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Effects of media composition on substrate removal by pure and mixed bacterial cultures

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Abstract. Continuous culture experiments with identical experimental designs were run with a mixed microbial community of activated sludge origin and an axenic bacterial culture derived from it. Each culture received 2-chlorophenol (2-CP) at a concentration of 160 mg/L as COD and L-lysine at a concentration of 65 mg/L as COD. A factorial experimental design was employed with dilution rate and media composition as the two controlled variables. Three dilution rates were studied: 0.015, 0.0325, and 0.05 h^{-1}. Media composition was changed by adding four biogenic compounds (butyric acid, thymine, glutamic acid and lactose) in equal COD proportions at total concentrations of 0, 34, 225, and 1462 mg/L as COD. The measured variables were the effluent concentrations of 2-CP as measured by the 4-aminoantipyrene test and lysine as measured by the o-diacetylbenzene procedure. The results suggest that community structure and substrate composition play important roles in the response of a microbial community to mixed substrates. The addition of more biogenic substrates to the axenic culture had a deleterious effect on the removal of both lysine and 2-CP, although the effect was much larger on lysine removal. In contrast, additional substrates had a positive effect on the removal of 2-CP by the mixed community and much less of a negative effect on the removal of lysine. The dilution rate at which the cultures were growing had relatively little impact on the responses to the additional substrates.

Abbreviations: COD – chemical oxygen demand; 2-CP – 2-chlorophenol; DOC – dissolved organic carbon; MDL – method detection limit; SS – suspended solids

Introduction

Although most studies on the biodegradation of xenobiotic organic pollutants are performed in single substrate experiments, biodegradation in natural and engineered systems seldom involves single substrates. Rather, the pollutant of interest is normally present in a complex milieu of organic chemicals of both biogenic and xenobiotic origin. This suggests that predictions of the fate of organic pollutants must consider the impact of the presence of other organic compounds. Unfortunately, substrate interactions are often complex, involving effects on both enzyme synthesis and activity, and this makes it difficult to develop mathematical models for engineering use. For example, the response of a two-substrate, single-organism continuous culture was found to be more complicated than most models would predict (Machado & Grady 1989), even though the experiments were conducted in a manner that minimized events that act on enzyme synthesis, such as catabolite repression. Furthermore,

biodegradation in both natural and engineered systems usually involves mixed microbial communities and the interactions among the organisms in those communities adds another level of complexity to the problem. This suggests that efforts to model systems of practical complexity may be premature at this time.

If researchers are to be successful in the formulation of mathematical models capable of predicting the fate of xenobiotic compounds in natural and engineered environments, data must be available upon which model development can be based. Unfortunately, few studies have observed the responses of microbial cultures to various substrate mixes under carefully controlled growth conditions. In particular, few studies have been done in continuous culture where the microorganisms are kept under carbon-limited conditions at low specific growth rates, even though they mimic more closely than batch cultures the circumstances to be found in nature and in engineered systems. However, one continuous culture study (Law & Button 1977) showed that as media complexity was increased while the influent total carbon concentration was maintained relatively constant, there was a decrease in the output concentration of a biogenic marker substrate. This suggests that multicomponent substrate systems may be advantageous to the achievement of maximum pollutant destruction. Since many significant pollutants are xenobiotic in structure, it is important to ask if they will behave in the same way. Furthermore, what will happen as the total carbon flux through the system is increased? If biogenic substrates were added in low concentration relative to a marker substrate, they would add little to the total energy flow through the system, and thus may have little impact. However, as the relative amount of energy provided by additional biogenic substrates increased, the impact on the biodegradation of a marker substrate might change because of the diminished importance of the marker substrate to the total energy flow. The available literature provides little information relative to such questions.

In order to contribute to the data base on the biodegradation of organic compounds in multicomponent mixtures, a study was conducted to ascertain how the relative contribution of a marker substrate to the total energy available in a complex media influenced its biodegradation in a continuous culture bioreactor. To make the results relevant to researchers concerned with predicting the fate of organic compounds in natural and engineered environments, four questions were identified as being of primary importance. First, is the output concentration of a marker substrate from a steady-state bioreactor operated at constant specific growth rate influenced by that substrate's contribution to the total energy available in the feed? Second, is the response of a xenobiotic marker substrate similar to the response of a biogenic marker substrate? Third, does the steady-state specific growth rate imposed on the culture influence the response? Fourth, does the response of an axenic bacterial culture accurately represent the response of a mixed microbial community from which it was derived? The purpose of this paper is to report the results of that study.

Materials and methods

Experimental plan

In order to address all of the questions asked above, experiments were conducted with both a mixed microbial community and an axenic bacterial culture derived from it, using the same basic experimental plan for each. All experiments were run in steady-state continuous flow bioreactors (chemostats) operated at low dilution rates (flow rate divided by reactor volume) to minimize the effects of metabolic controls like catabolite repression (Grady & Gaudy 1969). A 3×4 factorial experimental design was used, with three levels of dilution rate (0.015, 0.0325, and 0.050 h^{-1}) and four levels of media composition as the independent variables. The measured experimental variables were the concentrations of two marker substrates in the chemostats.

The two marker substrates were 2-chlorophenol (2-CP) and L-lysine. 2-Chlorophenol is a xenobiotic compound on the U.S. EPA priority pollutant list. It is aerobically biodegradable by both pure and mixed cultures of bacteria via metabolic pathways typical of aromatic compounds, leading ultimately

to acetyl-CoA (Rochkind-Dubinsky et al. 1987). L-Lysine is a readily biodegradable biogenic compound with a relatively complex catabolic pathway, leading ultimately to succinic acid (Clarke & Ornston 1975). One factor that contributed to the selection of these compounds is that they share no metabolic intermediates prior to the TCA cycle. Their concentrations in the feeds to the chemostats were fixed, with 2-CP being present at a concentration of 160 mg/L as chemical oxygen demand (COD) (98 mg/L as 2-CP or 0.77 mM) and lysine at a concentration of 65 mg/L as COD (42.5 mg/L as lysine or 0.29 mM). COD was used to express the concentrations of all organic compounds because it is a measure of the energy available in them (Grady & Lim 1980).

The total energy content of the feed was altered by simultaneously adding four additional biogenic compounds: lactose, butyric acid, glutamic acid and thymine, representing carbohydrates, fatty acids, amino acids and nucleic acids, respectively. They were added in equal proportions as COD; thus each provided an equal amount of energy to the biomass. A mixture containing 1.0 mg/L as COD of each compound would contain 0.89 mg/L of lactose (0.0026 mM), 0.55 mg/L of butyric acid (0.0062 mM), 1.02 mg/L of glutamic acid (0.0069 mM), and 0.56 mg/L of thymine (0.0057 mM). The base media contained only 2-CP and lysine as carbon and energy sources (zero additional biogenic compounds). To achieve the other three levels of media composition, the additional biogenic compounds were added to the base media at total COD concentrations of 34, 225, and 1462 mg/L, representing 15%, 100% and 650% of the sum of the CODs provided by the two marker substrates. These values were chosen to allow investigation over a broad range of concentrations and each step represented a 5.5-fold increase.

Microbial cultures

Mixed microbial community. A continuous enrichment culture capable of degrading 2-CP and lysine was developed in a chemostat using inocula from several sources, including wastewater treatment facilities, and was maintained throughout the experimental period as a seed culture at a dilution rate of 0.05 h^{-1} on a feed containing 65 mg/L as COD of 2-CP and 160 mg/L as COD of lysine. Prior to the initiation of the experimental program, a portion of the seed culture was harvested by centrifugation and stored in a solution of 15% glycerol at –70 °C for use during startup of each experiment.

The nature of the seed culture was investigated twice by selecting isolates and characterizing them with respect to colony morphology on plates, their ability to use 2-CP and lysine, and their resistance/susceptibility to 19 antibiotics (Mackey 1989). The first investigation yielded 10 distinct groups of isolates that differed significantly in their characteristics. One group could use both 2-CP and lysine as sole carbon sources, three could use lysine but not 2-CP, two could use 2-CP but not lysine, two could use lysine and could degrade 2-CP in the presence of lysine, and two groups could use neither substrate. The second investigation yielded 13 groups of isolates. One group could use both 2-CP and lysine as sole carbon sources, six could use lysine but not 2-CP, three could use 2-CP but not lysine, and three groups could use neither substrate. Although no attempt was made to identify the isolates, two things are evident. First, the culture was very diverse. Second, specialists were more dominant than generalists.

Axenic culture. The bacterium used in the axenic culture experiments was an isolate from the mixed culture capable of using both 2-CP and lysine as sole carbon sources. Its growth on 2-CP was characterized by a maximum specific growth rate ($\hat{\mu}$) of 0.34 h^{-1}, a half saturation coefficient (K_S) of 165 µg/L as COD of 2-CP, and a yield (Y) of 0.24 mg biomass/mg COD removed (Loven 1985). Growth on lysine occurred with a $\hat{\mu}$ value of 0.11 h^{-1} and a Y value of 0.30 mg biomass/mg COD removed (Lu 1987). The value of K_S for lysine was not measured.

The isolate was maintained on agar slants and its purity was routinely checked before and during all experiments by observing its susceptibility/resistance pattern to 19 antibiotics. During each experiment, each time a sample was collected 0.1 mL of reactor contents was spread evenly onto plates containing R3A media (Reasoner & Geldrich 1985), al-

lowing a lawn of bacteria to develop. Immediately after plating, antibiotic discs were placed on the surface of the plates. Because of the diffusion of the antibiotics into the agar, a concentration gradient is established around each disc and the extent to which the bacteria can grow into the circular zone is a measure of its resistance to the antibiotic. The isolate was susceptible to 13 of the antibiotics used and this provided the basis for easy detection of contamination during the continuous culture experiments. It is highly unlikely that a contaminant would be susceptible to all of the antibiotics to which the isolate was susceptible. Consequently, any contaminant that invaded the culture would be likely to grow in the clear zone around at least one of the antibiotic discs. Because the culture was plated without dilution, contaminants could be detected as individual colonies in clear zones when they comprised less than 10^{-7} of the total population. This sensitivity allowed contamination to be detected before it had an effect on reactor performance.

Growth media

The mineral salts media used as feed during the continuous culture experiments had the following composition (all as mg/L): $CaCl_2$, 8.0; $FeCl_3 \cdot 6H_2O$, 6.0; H_3BO_3, 0.06; $MgSO_4 \cdot 7H_2O$, 60.0; $MnSO_4 \cdot H_2O$, 1.8; $Na_2MoO_4 \cdot 2H_2O$, 0.2; $(NH_4)_2 \cdot SO_4$, 1000.0; KH_2PO_4, 272.0. In addition, each 12 liter bottle of media contained one liter of dechlorinated tap water to provide additional micronutrients and 12 mL of concentrated HCl to lower the pH and reduce precipitation. The mineral salts media was sterilized by autoclaving whereas the organic substrates were filter sterilized and added to the sterile mineral salts after cooling.

The media used to grow the axenic culture inocula was similar to the continuous culture media except for the elimination of the HCl and the addition of 0.05 M phosphate buffer to maintain the pH near neutrality.

Reactor system

All continuous culture experiments were performed in 14 liter reactor vessels (Model M1014–7260, New Brunswick Scientific Co.) containing a liquid volume of 10 liters. They were mixed with three six-bladed turbine impellers, which also served to disperse the incoming air for efficient oxygen transfer. The oxygen transfer rate was sufficient to maintain the dissolved oxygen concentration above 2.0 mg/L under all conditions studied. The incoming air was sterilized through a series of filters that also removed moisture, aerosols, particulate matter, and organic gases. The outgoing air passed through a condenser that returned the water vapor to the reactor. Feed was delivered to the reactor with a peristaltic pump (Model AL-4E, Sigmamotor, Inc.) at the rate required to maintain the desired dilution rate. Effluent was withdrawn with a similar pump from beneath the liquid surface through a Y-tube (Koopman et al. 1980). The pH of the reactor was maintained at 7.00 ± 0.05 units by using an automatic control system (Model 5977–20, Horizon Ecology, Inc.) which added 0.5 N HCl or 1.0 N NaOH solutions as needed.

Reactor startup

Mixed microbial community. Each experiment was started in a way that maintained maximum culture diversity, although the technique was slightly different for experiments with and without the additional biogenic compounds.

For experiments without additional biogenic compounds, a second stage chemostat was started from the seed culture chemostat by directing the effluent from the seed culture into an identical reactor system. During the fill period, fresh feed identical to that entering the seed culture was also added to the second-stage chemostat at a rate that kept the specific growth rate of the microbes in the second-stage chemostat identical to the specific growth rate in the seed culture; i.e., 0.05 h^{-1} (Grady & Lim 1980). This minimized any changes in species diversity during the transfer. Once the experimental reactor was filled, the flow of effluent from the seed culture

was stopped and the feed flow to the experimental reactor was adjusted to give the desired dilution rate for the particular experiment. At that time, previously frozen and stored biomass from the seed culture was thawed, washed twice in mineral salts media, grown overnight on media containing 200 mg/L as COD of lysine and 8 mg/L as COD of 2-CP, and added to the experimental chemostat. The media composition was then adjusted over a period of one residence time (reactor volume divided by flow rate) to raise the 2-CP concentration to 160 mg/L as COD and reduce the lysine concentration to 65 mg/L as COD. The reactor was then allowed to operate for at least three residence times before sampling began.

The procedure for starting experiments with the additional biogenic compounds was very similar to that described above, with three exceptions. First, the shift in the media composition to increase the 2-CP concentration to 160 mg/L as COD and decrease the lysine concentration to 65 mg/L as COD was done over two residence times rather than one. Second, the additional biogenic compounds were added at a total COD concentration of 34 mg/L during the time that the 2-CP concentration was being increased. Third, experiments at a given dilution rate with higher additional biogenic compound concentrations were started from the previous experiment at that dilution rate. At the completion of sampling for the experiment with 34 mg/L as COD of additional biogenic compounds, the total concentration of those compounds in the media was changed to 225 mg/L as COD in a single step. After operation for one residence time, previously frozen seed was added in the manner described previously. The reactor was then allowed to operate for at least three residence times before sampling began. After the completion of sampling, the same procedure was used to raise the feed biogenic compound concentration to 1462 mg/L as COD.

Axenic culture. To start each axenic culture experiment, biomass was removed from a slant and used to inoculate a tube containing 9 mL of lysine mineral salts media. This was shaken on a shaker table until appreciable growth had occurred and was then transferred aseptically to a chemostat vessel containing 4.5 liters of media for initial growth in batch mode.

For experiments without additional biogenic compounds, the media during the initial batch growth period contained 225 mg/L as COD of lysine as sole carbon source. As soon as appreciable growth had occurred, continuous operation was initiated with feed containing 50 mg/L as COD of 2-CP and 160 mg/L as COD of lysine. During the fill period, the feed rate was regulated by computer so that the feed rate divided by the reactor volume was maintained at a value equal to the dilution rate to be used in the experiment. Upon exhaustion of the bottle of media, it was replaced with one containing 160 mg/L as COD of 2-CP and 65 mg/L as COD of lysine. The chemostat was allowed to operate for at least three residence times after filling or until biomass absorbance readings indicated that the system had achieved steady state. Sampling was then begun.

The protocol for starting the experiments with the additional biogenic compounds was essentially the same as that described above, with two exceptions. First, after appreciable batch growth had occurred, continuous culture was initiated with media containing 30 mg/L as COD of 2-CP, 195 mg/L as COD of lysine and the appropriate concentration of biogenic compounds. Second, for experiments with the highest additional biogenic compound concentration, initial batch growth was accomplished on media containing 1462 mg/L as COD of lysine.

Reactor operation

Once stability was achieved, the chemostats were operated and sampled over a period of three residence time to establish their steady-state performance under the imposed conditions. Regardless of the type of culture, the media feed rates were calibrated daily and the tubing that passed through the pump head was inspected for any signs of wear and replaced when necessary. Other operational procedures depended upon the type of culture being studied. When the mixed culture was under investigation, the bioreactor contents were transferred to

a clean vessel every day and all reactor appurtenances were cleaned at the time of transfer, thereby preventing significant wall growth. The pH probe and meter were also calibrated at that time. Because the axenic bioreactor could not be opened without contaminating it, its walls were cleaned daily with a magnet to minimize wall growth, which was never significant. The pH meter was calibrated daily by removing a sample from the chemostat, checking its pH with another meter, and adjusting the bioreactor meter as required. As mentioned earlier, the axenic culture was checked for contamination each time a sample was taken. If contamination was detected early in an experiment, the experiment was terminated and restarted. If contamination was detected late in an experiment, the experiment was also terminated but the data collected prior to detection of contamination were considered to be valid and used to establish steady-state performance.

Sampling procedure

During each experiment, 12 (mixed culture) or 13 (axenic culture) samples were removed from the chemostats at equal intervals over three residence times via the reactor sampling port. Samples to be used for quantification of the marker substrate concentrations were immediately pressure filtered through two overlying prewashed glass fiber filters (Catalog No. 66078, Gelman Science, Inc.) that had been selected because they did not alter the concentrations of the two marker substrates. The filtration step removed the majority of the bacteria and stopped significant substrate removal, thereby allowing sample preparation to proceed without fear of additional substrate loss. The total elapsed time between sample withdrawal and filtration was normally less than ten seconds. To remove any remaining bacteria, the filtered samples were centrifuged for 20 min at 16,270 xg (mixed culture) or for 15 min at 27,000 xg (axenic culture) at 4 °C (Sorvall Model RC2B Centrifuge). The centrate was carefully decanted and stored at –15 °C for future analysis. Additional samples were removed for other analyses, such as biomass (absorbance and suspended solids, SS), COD, and dissolved organic carbon (DOC). Duplicate analyses for 2-CP and lysine were run on each sample collected during the axenic culture experiments, but single analyses were run during the mixed culture experiments.

Analytical techniques

2-Chlorophenol. 2-Chlorophenol was quantified by the 4-aminoantipyrene technique of Yang & Humphrey (1975) for total phenolic compounds. Color was allowed to develop for at least 20 minutes after addition of the 4-aminoantipyrene reagent and the absorbance was measured against a distilled water blank using a dual beam spectrophotometer (ACTA C III, Beckman Instruments, Inc.) at a wavelength of 505 nm using a 4 cm lightpath and a slit width of 2 mm. All measurements were made within 50 minutes of the addition of the 4-aminoantipyrene reagent, over which time the color was stable (Philbrook 1984). Analysis of eight replicate samples of a solution containing 26 μg/L yielded a standard deviation of 1.1 μg/L, suggesting that the method detection limit (MDL) was approximately 3.3 μg/L (Clesceri et al. 1989).

L-Lysine. Lysine was quantified with the procedure described by Roth & Jeanneret (1972), in which lysine reacts with o-diacetylbenzene in the presence of 2-mercaptoethanol to yield a highly fluorescent product. The only other commonly found amino acids that give the same reaction are ornithine and glycine, which were not supplied in the media and thus were expected to be low. Samples were read in a fluorescence spectrophotometer (Perkin-Elmer Model 650–40) at wavelengths of 350 and 445 nm for excitation and emission, respectively. A slit width of 10 nm was used for both excitation and emission. The variance of the assay was determined by analyzing eight replicate samples of a solution containing 269 μg/L of lysine. The standard deviation was found to be 10 μg/L, suggesting that the MDL was approximately 30 μg/L (Clesceri et al. 1989).

Other analyses. The absorbance of the biomass sus-

pension was measured with a Spectronic 20 (Bausch & Lomb) spectrophotometer using a 1 cm light path and a wave length of 540 nm. The concentration of suspended solids in the bioreactors was measured by centrifuging an 80 mL sample for 20 min at 16,270 xg (mixed culture) or for 15 min at 27,000 xg (axenic culture); pouring the centrate through a prewashed and tared 47 mm diameter, 0.45 μm pore size membrane filter (Gelman GN Metricel, No. 63069); dislodging and washing the pellet onto the filter; filtering to dryness; drying at 103 °C for 2.5 hr; cooling; and weighing on a closed pan balance (Mettler Type 6). Soluble COD was measured by the closed reflux technique as described in Clesceri et al. (1989) (Method 5220 C). Titration was done to a potentiometric endpoint using a platinum electrode. Dissolved organic carbon (DOC) was determined by the combustion-infrared method using a Beckman Model 915B total organic carbon analyzer (Clesceri et al. 1989, Method 5310B). Inorganic carbon was removed by acidification and sparging with nitrogen gas.

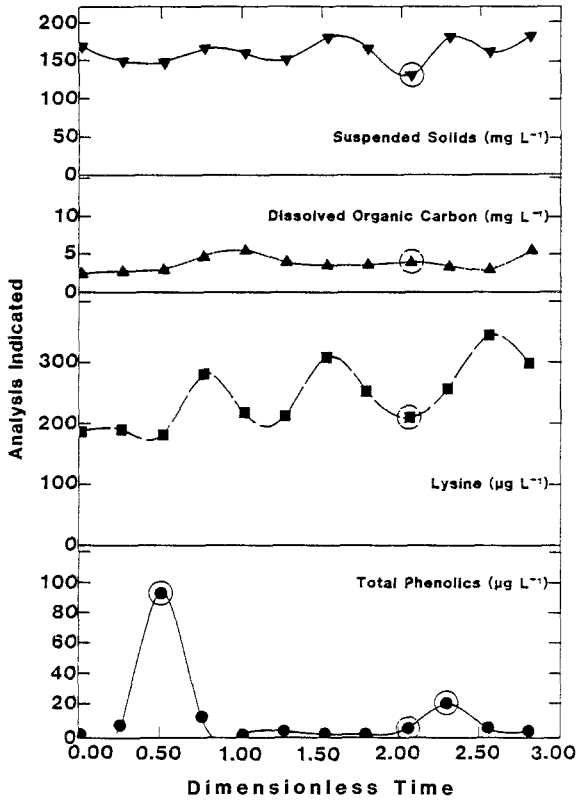

Fig. 1. Typical data set from the mixed microbial community.

Data analysis

Mixed microbial community. Figure 1 shows a typical data set from a mixed culture experiment. Dimensionless time is the actual time from the initiation of sampling divided by the reactor residence time. Although our interest here is only in the 2-CP (total phenolics) and lysine data, the SS and DOC concentrations are shown as general indicators of reactor performance. Examination of the data reveals that while all of the measured variables showed some degree of variability due to the mixed nature of the microbial community, the marker substrates were more variable than the SS and DOC. This is due in part to the fact that their concentrations were on the order of μg/L, whereas the concentrations of SS and DOC were on the order of mg/L.

Certain data points, indicated by circles around them, were deleted from the complete data sets to form adjusted data sets. Statistical analyses were then performed on both the complete and the adjusted data sets and conclusions about the significance of observed effects were only reached after considering the results from both sets of analyses. The rationale for this approach is as follows. Consider the total phenolics data point at a dimensionless time of 0.5. It is evident that it is not representative of overall reactor performance. Thus, its inclusion in the average would skew the estimate of the steady-state performance of the chemostat and perhaps lead to a false conclusion about the effects of media composition or dilution rate. However, its deletion cannot be justified on objective grounds; i.e., no known error occurred during collection or analysis of the sample. Faced with this dilemma, we decided to base all conclusions about the mixed culture experiments on two sets of statistical analyses; one with and one without such outliers. Examination of Fig. 1 reveals that some of the data points are encircled by solid lines whereas others are encircled by dashed lines. Data points surrounded by solid circles were judged to be outliers in their own right. Data points encircled with dashed lines were

deleted because one of the variables indicative of overall reactor stability (SS or DOC) was considered to be an outlier at that time. A deviant SS or DOC value indicates an overall system perturbation which is likely to have influenced the concentrations of the marker substrates as well. The adjusted data sets were formed prior to analysis of the data for significant effects and no alterations to the sets were made after those analyses were begun.

Axenic culture. Although the chemostats were allowed to operate for three residence times before the initiation of sampling, perusal of the data revealed that in some cases steady state had not been attained before sampling began. Consequently, it was necessary to examine the data to establish when steady state had been established. First, the steady-state period was limited to those samples taken before the detection of contamination, if it occurred. Because the antibiotic screening technique allowed contaminants to be detected before they comprised a significant fraction of the population, it is unlikely that they had a significant effect on the marker substrate concentrations in the chemostats before their detection. After eliminating all samples after a contaminant was detected, the mean and standard deviation was calculated for the DOC data because it was considered to be a sensitive indicator of bioreactor steady state. The DOC concentration in the first sample was then examined. If it was within one standard deviation of the mean, steady state was assumed to have been established when sampling began and all samples were used in the data analysis. If the DOC concentration in the first sample was not within one standard deviation of the mean, that sample was discarded and the second sample was examined. The procedure was repeated as needed to establish the start of steady state.

Figure 2 shows a typical data set for the axenic culture. Steady state was established before sampling was begun, but contamination occurred during the last residence time, limiting the steady-state data set to nine samples.

Statistical analyses. To determine whether the two independent variables, dilution rate and media composition, had significant effects on the concentrations of the marker substrates in the chemostats, analyses of variance were run on the data from the steady-state regions using the general linear model (GLM) procedure in SAS (SAS Institute 1982). The TUKEY, SNK and Student's T range tests were used to determine whether the means from individual experiments were significantly different from each other (SAS Institute 1982). When comparisons were made between several experiments to determine whether the addition of the biogenic compounds had a significant effect, only those experiments conducted at a given dilution rate were compared.

Results

Mixed microbial community

The mean concentrations of the marker substrates in the chemostats containing the mixed microbial community are shown in Table 1, along with the standard deviations. Analysis of variance showed that both dilution rate and media composition had effects on the lysine concentration at the 99% level of confidence for both the complete and adjusted data sets. The interaction between the two independent variables also had an effect at the same confidence level for the complete data set, but not for the adjusted one. Only media composition and its interaction with the dilution rate had significant effects (99% level) on the 2-CP concentration for the complete data set, but media composition, dilution rate and their interaction all had significant effects (99% level) for the adjusted data set. Thus, it is apparent that media composition has an effect on the removal of both biogenic and xenobiotic organic compounds by mixed microbial communities growing at constant specific growth rate in continuous culture reactors. Furthermore, the dilution rate at which the chemostat is operated influences that effect.

Analysis of variance only tells us that an effect exists, it does not tell us what it is. To help establish the nature of the effects, Fig. 3 presents the concentrations of the marker substrates in the adjusted data set in graphical form. Only the adjusted data set is

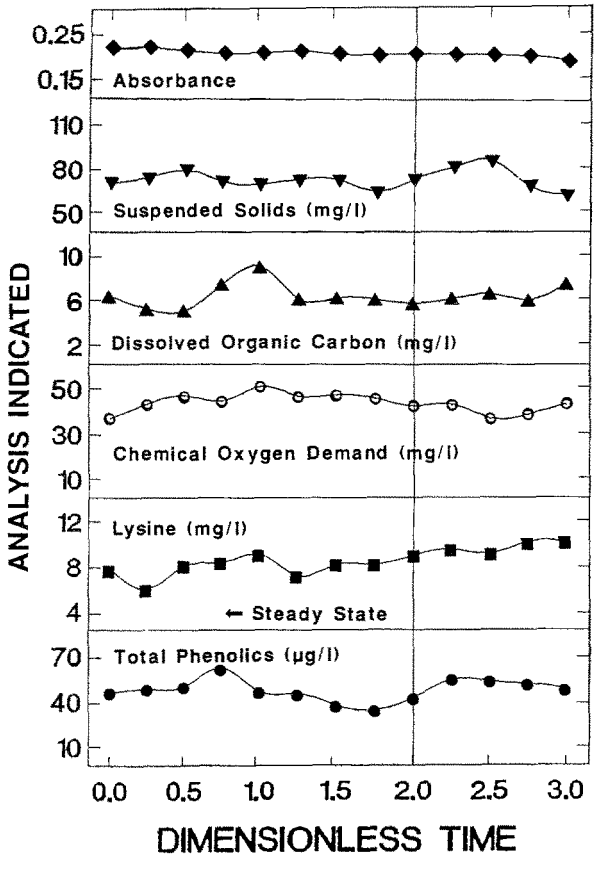

Fig. 2. Typical data set from the axenic culture.

presented because the relative effects were similar in the two, although the magnitudes were different (see Table 1). The cross-hatches on the bars represent significant differences (95% level of confidence) between mean concentrations at a given dilution rate. Bars with cross-hatching that rises to the right represent concentrations in chemostats receiving additional biogenic compounds that were significantly different from concentrations in chemostats without those compounds (i.e., different from the open bars on the far left of each group). Bars with cross-hatching that rises to the left represent concentrations that were significantly different from the concentrations in chemostats receiving media with the next lower concentration of additional biogenic compounds (i.e., different from the bars immediately to the left). Consequently, any bioreactor receiving 34 mg/L as COD of additional biogenic compounds that had a lysine or 2-CP concentration that was significantly different from the chemostat receiving no additional biogenic compounds is represented by a bar that is cross-hatched in both directions.

Consider first the effect of adding the biogenic compounds, without regard to the amount added (i.e., consider cross-hatching that rises to the right). Examination of the figure reveals that 2-CP and lysine behaved in different ways. Of the nine cases studied, the addition of the four biogenic compounds to the media resulted in a significantly lower 2-CP concentration in the bioreactor in five cases and a significantly higher concentration in only one

Table 1. Concentrations of marker substrates in mixed culture chemostats.

Dilution rate h^{-1}	Influent biogenics mg/L as COD	L-Lysine $\bar{x} \pm s$ µg/L	# of samples	2-Chlorophenol $\bar{x} \pm s$ µg/L	# of samples
Complete data set					
0.015	0	114 ± 42	12	<MDL	12
0.015	34	228 ± 91	12	<MDL	12
0.015	225	246 ± 54	12	12 ± 26	12
0.015	1462	300 ± 35	12	40 ± 65	12
0.033	0	140 ± 52	12	18 ± 6	12
0.033	34	439 ± 43	12	5 ± 4	12
0.033	225	250 ± 155	12	6 ± 13	12
0.033	1462	528 ± 61	12	17 ± 21	12
0.050	0	482 ± 105	5[a]	98 ± 101	6[a]
0.050	34	402 ± 8	12	64 ± 67	12
0.050	225	396 ± 24	12	44 ± 67	12
0.050	1462	486 ± 12	12	21 ± 11	12
Adjusted data set					
0.015	0	104 ± 7	7	<MDL	7
0.015	34	195 ± 37	10	<MDL	12
0.015	225	249 ± 55	11	3 ± 4	9
0.015	1462	291 ± 19	11	15 ± 5	10
0.033	0	132 ± 48	11	17 ± 5	11
0.033	34	430 ± 31	11	5 ± 4	12
0.033	225	418 ± 41	4	<MDL	10
0.033	1462	499 ± 28	9	9.5 ± 9	10
0.050	0	472 ± 118	4[a]	36 ± 13	3[a]
0.050	34	404 ± 7	9	30 ± 5	6
0.050	225	396 ± 24	12	16 ± 8	10
0.050	1462	487 ± 13	10	21 ± 12	10

[a] A green pigment was produced during a portion of the experiment and data collected during that time were excluded from analysis.

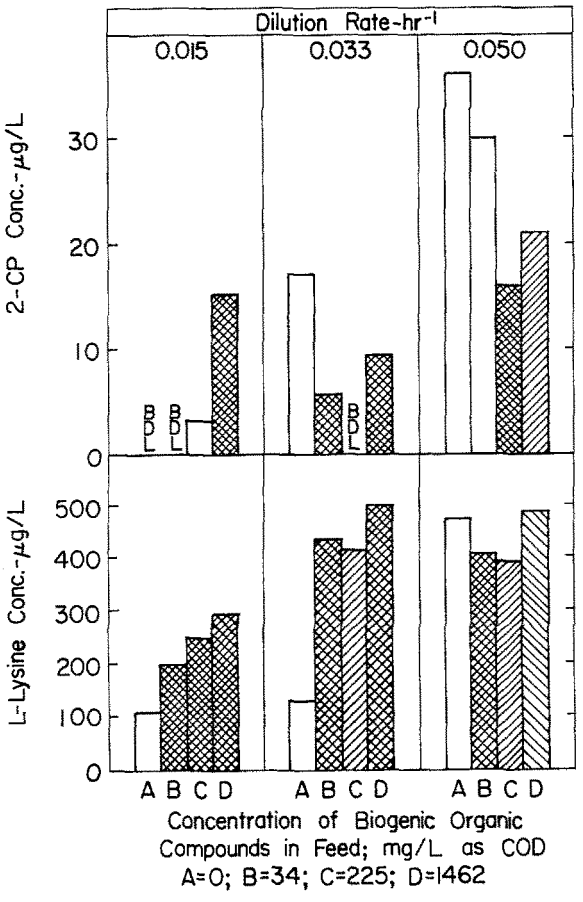

Fig. 3. Effects of dilution rate and media composition on the concentrations of 2-CP and L-lysine in chemostats containing a mixed microbial community. BDL = below method detection limit. Bars with cross-hatching that rises to the right were significantly different from bar A. Bars with cross-hatching that rises to the left were siginificantly different from the bar to the immediate left.

case. No significant effect was observed in three cases, but two of those were at the lowest dilution rate when the 2-CP concentration was already below the method detection limit of 3 µg/L. Therefore, in general, the addition of biogenic organic compounds tended to improve the removal of 2-CP. Furthermore, higher dilution rates tended to favor a reduction in 2-CP concentration when additional biogenic compounds were added to the feed, perhaps because at the lowest dilution rate 2-CP was removed to very low levels even without additional substrates. Conversely, in six of nine cases, addition of biogenic organic matter to the media caused the concentration of lysine in the bioreactors to rise. It also caused it to fall in two cases, but had no significant effect in one. Thus, in general, the addition of biogenic organic matter tended to retard the biodegradation of lysine, causing its concentration in the chemostats to rise. Furthermore, the effect was more pronounced at lower dilution rates.

Next, consider the effect of increasing the amount of biogenic organic matter added (i.e., consider cross-hatching that rises to the left). There were three levels of increase, $0 \rightarrow 34$, $34 \rightarrow 225$, and $225 \rightarrow 1462$, with the latter two each representing a 5.5 fold increase in concentration. Examination of the upper portion of Fig. 3 reveals that the magnitude of the increase had no clearly discernable effect on the effluent 2-CP concentration. Thus, while adding biogenic substrate to the influent generally resulted in better 2-CP removal, the effect of incremental increases in the amount remains unclear. In contrast, the effect on lysine removal was much more systematic. At the two lower dilution rates, the $0 \rightarrow 34$ increase caused the concentration of lysine in the chemostat to increase but further increases in the amount of biogenic matter in the feed had relatively little effect. However, at the highest dilution rate, the addition of biogenic organic matter had relatively little effect on the removal of lysine, regardless of the amount added.

In considering these results, it should be recalled that all experiments were started in such a way that the microbial communities were likely to be very similar, in spite of the fact that they were mixed communities. Nevertheless, differences in the gross morphological characteristics of the cultures were observed as the dilution rate and media biogenic compound concentrations were changed. Thus, population dynamics may well have influenced the results. Some insights into their effects may be obtained by examining the data from the axenic culture.

Axenic culture

The concentrations of the marker substrates in the

axenic culture chemostats are shown in Table 2. No data are available for the highest biogenic compound concentration at the lowest dilution rate. Under every condition studied, the concentrations were higher than they were for the mixed culture, particularly for lysine. This suggests that other bacteria in the mixed community had strong effects on system performance. As with the mixed community, analysis of variance showed that both dilution rate and media composition had significant effects on the concentrations of 2-CP and lysine in the chemostats at the 99% level of confidence, as did their interaction.

Figure 4 presents the data in the same manner as Fig. 3 to allow visualization of the effects and to aid in data interpretation. Examination of the figure reveals that the response of the 2-CP concentration to the addition of biogenic organic matter was different from that observed with the mixed microbial community. In five of the eight cases studied, the 2-CP concentration increased in a statistically significant manner when the additional biogenic compounds were added. There were no significant changes in the other three cases, all of which involved the smallest addition. Thus, in general, the addition of biogenic organic matter to the feed to axenic culture chemostats retarded the removal of 2-CP, whereas it had the opposite effect on chemostats containing mixed microbial communities. In contrast, the response of lysine to the addition of the other biogenic compounds was similar to that of the mixed community, although the effect was much stronger. In all eight cases studied, adding other biogenic compounds to the media caused the lysine concentration to increase drastically, with the increase exceeding an order of magnitude in most cases. These results strongly suggest that other bacteria in the mixed community were influencing the removal of 2-CP and lysine, which is consistent with observations that specialists outcompeted this generalist in controlled mixed cultures (Lu 1987; Lu & Grady 1988).

Next, consider the effect of the amount of biogenic organic matter added. At all dilution rates, the addition of a small amount of biogenic organic matter had no significant effect on the concentration of 2-CP in the chemostats, but further increases in the amount added significantly increased the concentration. This is a much more distinct effect than was evident in the mixed community and suggests that as more of the culture's energy needs are met by biogenic organic compounds, there is a decrease in the activity or quantity of the enzyme system responsible for the degradation of 2-CP. Although it is clear that adding biogenic organic matter had a deleterious effect on lysine removal, the effects of adding greater amounts were less distinct, except at the highest dilution rate, where additional amounts had progressively more negative impacts.

As the amount of biogenic compounds in the feed was increased, the amount of biomass in the reactors increased even though the steady-state specific growth rate was held constant at each dilution rate. As a consequence, the rate of 2-CP or lysine removal per unit mass of bacteria decreased at the same time the concentration of 2-CP or lysine surrounding the bacteria increased. Classical microbial and enzyme kinetics would suggest that a decrease in unit rate should be accompanied by a decrease in the substrate concentration surrounding the bacteria. Thus, it is clear that additional research is needed to improve our understanding of the control of

Table 2. Concentrations of marker substrates in axenic culture chemostats.

Dilution rate h^{-1}	Influent biogenics mg/L as COD	L-Lysine $\bar{x} \pm s$ µg/L	# of samples	2-Chlorophenol $\bar{x} \pm s$ µg/L	# of samples
0.015	0	450 ± 7	12	37 ± 14	12
0.015	34	7630 ± 930	9	43 ± 8	9
0.015	225	2780 ± 40	7	96 ± 26	7
0.015	1462	–[a]	–[a]	–[a]	–[a]
0.033	0	420 ± 10	7	28 ± 5	7
0.033	34	7160 ± 730	9	24 ± 1	9
0.033	225	7600 ± 1730	6	51 ± 8	6
0.033	1462	6410 ± 2930	11	62 ± 9	11
0.050	0	470 ± 33	10	45 ± 4	10
0.050	34	4000 ± 170	12	33 ± 13	12
0.050	225	6520 ± 540	11	60 ± 13	11
0.050	1462	17620± 1500	7	91 ± 28	7

[a]No data.

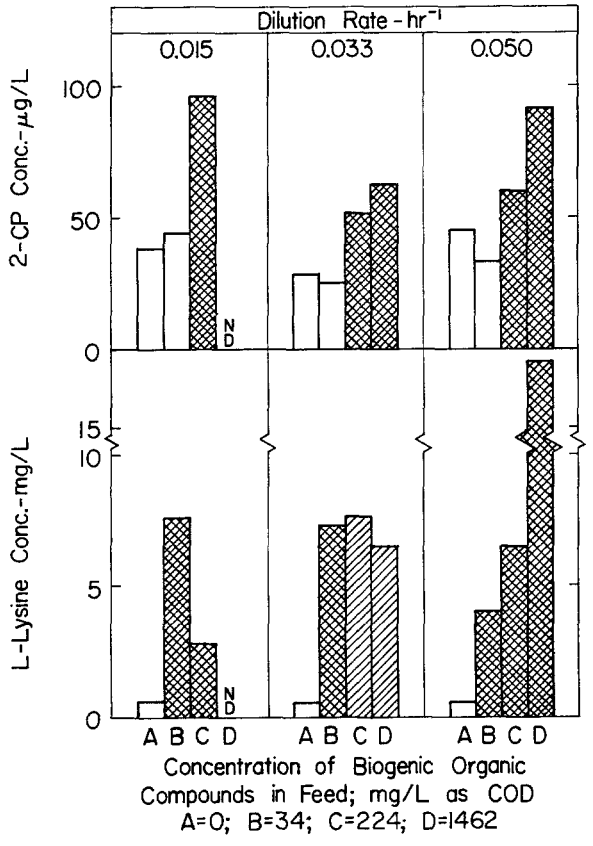

Fig. 4. Effects of dilution rate and media composition on the concentrations of 2-CP and L-lysine in chemostats containing an axenic culture. ND = no data. Bars with cross-hatching that rises to the right were significantly different from bar A. Bars with cross-hatching that rises to the left were siginificantly different from the bar to the immediate left.

degradative pathways in bacteria simultaneously degrading multiple substrates.

Discussion

The first question that comes to mind when viewing the results is how the analytical procedures affected them. Neither of the assays used for the marker substrates was specific for those compounds alone, although both are very limited in their reactivities. Thus, one must question whether they were adequate measurements of the marker substrates or whether their nonspecificity contributed to the apparently complex nature of the observations. While it is possible that other reactive compounds may have been present, the fact that none were present in the media suggests that the reactive material measured in the chemostats was either residual substrate or metabolic intermediates released by the bacteria. Metabolic intermediates tend to be very diverse in nature, and thus the percentage that is likely to be reactive in the two assays is small. If the reactive materials were predominantly metabolic intermediates, rather than residual substrate, we would expect them to be present in constant proportion to the total amount of soluble organic matter in the reactors. Two tests were used to measure that organic matter during this research, DOC during the mixed culture experiments and both DOC and COD during the axenic culture experiments. Table 3 lists the concentrations of the marker substrates in the mixed culture chemostats expressed as a ratio of the DOC present. Examination of the ratios reveals that they were not constant, but rather varied in a fairly systematic manner as the amount of influent biogenic organic matter was changed. For lysine, the ratio generally increased when the lowest quantity of biogenic organic matter was added to the feed, but then decreased as more was added. For 2-CP, the ratio generally decreased as more biogenic organic matter was added. Table 4 shows similar data for the axenic reactor expressed as a ratio of the COD present (the validity of our DOC data for these experiments was questionable, although they showed similar trends). The lysine ratios varied in the same manner as the ratios in the mixed culture. With the exception of the 2-CP values for the intermediate dilution rate, the 2-CP ratios also were not constant, but rather reached a maximum at the intermediate biogenic compound feed concentration. These results strongly suggest that the lysine and 2-CP tests were not simply measuring the effects of soluble microbial intermediates. Thus, we may conclude that the observed responses were reflective of the responses of the marker substrates.

The mixed microbial community achieved lower concentrations of the marker substrates than the axenic culture. Thus, although the organism studied in the axenic culture was part of the mixed community, it is clear that other microorganisms in that community were capable of degrading the marker

substrates to lower levels. Studies of the mixed community revealed that it contained specialists that could degrade only one of the marker substrates (Mackey 1989) and it is likely that those specialists were responsible for the better performance of the mixed community. Indeed, defined mixed culture experiments at similar dilution rates revealed that three-membered cocultures of this generalist and two specialists growing on 2-CP and lysine resulted in the predominance of the specialists (Lu 1978; Lu & Grady 1988). The mixed community also responded in somewhat different ways than the axenic culture to the addition of the biogenic compounds. This, too, is probably due to the presence of the specialists in the community. In the axenic culture, the bacteria that are degrading 2-CP and lysine must also degrade the additional biogenic compounds. In contrast, the mixed community contained bacteria that could degrade neither 2-CP nor lysine and they were free to attack the added biogenic compounds without directly affecting 2-CP or lysine biodegradation by the specialists. These results suggest that it may be difficult to extrapolate the results from axenic cultures to mixed communities from which they were derived.

The experiments with the axenic culture were done to eliminate the impact of population dynamics from the results. However, even without that factor the results were complex, indicating that the control of enzyme synthesis and function in mixed substrate environments is complex. One mechanism known to control enzyme levels is catabolite repression, but it generally occurs in carbon-limited cultures only when the specific growth rate is high and the cells are saturated with ATP (Neidhardt et al. 1990). The bacterium studied had a $\hat{\mu}$ value of 0.34 h^{-1} on 2-CP (Loven 1985) and 0.11 h^{-1} on lysine (Lu 1987), whereas the highest dilution rate studied here was 0.05 h^{-1}, which is sufficiently below the $\hat{\mu}$ values to preclude catabolite repression. It is interesting to note, however, that the observation that the reactor 2-CP concentrations were lower than the reactor lysine concentrations is consistent with the higher observed $\hat{\mu}$ value on 2-CP.

The biomass in the reactors increased in proportion to the total amount of organic matter (COD) added. Available kinetic models for multicomponent substrates suggest that the concentration of either marker substrate should have been driven to lower values as the biomass concentration in the chemostats increased (Machado & Grady 1989), yet that did not happen. Rather, the concentrations of the marker substrates in the axenic culture general-

Table 3. Concentrations of marker substrates in mixed culture chemostats expressed as a function of the concentration of dissolved organic carbon (DOC) present (adjusted data set).

Dilution rate h^{-1}	Influent biogenics mg/L as COD	L-Lysine $\frac{\mu g\ Lysine}{mg\ DOC}$	2-Chlorophenol $\frac{\mu g\ 2\text{-}CP}{mg\ DOC}$
0.015	0	61	<MDL
0.015	34	108	<MDL
0.015	225	66	0.8
0.015	1462	32	1.6
0.033	0	43	5.5
0.033	34	108	1.2
0.033	225	47	<MDL
0.033	1462	25	0.5
0.050	0	98	7.5
0.050	34	75	5.6
0.050	225	46	1.9
0.050	1462	39	1.7

Table 4. Concentrations of marker substrates in axenic culture chemostats expressed as a function of the concentration of Chemical Oxygen Demand (COD) present.

Dilution rate h^{-1}	Influent biogenics mg/L as COD	L-Lysine $\frac{\mu g\ Lysine}{mg\ COD}$	2-Chlorophenol $\frac{\mu g\ 2\text{-}CP}{mg\ COD}$
0.015	0	10	0.8
0.015	34	177	1.0
0.015	225	70	2.4
0.015	1462	–[a]	–[a]
0.033	0	12	0.8
0.033	34	256	0.9
0.033	225	129	0.9
0.033	1462	77	0.8
0.050	0	17	1.6
0.050	34	182	1.5
0.050	225	217	2.0
0.050	1462	107	0.6

[a]No data.

ly increased. Since all of the bacteria in the axenic culture had the capability to use the marker substrates, this suggests that the utilization rate per unit of bacteria decreased even though the substrate concentration in the media surrounding the bacteria (i.e., in the chemostat) increased. Although no enzyme assays were done as part of this research, another study with an enrichment culture revealed that the levels of lysine degrading enzymes per unit of protein decreased during continuous culture when another substrate was added to the media (Grady & Gaudy 1969), suggesting that the same probably happened here. Regardless of the mechanism, it is clear that either the quantity or the activity of the transport or degradative enzymes in the experiments reported herein must have been controlled by something other than the available substrate concentration. It is even conceivable that stochastic processes may be the major determinant of residual substrate concentrations when bacteria are utilizing several substrates simultaneously while growing at low specific growth rates, and they may have contributed to the apparent complexity of the responses observed herein.

Although a number of papers have addressed the question of dual substrate removal (see Machado & Grady 1989), very few studies have been performed to investigate the steady-state performance of either pure or mixed cultures receiving more than two substrates. Thus, it was difficult to find many reports to which to compare these results. Furthermore, the vast majority of reports on mixed substrate removal involve batch growth experiments in which the bacteria are challenged with substrates at higher concentrations than those present at steady state in continuous culture. Because the applicability of such reports to the results reported here is questionable, they have not been included.

The most relevant paper in the literature is that of Law & Button (1977) who studied the metabolism of glucose by a marine coryneform bacterium when additional substrates were added to the feed to a chemostat at levels similar to that of the glucose. When arginine was added, the steady-state glucose level dropped from 210 μg/L to 100 μg/L; when both arginine and glutamic acid were added, it dropped to 50 μg/L; and finally, when a mixture of 21 amino acids was added, the glucose concentration dropped to 10 μg/L. In contrast, in our axenic culture experiments, the addition of the biogenic compounds in increasing amounts either had no effect on the 2-CP concentration or caused it to rise, regardless of the dilution rate employed. The response of the lysine was more complex, being influenced by the dilution rate as well. At the lowest dilution rate, the lysine concentration dropped as more biogenic compounds were added; at the intermediate dilution rate, it was unaffected by the amount of biogenic material added; at the highest dilution rate it increased as more biogenic matter was added. Law & Button (1977) suggested that small numbers of substrates might cause abnormal behavior in some bacteria because they limit the crossover capacity of metabolic routes. This, in concert with our results, suggests that it might be best to investigate the metabolism of organic pollutants in multisubstrate environments if an accurate determination of their fate is to be made.

Several researchers have attempted to model multicomponent substrate utilization, generally in a two substrate environment (Nikolajsen et al. 1991; Papanastasiou & Maier 1983; Pavlou 1987; Turner et al. 1988; Yoon et al. 1977). However, none have addressed the application of their models to the situation investigated here. Furthermore, the nature of the marker substrate responses, particularly in the mixed microbial community, raises the question of whether any of the models is able to mimic such a response. In fact, modeling may only be capable of showing general trends, and may never be able to depict the subtleties observed herein, especially since the effluent substrate concentrations may have been influenced by stochastic processes, which most models don't consider. Consequently, it appears that much more research is needed before environmental scientists and engineers will be able to predict with certainty the fate of individual pollutants in complex mixtures.

Summary and conclusions

Despite the complex response of the bioreactors, conclusions can be reached on each of the four questions posed in the 'Introduction'.

- When the two marker substrates were the only organic compounds supplied to the bioreactors their effluent concentrations were different than when additional biogenic substrates were supplied. Furthermore, the quantity of the additional biogenic substrates supplied had less impact than their presence or absence. This suggests that studies on the biodegradation kinetics of individual organic compounds should be done in complex media if they are to have relevance to the fate of those compounds in natural or engineered systems.
- The two marker substrates exhibited qualitatively different responses to the presence of alternative substrates when they were being degraded by the mixed culture but not when they were being degraded by the axenic culture. Although one marker substrate was xenobiotic and the other was biogenic, it would be premature to conclude that the differences in their responses were related to their origin. However, in order to be able to generalize, the question is worthy of additional study.
- Although analyses of variance indicated that the steady-state specific growth rate of the cultures had an effect on the responses of the two marker substrates to the addition of other biogenic substrates, examination of Figures 3 and 4 revealed that the effect was much less than the effect associated with the presence or absence of the additional substrates.
- The response of the axenic bacterial culture to changes in media composition was not representative of the response of the mixed microbial community, even though it was derived from it. Consequently, biodegradation studies conducted with pure bacterial cultures may have little utility for predicting the concentrations of organic compounds in the effluents from bioreactors containing mixed microbial communities.

Taken together, the results of this research have demonstrated that the ability of bacterial communities to degrade organic compounds to low levels is influenced by the nature of the feeds they are receiving. Consequently, until more is learned about substrate and microbial interactions, studies to predict the output concentrations from wastewater treatment systems must be done with bioreactor feeds containing mixtures of organic compounds indicative of those in the wastewater to be treated.

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