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Reductive dehalogenation of chlorinated aromatic and aliphatic hydrocarbons under anaerobic conditions

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ABSTRACT

REDUCTIVE DEHALOGENATION OF CHLORINATED AROMATIC AND ALIPHATIC HYDROCARBONS UNDER ANAEROBIC CONDITIONS

by
Monica Turner Togna

The process of reductive dehalogenation involves the removal of a halogen substituent from a molecule with the concurrent addition of electrons to the molecule, resulting in a more reduced and often less toxic product. Anaerobic bacteria have the potential to utilize hazardous chlorinated aromatic and aliphatic hydrocarbons as electron acceptors in metabolic reductive dehalogenation processes.

In experiments performed with chlorinated aromatic compounds a highly enriched anaerobic culture stoichiometrically converted 2,4,6-trichlorophenol (2,4,6-TCP) to 4-monochlorophenol. Dehalogenation occurred only in alkaline media (pH 8-9) at concentrations of 2,4,6-TCP up to 1 mM. Data indicated that the dehalogenating organism did not fit into any of the typical metabolic classifications of anaerobic bacteria: methanogenic, sulfidogenic, nitrate-reducing, metal-reducing, or fermentative. Data suggested that dehalogenation was linked to growth and proceeded as a respiratory process. The organism was capable of utilizing a number of supplementary chlorinated compounds as electron acceptors, in addition to the 2,4,6-trichlorophenol.

Experiments performed with chlorinated aliphatic compounds involved soil microcosms from a perchloroethylene (PCE) contaminated site. The approach was to provide slowly fermentable compounds, which are not widely used by bacteria, as a

source of low potential electrons. The data obtained show that N-Z-Soy Peptone, xanthan gum, polyethylene glycol-60, Tween-80, xanthine, crude DNA, and a volatile fatty acid mix were all able to support dehalogenation as far as *cis*-dichloroethylene. Additionally, the data show that xanthan gum was able to carry the dehalogenation process past dichloroethylene to vinyl chloride, with no perchloroethylene or trichloroethylene remaining. The active population was able to dehalogenate up to 250 μM PCE (about 40 ppm). Inhibitor experiments performed with molybdate and bromo-ethane sulfonic acid suggested that part of the active population consisted of sulfidogenic bacteria, while methanogens did not play a significant role in the dehalogenation activity.

Taken together the results of these studies investigating the reductive dehalogenation of chlorinated aromatic and aliphatic compounds under anaerobic conditions demonstrate that bacteria which play significant roles in the dehalogenation processes come from diverse metabolic backgrounds which include fermentative, sulfidogenic, and actual chlorinated-compound-respiring organisms.

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by
Monica Turner Togna

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This thesis is dedicated to
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CHAPTER ONE

INTRODUCTION

The process of reductive dehalogenation involves the removal of a halogen substituent from a molecule with the concurrent addition of electrons to the molecule, resulting in a more reduced and often less toxic product. Anaerobic bacteria have the potential to utilize chlorinated aromatic and aliphatic hydrocarbons as electron acceptors in metabolic reductive dehalogenation processes. In this manner anaerobic bacteria can be employed in the dehalogenation and detoxification of chlorinated compounds classified as hazardous waste.

The dechlorination of polychlorinated aromatic compounds under anaerobic conditions has been demonstrated in enrichment cultures (Gibson and Suflita, 1986; Mikesell and Boyd, 1986; Madsen and Aamand, 1992; Holliger et al., 1992; Nicholson et al., 1992) and with only four pure cultures (Shelton and Tiedje, 1984; Madsen and Licht, 1992; Utkin et al., 1994; and Cole et al., 1994).

The most extensively studied pure culture, *Desulfomonile tiedjei*, is an oxygen sensitive sulfidogen isolated from a methanogenic enrichment with 3-chlorobenzoate as its target. *Desulfomonile tiedjei* requires naphthoquinone for growth and utilizes pyruvate, formate or hydrogen as electron donors for the dehalogenation of a variety of aromatic chlorine-containing compounds (Stevens *et al.* 1988). The second organism, DCB-2 (renamed *Desulfito hafneise*), is an oxygen tolerant Gram-positive spore forming organism (Madsen and Licht, 1992; Christiansen and Ahring, 1996). DCB-2 grows in salts

medium supplemented with yeast extract and pyruvate, and dehalogenates a variety of chlorophenols only under anaerobic conditions. The third organism, *Desulfitobacterium dehalogenans*, is a motile, Gram-positive, rod-shaped bacterium which is capable of dechlorinating 2,4-dichlorophenol and 3-chloro-4-hydroxyphenylacetate (Utkin et al., 1994). It is capable of dechlorination under a nitrogen atmosphere which contains up to 2% air, indicating that it is resistant to microaerophilic conditions. It has not been shown whether or not the organism can utilize the O_2 as an electron acceptor. The last isolate is a Gram-negative facultative organism, closely related to the myxobacteria. It is capable of *ortho-* dehalogenation of monochlorophenol, but dehalogenation is decreased or blocked by additional chlorines at other positions (Cole et al., 1994). The chlorinated aromatic portion of this investigation focused on the characterization and optimization of an anaerobic enrichment culture derived from a two-step sequential anaerobic-aerobic reactor system which stoichiometrically converted 2,4,6-trichlorophenol to 4-monochlorophenol.

In addition to chlorinated aromatic compounds, chlorinated aliphatic compounds are industrially important chemicals which are widely distributed in the environment. Perchloroethylene (PCE) is a toxic contaminant originally introduced into the environment through widespread industrial use as an extractant, solvent, and dry-cleaning chemical. It is one of the most frequently found compounds at Superfund sites and in contaminated groundwater, and it is classified as a listed volatile organic contaminant under the Safe Drinking Water Act amendments of 1986. Its carcinogenicity, toxicity, and persistence in the environment make it a target of concern for researchers.

PCE has been found to be attacked under anaerobic conditions through the process of reductive dehalogenation. Research has shown that dehalogenation of PCE can result in the accumulation of different end-products when anaerobic organisms are grown under various condition. This reductive dehalogenation process can result in the accumulation of various chlorinated products and mixtures including trichloroethylene (Fathepure and Boyd, 1988; Gibson and Sewell, 1992), dichloroethylene (Bagley and Gossett, 1990; Gibson et al., 1994), vinyl chloride (Vogel and McCarty, 1985; DiStefano et al., 1992), ethylene (DiStefano et al., 1991 and 1992; Freedman and Gossett, 1989; Holliger et al., 1993; Wild et al., 1995), and ethane (de Bruin et al., 1992). These studies indicate that in addition to the methanogenic cometabolic degradation of PCE, anaerobic bacteria can use reductive dehalogenation of PCE as an energy-producing respiratory process. This respiratory ability, coupled to growth, has been proven in pure culture with *Dehalobacter restrictus, Dehalospirillum multivorans,* and *Desulfitobacterium* sp. strain PCE1 (Holliger et al., 1993; Schumacher and Holliger, 1996; Neumann et al., 1994; Scholz-Muramatsu et al., 1995; Gerritse et al., 1996). This capability could give indigenous bacteria a competitive edge for the *in-situ* treatment of PCE contaminated environments; contingent on the existence of the proper nutritional conditions which would allow the process to take place.

The step-wise reductive dehalogenation of PCE to ethylene (via TCE, DCE, and VC) can occur if a source of low potential electrons is provided. The approach of this study was to provide slowly fermentable compounds, which are not widely utilized by bacteria, as a source of these low potential electrons. The slowly fermentable compounds and their products were chosen based on the redox potential of the expected oxidations within

fermentative pathways. The compounds are expected to release a steady stream of low potential electrons which will allow the reductive dehalogenation of PCE to proceed, ultimately resulting in a more reduced and less toxic product. This investigation included microcosm studies on thirteen of these slowly fermentable compounds to study the effects of these electron donors on PCE dehalogenation rate and the extent of dehalogenation.

The specific objectives of this investigation was to increase the rate of *in situ* dehalogenation of perchloroethylene (PCE), trichloroethylene (TCE), and dichloroethylene (DCE), while avoiding the accumulation of highly toxic vinyl chloride (VC) as an intermediate product. In order to meet these objectives, the experimental set-up of this investigation was designed to manipulate the metabolic pathways of fermentative bacteria, which typically compromise the dominant populations in anaerobic environments.

The present investigation was undertaken to learn more about the microbiology of reductive dehalogenation of chlorinated aromatic and aliphatic compounds of industrial interest, in order to better understand and optimize biological treatment processes.

CHAPTER TWO

LITERATURE REVIEW: METABOLIC TYPES OF BACTERIA WHICH PERFORM ANAEROBIC REDUCTIVE DEHALOGENATION

2.1 Reductive Dehalogenation

When examining anaerobic bacteria and their means of energy production most research has centered on methanogens, nitrate-reducing bacteria, sulfate-reducing bacteria, and some metal-reducing bacteria. In the past ten years another process of energy production has been proposed and is slowing being studied and elucidated. This process is reductive dehalogenation. Reductive dehalogenation involves the removal of a halogen substituent of a molecule with the simultaneous addition of electrons to the molecule. There are two mechanisms by which this can be accomplished: hydrogenolysis and vicinal reduction (Mohn and Tiedje, 1992).

In hydrogenolysis the halogen substituent is replaced with a hydrogen atom. This method can be used to reduce alkyl or aryl halides. In vicinal reduction (dihaloelimination) two halogen substituents are removed from adjacent carbons and an additional bond is formed between the carbons. This process can be used to reduce only alkyl halides. Both methods require an electron donor (reductant) and an electron acceptor.

A wide variety of organisms of various metabolic types have demonstrated the ability to perform reductive dehalogenation under anaerobic conditions. Understanding of the details of this mechanism is still in a very early stage of development. Much of the knowledge presently available in the literature has been from research on one organism: *Desulfomonile tiedjei.*

2.2 *Desulfomonile tiedjei*, a Unique Dehalogenating Organism

Desulfomonile tiedjei is an obligately anaerobic bacterium which has the capability to reductively dehalogenate 3-chlorobenzoate through hydrogenolysis. It is described as a slow growing, non-spore forming, Gram negative rod. In addition it has an unusual collar-like structure (Shelton and Tiedje, 1984).

As *D. tiedjei* has been formally characterized as a sulfidogenic bacterium, its sulfate-reducing and reductive dehalogenating respiratory capabilities will be discussed and compared.

2.2.1 *Desulfomonile tiedjei* as a Sulfidogen

In dissimilatory sulfate reduction, sulfate serves as terminal electron acceptor for anaerobic oxidation of hydrogen and organic compounds. Bacteria which are capable of sulfate reduction have been found to be nutritionally, physiologically, and morphologically diverse. This is the case for one of the most recently identified sulfidogenic bacteria, originally referred to as DCB-1 for dechlorinating bacterium one (since re-named as *Desulfomonile tiedjei*).

2.2.1.1 Electron Acceptors: When attempting to classify this organism Stevens et al. (1988) tested a variety of electron acceptors to determine substrate range and improve growth potential. In these experiments thiosulfate showed improved growth. Sulfite showed improved but slow growth (0.5mM) or a long lag period plus slow growth (10mM). Low concentrations of sulfate (2mM) also showed slightly improved growth after a long lag period. These findings suggested that DCB-1 was a sulfidogenic bacterium.

2.2.1.2 Electron Donors: As is characteristic of many sulfidogens *D. tiedjei* shows a limited substrate range. With sulfate as an electron acceptor the following support growth: carbon monoxide, lactate, pyruvate, butyrate, and 3-methoxybenzoate (Mohn and Tiedje, 1990a).

2.2.1.3 Thiosulfate Disproportionation: It was found that when formate or other electron donors were omitted from media, *D. tiedjei* could grow by disproportionating thiosulfate to sulfide and small amounts of sulfate. Apparent growth rates on thiosulfate were similar with and without formate (Mohn and Tiedje, 1990a).

2.2.1.4 Fermentative Growth: The ability of *D. tiedjei* to grow fermentatively was tested in buffered media with 10 mM pyruvate. It was found that growth only occurred when an electron donor was added to the media. CO_2 was found to be suitable to fill this role. Results indicated that pyruvate oxidation was balanced by CO_2 reduction to acetate and cell material (Mohn and Tiedje, 1990a).

2.2.1.5 Spectral Evidence: A further link of *D. tiedjei* to sulfidogenic bacteria was the spectral evidence indicating a *c*-type cytochrome and sulfite reductase (desulfoviridin) (Stevens et al., 1988). The cytochrome spectra of *D. tiedjei* matched that of *Desulfovibrio* strain DG-2 with absorption maxima at 420, 521, and 553nm characteristic of c-type cytochromes. The absolute spectra also showed a peak at 630nm. This corresponds to desulfoviridin (sulfite reductase) which is used as a marker for sulfidogen identification.

2.2.2 *Desulfomonile tiedjei* As A Dechlorinating Organism

2.2.2.1 Electron Donors: Hydrogen and Formate: Sulfate-reducing bacteria are able to use a variety of chemical compounds as electron donors: lactate, hydrogen or formate, acetate, propionate, butyrate and higher straight chain fatty acids, branched chain fatty acids, monovalent alcohols, dicarboxylic acids, and aromatic compounds (Widdel, 1988).

It has been proposed that *Desulfomonile tiedjei* couples formate oxidation and H_2 oxidation to dechlorination of 3-chlorobenzoate (Mohn and Tiedje, 1990b). To prove this hypothesis, Mohn and Tiedje performed experiments to monitor the consumption of formate and the consequent production of chloride ions. Experiments were set up with

and without the addition of *Propionibacterium* culture fluid (which replaced rumen fluid as the source of an undefined nutrient, later found to be 1,4-naphthoquinone). Their data show that when 3-chlorobenzoate is present in the media more formate is consumed, more CO_2 is produced, and the 3-CB is stoichiometrically converted to benzoate (Mohn and Tiedje, 1990b). These data support the hypothesis that formate oxidation is coupled to 3-chlorobenzoate reduction.

Dehalogenation activity on other compounds (*ortho* and *para* Br, I, and Cl substituted) has been found to be inducible by meta-substituted compounds (Mohn and Tiedje, 1992).

2.2.2.2 Electron Transport Chains: Sulfidogenic bacteria utilize sulfate, sulfite, thiosulfate, tetrathionate, or elemental sulfur as ultimate electron acceptors in energy metabolism. The reduction of these compounds is mediated by an electron transport system involving dehydrogenases, reductases, and a series of electron carriers.

Sulfate bacteria use different combinations of these compounds in order to channel electrons through their membranes and to their ultimate electron acceptors.

DeWeerd et al. (1991) proposed that *D. tiedjei* uses reductive dehalogenation as a novel type of anaerobic respiration. They showed that sulfite reduction and dehalogenation were inhibited by the same respiratory inhibitors suggesting that the two processes may share part of the same electron transport chain. Metronidazole, a ferredoxin inhibitor, completely inhibited hydrogen metabolism and dehalogenation. Because of this they proposed that ferredoxin is part of the electron transport chain between hydrogenase or formate dehydrogenase and 3-chlorobenzoate. Hydrogen uptake

was also inhibited by selenate and molybdate in the presence of sulfate or 3-CB. These are normally used to block sulfate uptake in cells, suggesting that 3-CB is transported to the cells similarly to sulfate.

Very little is known about the electron transport chain of *D. tiedjei*. As stated before *D. tiedjei* is known to contain c-type cytochromes (c3) and desulfoviridin. It has also been reported that it has a requirement for 1,4-naphthoquinone (this was found to be the undefined nutrient in rumen fluid and *Propionibacterium* culture fluid) (Mohn and Tiedje, 1992). No purpose or activity has been reported for this compound, but based on its structure it is possible that it is being used as an electron carrier, similar to menaquinone in respiratory energy conservation.

2.2.2.3 Formate and Hydrogen Oxidation Linked to ATP Production: While researching the energy metabolism of *D. tiedjei*. Dolfing measured growth yield (as protein produced) and its relationship to 3-CB dehalogenation (Dolfing, 1990; Dolfing and Tiedje, 1987). He was able to demonstrate that growth yield was linked to the amount of 3-CB dechlorinated. Dolfing proposed that this was because dechlorination provides energy for growth by coupling dehalogenation to ATP production. To demonstrate this he set up experiments where 3-CB was added to energy starved cells and monitored their activity and ATP production. The addition of 3-CB stimulated the production of ATP. To show that this was linked to dehalogenation of the 3-CB, he

performed an additional experiment and monitored ATP production and the appearance of benzoate from 3-CB. When 3-CB was added to the cultures their ATP levels drastically increased as benzoate was formed from the dehalogenation of 3-CB. This was proposed to be due to a chemiosmotic coupling of dehalogenation to ATP production.

Mohn and Tiedje (1991) propose that in *D. tiedjei* a chemiosmotic force couples the oxidation of formate and subsequent reduction of 3-CB with ATP production. In a series of experiments with uncouplers and inhibitors they demonstrated that dechlorination supported the formation of a proton-motive driven force which in turn supported ATP synthesis by a proton driven ATPase.

2.3 Reductive Dehalogenation as an Energy Yielding Alternate Respiratory Process

The data and results presented by Tiedje and his colleagues demonstrate that reductive dehalogenation is indeed a novel form of anaerobic respiration; in addition to the typical processes used by methanogens, nitrate-reducers, sulfate-reducers, and metal-reducers. The hypothesis that *Desulfomonile tiedjei* couples the reductive dehalogenation of 3-chlorobenzoate to formate oxidation and ATP production has been proven to be true by experimental evidence: formate oxidation, benzoate and Cl^- ion production, CO_2 production, disappearance of 3-CB, and ATP production. It has also been shown to be supported by a number of experiments involving chemiosmotic principles.

The concepts presented here can also be supported by work which was not directly related to research on *D. tiedjei.* Dolfing and Harrison (1992) performed experiments to determine the Gibbs free energy of formation of halogenated aromatic

compounds. They found that the redox couples for halogenated benzenes, benzoates, and phenols placed them in range of the redox couple NO_3^-/NO_2^-. This, in addition to data obtained with *D. tiedjei*, supports the potential role of halogenated compounds as energy yielding electron acceptors in anaerobic environments.

2.4 Metabolic Diversity in Cultures Performing Anaerobic Reductive Dehalogenation

In addition to *D. tiedjei* there have been a number of cultures, both pure and mixed, which have been shown to perform reductive dehalogenation under anaerobic conditions. The ability to couple this reductive dehalogenation to growth of the organism has also been demonstrated with some of these cultures.

One very interesting aspect of these anaerobic cultures which has received little attention is the vast metabolic diversity of the actual organisms which perform these dehalogenations. The ability to perform this type of anaerobic respiration has been demonstrated in sulfate-reducers, methanogens, nitrate-reducers, metal-reducers, and fermentative bacteria. (In many instances these metabolic characterizations have been found to overlap one another.) Additionally, bacteria are now being discovered which are believed to belong to a new metabolic group: bacteria which perform reductive dehalogenation as their natural respiratory process.

2.4.1 Reductive Dehalogenation in Sulfidogenic Cultures

In addition to *D. tiedjei*, a number of sulfidogenic bacteria have been described which reductively dehalogenate both aromatic and aliphatic hydrocarbons.

Desulfitobacterium dehalogenans has the ability to *ortho*-dechlorinate both 2,4-dichlorophenol and 3-chloro-4-hydroxyphenlacetate. This bacterium was isolated from methanogenic lake sediment and is characterized as an anaerobic, Gram-positive, rod-shaped bacterium. It reduces sulfite, thiosulfate, and sulfur to sulfide, as well as nitrate to nitrite and fumarate to succinate. It is able to perform reductive dechlorination in a nitrogen atmosphere of up to 2% oxygen, suggesting tolerance of microaerophilic conditions (Utkin et al. 1994).

Desulfitobacterium hafniense has the ability to perform *ortho*-dechlorination of phenols as well as *meta*-dechlorination of 3,5-dichlorophenol. This bacterium is an anaerobic, Gram-positive, endospore-forming, motile rod. It can reduce thiosulfate and sulfite to sulfide when grown on pyruvate, as well as performing Fe(III) reduction to Fe(II). Unlike other sulfidogens it is not able to reduce sulfate and contains no desulfoviridin (sulfite reductase). Cytochrome *c* is present, as is typical for sulfidogens. It has been shown to be able to conserve energy for growth from the reduction of 3-chloro-4-hydroxyphenylacetate to 4-hydroxyphenylacetate (Madsen and Licht 1992, Christiansen and Ahring 1996).

A strictly anaerobic organism, designated *Desulfitobacterium* sp. strain PCE1, was isolated and found to have the ability to reductively dehalogenate tetrachloroethylene. When grown with either succinate or propionate, dechlorination proceeded to vinyl chloride and ethene. Additionally, the organism was able to transform *ortho*-chlorinated phenolic compounds. The organism is a Gram-positive motile curved rod, with four lateral flagella, and contains cytochrome *c*. The bacterium was found to be able to grow fermentatively, as well as by respiration of sulfite, thiosulfate, and fumarate (Gerritse et al. 1996).

A 1987 study conducted with *Desulfitobacterium autotrophicans* revealed that this organism was able to perform reductive dehalogenation without having had any previous exposure to chlorinated solvents. It was able to quantitatively reduce 80 μM tetrachloromethane to trichloromethane and dichloromethane within 18 days. It was also able to reduce 1,1,1-trichloeoethane to 1,1-dichloroethane (Egli et al 1987, 1988).

In addition to the pure cultures described above, reductive dehalogenation has also been demonstrated with a sulfate-reducing mixed population. Lactate-fed cultures were able to reductively dehalogenate 92% of provided perchloroethylene to trichloroethylene and cis-1,2-dichloroethylene. Sulfide production, presumably from the included sulfate, was observed. Virtually no methane was produced and BES (bromoethane-sulfonic acid) did not inhibit dechlorination (Bagley and Gossett 1990).

2.4.2 Reductive Dehalogenation in Methanogenic Cultures

Methanosarcina sp. Strain DCM has been shown to be able to reductively dechlorinate perchloroethylene to trichloroethylene while growing on methanol, acetate, methylamine, and trimethylamine. Methane and trichloroethylene were produced simultaneously; dechlorination ceased when methane production stopped. This clear link between dechlorination and methane production suggested that the dechlorination was proceeding as a cometabolic activity (Fathepure and Boyd 1988).

Further research revealed that *Methanosarcina* sp. Strain DCM and another pure culture, *Methanosarcina mazei*, were capable of performing reductive dehalogenation of carbon tetrachloride, chloroform, and bromoform. For both cultures the extent of dechlorination was proportional to the amount of methane produced (Mikesell and Boyd 1990).

Complete reductive dehalogenation of perchloroethylene has been demonstrated in a mixed culture under methanogenic conditions. In studies performed with a continuous-flow fixed-film bioreactor, Vogel and McCarty demonstrated that 24% of radio-labeled PCE was completely mineralized to CO_2, via trichloroethylene, dichloroethylene, and vinyl chloride under methanogenic conditions (Vogel and McCarty 1985).

Enrichment cultures of PCE and TCE degrading organisms have been shown to reductively dechlorinate tetrachloroethylene and trichloroethylene to ethylene under methanogenic conditions. The resultant ethylene is an environmentally acceptable

compounds. The activity could be supported when methanol, hydrogen, formate, acetate, or glucose where supplied as electron donors. BES (2-bromoethanesulfonate) inhibited dechlorination (Freedman and Gossett 1989).

An anaerobic mixed culture was found to dechlorinate perchloroethylene to vinyl chloride using hydrogen as an electron donor. After 14 - 40 days the culture would cease dechlorination unless it was amended with the supernatant from a methanol fed culture. The results obtained from this particular study suggest that the hydrogen-utilizing dehalogenating organisms were dependent upon a metabolite(s) from the methanol-fed culture (DiStefano et al. 1992). This example demonstrates that a dehalogenator derived from a methanogenic culture may not necessarily be a methanogen, but rather an organism dependent upon the metabolic products derived from a diverse methanogenic culture. This proved to be the case for *D. tiedjei*, which is a sulfidogen which was isolated from a methanogenic consortium.

Another culture enriched from a methanogenic aquifer demonstrated the ability to reduce the aromatic pesticide 2,4,5-trichlorophenoxyacetic acid to 2,4- and 2,5-dichlorophenoxyacetic acid. Long term incubation of these samples eventually led to the formation of monochlorophenoxyacetic acid as well as di- and mono-chlorophenols and phenol. Dehalogenation did not occur in samples pre-treated with BES, but the addition of methanol partially relieved this condition (Gibson and Suflita 1990). Again, this demonstrates that dehalogenators derived from methanogenic cultures may or may not prove to be methanogens themselves. In this culture some evidence was found that the dehalogenator may be a sulfidogenic organism, but this has not been conclusively demonstrated.

2.4.3 Reductive Dehalogenation in Nitrate-Reducing Cultures

The genus *Alcaligenes* consists of aerobic organisms of which a few strains are capable of anaerobic respiration. This anaerobic growth is possible in the presence of nitrate or nitrite which act as alternate electron acceptors. *Alcaligenes denitrificans* NTB-1 is an organism which was found to have the capability of using either 4-chlorobenzoate, 4-bromobenzoate, 4-iodobenzoate, or 2,4-dichlorobenzoate as a sole carbon and energy source. What is interesting about this organism is that the initial step of 2,4-dichlorobenzoate degradation involved a reductive dehalogenation conversion to 4-chlorobenzoate. This occurred optimally with a headspace of 1.2% oxygen. Surprisingly, under anaerobic conditions very little conversion occurred. This behavior is being investigated for a link with the possible energy-dependent active transport of 2,4-dichlorobenzoate (van den Tweel et al. 1987).

A denitrifying *Psuedomonas* sp. (strain KC) was isolated which has the capability of reductively dehalogenating carbon tetrachloride. The products of this dehalogenation under denitrifying conditions were CO_2 and an unidentified water-soluble fraction, with little or no chloroform being produced. The addition of ferrous iron and cobalt to medium enhanced the growth of the strain while inhibiting the transformation of the carbon tetrachloride. The presence of oxygen or fumarate slowed the conversion of carbon tetrachloride (Criddle et al. 1990a).

Shewanella putrefaciens 200 is a non-fermentative bacterium which has the demonstrated ability to reductively dehalogenate tetrachloromethane to chloroform under anaerobic conditions. This culture is capable of NO_3^- and NO_2^- respiration as well as Fe(III) and trimethylamine oxide (TMAO) reduction. In a study of the competitive

effects of different electron acceptors on dehalogenation, tetrachloromethane dehalogenation by *Shewanella putrefaciens 200* was delayed until all nitrate and nitrite were consumed. Oxygen also inhibited reductive dehalogenation of the compound while the presence of Fe(III) and TMAO did not affect the dehalogenation rate. These results suggest direct inhibition of dechlorination by nitrogen oxides in the case of this very unique organism (Picardal et al. 1995).

2.4.4 Reductive Dehalogenation by Facultative Organisms

Escherichia coli K-12 is a facultative organism which is capable of reductively dehalogenating carbon tetrachloride under various electron acceptor conditions, which result in various pathways and rates of transformation. Oxygen and nitrate generally inhibited carbon tetrachloride dehalogenation, although it was dehalogenated to CO_2 in the presence of very low concentrations of oxygen (<1%). When grown under fumarate respiring conditions, CO_2, chloroform, and a non-volatile fraction were formed. Under fermentative conditions the predominant product was chloroform with almost no CO_2 being formed (Criddle et al. 1990b).

A novel facultative bacterium (2CP-1), isolated from sediment taken from a small stream near Lansing, MI, is capable of reductively dehalogenating 2-chlorophenol. 2CP-1 is described as a Gram-negative rod, 3 x 0.5 μm in size, and is catalase- and oxidase-negative. It forms small red colonies on anaerobic media. It is capable of *ortho*-dechlorination, chlorines at other positions blocked the activity. Growth yield

experiments demonstrated that the reductive dehalogenation was linked to growth of the organism. The bacterium is described as being most closely related to myxobacteria (Cole et al. 1994).

A facultative bacterium (strain MS-1) was isolated which is capable of reductively dehalogenating tetrachloroethene to cis-1,2-dichloroethene. Strain MS-1 has properties which relate it to the family *Enterobacteriaceae.* Oxygen, nitrate, and high concentrations of fermentable substrates (ex. glucose) inhibited dehalogenation (Sharma and McCarty 1996).

In the same study another closely related facultative organism, *Enterobacter agglomerans*, was also found to dehalogenate perchloroethylene to cis-dichloroethylene (Sharma and McCarty 1996).

2.4.5 Reductive Dehalogenation by Fermentative Organisms

The genus *Clostridium* consists of anaerobic fermentative bacteria, a few of which can grow in the presence of oxygen. They are unable to reduce sulfate. One bacterium, identified as a *Clostridium* sp., was isolated from the effluent of an anaerobic, suspended-growth bioreactor. The organism is capable of reductively dehalogenating 1,1,1-trichloroethane to 1,1-dichloromethane under anaerobic conditions. This *Clostrium* sp. is a Gram-positive, motile, endospore forming rod. The strain does not reduce nitrate or sulfate (Galli and McCarty, 1989).

One very interesting bacterium, *Dehalospirillum multivorans,* has been found to have the ability to grow anaerobically using hydrogen as an electron donor and PCE as an electron acceptor, resulting in DCE. This bacterium was enriched from an activated

sludge which had not previously been exposed to chlorinated ethenes and was isolated on a defined agar medium containing only pyruvate as a sole energy source. For this reason *D. multivorans* will be listed here with the fermentative bacteria capable of reductive dehalogenation. The organism is a Gram-negative anaerobic spirillum. It can grow with hydrogen or formate plus PCE as energy sources and acetate as a carbon source. Additionally it can utilize fumarate, and to some extent, nitrite as electron acceptors. Researchers working with this bacterium describe it as being able to couple the reductive dechlorination of PCE to growth, with catabolic PCE reduction representing a new type of anaerobic respiration (Neumann et al. 1994, Scholz-Muramatsu et al. 1995).

2.4.6 Bacteria Which Perform Reductive Dehalogenation as Their Natural Respiratory Process

Recently, a bacterium has been isolated which does not fit into any of the typical metabolic characterizations of anaerobes. This bacterium, *Dehalobacter restrictus,* is proposed to be a physiologically new type of bacterium which has evolved using perchloroethylene as its natural electron acceptor. The Gram-negative rod does not fit into any of the conventional classifications of methanogenic, sulfidogenic, metal-reducing, nitrate-reducing or fermentative bacteria. This culture (formerly known as PER-K23) reductively dechlorinates perchloroethylene, via trichloroethylene, to cis-1,2-dichloroethylene; it couples this reductive dechlorination to growth via oxidation of either hydrogen or formate. The culture does not grow in the absence of PCE or TCE, nor can it utilize O_2, NO_3^-, NO_2^-, SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$, S, or CO_2 as electron acceptors. It was not able to grow fermentatively on any of a number of compounds tested. Electron balances

showed that all electrons derived from hydrogen or formate utilization could stoichiometrically be accounted for in biomass and dechlorination products. This example is the first report of an anaerobe which utilizes a chlorinated compounds as its naturally occurring electron acceptor (Holliger et al. 1993, Schumacher and Holliger 1996).

2.5 Discussion

When reviewing the literature on anaerobic respiration it is evident that most research has centered on methanogenic, sulfidogenic, nitrate-reducing, and to some degree metal-reducing bacteria. In the past decade reductive dehalogenation has proven to be a unique alternate respiratory process which produces energy for growth, and in at least one case acts as a natural electron acceptor. As is evident from the literature cited, this dehalogenation process is performed by a wide variety of metabolic types of organisms. This leads one to believe that reductive dehalogenation is more than just an alternate respiratory process and is more widespread than originally thought. Interestingly, it has been found to be performed by cultures which were isolated and enriched for this activity as well as being performed by other isolated cultures which were not previously exposed to chlorinated compounds.

In addition to the traditional metabolic types stated, reductively dehalogenation will likely prove to be a unique metabolic classification of anaerobes with some overlap into the traditional classes (but a unique independent class, nonetheless).

When one looks at natural environments, it is not so far fetched an idea that halogenated compounds may be an organism's natural electron acceptor. Halogenated compounds are not new to the environment and the group is not exclusively made up of

human-introduced chemicals. Many sources of naturally occurring halogenated compounds are available to microorganisms. A 1967 article in *Science* reports on the formation of bromophenols as metabolites of red algae (Craigle and Grueling 1967). A 1977 report in *Tetrahedron* compiles an exhaustive list of halogenated compounds which are metabolites in marine environments. These include bromo-, iodo-, and chloro-compounds (including phenolics) produced by red and brown algae as well as by certain sponges. They range from polyhalogenated acetones to acetylenes, to terpenes, and numerous others (Faulkner 1977). Halogenated phenols and indoles have been attributed to thyroid production in acorn worms (Higa et al. 1980). Not only have these natural sources of halogenated compounds been reported, but their dehalogenation in natural sediments by unique anaerobic bacteria has also been documented (King 1988). This is true *in-situ* work.

It is very likely that bacteria have evolved with the ability to utilize these compounds, as anaerobic environments are typically electron acceptor limited and bacteria are unsurpassed in their abilities to adapt, conserve energy, and utilize what is available for most favorable nutritional gain.

Perhaps these bacteria were never widely studied because natural halogenated compounds are so diverse that there isn't a "typical" halogenated compound that would be noticed. Perhaps it is because their oxidized substrates or reduced products do not have an overwhelming smell (H_2S), appearance (iron deposition) or other characteristic.

When considering naturally chlorinated compounds' availability and potential as electron acceptors, one could argue that they were more likely than not to encourage the evolution of a specific class of bacteria with the ability to grow and produce energy from them.

In the same manner that Dr. Derek Lovley's work is changing researchers' perspective on the importance, ability, and widespread occurrence of iron-reducing bacteria, current and future research on reductive dehalogenation (begun by Tiedje, Mohn, Suflita, Schraa, Vogel, McCarty, Holliger, and others) will change the microbiology world's perspective on anaerobic classes of bacteria and their natural occurrence and abilities.

Reductive dehalogenation is not necessarily an "alternate" respiratory process at all.

CHAPTER THREE

CHARACTERIZATION OF AN ENRICHED ANAEROBIC CULTURE THAT REDUCTIVELY DEHALOGENATED 2,4,6-TRICHLOROPHENOL TO 4-MONOCHLOROPHENOL AS A GROWTH-LINKED RESPIRATORY PROCESS

3.1 Materials and Experimental Methods

3.1.1 Materials

All chlorinated chemicals used in this investigation were reagent grade chemicals obtained from Sigma Chemical Company (St. Louis, MO) with the exception of 3-chloro-4-hydroxyphenylacetic acid, which was obtained from ICN Biochemicals (Cleveland, OH). Borosilicate glass test tubes, aluminum seals, and 20 mm butyl rubber stoppers were obtained from Bellco Glass Company (Vineland, NJ). Teflon faced butyl rubber stoppers were obtained from The West Company (Phoenixville, PA). The COY anaerobic chamber was obtained from Coy Laboratory Products (Grass Lake, MI). The HPLC (high pressure liquid chromatography) column used for chlorinated compound analysis was obtained from Alltech Associates Inc. (Deerfield, IL). Media ingredients, syringes, sample vials, and all other labware were obtained from Fisher Scientific (Springfield, NJ).

3.1.2 Analytical Methods

Chlorophenols were analyzed by HPLC using a Waters 715 Ultra Wisp, equipped with a 600E Controller and a 484 Tunable Absorbance UV Detector. The detector was set at a wavelength of 280 nm. The HPLC was outfitted with an Econosphere C18 5u 150mm x

4.6 mm reverse phase column (Alltech catalog # 70070). The column was housed in a temperature control casing set at 30°C. The mobile phase was methanol plus 1% acetic acid combined with water plus 1% acetic acid in various ratios (50:50, 60:40, 40:60). Samples were acidified with concentrated hydrochloric acid and stored at 5°C until analyzed.

3.1.3 Consortium Growth and Purification

3.1.3.1 Source of Inoculum: The inoculum was obtained from the anaerobic component of a sequential anaerobic-aerobic reactor system which completely mineralized 25ppm (0.126 mM) 2,4,6-trichlorophenol (2,4,6-TCP) (Armenante et al., 1992; Kafkewitz et al., 1992). The reactor population originated from anaerobic sewage digester fluid and was gradually shifted to a chloride-free defined medium containing formate and acetate as oxidizable carbon sources. The anaerobic portion of the reactor stoichiometrically converted the 2,4,6-TCP to 4-monochlorophenol (MCP), which accumulated.

3.1.3.2 Anaerobic Medium: The composition of the medium was as follows (in g/L of deionized water, unless otherwise stated): K_2HPO_4, 0.225; KH_2PO_4, 0.45; $(NH_4)_2SO_4$, 0.20; $MgSO_4 \cdot 7H_2O$, 0.09; $FeSO_4 \cdot 7H_2O$, 0.002; sodium formate, 2.0; sodium acetate, 2.5; and $NaHCO_3$, 2.5. Resazurin (0.0001%) was added as an indicator of redox status. Varying concentration of 2,4,6-TCP were added from a 10 mM stock solution in 0.05 N NaOH. Initial experiments were performed without added reducing agents, later experiments contained various reducing agents as noted separately. Where noted,

trypticase (1% wt./vol.) was added to media as a nutrient source. The pH of the medium was maintained by inclusion of an organic buffer (either BICINE, pK_a 8.3, or AMPSO, pK_a 9.0) in 30 mM concentrations.

Ingredients of the medium were added to an Erlenmeyer flask and boiled for fifteen minutes. Sodium bicarbonate and an organic buffer were then added to the medium which was boiled and degassed for an additional fifteen minutes using a gassing manifold supplying an oxygen free gas mixture of N_2:CO_2 (80:20). Traces of oxygen were removed from the manifold gas by passing through a Sargent-Welch copper oven. The medium was then removed from heat and degassed for an additional fifteen minutes. Except as noted in earliest enrichment experiments, the flask was then sealed and transferred into a COY anaerobic chamber where media were dispensed under a N_2:H_2 (95:5) atmosphere. Standard 18 x 150 mm borosilicate glass crimp seal tubes or 160 ml serum bottles were used for all experiments. Culture vessels were sealed with butyl rubber stoppers and aluminum crimp seals inside of the anaerobic chamber and then returned to the manifold where they were evacuated and refilled with N_2:CO_2 three times and autoclaved. Additions to, and sampling from, the cultures were performed by syringe, purged three times with sterile N_2:CO_2. Cultures were incubated at 30°C in the dark.

3.1.3.3 Enrichment and Cultivation: Anaerobic medium, containing BICINE as a buffer (pH 8.7) and 0.125 mM 2,4,6-TCP, was prepared as described above and dispensed into gas-purged serum bottles. No reducing agent was added to the medium.

Four milliliters of liquid from the anaerobic portion of the sequential reactor were added to the bottles which each contained approximately 100 ml of medium. These bottles were incubated in the dark for two weeks at 30°C. The bottles were then used as inocula for nutrient agar spread plates which contained 0.125 mM 2,4,6-TCP. The agar plates were allowed to degas inside of a candle jar for 48 hours before inoculation. The inoculated plates were then incubated inside of the candle jar for one week until colonies appeared. Colonies from these plates were picked with an inoculating needle and inoculated into standard 20 ml test tubes (non-crimp seal tubes) containing 15 ml of degassed medium as previously described. No reducing agent was added to this medium which was generally pink (indicating a redox potential greater than -51 mV) after autoclaving. These tubes were incubated inside of the candle jar in the dark at 30°C. After two weeks the test tubes were moved into the anaerobic chamber and incubated at 30°C. One ml samples from these tubes were removed and analyzed by HPLC weekly for signs of dehalogenation. In addition, each week one ml of a 10x anaerobic formate and acetate stock solution was added to each tube.

*3.1.3.4 **Purification and Isolation Attempts:*** Isolation of the active dehalogenating constituent of the consortium was attempted numerous times using the spread plate to liquid culture transfer technique described in the "Enrichment and cultivation" section. In addition, over 150 solid to liquid culture transfers were attempted using continuous, strictly anaerobic technique and incubation. Spread and streak plates were incubated both in the anaerobic chamber under N_2:H_2 and in gas-pak jars under N_2:CO_2.

In addition to solid-to-liquid isolation attempts, a series of dilution-to-extinction purification attempts were made. Five most-probable-number (MPN) sets (5 x 10 tube) were set up under strictly anaerobic conditions with a high concentration of 2,4,6-TCP (1 mM) and pH levels near the top of the dehalogenating range (pH 8.7 to 8.8). These sets were sequentially inoculated with the most dilute tube showing dehalogenation activity from the previous MPN in order to attempt to dilute out any non-essential contaminant populations.

3.1.4 Growth Parameters

3.1.4.1 Effects of pH on Dehalogenation of 2,4,6-TCP: The active pH range for the dehalogenating population was evaluated by the inclusion of a number of different organic buffers (30 mM): MOPS (pK_a 7.2), TES (pK_a 7.4), TRICINE (pK_a 8.1), BICINE (pK_a 8.3), TAPS (pK_a 8.4), AMPSO (pK_a 9.0), CHES (pK_a 9.3), or no included buffer (Cote and Gherna, 1994). These represented a pH range of 7.2 to 8.7. The medium contained 0.8 mM 2,4,6-TCP and was reduced with 0.8 μM cysteine·sulfide. The cultures were tested in triplicate plus one autoclaved, uninoculated control.

3.1.4.2 Effect of Temperature on Dehalogenation: Medium was prepared using the PRAS (pre-reduced anaerobically sterilized) technique, using AMPSO (30 mM), trypticase (1% wt./vol.), 0.5 mM 2,4,6-TCP, and 0.05% sodium thioglycolate. Temperature range and optimum for dehalogenation were assayed at 20, 25, 30, 35, and 40°C. The cultures were tested in triplicate plus one autoclaved, uninoculated control. These were sampled approximately every 24 hours for HPLC analysis of chlorophenols.

3.1.4.3 Effects of Oxygen Level on Dehalogenation: In order to determine the effect of oxygen levels on dehalogenation an incremental air-headspace experiment was set up and monitored for turbidity and dehalogenation activity. Medium was prepared using the PRAS technique described, using BICINE (30 mM), 0.254 mM 2,4,6-TCP, 2 mM lactate, and 0.025% cysteine-sulfide as a reducing agent. Size 18 ml crimp sealed tubes were filled with 10 ml of medium. Aliquots of headspace were removed by syringe and replaced with equal amounts of air, from normal room atmosphere, in the following amounts (ml): 0, 0.5, 1.0, 1.5, 2.5, 3.5, 5.0, 7.0, and 10.0. The tubes were then autoclaved and inoculated. The cultures were tested in triplicate plus one autoclaved, uninoculated control. The cultures were monitored for turbidity and sampled for HPLC analysis of chlorophenols.

3.1.4.4 Effect of Redox Level on Dehalogenation: The effect of initial redox level on dehalogenation activity and lag time was tested. Medium was prepared using the PRAS technique described, using AMPSO (30 mM), trypticase (1% wt./vol.), and 0.5 mM 2,4,6-TCP. Triplicate samples, plus one uninoculated control, were reduced using one of the reducing agents listed in Table3.1 (Cote and Gherna, 1994).

3.1.5 Carbon and Nutrient Sources

3.1.5.1 Carbon Source / Electron Donor Requirements: The basal medium described above was modified to an inorganic, carbonate buffered medium by exclusion of formate, acetate, and organic buffers. The medium was prepared and dispensed anaerobically in

two sets: one which included 2,4,6-TCP at a concentration of 0.126 mM (25ppm), and one which included no added chlorinated compound. The medium was supplemented with the following organic compounds (30 mM) for evaluation as potential carbon sources and electron donors: acetate, formate, ethanol, glycine, BICINE, methanol, succinate, lactate, glycerol, pyruvate, trypticase (1% wt./vol.), L-glutamate, fructose, glucose, sucrose, lactose, casamino acids, or acetate (25 mM) plus formate (20 mM). Each substrate was tested in triplicate plus one uninoculated control. As an additional control, one set of test tubes had no organic compound added. Each culture vessel contained 10 ml of medium and was inoculated with 0.2 ml of an actively dehalogenating culture. The small inoculum size was used to minimize carry over of organic compounds from the inoculum source.

*3.1.5.2 **Supplemental Carbon Sources:*** In order to enrich for the dehalogenating constituents of the population an experiment was set up in which the basic medium was amended with supplemental carbon sources, in addition to the formate and plus acetate normally present. A concentrated medium was prepared by dissolving one liter ingredient amounts in 900 ml of liquid. PRAS (pre-reduced anaerobically sterilized) medium was prepared as described, with 0.05% thioglycolate as the reducing agent, 30 mM AMPSO (pH 8.4), and 1% wt./vol. trypticase.

Anaerobic stock solutions of 500 mM concentrations were prepared of the following supplemental carbon sources: sucrose, maltose, cellobiose, xylose, β-hydroxybutyrate, and succinate. An anaerobic 10x stock solution was prepared of the

following fatty acids: propionic, 0.6 ml/L; n-butyric, 0.4 ml/L; methyl butyric, 0.1 ml/L; iso-butyric, 0.1 ml/L; n-valeric, 0.1 ml/L; methyl valeric, 0.1 ml/L; and iso-valeric, 0.1 ml/L. One ml aliquots of these stock solutions were each filter-sterilized and added to 9 ml of concentrated medium, resulting in 50 mM final concentrations of supplements or the fatty acid concentrations stated above, and normal medium concentration. Formate and acetate concentration were also tested at double their normal concentrations of 2.0 and 2.5 g/L (4.0 and 5.0, respectively).

This experimental procedure was performed in triplicate with sets containing either 0.5 mM 2,4,6-TCP, 5 mM 3 chloro-4-phenoxyacetic acid (3Cl-4-OHPA), or no added chlorinated compound. Each culture vessel contained 10 ml of medium and was inoculated with 1 ml of an actively dehalogenating culture.

*3.1.5.3 **Supplemental Nutrient Sources:*** To determine the effect of various nutrient supplements on dehalogenation an experiment was set up with individual supplements added to the basal medium. The medium was prepared with PRAS technique and contained 30 mM AMPSO (pH 8.4) and 0.5 mM 2,4,6-TCP. The medium was reduced with 0.025 % sodium sulfide (Na_2S).

A stock solution of the following vitamin mix was prepared and filter sterilized (mg/L): pyridoxine·HCl, 10; thiamine·HCl, 5; riboflavin, 5; calcium pantothenate, 5; thioctic acid, 5; ρ-aminobenzoic acid, 5; nicotinic acid, 5; vitamin B_{12}, 5; biotin, 2; folic acid, 2; and mercaptoethanesulfonic acid, 10. This stock solution was stored at 5°C and tested as a supplement at 10 ml/ L which was aseptically added to culture vessels after autoclaving.

A trace metals / minerals stock solution was prepared containing the following (g/L): $MnSO_4$, 1.0; $Fe(NH_4)_2(SO_4)_2$, 0.8; $CoCl_2$, 0.2; $ZnSO_4$, 0.2; $CuCl_2$, 0.02; $NiCl_2$, 0.02; Na_2MoO_4, 0.02; Na_2SeO_4, 0.02; Na_2WO_4, 0.02; $CaCl_2$, 0.3, and Na_3VO_4, 0.1. This stock solution was filter sterilized, stored at 5°C, and tested as a supplement at 1 ml/L which was aseptically added to culture vessels after autoclaving. Rumen fluid was collected from a fistulated cow at the AgBiotech Center of Rutgers University in New Brunswick. This fluid was centrifuged at 200 rpm for 20 minutes and the decant was filter sterilized and stored at 5°C. This was tested as a supplement at 1 ml/L.

Succinate and monosodium glutamate were tested as supplements at concentrations of 10 mM. Yeast extract and trypticase were each tested as supplements at 1% (wt./vol.). These were pre-weighed and added as dry ingredients to culture vessels before the medium was dispensed.

A separate set of tubes was tested containing 50% freshly prepared medium and 50% autoclaved medium which had previously been used to grow the dehalogenating population. This was to determine whether the lag time before the onset of dehalogenation was due to production of an essential nutrient by a member of the consortium.

Cultures were tested in triplicate plus one uninoculated, autoclaved control. An additional control set was tested with no added supplement. Each culture vessel contained 10 ml of medium and was inoculated with 0.2 ml of an actively dehalogenating culture.

3.1.5.4 Role of Trypticase in Growth and Dehalogenation: An experiment was set up to determine whether trypticase was serving as a carbon source or as a nutrient source for the active population. Effect on lag time and dehalogenation rate was monitored to determine if there was any correlation with trypticase concentration. The following anaerobic stock solutions were prepared:

Solution A (grams in 500 ml dH_2O): (2x normal concentrations:) K_2HPO_4, 0.225; KH_2PO_4, 0.45; $(NH_4)_2SO_4$, 0.20; $MgSO_4 \cdot 7H_2O$, 0.09; $FeSO_4 \cdot 7H_2O$, 0.002; sodium formate, 2.0; sodium acetate, 2.5, (normal concentrations:) AMPSO, 2.5g; $NaHCO_3$, 2.5, plus 1 mM 2,4,6-TCP and 0.05% sodium thioglycolate

Solution B (grams in 500 ml dH_2O): AMPSO, 2.5g; $NaHCO_3$, 2.5

Trypticase solution: (in 100 ml of solution B) 5 g of trypticase

Vitamin mix 1: (50 ppm each) niacin, biotin, folic acid, thiamine, and pantothenic acid

Vitamin mix 2: (μg/L) ρ-aminobenzoic acid, 8.3; nicotinic acid, 8.3; pyridoxine·HCl, 16.7; riboflavin, 8.3; thioctic acid, 8.3; and cyanocobalamin, 1.7

Trace minerals/metals mix: (see supplemental nutrient source section for recipe)

The stock solutions were utilized in the combinations reported in Table 3.2. All sets were performed in triplicate plus one autoclaved, uninoculated control. Each 10 ml tube was inoculated with one ml from an actively dehalogenating culture.

3.1.6 Energy Metabolism

3.1.6.1 Tolerance Level of 2,4,6-TCP as an Electron Acceptor: 2,4,6-TCP toxicity was assayed in cultures grown in cysteine·sulfide reduced medium containing AMPSO as buffer (pH 8.4) and excess lactate as a carbon source. 2,4,6-TCP was tested in concentrations of 0.25, 0.50, 0.75, 1.0, 1.2, 2.0, and 3.0 mM.

3.1.6.2 Utilization of Alternate Electron Acceptors: In attempt to stimulate growth and determine the effect of alternate electron acceptors on the active population an experiment was set up with individual acceptors and monitored for turbidity and inhibition or stimulation of dehalogenation. Media were prepared in duplicate with AMPSO (30 mM) using PRAS technique with one set containing 0.5 mM 2,4,6-TCP and one set containing no added chlorinated compound. The media were reduced with Na_2S (0.025%). The following electron acceptors were tested at a concentration of 10 mM: fumarate, sodium sulfate, MnO_2, esculin, KNO_3, thiosulfate, trimethylamine-n-oxide, and DMSO. Because of its toxicity at higher concentrations, $FeSO_4$ was tested at a concentration of 1 mM plus 1 mM citrate as a chelating agent. Control sets were run which contained no added electron acceptor. The cultures were tested in triplicate plus one autoclaved, uninoculated control. Inocula size was 0.2 ml into 10 ml of medium.

In a similar experiment, the following electron acceptors were tested in low concentration (0.5 mM): KNO_3, Na_2SO_4, thiosulfate, and Fe_2O_3 plus citrate. The medium was reduced with sodium thioglycolate and contained 0.4 mM 2,4,6-TCP. Inocula size

was 1 ml into 10 ml medium. Cultures were monitored for turbidity and dehalogenation. Cultures which included KNO_3 in the medium were also monitored for accumulation of nitrite and disappearance of nitrate.

3.1.6.3 Effects of Iron Plus Different Chelating Agents: To determine the role of iron in dehalogenation an experiment was set up to test the following possibilities: ferric iron as an electron acceptor, ferrous iron as a trace nutrient, and chelating agents' effect on iron availability. The chelating agents used were citrate, ethylenediaminetriacetic acid (EDTA), and nitrilotriacetic acid (NTA). AMPSO buffered medium containing 0.4 mM 2,4,6-TCP and trypticase (1% wt./vol.) was prepared according to PRAS technique and reduced with thioglycolate. Inocula size was 3 ml into 11 ml media. The cultures were tested in triplicate plus one autoclaved, uninoculated control. The combinations reported in Table 3.3 were tested.

3.1.6.4 Effect of Ferric Iron Compounds with and without a Chelating Agent: To determine the effect of different ferric iron compounds on dehalogenation an experiment was set up with the following compounds (0.5 mM): Fe_2O_3 plus citrate, Fe_2O_3 plus NTA, ferric chloride with and without NTA, ferric sulfate with and without NTA, ferric nitrate with and without NTA, ferric citrate with and without NTA, and crystal ferric oxide with and without NTA. AMPSO buffered medium containing 0.4 mM 2,4,6-TCP and trypticase (1% wt./vol.) was prepared according to PRAS technique and reduced with thioglycolate. Inocula size was 3 ml into 11 ml media. The cultures were tested in triplicate plus one autoclaved, uninoculated control.

3.1.6.5 Halogenated Compounds Utilized as Electron Acceptors: Medium was prepared, according to PRAS technique, containing AMPSO, 1% trypticase, and 0.15 mM 2,4,6-TCP. The medium was reduced with thioglycolate and dispensed into crimp sealed tubes in 9 ml volumes. Anaerobic stock solutions were made of the compounds listed in Table 3.4 (1 mM).

One ml aliquots of these stock solutions were added to each test tubes resulting in a final concentration of 0.1 mM of the individual halogenated compound being tested. Test tubes were inoculated with 1 ml of an actively dehalogenating culture. Chloroethylenes were analyzed by GC; chlorobenzenes and chlorobenzoic acids were analyzed by HPLC at 254 nm, while remaining compounds were analyzed by HPLC at 280 nm as described in the analytical methods section.

3.1.6.6 Effect of Inhibitors and Antibiotics: In order to provide information on the metabolic pathways utilized by the dechlorinating population a study was set up with individual substrates which are known to be inhibitory to the growth of different types of microbes. These include BES (bromoethane sulfonic acid: a methanogen inhibitor), molybdate (a sulfidogen inhibitor), metronidazole (a ferredoxin inhibitor), vancomycin (a broad based antibiotic) and chloramphenicol (an inhibitor of protein synthesis). 100x stock solutions (100 mg/10ml) of these compounds were prepared in anaerobic dH_2O, or ethanol (chloramphenicol) and filter sterilized. These were stored at 5°C.

Medium was prepared, according to PRAS technique, which contained AMPSO, 1% trypticase, and 0.5 mM 2,4,6-TCP. The medium was reduced with thioglycolate. The medium was dispensed into two sets of crimp sealed test tubes in 10 ml volumes. To the first set of test tubes, inhibitors and antibiotics were added (0.1 ml) at time zero (inoculation) and the cultures were monitored for dehalogenation. The second set of test tubes was inoculated but did not have any inhibitors or antibiotics added until dehalogenation had begun, at day 3 of incubation. The cultures were tested in triplicate plus one autoclaved, uninoculated control. Also, one set of controls was run with no added test compounds.

3.1.6.7 Dehalogenation Linked Growth: An experiment was set up to look for a link between dehalogenation and population size. The medium was prepared according to PRAS technique with 30 mM AMPSO and 1% trypticase, it was then reduced with thioglycolate. A portion of the medium and an anaerobic stock solution of 3Cl-4-OHPA were dispensed into three serum bottles resulting in concentrations of 1 mM, 4 mM, and 8 mM 3Cl-4-OHPA in 100 ml media.

3Cl-4-OHPA was then added to the remaining medium, resulting in a 3 mM concentration. This medium was then dispensed into 3 sets of 5 x 10 tubes MPN series'.

The serum bottles were inoculated with 5 ml from an active 2,4,6-TCP dehalogenating culture and monitored. When HPLC analysis showed that 50% of the 3Cl-4-OHPA in a bottle was dehalogenated the culture was immediately used as inocula for one of the MPN sets. The inoculated MPN tubes were incubated for three weeks and analyzed for dehalogenation.

3.2 Results and Discussion

3.2.1 Consortium Growth and Purification

Spread plates incubated inside of the candle jar showed at least five distinct colony types: large pink opaque, small white opaque, large white clear, and medium and large yellow clear, indicating the presence of a very mixed population in initial reactor samples. When non-crimp seal tubes inoculated with these colonies were incubated in a candle jar for two weeks, the degassed media remained pink with respect to the resazurin indicator dye. The tubes were transferred into the anaerobic chamber and after approximately one week about 1/3 of the tubes turned colorless with respect to the resazurin indicator dye. This suggested that either the cultures were using the trace amounts of oxygen present in the media or that the oxygen was slowly diffusing out of the media and being converted to water by the palladium catalysts in the chamber. It is likely that the cultures which turned colorless were actually consuming the oxygen, as none of the uninoculated control tubes lost their oxidized pink color. After 8 weeks in the chamber, 10 weeks after inoculation, the level of 2,4,6-TCP decreased and a 2,4-dichlorophenol peak was detected by HPLC in one of the colorless test tubes. This tube was used as the initial source of inocula for all subsequent experiments.

None of the other colorless or pink tubes in the chamber produced any dehalogenation products nor could subsequent isolation attempts reproduce the solid to liquid dehalogenation activity. No turbidity was seen in any of the test tubes.

Additional solid to liquid isolation attempts were made using continuous, strictly anaerobic technique and incubation. Spread and streak plates were incubated both in the anaerobic chamber under N_2:H_2 and in gas-pak jars under N_2:CO_2. None of the transfers yielded dehalogenating cultures.

MPN tube dilution-to-extinction purification attempts yielded more favorable results. Tubes set up with high concentration 2,4,6-TCP (1 mM) and pH levels near the top of the dehalogenating range (pH 8.7 - 8.8) screened out a number of the non-dehalogenating contaminants in the culture. Microscopic examination typically showed three morphological types: a flagellated cocci, a non-motile very short rod, and very few non-motile longer rod. These cultures grew with formate plus acetate as the sole carbon/electron donor sources with no added vitamins or nutrients. MPN results showed that the dehalogenating organism was present in these cultures in very low numbers, originally 25 to 250 organism per ml of culture media. Subsequent dilution-to-extinction experiments screened out the flagellated organism, now believed to be a nitrate-reducing bacterium which provided an essential nutrient to the consortium. After this dilution-to-extinction, the culture could only be grown with 1% trypticase or with a vitamin mix added to the culture medium.

Microscopic examinations of stable enrichment cultures established over multiple generations consistently reveal mostly Gram-negative non-motile short oval rods and fewer Gram-negative non-motile larger rods (2.5 x larger).

3.2.2 Growth Parameters

3.2.2.1 Effects of pH on Dehalogenation of 2,4,6-TCP: The effect of pH on dehalogenation was determined by the inclusion of a number of Good buffers in the culture medium. These buffers were chosen because of their high solubility, impermeability to biological membranes, minimal interaction with mineral cations, and non-toxic characteristics. Figure 3.1 displays the effect of pH on the dehalogenation of 0.8 mM 2,4,6-TCP. The data are representative of triplicate cultures. In other experiments it was found that the apparent minimum pH for dehalogenation was 7.8 and the apparent maximum was 9.0. These data indicate that the dehalogenation is performed by a moderately alkalophilic bacterium which is active over a relatively narrow pH range. These may be the limits of the active range for the dehalogenase enzyme responsible for the dechlorination step.

3.2.2.2 Effect of Temperature on Dehalogenation: Figure 3.2 shows the effect of incubation temperature on dehalogenation. The data indicates that the dehalogenating organism has a temperature optimum of 30°C and operates over a somewhat narrow temperature range of 25 to 35°C, possibly because the dehalogenase enzyme is denatured at higher temperatures. The 30°C optimum temperature is normal for many anaerobic bacteria.

3.2.2.3 Effect of Oxygen on Dehalogenation: In experiments which had increasing amounts of oxygen added (in the form of air) all cultures with 1 or more ml's of air added initially turned pink in color, indicating redox levels above -51mV. After 48 hours of incubation, all cultures had become colorless with respect to resazurin. Turbidity measurements could be taken of cultures with greater than 1 ml of air added and reflected an increase in turbidity with increasing oxygen levels. However, none of these turbid cultures resulted in dehalogenation activity. Sub-cultures from these tubes inoculated into fresh anaerobic media also resulted in absence of dehalogenation activity, suggesting that the turbidity was due to an aerobic or facultative contaminant in the consortium.

All tubes to which either zero or 0.5 ml increments of air were added resulted in dehalogenation of the 2,4,6-TCP to 4-MCP. These data suggest that the dehalogenating organism(s) is an oxygen tolerant anaerobe which dehalogenates only under anaerobic conditions, as defined by a reduced resazurin state of the medium. The lack of dehalogenation activity after oxygen depletion in higher O_2 cultures suggests that the contaminant population depleted carbon sources and/or other essential nutrients which discouraged the growth and viability of the dehalogenating population.

3.2.2.4 Effect of Redox Level on Dehalogenation: Stringent anaerobic bacteria require not only the absence of oxygen but also a low oxidation-reduction level of the medium. For example, methanogens require a redox level of approximately -300 mV in order to grow, this is considerably lower than the redox level of media which merely has oxygen excluded, <-51 mV. Figure 3.3 shows the effect of various initial redox levels on the dehalogenation of 2,4,6-TCP. The data show that this particular culture prefers a redox

level at the upper end of the anaerobic range (-51 to -100 mV), as opposed to more highly reduced conditions. It is possible that the more reduced conditions cause the precipitation of an essential metal or other nutrient, thus delaying or inhibiting dehalogenation. Another possibility is that the dehalogenating organism is sensitive to the sulfur component of the reducing agents, which can be somewhat toxic.

3.2.3 Carbon and Nutrient Sources

3.2.3.1 Carbon Source / Electron Donor Requirements: Reductive dehalogenation requires an electron donor. The only organic compounds in the feed of the anaerobic reactor were formate, acetate, BICINE, and 2,4,6-TCP. In the inorganic, carbonate buffered medium fructose, lactate, glycerol, and trypticase supported stoichiometric conversion of 0.126 mM 2,4,6-TCP to MCP within 10 days, as did the combination of formate plus acetate, the mixed substrates used in the reactor feed (Figure 3.4 and Figure 3.5). Acetate, succinate, L-glutamate, glucose, and lactose resulted in complete conversion of 2,4,6-TCP to MCP within 3012days. Pyruvate, formate, ethanol, glycine, and BICINE supported only slight dehalogenation after 30 days. Test tubes containing methanol, sucrose, or casamino acids showed no signs of dehalogenation after 30 days of incubation. Sterile controls and inoculated controls that lacked electron donors and carbon sources showed no loss of 2,4,6-TCP and no appearance of either DCP or MCP.

None of the tested compounds produced turbidity in any of the test tubes. In addition, no turbidity was seen in any of the test tubes from the second set in this experiment, the second set being an identical experiment set up without the inclusion of

2,4,6-TCP or any other electron acceptors. Inocula taken from test tubes of the second set failed to perform dehalogenation in medium which included 2,4,6-TCP, suggesting that the dehalogenating organism was not a fermentative bacterium.

3.2.3.2 Supplemental Carbon Sources: In order to enrich for the dehalogenating constituents of the culture an experiment was set up in which the basic medium was amended with supplemental carbon sources, in addition to the formate and acetate normally present. The supplements were tested in media which contained either 2,4,6-TCP, 3-Cl-4OHPA, or no added chlorinated compound. Table 3.5 shows the results of the12supplements on dehalogenation and turbidity.

Cultures grown with cellobiose, maltose, and xylose all produced high turbidity within 24 hours but had no dehalogenation activity. Sub-culturing from these cultures also resulted in the absence of dehalogenation activity. This suggests that the culture contained a sugar-fermenting contaminant which was depleting nutrients and out competing the dehalogenating organism. Microscopic examination of these cultures showed a uniform morphology of gram negative short oval rods which were non-motile. These are consistent with one of the morphologies routinely observed in the stable enrichment culture. These will be referred to as "sugar-bugs."

The β-hydroxybutyrate and fatty acid mixture cultures showed slight turbidity with normal dehalogenation activity as compared to controls. Microscopic examination showed an increased presence of the "sugar-bugs" as well as the normal few examples of the longer rod shaped organism.

*3.2.3.3 **Supplemental Nutrient Sources:*** The benefits of providing supplemental nutrient sources for bacteria are well known. Additionally, it has been shown that dehalogenation activity and extent could be stimulated by the addition of certain nutrients including propionic bacteria fluid, short chain alcohols and acids, pyruvate, yeast extract, trypticase, butyrate, and rumen fluid (Mohn and Tiedje, 1992; Armenante et al., 1995; Fava et al., 1995; Gibson and Suflita, 1990; Kuhn et al., 1990). Rumen fluid alone has been shown to be an excellent source of supplemental amino acids, carbohydrates, fatty acids, peptides, vitamins, and intermediate metabolites (Cote and Gherna, 1994).

To determine the effect of various nutritional supplements on dehalogenation an experiment was performed with individual supplements added to the basal medium. The results of this experiment are shown in Table 3.6.

What is interesting about these data is the noticeably shorter lag time before the onset of dehalogenation in cultures grown with either yeast extract, trypticase, or 50:50 media (50% freshly prepared media and 50% sterilized media which had been previously used to grow the dehalogenating culture). These data suggest that an essential nutrient for dehalogenating population may have been initially absent in the basal medium and had to be synthesized by either the dehalogenator or a contaminant bacterium. When trypticase, yeast extract, or 50:50 media were present this essential nutrient (or a precursor of it) was available to the dehalogenator at an earlier time than in those cultures grown without the supplement. If the essential nutrient was being supplied by a

contaminant population it would explain the difficulty in isolation attempts. The dehalogenating bacterium can not be isolated until all essential nutrients for growth and dehalogenation are included in the basal medium.

Another explanation for the shorter lag time in the case of trypticase and yeast extract is the possibility that these mixed supplements are providing a more suitable carbon source for growth of either the dehalogenating population or symbiotic organism. These two possibilities are addressed in the next section.

In the culture which included succinate as a supplement dehalogenation had not begun after 20 days of incubation. This is consistent with the results of the supplemental carbon source experiment which showed a delay in the onset of dehalogenation when succinate was supplied as an alternate carbon source in a concentration of 50 mM. In this experiment succinate was tested in a concentration of 10 mM and a much smaller inocula was used, which would explain the longer lag time. The culture was not tested for dehalogenation after 20 days.

3.2.3.4 Role of Trypticase in Growth and Dehalogenation: In carbon source and supplemental nutrient source experiments trypticase was seen to stimulate the onset and rate of dehalogenation as compared to acetate/formate control cultures. Two possible explanations for this activity are the use of trypticase as a more suitable carbon source and the possibility that trypticase contains an essential nutrient for dehalogenation which, in the absence of trypticase, must be synthesized by the culture before dehalogenation can begin. In order to determine the role of trypticase in dehalogenation an experiment was performed which correlated the concentration of trypticase in media with dehalogenation

activity, lag time, and rate. The tested values were 1, 0.75, 0.5, 0.25, 0.10, 0.05, 0.01, and 0% trypticase. Also tested were a vitamin mix and a metals/minerals mix. The results of this experiment are shown in Figure 3.6 and Figure 3.7.

Initial dehalogenation rates for all trypticase concentrations from 1% down to 0.01% trypticase are very similar, with 0.05 and 0.01% trypticase cultures showing a slightly decreased rate after day 5. Triplicate cultures grown with 0.00% trypticase shown interesting results. One culture exhibited a dehalogenation rate similar to the trypticase cultures. A second culture showed dehalogenation but at a much slower rate, and the last triplicate did not show dehalogenation activity. These data suggest that the trypticase was not serving as a carbon source, if it had been one would expect dramatically different rates and lag times for dehalogenation with the different concentrations. It appears that the trypticase was providing an essential nutrient for dehalogenation which was needed in a very small concentration. The fact that the dehalogenation rate in the 0.05 and the 0.01% cultures slowed after day 5 indicates that at these low concentrations the essential nutrient becomes a limiting factor.

In the culture grown with the minerals/metals mix dehalogenation took place in two of the triplicates at a reduced rate and did not occur at all in the third culture. This suggests that the essential nutrient carried over from the inocula was being diluted out and was a limiting factor.

The most interesting results are those from the cultures grown with the vitamin mix (different in composition from the mix tested in the supplemental nutrients experiment). The rates of dehalogenation in all three of the triplicates are nearly identical to those of the 1% trypticase cultures. This suggests that the essential nutrient for dehalogenation

could be derived from the vitamin mix as well as from the trypticase. Subsequent culturing from these tubes retained the ability to grow with the vitamin mix as its nutrient source in place of the trypticase. Cultures would only dehalogenate when vitamin mix A and B were both included in the medium, suggesting that it was not a simple vitamin requirement.

It has been reported that *D. tiedjei* has a requirement for 1,4 naphthoquinone in order for dehalogenation to occur. Because of its structure (similar to menaquinone) it is reasonable to theorize that this compound could be used as a component of a respiratory electron transfer chain. (Dehalogenation in *D. tiedjei* has been shown to be linked to formate oxidation in respiration) (Stevens et al., 1988). Considering the possibility of this theory, the complex vitamin requirement in this 2,4,6-TCP dehalogenating culture may be due to the necessary synthesis of a respiratory chain component in order for dehalogenation to occur. The role of dehalogenation in the energy metabolism of this culture is considered in the next section.

3.2.4 Energy Metabolism

Reductive dehalogenation requires an electron acceptor, which in most anaerobic environments constitutes the limiting factor for growth. This 2,4,6-TCP dehalogenating culture has been found to stoichiometrically dechlorinate 2,4,6-trichlorophenol to 4-monochlorophenol at substrate concentrations of up to 1 mM. It is hypothesized that this culture is capable of utilizing 2,4,6-TCP as electron acceptor in the process of reductive

dehalogenation as an energy producing metabolic activity. The experiments discussed in this section address this hypothesis and study the energy metabolism of the active population.

3.2.4.1 Utilization of Alternate Electron Acceptors: In attempt to stimulate growth and determine the effect of alternate electron acceptors on the active population an experiment was set up with individual acceptors (with and without 2,4,6-TCP) and monitored for turbidity and effect on dehalogenation, either stimulation or inhibition. In the first series of experiments various electron acceptors were tested at concentrations of 10 mM, and $FeSO_4$ (plus citrate) was tested in a concentration of 1 mM due to its possible toxicity at higher concentrations. After 16 days dehalogenation had taken place only in the control culture (no added acceptor) and in the $FeSO_4$ culture, which had a shorter lag time than the control. This lack of dehalogenation activity may have been due to the toxicity of the electron acceptors at high concentrations, with the exception of fumarate. The absence of dehalogenation activity within 16 days in the presence of 10 mM fumarate is consistent with the long lag time seen in cultures grown with either 10 or 50 mM succinate. A similar experiment was then performed12with lower concentrations of electron acceptors (0.5 mM), the results of which are shown in Figure 3.8.

Nitrite assays in the KNO_3 culture showed no accumulation of nitrite and on day 14 nitrate was reduced to nitrite with zinc and analyzed. The assay showed that nearly all of the original nitrate (93%) had been utilized by the inoculated cultures, while the uninoculated control showed no loss of nitrate. (Cote and Gherna, 1994) These cultures exhibited no dehalogenation after 30 days of incubation. This suggests that one of the

original contaminants in the culture was a nitrate reducing bacterium which depleted the nutrient supply of the medium before dehalogenation began. (This bacterium is believed to be the motile coccus that was serially diluted out of the culture during MPN experiments.)

Cultures containing sodium sulfate and thiosulfate exhibited dehalogenation rates which were much slower than that of the control cultures. This may be due to the possible toxicity of the sulfur components of these compounds or it may be due to competition for nutrients between the dehalogenating organism and a contaminant population (possibly the nitrate reducing bacterium as some nitrate reducers are also able to reduce sulfate).

The most interesting aspect of this experiment is that, again, the iron containing cultures (in this case Fe_2O_3 plus citrate) had a shorter lag time for the onset of dehalogenation than did the control cultures. It is possible that the iron was being utilized as an alternate electron acceptor resulting in an increase in the population size of the dehalogenating organism. It is also possible that the added citrate was the actual cause for the decrease in lag time, due to its use as a chelating agent or as an alternate carbon source. These possibilities are addressed in the next two sections.

3.2.4.2 The Role of Iron and Chelating Agents in Dehalogenation: To determine the role of iron in dehalogenation an experiment was set up to test the following possibilities: ferric iron as an electron acceptor, ferrous iron as a trace nutrient, and chelating agents'

effect on iron availability. The chelating agents used were citrate, ethylenediaminetriacetic acid (EDTA), and nitrilotriacetic acid (NTA). The results of this experiment are shown in Figure 3.9.

In this experiment, no significant difference was seen in cultures grown with iron compounds alone or with citrate in comparison to control cultures. The decrease in lag time seen in the previous electron acceptor experiment with Fe_2O_3 plus citrate was not sustainable in subsequent cultures. An increase in lag time or absence of dehalogenation was seen in all cultures grown with the chelating agents NTA and EDTA. This may have been due to over-chelation resulting in metal starvation. The chelating agents may have bound the metals so tightly that they became inaccessible to the bacteria. This is the opposite of metal precipitation by reducing agents but it has a similar consequence for the bacteria, the unavailability of essential trace metals.

To determine the effect of different ferric iron compounds on dehalogenation an experiment was set up with the following compounds (0.5 mM): Fe_2O_3 plus citrate, Fe_2O_3 plus NTA, ferric chloride with and without NTA, ferric sulfate with and without NTA, ferric nitrate with and without NTA, ferric citrate with and without NTA, and crystal ferric oxide with and without NTA. The results are shown in Figure 3.10.

The results of this experiment with different types of ferric iron are consistent with those obtained in the previous iron experiment: there was no appreciable difference in cultures with supplemental iron alone versus controls with no added electron acceptor, and cultures which contained NTA exhibited an increase in dehalogenation lag time and a decrease in rate which is attributed to over-chelation of trace metals.

3.2.4.3 Halogenated Compounds Utilized as Electron Acceptors: Table 3.7 lists the halogenated compounds which were dehalogenated by the 2,4,6-TCP dechlorinating culture and the results of the study. The culture demonstrated both ortho- and meta-dehalogenation, no para- dehalogenation was observed. The following chlorophenols were not dehalogenated within the 30 days of incubation: pentachlorophenol, 2,4,5-trichlorophenol, 2,5-dichlorophenol, 3,4-dichlorophenol, 4-monochlorophenol, and 2-monochlorophenol. The structural analogs 2,4,6-tribromophenol and 2,4-dichloroaniline were each dehalogenated to a singly chlorinated product. Interestingly the singly chlorinated compound 3-Cl-4-OHPA was dehalogenated to a non-chlorinated product; an activity not demonstrated with monochlorophenols.

The culture was not able to utilize any of the twelve chlorobenzenes or chlorobenzoic acids tested under these conditions, nor did it transform either perchloroethylene or trichloroethylene.

3.2.4.4 Effect of Inhibitors and Antibiotics on Metabolism: In order to provide information on the metabolic pathways utilized by the dechlorinating population a study was set up with individual substrates which are known to be inhibitory to the growth of different types of microbes. These include BES (bromoethane sulfonic acid: an inhibitor of methyl coenzyme M reductase, the enzyme which catalyzes the final step in methanogenesis), molybdate (a sulfidogen inhibitor), metronidazole (a ferredoxin inhibitor), vancomycin (a broad based12antibiotic) and chloramphenicol (a bateriostatic compound).12BES and molybdate showed no effect on dehalogenation as compared to

control cultures without added inhibitors, suggesting that the dehalogenating organism is neither a methanogen nor a sulfidogen and that the dehalogenating culture is not influenced by organisms belonging to either of these metabolic groups. Both antibiotics vancomycin and chloramphenicol inhibited dehalogenation when added at time=0. When these antibiotics were added to the cultures on day 4, after the onset of dehalogenation, vancomycin stopped dehalogenation but chloramphenicol did not. The vancomycin most likely killed all of the bacterial cells whereas the chloramphenicol only stopped growth. Since the chloramphenicol did not kill the cells which had already begun the dehalogenation activity, the process continued.

One very interesting aspect of these data was the fact that metronidazole, when added at either time=0 or after the onset of activity, proceeded to block dehalogenation. Metronidazole is a ferredoxin inhibitor and its inhibition of activity suggests that dehalogenation may be respiratory linked. When respiration was inhibited, via ferredoxin inhibition, the dehalogenation ceased.

3.2.4.5 Dehalogenation Linked Growth: An MPN experiment series was set up to look for a link between dehalogenation and resulting population size (expressed as biomass concentration). Results are tabulated in Table 3.8. The biomass concentration numbers reported were calculated from established MPN statistical data tables (Harrigan and McCance, 1976). These tables have a number of built in assumptions, including the assumption that when diluting to extinction a single viable cell inoculated into a tube will result in a positive, observable reading. Because of the complex nature of anaerobic consortia and the extremely large 95% confidence12limit ranges, this author hesitates to

draw any conclusions based on the concrete numbers obtained from this type of study. Instead, it is more reliable to focus on the trends observed with these data, rather than the absolute values of growth yield.

The general values reported above agree with earlier observations that dehalogenation activity can be demonstrated in cultures which do not exhibit visible turbidity. Turbidity measurements are typically possible with cultures which contain between one hundred thousand and one million cells per ml of culture medium. As expected, the biomass numbers calculated for the dehalogenating population of this culture fall below this visible turbidity range.

The most interesting aspect of these data is the observation that biomass numbers increase as a function of increasing initial concentrations of chlorinated electron acceptors. This observation supports the theory that growth of the dehalogenating population may be linked to the execution of the dehalogenating activity. The plausibility of this theory is strengthened by the fact that in the case of *D. tiedjei* the oxidation of formate linked to the reduction of 3-chlorobenzoate has been proven to provide energy for growth of the organism. This link is clearly demonstrated in this pure culture but similar observations of dehalogenation-linked growth have also been observed in anaerobic mixed cultures (Holliger et al., 1992).

At first observation, the above values seem to indicate that the dehalogenating population obtains much more useful energy from the dechlorination of 2,4,6-TCP than from the dechlorination of greater concentrations of 3Cl-4-OHPA. This can not be conclusively stated, though, because not enough is known about the actual dehalogenation metabolism of the population. The apparent growth yields of the two

dehalogenation activities can not be directly compared. The enzyme systems for the dehalogenation of the two compounds need to be studied in more detail, preferably when the organism(s) is in pure culture. It is not known whether the dehalogenations involve the same or separate enzyme systems, nor is the effect of toxic metabolites, competitive electron acceptors, or other medium ingredients or requirements clear. Again, the only observation which is valid at this time is the trend for increased concentrations of electron acceptors to produce higher apparent biomass concentrations.

3.3 Conclusions

The TCP dechlorinating culture studied in this investigation is a very interesting example of a population of bacteria which has properties that make it very distinct from others found in the literature. The results also suggest some very interesting and new ideas about the process and potential of anaerobic bacteria performing reductive dehalogenations.

3.3.1 Unique Aspects of This Enrichment Culture

*3.3.1.1 **Enrichment:*** The manner in which this culture was enriched was very different from methods generally reported in the literature. An oxygen shocking technique was employed in the original bio-reactor design which resulted in the enrichment of an oxygen tolerant anaerobic population. Additionally, initial experiments and isolation attempts were performed under micro-aerophilic conditions with no added reducing agent. This also encouraged the cultivation of the oxygen tolerant organisms. As a result of these techniques, experiments dealing with oxygen levels and redox levels indicate that

the active dehalogenating population prefers the less-reduced end of the anaerobic range of environmental conditions. This enrichment procedure encouraged the cultivation of a population believed to be distinct from others found in the literature which were typically enrich under methanogenic conditions. With the employment of strict anaerobic technique, in this investigation dehalogenating population numbers showed steady increase with a corresponding decrease in lag time and increase in dehalogenation rate.

3.3.1.2 TCP Dehalogenation: What is noteworthy about these data is the rate of TCP dehalogenation by a population which is extremely small. After the initial lag phase, cultures inoculated with fewer than 100 dehalogenating cells converted 0.85 mM TCP to MCP within 5 days when 1% trypticase was provided as carbon and electron source, and within about 8 days (after the lag phase) when acetate and formate were provided. By contrast, Fathepure and Vogel (1991) have described an anaerobic biofilm reactor (50x 2.5 cm) that, after 6 to 8 weeks adaptation and 4 to 6 weeks of steady state operation with continuous feeding of the target compound, required 1.5 days to convert 0.26 μM (75ppb) hexachlorobenzene to dichlorobenzene. Nicholson et al. (1992) have described batch experiments of a pentachlorophenol (PCP) acclimated methanogenic consortium in which 5.84 μM 2,4,6-TCP was partially transformed (66%) to 2,4-DCP in 1.5 days. The rate of transformation can be approximated at 3.34 μM in 24 hours for the 2.5 liter batch reactor.

Additionally, the enriched population studied in this investigation has shown a very high level of TCP tolerance and dehalogenation as compared to others found in the

literature. The dehalogenating isolate designated DCB-2 (*Desulfitobacterium hafieinse)* shows a dehalogenation activity limit of 0.3 mM TCP as compared to the capability to dehalogenate 1 mM TCP reported here (Madsen and Licht, 1992; Togna et al., 1995).

3.3.1.3 Anaerobic Metabolism: Data from the carbon source, iron, electron acceptor, and inhibitor experiments provide information about the metabolism performed by this culture and its relationship to dehalogenation activity. The data presented in this study suggests that this culture does not fit into any of the typical metabolic classes of anaerobic bacteria: methanogenic, sulfidogenic, nitrate-reducing, metal-reducing, or fermentative. Data obtained from this organism show that it is unable to use any of the typical anaerobic electron acceptors, yet it is able to dehalogenate a number of chlorinated phenols, bromophenol, chloroaniline, and 3-chloro-4-hydroxyphenylacetate.

These data support the possibility of a new metabolic class of anaerobes, those which perform reductive dehalogenations as their natural respiratory pathway. The existence of naturally occurring halophenols and their documented dehalogenation provide support for this theory (King, 1988).

Additionally, one organism, PER-K23, has been isolated which has been shown to use perchloroethylene as its natural electron acceptor (Holliger et. al., 1995). The ability to use reductive dehalogenation as a respiratory process has been established in *D. tiedjei* and in a number of other cultures.

*3.3.1.4 **Observations Which Suggest Link to Respiration:*** A number of the results obtained from this study support the hypothesis that this culture is able to use reductive dehalogenation as a respiratory process. In experiments which varied the amount of trypticase available to the culture the same lag time was observed with either trypticase or a substituted vitamin mix. The lag time suggests that the organism is not simply using a vitamin but rather synthesizing another essential compound from it or the trypticase. Considering *D. tiedjei's* requirement for 1,4-naphthoquinone it is possible that this culture requires a vitamin which it can use to synthesize a respiratory chain component, possibly a quinone-structure compound (menaquinone, naphthoquinone, etc.).

Another result which suggest a link between dehalogenation and respiration is the fact that when respiration was blocked via ferredoxin inhibition with metronidazole dehalogenation ceased. Lastly, MPN data trends show that increased concentrations of chlorinated compound electron acceptors correspond to an increase in growth yields.

Taken together, these data support the hypothesis that this culture performs reductive dehalogenation as a growth-linked respiratory process. When the organism is isolated in pure culture additional experiments can be performed to confirm this hypothesis.

3.3.2 Significance of these Findings

The above observations suggest that the population studied consists of a distinct dehalogenating organism which is capable of respiratory-linked reductive dehalogenation, possibly as its natural metabolism.

A broader application of this work is that the reductive dehalogenation potential of microbes may be more widespread than is currently thought. Reductive dehalogenation has been shown to be performed by a wide variety of metabolic types of organisms: facultative organism, oxygen tolerant anaerobes, and various strict anaerobes of different anaerobic metabolic types (see chapter two for detailed discussion). Data obtained with this culture as well as those cited from the literature provide evidence that reductive dehalogenation is a capability which is the basis for a distinct metabolic class rather than just an interesting alternate metabolic pathway for anaerobes.

CHAPTER FOUR

THE ROLE OF FERMENTATIVE BACTERIA IN THE REDUCTIVE DEHALOGENATION OF CHLORINATED ALIPHATIC COMPOUNDS

4.1 Materials and Methods

4.1.1 Materials

The chlorinated compounds used in this study, including perchloroethylene, trichloroethylene, and vinyl chloride were obtained from Aldrich Chemical Company, Milwaukee, WI. Dichloroethylene and analyzed standards were obtained from Sigma Chemical Company, St. Louis, MO. Stock solutions of these compounds were prepared by adding the individual compounds to anaerobic distilled water in 160 ml serum bottles with identical stir-bars previously placed inside. These were crimp sealed with Teflon lined rubber stoppers and stirred overnight to ensure that the compounds were completely solubilized and well mixed. Scott Specialty Gas standards for carbon dioxide (pure), methane (pure), and ethylene (100 ppm in helium) were obtained from Supelco Inc., Bellafonte, PA.

The fermentation substrates tested as primary substrates were xanthan gum, Tween-80, Brij-52, xanthine, uric acid, N-Z-Soy Peptone (an enzymatic hydrolysate of peptides), crude DNA, hypoxanthine, karaya gum, polyethylene glycol-60, methanol, and a volatile fatty acid mixture (an anaerobic 10x stock solution prepared of the following: propionic, 0.6 ml/L; n-butyric, 0.4 ml/L; methyl butyric, 0.1 ml/L; iso-butyric, 0.1 ml/L; n-valeric, 0.1 ml/L; methyl valeric, 0.1 ml/L; and iso-valeric, 0.1 ml/L.).

All chemicals were obtained from Sigma Chemical Company, St. Louis, MO. The known structures of these complex primary substrates can be found in Appendix Three (Structures of primary substrates tested in chlorinated aliphatic compound experiments).

All experiments were performed inside of borosilicate glass tubes, obtained from Bellco Glass Company (Vineland, NJ), which were stoppered with aluminum crimp seals and Teflon lined rubber stoppers obtained from The West Company (Phoenixville, PA).

Glass beads, used in sampling, were obtained from Fisher Scientific (Springfield, NJ). Millex nylon 0.22 μm filters were obtained from Fisher Scientific, Springfield, NJ.

4.1.2 Source of Inocula

The microbes utilized in this investigation were obtained from soil taken from a PCE contaminated site. The industrial site had been initially exposed to PCE 15 years prior to sampling. PCE could no longer be detected in the soil, but the presence of dichloroethylene (DCE) and vinyl chloride (VC) suggested that reductive dehalogenation had taken place to some extent but had ceased, possibly due to the lack of an essential nutrient (vitamins, minerals, electron donors, carbon source).

An anaerobic soil slurry was prepared by weighing out 70 grams of the soil (inside of the anaerobic chamber) and diluting this to 500 ml with anaerobic media. This was then well mixed with a magnetic stir-bar inside of the anaerobic chamber.

4.1.3 Anaerobic Medium

The composition of the medium was as follows:

Solution One (per liter media): K_2HPO_4, 0.1 g; KH_2PO_4, 0.1 g; NaCl, 0.2 g; NH_4Cl, 0.1 g; resazurin, 0.1 ml of a 0.1% solution

Solution Two (per liter of media): $MgSO_4$, 0.01 g; $CaCl_2$, 0.01 g

Additional ingredients (per liter of media): $NaHCO_3$, 0.6 g; NaS, 0.05%

Solution one ingredients were added to an Erlenmeyer flask and boiled for fifteen minutes. Sodium bicarbonate and sodium sulfide were then added to the medium which was boiled and degassed for an additional fifteen minutes using a gassing manifold supplying an oxygen free gas mixture of N_2:CO_2 (80:20). Traces of oxygen were removed from the manifold gas by passing through a Sargent-Welch copper oven. The medium was then removed from heat and allowed to cool while degassing for an additional fifteen minutes. Solution two ingredients were added during this time. The flask was then sealed and transferred into a COY anaerobic chamber where media were dispensed under a N_2:H_2 (95:5) atmosphere. The pH of the medium was 7.0.

4.1.4 Microcosm Set-up

All media were dispensed inside a COY anaerobic chamber into 160 ml serum bottles. Each serum bottle contained 135 ml of the basal media, 20 ml of the soil slurry, 4 ml of a PCE stock solution (to bring the final concentration to 20 micromolar PCE), and an individual fermentation substrate being tested at a concentration of 4 millimolar. Molecular weights used for N-Z-Soy Peptone, Brij-52, and xanthan gum were averages supplied by the manufacturer of 1310, 330, and 180 g/L, respectively. Each substrate was

tested in triplicate plus one autoclaved control containing soil, and one autoclaved control without soil. The serum bottles were crimp sealed with Teflon-lined rubber stoppers and incubated at 25°C.

An additional study was also performed with individual compounds which are known to be inhibitory to certain metabolic types of bacteria. These included BES (bromo-ethane sulfonic acid: an inhibitor of methanogenesis), molybdate (an inhibitor of sulfidogens), and vancomycin (a broad based antibiotic). The inhibitory compounds were added in concentrations of 100 μg/ml of media. The effect of these compounds was tested using the soil slurry as an inocula and also using an actively dehalogenating culture as the inocula.

In addition to these microcosms, a study was performed to determine the PCE tolerance level of the actively dechlorinating population. This was performed using N-Z-Soy Peptone as the primary substrate with different concentrations of PCE (25, 65, 125, and 250 micromolar).

4.1.5 Sampling Procedure and Preparation

Three milliliter samples were aseptically drawn from serum bottles using a sterile-nitrogen-purged gas/liquid tight syringe. Sample aliquots of 2 ml were added to 12 ml of distilled water in a 15 ml serum bottle and crimped sealed with a Teflon-lined rubber stopper. The actual headspace volume in the sealed vessel was < 1 ml. These samples were then analyzed by purge and trap gas chromatography to detect and quantify the

presence of chlorinated compounds. The remaining 1 ml aliquots of each sample were placed in glass screw-cap vials and immediately frozen for later analysis of fermentation acids and alcohols.

After sampling, the original 160 ml serum bottles were transferred into the anaerobic chamber. The punctures stoppers were individually removed and the 3 ml sample volume was replaced by the addition of approximately ninety 7 mm autoclaved glass beads. The bottles were then sealed with a new autoclaved Teflon-lined rubber stopper. Each individual bottle was processed (as described) as quickly as possible to minimize loss of substrates and products due to volatilization. The sealed bottles were then returned to 25°C incubators. All autoclaved controls were similarly processed in separate sets using autoclaved glass syringes with Teflon-tipped plungers.

For fermentation acid product analysis, samples were thawed and filtered with Millex 0.22 μm nylon filter discs. Ten microliters of 85% phosphoric acid were added to 0.75 ml samples in vials and immediately analyzed by HPLC (high pressure liquid chromatography).

For fermentation alcohol product analysis, samples were thawed and 1 ml aliquots were diluted with 10 ml of distilled water in 15 ml serum bottles. These 11 ml volume samples were then acidified with 15 μl of 85% phosphoric acid and sealed with Teflon-lined rubber stoppers. These were then analyzed by gas chromatography pressure-balanced headspace sampling using an electronic-pressure-controlled on-column injector.

4.1.6 Chemical Analysis

4.1.6.1 Chlorinated Compounds: Chlorinated compounds were analyzed via gas chromatography. A Tekmar 16 position purge and trap (model 2000 with A/S 2016 autosampler) was used to extract the compounds from the liquid samples. The 5 ml samples were heated at 80°C while being purged with helium, set at 50 ml/minute, for 12 minutes. The samples were then desorbed from the Tenax K adsorbant (250°C for 6 minutes). The purged samples were then analyzed via a Varian 3400 GC equipped with an HP-624 capillary column (30 m x 0.53 mm x 3.0 μm film thickness) using helium as the carrier gas (13 ml/min.). The auxiliary gas and hydrogen gas were set at 17 and 100 ml/min.. The oven temperature was set at 150°C, the injector at 150°C, and the detector at 200°C. The samples were detected using an electrolytic conductivity detector (ELCD) (DI Corporation model 4420) which used n-propanol as an electrolyte.

4.1.6.2 Fermentation Acids: Fermentation acids were analyzed using a Waters 600 HPLC equipped with a Waters 600E system controller and a Waters 717 autosampler. The column used was a Supelcogel C-610H, 30 cm x 7.8 mm i.d. column housed inside of a column jacket set at 30°C. The mobile phase was 0.1% phosphoric acid run at 0.5 ml/min., sparging with 20 ml/min. of helium.

4.1.6.3 Fermentation Alcohols: Alcohols were monitored using a Hewlett-Packard 5890 Series II gas chromatograph equipped with an flame ionization detector (FID). The GC was outfitted to perform pressure-balanced headspace sampling using an electronic-

pressure-controlled on-column injector (Wessels and Schwinger, 1995). The sample bath was set at 80°C, the oven was set at 60°C, and the thermostating temperature was 80°C. The hydrogen/air flow was 30/400 ml/min. and the carrier gas was helium set at 25 ml/min..

4.1.6.4 Gas Analysis: Methane and ethylene gases were analyzed via a Hewlett-Packard 5890 Series II GC equipped with an FID detector. The GC was equipped with an Alltech Hayesep D, mesh 80/100, 10' x 1/8" o.d. x 0.085" i.d. packed column. The column and injector temperature was 45°C and the detector temperature was 200°C. The carrier gas was helium set at 25 ml/min., and the hydrogen/air flow rate was 30/400 ml/min..

4.2 Results and Discussion

4.2.1 PCE Dechlorination

4.2.1.1 Dechlorination with Different Primary Substrates: Figures 4.1 through 4.3 show the effect of the different compounds tested as primary substrates for PCE dechlorination. The data are averages of triplicates. The graphs demonstrate the effect of the different slowly fermentable substrates on the lag time, extent, and rate of dechlorination. The data show that N-Z-Soy Peptone, xanthan gum, polyethylene glycol-60 (PEG-60), Tween-80, xanthine, crude DNA, and the volatile fatty acid mix (VFA) were all able to support dehalogenation of PCE.

Figures 4.4(a) through 4.16(a) show the dechlorination of PCE and appearance and disappearance of daughter products for each of the individually tested substrates. Most notably, the graphs demonstrate the difference between a readily fermentable substrate (N-Z-Soy Peptone - a mixture of peptides, Figure 4.5a) and a more slowly fermentable substrate (xanthan gum, Figure 4.4a) which provides a steady supply of low potential electrons. The low potential electrons supplied by xanthan gum were able to carry the dechlorination of PCE past cis-DCE to vinyl chloride, with no PCE or TCE remaining. No ethylene was detected in any of the samples. Subsequent sampling showed that no ethylene was produced in microcosms which were incubated for over 250 days.

4.2.1.2 PCE Tolerance Level: In addition to the above microcosms, a study was initiated to determine the PCE tolerance level of the actively dechlorinating population. This was performed using N-Z-Soy Peptone as the primary substrate with different concentrations of PCE (from 25 to 250 μM). The active population was found to be able to tolerate and dechlorinate the highest concentration tested, 250 μM. This corresponds to approximately 40 ppm PCE.

4.2.2 Metabolic Pathway Information

4.2.2.1 Fermentation Products: Figures 4.4(b) through 4.16(b) show the appearance and disappearance of different acids produced in microcosms supplied with different slowly fermentable primary substrates. Tables 4.1 through 4.3 list the different trace

alcohols detected in each microcosm and also the gases produced. These tables summarize the fermentation products produced from each primary substrate and any correlation with dehalogenation activity for each substrate.

*4.2.2.2 **Visual Observations:*** Tables 4.1 through 4.3 also show the visual color changes observed for each individual substrate tested. In each case where PCE was dehalogenated, the change from the original medium brown color to black corresponded with the onset of dehalogenation activity. This black color change suggests a possible role of sulfidogenic bacteria in dehalogenation.

*4.2.2.3 **Effects of Inhibitors on Dehalogenation Activity:*** An additional study was also performed with individual compounds which are known to be inhibitory to certain metabolic types of bacteria. These included BES (bromo-ethane sulfonic acid: an inhibitor of methanogenesis), molybdate (an inhibitor of sulfidogens), and vancomycin (a broad based antibiotic). The effect of these compounds was tested using the soil slurry as an inocula and also using an actively dehalogenating culture as the inocula. As expected the vancomycin samples showed no dechlorination. The BES samples showed dechlorination which was comparable to the control set (no compound added) which suggests that the dechlorinating population was neither compromised of nor dependent on methanogenic bacteria. Interestingly, the molybdate completely inhibited dechlorination in both the soil samples and the culture inocula samples. This suggests that the active population may be made up of sulfidogenic bacteria. These sulfidogens may be the actual fermentative bacteria which are utilizing the slowly fermentable substrates and

then switching to respiratory growth coupled to the PCE dechlorination. Another possibility is that the sulfidogenic bacteria are a separate population which is dependent upon the fermentative bacteria for their steady supply of low potential electrons (from the primary substrates) which they are then able to couple to PCE dechlorination. Another scenario would be one where the dehalogenating population is dependent upon the sulfidogenic bacteria; it is possible that the sulfidogenic bacteria maintain the redox value of the medium at a level which allows the dehalogenating bacteria to grow and/or perform reductive dehalogenation.

4.3 Conclusions

The data acquired is sufficient to allow for some general conclusions and to refine the original working hypothesis. The data obtained in this investigation show that the slowly fermentable substrates N-Z-Soy Peptone, xanthan gum, PEG-60, Tween-80, xanthine, crude DNA, and a VFA mix were all able to support the dehalogenation of PCE at least as far as *cis*-DCE. Additionally, the data demonstrate the difference between a readily fermentable primary substrate (N-Z-Soy Peptone) and a more slowly fermentable substrate (xanthan gum) which provided a slow, steady supply of low potential electrons. The electrons supplied by xanthan gum were able to carry the dehalogenation process past *cis*-DCE to vinyl chloride, with no PCE or TCE remaining. This supports the original working hypothesis that the extent of dehalogenation can be influenced by the selection of a primary substrate which will provide a slow constant source of low potential electrons, allowing dehalogenation to proceed past its typical stopping point of DCE. Subsequent work performed by members of this research group (using the same

inocula source as in this study) has shown that when provided with combination of succinate and butyrate this culture was able to partially carry dehalogenation past vinyl chloride to ethylene (Nelson Villarante unpublished thesis work, University of the Philippines, 1996). The key to *in-situ* success with this culture lies in finding a primary substrate which will produce a steady supply of suitable secondary metabolites (possibly succinate and butyrate) to carry PCE dehalogenation to completion, resulting in stoichiometric conversion to ethylene.

In addition to the dehalogenating data, this study has provided some accompanying information about the metabolic types of organisms which constitute the active population. Most obvious was the visual color change in the microcosms which correlated with dehalogenation activity. This change from medium brown to black suggested the involvement of sulfidogenic bacteria in dehalogenation. Their involvement was confirmed in experiments using molybdate as an inhibitor of sulfidogenesis, which proceeded to block dehalogenation. From this study it can not be confirmed whether or not the sulfidogens are the actual dehalogenating bacteria, but it is clear that they are an essential constituent of the active consortium.

Inhibitor studies involving BES revealed that methanogens were not involved in the dehalogenation activity; additionally, in the initial microcosms there was no correlation between methane production or absence and dehalogenation ability.

When viewing this reported data along with the recent literature on PCE degradation, it is evident that reductive dehalogenation is performed under a wide variety of conditions and that diverse metabolic types of bacteria play roles in the degradation activity and resultant extent of dehalogenation. Methanogens have been well documented

as bacteria capable of reductive dehalogenation of PCE, largely as a cometabolic activity (Vogel and McCarty, 1985; Belay and Daniels, 1987; Fathepure and Boyd, 1988; Freedman and Gossett, 1989; de Bruin et al., 1992). A link between sulfidogenic bacteria and dehalogenation has been shown in this investigation; additionally, Bagley and Gossett (1990) have shown PCE dechlorination activity in a sulfate-reducing enrichment culture.

In addition to these methanogenic and sulfidogenic cultures, dehalogenation of PCE has been reported to be a primary respiratory process for both mixed and pure cultures of anaerobes including *Dehalobacter restrictus, Dehalospirillum multivorans, Desulfitobacterium* sp. strain PCE1, and an H_2-utilizing enrichment culture (Holliger et al., 1993; Schumacher and Holliger, 1996; Neumann et al., 1994; Scholz-Muramatsu et al., 1995; Gerritse et al., 1996; Maymo-Gatell et al., 1995).

The enhancement of reductive dehalogenation of chlorinated compounds (including PCE) through the addition of common fermentation products such as short chain organic acids and alcohols, and mixed fatty acids suggests a possible role for fermentative bacteria in *in-situ* processes (Gibson and Suflita, 1990;Gibson and Sewell, 1992; Gibson et al., 1994). This investigation provides an introduction into the potential role of fermentative in the degradation of PCE, which is especially promising for the area of *in-situ* treatment of PCE or TCE. This involves starting at the beginning of the ecological electron chain, with the fermentative bacteria, and duly manipulating the extent and end products of dehalogenation. The key lies in working both backward and forward to determine primary substrates for a site which will allow fermentative bacteria to produce the desired secondary substrates which can then be used for dehalogenation.

This study demonstrates the potential of this approach, now it is necessary to systematically test primary substrates to find ones suitable for providing the steady source of low potential electrons needed for dehalogenation to proceed most effectively.

In order for *in-situ* work to be most successful, it may be necessary for laboratory research to focus more on the microbial ecology and interactions of a site rather than trying to target only one particular metabolic type of organism (ex. methanogens). Gently directing the natural ecology of a site may provide to be more effective, predictable, and sustainable for *in-situ* remediation.

CHAPTER FIVE

GENERAL DISCUSSION

The main purpose of this thesis inquiry was to learn more about the process of reductive dehalogenation as performed by anaerobic bacteria. The investigation included research on the dechlorination of both aromatic (TCP) and aliphatic (PCE) compounds in both an enriched culture and in undefined microcosms. From these studies a number of conclusions can be drawn, which result in the formation of two primary hypotheses: (1) in addition to the traditional metabolic types of anaerobic bacteria studied it is likely that organisms have evolved which use naturally occurring halogenated compounds as their principal electron acceptors, (2) in the case of *in-situ* biodegradation it may be more effective to consider the microbial ecology of a site rather than trying to focus on the manipulation of a single metabolic class of anaerobic organisms. These are two particularly interesting points which deserve further discussion and investigation.

5.1 Reductively Dehalogenating Bacteria as a Distinct Metabolic Class

As reviewed in chapter one, there is a wide diversity of halogenated compounds which occur in natural environments as a result of the metabolic processes of marine organisms, fungi, insects, etc.. Because of their unique ability to adapt and use whatever is available to their nutritional advantage, it is likely that bacteria have developed with the ability to use these compounds as electron donors (which are typically limiting factors in anaerobic environments.) In fact, it has been shown that

bacteria do indeed have this ability to reductively dehalogenate naturally occurring compounds in the environment (King, 1988). Additionally, it has been proposed and demonstrated in pure culture that at least one bacterium uses a chlorinated compounds as its natural electron acceptor. This bacterium, *Dehalobacter restrictus* (formerly PER-K23), uses perchloroethylene as its electron acceptor. It has been unable to use any other electron acceptor tested, including the traditional anaerobic electron acceptors on which metabolic classification is based. Also, it is unable to grow fermentatively on any of a number of compounds tested (Holliger et al, 1993; Schumacher and Holliger, 1996).

The bacteria studied in this TCP investigation appear to fall into a similar category. They will reductively dehalogenate 2,4,6-trichlorophenol, and a number of other halogenated compounds, in a act which is likely a growth-linked respiratory process. Interestingly, they have been unable to utilize any of the traditional electron acceptors on which anaerobic bacteria typically rely.

Upon isolation, it is likely that the dehalogenating bacterium in this culture will prove to be one which evolved using a naturally occurring halogenated compound as its primary electron acceptor. One obstacle complicating the isolation of this organism seems to be in defining the exact nutritional requirements of the organism. At this time it seems that the dehalogenating organism may have a requirement for an as yet undefined carbon source or other nutrient in order to achieve turbidity in laboratory cultures.

In a review by Mohn and Tiedje (who have been involved in much of the research which has elucidated reductive dehalogenation as a respiratory process in anaerobic bacteria) the authors point out that reductively dehalogenating bacteria often have unusual nutritional requirements not seen with other classes of anaerobes (ex. *D. tiedjei*'s requirement for 1,4-naphthoquinone; *D. restrictus'* requirement for a fermented yeast extract solution) (DeWeerd et al., 1991; Holliger et al., 1993). The review also points out that reductively dehalogenating organisms often can not be cultivated on typical rich media formulations used for more familiar types of anaerobes (Mohn and Tiedje, 1992).

The availability of naturally occurring halogenated compounds and the unique nutritional requirements and activities of known reductively dehalogenating bacteria suggest that these bacteria may have evolved as a unique group of respiratory anaerobes which need to be distinguished in their own metabolic class.

5.2 Focus on Microbial Ecology and Interactions as a Tool for *In-situ* Biodegradation Studies

The approach of the PCE investigation in this thesis was to provide slowly fermentable compounds, which are not widely used by bacteria, as a source of low potential electrons to be coupled to the reductive dehalogenation of perchloroethylene. The microcosm experiments were designed to attempt to utilize the metabolic pathways of fermentative bacteria, which typically compromise the dominant populations in anaerobic environments, in order to optimize the dechlorination process. Results of this study demonstrated that the bacteria in these cultures could indeed utilize the slowly

fermentable substrates as electron sources which carried out the dehalogenation of PCE to varying extents. Interestingly, inhibitor studies showed that in addition to these fermentative bacteria sulfidogenic bacteria also played a vital role in the dehalogenation process. It is not clear from this study whether the sulfidogens are the actual dehalogenators or fermentative bacteria themselves, or if they are somehow nutritionally dependent upon one another. It seems most likely that the fermentative bacteria are breaking down the primary substrates into secondary substrates which are eventually used as, or transformed into, electron donors for dehalogenation. As stated in the conclusions section, the key lies in finding primary substrates which will produce a steady supply of suitable resultant substrates which will allow dehalogenation to proceed optimally. This is a good example of how focusing on bacterial interactions may yield more favorable results than focusing on one metabolic type of anaerobe.

A paper by Gibson and Suflita yields supporting data. The study, performed with a chlorophenoxyacetate dehalogenating culture, demonstrated that the addition of butyrate to cultures increased the transformation of mono- and di-chlorinated phenoxyacetate from less than 37% to more than 78%. The study also found that the addition of short-chain organic acids and alcohols (butyrate, crotonate, ethanol, or propionate) would increase the amount of 3-chlorophenoxyacetate transformed to over 2 - 5 times that transformed in unamended cultures. The authors concluded that reductive processes can be stimulated by the oxidation of reduced cosubstrates (Gibson and Suflita, 1993). These reduced cosubstrates could be supplied by consortia bacteria other than the active dehalogenator in a manner similar to that believed to be functioning in the fermentation substrate investigation of this thesis research. In these

two cases, isolation of the active bacterium could yield useful information at the possible expense of decreased activity. Consequently, when investigating the possibility of *in-situ* work it is especially important to look at the interactions and microbial ecology of a site rather than trying to focus only on a specific metabolic type of organism.

5.3 Implications of these Findings

Microbiological evaluation of individual bacterial culture components is absolutely necessary for the long term goal of understanding and optimizing biodegradation processes and abilities. At the same time, the fields of microbiology and environmental science are moving towards more *in-situ* applications of these processes. Therefore, while performing these studies it is necessary to keep in mind the microbial ecology of a site including: the possibility of naturally-occurring chlorinated-compound dehalogenating organisms, unique nutritional requirements of dehalogenating organisms, the potential roles of fermentative bacteria, as well as other bacterial interactions.

Reductively dehalogenating bacteria, with their unusual electron donors, electron acceptors, nutritional requirements, and consortia interactions, are in the process of emerging as a distinct class of anaerobes. Further study of both pure cultures and natural consortia is necessary in order to realize the full potential of these organisms for both *in-situ* and *ex-situ* biodegradation applications.

APPENDIX ONE

TABLES AND FIGURES FOR EXPERIMENTS INVOLVING CHLORINATED AROMATIC COMPOUNDS

Table 3.1 Reducing agents used in redox experiments.

Reducing Agent	E_0' (mV)	Concentration in media
No added agent	>-51	-
Sodium thioglycolate	<-100	0.050%
Cysteine-sulfide	-200	0.025%
$Na_2S \cdot 9H_20$	-270	0.025%

Table 3.2 Combinations of experiments using trypticase.

Tube (Trypticase %)	Solution A	Solution B	Trypt. Solution	Vitamin Mix 1	Vitamin Mix 2	Minerals Mix
1 (1.00%)	5 ml	3.00 ml	2.00 ml	-	-	-
2 (0.75%)	5 ml	3.50 ml	1.50 ml	-	-	-
3 (0.50%)	5 ml	4.00 ml	1.00 ml	-	-	-
4 (0.25%)	5 ml	4.50 ml	0.50 ml	-	-	-
5 (0.10%)	5 ml	4.80 ml	0.20 ml	-	-	-
6 (0.05%)	5 ml	4.90 ml	0.10 ml	-	-	-
7 (0.01%)	5 ml	4.98 ml	0.02 ml	-	-	-
8 (0.00%)	5 ml	5.00 ml	-	-	-	-
9 (0.00%)	5 ml	4.90 ml	-	0.05 ml	0.05 ml	-
10 (0.00%)	5 ml	4.95 ml	-	-	-	0.05 ml

Table 3.3 Combinations of experiments using iron plus different chelating agents.

Tube	Fe_2O_3	$FeSO_4$	Citrate	EDTA	NTA
1	-	-	-	-	-
2	-	-	**0.5 mM**	-	-
3	-	-	-	**0.5 mM**	-
4	-	-	-	-	**0.5 mM**
5	**0.5 mM**	-	-	-	-
6	-	**0.5 mM**	-	-	-
7	-	**0.5 mM**	**0.5 mM**	-	-
8	**0.5 mM**	-	**0.5 mM**	-	-
9	**0.5 mM**	-	-	**0.5 mM**	-
10	**0.5 mM**	-	-	-	**0.5 mM**

Table 3.4 Chlorinated compound stock solutions used in dehalogenation experiments.

Prepared in dH2O:	Prepared in 0.05 N NaOH:	Prepared in methanol:
chlorobenzoic acids:	chlorophenols:	chlorobenzenes:
2-CBA	2-MCP	1,2-DCB
3-CBA	4-MCP	1,4-DCB
4-CBA		
	2,3-DCP	1,2,3-TCB
2,5-diCBA	2,4-DCP	1,2,4-TCB
2,6-diCBA	2,5-DCP	1,3,5-TCB
3,4-diCBA	2,6-DCP	
	3,4-DCP	1,2,3,4-tertCB
perchloroethylene	2,3,4-2,4,6-TCP	
trichloroethylene	2,3,6-2,4,6-TCP	2,4-dichloroaniline
	2,4,5-2,4,6-TCP	
	2,4,6-2,4,6-TCP	
2,4,6-tribromophenol		
	pentachlorophenol	
3-chloro-4-OHPA		

Table 3.5 Effect of supplemental carbon sources on dehalogenation and turbidity.

Supplemental Carbon Source	2,4,6-TCP (dehalogenation)	3Cl-4-OHPA (dehalogenation)	Turbidity: 2,4,6-TCP/3Cl-4-OHPA	Turbidity With No Chlorinated Compound Added
control (acetate/formate)	standard dehalogenation (days 4-6)	standard dehalogenation (days 4-5)	none	none
sucrose	standard	standard	none	none
cellobiose	none	none	both high	both high
maltose	none	none	both high	both high
xylose	none	none	both high	both high
b-hydroxybutyrate	standard	standard	both slight	both slight
succinate	longer lag time (proceeded days 6-8)	longer lag time (proceeded days 5-7)	none	none
fatty acid mix	standard	standard	both slight	both slight
2x acetate/formate	standard	standard	none	none

Table 3.6 Effect of supplemental nutrients on dehalogenation and turbidity.

Supplemental nutrients	Dehalogenation	Turbidity
control (acetate/formate)	standard (days 8-15)	none
vitamin mix	standard	none
metals/minerals	standard	none
rumen fluid	standard	none
succinate	none (in 20 days)	none
monosodium glutamate	longer lag time (days 13-20)	none
yeast extract	shorter lag (days 7-13)	none
trypticase	shorter lag and time (days 7-11)	none
50:50 media	shorter lag and time (days 7-11)	none

Table 3.7 Halogenated compounds dehalogenated by the 2,4,6-TCP dechlorinating culture.

Compound	Percent dehalogenation after 8 days	Percent dehalogenation after 20 days	Percent dehalogenation after 30 days	Products of dehalogenation
2,3,4-trichlorophenol	71	90.3	96.3	3,4-DCP
2,3,5-trichlorophenol	80	95	97.3	3,5-DCP
2,4,6-trichlorophenol	100	100	100	4-MCP via 2,4-DCP
2,6-dichlorophenol	0	92.5	94	2-MCP
2,3-dichlorophenol	0	92.7	95.6	3-MCP
2,4-dichlorophenol	50	100	100	4-MCP
2,4,6-tribromophenol	30	46	100	4-MBP via 2,4-DBP
2,4-dichloroaniline	20	70	100	4-chloroaniline
3-Cl-4-OHPA	80	100	100	4-hydroxyphenylacetate

Table 3.8 Effect of initial electron acceptor concentration on biomass number concentration.

Initial Electron Acceptor Concentration	Growth Yield (cells/ml)	95% Confidence Limit
0.25 mM 2,4,6-TCP	**4930**	**2350 - 10,340**
0.5 mM 2,4,6-TCP	**23,970**	**20,060 - 59,490**
1 mM 2,4,6-TCP	**49,300**	**23,500 - 103,400**
1 mM 3Cl-4-OHPA	**1723**	**927 - 3202**
4 mM 3Cl-4-OHPA	**10,700**	**4000 - 28,500**
8 mM 3Cl-4-OHPA	**32,500**	**16,200 - 65,000**

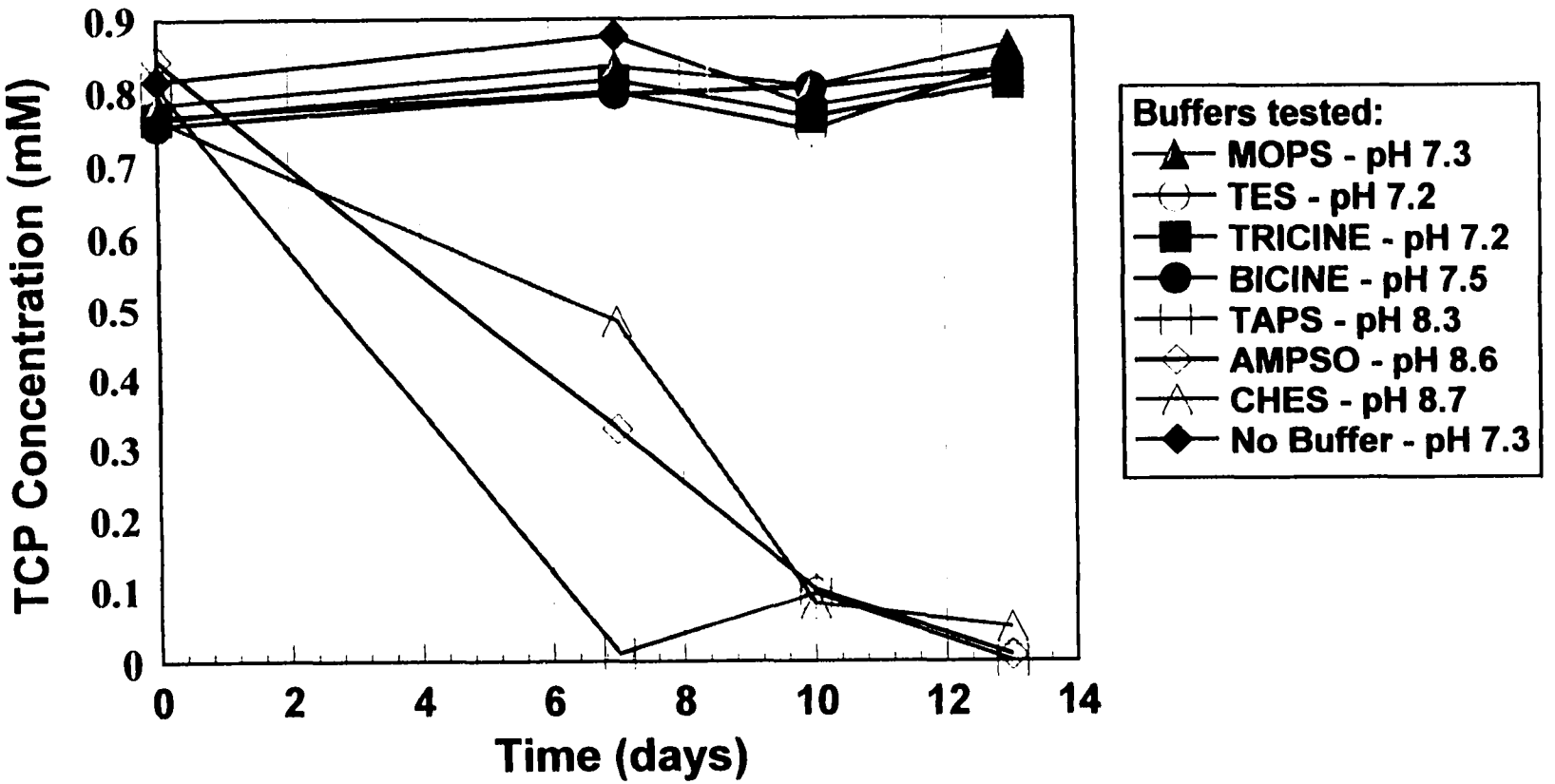


Figure 3.1 Effect of pH on the Dehalogenation of 2,4,6-Trichlorophenol

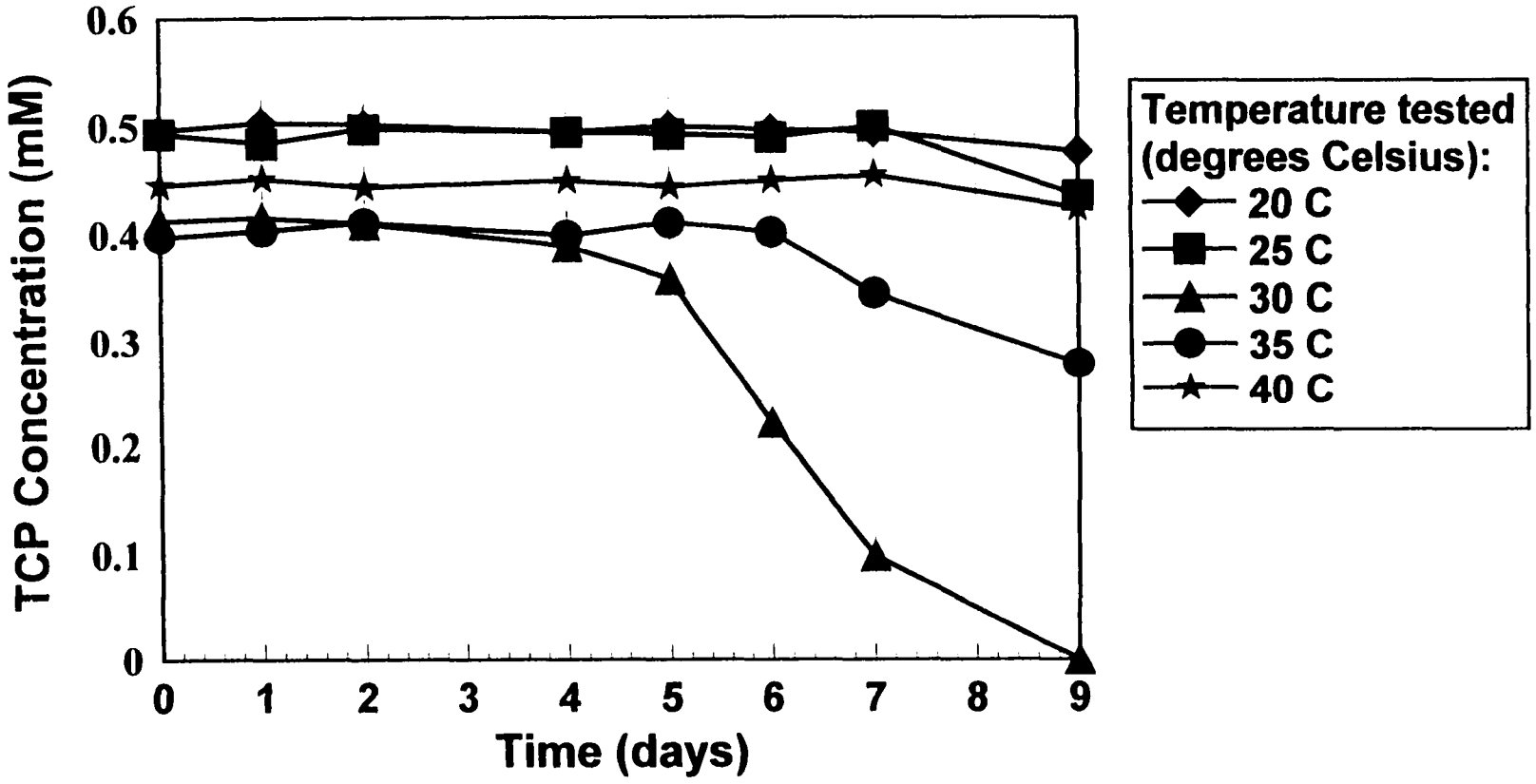


Figure 3.2 Effect of Temperature on 2,4,6-Trichlorophenol Dehalogenation

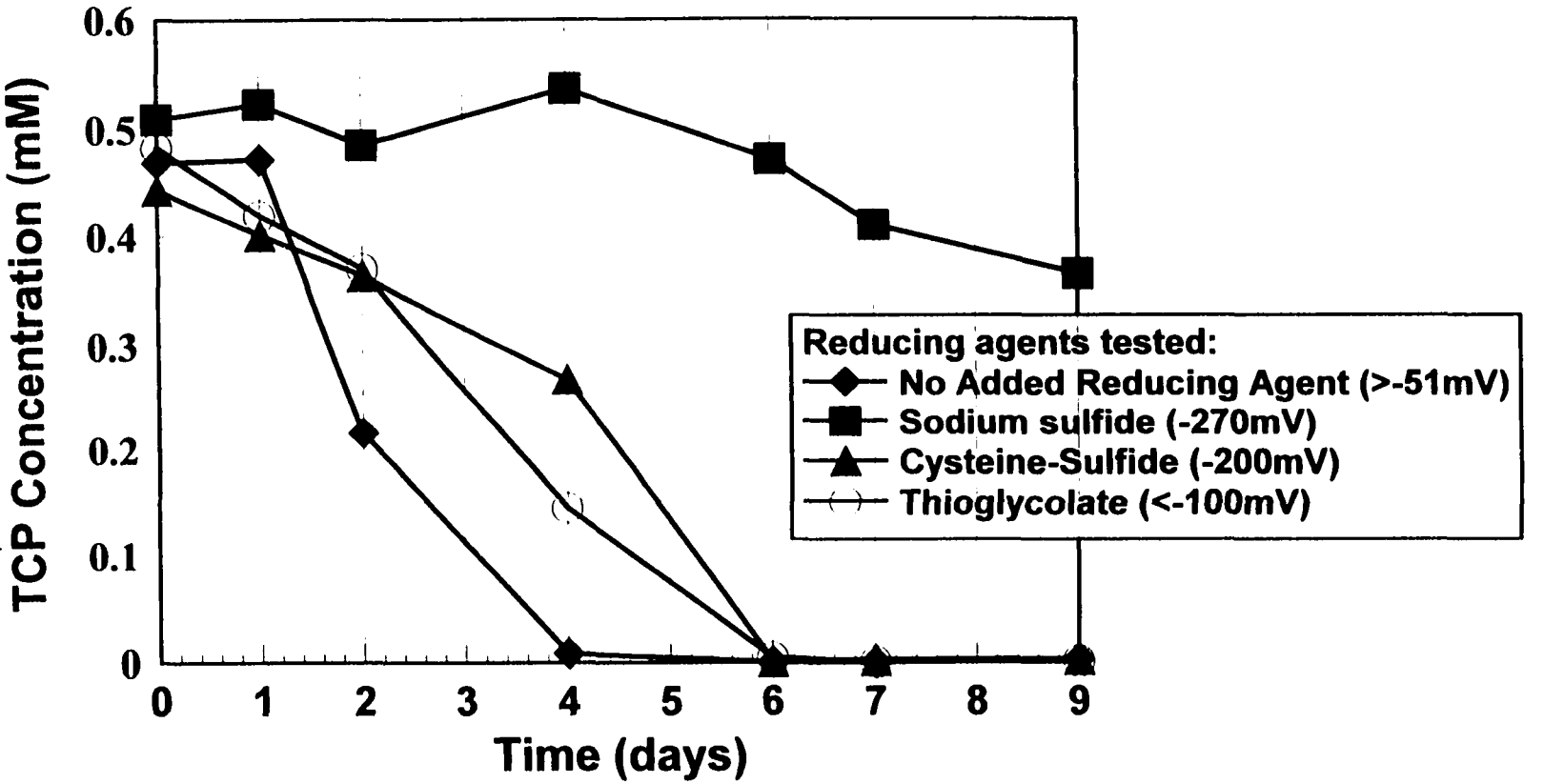


Figure 3.3 Effect of Initial Redox Level on 2,4,6-Trichlorophenol Dehalogenation

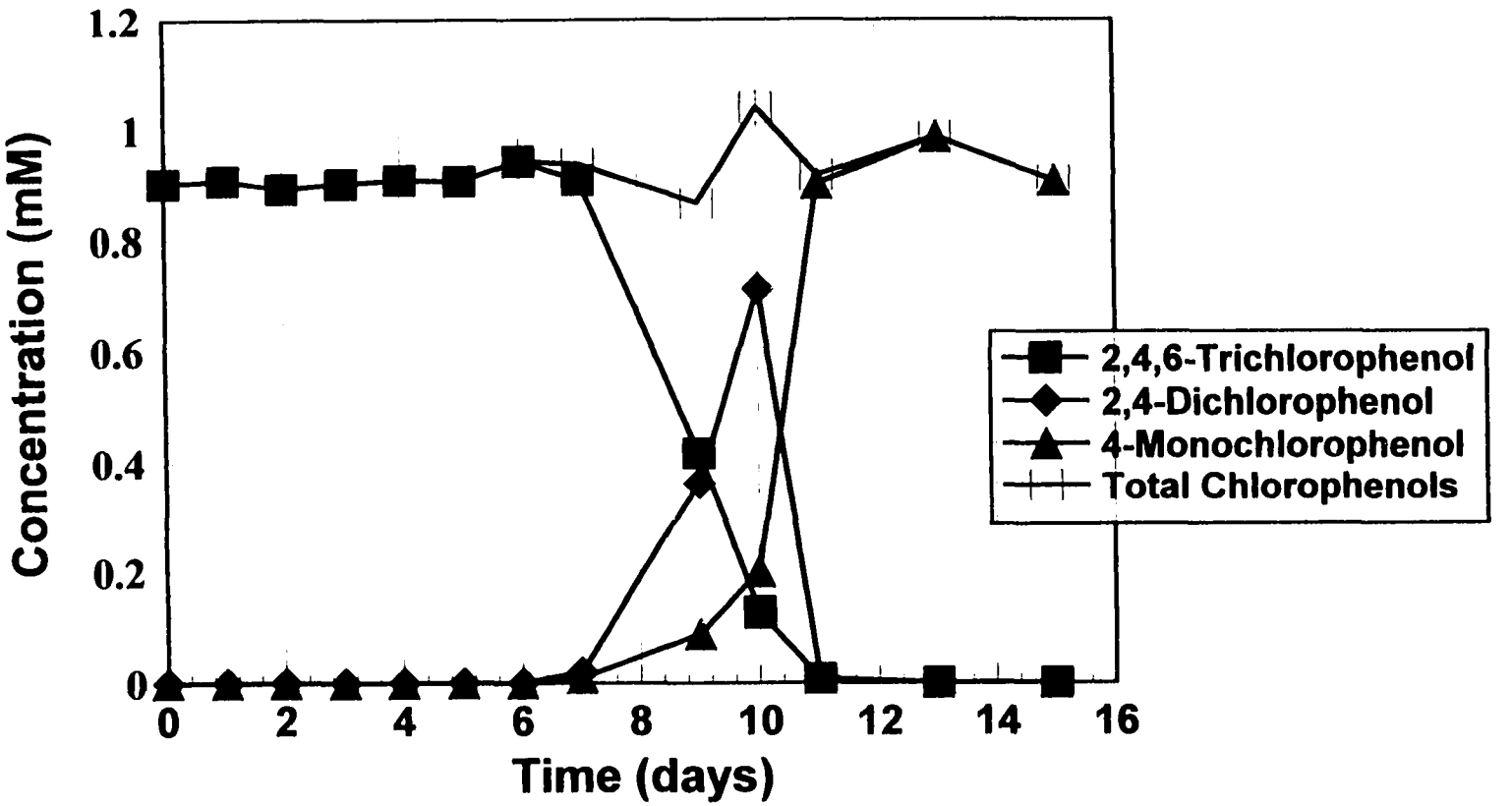


Figure 3.4 2,4,6-Trichlorophenol Dehalogenation with Trypticase

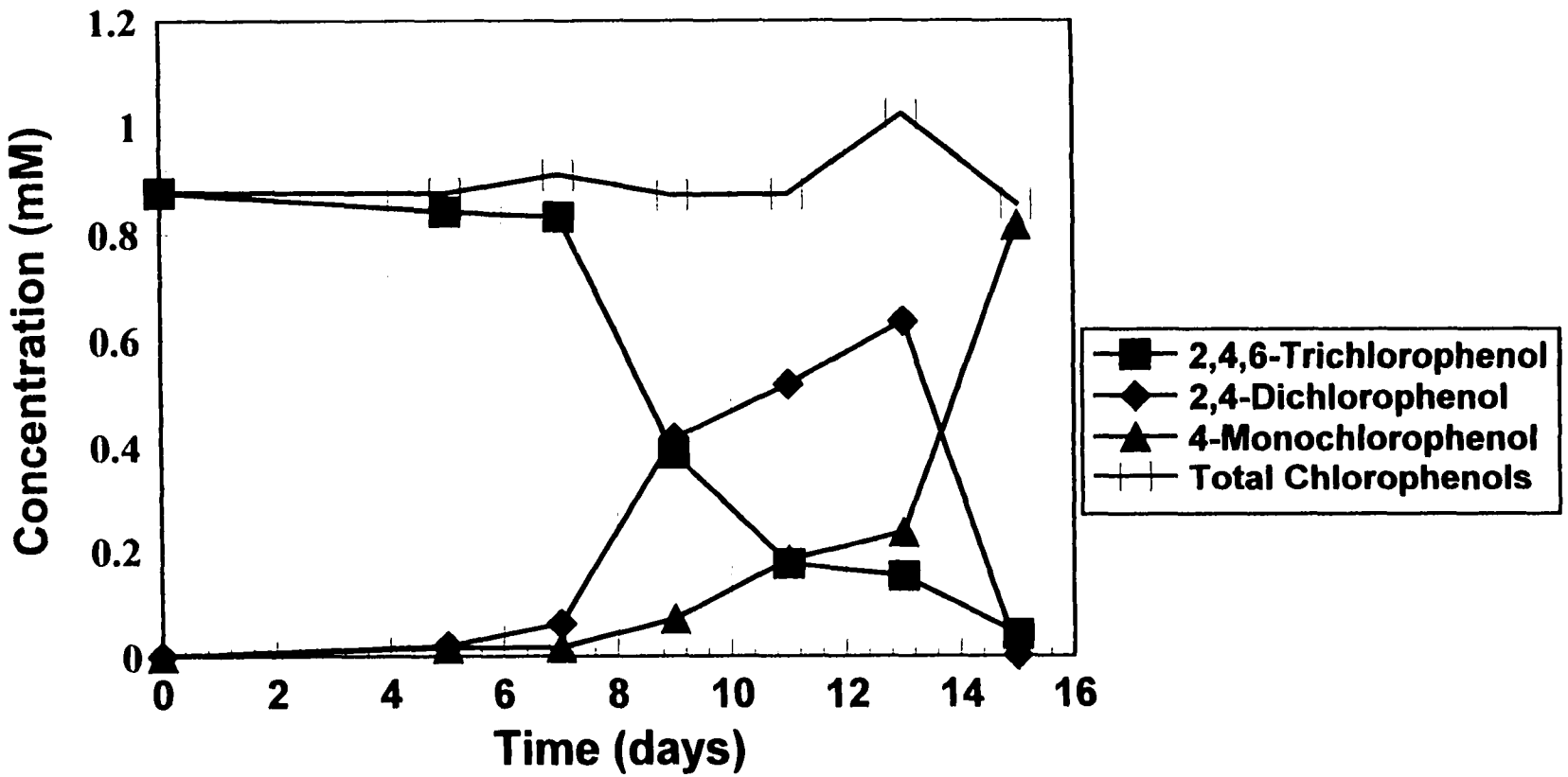


Figure 3.5 2,4,6-Trichlorophenol Dehalogenation with Acetate plus Formate

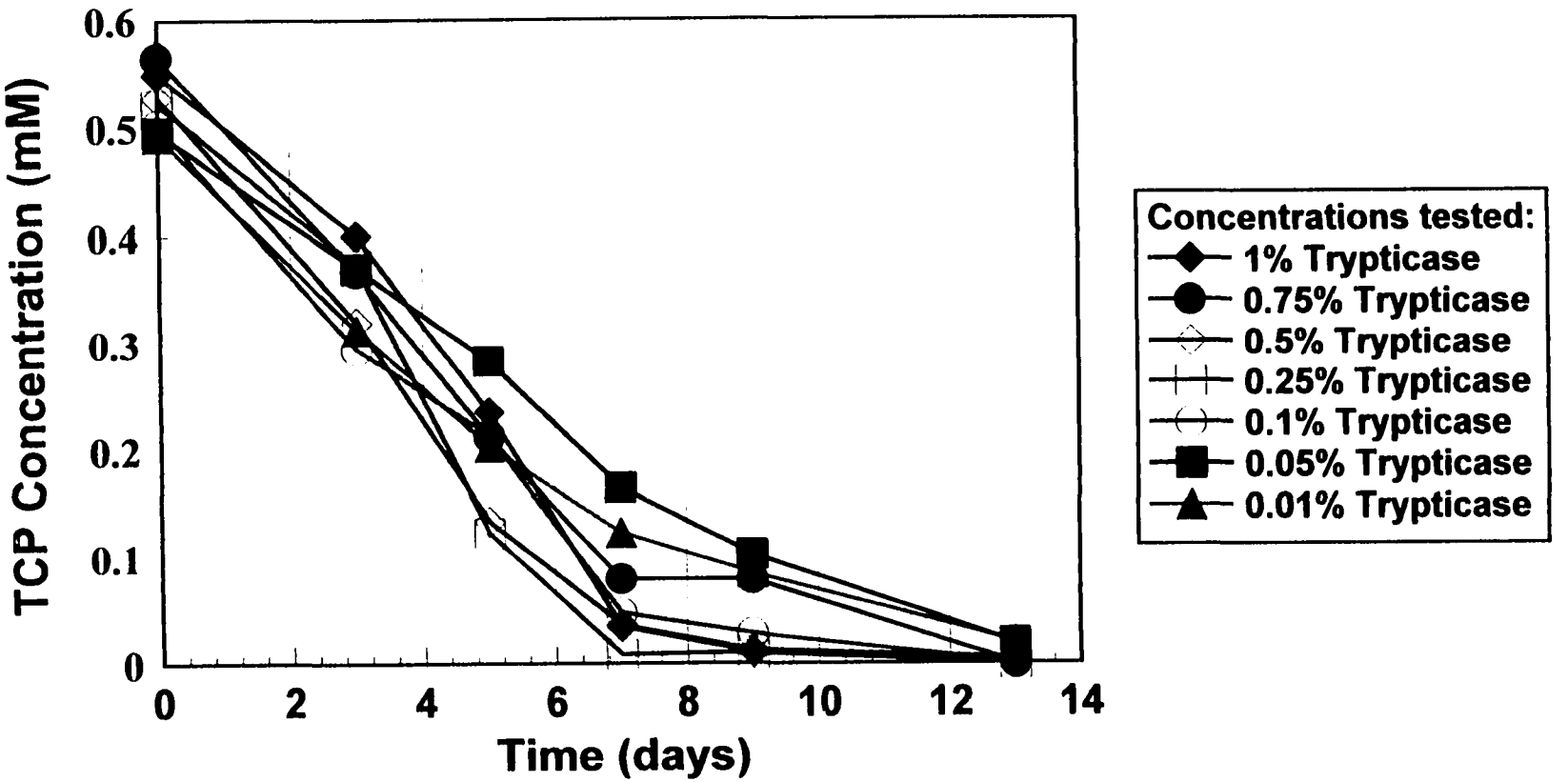


Figure 3.6 Effect of Trypticase (Concentrations: 0.01% -1%) on Dechlorination of 2,4,6-TCP

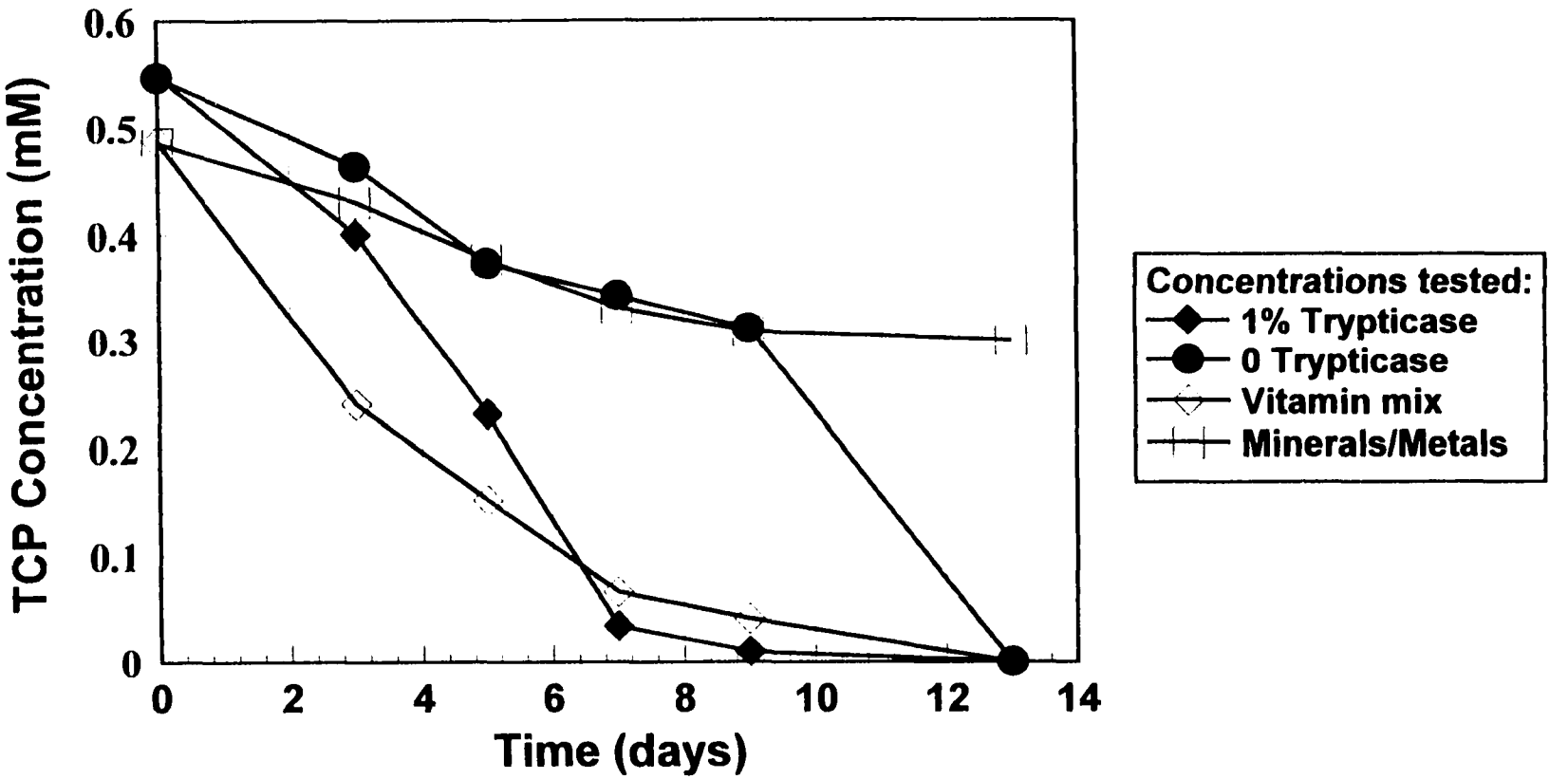


Figure 3.7 Effect of Vitamins and Minerals/Metals Mixture on the Dechlorination of 2,4,6-TCP

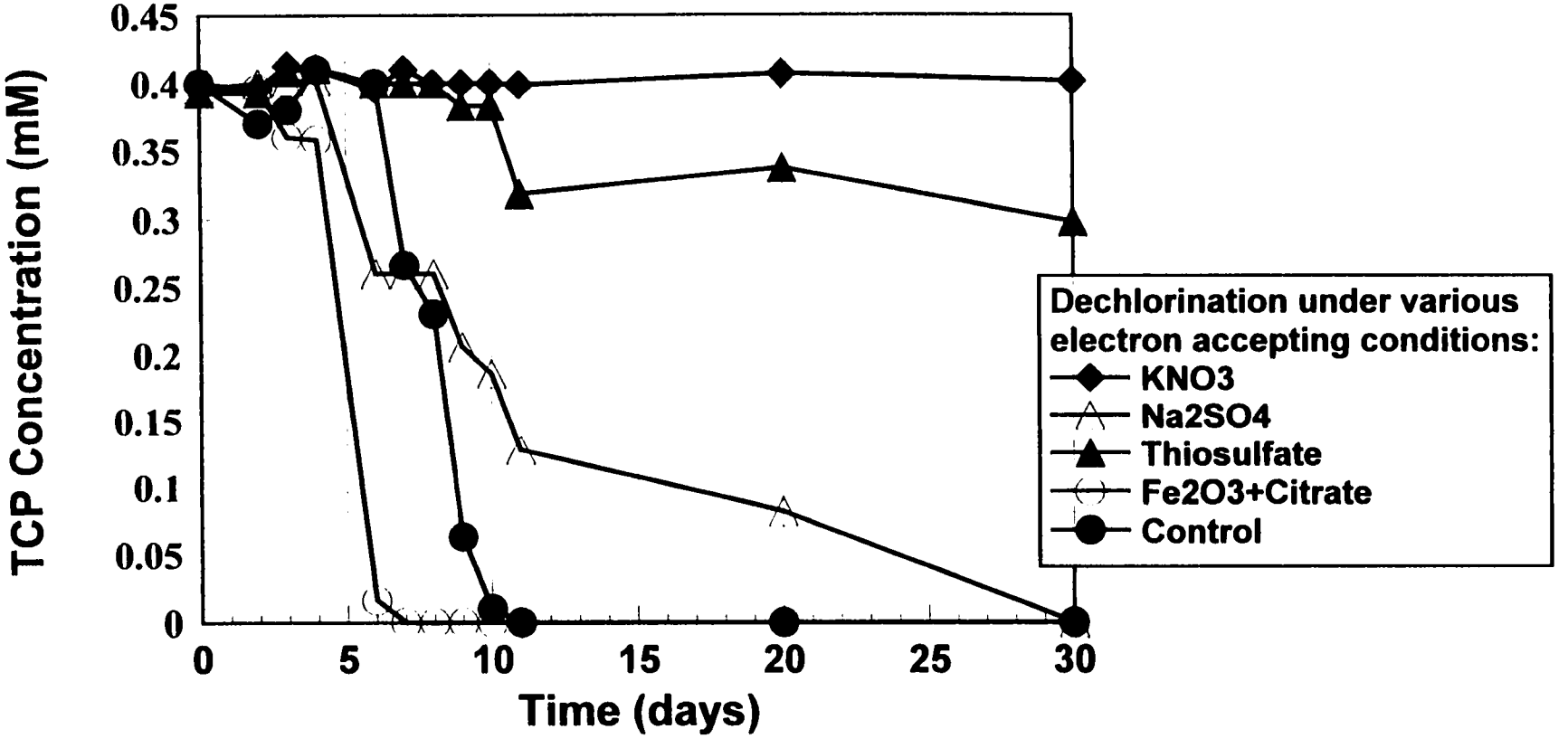


Figure 3.8 Effect of Electron Acceptors on 2,4,6-Trichlorophenol Dehalogenation

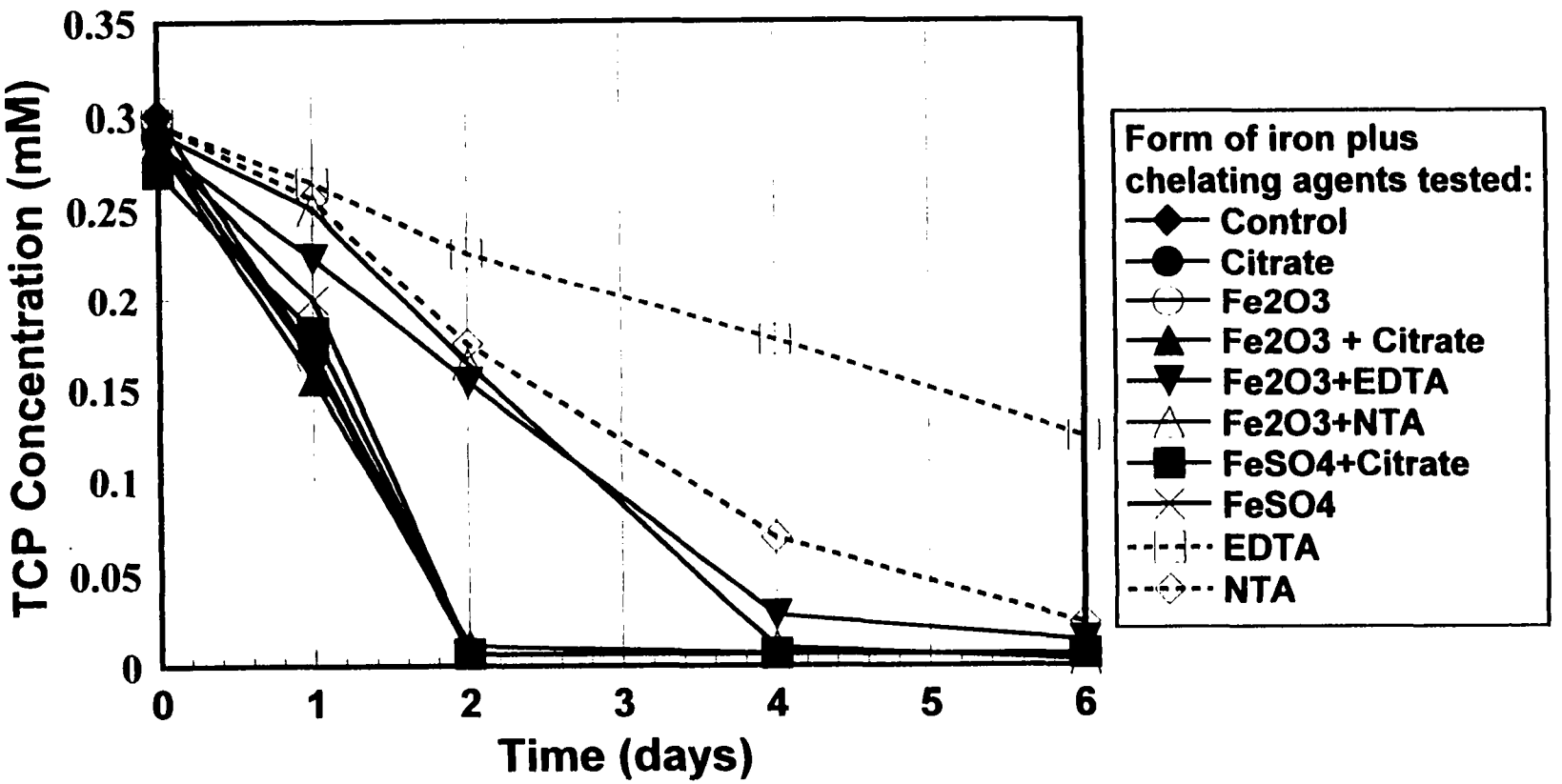


Figure 3.9 Effect of Iron with Different Chelating Agents on 2,4,6-Trichlorophenol Dehalogenation

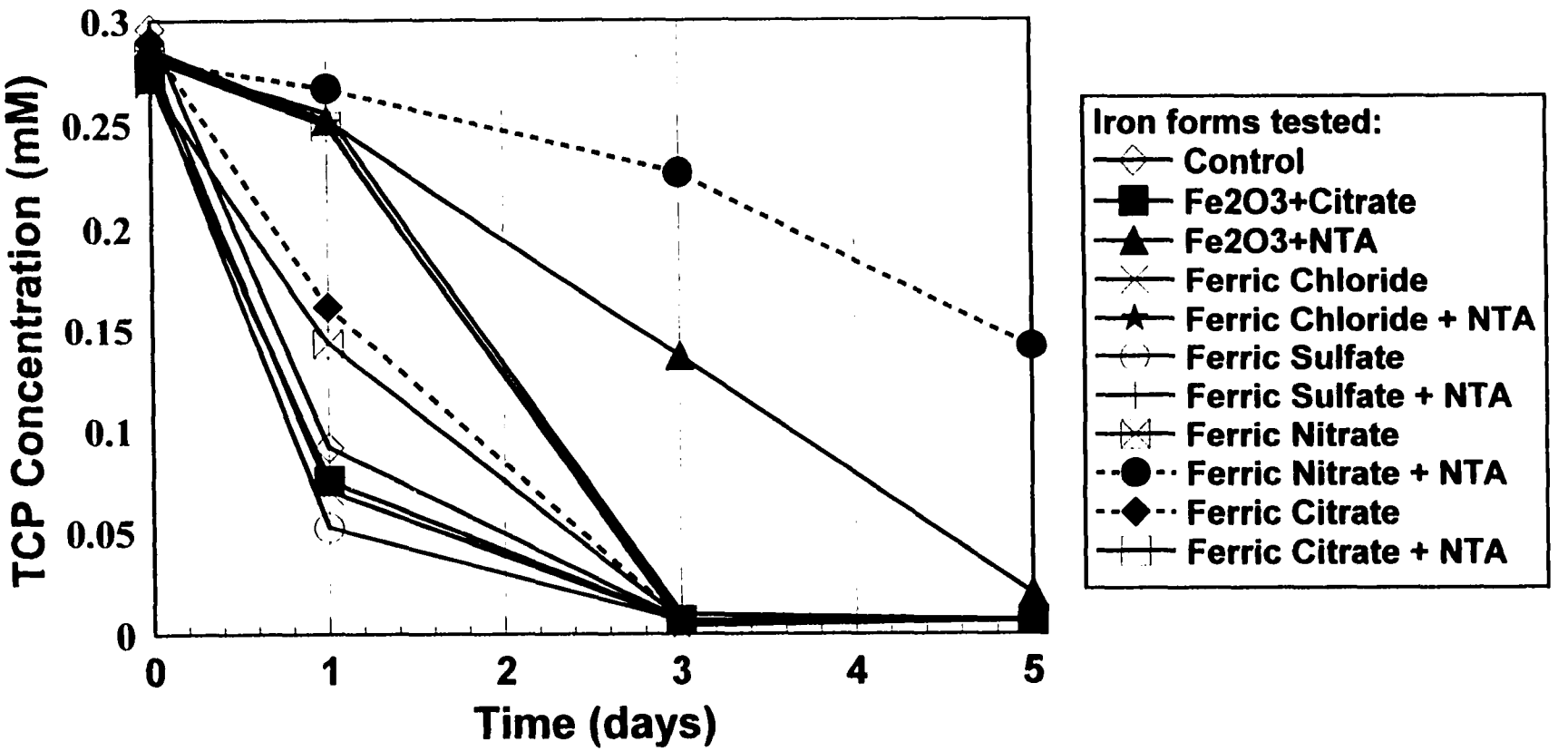


Figure 3.10 Effect of Different Types of Iron w/wo Chelating Agents on 2,4,6-Trichlorophenol Dehalogenation

APPENDIX TWO

TABLES AND FIGURES FOR EXPERIMENTS INVOLVING CHLORINATED ALIPHATIC COMPOUNDS

Table 4.1 Summary of Observations (Set one)

Compound	Acids Produced	Alcohols Produced	Methane Produced	Visual Observation	Chlorinated Compounds Produced	Comments
No Substrate	trace acetic	trace methanol and propanol	none	brown	none	
Methanol	none	methanol decreases from 4 mM to 0.5 mM; trace ethanol and propanol	none	brown	none	
Hypo-xanthine	up to 4 mM acetic; up to 0.5 mM propionic; trace succinic	trace butanol, propanol, methanol	none	brown	none	
Uric Acid	up to 7.8 mM acetic; up to 2 mM formic	trace methanol	none	brown	none	
N-Z-Soy Peptone	up to 25 mM iso-butyric; up to 10 mM acetic; trace propionic and succinic	up to 1.3 mM methanol, 0.8 mM ethanol; 0.77 mM propanol; 0.26 mM butanol	none	black appearance starting with sampling period prior to onset of dehalo. activity	cis-DCE	acetate level steady at 4 mM during dehalo. activity; steady at 8 mM after dehal. activity ceases; substantial amounts of unidentified compounds detected in acid analysis during dehal. activity
Xanthan Gum	up to 2.5 mM acetic; up to 0.5 mM succinic	up to 0.28 mM propanol	3.6 mM	black appearance starting with sampling period prior to onset of dehalo. activity	cis-DCE, vinyl chloride	higher acetate levels during dehal. from PCE to DCE; less acetate and trace succinate during dehalo. From DCE to VC

Table 4.2 Summary of Observations (Set two)

Compound	Acids Produced	Alcohols Produced	Methane Produced	Visual Observation	Chlorinated Compounds Produced	Comments
Polyethylene Glycol-60	up to 13 mM acetic	trace butanol	5.19 mM	black appearance starting with sampling period prior to onset of dehalo. activity	cis-DCE	appearance and disappearance of butanol and TCE coincide
Tween-80	up to 1.2 mM acetic	trace butanol, propanol, methanol	3.9 mM	black appearance starting with sampling period prior to onset of dehalo. activity	cis-DCE	appearance and disappearance of butanol and TCE coincide
Brij-52	none	trace methanol and propanol	none	gray/brown	none	
Xanthine	up to 3 mM acetic; trace succinic and formic	trace methanol and propanol	0.4 mM	gray/brown	cis-DCE	
Karaya Gum	trace succinic	up to 0.8 mM methanol, trace propanol	none	gray/brown	none	

Table 4.3 Summary of Observations (Set three)

Compound	Acids Produced	Alcohols Produced	Methane Produced	Visual Observation	Chlorinated Compounds Produced	Comments
Crude DNA	**up to 4 mM propionic**	**up to 1.3 mM methanol, trace propanol**	**0.34 mM**	**black appearance starting with sampling period prior to onset of dehalo. activity**	**cis-DCE**	
VFA Mix	**trace succinic**	**trace propanol, high methanol (cannot calibrate because of methane overlap)**	**0.33 mM**	**turned gray, then turned black with sampling period prior to onset of dehalo. activity**	**cis-DCE**	

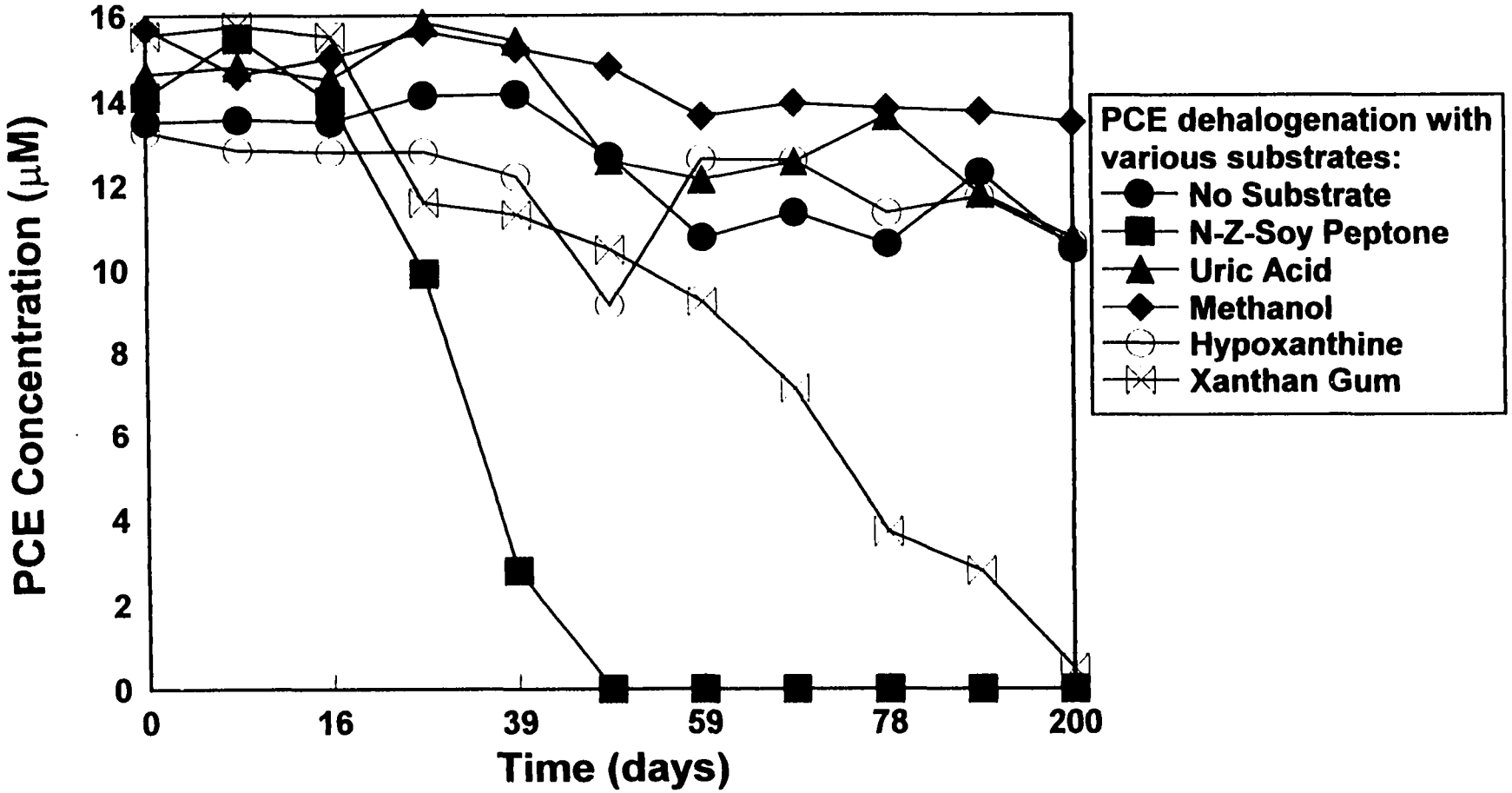


Figure 4.1 Effect of Primary Substrates on the Dehalogenation of Perchloroethylene

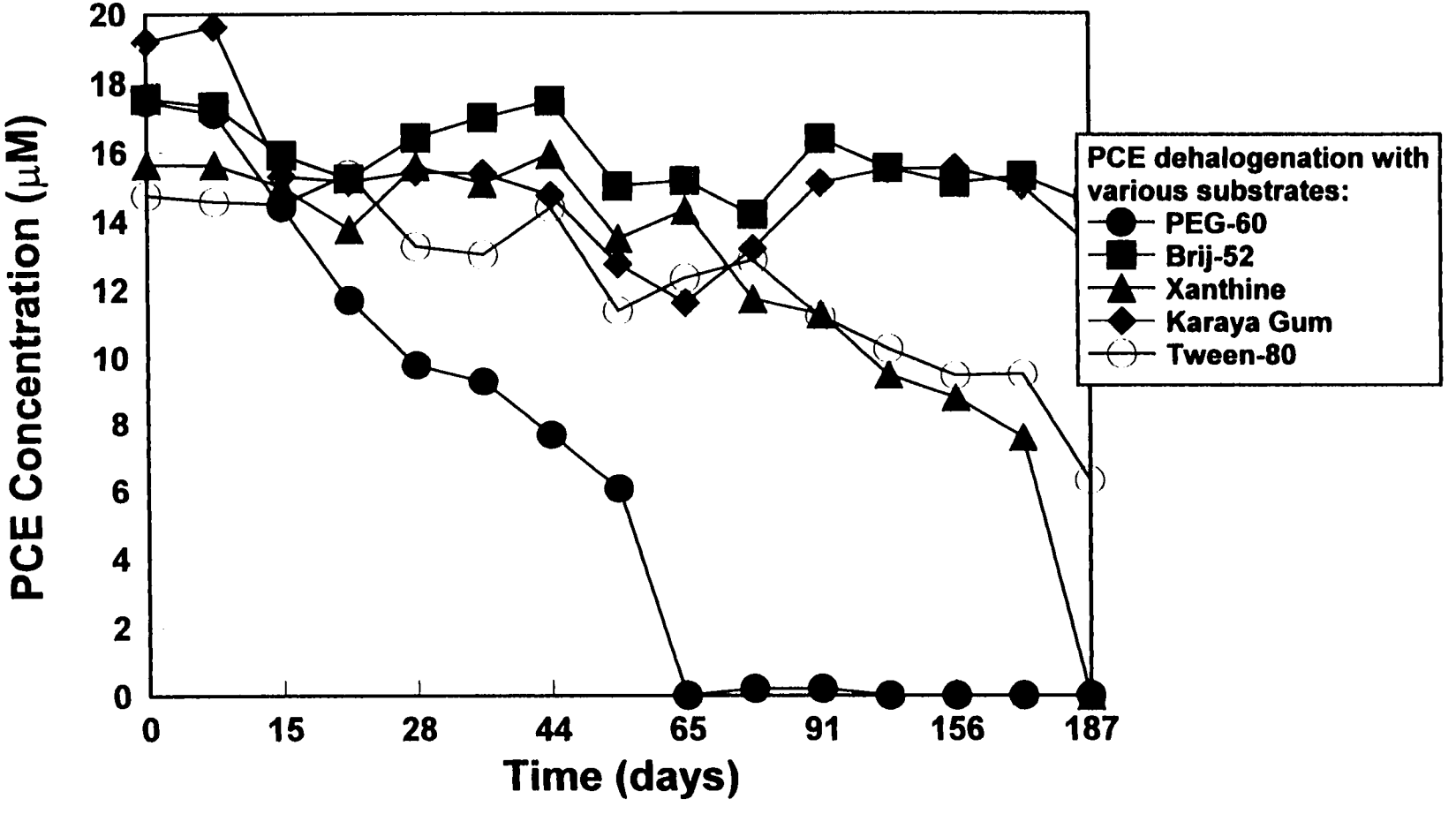


Figure 4.2 Effect of Primary Substrates on the Dehalogenation of Perchlororthylene

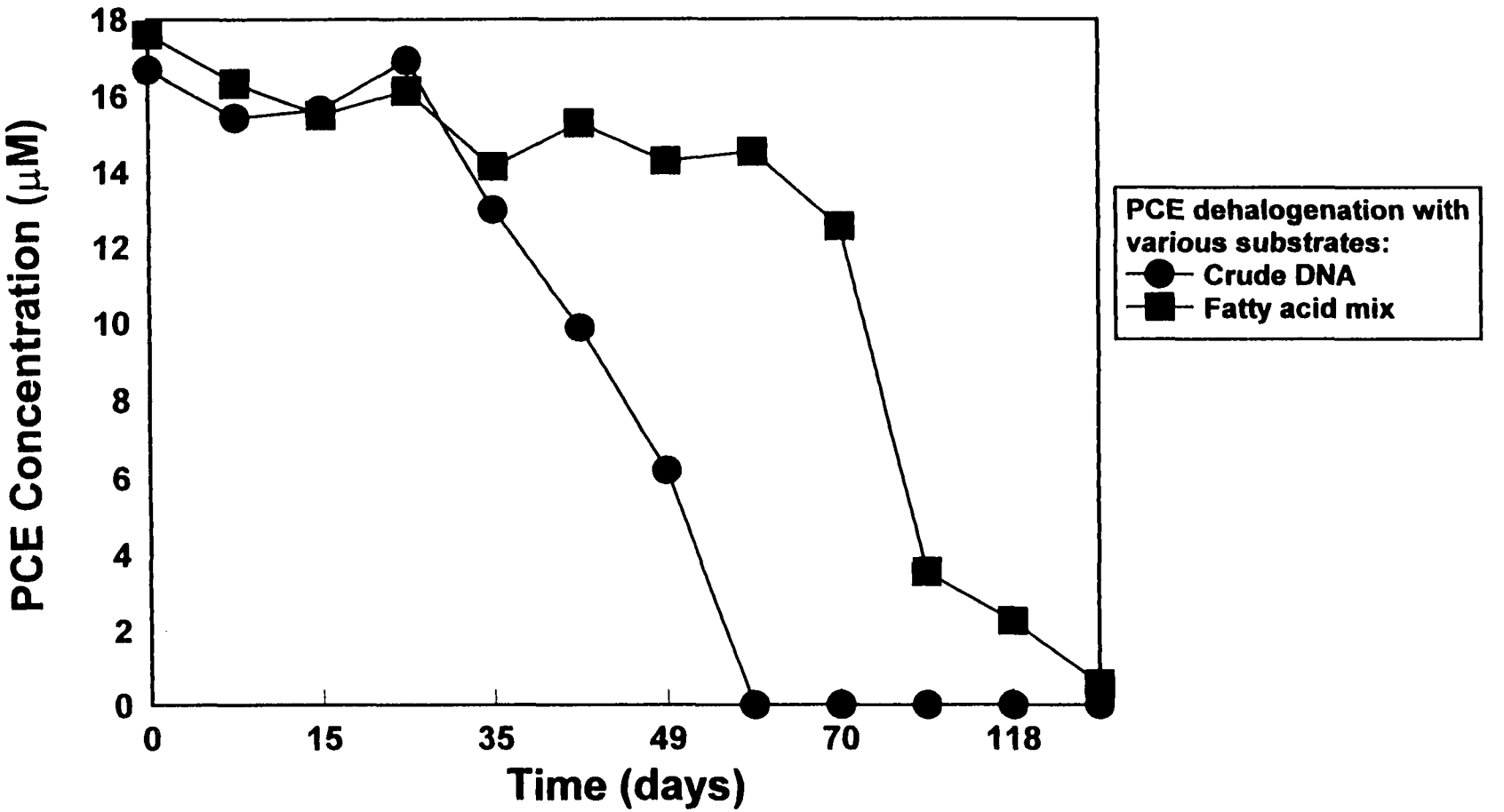


Figure 4.3 Effect of Primary Substrates on the Dehalogenation of Perchloroethylene

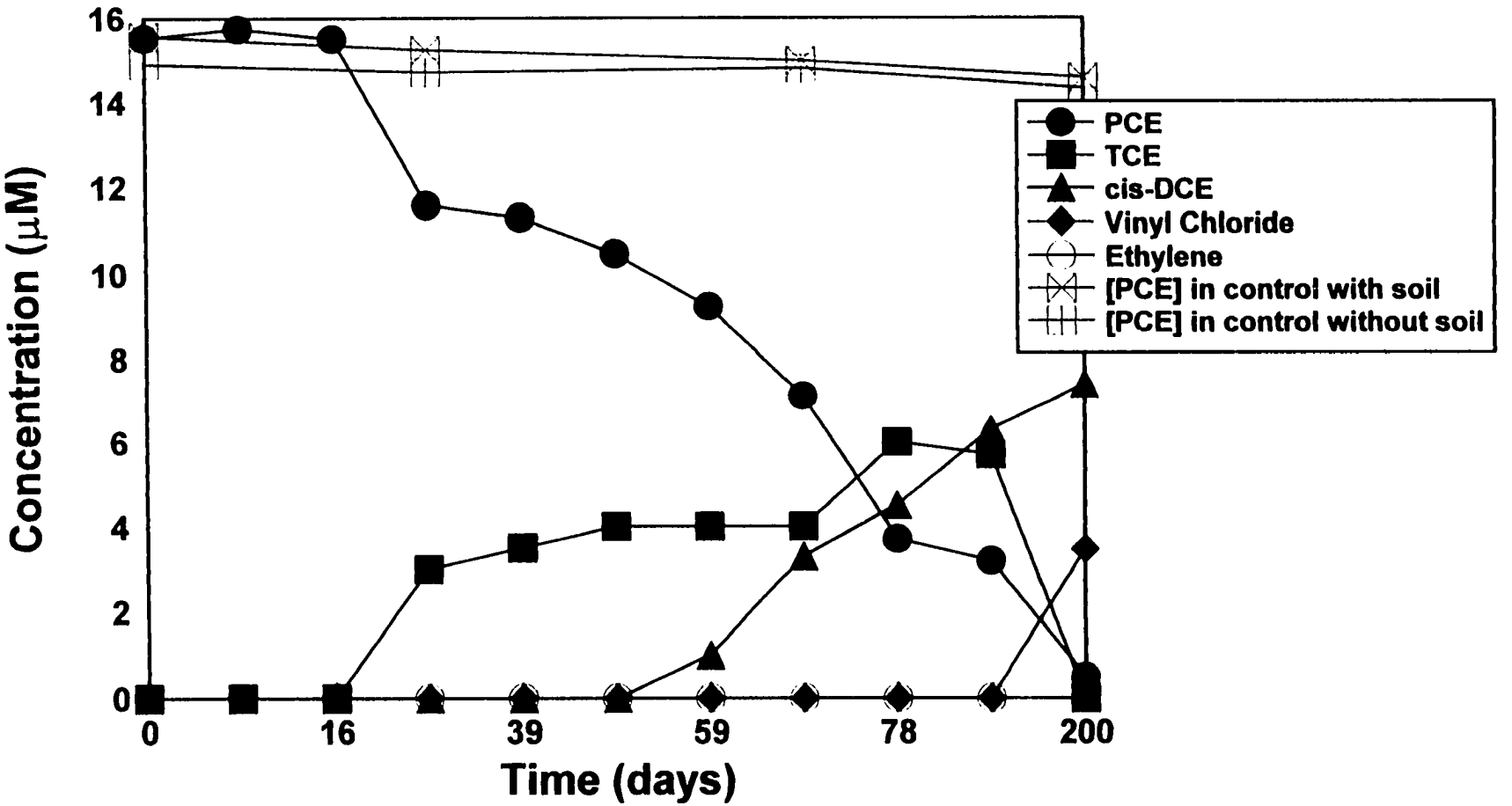


Figure 4.4(a) Effect of Xanthan Gum as Primary Substrate for the Dehalogenation of Perchloroethylene

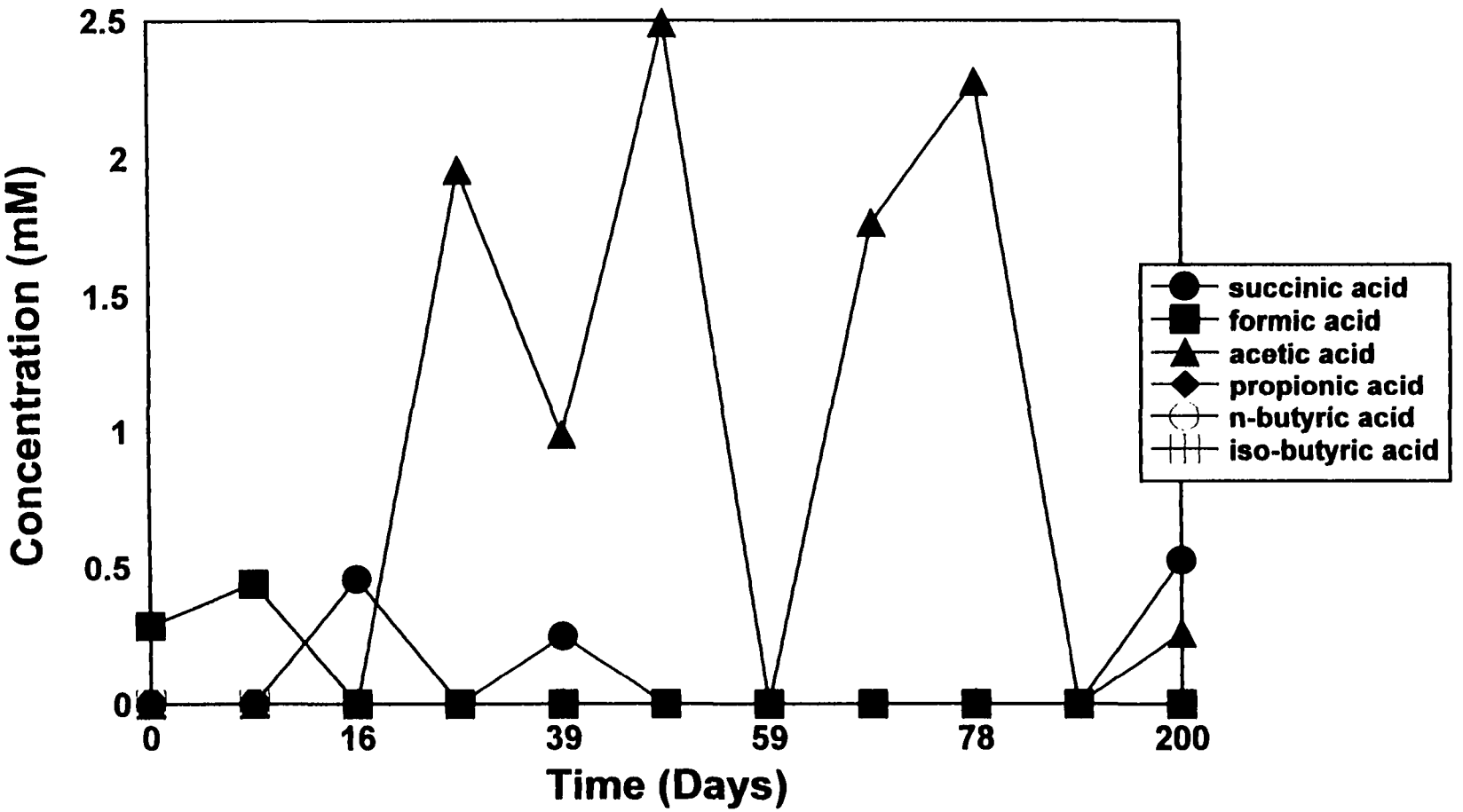


Figure 4.4(b) Fermentation Products with Xanthan Gum as Primary Substrate

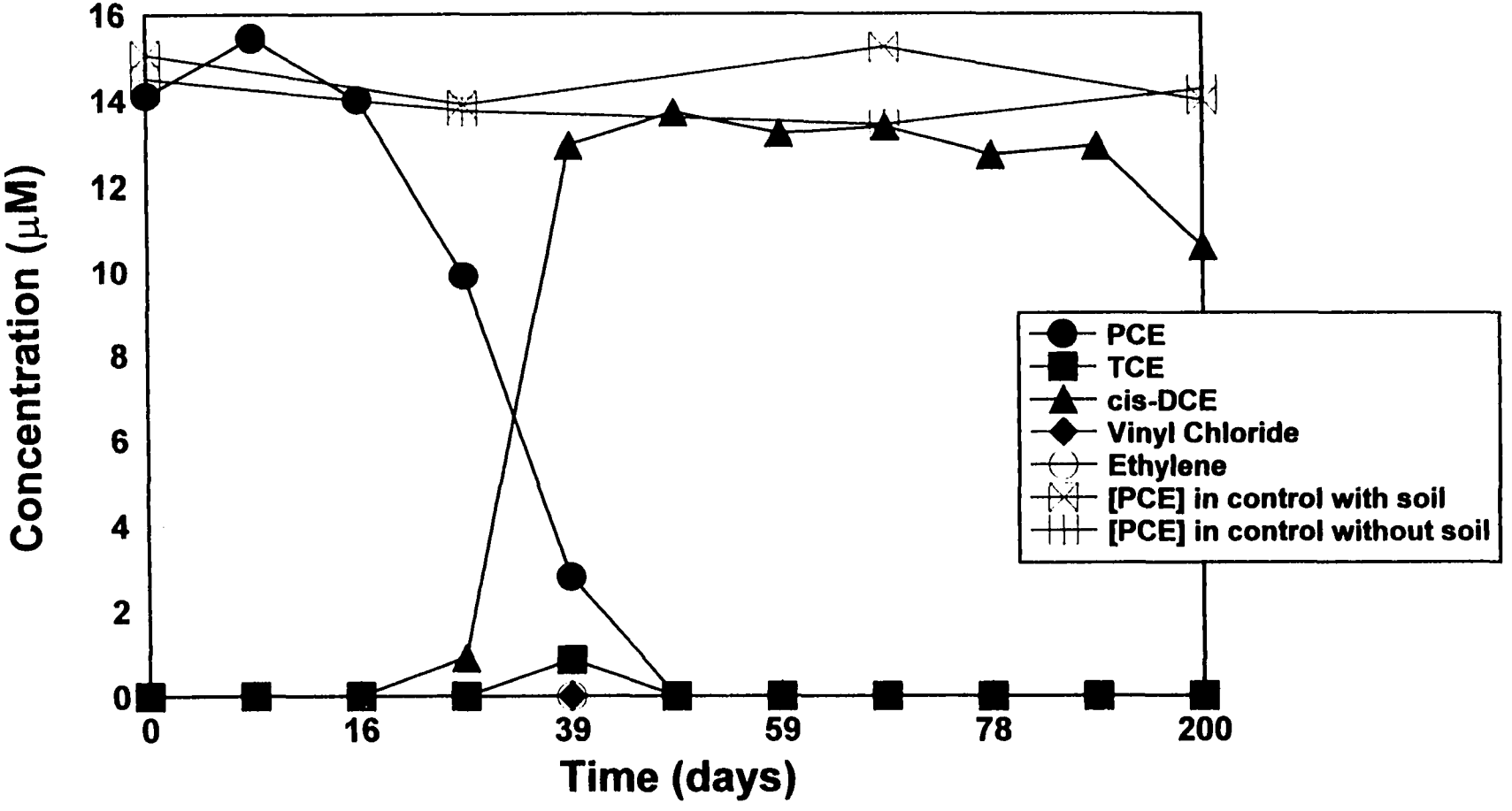


Figure 4.5(a) Effect of N-Z-Soy Peptone as Primary Substrate for the Dehalogenation of Perchloroethylene

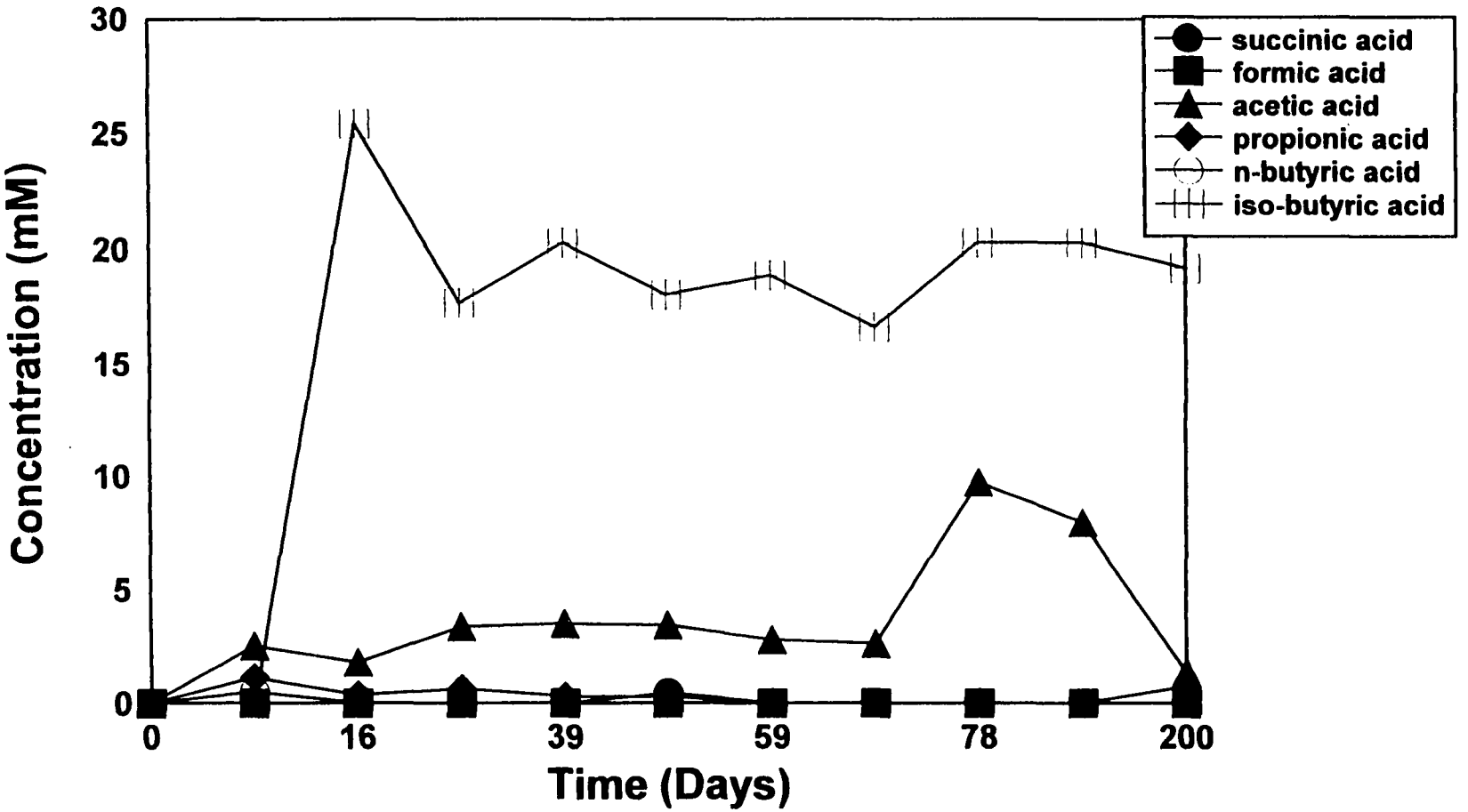


Figure 4.5(b) Fermentation Products with N-Z-Soy Peptone as Primary Substrate

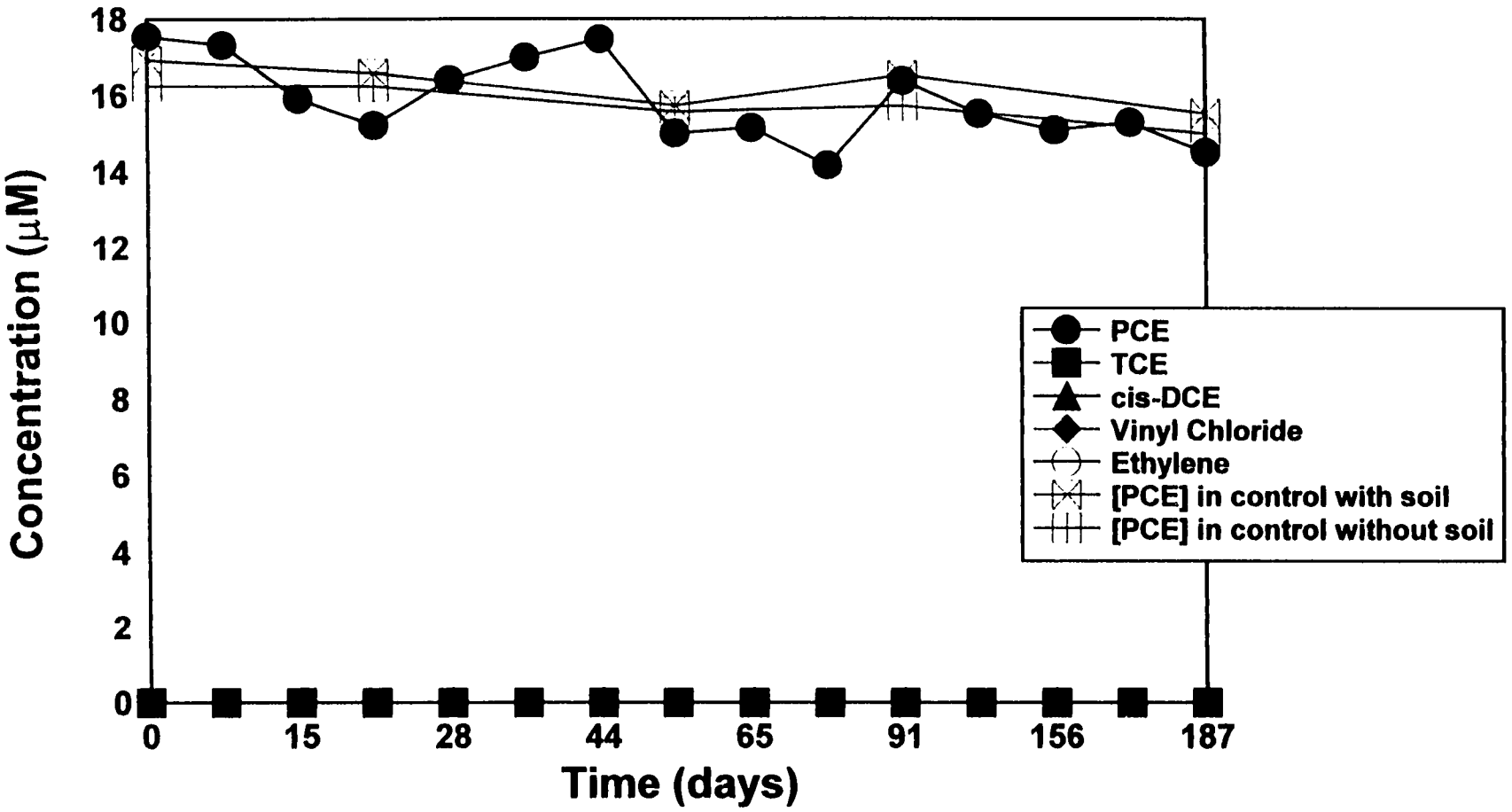


Figure 4.6(a) Effect of Brij-52 as Primary Substrate for Dehalogenation of Perchloroethylene

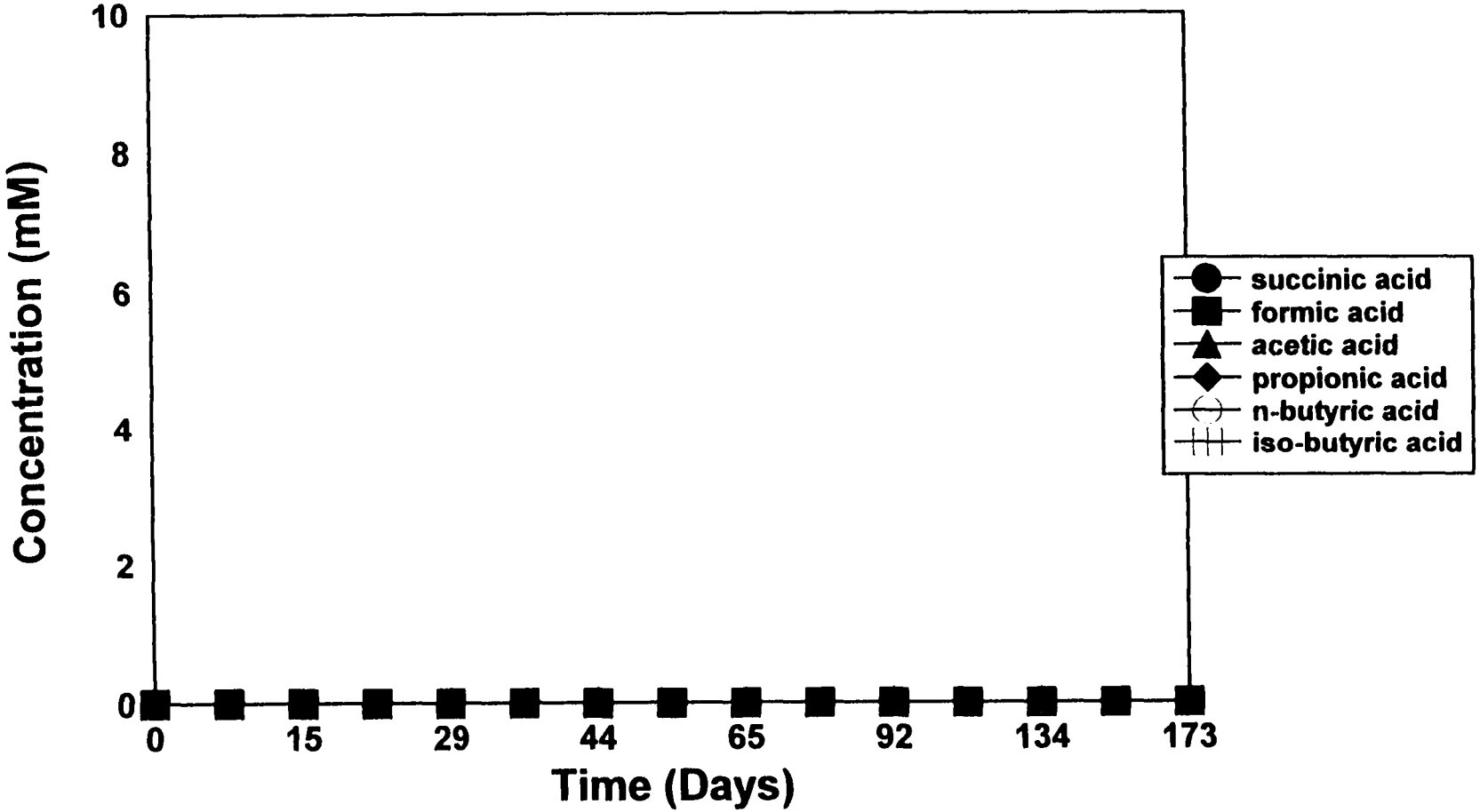


Figure 4.6(b) Fermentation Products with Brij-52 as Primary Substrate

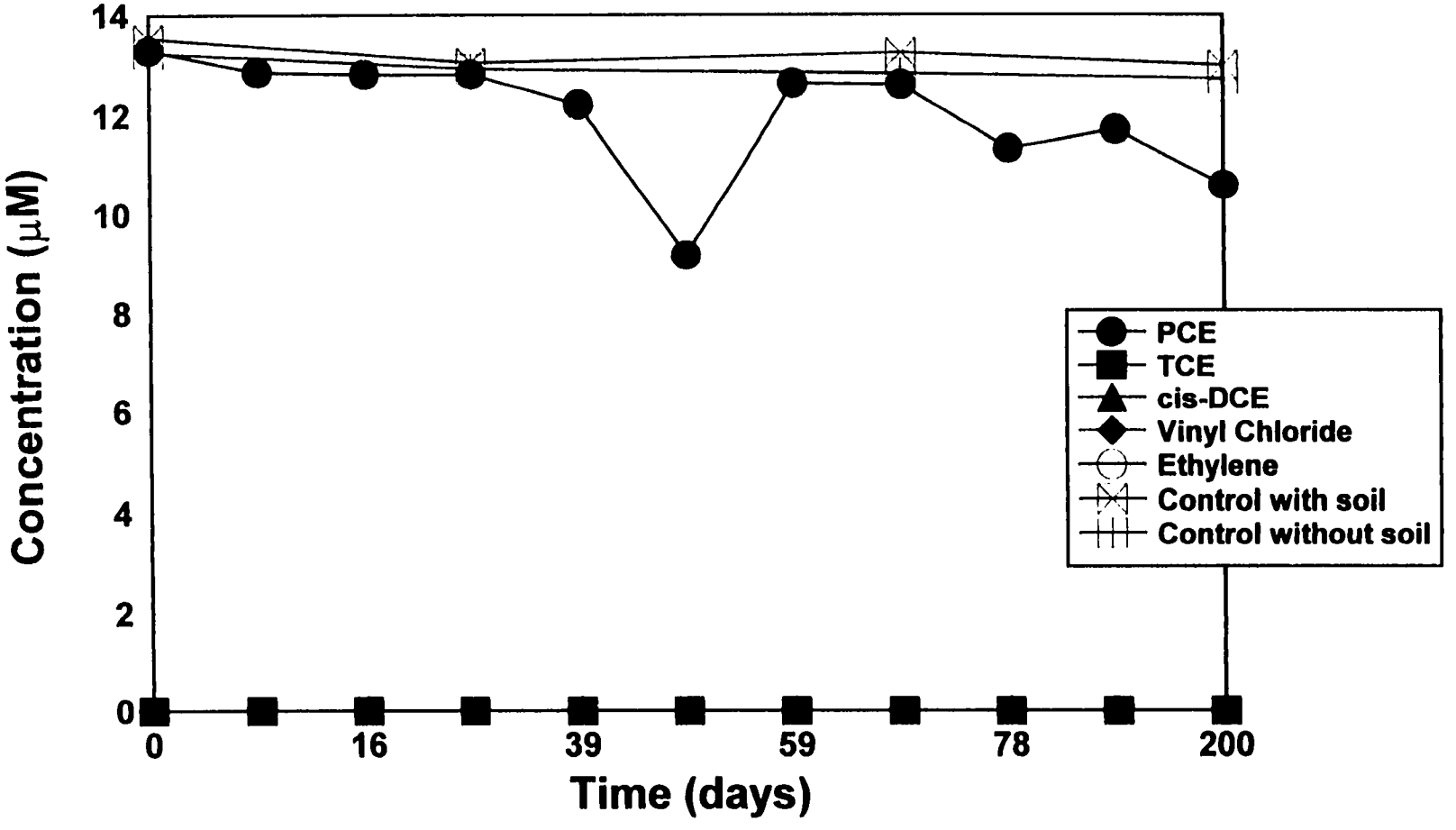


Figure 4.7(a) Effect of Hypoxanthine as Primary Substrate for the Dehalogenation of Perchloroethylene

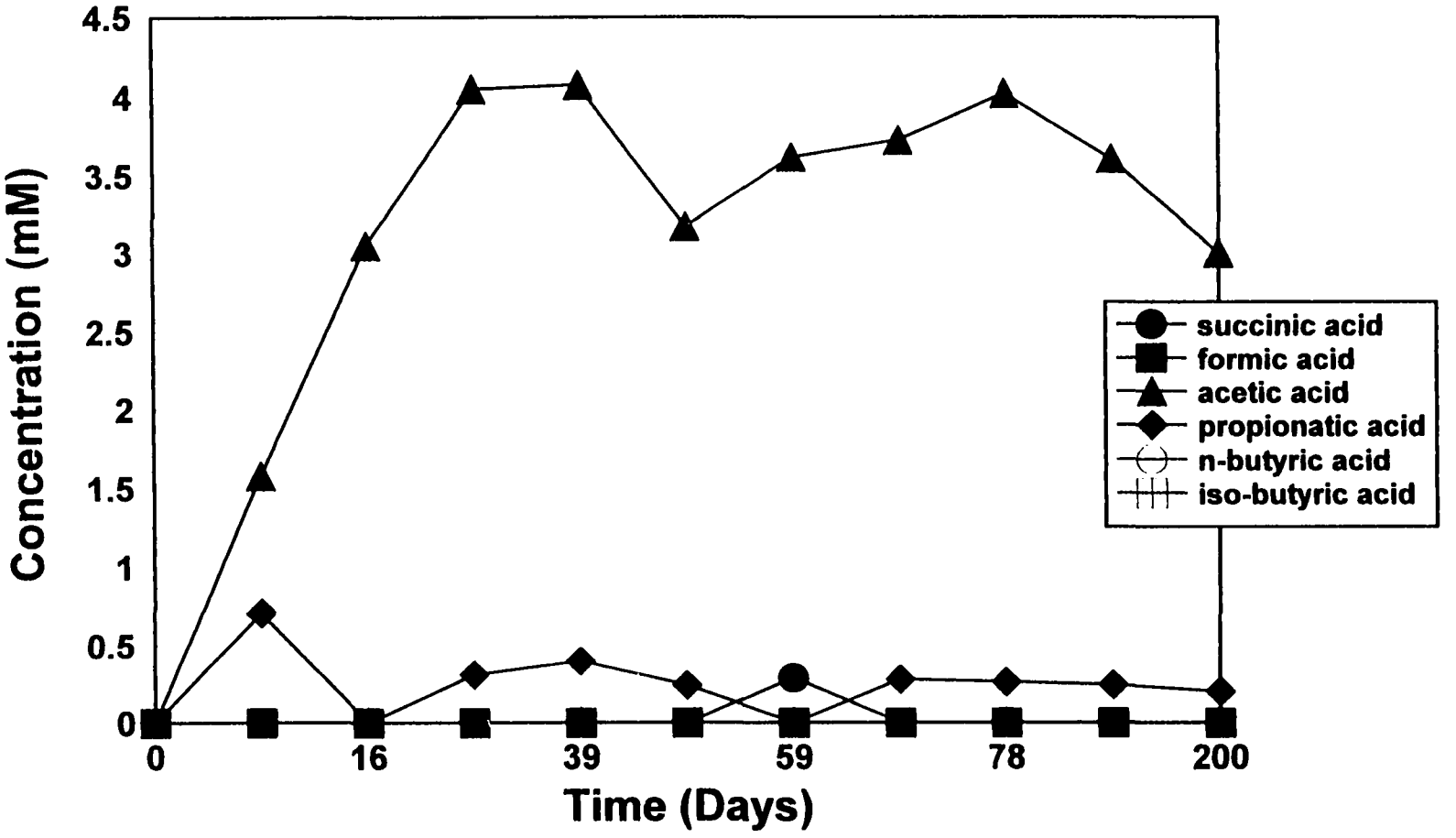


Figure 4.7(b) Fermentation Products with Hypoxanthine as Primary Substrate

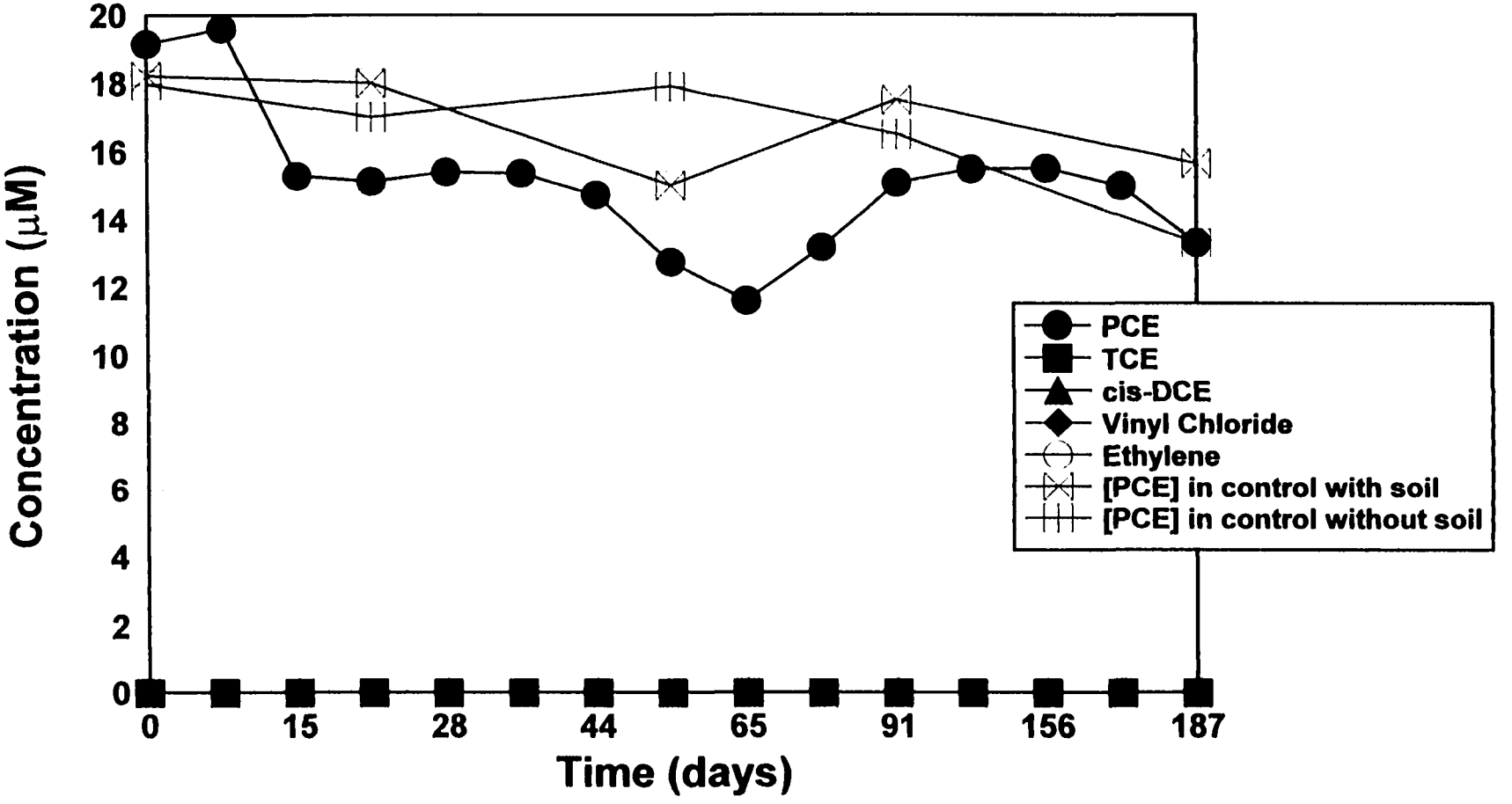


Figure 4.8(a) Effect of Karaya Gum as Primary Substrate for the Dehalogenation of Perchloroethylene

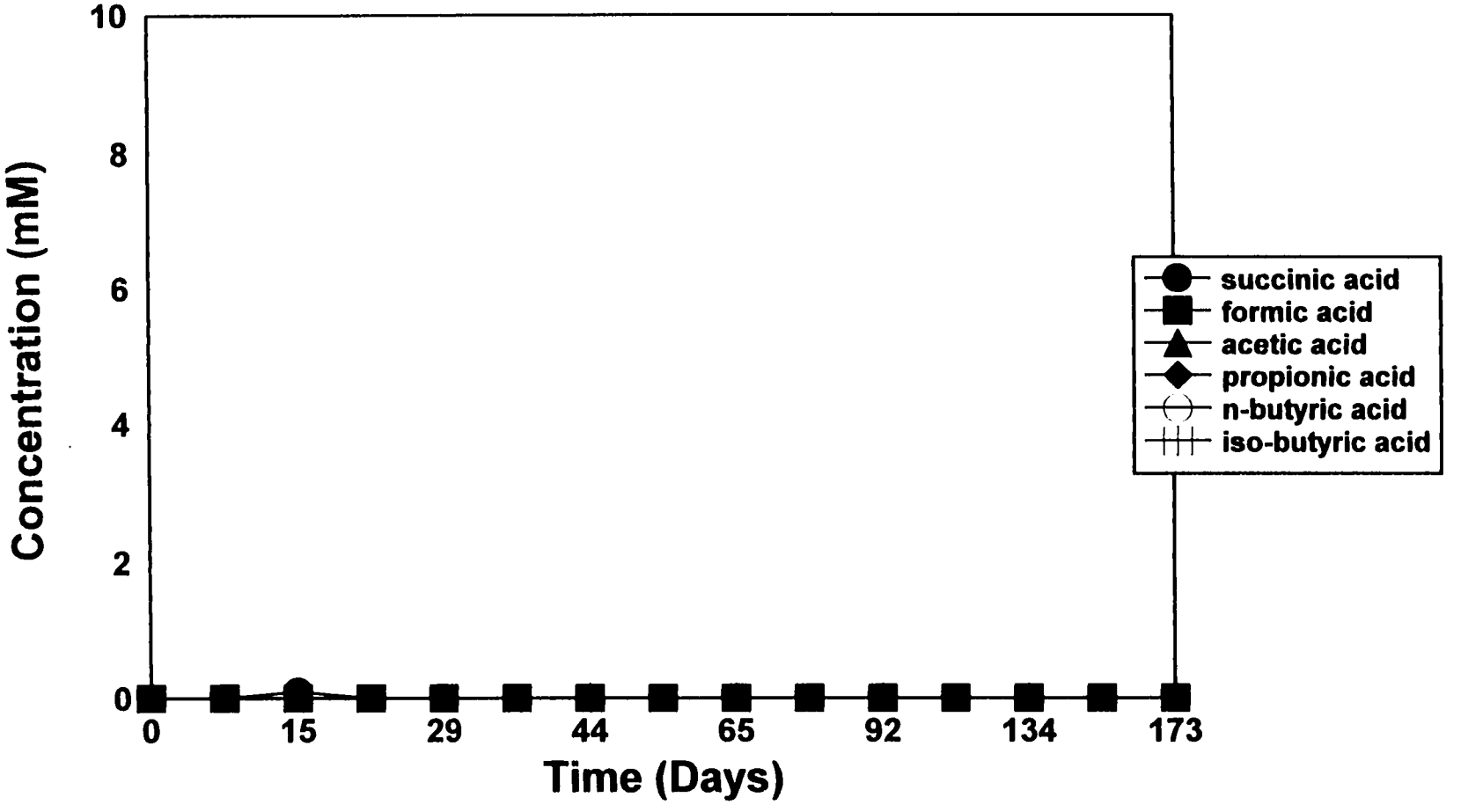


Figure 4.8(b) Fermentation Products with Karaya Gum as Primary Substrate

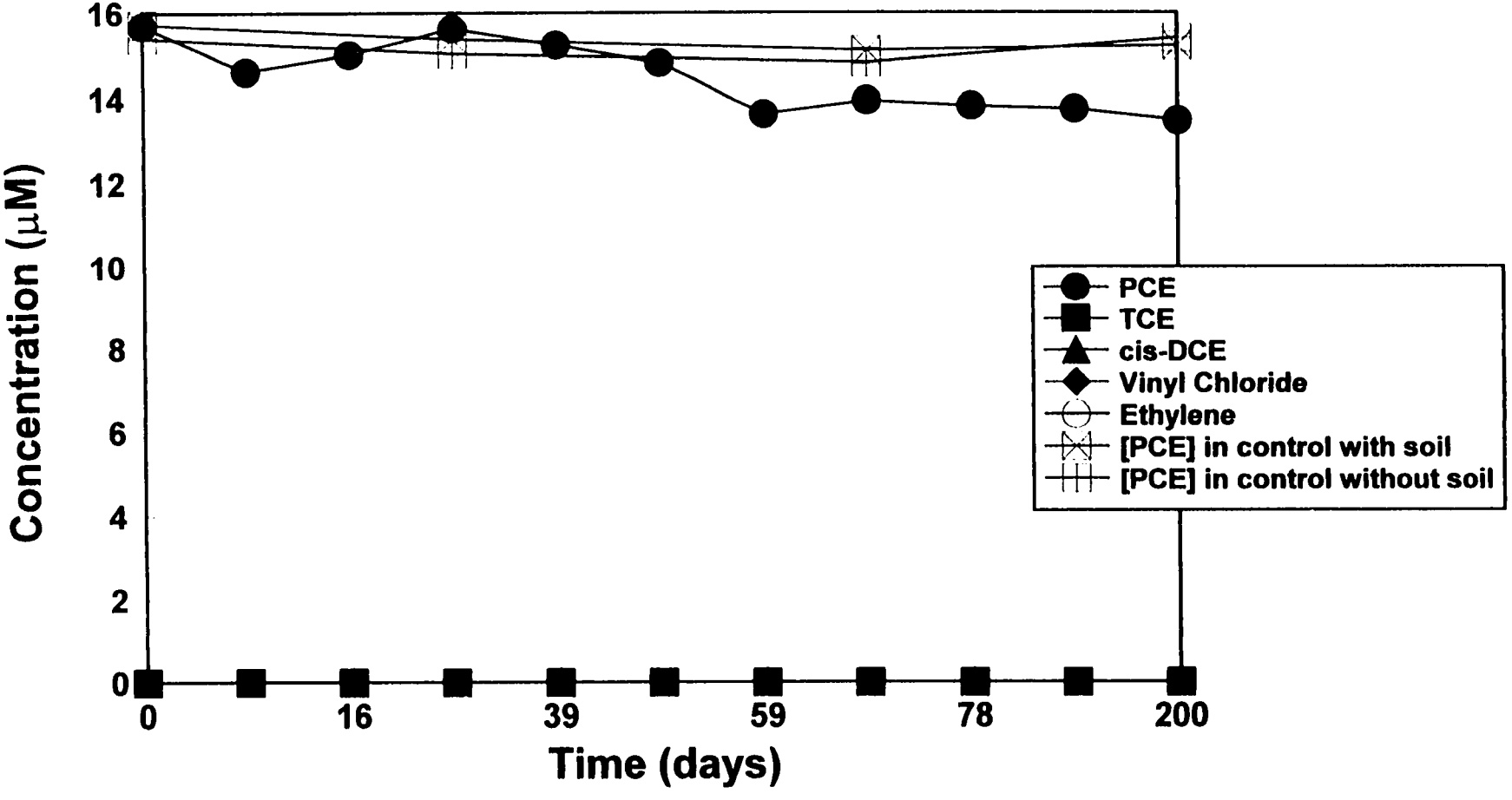


Figure 4.9(a) Effect of Methanol as Primary Substrate for the Dehalogenation of Perchloroethylene

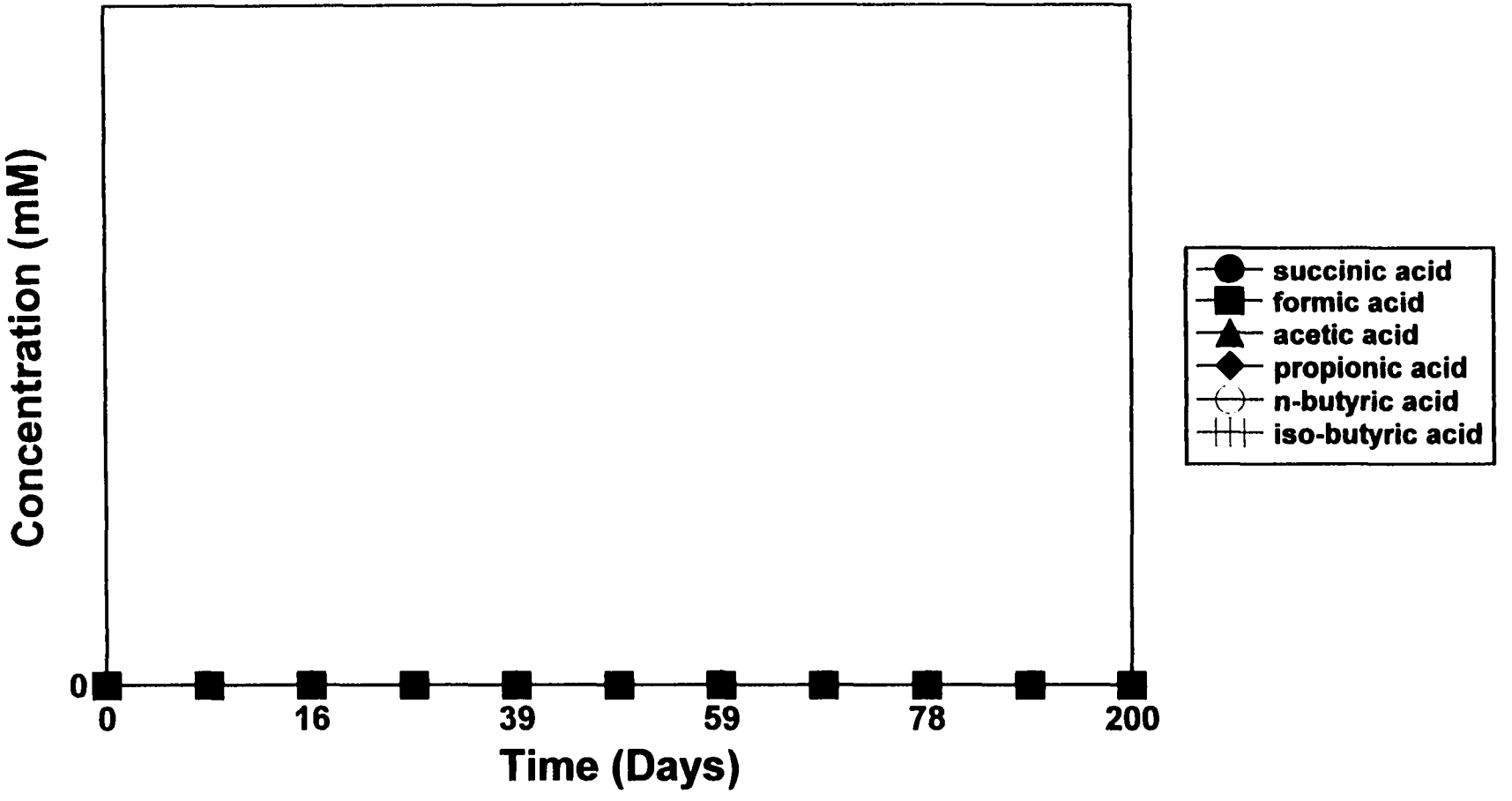


Figure 4.9(b) Fermentation Products with Methanol as Primary Substrate

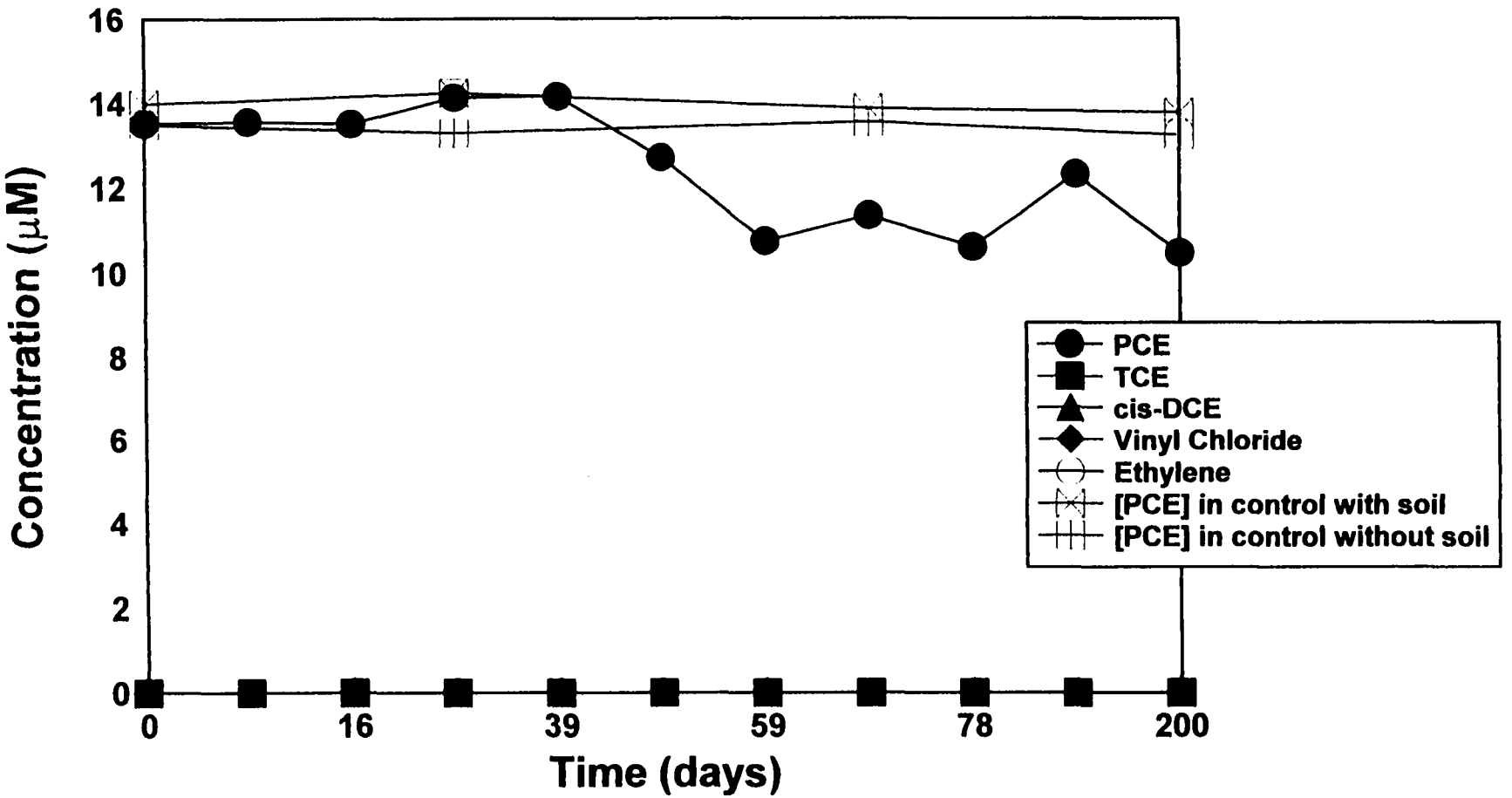


Figure 4.10(a) Effect of No Added Primary Substrate on Dehalogenation of Perchloroethylene

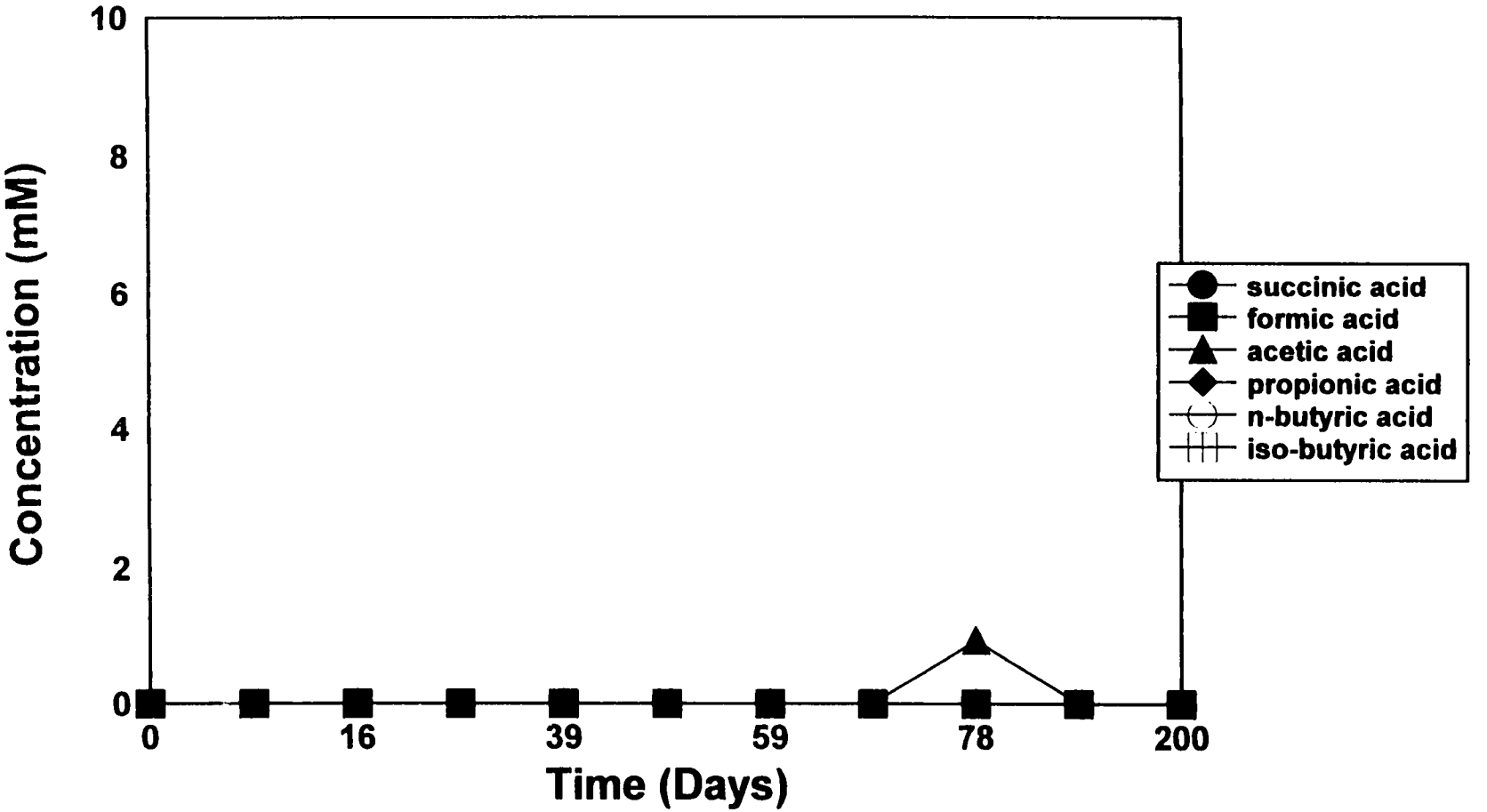


Figure 4.10(b) Fermentation Products with No Added Primary Substrate

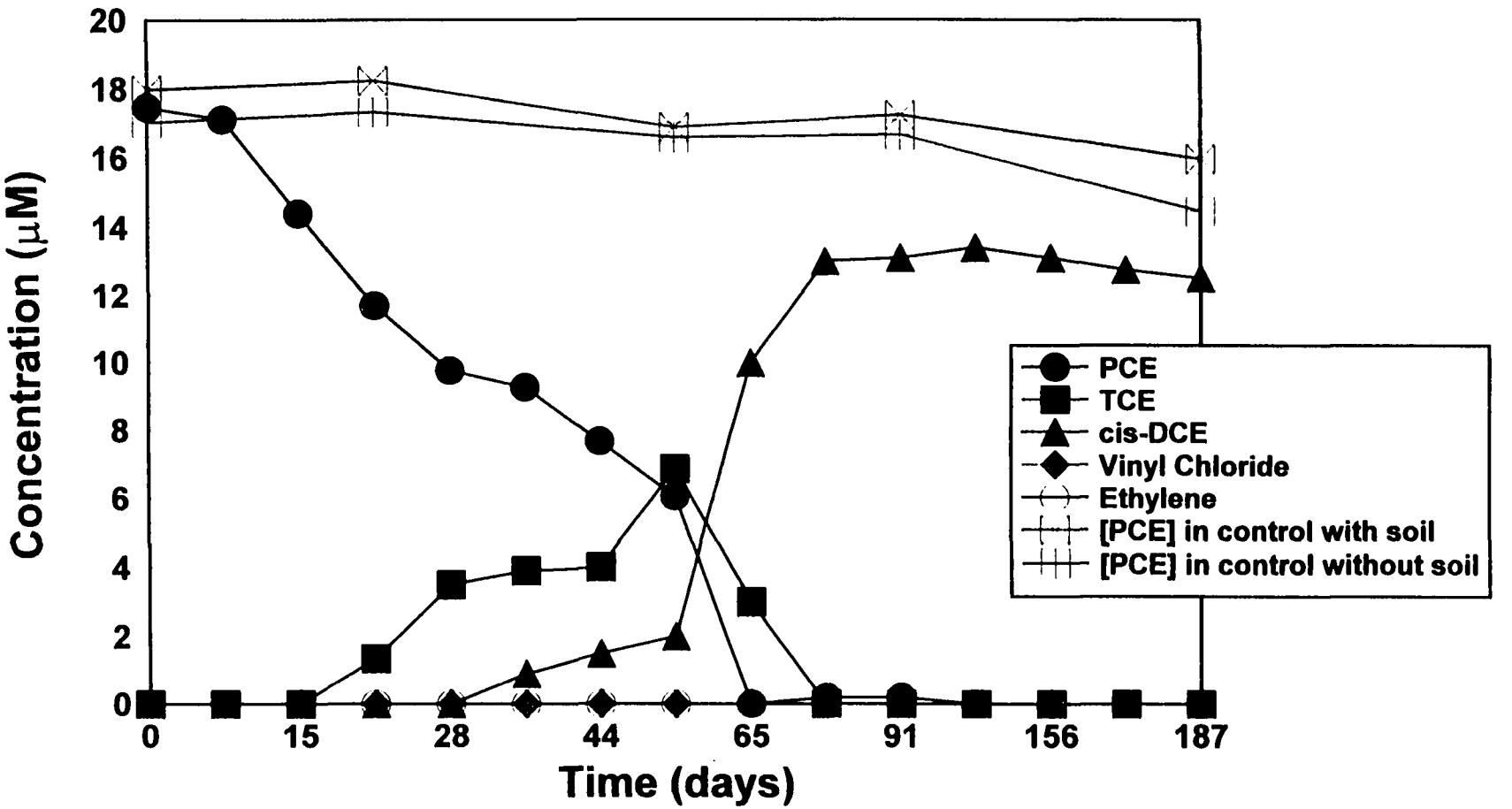


Figure 4.11(a) Effect of Polyethylene Glycol-60 as Primary Substrate for the Dehalogenation of Perchloroethylene

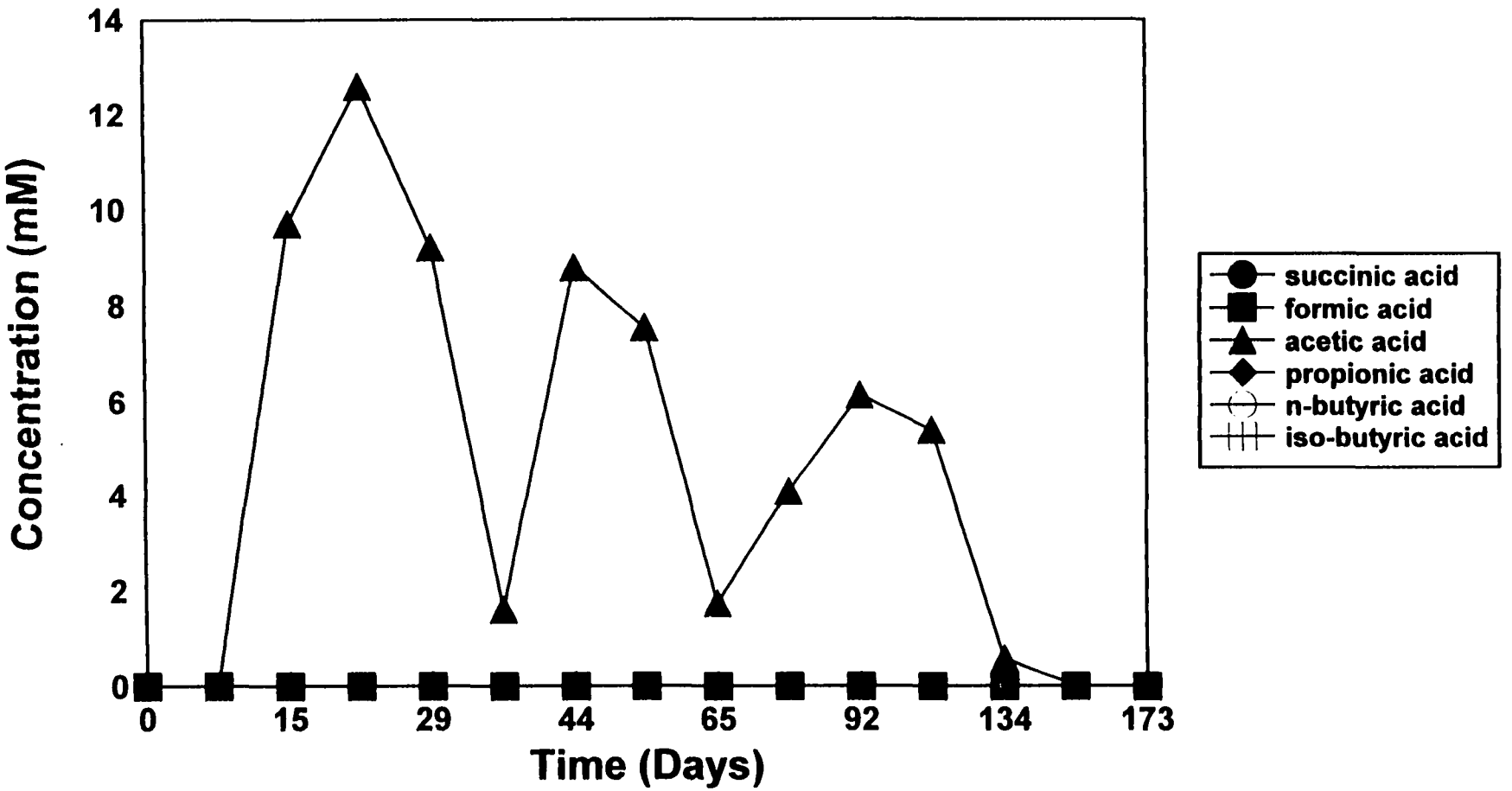


Figure 4.11(b) Fermentation Products with Polyethylene Glycol-60 as Primary Substrate

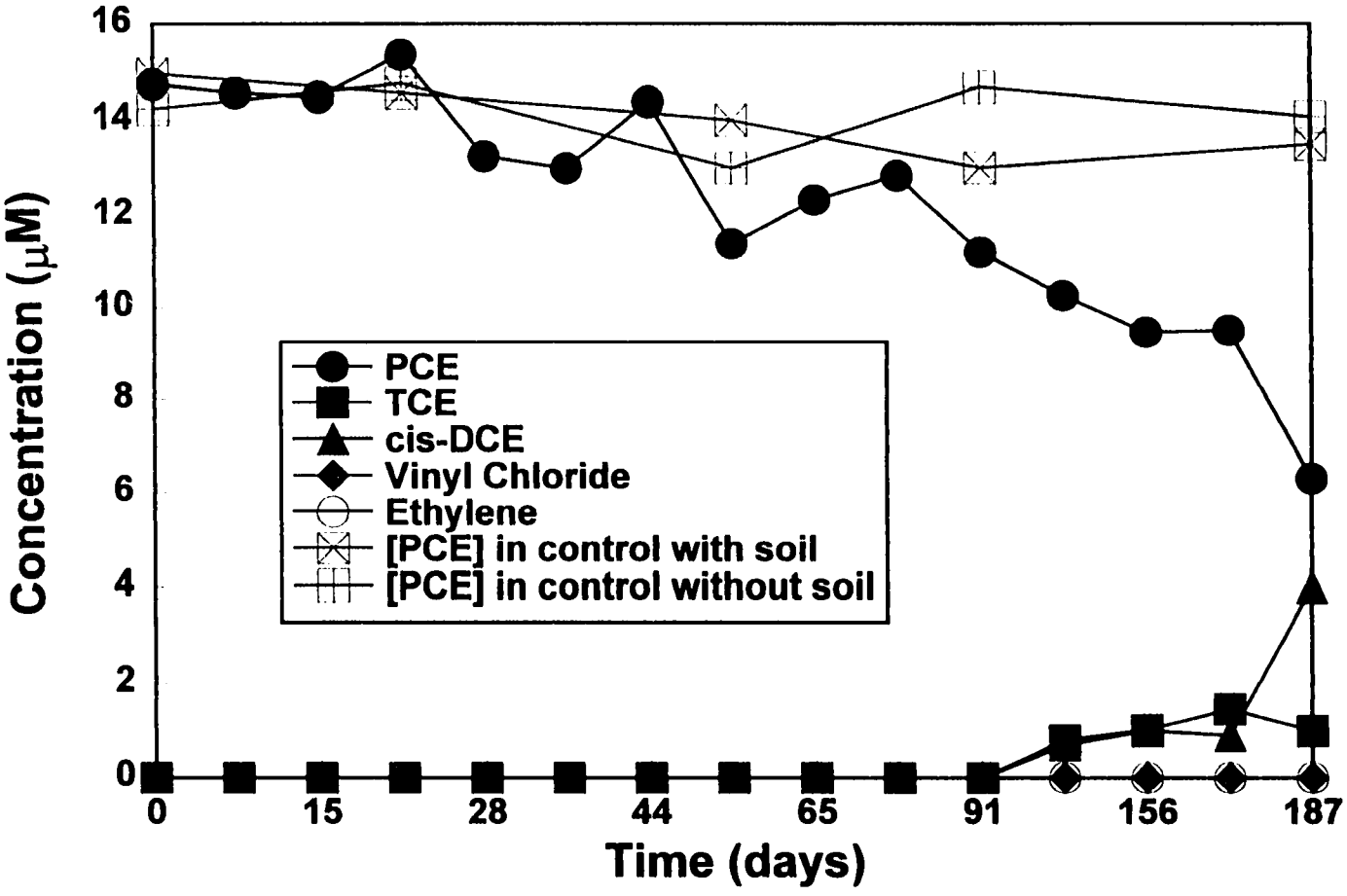


Figure 4.12(a) Effect of Tween-80 as Primary Substrate for the Dehalogenation of Perchloroethylene

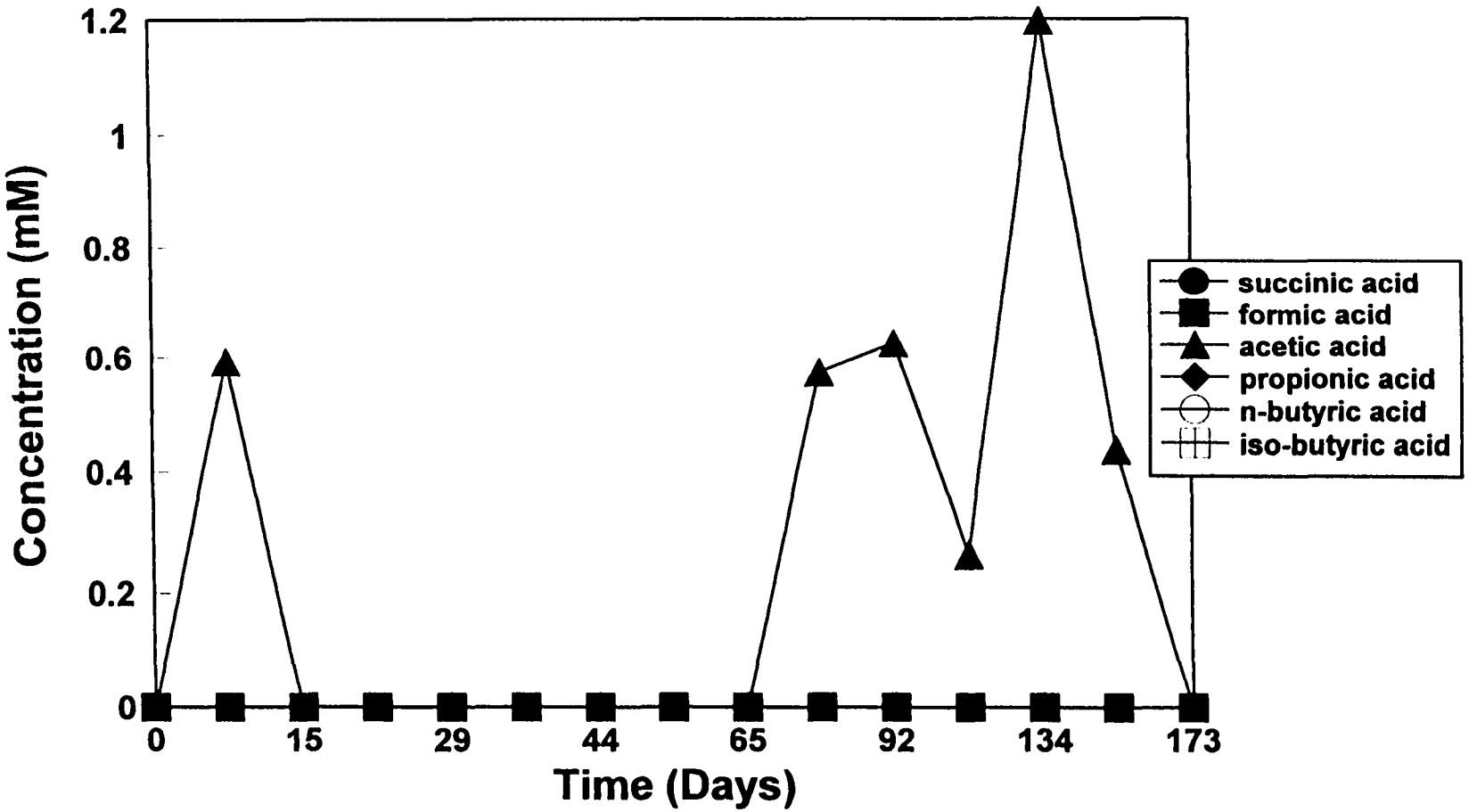


Figure 4.12(b) Fermentation Products with Tween-80 as Primary Substrate

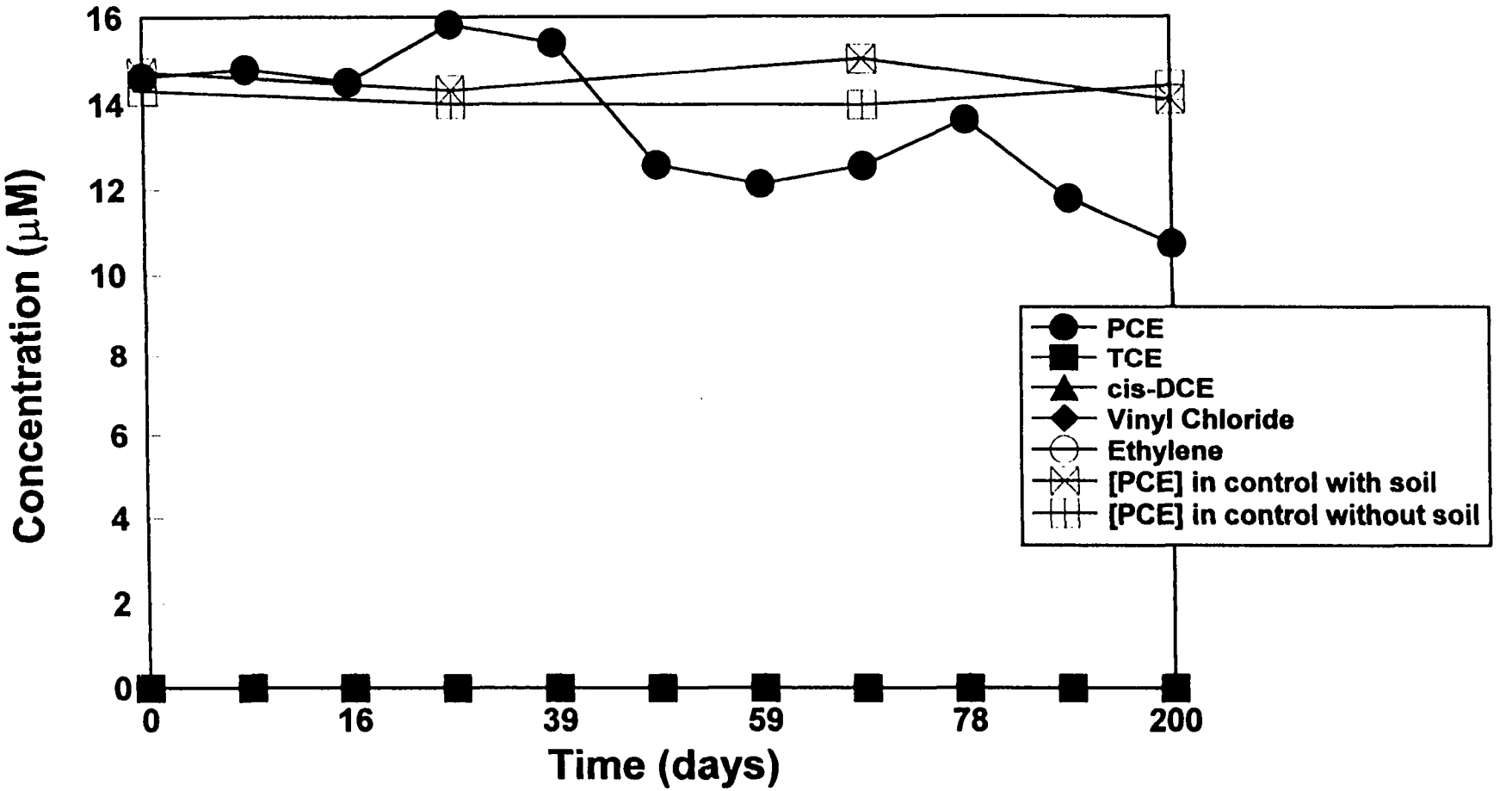


Figure 4.13(a) Effect of Uric Acid as Primary Substrate for the Dehalogenation of Perchloroethylene

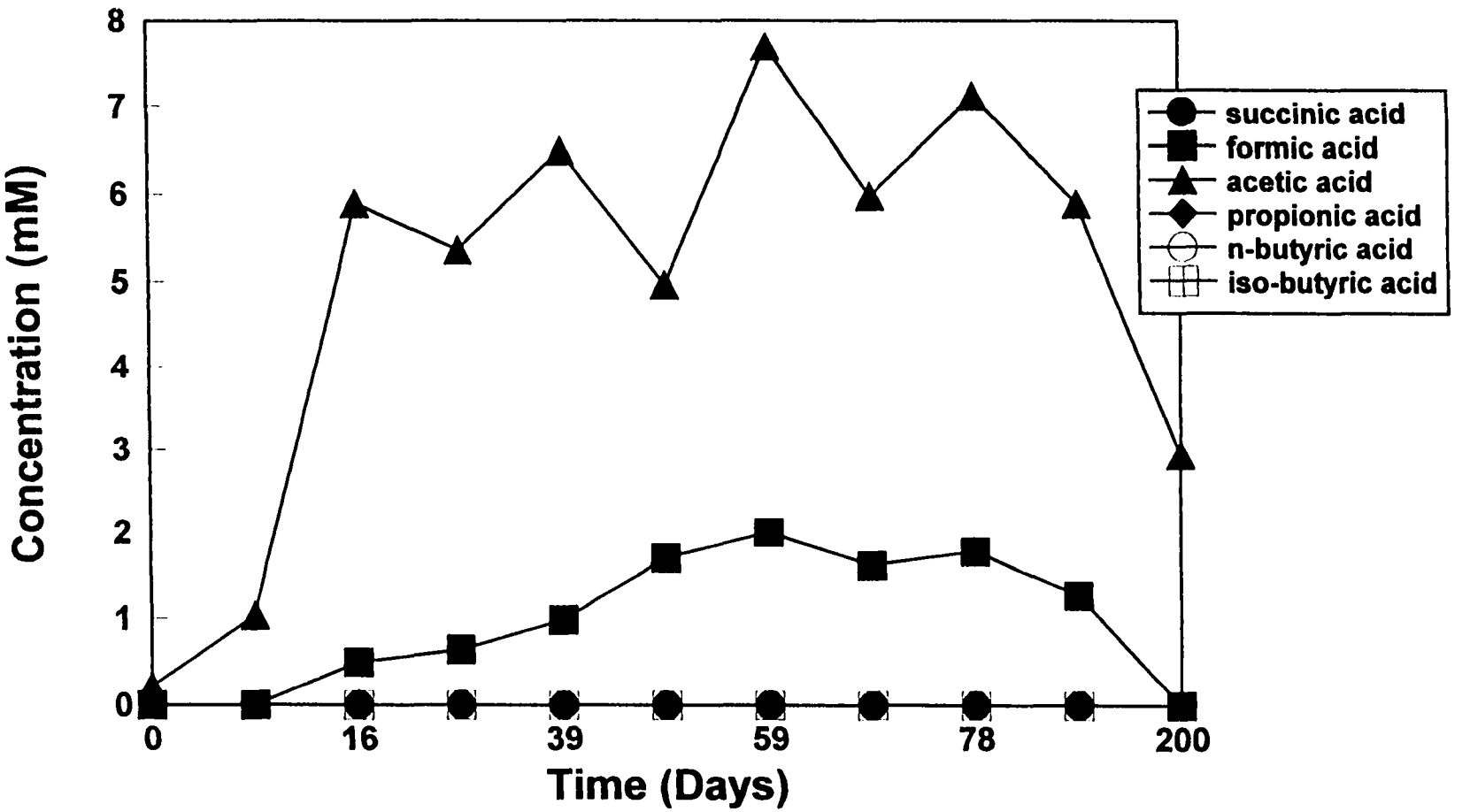


Figure 4.13(b) Fermentation Products with Uric Acid as Primary Substrate

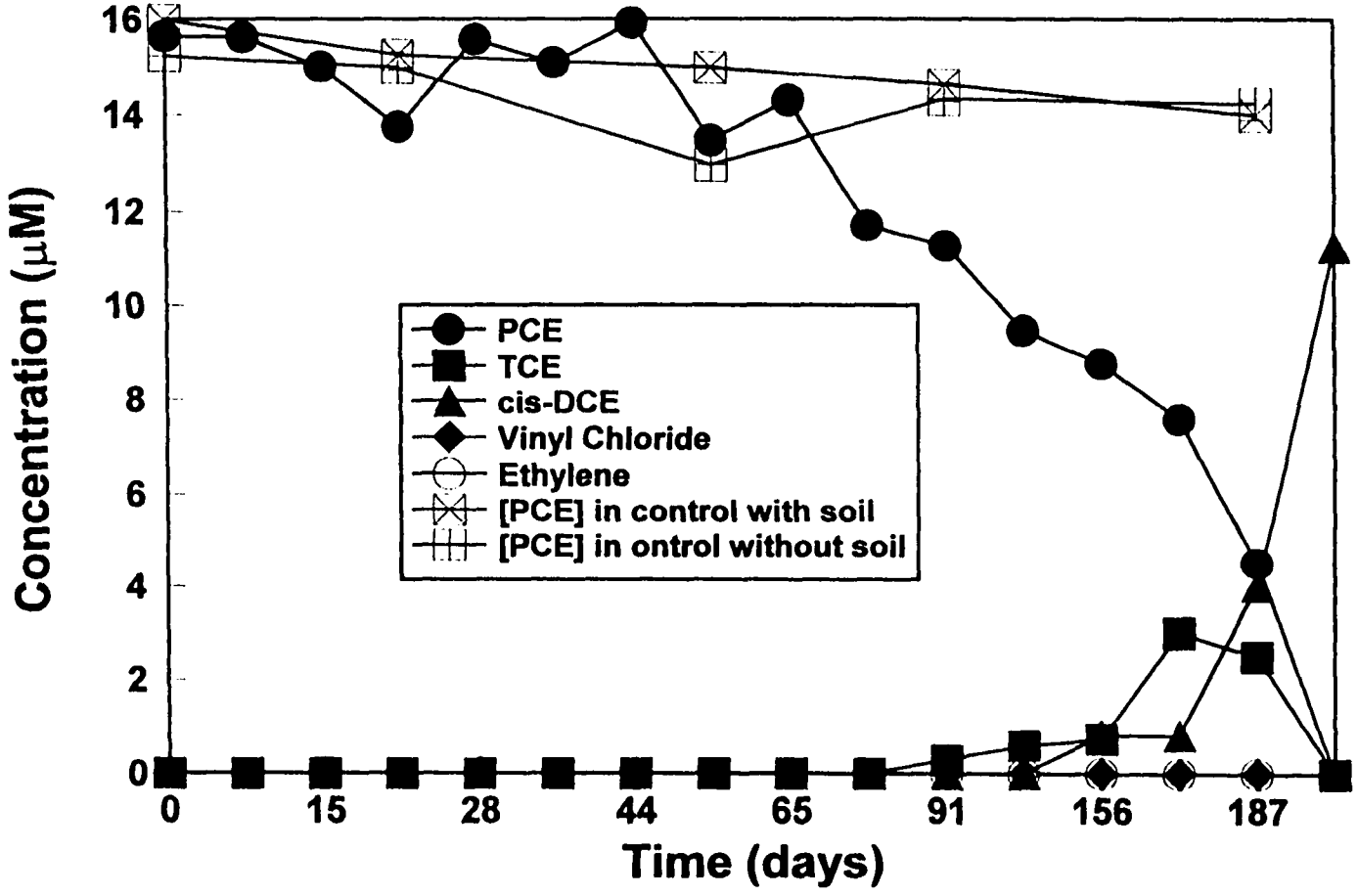


Figure 4.14(a) Effect of Xanthine as Primary Substrate for the Dehalogenation of Perchloroethylene

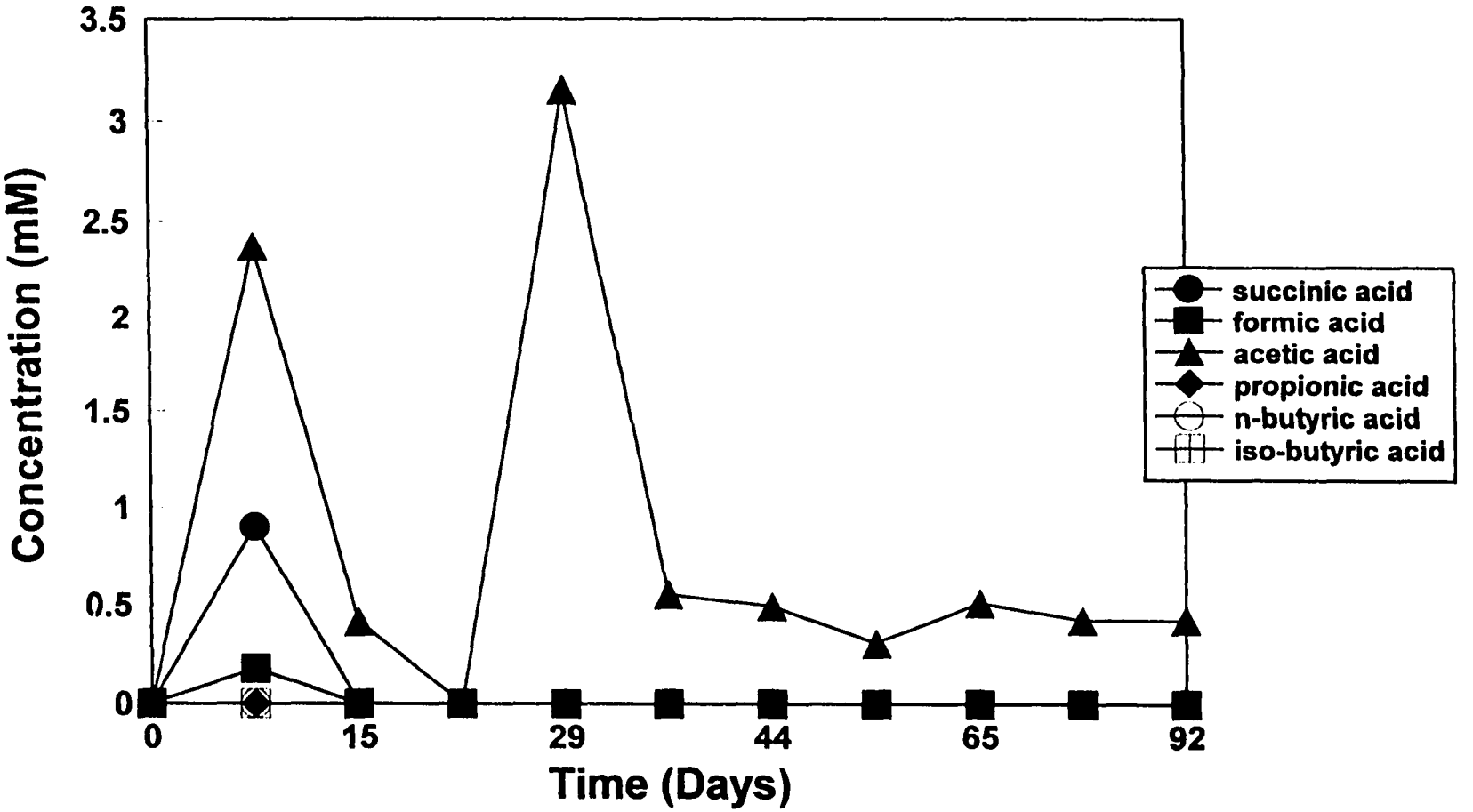


Figure 4.14(b) Fermentation Products with Xanthine as Primary Substrate

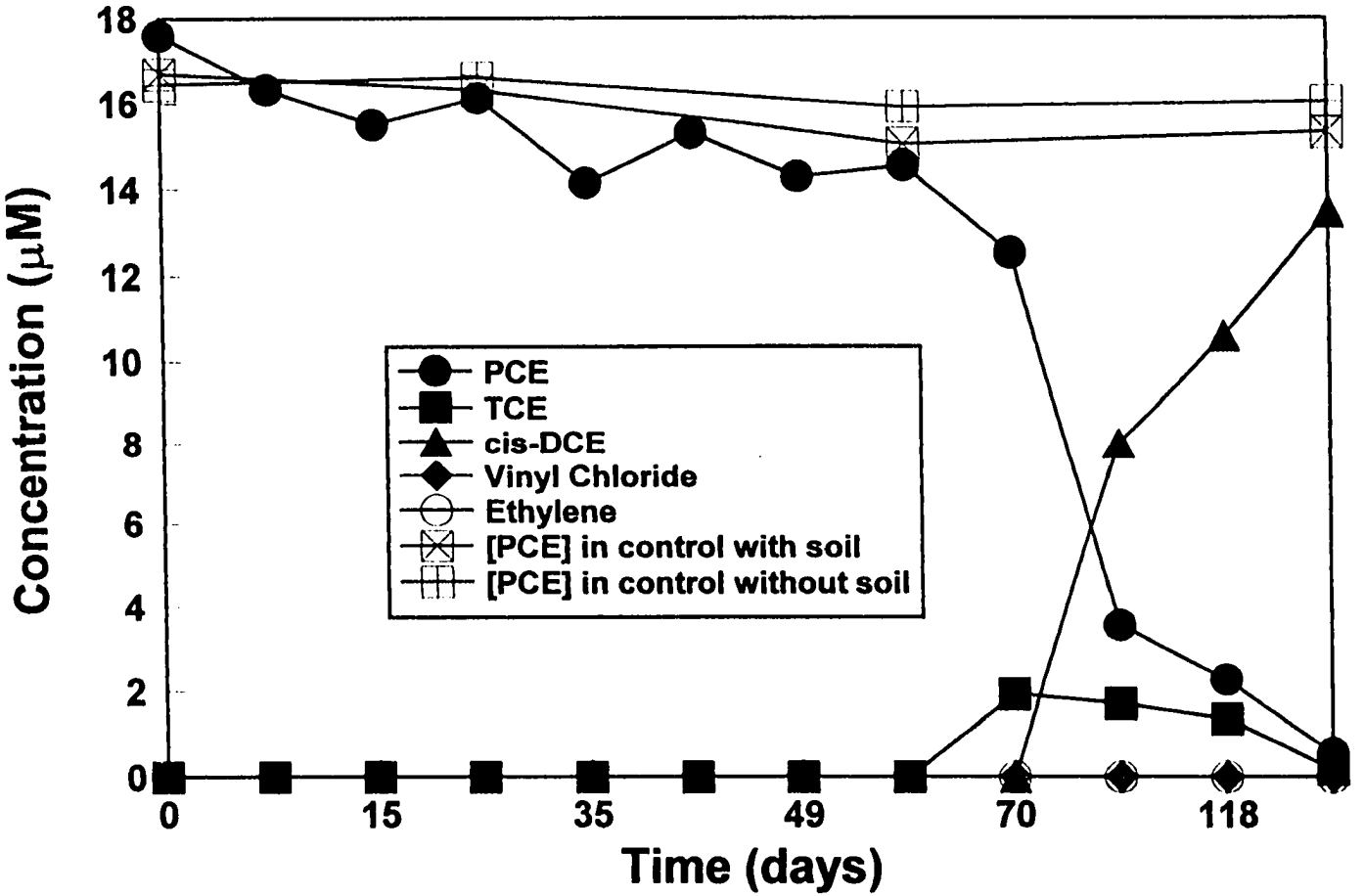


Figure 4.15(a) Effect of Volatile Fatty Acid Mix as Primary Substrate for the Dehalogenation of Perchloroethylene

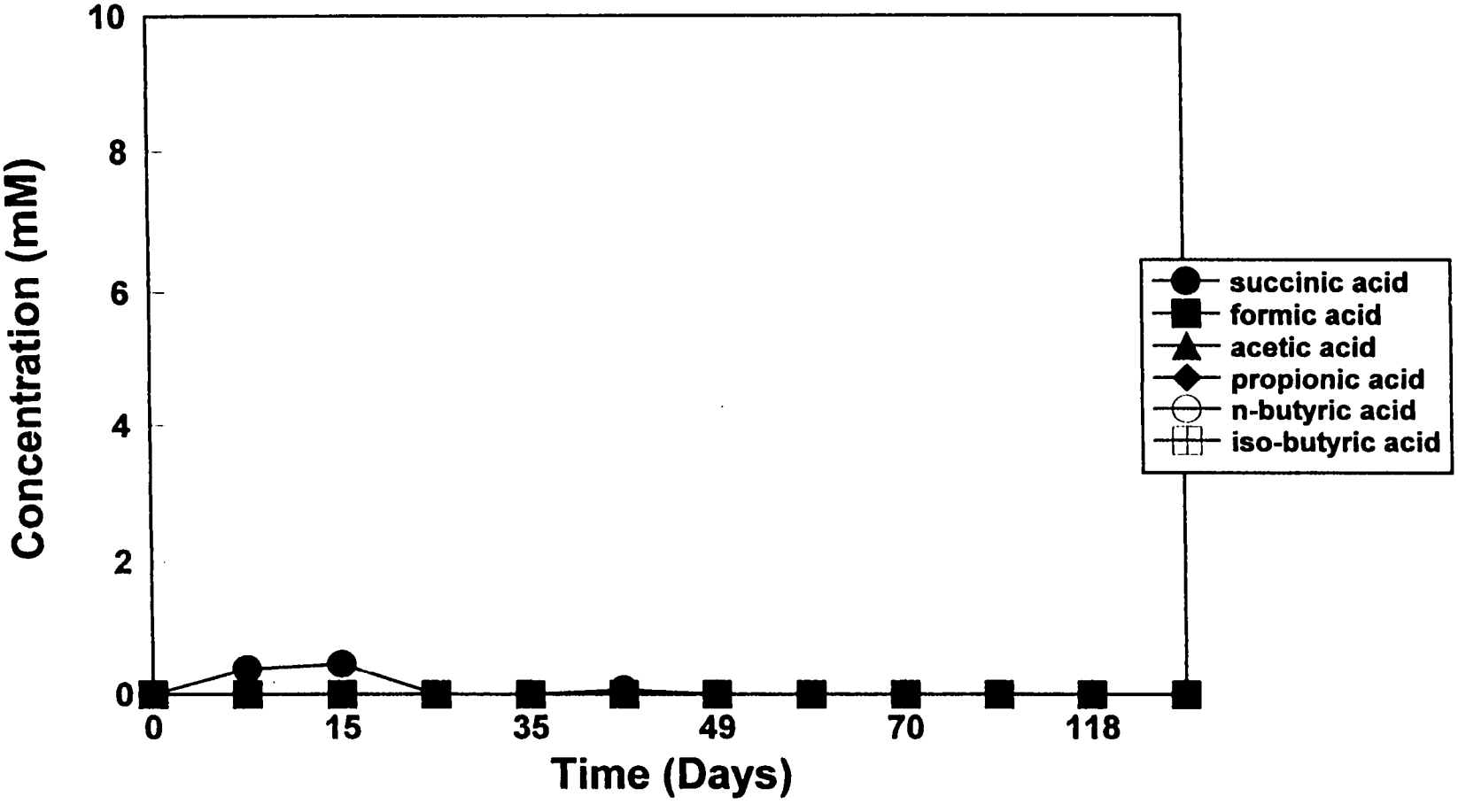


Figure 4.15(b) Fermentation Products with Volatile Fatty Acid Mix as Primary Substrate

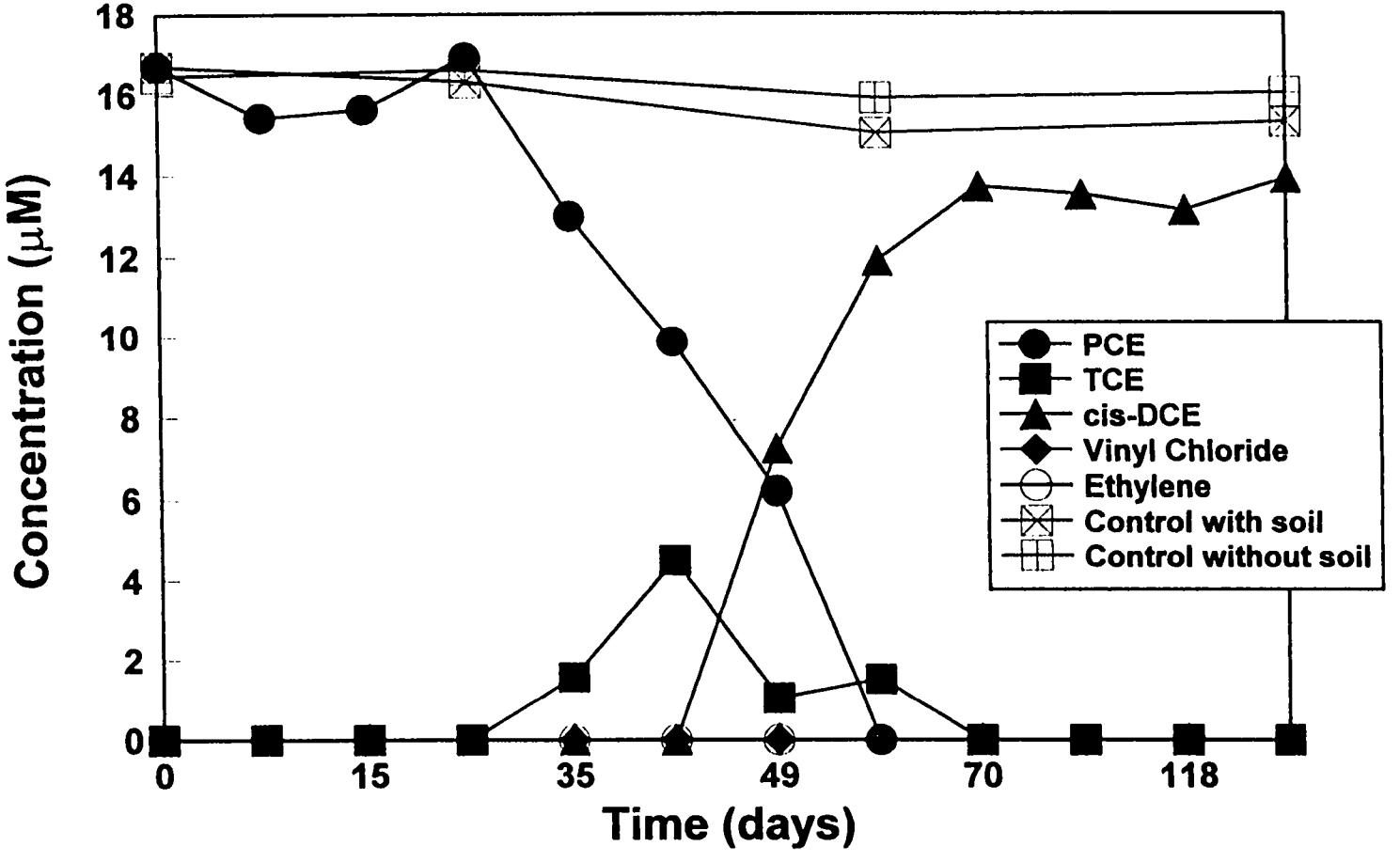


Figure 4.16(a) Effect of Crude DNA as Primary Substrate for the Dehalogenation of Perchloroethylene

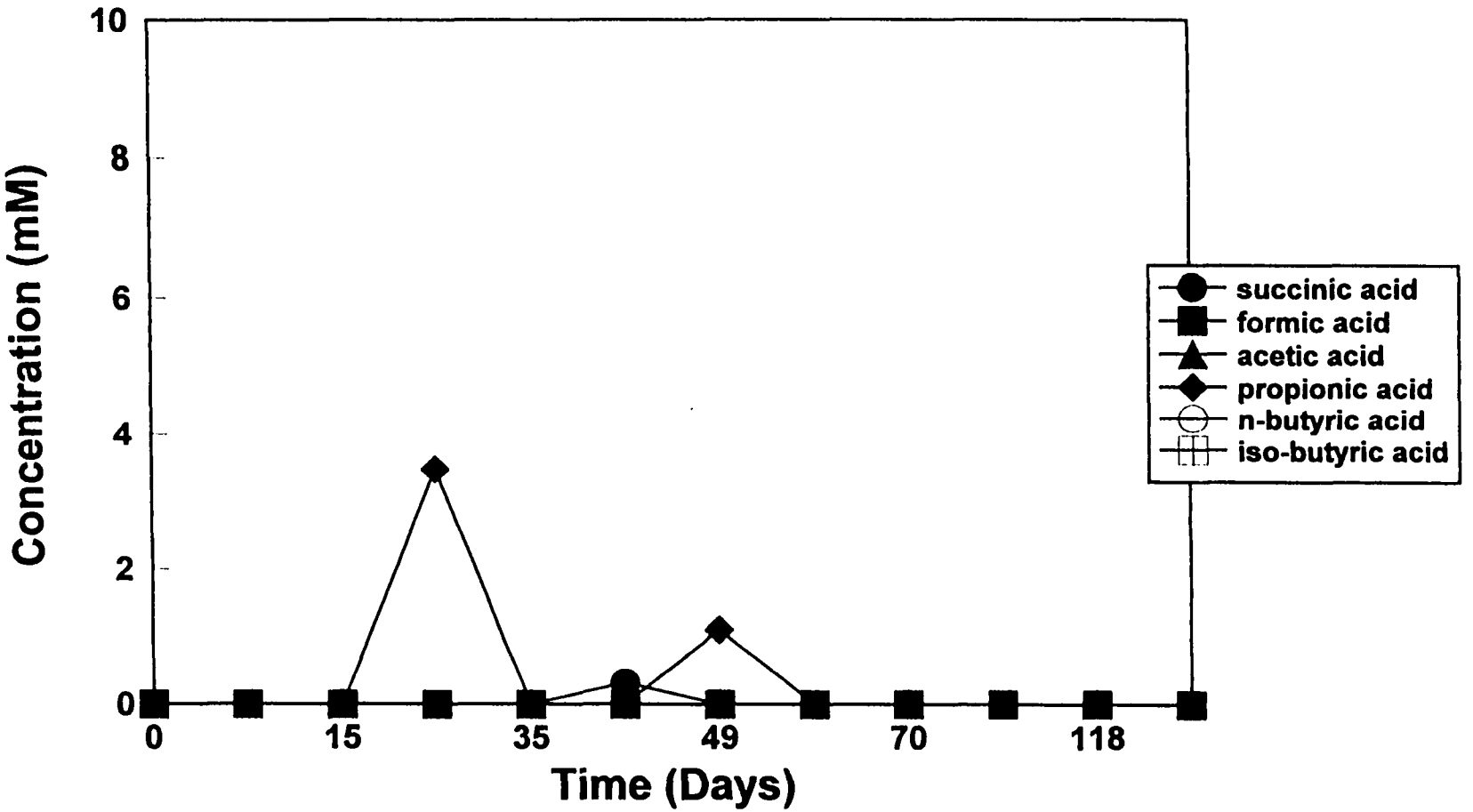
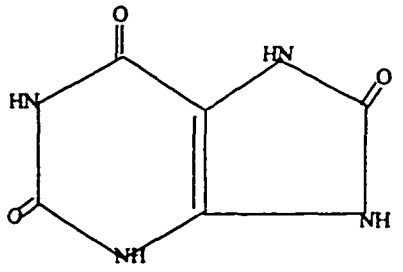


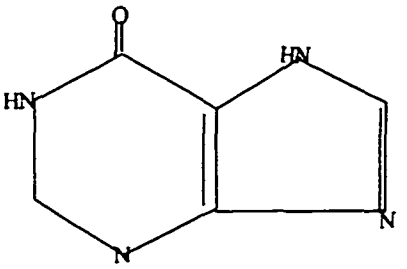
Figure 4.16(b) Fermentation Products with Crude DNA as Primary Substrate

APPENDIX THREE

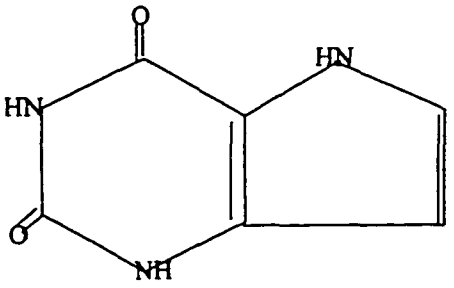
STRUCTURES OF PRIMARY SUBSTRATES TESTED IN CHLORINATED ALIPHATIC COMPOUND EXPERIMENTS



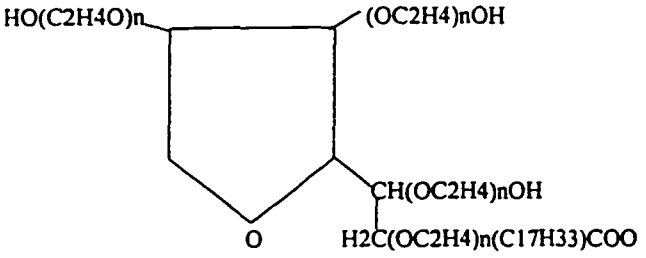
Uric Acid



Hypoxanthine



Xanthine



Tween-80

Figure 4.17 Known Structures of Primary Substrates Tested in Chlorinated Aliphatic Compound Experiments

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