APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1996, p. 2953–2960 Vol. 62, No. 8
0099-2240/96/$04.00+0

Effects of Phenol Feeding Pattern on Microbial Community Structure and Cometabolism of Trichloroethylene

CHIEN-CHUN SHIH,[1] MARY ELLEN DAVEY,[2,3] JIZHONG ZHOU,[2,4] JAMES M. TIEDJE,[2,3,4] AND CRAIG S. CRIDDLE[1,2]*

Department of Civil and Environmental Engineering,[1] National Science Foundation Center for Microbial Ecology,[2] Department of Microbiology,[3] and Department of Crop and Soil Science,[4] Michigan State University, East Lansing, Michigan 48824

Received 9 February 1996/Accepted 3 June 1996

Cometabolism of trichloroethylene (TCE) by phenol-fed enrichments was evaluated in four reactors with distinct phenol feeding patterns. The reactors were inoculated from the same source, operated at the same average dilution rate, and received the same mass of phenol over time. Only the timing of phenol addition differed. Reactor C received phenol continuously; reactor SC5 received phenol semicontinuously—alternating between 5 h of feed and 3 h without feed; reactor SC2 alternated between 2 h of feed and 6 h without feed; and reactor P received a single pulse every 24 h. The structure of the enrichments and their capacity for TCE transformation were analyzed. In long-term operation, reactors C and SC5 were dominated by fungi, had higher levels of predators, were more susceptible to biomass fluctuations, and exhibited reduced capacity for TCE transformation. Reactors P and SC2 were characterized by lower levels of fungi, higher bacterial biomass, higher concentrations of TCE-degrading organisms, and higher rates of TCE transformation. After 200 days of operation, rates of TCE transformation increased 10-fold in reactor P, resulting in TCE transformation rates that were 20 to 100 times higher than the rates of the other reactor communities. The cause of this shift is unknown. Isolates capable of the highest rates of TCE transformation were obtained from reactor P. We conclude that cometabolic activity depends upon microbial community structure and that the community structure can be manipulated by altering the growth substrate feeding pattern.

A wide range of chemicals can be detoxified by cometabolism. To sustain cometabolic reactions, a growth substrate must be added, either continuously or periodically. In mixed cultures, it is not clear how different methods of growth substrate addition will affect community structure and cometabolic populations. The pattern and timing of growth substrate addition are likely to change the structure of the microbial community and its propensity for cometabolism. This could be of particular importance in the design and operation of reactor systems for cometabolism. To explore this issue, trichloroethylene (TCE) was selected as a nongrowth substrate, and phenol was selected as the growth substrate. Certain phenol- and toluene-degrading *Pseudomonas* species rapidly cometabolize TCE (6, 12, 19), but this ability is not common to all phenol and toluene degraders (4). Consequently, enrichments that are fed phenol or toluene may differ in their capabilities for cometabolism because of differences in the species present. To test this hypothesis, phenol and TCE degradation activities were monitored in four reactors fed the same daily mass of phenol, but with different feeding patterns (frequency and feeding rate). The structure and TCE degradation activity of the resulting microbial communities were subsequently analyzed.

MATERIALS AND METHODS

Chemicals. TCE (99% purity) was obtained from Aldrich Chemical Co., Milwaukee, Wis. Chemicals for preparation of media and analyses were American Chemical Society reagent grade and were purchased from Aldrich or Sigma Chemical Co.

Medium and culture. Phenol feed medium contained the following (per liter of deionized water): 2 g of phenol, 2.13 g of Na_2HPO_4, 2.04 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, 0.067 g of $CaCl_2 \cdot 2H_2O$, 0.248 g of $MgCl_2 \cdot 6H_2O$, 0.5 mg of $FeSO_4 \cdot 7H_2O$, 0.4 mg of $ZnSO_4 \cdot 7H_2O$, 0.002 mg of $MnCl_2 \cdot 4H_2O$, 0.05 mg of $CoCl_2 \cdot 6H_2O$, 0.01 mg of $NiCl_2 \cdot 6H_2O$, 0.015 mg of H_3BO_3, and 0.25 mg of EDTA. The pH of the medium was 6.8.

Reactors and reactor operating conditions. Four glass vessel reactors were used to examine the effects of phenol addition. (i) Reactor C, a continuously fed reactor, continuously received 200 ml of phenol feed each day. (ii) Reactor SC5, a semicontinuously fed reactor, alternated between periods of feed (66.7 ml of phenol feed in 5 h) and periods of fasting (3 h). (iii) Reactor SC2, a second semicontinuously fed reactor, alternated between shorter periods of feed (66.7 ml in 2 h) and no feed (6 h). (iv) Finally, reactor P, a pulse-fed reactor, received 200 ml of phenol feed as a single daily pulse immediately after removal of the same volume of liquid from the reactor. Each reactor received 200 ml of phenol feed medium daily, resulting in a phenol loading rate of 0.2 g/liter/day and an average dilution rate of 0.1 day^{-1} (hydraulic residence time of 10 days). Each reactor was operated with daily wasting of 200 ml to maintain a constant liquid volume of 2 liters.

Reactor vessels (Wheaton 2-L Double-Sidearm Celstir; Wheaton no. 356806) were vigorously stirred by Teflon paddles attached to a spinning shaft and driven by a Teflon-coated stir rod coupled magnetically to a magnetic stir plate under the vessel. Defined phenol medium was provided by syringe pumps. An air pump supplied oxygen (dissolved oxygen [DO], >2 mg/liter) through a glass diffusion tube. All reactors were operated at 21.5 ± 1.0°C.

Inoculum. Each reactor was inoculated on the same day with a subsample from the same phenol-degrading enrichment. The enrichment was obtained by seeding a chemostat with activated sludge from a municipal wastewater treatment plant (East Lansing, Mich.) and providing phenol feed medium for 2 months at a dilution rate of 0.1 day^{-1}. The enrichment was maintained at 21.5 ± 1.0°C. Microscopic examination revealed a diverse microbial community, including flocs of spherical and rod-shaped bacteria, filaments, and distinctive predators, including protozoa and rotifers.

Evaluation of TCE transformation kinetics. Samples were periodically removed from the four phenol-fed reactors just before the beginning of the feed to evaluate TCE transformation activity. Two to 5 ml of culture was transferred to a 20-ml glass vial, crimp sealed with Teflon-coated butyl rubber stoppers, and spiked with 10 to ~50 μl of aqueous TCE stock solution (~1,000 mg/liter) to give the desired initial TCE concentration. Vials were incubated at 21.5 ± 1.0°C on a rotatory shaker at 120 rpm. Periodically, 0.1 ml of headspace gas was withdrawn with a Precision gas-tight syringe and injected into the injection port of a Hewlett-Packard 5890A gas chromatograph equipped with a 30-m (length) 0.53-mm (inside diameter) DB624 capillary column (Alltech no. 93532) and a flame ionization detector (helium carrier flow rate of 12 ml/min). The gas

* Corresponding author. Fax: (517) 355-0250. Electronic mail address: criddle@ce.msu.edu.

chromatography oven was operated isothermally at 90°C, and both the detector and the injector were maintained at 250°C. TCE concentrations were determined by comparison of the peak areas of samples with the peak areas of external standards. Aqueous phase TCE concentrations were calculated with a dimensionless Henry's law constant of 0.32 at 21.5°C (8). The initial concentration of TCE added was estimated from triplicate sterile controls after equilibration. The total sample volume withdrawn from each vial was less than 5% of the total headspace volume. The coefficient of variance on TCE measurements was 1 to 5% for triplicate samples.

Data from the assays described above were fit to three different kinetic models with the statistical package SYSTAT 5.2.1. A first-order rate expression (first order in TCE concentration) fit the data for each of the four reactors with an average r^2 of >0.95; a zero-order rate expression did not fit the data, and saturation kinetics only applied at high TCE transformation rates (15). Transformation rates were also proportional to the biomass concentration. On the basis of these observations, TCE transformation kinetics are assumed to be first order with respect to TCE concentration and first order with respect to biomass concentration. A second-order rate coefficient, k' (first order with respect to TCE and biomass concentrations), was used for comparison of TCE transformation rates. The corresponding mass balance expression was

$$\frac{dC}{dt} = -k'CX$$

where C is the concentration of TCE in the aqueous phase and X is the concentration of biomass. Assuming that cell concentration does not change appreciably during the assay, the integrated form of equation 1 is

$$lnC = inC_0 - k'Xt$$

where C_0 is the initial concentration of aqueous TCE and t is time.

Measurement of pH, DO, OD, phenol, and total suspended solids. DO, pH, and optical density (OD) (A_{600}) were routinely monitored with a DO probe (Orion model 97-08), pH meter (Orion 720), and spectrophotometer (Shimadzu UV-160), respectively. Phenol was assayed by liquid chromatography on a Gilson high-performance liquid chromatograph (Gilson Medical Electronics, Inc., Middleton, Wis.) equipped with a Whatman C_{18} column (250 mm [length] by 4.6 mm [inside diameter]) (Alltech no. 46211502). The flow rate of the mobile phase (40% acetonitrile plus 60% deionized water containing 0.1% H_3PO_4) was 1 ml/min. A 20-μl injection loop was used for sample injection. Phenol was detected by a UV detector at 235 nm. The detection limit was 0.5 mg/l. A typical coefficient of variance for phenol measurement for five samples was less than 5%. Suspended solids were measured by dry weight according to *Standard methods for the Examination of Water and Wastewater* (10), but 2-μm-pore-size membrane filters were substituted for glass fiber filters.

Identification and quantification of filaments. Identification of filaments was performed according to the identification keys of Eikelboom and van Buijsen (5) and Storm and Jenkins (16). Filamentous organisms were quantified by total extended filament length (TEFL), as suggested by Sezgin and Jenkins (14).

Rotifer enumeration. Rotifers are distinguished from protozoa by their size (100 to ~500 μm) and morphological characteristics (3). To enumerate rotifers, a 10-μl cell suspension was evenly spread over an area of 1.5 cm^2 on a glass slide and examined under ×100 magnification by a phase-contrast microscope (Olympus BH2) equipped with a micrometer scale in ocular. The numbers of rotifers present in the 0.5-cm^2 viewing area were counted. The number of rotifers in a 1-ml sample was calculated and corrected for viewing area and dilution factor. For triplicate samples, this method gave an average coefficient of variation of 30% for the number of rotifers in a sample containing 1,000 to 10,000/ml.

Enumeration of phenol utilizers and TCE degraders. A most probable number (MPN) procedure was developed to estimate the number of phenol and TCE degraders. Phenol utilizers were identified by their ability to grow on phenol, and TCE degraders were identified by their ability to degrade TCE after growth on phenol. It was assumed that all TCE degraders are phenol utilizers. Some support for this assumption was obtained in preliminary experiments: phenol-grown cells degraded TCE, but catechol-grown cells from the same inoculum did not. To enumerate cells by MPN, 10 ml of cell suspension was removed from the reactors at the end of the phenol feeding period and briefly sonicated (30 s, 25 W) to disperse floc and to dislodge attached cells from the filaments (2). Dilutions (10^{-3} to 10^{-8}) of the dispersed cell suspension were prepared in mineral medium. A 1-ml inoculum was added to a 20-ml glass vial with 9 ml of phenol feed medium (phenol concentration, 100 mg/liter). TCE was added to give a concentration of 1 mg/liter in the aqueous phase. Vials were sealed with Teflon-coated septa and incubated at 21°C on a rotary shaker at 120 rpm. Control vials were prepared with sterile medium. After 10 days, the OD at 600 nm (OD_{600}), phenol concentration, and TCE concentration were measured. A vial was scored as phenol positive at an OD_{600} of >0.1 or when more than 50% of the phenol had disappeared. A vial was scored as TCE positive when the amount of TCE remaining was less than 50% of that in the control vials.

Pure culture isolation and characterization. Ten milliliters of sample was collected from each of the four reactors on day 425. The samples were diluted in phosphate buffer (1.0 g of K_2HPO_4 and 0.75 g of KH_2PO_4 [pH 7.2]). Ten-fold dilutions were prepared in a total volume of 10 ml. The samples were vortexed for 5 min before dilution. The samples were also vortexed briefly at each dilution step before transfer. The dilutions were plated on R2A agar and incubated for 4 days at 25°C. Representatives of the various colony morphologies were counted, picked, and transferred to R2A plates for isolation. To ensure the purity of each isolate, the colonies were restreaked five times. To screen for different genotypes, the DNA from each isolate was amplified with repetitive palindromic (REP) sequences, and the products were separated by agarose gel electrophoresis (18). Each isolate with a different REP-PCR pattern was regrown in 20-ml glass vials with 10 ml of mineral medium containing 100 mg of phenol per liter and 1 mg of TCE per liter for 10 days. The increase in OD_{600} over the incubation period was used to determine whether the isolate can grow on phenol. TCE cometabolism was evaluated by monitoring TCE removal. For the phenol and TCE degradation assays, each isolate was regrown and tested in five replicate vials.

Assay for catechol ring fission pathway. The production from catechol of the yellow product, α-hydroxymuconic semialdehyde (α-HMS), or β-ketoadipate was used as a test for the presence of *meta* or *ortho* ring fission pathways, respectively (6, 7). Ten milliliters of cell suspension was concentrated to 2 ml (~5,000 mg [dry weight]/liter) by centrifugation. Concentrated cells (0.5 ml) were resuspended in 2 ml of 0.2 M Tris buffer (pH 8.0), supplemented with 0.5 ml of toluene to solubilize the cell membranes, and shaken with 0.2 ml of a 1.0 M catechol solution. The appearance of a yellow color within a few minutes is indicative of *meta* cleavage activity. After 1 h of incubation, 1 g of $(NH_4)_2SO_4$, 1 drop of 1% sodium nitroprusside (nitroferricyanide), and 0.5 ml of ammonia solution (28 to 30%) were added. A purple color indicates *ortho* cleavage activity.

RESULTS

TCE transformation. The reactor communities displayed significantly different TCE removal rates during the period of operation (Fig. 1). TCE degradation rates in reactor P increased over time. For the initial 75 days, k' values as low as 0.0054 ± 0.0011 liter/mg/day were observed. From day 75 to day 220, k' doubled to 0.011 ± 0.001 liter/mg/day. After day 250, removal rates increased 10-fold to a k' of 0.11 ± 0.03 liter/mg/day. At this point, saturation kinetics (maximum specific rate of transformation, 0.10 ± 0.032 mg of TCE per mg of cell [dry weight]/day; K_s, 0.35 ± 0.08 mg/liter) provided a more accurate description of TCE transformation kinetics. Degradation rates observed after day 250 were comparable to rates of methanotrophic consortia (11). During the period of increasing TCE transformation rates, the biomass concentration did not increase.

Moderate TCE degradation rates were observed for reactor C during the initial 25 days of operation (k' = 0.013 ± 0.004 liter/mg/day). Rates decreased thereafter and remained low for the remainder of the operating period (k' = 0.0036 ± 0.0018 liter/mg/day). The rates ultimately observed in reactor C were far below than those of reactor P. For cells harvested from reactor SC2, k' varied from 0.015 to 0.005 liter/mg/day. Changes in TCE removal rates roughly correlated with changes in biomass concentration. For organisms from reactor SC5, removal of TCE was optimal from day 10 to day 35 with a k' of 0.021 ± 0.003 liter/mg/day. After day 35, TCE degradation rates and biomass levels decreased. After day 80, k' decreased to 0.0026 ± 0.0009 liter/mg/day, and the biomass concentration varied cyclically from 500 mg/liter to 1,000 mg/liter. TCE transformation rates were low and remained at 0.002 to 0.003 liter/mg/day after day 80. Second-order rate coefficients for reactor C were similar to those of reactor SC5. Typical TCE degradation curves for each reactor community after day 250 are illustrated in Fig. 2. A transition to higher rates of TCE degradation was observed with more pulse-like feeding patterns (SC2 and P).

Phenol degradation. The results of the catechol ring fission assay were positive for α-HMS, indicating that phenol degradation proceeded by the *meta*-cleavage pathway in all reactors.

Phenol concentrations in reactor C and the semicontinuously fed reactors (SC2 and SC5) were below the phenol detection limit (0.5 mg/liter). In reactor P, the phenol concentration decreased from 200 mg/liter to 0 mg/liter within 2 to ~6 h after the pulse. On day 30, phenol degradation in reactor P was

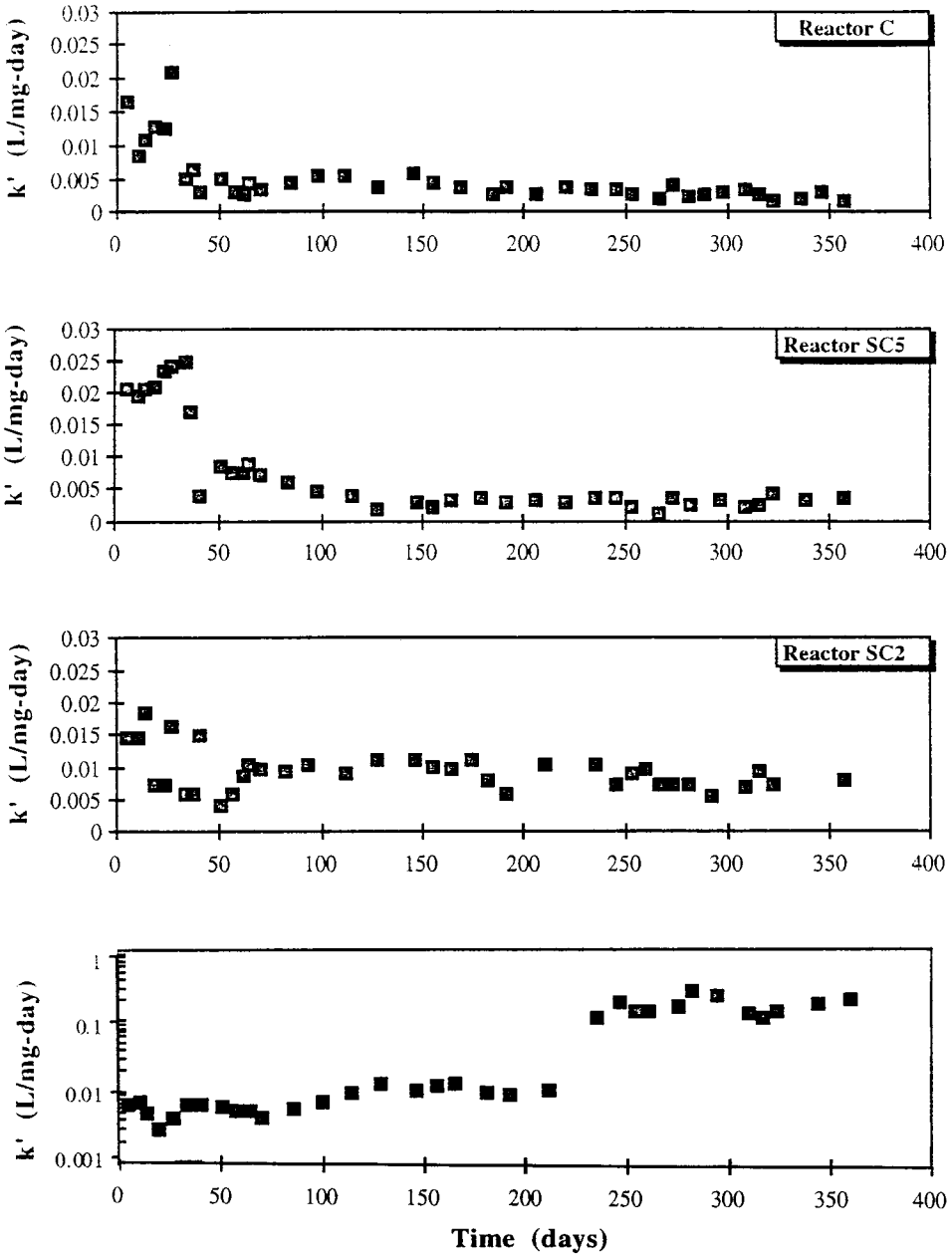

FIG. 1. Long-term variations in the second-order rate coefficients for TCE removal for each reactor enrichment community. Note the log scale for reactor P (bottom).

zero order with respect to the phenol concentration, with a rate coefficient of $0.051 \pm 0.008\ h^{-1}$ ($r^2 > 0.99$). After day 250, the rate of phenol degradation in reactor P more than doubled to $0.135 \pm 0.010\ h^{-1}$. The zero-order pattern of phenol utilization observed in this work was similar to the results of Tischler and Eckenfelder (17). Substrate inhibition kinetics, such as those reported by Grady (9), were not observed, nor was there any evidence of delayed degradation of consecutive phenol pulses (13). Phenol degraded repeatedly without detectable inhibition.

Changes in total biomass concentration. Total biomass concentrations remained relatively stable in reactor P (concentration of suspended solids, 913 ± 126 mg/liter) and reactor SC2 (concentration of suspended solids, 990 ± 170 mg/liter) (Fig.

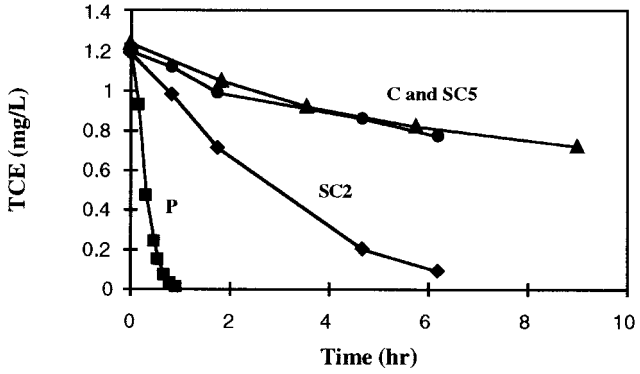

FIG. 2. Typical pattern of TCE degradation in batch experiments with cells from each reactor enrichment community.

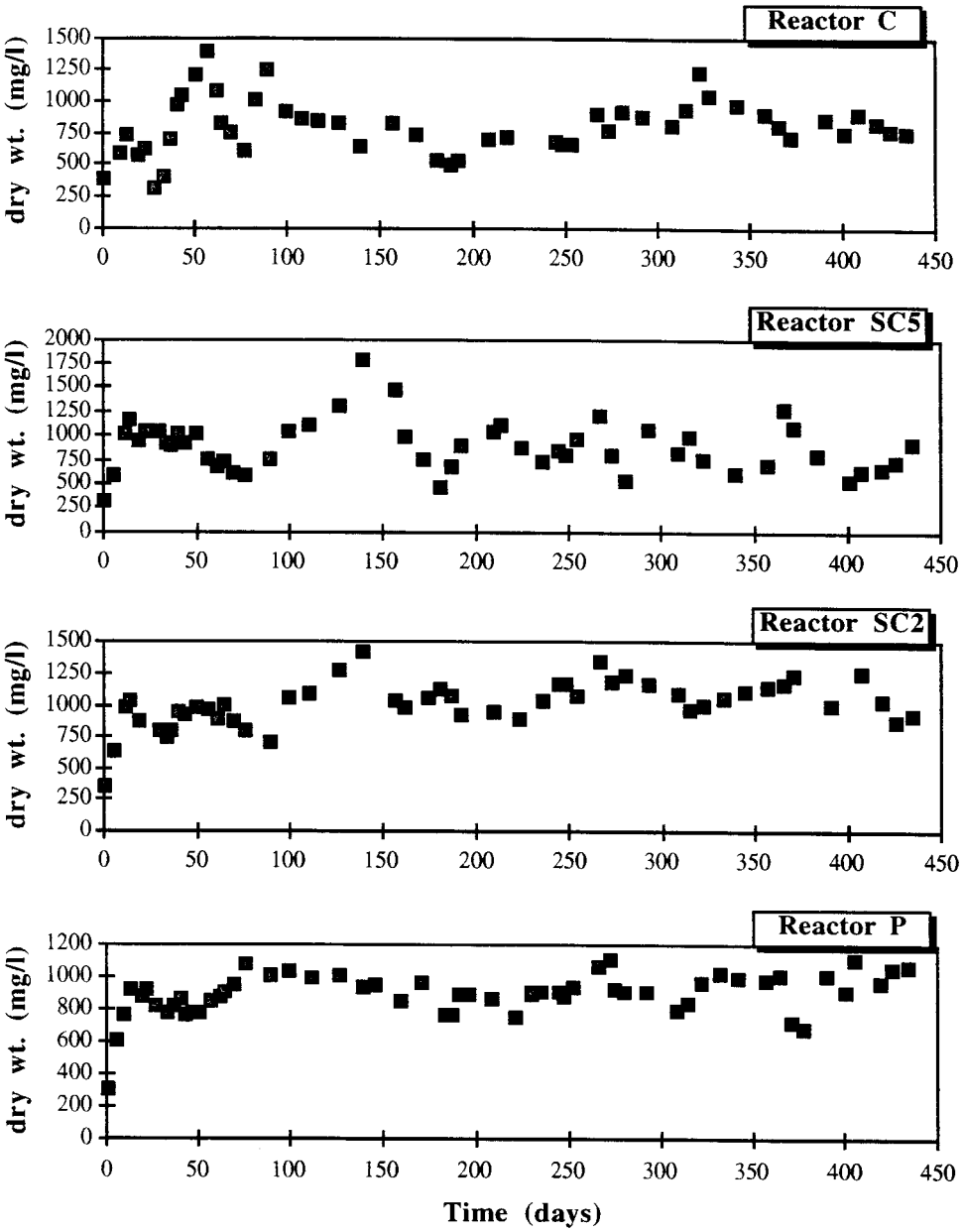

FIG. 3. Long-term variations in biomass concentration for each reactor enrichment community. Samples were taken at the end of the phenol addition period.

3). Significant fluctuations were observed in reactor C (300 to 1,400 mg/liter, with an average of 835 ± 248 mg/liter) and reactor SC5 (850 ± 200 mg/liter). Reductions in the concentration of suspended solids were accompanied by decreased TCE degradation rates in reactors C, SC2, and SC5.

Microscopic inspection. The microbial community of all four reactors contained bacteria, fungi, protozoa, and rotifers. A succession of organism morphologies was observed. Prior to day 35, the dominant species in reactor SC5 were unattached 1- to 2-μm-diameter motile gram-negative rod-shaped bacteria. From day 35 to day 80, a flocculant biomass appeared. After day 80, the dominant organisms were filaments with an average length of 500 to 900 μm and 2 to 4 μm in diameter, with visible septa and true branches. Some bacteria were attached to the filaments. For reactors C and SC2, a similar morphological succession was observed. From day 35 to day 40, the floc structure of reactor SC2 changed from flocculant to filamentous. This coincided with a color change from straw yellow to white. After day 50, reactors SC5, C, and SC2 were dominated by filamentous microorganisms (Fig. 4). The filamentous organisms were identified as fungi. The filaments were composed of true branches, straight or slightly bent and >200 μm in length, sheathless with visible crosswalls, and containing rectangular cells >2.5 μm in diameter with a negative Neisser stain. In reactor P, fungi were much less common, and a distinctive floc structure was observed (Fig. 5).

Fungi were quantified by TEFL. As shown in Table 1, TEFL values ranged from 1.7×10^7 to 2.2×10^8 μm/mg of dry weight. Reactor C and SC5 had 10 times higher TEFL values than reactor P—filaments made up 60 to 95% of the total

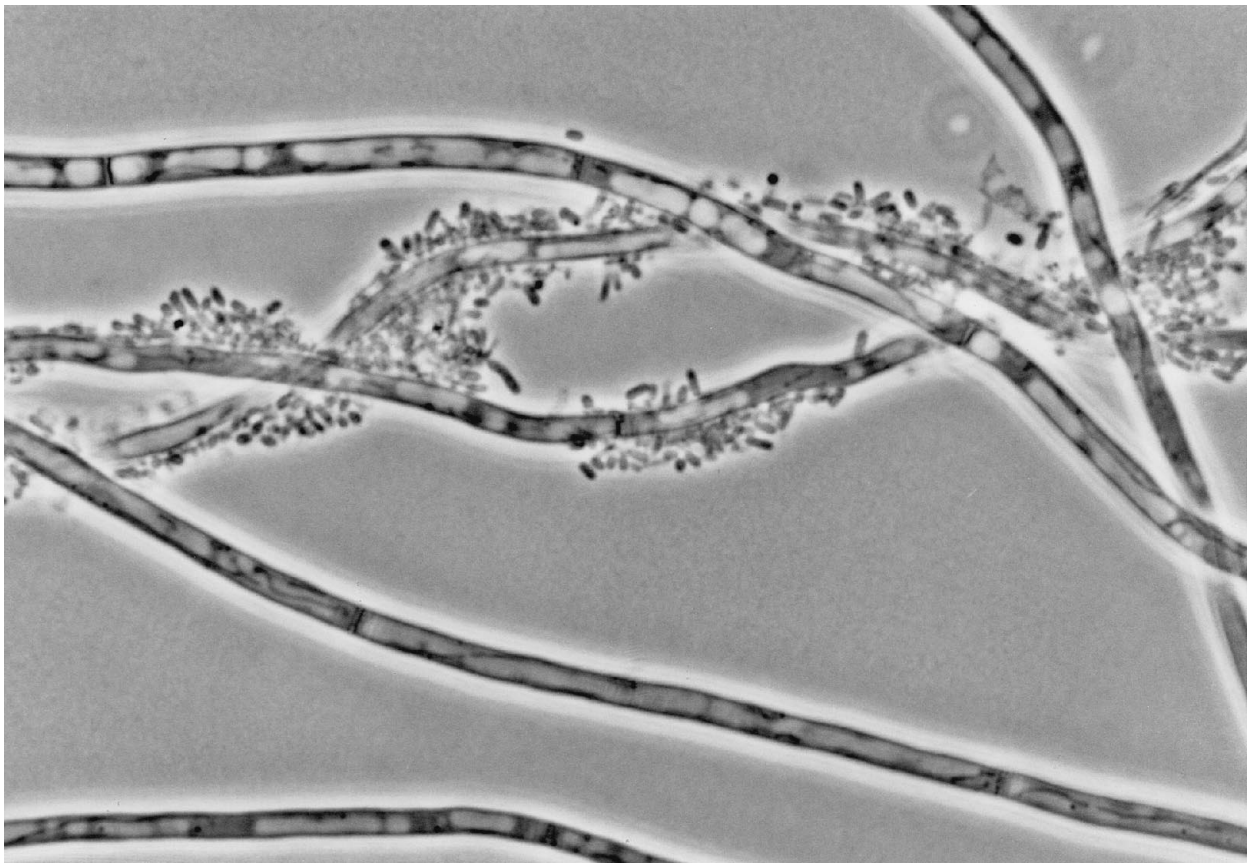

FIG. 4. Enrichment community in reactor SC5.

biomass in reactor C compared with only 5 to 10% of the total biomass in reactor P. Rotifers, principally of the order *Bedellodia*, were present in relatively stable numbers (1,000 to 100,000/ml), depending on the feeding pattern. Rotifers were present in much higher concentrations in reactors C, SC2, and SC5 than in reactor P.

Enumeration of phenol utilizers and TCE degraders. MPN values for phenol utilizers ranged from 2.2×10^6 to 60×10^6 ml^{-1} for all reactors, and the MPN values for TCE degraders ranged from 0.34×10^6 to 40×10^6 ml^{-1} (Table 2). Protozoa and rotifers were present in all reactors. Their existence might be responsible for the fluctuation of biomass and TCE transformation. The dominant protozoa were ciliates, and their population varied (10^2 to $\sim 10^7$/ml) over the period of operation. The number of ciliates appeared to be inversely proportional to total suspended solids. Reactor P had the highest number of phenol utilizers and TCE degraders ($\sim$10 to 100 times higher than those of the other reactors). Reactors C, SC2, and SC5 were not significantly different, with approximately 2×10^6 phenol utilizers ml^{-1} and 0.3×10^5 TCE utilizers ml^{-1}. MPN enumeration results were consistent with the measured TCE transformation rates. The reactors with the highest TCE transformation rates also had highest concentration of TCE degraders. The MPN method provided an approach for estimating the number of cometabolizing organisms, but the method was time-consuming and required exhaustive effort to draw statistically valid conclusions. For samples with the same order of magnitude MPN value, each dilution required as many as 100 tubes to provide statistically distinguishable results. MPN measurements for reactor C, SC2, and SC5 were indistinguishable in the typical five-tube counting procedure recommended by *Standard Methods for the Examination of Water and Wastewater* (10). A further disadvantage of the MPN method was the need for a completely dispersed sample for dilution. This was problematic for filamentous or flocculant growth.

Pure culture isolation and characterization. One hundred forty-six bacterial isolates, representing various colony morphologies on R2A medium, were obtained from the four reactors. The isolates were characterized according to their PCR-REP patterns, phenol degradation capability, and TCE degradation capability (Table 3). Forty-seven of the isolates had distinct REP-PCR patterns. While 23 and 14 distinct isolates were obtained from reactors P and SC2, only 11 and 5 isolates were obtained from reactors C and SC5 (Table 3). This suggests that the microbial communities were more diverse in reactors P and SC2 than in reactors C and SC5. Isolates from reactors C and P had completely different REP-PCR patterns. An isolate that was dominant in reactor C had the same REP-PCR pattern, colony morphology, and efficiency of TCE removal as an isolate present in low concentrations in reactor SC5. The dominant isolate in reactor SC5 was also present in significant numbers in reactor C. One isolate was present in both reactors SC2 and C in low numbers.

Of the 47 distinct isolates, 13 tested positive for phenol degradation. Of these, 10 were able to cometabolize TCE. Three phenol-degrading strains from reactor P exhibited strong TCE degradation activity, two phenol-degrading strains from reactor SC2 exhibited moderate activity (Table 4), and all of the phenol-degrading strains from reactors C and SC5 de-

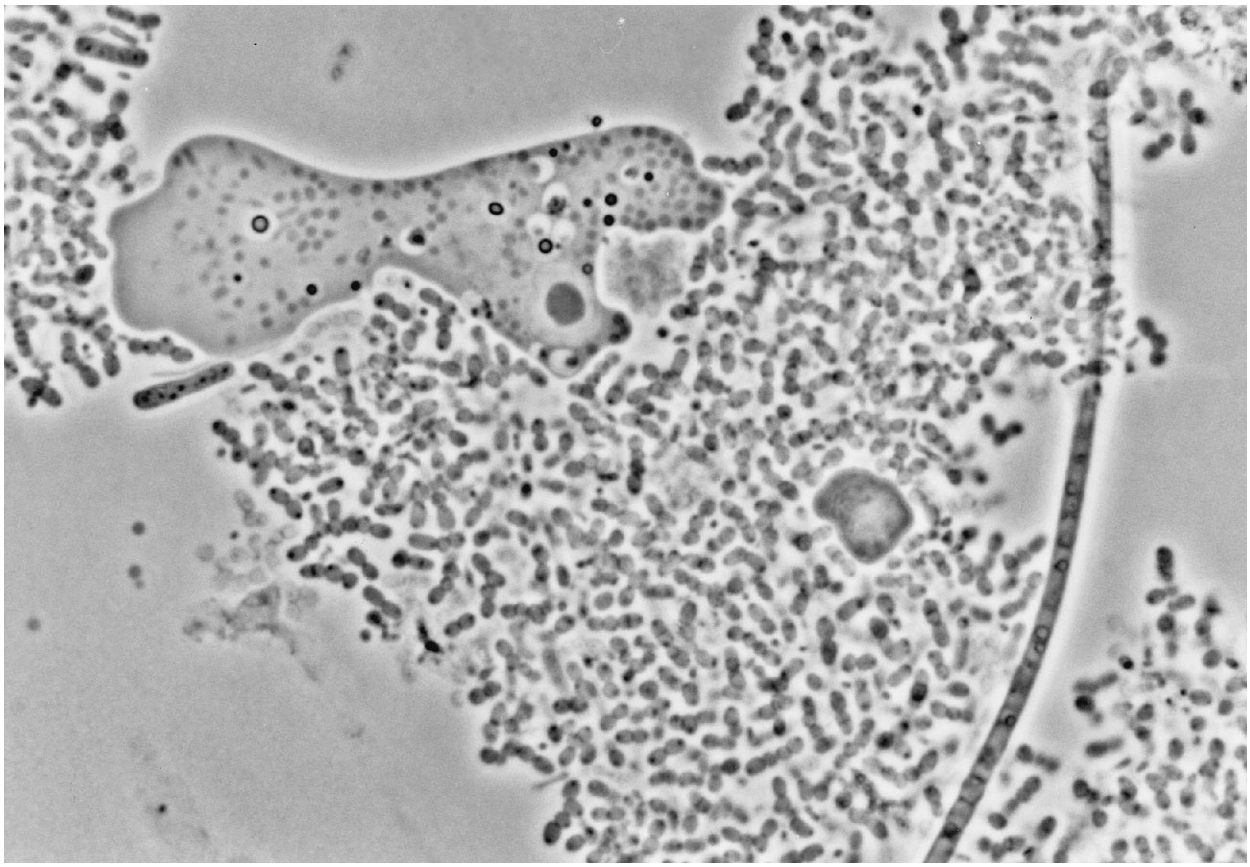

FIG. 5. Enrichment community in reactor P.

graded TCE poorly or not at all (Table 4). These results suggest that the growth substrate feeding patterns dramatically changed the physiology and functions of microbial populations and thus the cometabolic capabilities of the microbial communities.

Effect of phenol feeding pattern on reactor pH and DO. There was little difference in the pH values of the four reactor communities. During start-up, pH decreased from 6.9 to 6.3. After 50 days, pH values stabilized as follows: reactor C, 6.47 ± 0.10; reactor SC5, 6.51 ± 0.06; reactor SC2, 6.60 ± 0.08; reactor P, 6.51 ± 0.09. Ammonia-oxidizing bacteria can contribute to pH reduction and TCE removal, but their role was apparently minor, as indicated by the low levels of nitrate (0 to 10 mg/liter) and nitrite (0 to 3 mg/liter) detected in the reactors.

Aeration levels for all reactors sustained DO levels in excess of 2 mg/liter at all times. These levels are considered sufficient to avoid oxygen limitation (1). However, variations in phenol feeding patterns did mirror changes in DO concentrations. The continuously fed reactor had the highest and most stable DO concentration of 7.5 mg/liter. DO concentrations in the other reactors varied from 7.5 to 2.0 mg/liter, depending on the operating mode. The lower DO concentrations were observed during periods of phenol feed.

DISCUSSION

The results of this study demonstrate that growth substrate feeding patterns have a significant effect on the selection of microorganism types and concentrations and can dramatically change the cometabolic capabilities of a microbial community. TCE degraders were initially present in reactors C and SC5. However, long-term maintenance of these feeding patterns selected against members of the community that were capable of TCE degradation, favoring outgrowth of filamentous micro-

TABLE 1. Quantification of filaments and rotifers (days 275 and 290)

Reactor	Total no. of filaments/mg	Total length of filaments (μm/mg)	Filament length (μm)	% of filament mass in total biomass[a]	No. of rotifers/ml
P	$(1.9 \pm 0.5) \times 10^4$	$(1.7 \pm 0.5) \times 10^7$	900 ± 400	5–10	820 ± 350
SC2	$(1.7 \pm 0.7) \times 10^5$	$(8.0 \pm 4.2) \times 10^7$	500 ± 400	30–50	2,500 ± 750
SC5	$(2.2 \pm 0.6) \times 10^5$	$(2.2 \pm 1.0) \times 10^8$	1,000 ± 700	70–95	4,100 ± 1,300
C	$(2.9 \pm 0.7) \times 10^5$	$(1.7 \pm 0.5) \times 10^8$	600 ± 300	60–95	11,600 ± 2,100

[a] The percentage of filaments in the total biomass was estimated with the culture dry weight and an estimation for fungal dry weight assuming filament diameters of 2 to 3 μm and a density of 1 g/cm^3.

TABLE 2. Phenol and TCE degraders enriched by different feeding patterns (day 192)

Reactor	MPN (no. of cells/g [dry wt] of cells) [95% CI][a]	
	Phenol degraders	TCE degraders
P	6.7×10^{10} [2.2×10^{10}–2.0×10^{11}]	4.5×10^{10} [1.5×10^{10}–1.4×10^{11}]
SC2	3.3×10^{9} [1.1×10^{9}–1.0×10^{10}]	2.2×10^{8} [7.3×10^{7}–6.6×10^{8}]
SC5	3.3×10^{9} [1.1×10^{9}–1.0×10^{10}]	4.5×10^{8} [1.5×10^{7}–1.4×10^{9}]
C	5.4×10^{9} [1.8×10^{9}–1.6×10^{10}]	1.7×10^{9} [5.4×10^{8}–5.1×10^{10}]

[a] CI, confidence interval.

organisms, especially fungi, that were incapable of TCE degradation or degraded TCE slowly.

There are several possible explanations as to why fungi would be favored over bacteria in reactors C and SC5. Competition for low levels of phenol may have favored filamentous growth (14). Bacterial predation may also have favored fungal growth. Significant biomass fluctuations were observed in reactors C and SC5 (Fig. 3), and higher levels of predators were observed in these reactors than in reactor P (Table 1). The paucity of predators in reactor P might be due to the inhibitory action of high phenol concentrations on predators. Visual inspection of the reactor communities suggested that phenol degradation intermediates might have contributed to biomass fluctuations or that biomass fluctuations affected mineralization of phenol. A color switch occurred in reactors C and SC2. From day 25 to day 40, reactor C changed color from white to yellow, and biomass decreased from 750 mg/liter to 250 mg/liter. The yellow may have been due to the accumulation of α-HMS, a yellow intermediate of phenol degradation. After 2 more weeks of operation, the yellow disappeared, and white was again observed. Brief interruptions in aeration to reactor C also triggered the appearance of yellow. These observations suggest that the yellow color resulted from incomplete oxidation of phenol. The appearance of yellow correlated with decreases in the cell concentration and the TCE transformation rate. The yellow was not removed over a 3-day period when a filtered sample from reactor C was incubated with cells drawn from other reactors (reactors P, SC2, and SC5).

Characterization of isolates from the four reactors indicated that the microbial communities in the four reactors were different and complex. Only 28% of all isolates were able to utilize phenol, and only 21% of all isolates were capable of appreciable TCE transformation (Tables 3 and 4). It is probable that some organisms that were unable to degrade phenol under the chosen assay conditions might still be able to degrade phenol under other conditions: if, for example, growth factors provided by other community members were available or if the phenol concentration of the assay were reduced. The phenol concentration used might have been excessive for some isolates, especially those from reactors C and SC5, because these communities were not adapted to high concentrations of phenol. It is also possible that some of the isolates were unable to use phenol because they use other substrates for growth, such as intermediates in the phenol degradation pathway or substances released by cell lysis. Nevertheless, the results of the isolate studies were consistent with other measures of community function, such as TCE transformation and phenol utilization.

Of interest is the fact that reactor P had more bacterial diversity (Table 3), and perhaps for that reason, also contained more organisms capable of rapid phenol and TCE transformation (Table 4). It is also likely, though, that the pulse feeding pattern selected for cells with higher specific rates of substrate degradation for both TCE and phenol. Cells from reactor P quickly degraded phenol, while cells from continuously and semicontinuously fed reactors exhibited a lag phase and lower rates of degradation. These observations might be explained by differences in community structure or in the enzymes present. In general, a lag period occurred whenever cells were exposed to phenol concentrations that were significantly greater than the levels to which the cells had adapted. A lag period was even observed for cells from reactor P when the added phenol concentration exceeded 400 mg/liter. TCE transformation rates followed the same general pattern as phenol transformation rates, and the degradation activities of the isolates reflected patterns found in the parent enrichments: isolates from

TABLE 3. Community diversity of the four reactors as revealed by the number of bacterial isolates showing distinct REP-PCR patterns

Reactor	Total no. of distinct isolates	No. of distinct phenol degraders	No. of distinct strong TCE degraders[a]
P	20	4	3
SC2	14	6	0
SC5	4	1	0
C	9	2	0

[a] Strong TCE degraders are defined as isolates capable of removing >50% of the TCE after a 10-day incubation in 20-ml glass vials containing 10 ml of phenol medium (phenol concentration, 80 mg/liter) and spiked to 1.0 mg of TCE per liter. A loopful of the inoculum was transferred from R2A agar and incubated on a 120-rpm rotary shaker at room temperature.

TABLE 4. Characteristics of phenol-degrading isolates from different reactor communities[a]

Reactor	Isolate designation	Colony morphology	Phenol degradation	% TCE removal
C	C-1	White, pearl[b]	+	13
	C-2	White, small	+	11
SC5	SC5-1	White, pearl[b]	+	15
SC2	SC2-3	White, large[b]	+	45
	SC2-4	White, large[b]	+ (yellow)	0
	SC2-6	White, small	+	38
	SC2-7	Clear, small, waxy	+	0
	SC2-8	White, spreading	+ (yellow)	0
	SC2-9	Opaque	+	15
P	P-1	White, large	+	11
	P-4	White, brown center, hard[b]	+	95
	P-21	White, large	+	94
	P-23	White, large	+	54

[a] See the legend to Table 3 for the TCE degradation assay conditions. Phenol utilization was determined by measurement of the changes in OD before and after incubation.

[b] Dominant colony morphology.

reactors P and SC2 exhibited higher rates of TCE and phenol degradation than isolates from reactors SC5 and C.

The results of this study indicate that the manner in which growth substrate is added profoundly alters bioreactor ecology and may be critical to the performance of reactors designed to achieve cometabolism. Communities enriched with continuous or protracted feeding intervals (reactor C and SC5) exhibited limited long-term capacity for TCE transformation. Communities enriched with short feeding intervals (reactors P and SC2) maintained higher TCE transformation rates. Pulse feeding resulted in a more stable and diverse bacterial biomass concentration, presumably because of the decrease in predators. Additional research is needed to explain whether bacterial isolates from the pulse-fed reactor community exhibited higher rates of TCE transformation than bacterial isolates from the other reactor communities. Research is also needed to explain the sudden step increase in TCE transformation rates in reactor P in the absence of any direct selective pressure for TCE transformation.

ACKNOWLEDGMENTS

This work was funded in part under grant R-817873 from the U.S. Environmental Protection Agency; in part by the Great Lakes and Mid-Atlantic Hazardous Substance Research Center under grant R-819605 from the Office of Research and Development, U.S. Environmental Protection Agency; and in part by the NSF Center for Microbial Ecology under National Science Foundation grant BIR-9120006. Partial funding for the Mid-Atlantic Hazardous Substance Research Center was provided by the State of Michigan Department of Natural Resources.

REFERENCES

1. **Bailey, J. E., and D. F. Ollis.** 1986. Biochemical engineering fundamentals, 2nd ed., McGraw-Hill, New York.
2. **Banks, C. J., and I. Walker.** 1977. Sonication of activated sludge flocs and the recovery of their bacteria on solid media. J. Gen. Microbiol. **98:**363–368.
3. **Calaway, W. T.** 1968. The metazoa of waste treatment processes—rotifers. J. Water Pollut. Control Fed. **40:**R415.
4. **Dabrock, B., J. Riedel, J. Bertram, and G. Gottschalk.** 1992. Isopropylbenzene (cumene)—a new substrate for isolation of trichloroethene-degrading bacteria. Arch. Microbiol. **158:**9–13.
5. **Eikelboom, D. H., and H. J. J. van Buijsen.** 1981. Microscopic sludge investigation manual. TNO Research Institute for Environmental Hygiene, Delft, The Netherlands.
6. **Folsom, B. R., P. J. Chapman, and P. H. Pritchard.** 1990. Phenol and trichloroethylene degradation by *Pseudomonas cepacia* G4: kinetics and interactions between substrates. Appl. Environ. Microbiol. **56:**1279–1285.
7. **Gerhardt, P., R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.).** 1994. Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C.
8. **Gossett, J. M.** 1987. Measurement of Henry's law constants for C1 and C2 chlorinated hydrocarbons. Environ. Sci. Technol. **21:**202–208.
9. **Grady, C. P. L., Jr.** 1985. Biodegradation: its measurement and microbiological basis. Biotechnol. Bioeng. **27:**660–674.
10. **Greenberg, A. E., R. R. Trussell, and L. S. Clesceri (ed.).** 1989. Standard methods for the examination of water and wastewater, 17th ed. American Public Health Association, Washington, D.C.
11. **Henry, S. M., and D. Grbić-Galić.** Effect of mineral media on trichloroethylene oxidation by aquifer methanotrophs. Microb. Ecol. **20:**151–169.
12. **Nelson, M. J. K., S. O. Montgomery, W. R. Mahaffey, and P. H. Pritchard.** 1987. Biodegradation of trichloroethylene and involvement of an aromatic biodegradative pathway. Appl. Environ. Microbiol. **53:**949–954.
13. **Okaygun, M. S., and A. Akgerman.** 1992. Microbial dynamics in a continuously stirred tank reactor with 100% cell recycle. Water Environ. Res. **64:** 811–816.
14. **Sezgin, M., and D. Jenkins.** 1978. A unified theory of filamentous activated sludge bulking. J. Water Pollut. Control Fed. **2:**362–381.
15. **Shih, C.-C.** 1995. Experimental and numerical evaluation of cometabolism in sequencing batch reactors. Ph.D. dissertation. Michigan State University, East Lansing.
16. **Storm, P. F., and D. Jenkins.** 1984. Identification and significance of filamentous microorganisms in activated sludge. J. Water Pollut. Control Fed. **56:**449–458.
17. **Tischler, L. F., and W. W. Eckenfelder.** 1969. Linear substrate removal in the activated sludge process, p. 361–374. *In* Advances in water pollution research. Pergamon Press, Oxford.
18. **Versalovic, J., M. Schneider, F. J. deBruijn, and J. R. Lupski.** 1994. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol. Cell. Biol. **5:**25–40.
19. **Wackett, L. P., and D. T. Gibson.** 1988. Degradation of trichloroethylene by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. Appl. Environ. Microbiol. **54:**1703–1708.