred into 10 ml RAMM containing 2.5 mM $SO_4^=$ and either 2.5 mM phenol or 10 mM benzoate. The cultures were maintained by periodic transfers of 5% of the volume into fresh media. Strict anaerobic techniques were employed throughout. Incubations were at 37°C and in the dark.

Characterization. The substrate range for the methanogenic phenol enrichment was determined by transferring 50 ml of active culture into triplicate serum bottles containing 5 mM of either phenol, benzoate, 4-hydroxybenzoate, phenylacetate or p-cresol. Methane production was periodically monitored.

Fermentation balance and time course studies for the two isolates were performed in triplicate by transferring 5% of a turbid co-culture into RAMM containing 2.5 mM $SO_4^{=}$ and either 2.5 mM phenol or 10 mM benzoate. Periodic samples of the aqueous phase were taken and frozen until analysis.

Chemical Analysis. Phenol and benzoate were quantified by using a Waters high pressure liquid chromatography system consisting of a Model 6000A pump and a model 441 *UV* absorbance detector set at 280 nm. The sample injection valve (Rheodyne 7125) was fitted with a 20-ul loop. The analytical column was a Waters Radial PAK C-18 cartridge held in a RCM-100 Radial Compression Module. Peak areas were measured with a Hewlett Packard 3390A integrator. The mobile phase was a 3:7 mixture of acetonitrile: 5% acetic acid to give retention times of approximately three minutes. The mobile phase flow rate was set at 2.0 ml/min. The samples were thawed and filtered through a 0.45 um HA Millipore filter before analysis.

Acetate was quantified by using a PE-9000 gas chromatograph equipped with a flame ionization detector and a 2-m glass column (2 mm ID) packed with Carbopack C-0.3% Carbowax 20 M-0.1% H_3PO_4. The oven temperature was 90°C and the nitrogen carrier gas flow was set at 30 to 40 ml/min. Samples were acidified with formic acid (20% by volume) before injection.

Methane was quantified by injecting 0.2 ml of headspace gas into a Carle gas chromatograph equipped with a porapack Q column and a microthermistor detector. Protein was analyzed according to the procedure of Lowry *et al.*, (1951).

Results

Phenol Enrichment. The phenol enrichment contained three dominant morphological types, tentatively identified by their morphology as: 1) a putative phenol-oxidizing bacterium, 2) a *Methanothrix sp.* and 3) a hydrogen-utilizing methanogen (Figures 29 and 30). The phenol-oxidizing bacterium was a Gram-negative oval shaped rod, 0.8 um by 1.2-3.3 um in length. A thin section electron micrograph of the phenol-oxidizing bacterium is shown in Figure 31.

The methanogenic phenol enrichment was also able to degrade other aromatic substrates including benzoate, 4-hydroxybenzoate, phenylacetate and p-cresol as determined by the production of methane. The substrate p-cresol exhibited a longer lag time before the onset of methane production than the other aromatic substrates tested (Figure 32).

Isolate P1. Isolate P1 was obtained on phenol from the sulfidogenic phenol enrichment. It was morphologically similar to the putative phenol-oxidizer found in the enrichments. P1 in co-culture with *Desulfovibrio G11* degraded phenol to approximately equal molar amounts of acetate (Figure 33). The generation time was

Figure 29. Phase contrast photomicrograph of the methanogenic phenol-degrading enrichment depicting the proposed phenol-oxidizing microorganism (2000X).

Figure 30. Phase contrast photomicrograph of the methanogenic phenol-degrading enrichment (2000X). The microorganisms found in the enrichment include: A) a proposed phenol-oxidizing bacterium, B) a *Methanothrix sp.*, and C) a presumed hydrogen-utilizing methanogen.


A
C
B

Figure 31. **Thin section electron micrograph of the proposed phenol-oxidizing microorganism taken from the methanogenic phenol-degrading enrichment culture (95,000X).**

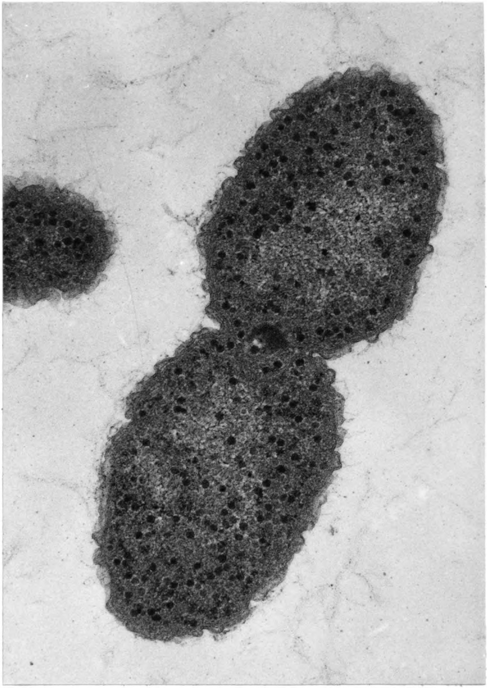


Figure 32. Methane production from various aromatic substrates in the phenol-degrading enrichment culture.

Figure 32. PHENOL ENRICHMENT

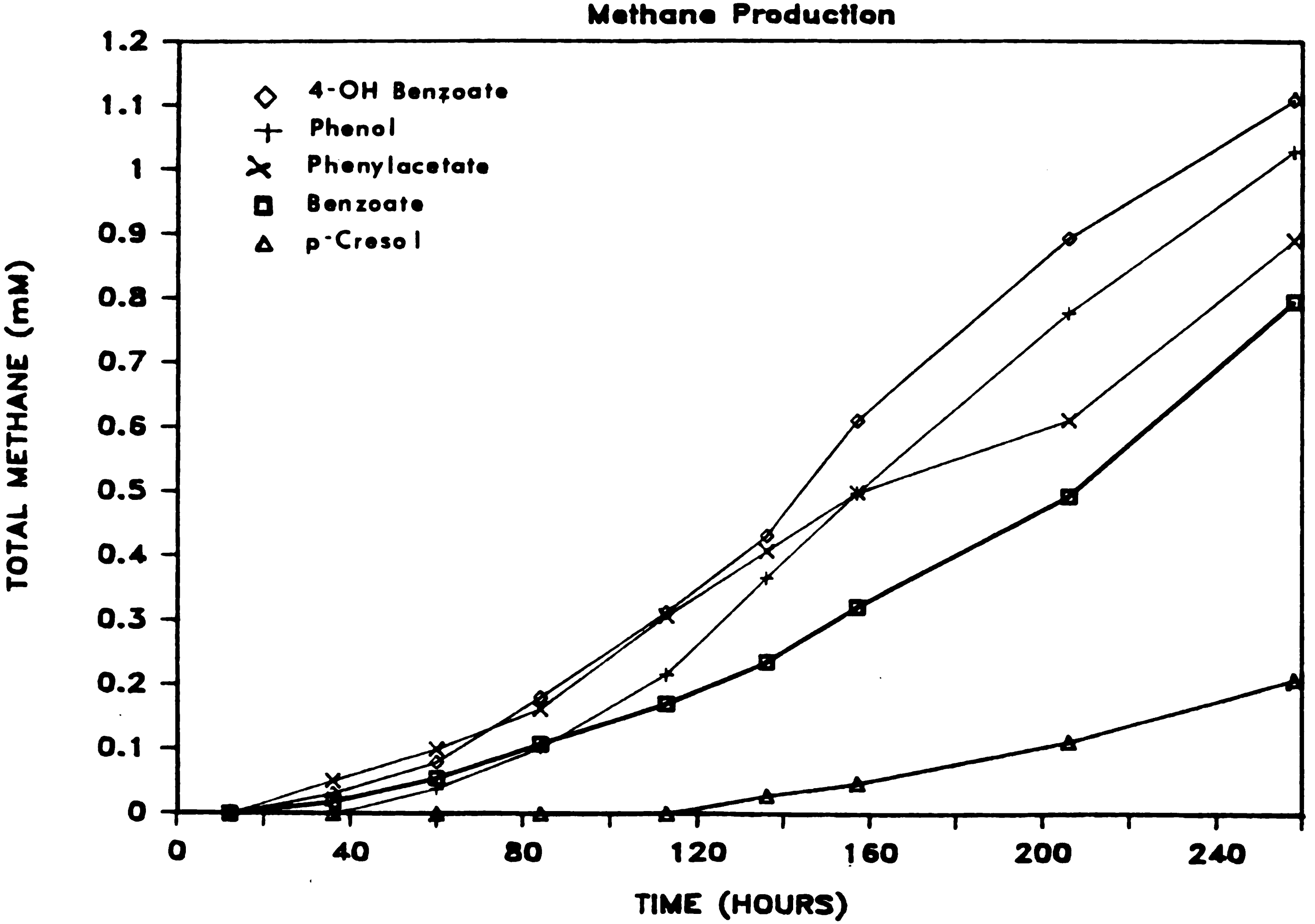
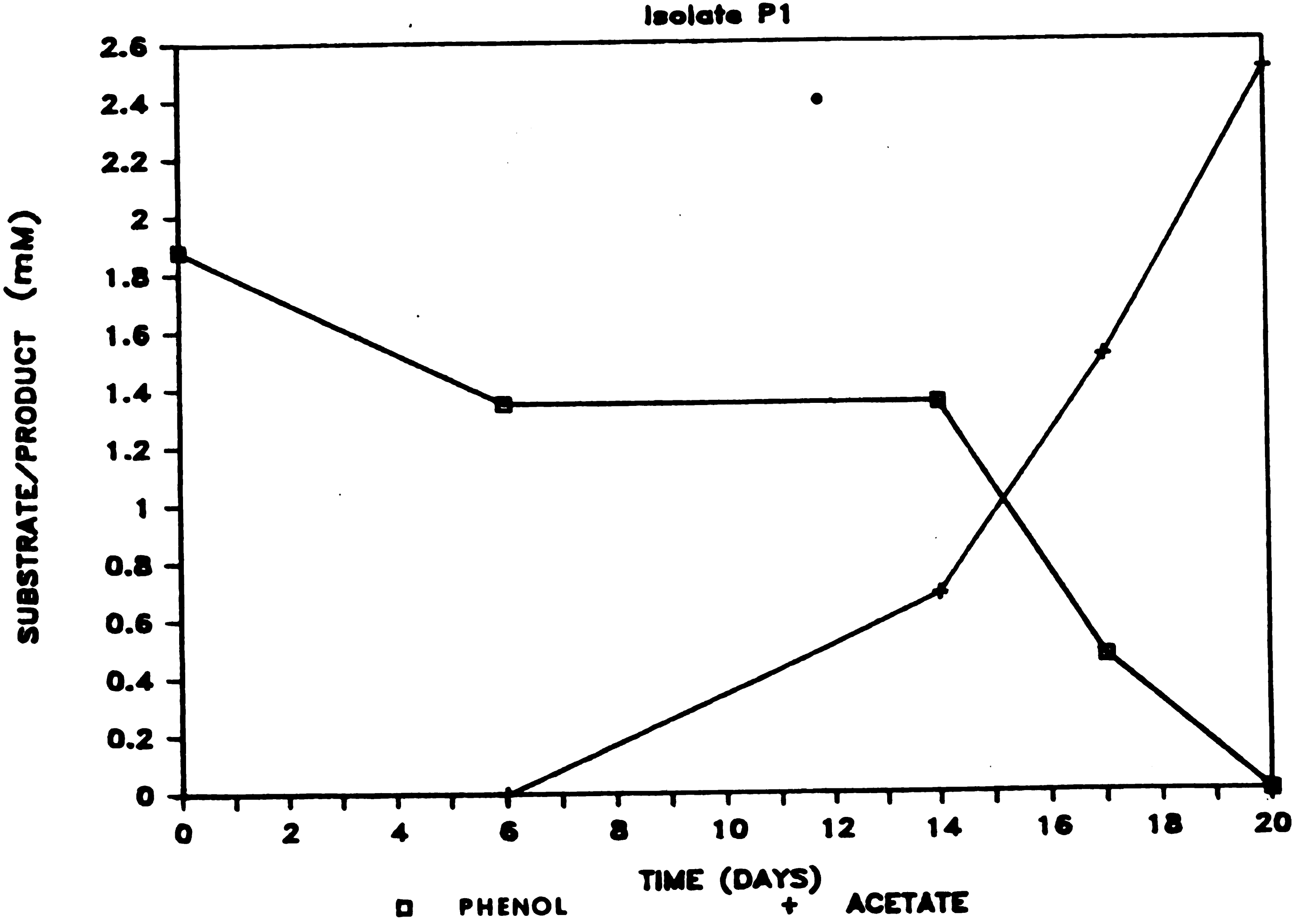


Figure 33. Phenol degradation by isolate P1 in co-culture with *Desulfovibrio G11*.

Figure 33. PHENOL DEGRADATION TIME COURSE



approximately two to three days. Pl was also able to degrade benzoate (data not reported). Other aromatic substrates were not tested.

Isolate B4. Isolate B4 was obtained on benzoate from sulfidogenic benzoate enrichments. The morphology was slightly different from the phenol-oxidizer observed in the methanogenic phenol enrichment and isolate Pl. The isolate was similar in morphology to the anaerobic benzoate-degrader isolated by Mountfort and Bryant (1982). Isolate B4 was 0.5 um by 1-1.5 um in length. Isolate B4 in co-culture with *Desulfovibrio G11* degraded one mole of benzoate to approximately three moles of acetate. Growth was determined by the dissappearance of substrate and the accumulation of both acetate and protein (Figure 34). The estimated generation time was four days. The co-culture was not able to utilize phenol (data not reported). Other aromatic substrates were not tested.

Discussion

There has been only one previous report of the isolation of anaerobic phenol-oxidizing bacteria (Barik *et al.*, 1985). Phenol-oxidizing microorganisms may be difficult to isolate in pure culture for several reasons. First, anaerobic techniques must be employed which are often difficult or cumbersome. Second, isolation of an anaerobic phenol-oxidizer requires co-culture with hydrogen-consuming microbes. The establishment of these two organisms together on a roll tube may be difficult. Third, phenol is a toxic substrate and may inhibit growth of the same organisms that are capable of degrading it.

Figure 34. **Benzoate degradation** by isolate B4 in co-culture with *Desulfovibrio G11*.

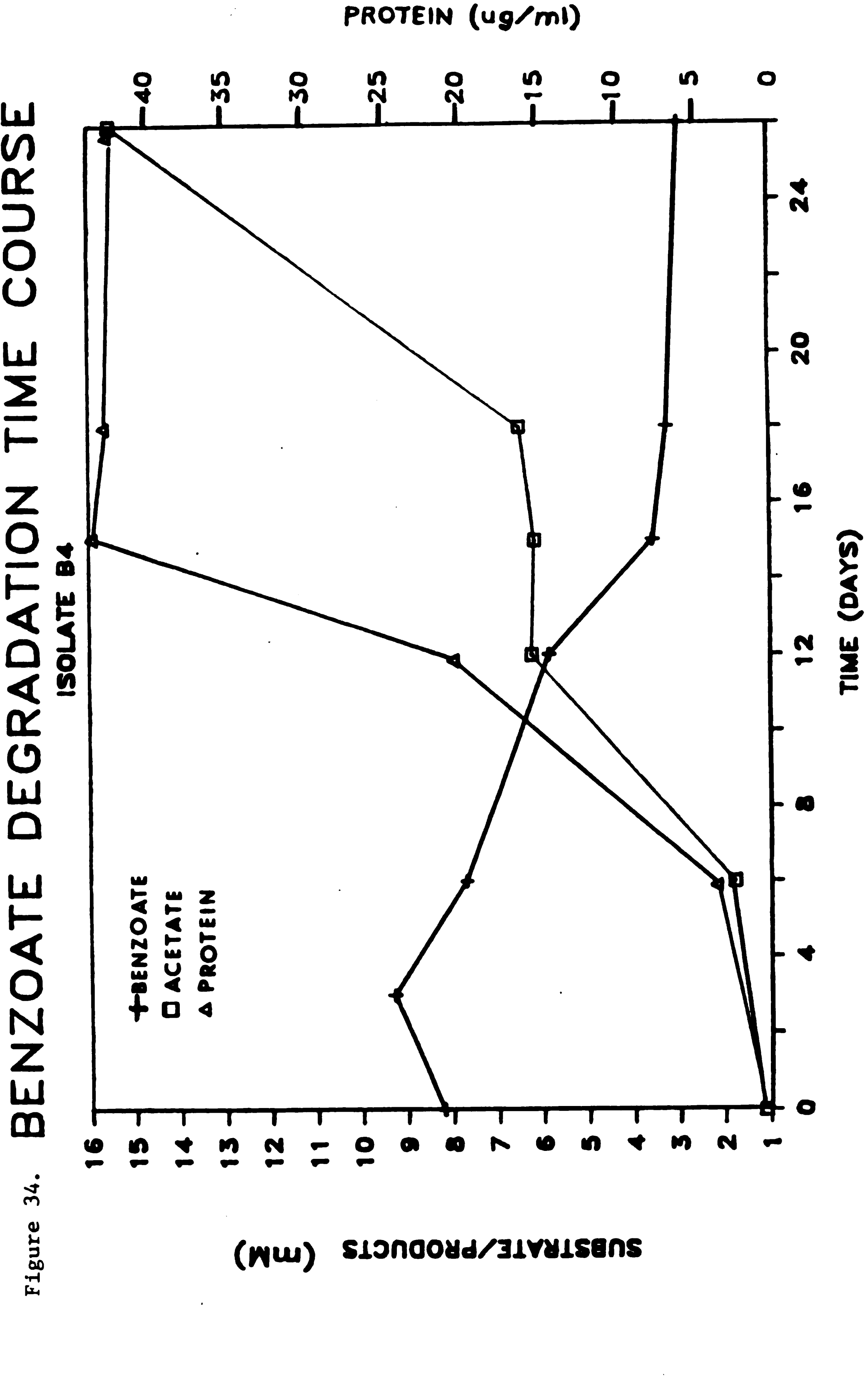

BENZOATE DEGRADATION TIME COURSE
ISOLATE B4
+BENZOATE
□ ACETATE
△ PROTEIN
SUBSTRATE/PRODUCTS (mM)
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
PROTEIN (ug/ml)
40
35
30
25
20
15
10
5
0
TIME (DAYS)
0
4
8
12
16
20
24

Figure 34.

We attempted to alleviate the difficulty of placing the two members of the co-culture in the necessary proximity to each other on the roll tube by first inoculating the roll tube with an established sulfidogenic enrichment and by providing additional pure-culture sulfidogen as part of the inoculum to each roll tube. We utilized two approaches in attempts to overcome the toxicity of the substrate, phenol. First, we attempted to isolate phenol-oxidizing bacterium on phenol at a low substrate concentration of 2.5 mM. Second, we attempted to isolate a phenol-oxidizing bacterium on an alternative substrate such as benzoate. We chose benzoate as the alternative substrate because the original methanogenic phenol enrichment readily utilized benzoate.

We were successful in obtaining a pure culture of a phenol-oxidizing bacterium, isolate P1, by isolating the organism on a low concentration of phenol from sulfidogenic phenol enrichments. The organism also utilizes benzoate as a growth substrate, as predicted. We also isolated an organism on the alternative substrate, benzoate. However, all attempts at either growth of the organism on phenol or degradation of phenol when added to active benzoate-grown cultures failed.

There are several plausible explanations for these results. First, we may have selected for a benzoate-oxidizing bacterium by the establishment of the sulfidogenic benzoate enrichment. Indeed, the morphology of isolate B4 appears to be different than the phenol-oxidizer observed in the methanogenic phenol enrichments or isolate P1. The microbes, however, have similarites and any differences may be due to morphological changes during the growth period. The second explanation for the results may be that through repeated cultivation of isolate B4 on benzoate, a mutation occurred or there was a loss of a plasmid which encoded for phenol-oxidative enzymes. At this point, there is insufficient data to verify any of the explanations presented.

Isolates P1 and B4 have only been isolated once on roll tubes. To insure purity, these microorganisms should be further purified by a second and possibly third isolation on roll tubes. Further, purity of both cultures should be checked by inoculation into a rich, complex media.

LIST OF REFERENCES

LIST OF REFERENCES

Ahlborg, U.G., and T.M. Thunberg. 1980. Chlorinated phenols: occurrence, toxicity, metabolism, and environmental impact. CRC Critical Reviews in Toxicology 7:1-34.

Arsenault, R.D. 1976. Pentachlorophenol and contained chlorinated dibenzodioxins in the environment. Proc. Am. Wood Preserv. Assoc. 72:122-148.

Attaway, H.H., N.D. Camper, and M.J.B. Paynter. 1982. Anaerobic microbial degradation of diuron by pond sediment. Pest. Biochem. Physiol. 17:96-101.

Attaway, H.H. III, M.J.B. Paynter and, N.D. Camper. 1982a. Degradation of selected phenylurea herbicides by anaerobic pond sediment. J. Environ. Sci. Health B17(6):683-699.

Bakker, G. 1977. Anaerobic degradation of aromatic compounds in the presence of nitrate. FEMS Lett. 1:103-108.

Balba, M.T., N.A. Clarke, and W.C. Evans. 1979. The methanogenic fermentation of plant phenolics. Biochem. Soc. Trans. 7:1115-1116.

Balba, M.T., and W.C. Evans. 1977. The methanogenic fermentation of aromatic substrates. Biochem. Soc. Trans. 5:302-304.

Barik, S., W.J. Brulla, and M.P. Bryant. 1985. PA-1, a versatile anaerobe obtained in pure culture, catabolizes benzenoids and other compounds in syntrophy with hydrogenotrophs, and P-2 plus *Wolinella sp*. degrades benzenoids. Appl. Environ. Microbiol. 50:304-310.

Barker, P.S., F.P. Morrison, and R.S. Witaker. 1965. Conversion of DDT to DDD by *Proteus vulgaris*, a bacterium isolated from the intestinal flora of a mouse. Nature 205:621-622.

Binot, R.A., T. Bol, H.-P. Naveau, and E.-J. Nyns. 1983. Biomethanation by immobilized fluidised cells. Water Sci. Tech. 15:103-115.

Bouwer, E.J., B.E. Rittmann, and P.L. McCarty. 1981. Anaerobic degradation of halogenated 1- and 2-carbon organic compounds. Environ. Sci. Technol. 15(5):596-598.

Bouwer, E.J., and P.L. McCarty. 1983. Transformation of 1- and 2-carbon halogenated aliphatic organic compounds under methanogenic conditions. Appl. Environ. Micobiol. 45:1286-1294.

Boyd, S.A., D.R. Shelton, D. Berry, and J.M. Tiedje. 1983. Anaerobic biodegradation of phenolic compounds in digested sludge. Appl. Environ. Microbiol. 46:50-54.

Boyd, S.A., and D.R. Shelton. 1984. Anaerobic biodegradation of chlorophenols in fresh and acclimated sludge. Appl. Environ. Microbiol. 47:272-277.

Crosby, D.G. 1981. Environmental chemistry of pentachlorophenol. Pure Appl. Chem. 53:1052-1080.

Dorn, E., and H.-J. Knackmuss. 1978. Chemical structure and biodegradability of halogenated aromatic compounds. Two catechol 1,2-dioxygenases from a 3-chlorobenzoate-grown *Pseudomonad*. Biochem. J. 174:73-84.

Dorn, E., and H.-J. Knackmuss. 1978a. Chemical structure and biodegradability of halogenated aromatic compounds. Substituent effects on 1,2 dioxygenation of catechol. Biochem. J. 174:85-94.

Edgerton, T.R., R.F. Moseman, and L.H. Wright. 1981. Method development for the assessment of possible human exposure to pesticides and industrial chemicals. U.S. EPA-600/S1-81-024. Wash. D.C.

Environmental Protection Agency, (U.S.). 1980. Ambient water quality criteria for chlorinated phenols. EPA 440/5-80-032. Wash. D.C.

Evans, W.C. 1977. Biochemistry of the bacterial catabolism of aromatic compounds in anaerobic environments. Nature 270:17-22.

Fedorak, P.M., and S.E. Hrudey. 1984. Batch anaerobic methanogenesis of phenolic coal conversion wastewater. Water Sci. Technol. 17:143-154.

Ghosal, D., I.-S. You, D.K. Chatterjee, and A.M. Chakrabarty. 1985. Microbial degradation of halogenated compounds. Science 228:135-142.

Godsy, E.M., D.F. Goerlitz, and G.G. Ehrlich. 1986. Effects of pentachlorophenol on methanogenic fermentation of phenol. Bull. Environ. Contam. Toxicol. 36:271-277.

Guenzi, W.D., and W.E. Beard. 1967. Anaerobic biodegradation of DDT to DDD. Science 156:1116-1117.

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

Hakulinen, and M. Salkinoja-Salonen. 1982. Treatment of pulp and paper industry wastewaters in an anaerobic fluidised bed reactor. Process Biochem. 17(2): 18-22.

Hakulinen, R., S. Woods, J. Ferguson, and M. Benjamin. 1985. The role of facultative anaerobic microorganisms in the anaerobic biodegradation of chlorophenols. Water Sci. Tech. 17:289-301.

Healy, J.B. Jr., and L. Y. Young. 1978. Catechol and phenol degradation by a methanogenic population of bacteria. Appl. Environ. Microbiol. 35:216-218.

Holladay, D.W., C.W. Hancher, C.D. Scott, and D.D. Chilcote. 1978. Biodegradation of phenolic waste liquors in stirred-tank, packed-bed, and fluidized-bed reactors. J. Water Pollut. Cont. Fed. 50:2573-2589.

Horowitz, A., J.M. Suflita, and J.M. Tiedje. 1983. Reductive dehalogenations of halobenzoates by anaerobic lake sediment microorganisms. Appl. Environ. Microbiol. 45:1459-1465.

Ide, A., Y. Niki, F. Sakamoto, I. Watanabe, and H. Watanabe. 1972. Decomposition of pentachlorophenol in paddy soil. Agr. Biol. Chem. 36(11):1937-1944.

Jagnow, G., K. Haider, and P.-Chr. Ellwardt. 1977. Anaerobic dechlorination and degradation of hexachlorocyclohexane isomers by anaerobic and facultative anaerobic bacteria. Arch. Microbiol. 115:285-292.

Jewell, W.J. 1982. Anaerobic attached film expanded bed fundamentals, p.17-42. *In* Proceedings of the First International Conference in Fixed Film Biological Processes. University of Pittsburgh, Pittsburgh, PA.

Keith, L.H., and W.A. Telliard. 1979. Priority pollutants I: a perspective view. Environ. Sci. Technol. 13(4):416-423.

Kirkpatrick, D., S.R. Biggs, B. Conway, C.M. Finn, D.R. Hawkins, T. Honda, M. Ishida, and G.P. Powell. 1981. Metabolism of N-(2,3-dichlorophenyl)-3,4,5,6-tetrachlorophthalamic acid (Techlofthalam) in paddy soil and rice. J. Agric. Food Chem. 29:1149-1153.

Klass, D.L. 1984. Methane from anaerobic fermentation. Science 223:1021-1028.

Kleopfer, R.D., D.M. Easley, B.B. Haas Jr., T.G. Deihl, D.E. Jackson, and C.J. Wurey. 1985. Anaerobic degradation of trichloroethylene in soil. Environ. Sci. Technol. 19:277-280.

Knackmuss, H.-J. 1981. Degradation of halogenated and sulfonated hydrocarbons, p. 189-212. *In* T. Leisinger, R. Hutter, A.M. Cook and J. Nuesch (eds.), Microbial Degradation of Xenobiotics and Recalcitrant Compounds. Academic Press, London.

Kobayashi, H., and B.E. Rittmann. 1982. Microbial removal of hazardous organic compounds. Environ. Sci. Technol. 16(3):170A-181A.

Krumme, M.L., and S.A. Boyd. 1986. Isolation of an anaerobic, obligately syntrophic phenol-oxidizing bacterium. Abstr. ASM Ann. Mtg. p.286.

Kuwatsuka, S., and M. Igarashi. 1975. Degradation of pentachlorophenol in soils. II. The relationship between the degradation of PCP and the properties of soils, and the identification of the degradation products of PCP. Soil Sci. Plant Nutr. 21(4):405-414.

Lindberg, S., and L.-B. Lund. 1980. A "non-polluting" bleach plant. Tappi 63(3):65-67.

Linkfield, T. G. 1986. Anaerobic reductive dehalogenation: The lag phase preceding haloaromatic dehalogenation, enrichment of sediment activity, and the partial characterization of a dehalogenating organism, strain DCB-1. Ph.D. Thesis, Michigan State University.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the folin reagent. J. Biol. Chem. 193:265-275.

McRae, I.C., K. Raghu, and T.F. Castro. 1967. Persistence and biodegradation of four common isomers of benzene hexachloride in submerged soils. J. Agr. Food Chem. 15:911-914.

McRae, I.C., K. Raghu, and E.M. Bautista. 1969. Anaerobic degradation of the insecticide lindane by *Clostridium sp.* Nature 221:859-860.

Mikesell, M.D., and S.A. Boyd. 1985. Reductive dechlorination of the pesticides 2,4-D, 2,4,5-T, and pentachlorophenol in anaerobic sludges. J. Environ. Qual. 14(3):337-340.

Mikesell, M.D., and S.A. Boyd. 1986. Complete reductive dechlorination and mineralization of pentachlorophenol by anaerobic microorganisms. *Submitted to* Appl. Environ. Microbiol.

Miskus, R.P., D.P. Blair, and J.E. Casida. 1965. Conversion of DDT to DDD by bovine rumen fluid, lake water, and reduced porphyrins. J. Agr. Food Chem. 13(5):481-483.

Moon, Y.H., and S. Kuwatsuka. 1984. Properties and conditions of soils causing the dechlorination of the herbicide benthiocarb (thiobencarb) in flooded soils. J. Pest. Sci. 9:745-754.

Mountfort, D.O., and M.P. Bryant. 1982. Isolation and characterization of an anaerobic syntrophic benzoate-degrading bacterium from sewage sludge. Arch. Microbiol. 133:249-256.

Murthy, N.B.K., D.D. Kaufman, and G.F. Fries. 1978. Degradation of pentachlorophenol in aerobic and anaerobic soil. J. Environ. Sci. Health. B14(1):1-14.

Olthof, M., and J. Oleszkiewicz. 1982. Anerobic treatment of industrial wastewaters. Chem. Eng. 89(23):121-126.

Paasivirta, J., K. Heinola, T. Humppi, A. Karjalainen, J. Knuutinen, K. Mantykoski, R. Paukku, T. Piilola, K. Surma-Aho, J. Tarhanen, L. Welling, and H. Vihonen, J. Sarkka. 1985. Polychlorinated phenols, guaiacols and catechols in environment. Chemosphere 14(5):469-491.

Robinson, J.A., R.F. Strayer, and J.M. Tiedje. 1981. Method for measuring dissolved hydrogen in anaerobic ecosystems: application to the rumen. Appl. Environ. Microbiol. 41:545-548.

Salkinoja-Salonen, M., M.-L. Saxelin, J. Pere, T. Jaakkola, J. Saarikoski. R. Hakulinen, and O. Koistinen. 1981. Analysis and biodegradability of organochlorine compounds released into the environment in bleaching effluents of kraft pulping, p. 1131-1164. *In* L.H. Keith (ed.), Advances in the Identification and Analysis of Organic Pollutants in Water Vol. 2. Ann Arbor Science Publishers, Ann Arbor, MI.

Salkinoja-Salonen, M.S., R. Hakulinen, R. Valo, J. Apajalahti. 1983. Biodegradation of recalcitrant organochlorine compounds in fixed film reactors. Water Sci. Tech. 15:309-319.

Salkinoja-Salonen, M., R. Valo, J. Apajalahti, R. Hakulinen, L. Silakoski, and T. Jaakkola. 1984. Biodegradation of chlorophenolic compounds in wastes from wood processing industry. 1984. p. 668-676. *In* M.J. Klug and C.A. Reddy (eds.), Current Perspective in Microbial Ecology. American Society for Microbiology, Washington D.C.

Shelton, D.R., and J. M. Tiedje. 1984. Isolation and partial characterization of bacteria in an anaerobic consortium that mineralizes 3-chlorobenzoic acid. Appl. Environ. Microbiol. 48:840-848.

Speece, R.E. 1983. Anaerobic biotechnology for industrial wastewater treatment. Envir. Sci. Technol. 17(9):416A-427A.

Stepp, T.D., N.D. Camper, and M.J.B. Paynter. 1985. Anaerobic microbial degradation of selected 3,4-dihalogenated aromatic compounds. Pesticide Biochem. and Physiol. 23:256-260.

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 218:1115-1117.

Sulflita, J.M., J. Stout, and J.M. Tiedje. 1984. Dechlorination of (2,4,5-trichlorophenoxy) acetic acid by anaerobic microorganisms. J. Agric. Food Chem. 32:218-221.

Switzenbaum, M.S. and W.J. Jewell. 1980. Anaerobic attached film expanded bed reactor treatment. J. Water Pollut. Cont. Fed. 52(7):1953-1964.

Tabak, H.H., C.W. Chambers, and P.W. Kabler. 1964. Microbial metabolism of aromatic compounds. I. Decomposition of phenolic compounds and aromatic hydrocarbons by phenol-adapted bacteria. J. Bacteriol. 87:910-919.

Tarvin, D., and A.M. Buswell. 1934. The methane fermentation of organic acids and carbohydrates. J. Am. Chem. Soc. 56:1751-1755.

Thomas, L.M. 1984. EPA fights hazardous waste. EPA J. 10(8):4-7.

Tiedje, J.M., S.A. Boyd, and B.Z. Fathepure. 1986. Anaerobic degradation of aromatic hydrocarbons. *In press*. Developments in Industrial Microbiol., Vol. 27.

Valo, R., V. Kitunen, M. Salkinoja-Salonen and, S. Raisanen. 1984. Chlorinated phenols as contaminants of soil and water in the vicinity of two Finnish sawmills. Chemosphere 13(8):835-844.

Valo, R., V. Kitunen, M. 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.

Van den Berg, L. 1984. Developments in methanogenesis from industrial wastewater. Can. J. Microbiol. 30:975-990.

Vogel, T.M., and P.L. McCarty. 1985. Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. Appl. Environ. Microbiol. 49:1080-1083.

Wedemeyer, G. 1967. Dechlorination of 1,1,1,-trichloro-2,2-bis (p-chlorophenyl) ethane by *Aerobacter aerogenes*. Appl. Microbiol. 15:569-574.

Weinbach, E.C., J. Garbus, and E.C. Clagett. 1965. The interaction of uncoupling phenols with mitochondria and with mitochondrial protein. J. Biol. Chem. 240:1811-1819.

Woods, S.L. 1985. The fate of chlorinated, hydroxylated and methoxylated benzenes in anaerobic wastewater treatment. Ph.D. Thesis. University of Washington.

Xie, T.-M., K. Abrahamsson, E. Fogelquist, and B. Josefsson. 1986. Distribution of chlorophenolics in a marine environment. Environ. Sci. Technol. 20(5):457-463.

Yamada, T., and T. Suzuki. 1983. Occurrence of reductive dechlorination products in the paddy field soil treated with CNP (Chlornitrofen). J. Pest. Sci. 8(4):437-443.

Young, L.Y., and M.D. Rivera. 1985. Methanogenic degradation of four phenolic compounds. Water Res. 19(10):1325-1332.

Zoro, J.A., J.M. Hunter, and G.C. Ware. 1974. Degradation of p,p'-DDT in reducing environments. Nature 247:235-237.