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Perchloroethylene Utilization by Methanogenic Fed-Batch Cultures

Acclimation and Degradation

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ABSTRACT

Anaerobic sludge granules from two different sources were maintained in serum bottles and fed at 3-d intervals in the presence of 0.2 ppm perchloroethylene (PCE). Following acclimation periods ranging from 48–79 d, PCE degradation was observed with 95% utilization in 3 d. Only granules amended with formate as a cosubstrate showed PCE-degrading activity, whereas those utilizing acetate, lactate, ethanol, and methanol remained PCE-inactive after 90 d. Both cosubstrate consumption and PCE degradation ceased when acetate replaced formate as the cosubstrate for formate-acclimated cultures. The total moles of chlorinated and nonchlorinated ethene moieties were found to remain constant over time, demonstrating conservation of mass. Trichloroethylene (TCE) and *cis*-1,2 dichloroethylene (*cis*-1,2 DCE) were identified as the major dechlorination end products. The absence of further dechlorination in the presence of a great excess of reducing equivalents (13,000 times that needed) supports the hypothesis that complete degradation of PCE to ethene is not solely dependent on excess reducing equivalents, but also requires the presence of an appropriate microbial consortium.

Index Entries: Perchloroethylene; degradation; biodegradation; methanogenic; acclimation; bioremediation; reductive dechlorination.

INTRODUCTION

First synthesized in 1821, perchloroethylene (PCE) has been used extensively as a solvent by the metal processing, electronics, pulp and paper, printing, textile, and dry cleaning industries *(1)*. Improper handling and storage have made this chemical a ubiquitous environmental pollutant, causing concern owing to its hazardous nature. PCE's omnipresence in the environment combined with its suspected carcinogenic nature has spawned widespread interest in developing techniques to treat groundwater and subsurface materials contaminated with this pollutant. Biodegradation appears to be one promising avenue.

Although the literature contains reports of biologically induced PCE degradation under a variety of conditions *(2–8)*, the process named "cosubstrate-dependent reductive dechlorination" remains incompletely understood. Reasons for variations in the extent of dechlorination observed, as well as information concerning

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the length of acclimation period, and the suitability of particular inocula and cosubstrates, continue to be unclear. This lack of basic knowledge concerning the biologically mediated transformation is an important barrier to the practical application of reductive dechlorination. In general, PCE dechlorination has been shown either to cease at dichloroethylene (DCE) *(6,9,10)* or to continue to ethene *(4,7,8)*. In the latter case, the transformation of vinyl chloride to ethene has often been identified as the rate-limiting step *(11)*. Since the process of reductive dechlorination relies on the presence of electron donors, it may be hypothesized that providing available electrons in great excess may produce a driving force sufficient to achieve complete degradation of PCE to ethene and/or ethane.

The research presented herein is part of an effort to elucidate further the reductive dechlorination phenomena and lay the groundwork for future process engineering studies. The specific goals of this work are divided into three categories: first, to document the effectiveness of several cosubstrates in facilitating reductive dechlorination by anaerobic sludge granules originating from two distinct sources, second, to quantify the acclimation period required prior to the onset of reductive dechlorination (in the literature, acclimation periods for undefined mixed cultures have ranged from several days to several months *[6,8,10]*), and third, to identify and quantify the end products of PCE degradation. In addition to establishing mass conservation, this result is interesting in the context of a literature characterized by dichotomous observations concerning the extent of PCE degradation.

MATERIALS AND METHODS

Materials

Glass serum bottles (250 mL) and quantities of reagent-grade PCE and trichloroethylene (TCE) (99.0% min) were acquired from VWR Scientific (Boston, MA). Practical-grade (>95%) 1,1 DCE, *cis*-1,2 DCE, *trans*-1,2 DCE, and ethene were obtained from Fluka (Buchs, Switzerland); 1000 ppm vinyl chloride in nitrogen and Teflon™-lined neoprene septa were purchased from Supelco (Bellefonte, PA). All other chemicals were reagent-grade and were acquired from VWR Scientific and/or Sigma (St. Louis, MO).

Source of Inoculum

Two distinct inocula were utilized in these experiments. The first inoculum was comprised of anaerobic sludge granules provided by Kevin Kennedy of the University of Ottawa. These granules, referred to as Ottawa sludge, were a mixture of biomass obtained from two anaerobic sludge blanket reactors—one having only been exposed to acetate during a decade of operation, another used to study the degradation of chlorophenols. Granules originating from a full-scale anaerobic sludge blanket reactor treating pulp and paper plant waste water formed the second inoculum. These later granules, denoted as New Brunswick sludge (NB), were provided by Irving Environment (New Brunswick, Canada).

Media Preparation

Solutions employed during these experiments are as follows. A buffer contained 2.78 g/L KH_2PO_4 and 7.18 g/L $K_2HPO_4 \cdot 3H_2O$. Mineral nutrient solutions contained 2.78 g/L KH_2PO_4, 7.18 g/L $K_2HPO_4 \cdot 3H_2O$, 4.5 g/L NH_4Cl, 0.15 g/L NaCl, 0.09 g/L $(NH_4)_2SO_4$, 0.037 g/L $CaCl_2 \cdot 2H_2O$, 0.037 g/L $MgCl_2 \cdot 6H_2O$, 0.5 mL/L of 2.0 g/L resazurin solution, and 2.9 mL/L trace mineral solution. The trace mineral solution was comprised of 2.00 g/L $FeCl_2$·$4H_2O$, 0.05 g/L $ZnCl_2$, 0.04 g/L $CuCl_2$·$2H_2O$, 0.10 g/L $NiCl_2$·$6H_2O$, 0.50 g/L $MnCl_2$·$4H_2O$, 0.03 g/L $AlCl_3$, 0.15 g/L $CoCl_2$·$6H_2O$, 0.05 g/L HBU_3, 0.50 g/L Na-EDTA, 0.068 g/L Na_2MoO_4·$2H_2O$, 1.0 mL/L HCl (concentrated), and was a modification of a previously published recipe *(12)*.

Mineral nutrient solutions were prepared by adding the aforementioned compounds to distilled water that had previously been autoclaved and sparged with nitrogen while cooling. The pH was adjusted using concentrated solutions of potassium hydroxide and/or hydrochloric acid. During the first 2 wk of the experiment, an initial pH value for mineral nutrient solutions was determined for each cosubstrate group such that the reaction broth pH approached 7.0 3 d after the cosubstrate was added. The initial pH values for mineral nutrient solutions for each cosubstrate were as follows: 6.5 acetate, 5.5. formate, and 7.0 methanol, ethanol, and lactate. Despite an initial pH of 5.5, the pH of formate-fed cultures approached 8.0 after 3 d of incubation. Mineral nutrient solutions were stored at 4°C, and were bubbled with nitrogen for approx 5 min prior to each use.

Substrate solutions contained 2.78 g/L KH_2PO_4, 7.18 g/L K_2HPO_4·$3H_2O$, 0.5 mL/L resazurin solution, 0.8 g/L yeast extract, and one of the following: 26.5 g/L $KC_2H_3O_2$, 168.0 g/L $KCHO_2$, 15.5 g/L $C_3H_6O_3$, 10.0 mL/L C_2H_6O, or 13.0 mL/L CH_3OH. These solutions were prepared by adding the first four chemicals to 3 L of water, and adjusting the pH to 7.0 using potassium hydroxide and/or hydrochloric acid. Appropriate amounts of cosubstrate were then added to 400-mL portions of the resulting solution. Addition of lactic acid required the pH to be again adjusted to 7.0, whereas all other cosubstrates had negligible influence on pH. Two hundred-milliliter portions of each substrate solution were autoclaved in 245-mL serum bottles and subsequently stored at 4°C. When diluted with 10 mL of inoculum and 65 mL of mineral nutrient solution, 10 mL of substrate solution resulted in final chemical oxygen demands (COD) of approx 1.0 g/L.

A PCE-saturated distilled water solution was prepared by combining 2 mL of PCE and 230 mL distilled water in a serum bottle sealed with a Teflon™-lined septa. The mixture was stirred for 24 h using a magnetic stirrer and was thereafter stored at 30°C.

Establishing and Feeding the Cultures

Batch Bottle Initiation

Using cosubstrate and inoculum as experimental variables, groups were defined as outlined in Table 1. Bottles were initiated by adding 10 mL of the appropriate sludge to 70 mL of mineral nutrient solution and 5 mL of substrate solution in a 245-mL serum bottle. Bottles were subsequently sealed and oxygen removed from the headspace by piercing the septa with a 30-gage needle, and repeatedly applying a vacuum and then pressurizing bottles with nitrogen at 2 psig (repeated three times and ending with nitrogen). Incubation at 30°C in an inverted position on an orbital shaker table running at 90 rpm with a 3/4-in. orbit radius followed. Duplicates were used in each group with the exception of groups III and IV, which were done in triplicate.

Feeding and Sampling

Typically, bottles were maintained in a fed-batch mode, with cosubstrate and PCE replenished every 3 d. During the morning of the last day of each cycle, bottles were analyzed for PCE content. After all bottles had been sampled, they were placed upright for several minutes, allowing granules to settle and resulting in minimal loss of biomass in the following steps.

Table 1
Experimental Matrix

Cosubstrate	Inoculum NB	 Ottawa
Acetate	Group I	Group II
Formate	Group III	Group IV
Lactate	Group V	Group VI
Ethanol	Group VII	Group VIII
Methanol	Group IX	Group X

Groups defined using inoculum source and cosubstrate as experimental variables.

Next, bottles were transferred to a fume hood where they were uncapped one by one and 60 mL of liquid were removed using a 60-mL syringe fitted with a 3-in. 13-gage needle. Liquid removed from selected bottles was employed in final (end of cycle) cosubstrate and pH analysis. Fifty-five milliliters of the appropriate mineral nutrient solution were added to each bottle, and the resulting mixture was sparged with nitrogen for 1 min. Following addition of 5 mL of the appropriate substrate solution, the bottle was sealed with a septa and oxygen purged from the headspace via the vacuum and pressurization technique previously mentioned.

Using a 1.0-mL Dynatech Series A-2 syringe fitted with a sideport needle (Supelco), 0.90 mL of liquid was removed from selected bottles to determine initial carbon source concentrations and replaced with 0.75 mL of buffer; 0.15 mL of liquid was removed from all other bottles, resulting in bottles with aqueous phases 0.15 mL short of 85 mL.

PCE addition was accomplished by using a 0.5-mL Dynatech Series A-2 syringe equipped with sideport needle to add 0.15 mL of PCE-saturated distilled water to each bottle. Resulting initial PCE concentrations were approx 0.2 ppm in the liquid phase. Bottles were subsequently returned to the shaker table and allowed to equilibrate for a minimum of 2 h before being sampled for initial PCE concentrations.

All bottles were maintained on 3-d cycles (with the exception of one 4-d cycle), consistent with the protocol described until d 91. Starting d 91, material balance experiments were performed on three bottles that had previously shown repeated losses of PCE. These bottles were fed in the usual manner, and concentrations of PCE, possible dechlorination byproducts, and cosubstrate were measured during the following 7 d. On the 2nd d of this experiment, 10 mL of liquid were removed from one bottle and replaced with 10 mL of a 42.5 g/L solution of sodium azide in distilled water. After the 7th d of this experiment, bottles were analyzed for methane content.

Following the material balance experiment, three bottles that had previously shown PCE activity when amended with formate were used to determine whether the observed dechlorination was cosubstrate specific. Baseline activity was established by feeding all bottles formate during an initial cycle. Subsequently, the cosubstrate for two of the bottles was changed to acetate, and concentrations of carbon source and PCE were determined at the beginning and end of the following four 3-d cycles.

Analytical Techniques

Analysis of Chlorinated Organics

Concentrations of chlorinated organics were determined by using a Dynatech Series A-2 syringe equipped with a push-button valve and a sideport needle to deliver a sample of headspace gas into a Shimadzu GC-9 fitted with dual Flame Ionization Detectors (FID). Medical-grade compressed air and practical-grade hydrogen (Phoenix Distributors, White River Junction, VT) were supplied to the FIDs. Carrier-grade helium (Phoenix Distributors) served as the carrier gas. Quantification was achieved by comparing sample peak heights with those of standards prepared by adding known amounts of suspected reaction byproducts to 245-mL serum bottles containing 85 mL of distilled water and nitrogen in the headspace.

PCE was analyzed by injecting a 0.5-mL sample onto a 8 ft. × 1/8 in. stainless-steel column packed with 1% SP-1000 on 60/80 Carbopack B preceded by a 6 in. × 1/8 in. precolumn containing an identical packing (Supelco). The oven, injector, and detector were operated isothermally at 220°C. The helium flow rate was 60 mL/min. The approximate detection limit for this technique was 6.0×10^{-9} mol/bottle.

Vinyl chloride, ethene, TCE, 1,1 DCE, and the total of *cis*-1,2 DCE and *trans*-1,2 DCE were measured by injecting a 1.0-mL gas sample into a 16 ft. × 1/8 in. stainless-steel column packed with 1% SP-1000 on 60/80 Carbopack B preceded by a 6 in. × 1/8 in. precolumn containing the same packing (Supelco). Helium was supplied at a flow rate of 30 mL/min. The injector and detector were operated isothermally at 220°C, while the oven started at 40°C for 1.75 min, ramped to 200°C at a rate of 15°C/min, and then remained at 200°C for 16 min. The approximate detection limits for this method were 6.2×10^{-9}, 2.3×10^{-9}, 6.5×10^{-9}, and 2.9×10^{-9} mol/bottle for TCE, 1,1 DCE, *cis*-1,2 DCE, and VC.

As *cis*-1,2 DCE and *trans*-1,2 DCE coeluted in the methods outlined above, a separate analysis for these two compounds was performed. One milliliter of headspace gas was injected onto a 8 ft. × 1/8 in. stainless-steel column packed with 20% SP-2100 on 80/100 Supelcoport (Supelco). The injector, detector, and column were operated isothermally at 220, 220, and 60°C, respectively. Helium served as the carrier gas at a flow rate of 30 mL/min. Since no *trans*-1,2 DCE was detected using this methodology, the response recorded using the aforementioned techniques was attributed to the *cis*-isomer. Approximate detection limits for the *cis*- and *trans*-isomers were 6.7×10^{-9} and 3.9×10^{-9} moles/bottle, respectively.

Analysis of Methane

Methane was analyzed by injecting a 0.5-mL headspace sample into a Perkin Elmer Series 900 Gas Chromatograph fitted with a 6 ft. × 1/8 in. stainless-steel column packed with HayeSep Q 80/100 (Alltech, Deerfield, IL). The injector, column, and detector were operated isothermally at 170, 110, and 190°C, respectively. Nitrogen was supplied as the carrier gas at a flow rate of approx 30 mL/min. Thermal conductivity was used as a detection method.

Analysis of Organic Acids and Short-Chain Alcohols

Cell broth concentrations of ethanol, acetate, lactate, formate, ethanol, and methanol were analyzed by delivering a 90-µL vol of sample fluid onto an Aminex HPX-87H high-pressure liquid chromatography column (Bio-Rad, Hercules, CA) equipped with a refractive index detector. A 0.01*N* sulfuric acid eluent was supplied to the column at a flow rate of 0.4 mL/min. The sample fluid was prepared by acidifying a 900-μL aliquot of reaction broth with 100 μL of 10% sulfuric acid (w/v), spinning it in a microfuge for 10 min, and transferring 720 μL of the resulting supernatant to an HPLC sample vial. Vials were capped, and either analyzed immediately or stored at 4°C until analysis was convenient.

RESULTS AND DISCUSSION

Acclimation of Formate-Amended Cultures

Figure 1 depicts "3-d conversions" during the first 91 d for formate-amended bottles. Three-day conversion is defined as the percentage of originally added PCE that remains at the end of a 3-d cycle, and thus each data point represents fractional change in concentration for a 3-d batch reaction. No appreciable PCE degrading activity was observed at the onset. However, bottles inoculated with Ottawa sludge and fed formate as a cosubstrate (Group IV) evidenced signs of activity on d 48, and by d 67 were degrading in excess of 95% of the original PCE. Acclimation of NB sludge amended with formate (Group III) occurred later. Appreciable losses of PCE for Group III bottles were first noted on d 79, and >90% conversion was observed by d 90. Three-day conversions for all other test groups remained within the shaded region of Fig. 1. In summary, both inoculum and cosubstrate appeared to influence the observed acclimation periods. Formate was the only effective cosubstrate under the conditions tested, and Ottawa sludge granules acclimated more rapidly than NB granules.

Stoichiometric Degradation by Biologically Active Cultures

Measured concentrations of chlorinated ethenes for the three bottles employed in the material balance experiment are summarized in Fig. 2. During this period, TCE and *cis*-1,2 DCE were the only detectable byproducts of PCE degradation. Bottles that were biologically active throughout the 7 d showed activity throughout the experiment, whereas the bottle amended with sodium azide showed little activity after its addition. PCE degrading activity of the azide-amended culture was approx 11% of that in the biologically active bottles. Although PCE utilization occurred in both of the biologically active bottles, their final product distributions differed. In one bottle, *cis*-1,2 DCE was the predominant end product, whereas in the other, an approximately equivalent number of moles of TCE and *cis*-1,2 DCE persisted on d 7. The differences in observed end products is most likely attributable to a higher PCE degrading activity in one of the cultures. As evidenced by its transient accumulation, TCE degradation was slower than PCE degradation, a result that is consistent with the literature. Complete dechlorination of PCE to ethene has been reported in some cases. However, our observations are consistent with another study that isolated cultures producing only TCE and *cis*-1,2 DCE from PCE *(7)*.

Since complete reductive dechlorination of PCE to ethene was not achieved, an available electron analysis was performed. In this analysis, reducing equivalents made available by yeast extract were neglected, and formate was considered the sole source of available electrons. Chlorinated ethenes as well as small amounts of acetate detected in the cell broth comprised the pool of electron acceptors. The source of the acetate is uncertain. However, homoacetogens may be responsible. Although its concentration increased throughout the 7 d, acetate levels remained below 10% of the initially added COD. Data for the available electron balance are outlined in Table 2. In all cases, <0.004% of the electrons donated by formate were

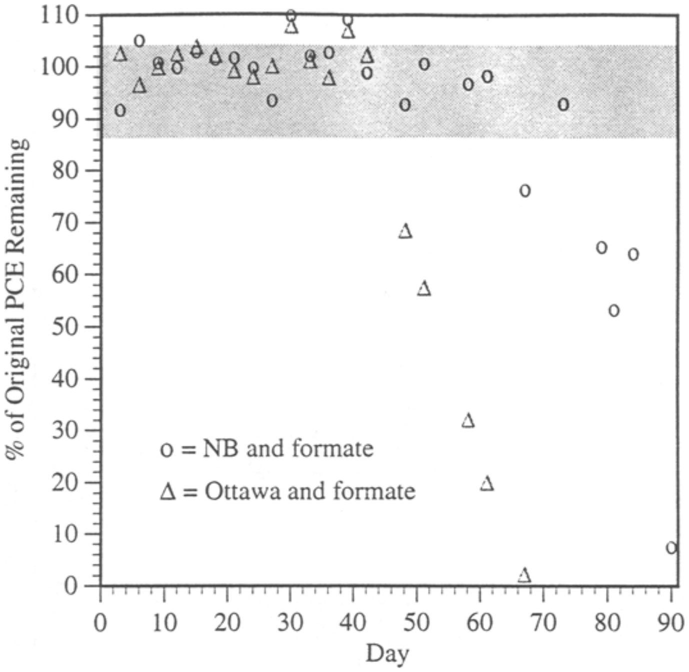

Fig. 1. Three-day conversions for formate-amended batch cultures exhibiting acclimation to PCE. Points represent the fraction of added PCE that remains at the end of 3 d of incubation. Gray region represents negligible activity.

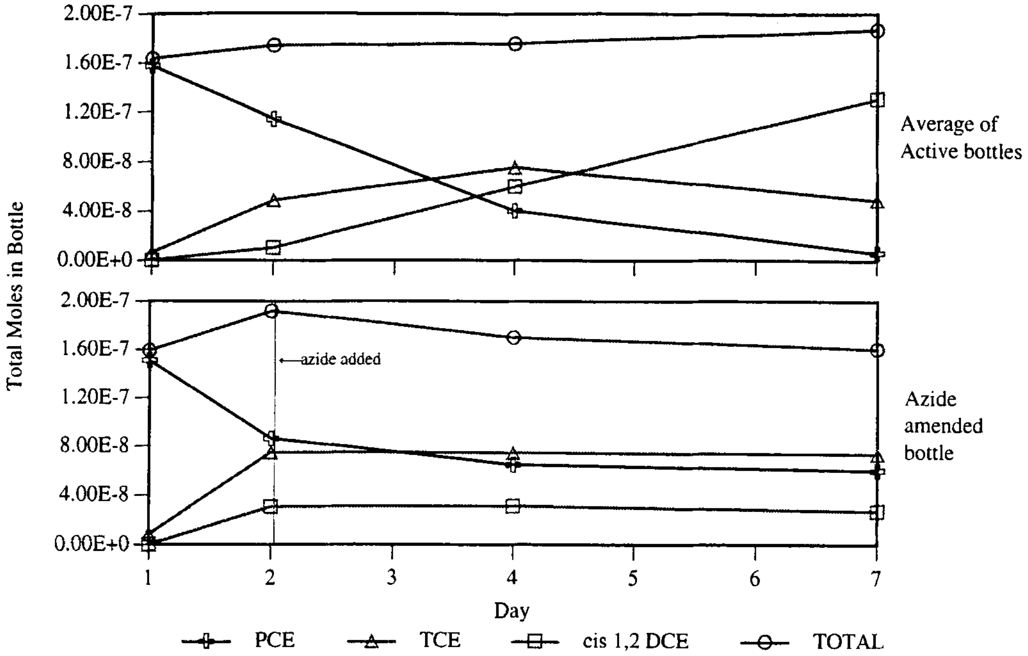

Fig. 2. Products of PCE degradation in acclimated formate-amended batch cultures. Cultures incubated and sampled during 7-d period beginning with the introduction of fresh PCE and growth media. Control culture inhibited using sodium azide on d 2.

Table 2
Available Electron (AE) Balance for Three Formate Amended Batch Cultures[a]

Bottle status	Compound	AE/mol, electron equivalent	Initial moles, d one	Final moles, d seven	AE used, electron equivalent	% AE used for dechlorination
Active	Formate	2	8.3×10^{-3}	0.0	-1.7×10^{-2}	0.004
	PCE	0	1.5×10^{-7}	6.5×10^{-9}	0.0	
	TCE	2	9.9×10^{-9}	9.5×10^{-9}	-1.0×10^{-9}	
	DCE	4	0.0	1.7×10^{-7}	6.6×10^{-7}	
	Methane	8	0.0	1.9×10^{-3}	1.5×10^{-2}	
	Acetate	8	0.0	9.8×10^{-5}	7.9×10^{-4}	
Azide amended	Formate	2	8.3×10^{-3}	0.0	-1.7×10^{-2}	0.001
	PCE	0	1.5×10^{-7}	6.0×10^{-8}	0.0	
	TCE	2	8.3×10^{-9}	7.3×10^{-8}	1.3×10^{-7}	
	DCE	4	0.0	2.7×10^{-8}	1.1×10^{-7}	
	Methane	8	0.0	1.9×10^{-3}	1.5×10^{-2}	
	Acetate	8	0.0	1.6×10^{-4}	1.2×10^{-3}	
Active	Formate	2	8.7×10^{-3}	0.0	-1.7×10^{-4}	0.003
	PCE	0	1.6×10^{-7}	5.9×10^{-9}	0.0	
	TCE	2	2.5×10^{-9}	9.0×10^{-8}	1.8×10^{-7}	
	DCE	4	0.0	9.7×10^{-8}	3.9×10^{-7}	
	Methane	8	0.0	1.9×10^{-3}	1.5×10^{-2}	
	Acetate	8	0.0	1.5×10^{-4}	1.2×10^{-3}	

[a] All batch cultures were incubated for 7 d after having been amended with formate on d 1; the control was inhibited via a sodium azide addition on d 2. The large fraction of electrons utilized in the production of methane speak to the prevalence of methanogenesis in these cultures. Dechlorination beyond DCE did not occur despite an excess of reducing equivalents.

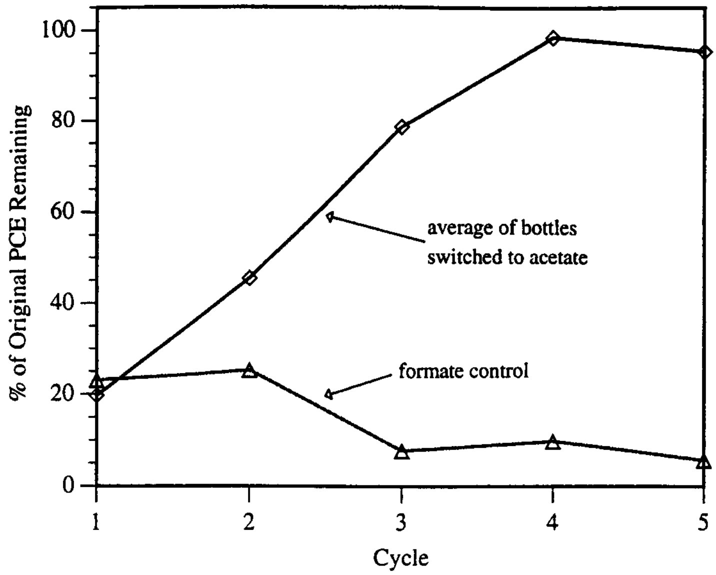

Fig. 3. Inability of acetate to sustain dechlorination when substituted for formate in previously acclimated cultures.

utilized in the dechlorinating process, and more than 13,000 times the required reducing equivalents were supplied. This is far in excess of previous studies, and supports the interpretation that a lack of reducing equivalents was not the cause of incomplete degradation by these cultures. A more plausible explanation is that complete degradation of PCE may be the result of a consortium rather than a single organism, and a lack of a certain microbial population resulted in the accumulation of *cis*-1,2 DCE. The prevalence of methanogenesis in these cultures is demonstrated by the large fraction of available electrons that were accounted for by methane production, ranging from 87–92%. In general, available electron balances were good, with recoveries ranging from 94–98%.

Formate-Dependent Degradation

Three-day conversions increased drastically following the substitution of acetate for formate and approached 100% by the third cycle (*see* Fig. 3). This was seen in conjunction with an absence of methane production and acetate utilization, suggesting negligible levels of acetoclastic methanogens in these cultures. The absence of acetoclastic organisms from these particular bottles was most likely owing to changes in the cultures over time since both sludges initially showed complete utilization of all cosubstrates tested.

CONCLUSIONS

The biodegradability of PCE by methanogenic anaerobic sludge granules was observed after an initial acclimation period consistent with that observed by other researchers *(6,10)*. The length of the acclimation process was dependent on culture conditions, including the source of inoculum and the type of cosubstrate supplied. Despite utilization of all cosubstrates tested, only formate-amended cultures displayed PCE degrading activity, with Ottawa and NB sludges evidencing activity on d 48 and 79, respectively. It is currently unclear why acetate was not able to facilitate PCE dechlorination in this experiment, since Ottawa granules provided acetate as a cosubstrate were previously shown to degrade PCE under a slightly different feeding protocol (data not shown).

Despite the presence of reducing equivalents in great excess, PCE was stoichiometrically converted to TCE and *cis*-1,2 DCE in these experiments. Further degradation to vinyl chloride and/or ethene was not observed. These results support the hypothesis that complete degradation of PCE to ethene and/or ethane may in some cases be dependent on the existence of the appropriate microbial consortium instead of on a single strain or a large pool of electron donors. This hypothesis is consistent with the isolation of two enrichment cultures exhibiting different dechlorinating capacities by DeBruin and Kotterman *(7)*; one culture was capable of transforming PCE to TCE, whereas the other affected conversion of DCE and VC to ethene.

Although our results suggest that anaerobic sludge granules may be quickly acclimated to PCE while being provided with a relatively inexpensive and harmless cosubstrate, increased understanding of the reductive dechlorination process is necessary prior to effective application of this type of biodegradation system. A greater understanding of how to ensure complete dechlorination of PCE, and thus avoiding the accumulation of harmful byproducts, is of crucial importance.

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REFERENCES

1. Keil, S. L. (1977), Chlorocarbons—Hydrocarbons, in *Encyclopedia of Chemical Technology*. Wiley-Interscience, New York.
2. Fathepure, B. Z. and Boyd, S. A. (1988), *Appl. Environ. Microbiol.* **54(12)**, 2976–2980.
3. Bagley, D. M. and Gossett, J. M. (1990), *Appl. Environ. Microbiol.* **56**, 2511–2516.
4. DiStefano, T. D., Gossett, J. M., and Zinder, S. H. (1991), *Appl. Environ. Microbiol.* **57(8)**, 2287–2292.
5. Kastner, M. (1991), *Appl. Environ. Microbiol.* **57(7)**, 2039–2046.
6. Sewell, G. W. and Gibson, S. A. (1991), *Environ. Sci. Technol.* **25**, 982–984.
7. DeBruin, W. P., Kotterman, M. J. J., Posthumus, M. A., Schraa, G., and Zehnder, A. J. B. (1992), *Appl. Environ. Microbiol.* **58(6)**, 1996–2000.
8. Narayanan, B., Suidan, M. T., Gelderloos, A. B., and Brenner, R. C. (1993), *Water Res.* **27(1)**, 181–194.
9. Fathepure, B. Z., Nengu, J. P., and Boyd, S. A. (1987), *Appl. Environ. Microbiol.* **53(11)**, 2671–2674.
10. Gibson, S. A. and Sewell, G. W. (1992), *Appl. Environ. Microbiol.* **58(4)**, 1392–1393.
11. Freedman, D. L. and Gossett, J. M. (1989), *Appl. Environ. Microbiol.* **55(9)**, 2144–2151.
12. Guiot, S. R., Gorur, S. S., and Kennedy, K. J. (1988), Nutritional and Environmental Factors Contributing to Microbial Aggregation During Upflow Anaerobic Sludge Bed-Filter (UBF) Reactor Start-Up. *Proceedings of the 5th International Symposium on Anaerobic Digestion, Bologna, Italy*. Pergamon, New York.