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Biodegradation of Mixed-Organic Wastes by Microbial Consortia in Continuous-Recycle Expanded-Bed Bioreactors

Tommy J. Phelps,*,† John J. Niedzielski,† Kenneth J. Malachowsky,† Richard M. Schram,† Stephen E. Herbes,‡ and David C. White*,†,‡,§

Institute for Applied Microbiology, University of Tennessee, 10515 Research Dr. Suite 300, Knoxville, Tennessee 37932-2567, Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831, and the Department of Microbiology, University of Tennessee, Knoxville, Tennessee 37996-0845

■ Microbial consortia enriched from subsurface sediments contaminated with chlorinated hydrocarbons proved capable of degrading mixed-organic wastes. Methane and/or propane as foodstock enabled aerobic mineralization of greater than 20 mg L^{-1} trichloroethylene (TCE) plus 1 mg L^{-1} vinyl chloride, benzene, and toluene in cell suspension or bioreactor experiments. The microbial consortia degraded 80–95% of TCE at 20 mg L^{-1} within 5 days in continuous-recycle expanded-bed bioreactors requiring 50–100 mol of foodstock/mol of TCE degraded. When the bioreactors were challenged with groundwaters contaminated with mixed-organic wastes, the microbial consortia degraded greater than 99% of the benzene, toluene, xylene, vinyl chloride, and nine chlorinated hydrocarbons, 85% of the TCE, and 60% of the tetrachloroethylene within 21 days, while requiring 80 μmol of methane plus propane per micromole of mixed-organic waste degraded. The potential for bioremediation of groundwater contaminated with mixed-organic wastes was demonstrated in laboratory reactors.

Introduction

Chlorinated and aromatic hydrocarbons are major components of mixed-organic wastes contaminating soils, sediments, and groundwaters (*1–6*). As a consequence of high usage and improper disposal technologies, many soils and subsurface aquifers are contaminated; some containing greater than 1000 mg L^{-1} chlorinated hydrocarbons, often in combination with other organic wastes (*2*, *6*, *7*). Remediation of subsurface contamination may require long treatment times and considerable expense (*3*, *4*, *8*). Some treatments often result in the mere transfer of wastes from poorly controlled environments to storage sites (*2*). Waste storage facilities or atmospheric venting may alleviate an immediate concern without detoxifying the waste. Remediation technologies that destroy or mineralize toxicants on-site would be more desirable than transfer of wastes from one environment to another.

Bacteria capable of utilizing aromatic hydrocarbons as sources for energy are well documented (*1*, *5*, *9*) and successful examples of bacterial remediation of contamination plumes have been demonstrated (*1*, *3*, *8*). Chlorinated alkenes such as trichloroethylene (TCE) are degraded fortuitously or cometabolically by methanotrophic (*10*, *11*) and heterotrophic microorganisms (*6*, *12*, *13*). Methane and propane have been shown to stimulate biodegradation of trichloroethylene in soil columns (*10*, *14*) and bioreactors (*15–17*). Several microorganisms have the capability to degrade specific chlorinated or aromatic compounds, but in this study we investigated the ability of microbial consortia to degrade a mixture of organic toxicants. Mixed-organic waste degradation in continuous-recycle expanded-bed bioreactors was successful. Biological degradation of organic waste from groundwater, which contained TCE, benzene, toluene, xylene, and several other chlorinated aliphatic hydrocarbons, is reported.

Materials and Methods

Bioreactor Design and Operation. Each bioreactor consisted of two borosilicate glass chromatography columns (Pharmacia, Piscataway, NJ) linked in series (Figure 1). The expanded bed consisted of 70 g of 60–80-mesh crushed glass and a liquid displacement of 45 mL. Bed expansion was 20 mL at a flow rate of 20 mL min^{-1}. Liquids flowed upward through the expanded bed, into the gas recharge column, and through the peristaltic pump. A detailed description of the bioreactor construction, maintenance,

† Institute for Applied Microbiology, University of Tennessee.
‡ Oak Ridge National Laboratory.
§ Department of Microbiology, University of Tennessee.

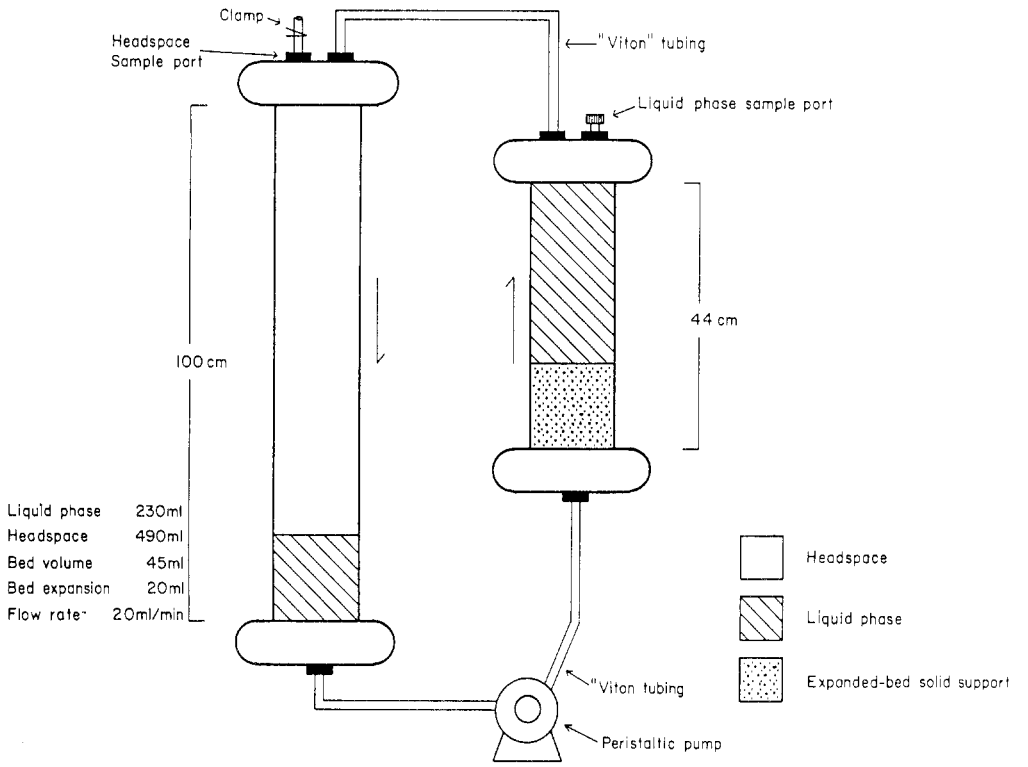

Figure 1. Diagram of expanded-bed bioreactor and gaseous recharge column. Recirculation was accomplished by peristaltic pumping through Viton tubing.

and operation has been given (*15*). Each bioreactor contained 230 mL of medium to which mineral salts and 2.0 mM phosphate/bicarbonate buffer were added, and the pH of the medium was 7.2. Temperature was maintained at 22 °C. Resazurin, 2 mg L^{-1}, was added as a redox indicator. Bioreactors were fed methane (5% v/v) and/or propane (3% v/v) as foodstock while oxygen served as the terminal electron acceptor (*15*). Methane and propane were added daily by syringe to maintain their respective concentrations unless stated otherwise. Each day samples were obtained from the headspace sampling port. Losses of TCE, methane, and propane from the inhibited control reactor averaged less than 5% of the initial concentrations after 20 days. The control reactor was inhibited with 0.2% sodium azide plus 0.5% formalin. Contaminated groundwater was obtained from a shallow monitoring well in a waste disposal site near Oak Ridge, TN. Field measurements indicated the groundwater had a pH = 6.5, temperature of 18 °C, oxidation–reduction potential of 26 mV, and dissolved oxygen concentrations of 1.2 mg L^{-1}.

Bacterial Cultures. Three consortia with TCE-degrading capabilities were used as inocula. Culture SRP was isolated from the Savannah River Site and was capable of degrading TCE at concentrations exceeding 100 mg L^{-1} (*6*). The PM-M culture contained mixtures of propane- and methane-oxidizing cultures obtained from soils near the vicinity of Ada, OK, in addition to the TCE-degrading consortium from SRP. The additional methane- and propane-oxidizing cultures added to the PM-M consortium appeared incapable of degrading 20 mg L^{-1} TCE. A methanotroph capable of degrading TCE at concentrations less than 1 mg L^{-1} (*11*) was also added to the PM-M consortium. Consortium SM-1 consisted of the SRP consortium plus the TCE-degrading methanotroph (*11*). Consortium SM-1 and PM-M had been maintained in the continuous-recycle bioreactors for >3 months (*15*). Culture maintenance and cell suspension studies utilized crimp-top serum vials with Teflon septa as previously described (*6*). Cultures for cell suspension studies were centrifuged and resuspended in 10 mL of 2 mM phosphate plus 10 mM bicarbonate-buffered medium at an approximate density of 1.0 g dry weight L^{-1}. Duplicate vials of controls and cell suspensions were sacrificed at each time point.

Analytical Procedures. TCE was analyzed by headspace analyses using a Hewlett-Packard 5890 gas chromatograph (GC) equipped with a 50 m × 0.2 mm Hewlett-Packard ultraperformance cross-linked methylsilicone column and an electron capture detector (*6*). Benzene, xylene, and toluene were analyzed from liquid-phase samples by use of a similar GC and column equipped with a flame ionization detector. Propane and methane in headspace gases were analyzed with a Shimadzu GC-9A equipped with a packed column and flame ionization detector. Standard calibration curves used the same headspace/liquid-phase ratio as the columns. Analyses of multiple chlorinated hydrocarbons were performed from water samples collected in 40-mL Teflon sealed vials in accordance with U.S. EPA procedure 624. Analyses were conducted by Oak Ridge National Laboratory (ORNL).

Results and Discussion

Cell Suspension Studies. Our laboratory previously demonstrated degradation of greater than 99% of 50 mg L^{-1} TCE to carbon dioxide by mixed populations of heterotrophic microorganisms (*6*). Nelson et al. (*12*) reported that heterotrophic TCE-degrading microorganisms could be induced by toluene, suggesting that microorganisms may possess the ability to simultaneously degrade mixed-organic wastes. Results in Table I show that cell suspensions of microbial consortia were capable of degrading mixed-organic wastes. Cell suspensions inhibited with 0.1% sodium azide and 0.5% formalin did not degrade the mixed-organic wastes in the 10-day experiment when compared to uninoculated controls. Increased benzene and toluene in the inhibited suspensions after 10 days was attributed to variability of the uninoculated and inhibited controls when compared to standard curves. Suspensions of the SRP consortium degraded >80% of the mixed-or-

Table I. Removal of Organic Contaminants by Cell Suspensions[a]

suspension	mixed-waste component[b] (initial concn) TCE (20 mg L^{-1})	vinyl chloride (1 mg L^{-1})	benzene (1 mg L^{-1})	toluene (1 mg L^{-1})
inhibited cells	26	11	+7	+27
SRP consortium	85	82	87	90
reactor consortium	87	96	93	99
SRP consortium maximum from separate expts	>99.99	99.9	>99	>99

[a] Experimental procedure: Stationary-phase cultures were centrifuged and resuspended into duplicate 10-mL volumes of 2 mM phosphate plus 10 mM bicarbonate-buffered basal medium to which one or more toxicants were added. Oxygen (10 mL) was added to each suspension and the 28-mL vials were sealed with Teflon septa. Results were analyzed after 10 days and the average value was reported. The SRP maximum values represent results obtained in similar experiments performed in different months. [b] Data reported as percent loss of initial concentration relative to controls containing no cells.

Table II. Utilization of TCE and Propane during Degradation of Mixed-Organic Wastes[a]

day	reactor	TCE, $\mu g\ L^{-1}$	TCE loss, μmol	propane loss, μmol
0	control	2200	0	0
	PM-M	2200	0	0
	SM-1	2200	0	0
3	PM-M	2000	0.5	700
	SM-1	1900	0.5	600
6	PM-M	1300	1.7	1300
	SM-1	1300	1.6	1300
15	PM-M	560	3.0	2000
	SM-1	420	3.1	2000
21	control	2400	0	70
	PM-M	440	3.3	2700
	SM-1	400	3.3	2600

[a] Experimental conditions: Each reactor (Figure 1) contained 230 mL of groundwater from a contaminated site. Control reactor was inhibited with 0.1% sodium azide plus 0.5% formalin. Culture PM-M contained the SRP consortium plus several methane- and propane-oxidizing cultures. Culture SM-1 consisted of the SRP consortium and a TCE-degrading methanotroph. Propane was maintained at 5% (v/v) and bioreactors were recirculated for 21 days.

ganic wastes in these experiments. In other experiments performed in different months, the SRP consortium maximally degraded >99.9% of the toxicants (Table I). The reactor consortium included the PM-M consortium plus uncharacterized microorganisms that colonized the reactor after it was challenged with groundwater containing a diverse microbial community in addition to mixed-organic wastes. The reactor consortium, which was inoculated with more methane- and propane-oxidizing cultures than the SRP consortium, appeared to degrade toxicants to a greater extent than the SRP consortium alone (Table I). In previous bioreactor experiments fed methane plus propane (*17*), TCE degradation by the PM-M consortium was significantly greater than the less complex SM-1 consortium (95% level by Student's t test) (*17*). Furthermore, the difference between the consortia was significantly greater than zero at the 99% confidence level with a Z score (standard error of the mean) of 3.4 (data not shown) (*17*). These results suggest that complex microbial consortia may have a greater ability to simultaneously degrade mixtures of organic wastes (*11–13, 17*) than pure cultures. However, the addition of pure cultures to a consortium may further enhance its ability to degrade mixtures of organic wastes.

Bioreactor Experiments. Previous reports described the ability of these consortia to degrade 20 mg L^{-1} TCE in expanded-bed bioreactors (*17*). Table II illustrates the utilization of TCE and propane after bioreactors were challenged with groundwaters contaminated with mixed-organic wastes. Laboratory analyses determined the bioreactors contained 2.2 mg L^{-1} TCE at the time of inoculation. TCE loss within the reactors averaged 115 $\mu g\ L^{-1}\ day^{-1}$ for the first 15 days versus 12 $\mu g\ L^{-1}\ day^{-1}$ for days 15–21. The TCE degradation rate thus decreased by 1 order of magnitude while propane consumption decreased 50%, possibly due to limiting availability of inorganic nutrients. However, in other experiments when the concentrations of phosphate, nitrate, and minerals were doubled, the propane consumption rate was linear for >8 days (data not shown) but the rate of TCE degradation decreased to <60% of the initial rate and 50% more propane was required per micromole of TCE degraded

Table III. Degradation of Mixed-Organic Wastes by a Propane-Fed Bioreactor

organic wastes	toxicant concn in reactors,[a] $\mu g/L$ control day 0	control day 0	control day 21	PM-M day 21	SM-1 day 21
vinyl chloride	4000	4400	1200	10	10
chloroethane	27	30	13	10	10
methylene chloride	10	11	9	1	1
1,1-dichloroethylene	690	780	5	<5	<5
1,1-dichloroethane	1100	1200	820	<5	<5
1,2-dichloroethylene	3000	3100	3600	29	13
1,2-dichloroethane	21	23	18	<5	<5
1,1,1-trichloroethane	1200	1300	940	15	6
trichloroethylene	1700	1900	2600	435	235
1,1,2-trichloroethane	52	54	46	<5	<5
tetrachloroethylene	2100	2300	2100	805	860
benzene	86	90	49	<5	<5
toluene	49	51	26	<5	<5
xylene	26	28	10	<5	<5

[a] Analyses performed by ORNL in accordance with EPA procedure 624. Values below detectable concentrations are noted by "<".

Table IV. Comparison of TCE and Mixed-Organic Waste Degradation in Recycled Bioreactors[a]

experiment	initial TCE concn, μg/L	total wastes degraded, μmol	foodstock consumed, μmol	foodstock/TCE loss, μmol/μmol
TCE control	20000	2	na[b]	na[b]
TCE with propane plus methane[c]	20000	31	3700	123 ± 16
TCE with propane and methane at day = 0 only[c]	20000	31	1730	55 ± 1
TCE with propane[c]	20000	29	2650	91 ± 12
TCE with propane plus methane	4000	4.1	860	215 ± 40
mixed organics	1700	32	2700	84
with propane	1900	34	2600	76

[a] Experimental design: Each reactor contained 230 mL of medium, which in the mixed-waste experiments was replaced with well water. Propane and methane were maintained at 3–5% (v/v) in the headspace. Experiments receiving propane and methane at day = 0 only did not receive additional methane or propane after initiation of the experiment. Standard deviations were calculated for those experiments repeated four times. [b] na, not applicable. [c] Data summarized from Phelps et al. (*17*).

(*17*). These results agreed with other studies (*16*, *17*) which suggested that microbial degradation of TCE in bioreactors becomes slower and less efficient as TCE concentrations decrease to approximately 500 μg L^{-1}.

Degradation of mixed-organic wastes in the expanded-bed total-recycle bioreactors is shown in Table III. The analyses performed by EPA procedure 624 showed TCE to be 1700–1900 μg L^{-1} in the reactors versus 2200 μg L^{-1} by headspace analysis. Variability between replicate samples analyzed by EPA procedure 624 rarely varied by more than 10%, as shown by the day = 0 controls. Results after 21 days are expressed as averages of duplicate analyses. Seven of the 14 toxicants present in the groundwaters were degraded to the detection limits of the methods employed. Vinyl chloride and 1,1-dichloroethylene were the only toxicants whose concentrations decreased more than 60% in the control reactor. In the experimental reactors vinyl chloride was reduced >99% to concentrations less than the detection limits. The EPA procedure 624 analyses, which were in agreement with Table II, showed that >80% of the TCE was degraded in both bioreactors. Dichloroethylenes and ethanes were decreased >99.5% to nondetectable concentrations.

Tetrachloroethylene decreased 60% in both test reactors, but less than 5% in the control reactor. In preliminary experiments in which cell suspensions of these consortia have been exposed to <0.6 mg L^{-1} tetrachloroethylene, loss of tetrachloroethylene has been observed under aerobic conditions. However, due to the low specific activity of [^{14}C]tetrachloroethylene, verification of degradation products has not been achieved (data not shown). It was also possible that tetrachloroethylene degradation in these bioreactors occurred via reductive dechlorination in anaerobic microniches as observed previously (*18*). Although the bulk-phase liquid remained oxidized, as monitored by the blue color of resazurin, it is likely that anaerobic microniches existed within biofilms.

Benzene, toluene, and xylene were also degraded in the bioreactors. Nelson et al. (*12*) reported that a heterotrophic toluene degrader could degrade TCE when induced with toluene. Inducers were not required for TCE degradation by these consortia (*6*), nor did the presence of methane and propane eliminate the degradation of aromatics and chlorinated hydrocarbons. In all, greater than 91% of the total mixed-organic contaminants were degraded. Chlorinated intermediates of TCE decomposition such as dichloroethylenes and vinyl chloride did not accumulate within the bioreactors. These results substantiated previous studies, which accounted for >60% of [^{14}C]TCE being converted to carbon dioxide by the SRP consortium (*6*).

Foodstock Utilization. Previous studies (*6*, *17*) showed that these consortia could use methane or propane as foodstock for bioremediation of chlorinated hydrocarbons, but efficiency per mole of foodstock and degradative rates were less with methane alone. Table IV compares the efficiency of TCE degradation and mixed-organic waste degradation in expanded-bed total-recycle bioreactors. When 20 mg L^{-1} TCE was the only contaminant present and propane plus methane were continuously available as foodstocks, each mole of TCE degraded required an average of 123 mol of foodstock. When no methane or propane was added, 10% of the TCE was lost during starvation of the consortia. During pulsed feeding of the consortia, when methane and propane were provided only at day = 0 and the reactors depleted their foodstocks, stable and reproducible TCE degradation proceeded for 5 days. Pulsed feeding reduced methane and/or propane consumption by half with no adverse affect on degradation of TCE with an efficiency of 55 mol of foodstock consumed/mol of TCE degraded (Table IV). When propane was the foodstock, 91 mol of propane were required per mole of TCE degraded. As the TCE concentration decreased, the TCE degradation rate and degradative efficiency decreased. At 4 mg L^{-1} TCE, 215 mol of propane plus methane were required per mole of TCE degraded versus 123 mol at higher TCE concentrations (Table IV). Degradation efficiencies for TCE from Table II correspond to an approximate foodstock/TCE ratio of 500 for the first 15 days versus a ratio of 5000 for the final 6 days; however, mixed-organic wastes were also degraded.

A total of 33 μmol of mixed-organic wastes (2.6 μmol of TCE plus 30.4 μmol of other contaminants) were degraded in the mixed-organic waste experiments requiring 2650 μmol of propane or 80 μmol of foodstock per micromole of contaminant degraded. If TCE had been the only organic waste, the expected foodstock/TCE ratio for TCE at 2 mg L^{-1} would have been >200 (Table IV). The observed ratio of 80 suggests that mixed-waste degradation may require fewer moles of foodstock than TCE degradation. Possible explanations for the apparent efficiency of mixed-waste degradation include the following: (1) less chlorinated hydrocarbons may be more readily degraded than TCE (*16*) or PCE, (2) degradative products may be available as energy or carbon sources, (3) toxicants such as benzene, xylene, or toluene could serve as energy sources. Bioremediation of gasoline and jet fuel plumes supports the notion that light aromatics can serve as substrates for bioremediation (*1*, *5*, *8*), and toluene is known to induce TCE degradation by *Pseudomonas* sp. (*12*). The groundwater used in these studies contained 161 μg L^{-1} light aromatics, an amount that would be insignificant as an energy source for the reactor biomass. The apparent efficiency of mixed-waste degradation by these consortia likely resulted from the less chlorinated aliphatics being more susceptible to oxidation (*4*); consequently they

may have readily undergone transformations by fortuitous or cometabolic processes (*16*).

Each bioreactor contained approximately 20 mg dry weight of biomass as estimated by phospholipid fatty acids (*17*). When TCE was degraded at a concentration of 20 mg L^{-1}, each milligram of biomass degraded an average of 40 μg of TCE each day with a maximum of 80 μg of TCE/mg of biomass each day (calculations not shown). During mixed-waste degradation at 2 mg L^{-1} TCE and 230 mL of groundwater per reactor, the daily TCE consumption was 1.3 μg of mixed-organic wastes (mg of biomass)$^{-1}$ day^{-1}. When considering all contaminants, the average degradation rate was 7 μg of mixed-organic wastes (mg of biomass)$^{-1}$ day^{-1}. Although the quantity of foodstock required per micromole of contaminant degraded was similar between the mixed-organic waste and TCE experiments, the biomass required more time to degrade the lower concentration of mixed-organic wastes. As shown in Tables II and III, several weeks were required to decrease the TCE concentration 80%, with little degradation occurring over the final 6 days. These results suggest that as the concentration of TCE decreases, degradation rates decrease dramatically. Results also suggest that highly chlorinated constituents, such as PCE and TCE, may decrease the degradation rate and the metabolic efficiency of mixed-waste bioremediation, while lesser chlorinated aliphatics or light aromatics may be degraded more rapidly and efficiently. Fortunately, degradation of many components of mixed-organic waste may not require additional nutrients or degradative time and may even contribute to foodstock pools and energy flow.

Conclusions

Bioremediation of mixed-organic wastes from contaminated groundwaters was demonstrated in laboratory bioreactors. The feasibility of remediating organic waste mixtures including PCE, TCE, less chlorinated aliphatics, benzene, toluene, and xylene from groundwaters by microbial consortia fed gaseous hydrocarbons was established. Degradation required less than 100 mol of methane or propane/mol of contaminant degraded. The closed-system reactors appeared well suited for perturbation and metabolic studies. Future plans include use of these systems to assess the resiliency and efficiency of bioremediation processes.

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Registry No. $H_2C{=}CHCl$, 75-01-4; $ClCH_2CH_3$, 75-00-3; CH_2Cl_2, 75-09-2; $Cl_2C{=}CH_2$, 75-35-4; Cl_2CHCH_3, 75-34-3; $ClCH{=}CHCl$, 540-59-0; $ClCH_2CH_2Cl$, 107-06-2; Cl_3CCH_3, 71-55-6; $Cl_2C{=}CHCl$, 79-01-6; Cl_2CHCH_2Cl, 79-00-5; $Cl_2C{=}CCl_2$, 127-18-4; benzene, 71-43-2; toluene, 108-88-3; xylene, 1330-20-7; methane, 74-82-8; propane, 74-98-6.

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