

DEVELOPMENT OF BACTERIAL CULTURES WHICH CAN METABOLIZE
STRUCTURAL ANALOGS OF DIOXIN

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by

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ABSTRACT

The compound 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), an impurity in the herbicides 2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid and the defoliant Agent Orange, is considered by many to be one of the most toxic substances known to man. Widely released into the environment via pesticide application, improper hazardous waste disposal, and various industrial processes, the hydrophobic TCDD accumulates in the food chain and has been found to be resistant to microbial biodegradation. In an effort to address this recalcitrance, a two-phase biodegradation strategy was conceptualized. The first phase will acclimate mixed anaerobic cultures to structural analogs of TCDD, while the second phase will utilize these acclimated inocula as a source of microorganisms in a TCDD biodegradation study. In order to develop an anaerobic biodegradation approach for soils and sediments contaminated with TCDD, methanogenic and denitrifying cultures were established on a variety of chloroaromatic substrates using an inoculum from Newtown Creek, New York. Cultures were amended with 2-chlorophenol, 3-chlorophenol, 4-chlorophenol, 2,3-dichlorophenol, 3,4-dichlorophenol, 4,5-dichlorophenol and catechol. In addition, select cultures were amended with a mixture of fatty acids in order to supply excess electron donor for reductive dechlorination. The effect of mixed substrates and a solubilizing agent, *n*-butanol, was also evaluated for its effect on culture activity. Biodegradation

was assessed by quantifying the disappearance of substrate, appearance of intermediates and the stoichiometric products of methanogenesis or denitrification.

Dehalogenation was observed under methanogenic conditions, with monochlorophenols producing phenol and dichlorophenols producing monochlorinated phenols and phenol.

While catechol was degraded under methanogenic conditions, based on gas production, the chlorinated catechol did not appear to undergo biodegradation under any condition,

Cultures amended with mixed substrates exhibited enhanced dehalogenation in the presence of n-butanol under methanogenic conditions and minimal degradation under denitrifying conditions. Biodegradation of TCDD adsorbed onto particles of gallium oxide is currently being investigated with an amalgamation of the active single-substrate methanogenic cultures.

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I. INTRODUCTION

Dioxins are a highly stable class of chlorinated aromatic compounds. Based upon high toxicity in select species, the compound 2,3,7,8-tetrachloro-*p*-dibenzodioxin (TCDD) is considered one of the most toxic substances known, having an LD₅₀ of 0.6 µg/kg in guinea pigs (Hanson, 1991). TCDD often occurs as an incidental impurity in the synthesis of herbicides like 2,4,5-trichlorophenoxyacetic acid, 2,4-dichlorophenoxyacetic acid and Agent Orange. Polychlorinated biphenyls (PCBs), besides being toxic in their own right, may also be contaminated with dioxins and may represent a potential source of dioxin following incineration. Chlorinated aromatic compounds like TCDD and PCBs have been inadvertently released into the environment via incinerator emissions, transformer fires, and inadequately-managed hazardous waste disposal sites (Hutzinger, et al, 1985; Hutzinger, et al., 1985).

To date, no studies to our knowledge have successfully demonstrated the anaerobic biodegradation or biotransformation of TCDD. Remarkably, a removal of a single chlorine atom from TCDD would reduce the LD₅₀ by several orders of magnitude (Nessel, 1992). The purpose of this study was to investigate a novel biodegradation approach for the dechlorination of TCDD-contaminated soil using mixed cultures acclimated to water-soluble TCDD analogs, including mono- and dichlorinated phenols and catechols. This paper

reports on the preliminary development of microbial cultures capable of dechlorinating many of the structural analogs studied.

A. TCDD Toxicity.

Aquatic life in general is susceptible to the toxic effects of TCDD contamination, even though the aqueous solubility of TCDD is less than 200 parts per trillion. Many invertebrates live in or near the sediments and extract nutrients from ingested sediment particles. Since TCDD has a high partition coefficient and is very lipophilic, these organisms can bioaccumulate the compound from the contaminated sediments, or the contaminant may be directly absorbed through permeable tissues. This accumulation at the lower trophic levels of the food chain causes higher organisms, including commercial fish, to develop even higher total body burdens of (Cooper, 1989). Uptake and subsequent toxicity of TCDD to aquatic life was demonstrated by Yokim et al. (1978). A silt loam soil treated with TCDD to a concentration of 0.1 ppm was introduced into an ecosystem consisting of water fleas, snails, and algae separated from mosquito fish through a permeable partition. TCDD concentrations in the water were stable at 3 parts per trillion after one day. Ratios of fish TCDD tissue concentration to water concentration ranged from two- to six-thousand, with death occurring after 15 days of exposure to TCDD-contaminated water. This study clearly demonstrates the

sensitivity of some species of fish to very low aqueous concentrations of TCDD.

B. Resistance to Biodegradation.

Besides being extremely toxic, many researchers have shown TCDD to be highly resistant to biodegradation. Ward and Matsumura (1978) found that TCDD was not significantly biodegraded when incubated with lake sediment and water, and a basal media. Kearney, et al. (1972) spiked a silty clay loam and a loamy sand with radiolabelled TCDD. Since they detected very little $^{14}\text{CO}_2$, as measured by radioassay, metabolism by indigent populations in the sediment was considered to be minimal. After 350 days incubation, recovery of TCDD by combustion was 52 to 89%. The loss was thought to be due to evaporation, although TCDD is not very volatile. Resistance of TCDD to microbial metabolism was also shown by Matsumura and Benezet (1973) when they revealed that only five of 100 strains of previously proven pesticide-metabolizing bacteria were capable of degrading TCDD.

Part of this resistance to biodegradation may be due in part to poor bioavailability. TCDD has a high octanol-water partition coefficient (K_{ow}) of $10^6.8$, which favors adsorption onto hydrophobic surfaces over dissolution in water (Shiu, et al., 1988). In lakes and rivers, most of the contamination would be expected to settle out and adsorb onto sediments. For example, in a partitioning study conducted by Ward and Matsumura (1978), approximately 95% of the TCDD added to a

mixture of clean lake water and sediment was found to be associated with the sediment. Organic matter in soil and sediment has many reactive sites that could potentially immobilize hydrophobic compounds. Decreased bioavailability due to adsorption to sediment may cause low biodegradation rates (Sims, et al., 1990).

C. Anaerobic Biodegradation Processes and Engineering Applicability.

Despite previous negative studies on the aerobic microbial transformation of TCDD, anaerobic biodegradation strategies may possess several distinctive advantages, especially in the *in situ* remediation of contaminated soils and sediments. Although relatively slow, *in situ* biodegradation processes are a cost-effective approach to site remediation. Transportation costs, potential spread of contaminants, and liability are minimized when contaminated materials do not have to be transported to an off-site treatment facility (Sims, et al., 1990). Biodegradation is also less controversial in the eyes of the public than alternative methods like on-site incineration, and would probably be more acceptable to residents neighboring a contaminated site.

Most sediments and soils are aerobic for only the first few centimeters below the surface. Below this depth, or in poorly-aerated systems, anaerobic processes predominate. Since water-insoluble compounds like TCDD and PCBs often

enter anaerobic environments by sedimentation, an understanding of the fates of these chemicals under anoxic conditions is important. Important anaerobic electron acceptors in the anaerobic environment include nitrate, sulfate and carbonate. Due to thermodynamic considerations, denitrification (NO_3^- reduction to N_2) precedes sulfate reduction (SO_4^- reduction to H_2S), which precedes methanogenesis (CO_2 reduction to CH_4). In the field, denitrifying consortia are usually found at redox potentials around -50 mV, while methanogenic consortia are found at redox potentials around -350 mV (Young, 1984). Engineering processes based on sulfate reduction may not be practical because by-products often include poisonous hydrogen sulfide gas. To date, dehalogenation has been demonstrated under methanogenic (Woods, et al., 1989; Hrudney, et al., 1987; Suflita and Miller, 1985; Kohring, et al., 1989; Mikesell and Boyd, 1985; and Zhang and Wiegel, 1990) and sulfate reducing conditions (Hagblom and Young, 1990), but not under denitrifying conditions. Dissimilation of these compounds under denitrifying conditions would be desirable, since 1) required nitrate concentrations could be balanced with regulated drinking water limits, 2) redox requirements are not as stringent, and 3) denitrifying bacteria are all facultative and hence tolerant to molecular oxygen.

As stated earlier, development of a feasible biodegradation process for TCDD biodegradation requires a stable microbial population that can dehalogenate aromatic

compounds. In this study, such cultures have been produced by acclimating microbial populations in sediments to various chlorinated substrates. The effects of single and mixed substrates will be examined, as well as the effect of a solubilizing agent.

II. APPROACH

A. Mixed Cultures.

Sediment from Newtown Creek, New York City was chosen as an inoculum source. It is anticipated that the effluents received by this stream and the contaminated nature of the sediments may have provided an important selective pressure for the enrichment of bacteria capable of metabolizing environmental toxicants. Several investigators (Sufilita, et al., 1982; Boyd and Shelton, 1984; Woods et al., 1988) have shown chloroaromatic degradation of chlorophenols and chlorobenzoates under methanogenic conditions. After an initial lag period, the initial reaction in the metabolism of chlorophenols, chlorobenzoates, and PCBs under anaerobic conditions was dehalogenation producing phenols, benzoates and lower-chlorinated PCBs. Once acclimation was achieved, subsequent dehalogenation activity occurred more rapidly.

B. TCDD Analogs.

Dioxins, as previously noted, have a low water solubility and limited bioavailability. These factors may contribute to the difficulty to the enrichment and

acclimation of bacteria with an ability to metabolize TCDD. In this study, polar structural analogs of TCDD were used to develop a dehalogenating bacterial consortia. The selection of these analogs was based upon a recent study which demonstrated the ether cleavage of dibenzo-*p*-dioxin in the presence of diphenyl ether to benzene, catechol, phenol and methane (Dawyer and Tiedje, 1986). While it is suspected that phenol is the product of diphenyl ether metabolism, these results suggest that one of two ether cleavage patterns may occur. Figures 1 and 2 present the products of symmetrical and asymmetrical ether cleavage.

Figure 1. Symmetrical cleavage of 2,3,7,8-TCDD.

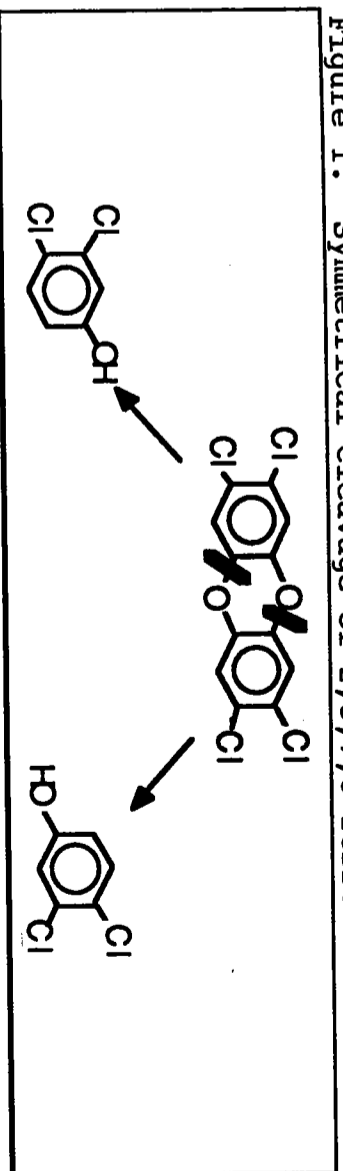
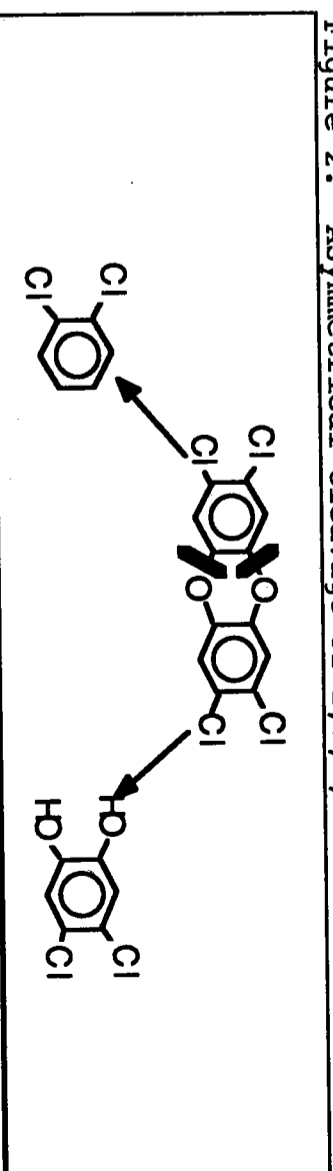


Figure 2. Asymmetrical cleavage of 2,3,7,8-TCDD.



Symmetrical cleavage involves breaking of the oxygen-carbon bond to produce two molecules of 2,3-dichlorophenol (2,3-DCP). Asymmetrical cleavage would produce 4,5-dichlorocatechol (4,5-DCC) and 1,2-dichlorobenzene (DCB). Subsequent dehalogenation of these products could produce 2-chlorophenol (2-CP), 3-chlorophenol (3-CP), 4-chlorophenol (4-CP), 3,4-dichlorophenol (3,4-DCP) and catechol (dihydroxybenzene). All of these substrates are considerably more water soluble than TCDD and were chosen as the substrates for acclimation of a dehalogenating consortia. DCB is sparingly soluble, and was not examined in this study.

C. Co-substrates and Solubilizing Agents.

Reducing equivalents (electrons) are required to reductively dehalogenate TCDD to *p*-dibenzo-dioxin. Based on some previous studies demonstrating that electron donors appear to enhance dehalogenation (Sufliita, 1990), an aliquot of co-substrates consisting of an equimolar mixture (1:1:1:1) of acetate(C2):propionate(C3):butyrate(C4):valerate(C5):caproate(C6) was added to particular cultures.

Additionally, the amendment of a co-solvent to the culture should lower the octanol-water partitioning coefficient (K_{ow}), theoretically shifting the equilibrium and releasing adsorbed TCDD for equilibrium dissolution in the aqueous phase. This shift should increase the aqueous concentration of TCDD, thereby increasing its bioavailability. In this study, *n*-butanol was added to some

cultures to investigate its potential as a solubilizing agent.

III. METHODS

A. Sediment Collection.

Inoculation for all cultures was made using a 2:1 (wt/vol) dilution of sediments collected from Newtown Creek. Sediment was collected in mid-June from the center of Newtown Creek about 200 yards off the East River, courtesy of the New York City Department of Environmental Protection Research Vessel during a routine monitoring run. The sediment was placed into 0.5 L glass containers leaving no headspace, and transported back to the lab within 24 hours.

B. Media Preparation.

The denitrifying media (DM) used is the same as that described by O'Connor and Young (1992) except that the purge gas was high purity helium (Matheson Gas, Rutherford, NJ). Briefly, the DM contained Na_2HPO_4 and KH_2PO_4 (29 and 19 mM respectively), NH_4Cl (5.6 mM), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.4 mM), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (9 mM), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (1.2 mM), KNO_3 (30 mM as sole electron acceptor) and 15 ml/L of trace elements and 10 ml/L vitamins (Owen, et al., 1979; O'Connor, et al., 1989). The methanogenic media (MM) was also made according to the method described by O'Connor and Young (1992). MM contained resazurin (1 ml of a 3.9 mM stock solution), $(\text{NH}_4)_2\text{HPO}_4$ (1.57 ml of a 20 mM stock), $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (1 ml of 1.86 mM stock), $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (1 ml of a 2.3 mM stock), NaHCO_3 (2.6 g) and 15 and

ten mL of trace vitamins and elements as discussed above. In these cultures, the amended NaHCO_3 functioned as the electron acceptor.

C. Culture Preparation and Monitoring.

Chlorinated phenols and catechol were purchased at the highest available purity from Fluka Chemical (Ronkonkoma, NY). The 4,5-dichlorocatechol was purchased from Helix Biotech Corporation (Richmond, British Columbia, Canada). Cultures amended with each of the chlorinated aromatic substrates (0.25 mM) were established under denitrifying and methanogenic conditions. These included 2-CP, 3-CP, 4-CP, 2,3-DCP, 3,4-DCP, 4,5-DCC and catechol. In addition, a set of mixed substrate cultures were established with 0.05 mM of each substrate. In both cases, all cultures were established in duplicate, and an autoclaved sterile control was also prepared. Background controls (cultures to which no substrates were added) were also established. Only with the mixed substrate cultures was butanol (10% v/v) examined as a co-substrate. Cultures were incubated at 37°C in the dark.

Approximately 1.0 mL of sample was routinely withdrawn from each culture for substrate determination. Samples were acidified with 50 μL of 10% HCl and centrifuged for 8 minutes. The supernatant was removed and filtered through a 0.45 μm hydrophilic syringe filter, and placed in 1.5 mL HPLC screw-top vials. Chlorinated substrates and phenol, a suspected metabolite, were analyzed by high-performance

liquid chromatography (HPLC). HPLC running solvents consisted of a solution of 2% acetic acid in deionized water and a solution of 2% acetic acid in methanol. Acetic acid solutions and a ramped concentration program were employed to optimize peak separation. The program consisted of 5 minutes at 70% water solution:30% methanol solution, ramp to 30% water solution:70% methanol solution over 20 minutes, hold for 5 minutes. The column was a 25.4 cm LC-18-DB obtained from Supelco, Inc. (Bellefonte, PA), and was held isothermally at 50°C in an SSI 505 LC Column Oven. The detector was a UV-spectrophotometer operated at 280 nm. This protocol separated all of the chlorophenols, as well as phenol.

Due to early difficulties encountered during analytical method development, the initial substrate concentrations described are nominal. When a substrate concentration fell below detection limits, the cultures were reamended to approximately the same initial substrate concentration.

Headspace gas was analyzed by packed column installed on an HP 5880A gas chromatograph. A 0.25 mL sample of headspace gas injected onto a Supelco 1-2382 packed column (5' x 1/8" stainless steel support, 45/60 Carboxen 1000) held isothermally at 200°C. The thermal conductivity detector allowed detection and quantitation of nitrogen, carbon dioxide, methane and N_2O in a single injection.

In order to ensure that denitrifying cultures were not nitrate limited, nitrate levels were monitored with a Dionex ion chromatograph .

IV. RESULTS

A. Monochlorinated Phenols.

Figures 3 - 5 show substrate depletion and gas production data for all methanogenic and denitrifying cultures amended with monochlorinated phenols (MCP). Substrate concentrations and methane and nitrogen production were plotted as a function of time. In all cases, excess gas production above that recorded for the background control, coupled with a loss of substrate, were the criteria for assessing biodegradability.

Under methanogenic conditions, 2-CP (Figure 3a) was metabolized within six weeks, producing phenol as an intermediate. After subsequent reamendment of the culture, complete degradation was again observed within six weeks incubation. Methane exceeded background production after phenol depletion began. Under denitrifying conditions (Figure 3b), phenol appeared after sixteen weeks incubation, after which the concentrations of 2-CP were observed to decrease sharply. Under methanogenic conditions, cultures amended with 3-CP (Figure 4a) did not produce phenol until after 18 weeks incubation. Again, methane production significantly exceeded background control levels only after phenol concentrations began to decline. Under denitrifying

conditions (Figure 4b), phenol was produced from 3-CP after 20 weeks incubation, eventually leading to enhanced nitrogen production over the background control. As shown in Figure 5a, cultures amended with 4-CP significantly inhibited methanogenesis relative to that observed in the background control. Conversely, under denitrifying conditions (Figure 5b), there was approximately a 50% loss of substrate over 20 weeks with little suppression of denitrification.

For methanogenic cultures amended with 2-CP and 3-CP, the gas production patterns generally revealed excess CH_4 and N_2 , while 4-CP was found to be persistent in the study. While many of the single substrate denitrifying cultures became nitrate depleted during the incubation period, methanogenesis did not occur and NO_3^- was reamended. For the denitrifying cultures amended with 2-CP and 3-CP, gas production patterns revealed that dinitrogen was produced in excess of the background control, while the 4-CP culture produced the same amount as the background control.

B. Dichlorinated phenols.

Sequential dehalogenation of dichlorinated phenols was observed, with monochlorinated phenols appearing before phenol. In the dehalogenation of 2,3-DCP under methanogenic conditions (Figure 6a), the ortho chlorine was removed first, producing 3-CP. Initial concentrations of 2,3-DCP were completely removed by week 6. Following 2,3-DCP removal, 3-CP

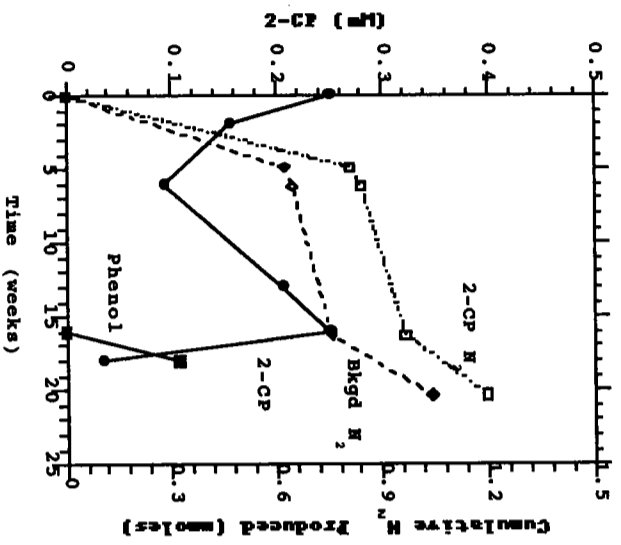
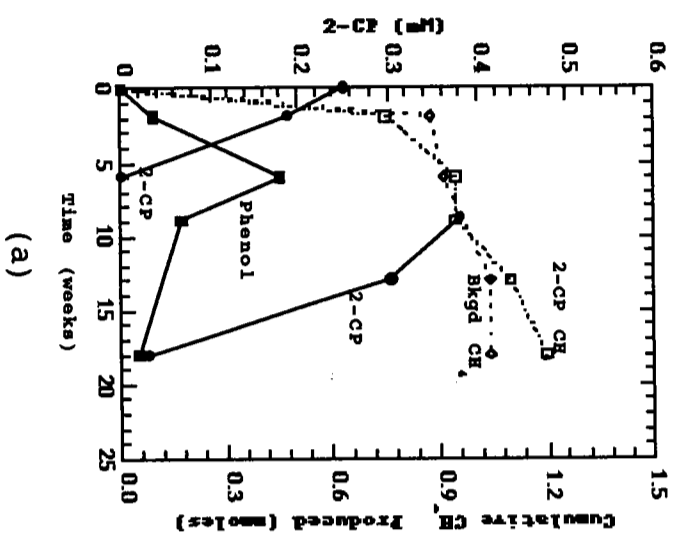


Figure 3. 2-CP: Methanogenic (a) and Denitrifying (b) Conditions

IV-22

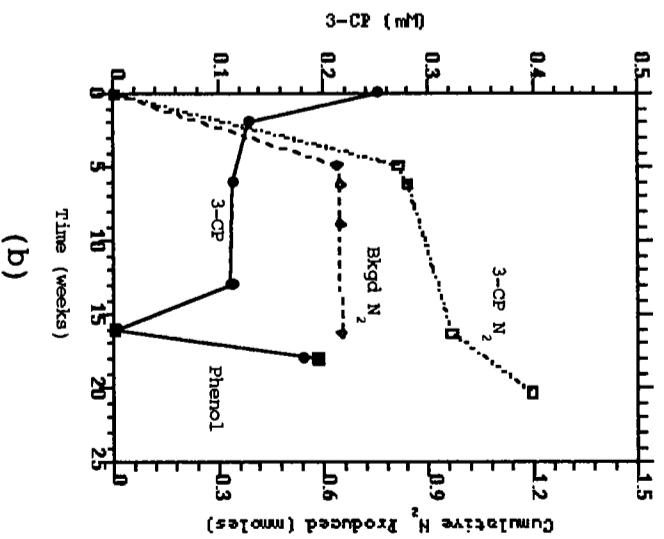
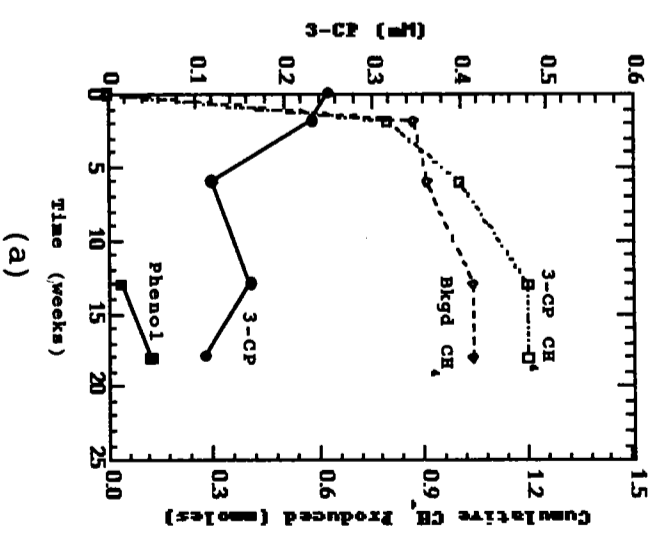
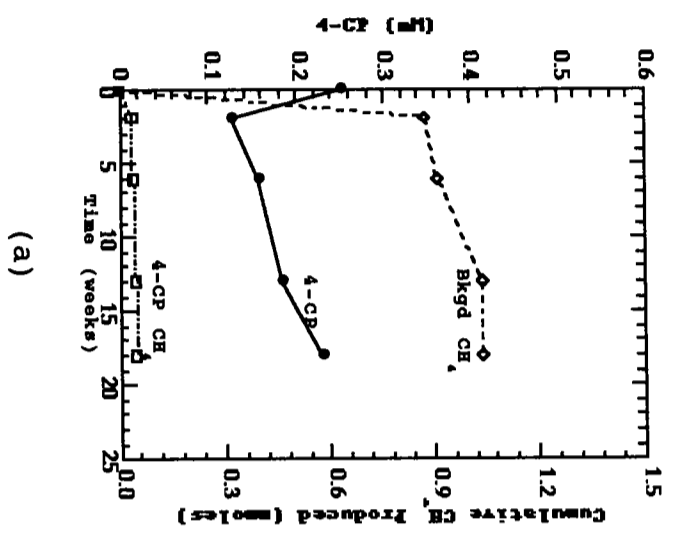
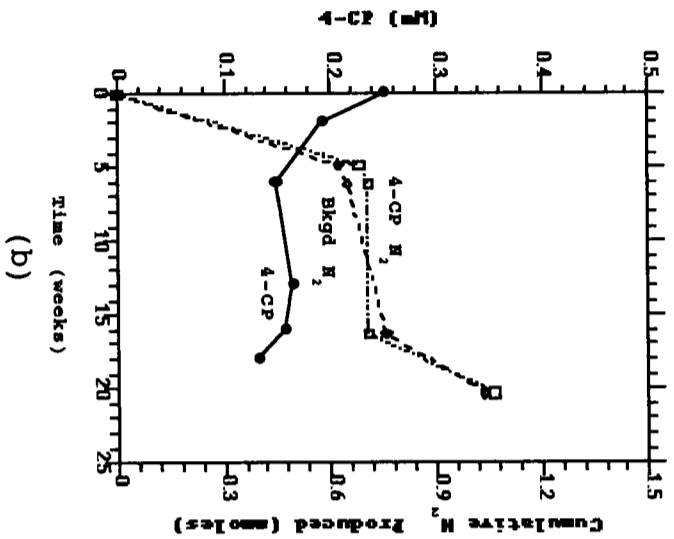


Figure 4. 3-CP: Methanogenic (a) and Denitrifying (b) Conditions

IV-23

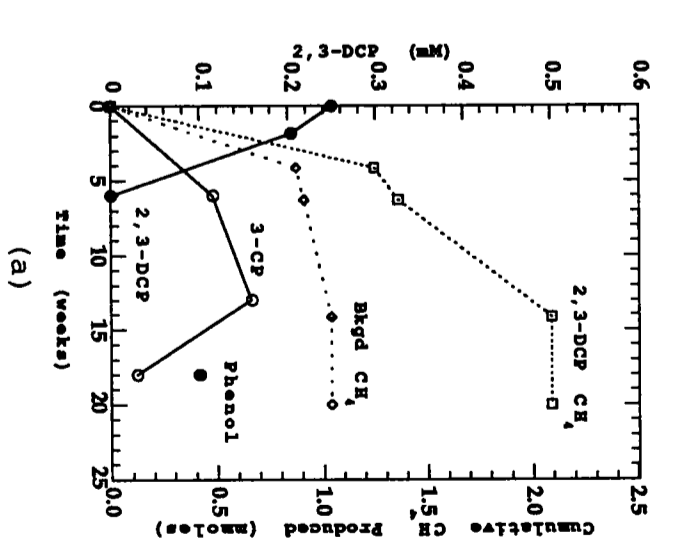


(a)

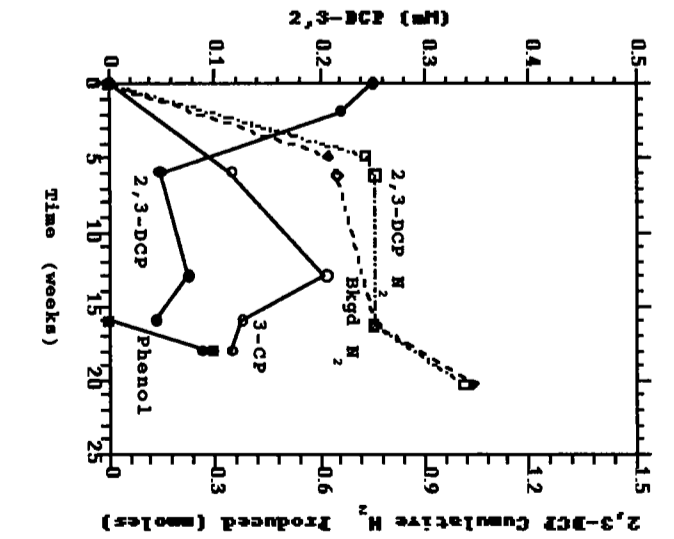


(b)

Figure 5. 4-CP: Methanogenic (a) and Denitrifying (b) Conditions



(a)

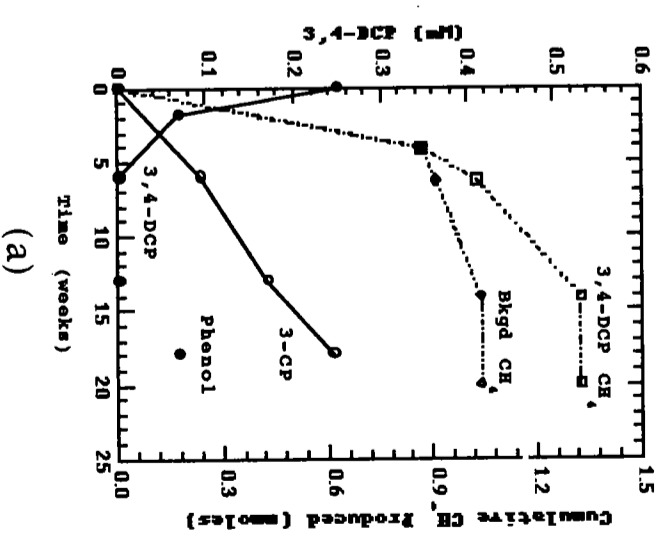


(b)

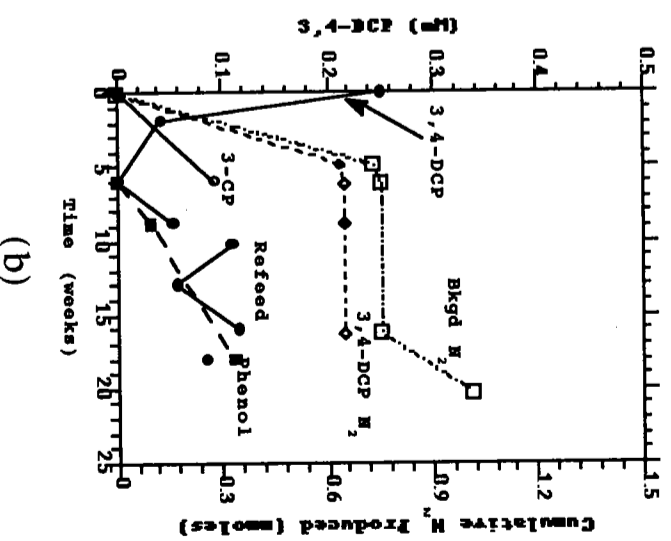
Figure 6. 2,3-DCP: Methanogenic (a) and Denitrifying (b) Conditions

and phenol concentrations were observed to increase, which was then followed by an increase in methane production above the background control. Under denitrifying conditions, similar observations were noted in that the ortho chlorine atom was preferentially removed producing 3-CP and eventually phenol. Since these cultures also became NO_3^- limited (no CH_4 was produced), these observations need to be reevaluated under conditions in which nitrate no longer becomes limiting.

Under methanogenic conditions, 3,4-DCP was rapidly biodegraded after six weeks acclimation, producing 3-CP as an intermediate (Figure 7a) and increased methane concentrations relative to the background control. As indicated by the appearance of 3-CP and phenol, it appears that degradation is occurring under denitrifying conditions. As with the 2,3-DCP denitrifying cultures, though, neither nitrate nor methane were detected by week 13, and conditions where nitrate does not become limiting need to be assessed. Dinitrogen production was also depressed relative to the background control. In both the 2,3-DCP and 3,4-DCP denitrifying cultures, there may be substrate degradation under denitrifying conditions. However, additional work on these cultures with more frequent assessment of nitrate levels at higher loadings must be completed before any definitive conclusions can be drawn.



(a)



(b)

Figure 7. 3,4-DCP: Methanogenic (a) and Denitrifying (b) Conditions

C. Catechols.

Gas production data from methanogenic and denitrifying catechol cultures are shown in Figures 8 and 9. The HPLC method used was not appropriate for catechol or 4,5-DCC analysis, and thus only gas data are shown for these cultures. Some methane in excess of background levels was produced in the catechol cultures (Figure 8a). Dinitrogen production initially paralleled, but later plateaued while control background levels increased (Figure 8b). Figures 9a and 9b show the gas production data for methanogenic and denitrifying cultures amended with 4,5-dichlorocatechol, and reveal that both methane and dinitrogen production levels were comparable to background levels.

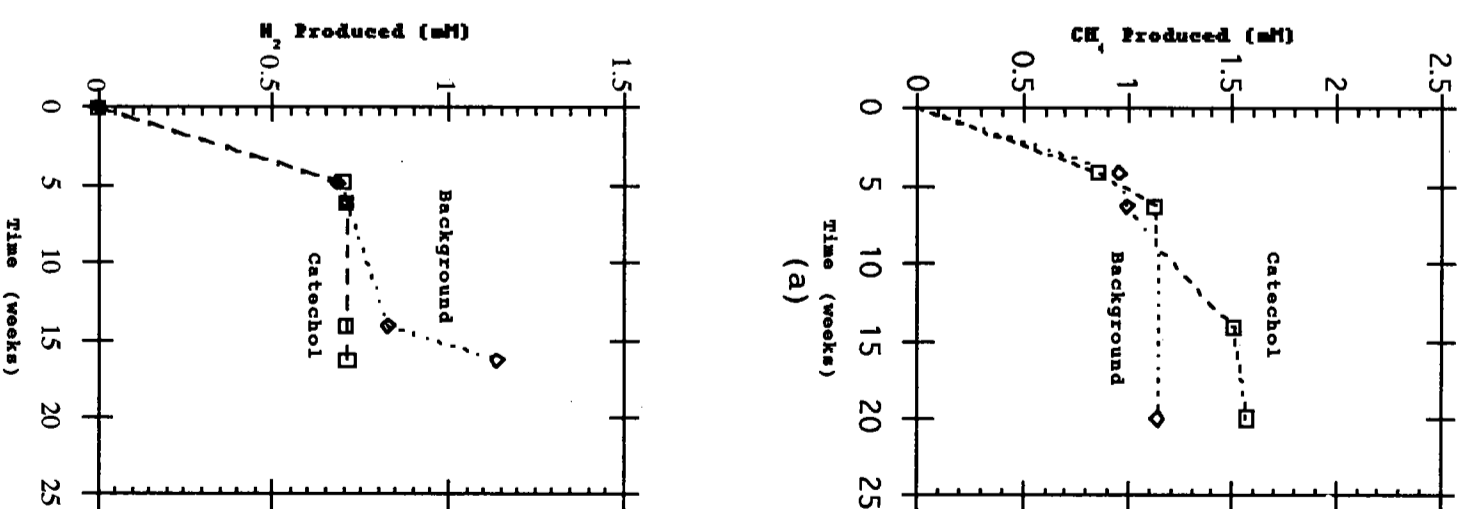


Figure 8. Catechol: Methanogenic (a) and Denitrifying (b) Conditions

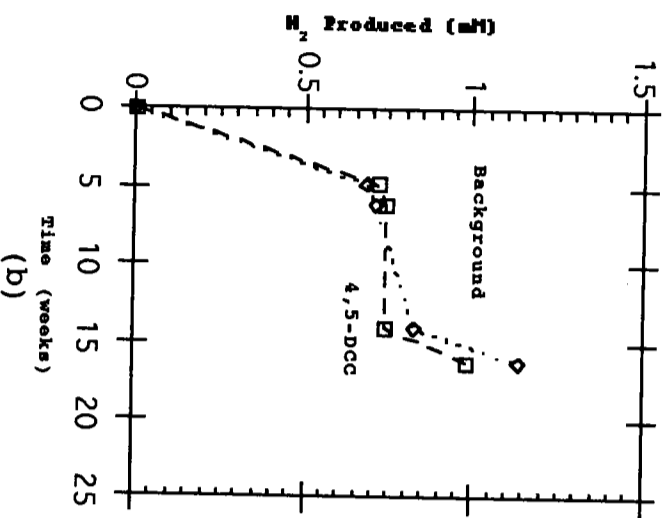
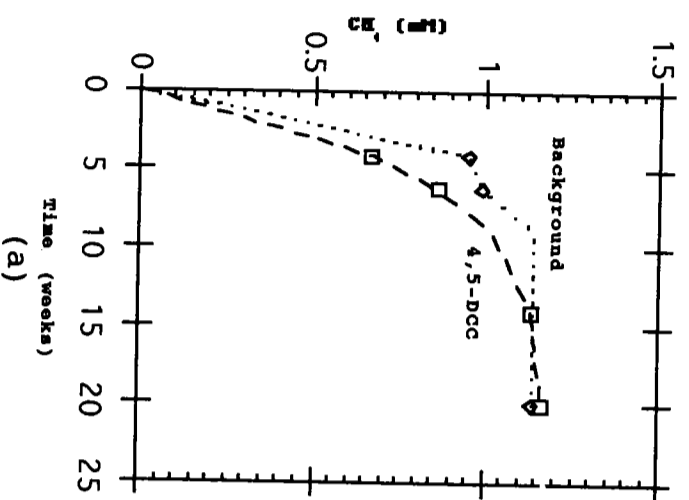


Figure 9. 4,5-DCC: Methanogenic (a) and Denitrifying (b) Conditions

D. Mixed Substrates.

Figures 10 - 14 show the results of mixed substrate amended cultures. Under methanogenic conditions (Figure 10), 2-CP was completely metabolized in the first two weeks. Concentrations of 2-CP, 3-CP and 2,3-DCP appeared to increase, presumably due to dechlorination of 2,3-DCP and 3,4-DCP. Concentrations of 4-CP appeared to be decreasing after 13 weeks incubation. Under denitrifying conditions (Figure 11), 2-CP concentrations declined after 13 weeks incubation and levels of other substrates were unchanged after 20 weeks incubation.

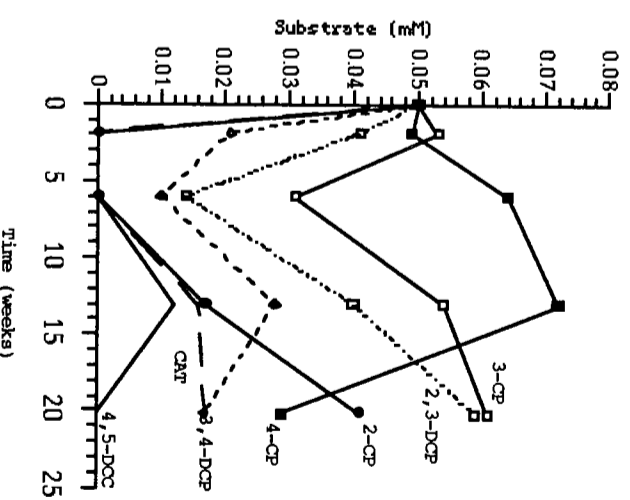


Figure 10. Mixed Substrates Under Methanogenic Conditions

regarding mixed substrate degradation under denitrifying conditions.

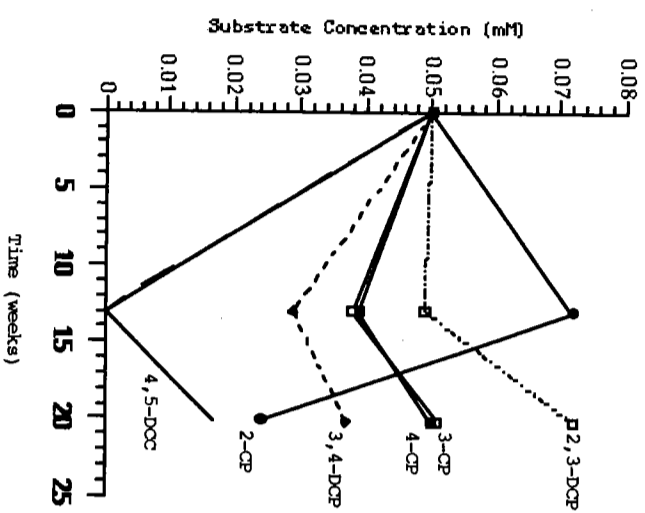


Figure 11. Mixed Substrates Under Denitrifying Conditions

The effect of butanol is shown in Figures 12 and 13 for methanogenic and denitrifying cultures, respectively. Concentrations of 2-CP in methanogenic cultures (Figure 12) initially increased and then decreased. In addition, 3-CP and 4-CP were depleted rapidly, then eventually reappeared after 13 weeks incubation. Phenol was never detected. n-Butanol may increase the aqueous solubility of these compounds, causing greater bioavailability and subsequently increased rates of metabolism and mineralization. Under denitrifying conditions (Figure 13), 2-CP and 4-CP concentrations were constant over 20 weeks incubation. Closer study of the noted disappearance of the other compounds is warranted before conclusions can be drawn

regarding mixed substrate degradation under denitrifying conditions.

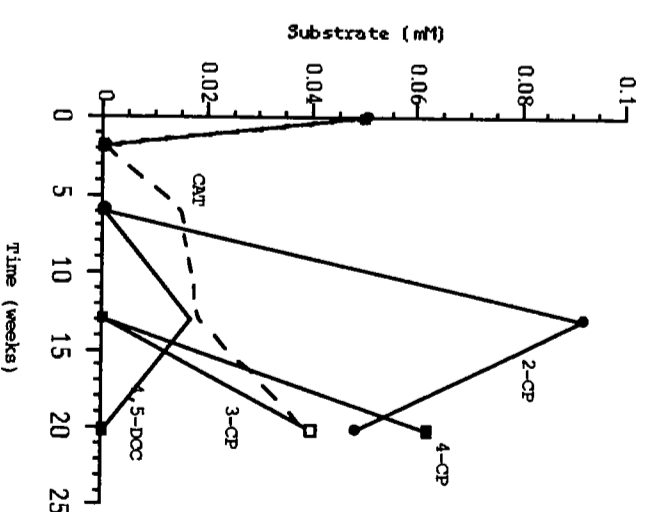


Figure 12. Mixed Substrates Under Methanogenic conditions with n-Butanol

