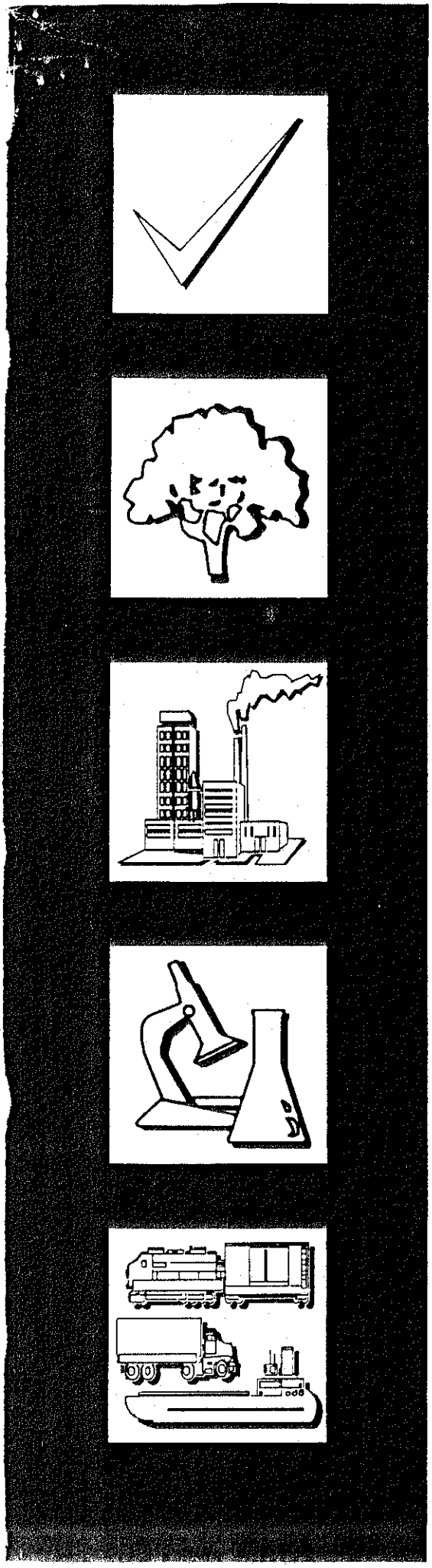

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A CO-METABOLIC APPROACH TO GROUNDWATER REMEDIATION*

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ABSTRACT

In support of the U.S. Department of Energy's (DOE) Integrated Demonstration (Cleanup of Organics in Soils and Groundwater at Non-arid Sites) at the Savannah River Site (SRS), Oak Ridge National Laboratory (ORNL) and the University of Tennessee (UT) are involved in demonstrations of the use of methanotrophs in bioreactors for remediation of contaminated groundwater. In preparation for a field demonstration at ORNL's K-25 Site in Oak Ridge, Tennessee, ORNL is conducting batch experiments, is operating a number of bench-scale bioreactors, has designed pretreatment systems, and has modified a field-scale bioreactor provided by the Air Force Engineering and Services Center for use at the site. UT is operating bench- scale bioreactors with the goal of determining the stability of a trichloroethylene-degrading methanotrophic consortia during shifts in operating conditions (e.g. pH, nutrient inputs, and contaminant mixtures). These activities are all aimed at providing the knowledge base necessary for successful treatment of contaminated groundwater at the SRS and K-25 sites as well as other DOE sites.

INTRODUCTION

Numerous chlorinated hydrocarbons pose serious threats to groundwater sources. Chlorinated hydrocarbons and organic solvents are widespread priority pollutants of groundwater, and of these trichloroethylene (TCE) is the most frequently reported contaminant at waste disposal sites on the U. S. Environmental Protection Agency (EPA) National Priority List (1). Concentrations of priority pollutants in excess of 1000 $mg \cdot l^{-1}$ have been reported beneath some U. S. Department of Energy (DOE) sites (2). TCE, a suspected carcinogen, is resistant to degradation in the subsurface environment, air stripping, carbon adsorption, and other traditional methods for removal of TCE from groundwater merely transfer TCE from one medium to another. Thus, DOE has a significant interest in developing new methodologies for the treatment of TCE contamination.

The DOE Integrated Demonstration (Cleanup of Organics in Soils and Groundwater at Non-arid Sites) at the Savannah River Site (SRS) is designed to address this type of groundwater contamination. Significant efforts under way at SRS and numerous DOE facilities, including Oak Ridge National Laboratory (ORNL), are aimed at demonstrating innovative treatment technologies for chlorinated organics at SRS. The activities at ORNL specifically focus on the remediation of a site in Oak Ridge, a seep at the K-25 Site, but also support the Integrated Demonstration by providing information on the generality of the bioremediation approaches being taken and data on operating conditions that are common to the technology not specific to the site. ORNL and the University of Tennessee (UT) are involved in demonstrations of the use of methanotrophs in bioreactors for remediation of contaminated groundwater and in determining conditions in the subsurface that will promote high rates of TCE degradation by methanotrophs.

Methanotrophs are bacteria capable of oxidizing one-carbon compounds as the sole source of carbon and energy. The first step in methane oxidation is catalyzed by methane monooxygenase (MMO). MMO has wide substrate specificity which results in fortuitous degradation of nongrowth substrates, including TCE (3–7). Transformation of compounds unable to support growth is referred to as cometabolism or cooxidation and is the basis for using methanotrophs in the degradation of TCE as well as numerous other recalcitrant organic contaminants.

The MMO of methanotrophs exists in two forms, soluble and particulate. The form of the enzyme present depends upon growth conditions (8); soluble MMO occurs under copper-limiting conditions ($< 4.8\ \mu M\ CuSO_4$), whereas the particulate enzyme is found with excess copper (9). Numerous investigators have found that production of the soluble enzyme is responsible for maximum TCE degradation (10), possibly because of its broader substrate specificity (11).

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The rate of methane and TCE oxidation may be limited by insufficient reducing power. It appears that both particulate and soluble MMO use NADH as the electron donor and growth yields of methanotrophs are likely to be NADH limited (12).

In addition to oxidation of methane and TCE, there is substantial evidence for methanotrophic degradation of ammonia to nitrate (9). The initial oxidation step is catalyzed by MMO. Because MMO is involved in oxidation of the three compounds, ammonia may compete for the active site on the enzyme and thus reduce both methane and TCE oxidation rates.

The effectiveness of different microbial consortia and operating conditions required for maximization of TCE degradation (nutrient levels, gas flow rates, etc.) are being evaluated in batch experiments and in bench-scale continuous-flow trickle-filter bioreactors at ORNL and in upflow expanded-bed bench-scale bioreactors at UT. The purpose of this paper is to present a summary of the results achieved to date in the determination of operating conditions for the field-scale bioreactor and a brief description of the design and anticipated use of the field-scale bioreactor.

MATERIALS AND METHODS

Characteristics of the Site Water

The seep water at K-25 contains numerous contaminants in addition to TCE, including anaerobic degradation products of TCE [e.g., dichloroethylenes (DCEs)] and compounds disposed of with the TCE, [e.g. tetrachloroethylene (PCE), toluene, other aromatics, numerous chlorinated and non-chlorinated organic compounds, and high levels of iron] (13). Pretreatment of the seep water may be necessary because of the high iron content of the water. Several pretreatment techniques designed to eliminate iron precipitation problems will be evaluated in bench-scale systems and in the field but are not discussed here.

Cultures and Culture Conditions

Cultures used at ORNL were obtained from methane enrichments of water samples from various contaminated sites, including one culture (KCMC) from a DOE site in Kansas City, Missouri, and two cultures, DT-1 and DT-2, from waste disposal areas at Oak Ridge Y-12 Plant (3). Two additional cultures were obtained from K-25 by incubating NATE mineral salts medium (7) with seep water (50% seep water, 50% mineral salts medium) containing 0.43 $mg \cdot L^{-1}$ TCE, 0.46 $mg \cdot L^{-1}$ methylene chloride, and methane, and by filtering seep water through 0.45 μM filters and incubating the filter in mineral salts medium with methane.

Cultures were prepared by transferring cells from stock plates to NATE medium, without copper and ammonia unless otherwise stated, in bottles with Teflon-lined septum caps. Sterile-filtered methane gas was injected to yield a 10% (vol/vol) methane headspace, and cultures were incubated at 22°C on a shaker table (75 RPM) for 5-7 days.

Experiments at UT used two TCE degrading consortia. The first consortium (SM) was established from an enrichment from the Savannah River Plant, Aiken, South Carolina (9,12). The second consortium (PM-M) was derived by combining the SM consortia, a methanotroph isolated from ORNL (7) and methane and propane using cultures obtained from the vicinity of Ada, Oklahoma (4). Consortia were maintained on a buffered mineral salts medium (12) containing 5% methane and 3% propane (vol/vol, headspace).

Batch Degradation Assays at ORNL

Batch experiments were run and analyzed similarly to those described previously (13). Cultures were centrifuged and the pellets suspended in NATE. Twelve mL aliquots were transferred to 40-mL vials. Water-saturated TCE and methane and/or formate were injected, and vials were inverted and incubated as described above. Hexane was injected to terminate degradation reactions and to extract TCE for quantification by gas chromatography (GC) using a Perkin-Elmer Sigma 2000 GC with an electron capture detector (ECD) and a 1% SP1000 60/80 Carbopac B column (Supleco).

Various conditions were evaluated for their effects on TCE degradation. Degradation by several consortia in mineral salts medium containing copper was compared with that in medium without copper. Ammonium chloride was omitted from the degradation medium to determine whether TCE removal would be increased. To assess the effect of additional reducing power on TCE oxidation, 20 mM sodium formate was added to the degradation media in another series of experiments. Growth of the consortia and the degradation experiments described above took place at 22°C. Because temperatures in the bioreactors may exceed 22°C, cultures were also tested for growth at 37°C. Because the bench-scale bioreactors were run at dilutions of the NATE media, a comparison was made of degradation in 1X and 0.1X NATE media. Cultures were grown in 1X media, and degradation was measured in either 0.1X or 1X media.

Bioreactors at ORNL

Six 5-cm idia glass laboratory-scale trickle-filter bioreactors are used in these experiments and are similar in concept to one described earlier (14). Five of the columns are packed with 1.6-cm polypropylene flexrings to a depth of 45 cm. One is packed with 0.48 cm idia polypropylene tubing 0.32 to 0.95 cm long. The reactors are fed 10x to 100x dilutions of the media described for the ORNL batch experiments. Ammonia has been removed from the nutrient feed to the bioreactors and replaced with nitrate.

TCE degradation is assessed by GC measurement of TCE in off-gas and liquid effluent. A Hewlett Packard model 5890A GC equipped with an ECD and a Megabore DB + 1 capillary column (J&W Scientific Corp.) was used.

Bioreactors at UT

Construction, maintenance, and operation of the total-recycle expanded-bed bioreactors operated at UT have been previously described (15). All manipulations used Teflon-plungered syringes for transferring solutions. Batch experiments used crimp-top tubes or serum vials sealed with Teflon-lined caps. All incubations were at ambient temperature (23°C). In TCE degradation experiments initial TCE concentration was 20 $mg \cdot l^{-1}$ (35 $\mu mol \cdot reactor^{-1}$). Medium was recycled for 5 days. Methane was added at 5% (vol/vol), and propane was added at 3% (vol/vol). Compounds and concentrations used in mixed- waste experiments are listed in Table I. Unless otherwise noted, data are presented as means of several bioreactors containing the SM and PM consortia.

TABLE I

Degradation of Mixed Organic Wastes by Propane/methane-fed Microbial Consortia

Organic wastes	Concentration[a] in bioreactors at day 0 ($mg \cdot l^{-1}$)	Mean loss in bioreactors after 21 days (%)	Mean loss in cell suspensions after 20 days (%)
vinyl chloride	4.0	>99	>99.9
1,1-dichloroethane	1.1	>99	N.D.b
1,1-dichloroethylene	0.7	>99	99
1,2-dichloroethylene	3.0	>99	N.D.
1,1,1-trichloroethane	1.2	>99	>99
trichloroethylene	2.6	90	>99.9
tetrachloroethylene	2.1	60	80
benzene, xylene	0.1	99	>99.9
toluene	0.05	>90	>99.9

[a]Concentrations based upon the model of all the toxicants present in the aqueous phase with headspace to liquid volume ratios of 2.0 2.5:1.
N.D. = not determined.

Analytical procedures are described in detail elsewhere (9,12,15,16). TCE and other chlorinated hydrocarbons not mentioned below were analyzed using a Hewlett Packard 5890 GC equipped with a capillary column and an ECD. Vinyl chloride, cis- and trans-1,2-dichloroethylene (DCE) were analyzed using a Schimadzu GC-9A GC equipped with a photoionization detector (HNU Systems, Newton, MA). Methane and CO_2 were assayed using a Schimadzu GC-8A gas chromatograph with a thermal conductivity detector, and radioactive CO_2 was determined by a gas proportional counter. Propane was analyzed using a Schimadzu GC-9A GC equipped with a flame ionization detector.

RESULTS AND DISCUSSION

Batch Experiments at ORNL

Elimination of copper from mineral salts medium at 1X trace metals concentration had no effect on TCE degradation by the KCMC culture. It was not surprising that TCE degradation did not increase under these conditions since soluble MMO production reportedly occurs at copper concentrations of 0.50 μM or less. However, achievement of those levels required extensive purification and decontamination measures (10). Alternatively, it may be possible to drive the system to copper limitation with less heroic efforts if high biomass levels are achieved. This is apparently the approach taken by Oldenhuis et al. (17). Because removal of trace amounts of copper from bioreactors in field situations is not feasible, we made no similar efforts to remove trace amounts of copper from glassware or the distilled, deionized water usually used in media preparation. As a possible means of circumventing the problem of contaminating copper, concentrations of trace metals other than copper were increased. This would, in effect, reduce the relative amount of copper available in the media. Doubling the trace metal concentration in the absence of copper had no effect on TCE degradation by KCMC (data not shown). Thus, simply removing the copper did not result in the high rates of degradation noted by other investigators (10) when copper is limited to very low levels.

KCMC cells cultured in 1X NATE exhibited the same level of TCE degradation when transferred into 0.1X medium as that occurring in 1X medium (Fig. 1). Similar results were obtained with the DT-1 culture (data not shown). No significant growth occurred when cells were cultured in 0.1X NATE. Thus, media concentration had no direct effect on TCE degradation but may have an indirect effect by limiting growth of the consortia. Limitation of growth in the bioreactors must be considered since nutrients are present at lower concentrations.

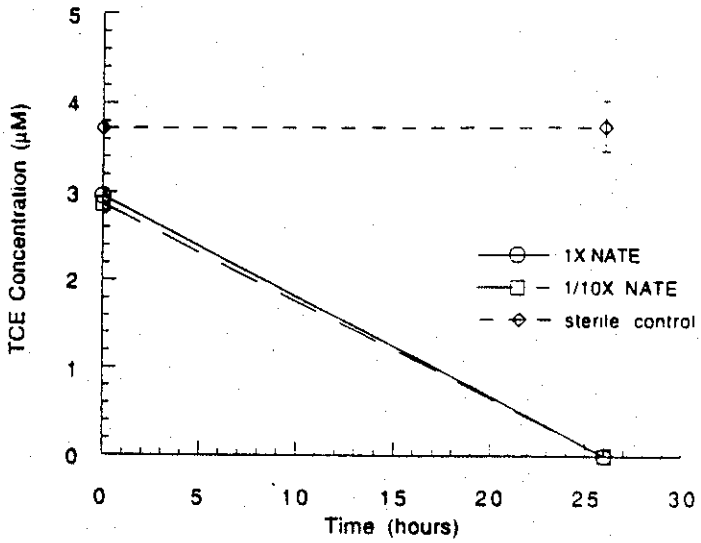

Fig. 1. Degradation of TCE in 1X and 0.1X NATE media, both without copper or ammonia, by methane using consortia in batch culture. Consortia were grown in 1X trace metals and transferred to fresh 1X or 0.1X media for the degradation assays.

As in previous experiments with a pure culture (18), the elimination of ammonium chloride from the medium resulted in significantly increased degradation of TCE by several consortia (data not shown). In experiments with 5X trace metals and without ammonia we saw increased TCE degradation by the K-25 culture. When ammonium chloride was omitted from

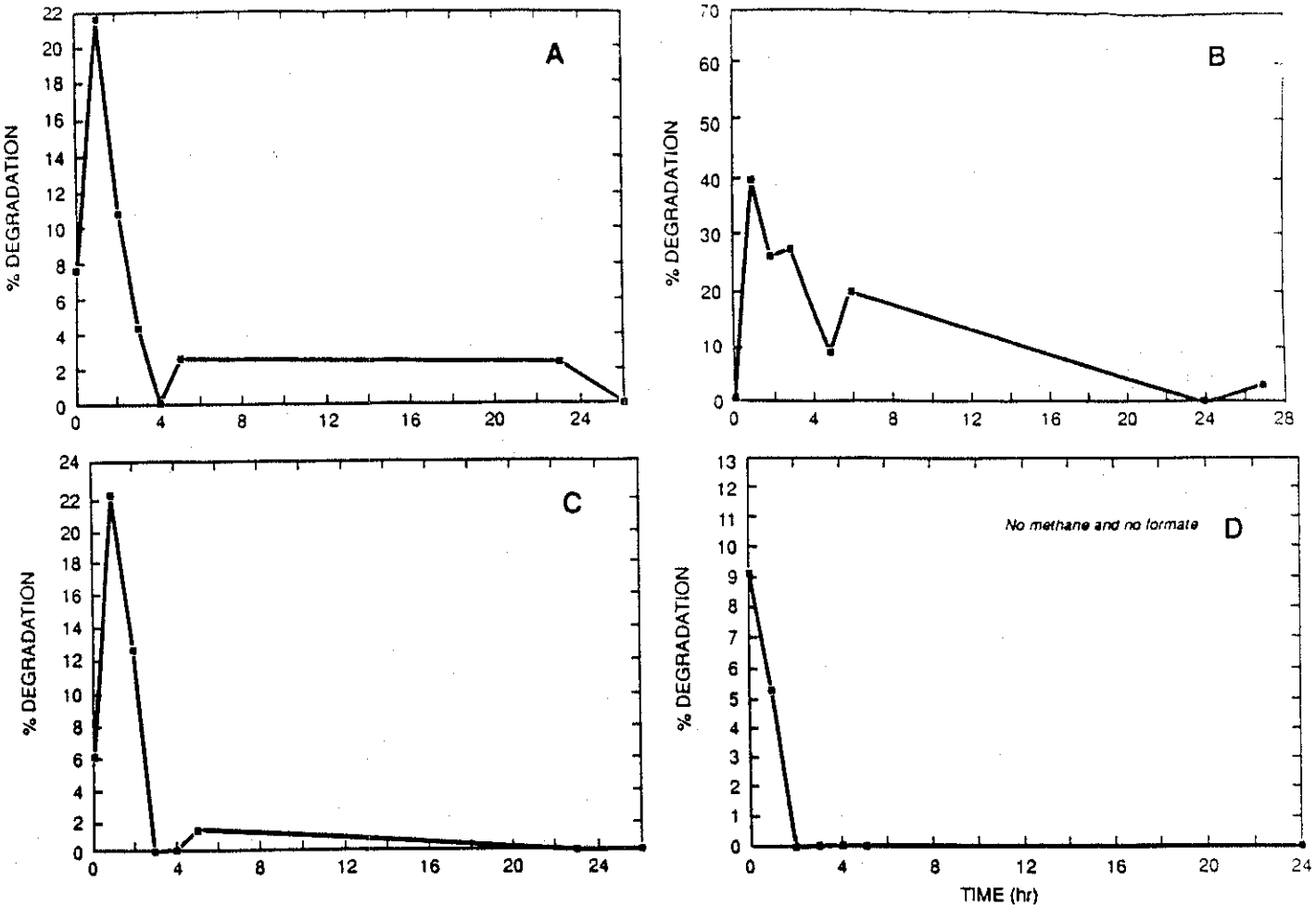

Fig. 2. TCE degradation response of continuous trickle-filter methanotrophic bioreactor to withdrawal of methane from the gas phase and simultaneous addition of 20 mM formate to the liquid phase. Panels (a), (b), and (c) are replicates; panel (d) is a control experiment in which methane was removed, but formate was not added.

the degradation mixture with the DT-2 culture degradation was doubled. The data strongly suggest that alleviating competitive inhibition of the MMO will substantially increase TCE degradation. After completion of these experiments, ammonium chloride was omitted from the plates used to isolate and maintain bacterial stocks and from the growth and degradation media.

Formate increased TCE degradation by KCMC over that occurring in the absence of both formate and methane (13), but in the batch experiments the effect was not generalizable over cultures (data not shown). For example, degradation by DT-2 was similar under all substrate conditions tested. Addition of both methane and formate decreased degradation by the K-25 and KCMC cultures. Other investigators have previously shown that under some conditions formate can increase TCE degradation (e.g. 19).

At an elevated growth temperature of 37°C, only the DT-1 culture exhibited vigorous growth. No visible turbidity was evident in either the DT-2 culture or the KCMC cultures after >7 days. DT-1 grows poorly at 22°C, often exhibiting no growth at this temperature. Preliminary results indicate that DT-1 does not degrade TCE as well as either DT-2 or KCMC.

ORNL Bench-Scale Bioreactors

Formate experiments with the ORNL bioreactors indicate a general short-term stimulation of TCE degradation with the addition of formate to the bioreactors (Fig. 2). In the absence of methane and formate the degradation rate dropped rapidly and did not show the short-term increase evident with the formate addition. Alternate pulsing of methane and formate may serve to maintain MMO production and provide sufficient reducing power for maximum TCE degradation.

UT Bench-Scale Bioreactors

The UT bioreactors (both the PM and SM consortia) removed 87±5% of the initial TCE added to reactors within 5 days. An mean of 2.1 mmol propane plus 1.5 mmol methane were oxidized for a substrate/TCE loss ratio of 123±16 (μmol/μmol). When substrate was added to the reactors only at Day 0 (pulsed-fed), twice as much TCE was consumed per mmol substrate oxidized. If reactors were replenished with respect to TCE and further starved, the TCE degradation rates dropped an order of magnitude, demonstrating that brief starvation resulted in increased TCE degradation efficiencies but frequent pulsed-feedings were required to maintain stable TCE degradation. Reactors fed either methane or propane alone degraded TCE but less effectively.

A variety of other toxic compounds were degraded, many to detection limits, in the total-recycle bioreactors (Table I). TCE degradation ceased at 0.3 mg·l^{-1}, resulting in a loss of only 90% of the initial TCE concentration while

dichloroethylenes and vinyl chloride, suspected intermediates of anaerobic TCE degradation, were degraded to the detection limits. Benzene, toluene, and xylene were degraded to detectable limits.

The bioreactor stability studies at UT have also demonstrated that pH is a critical factor in the TCE degradation by the PM and SM consortia. Elevation of the pH to >7.3 reduced TCE degradation by the two consortia from >80% to <20% indicating low resistance to pH disturbance. The resilience was also low; on these systems TCE degradation activity took days to weeks to recover. Examination of the effects of low pH excursions, as well as altered nutrient inputs and contaminant mixtures on the metabolism and physiology of the microbial community on these bioreactors, are in progress.

Progress on Field-Scale Bioreactors

The field-scale Air Force bioreactor has been modified to maximize the flexibility of the operational modes and has been moved to the K-25 site. The bioreactor consists of two 7-ft columns which are 16 in. in diameter. The modifications will allow the columns to be operated in series or in parallel and could allow treatments such as pulsing of formate. Operation of the field unit is scheduled to begin in the fall of 1991 under operating conditions established in the batch and bench-scale tests. In the fall of 1992 the operation of this system with methanotrophs will be compared with a bioreactor system that uses aromatic degraders that also can degrade TCE.

SUMMARY

The collaboration between ORNL and UT and the stepwise progression from batch tests to bench-scale bioreactors to a pilot-scale bioreactor in the field provides for testing of several types of cultures and conditions and will lead to improved rates of degradation during the demonstration. Results to date include the determination of the appropriate pH range for improved TCE degradation, the unsuitability of ammonia as a nitrogen source, the potential for a short-term positive result of formate addition (to at least some cultures), and the detrimental effect of elevated temperature on growth of most of the cultures. Continuation of this approach should also lead to greater applicability of the results to conditions at a range of DOE sites.

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