Reactor optimization of volatilized p-xylene metabolism
by Barbara Christine Vaughn

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Environmental Engineering
Montana State University

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The goal of this research was to evaluate the performance of vapor phase bioreactors in response to various process parameters, gas and liquid flow rates and xylene inlet concentration. The columns were packed with two different elements, diatomaceous earth (D.E.) pellets (porous) and glass spheres (non-porous).

Results indicated that when normalized to exterior packing surface area, the D.E. pellet reactor exhibited significantly higher degradation rates than the glass sphere reactor at comparable loading rates. Degradation rates increased with increased mass loading except at the highest gas flow rate. Maximum elimination capacity for the D.E. pellet reactor was 66 mg xylene/m^2 packing per hour with less than 2 millimeters pressure drop. Protein levels were significantly higher in the D.E. pellet reactor while total organic carbon levels were significantly higher in the glass sphere reactor. Both systems maintained maximum degradative capacity after five months of operation under changing organic loads and loss of pure culture.

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Date April 20, 1983

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ABSTRACT

VOC emissions are subject to increasingly strict regulations dictating a need for innovative, cost-effective control technologies. The degradation of volatile, higher molecular weight organic compounds such as those in the BTEX group are of special interest due to their association with hydrocarbon fuel spills. Certain bacteria are capable of metabolizing these contaminants as their sole carbon and energy source and the one used in this study, *Pseudomonas putida* Idaho, has been shown to be resistant to high concentrations of *p*-xylene.

Packed tower bioreactors offer several advantages over suspended cell systems for contaminant removal including continuous degradation, high volumetric reaction rates, reduced VOC stripping losses, and simplified downstream processing. Because they can operate as a semi-closed system, bacterial strains can be introduced that target degradation of specific pollutants without competition from indigenous soil or water microorganisms.

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Results indicated that when normalized to exterior packing surface area, the D.E. pellet reactor exhibited significantly higher degradation rates than the glass sphere reactor at comparable loading rates. Degradation rates increased with increased mass loading except at the highest gas flow rate. Maximum elimination capacity for the D.E. pellet reactor was 66 mg xylene/m^2 packing per hour with less than 2 millimeters pressure drop. Protein levels were significantly higher in the D.E. pellet reactor while total organic carbon levels were significantly higher in the glass sphere reactor. Both systems maintained maximum degradative capacity after five months of operation under changing organic loads and loss of pure culture.

INTRODUCTION

The Superfund Amendments and Reauthorization Act (SARA) emission summary for the petroleum and chemical industries reveals that the largest environmental releases of chemicals are volatile organic compounds (VOC's) into air. VOC's are emitted primarily from industrial operations involving organic solvents or hydrocarbon fuels. Because they can lead to a variety of health and environmental problems, VOC emissions are subject to increasingly strict regulations dictating a need for innovative, cost-effective control technologies.

Removal of volatile organic compounds from air streams is generally approached in two ways; sorption or reaction. Sorption is accomplished either by absorbing compounds into a solvent liquid (gas absorption) or adsorbing them onto the surface of a solid material, usually granulated activated carbon. Reaction processes include combustion, photochemical oxidation, and microbial degradation (Schroeder, 1990). Although the abiotic processes can achieve high elimination efficiencies, the disadvantages can be characterized by a relatively high cost (Know and Canter, 1988; Chang et al., 1989; Guensler, 1989; Kosky and Neff, 1988), a phase transfer of the pollution problem, and the formation of toxic products (Diks,1992).

Microbial degradation is an effective alternative particularly when the pollutant concentration is low and the volumetric flow rate is high which is often the case in odorous or toxic waste gas emissions (Leson and Winer,

1991). Biological processes operate at low pressures and temperatures and require low maintenance making them cost-effective. The pollutants are not just shifted into another phase but are ultimately eliminated by metabolizing the contaminants to carbon dioxide, water, and cellular constituents.

Although biological treatment of easily metabolized organic compounds has been used for decades to treat municipal and industrial waste, its use for recalcitrant compounds has been developed only in the past ten years. A number of microorganisms, primarily bacteria, have been isolated that show a high activity and stability towards several xenobiotic compounds. The biodegradation of volatile, higher molecular weight organic compounds such as those in the BTEX group (benzene, toluene, ethylbenzene and xylene) are of particular interest due to their association with hydrocarbon fuel spills (Apel et al.,1991). These aromatic compounds are readily biotransformed in aerobic environments (Leahy and Colwell, 1990). Certain Gram negative heterotrophic bacteria are capable of metabolizing these contaminants as their sole carbon and energy source and one in particular, *Pseudomonas putida* Idaho, has been shown to be resistant to high concentrations of xylene (Rogers et al., 1992).

When substrate biodegradation is desired, the formation of biofilms can be an advantage over traditional batch fermentation because it applies continuously operated processes (Characklis, 1990). In suspended cultures the cell retention time and the hydraulic retention time are coupled. The

immobilization of cells in a packed tower allows the accumulation of high biomass concentrations and thus high volumetric reaction rates, while the hydraulic residence time can be reduced below the washout condition for the suspended system (Diks, 1992). Because there is less liquid effluent in packed towers, downstream processing is generally simplified. Conventional liquid phase bioreactors use mechanical aeration which can result in significant VOC stripping losses to the air stream rather than biodegradation. Gas-in-water solubility limitations in chemostats often produce unacceptably low conversions (Rogers et al.,1992). Fixed film, vapor phase bioreactors provide a solution to these problems.

Industrial manufacturing practices produce dilute gaseous effluents that cannot be recovered or disposed of economically particularly in the case of mixed wastes such as spent scintillation cocktail. On-line processing employing vapor phase bioreactors would lower treatment costs, reduce user liability, and allow continuance of existing manufacturing processes (Rogers, et al., 1992).

Contaminated air streams are produced during soil venting of unsaturated subsurface material and air stripping of ground water pumped to the surface. Degradation of VOC's by vapor phase bioreactors at the wellhead provides a promising alternative for remediation on site. Diverse geochemistries of groundwaters present problems to biological-based systems for treating water; treating air would circumvent this complication (Canter et al., 1989). Pumping air instead of water also lowers processing costs. Another potential advantage

is the dilution of high concentrations of toxic organic compounds in water by the 10 to 1 or 20 to 1 air-to-water ratios of packed towers (Canter et al., 1989). Recent findings indicate many hydrocarbon-degrading bacteria in the environment are genetically unstable or physiologically impaired which can interfere with remediation effectiveness (Ridgway and Phipps, 1991; Ridgway, 1990). Because vapor phase bioreactors can operate as a semi-closed system, it may be possible to introduce recombinant or genetically improved bacterial strains into such a system to target degradation of specific hydrocarbon pollutants without competition from indigenous groundwater or soil microorganisms.

Before vapor phase bioreactors can be developed, fundamental studies are needed to optimize reactor design and operation. Practical applications of the process are limited, primarily due to a lack of empirical data. An enormous amount of literature has been published on physical aspects of packed bed reactors and on intrinsic microbial degradation kinetics but few studies are available that evaluate the mass transfer and reaction processes occurring in a single bioreactor system.

Goal and Objectives

The goal of this project is to evaluate the performance of a vapor phase bioreactor in response to various process parameters in order to produce a maximum feed rate to reactor volume ratio and a minimum contaminant breakthrough.

Specific objectives relevant to this goal follow: 1) Identify nutrient requirements and/or limitations of a xylene-degrading organism, *Pseudomonas putida* Idaho. 2) Compare mass transfer rates of xylene for two packings (one porous and one non-porous) under abiotic conditions. 3) Determine the response of inoculated vapor phase bioreactors to variations in gas flow rate, liquid flow rate, and influent vapor phase xylene concentrations. 4) Compare the relative importance of mass transfer and biodegradation kinetics in a vapor phase bioreactor.

BACKGROUND

Biofilters

The use of biological systems for elimination of volatile compounds can be found in literature as early as 1923 when Bach discussed control of H_2S emissions from sewage treatment plants. The systems originally built in the US were mostly "soil beds" where mineral soils were used as filter materials (Leson and Winer, 1991). Pomeroy (1963) received a US patent in 1957 for a soil bed concept and described a successful biofilter installation in California for the treatment of sewage-related odor including organic sulfides. The first systematic research on the biofiltration of H_2S was conducted by Carlson and Leiser in the early 1960's. They demonstrated that biodegradation rather than sorption accounted for odor removal in several soil filters (Carlson and Leiser, 1966). In 1988, a manufacturing facility of SC Johnson & Son Inc. was able to satisfy regulatory requirements for the reduction of VOC emissions from an aerosol can filling operation by using a prototype biofilter. The soil bioreactor reduced concentrations of propane, isobutane, and n-butane in a waste air stream by at least 90% (Kampbell et al.,1987).

Large-scale use of biofilters in the US has been sporadic due to low biodegradative capacity of the soils, correspondingly large space requirements of the beds, lack of regulatory programs, and little governmental support for research and development. In contrast, increasingly stringent regulatory

requirements and funding from the federal government in The Netherlands and West Germany have established biofiltration as a well used air pollution control (APC) technology. It is considered the best available control technology (BACT) in a variety of VOC and odor control operations such as chemical plants, foundries, print shops, and coating operations. These sources typically emit large volumes of waste gases that contain low concentrations (less than 1000 ppm as methane) of the target pollutant (Leson and Winer, 1991). Ottengraf and Van den Oever (1983), Hartenstein (1987), Kosky and Neff (1988), and Bohn (1989) have all reported excellent success in the removal of VOC's from various sources employing packed bed microbial processes.

A packed bed biofilter consists of one or more beds of a biologically active material, primarily mixtures based on compost, peat, or soil. Contaminated waste gas is vented through the filter which, given sufficient residence time, diffuses into biofilm immobilized on the filter particles. Aerobic degradation of the target pollutants will occur if the microorganisms are present that can metabolize them (Figure 1).

In order for a biofilter to operate efficiently the filter material must provide optimum environmental conditions for the microbial population, large reactive surfaces, and low pressure drops. Problems arise with the development of microbial growth which can result in plugging, high head losses, and compaction, requiring replacement of the filter material. The biodegradation of air pollutants often generates acidic by-products and end-

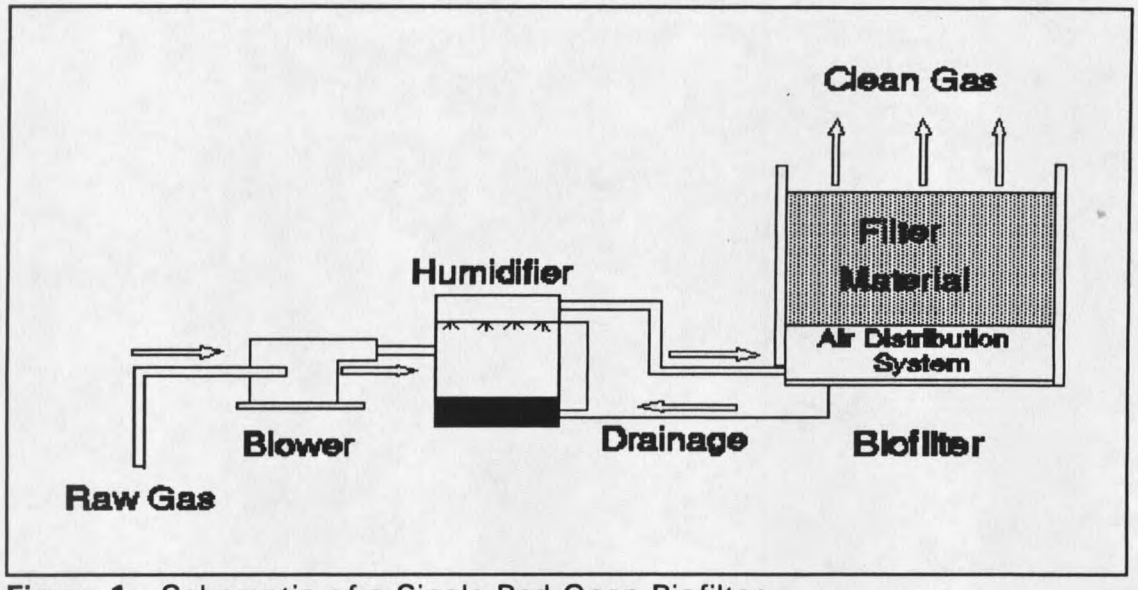

Figure 1. Schematic of a Single Bed Open Biofilter

products. Most bacteria and actinomycetes proliferate in a pH range between 7 and 8 (Atlas, 1981) so a resultant drop in pH can severely inhibit the resident population and reduce or eliminate the filter's degradative capacity. Because they lack a continuous liquid flow, biofilters cannot remove metabolic waste products or replace depleted nutrients. Removal of toxic compounds by adsorption to the filter material transfers rather than alleviates the environmental problem.

The approach to designing biofilter systems is usually empirical. Mobile pilot units are often used for treatability studies and the sizing of full scale systems. Predictive modelling of these systems is complicated for a variety of reasons. Because the surface area of the soil matrix cannot be determined, substrate depletion as a result of mass transfer from the gas to liquid phase, mass flux from the liquid to biofilm surface, and reaction within the biofilm

cannot be quantified. Soil may contain as many as 10^7-10^9 cells/g soil of naturally occurring microorganisms making enumeration of existing species and mass on a spatial and temporal basis impossible. This precludes development of genuine kinetic models of the biofilm. The metabolic activity of the biofilm may change in response to buildup of acid metabolites and reduction of nutrients resulting in an inherently "unsteady state" system. In a biofilter, it is difficult to differentiate between, and subsequently model, contaminant removal due to adsorption or to biodegradation.

Biological Trickling Filters

Biological trickling filters address some of the problems encountered in biofilter operation and design. They consist of columns with manufactured packing elements onto which a suitable microbial culture is immobilized. The specific surface area of the packing is high for maximum biofilm development with a large void fraction between elements for gas flow which minimizes the pressure drop. This also prevents obstruction of the bed due to biofilm growth and sloughing. The waste gas is forced through the packed bed, in co- or countercurrent flow with a continuously circulated water phase. Inert packing materials maximize bioavailability, minimize compaction, and prevent accumulation of pollutants in the filter material. A flowing liquid phase allows for continuous control of the physiological conditions, such as nutrient requirements, pH, temperature, and concentration of inhibiting compounds.

The increase in the specific wetted area optimizes gas-liquid mass transfer. An initially aseptic bioreactor system can be inoculated with selected organisms without competition from existing soil microbes.

Quantification of an overall net substrate flux from transport and reaction phenomena occuring at the microscale is facilitated by using packing materials of known geometry and surface area, aiding modelling efforts. Mass transfer characteristics can be measured independently by running the system abiotically. Intrinsic kinetic parameters (i.e. maximum growth and Monod constant) can be evaluated for a pure culture or known consortia. When continuous liquid flow and substrate availability are insured, steady state performance can be assumed implying the presence of a biofilm with constant activity, thickness, and contact area, independent of time.

One of the earliest uses of immobilized biofilms to degrade undesirable compounds was in a biological trickling filter. The technique was known in wastewater treatment by 1898 (Diks, 1992). The method was applied commercially in a plant constructed in Palm Springs, California, in 1960. At Avila Beach, California, a primary plant has operated a small trickling filter since 1968 solely for the deodorization of waste gas. Brown and Caldwell have designed "odor reduction towers" at six locations in California. Hydrogen sulfide removal efficiencies of 98 to 99% are attained (Pomeroy, 1982).

Unlike biofilters, commercial use of biological trickling filters for VOC elimination is limited outside the wastewater industry. The recent increase in

the availability and the stability of isolates capable of degrading xenobiotic compounds and the disadvantages of biofiltration systems have refocused attention on this process. The majority of studies employing microbial trickling filters and specifically vapor phase bioreactors for VOC removal have been conducted in the last five years. Most fundamental research has been done at the laboratory scale with little data available at the pilot or industrial scale.

Vapor Phase Bioreactors

To demonstrate the advantage of immobilized over suspended cell systems, Hill et al. (1991) compared the performance of batch, CSTR, and packed bed reactors for their ability to biodegrade liquid phase phenol with *Pseudomonas putida*. The processing rates for the packed bed bioreactor were an order of magnitude above those achieved by either batch or continuous, well-mixed bioreactors. Air stripping in the batch reactors resulted in losses of phenol of up to 25% while no loss of phenol was detected in the air stream of the packed bed reactor. Mass transfer and fluid mechanic parameters were measured with and without biomass and compared to traditional chemical engineering correlations. Comparisons differed by up to an order of magnitude. Hill concluded that actual column performance should be measured to prevent the use of incorrect functions in a model resulting in erroneous design calculations.

Kirchner et al. (1989) used a combination of bacterial monocultures fixed

on various carriers for degradation of a mixture of pollutants. By using different compounds (eg. acetone, propionaldehyde, napththalene and toluene, crude gas concentrations:5-35 ppm) the effect of the water solubility of the gaseous substances on removal efficiency was studied. They showed that the elimination capacity of the systems treating low solubility compounds was still high but less so than that of the more hydrophilic compounds. When the fixed cells were present in high densities, the reaction rate was independent of the cell count. The conversion of the pollutant did not depend to a significant extent on the carrier as long as it supported a high cell density. These and some other results indicated to Kirchner that the catalysed biological oxidation was limited by mass transfer, i.e., the biological reaction takes place comparatively rapidly and the mass transfer of the reactants through the liquid film is rate-determining (mass transfer regime) (Kirchner et al. 1987).

A later study by Kirchner, et al. (1991) focused on one of the systems described above; *Pseudomonas fluorescens* as the bacterial strain and propionaldehyde as the pollutant in a model bioreactor. Propionaldehyde is a relatively water-soluble substance. The carrier materials used were open-pore sintered glass, smooth glass, and plastic. The absorption and degradation of propionaldehyde was measured by systematic variation of the gas and liquid flow rates. The reactor was run in co- and countercurrent mode. Co-current mode has a potential disadvantage of limited mass transfer at the column outlet due to the concentration difference tending to zero. Results showed, however,

that the driving concentration gradient was practically independent of the mode of operation because the pollutant concentration in the liquid throughout the reactor remained very low. Tubes of sintered glass were compared with Raschig rings of a similar specific surface area. Although the tubes had advantages with regard to pressure loss and operational stability, the conversions were on average 20% lower, owing to poorer mass transfer (less turbulence in the oriented packing). At high pollutant loads, increasing inhibition (termed "retardation") of the biological degradation reaction was indicated. The cell density with the open-pore sintered glass was at least an order of magnitude higher than that for the plastic and smooth glass packings which resulted in improved degradation efficiencies. Intermittent operation of the bioreactor caused no significant decrease in conversion.

A microbial consortium that utilized methanol, butanol, acetonitrile, hexane, benzene, and toluene was selected by Oh and Bartha (1991) and fixed on a highly porous peat-perlite matrix packed into glass columns. Uninoculated columns served as a control for abiotic vapor removal by absorption alone. The experimental setup allowed the independent variation and measurement of solvent vapor concentrations and air flow rates. At very high solvent concentrations performance declined, presumably due to solvent toxicity. Performance also declined at very high flow rates, presumably because of insufficient retention time. Performance was steady around the maximum removal rate. In general, hydrophobic solvents were more difficult to remove

than water-miscible solvents.

Oh, et al. (1991) did a follow up study on methanol vapor removal using a similar experimental design. Steady state conditions for most experiments were attained after 7-10 days of operation. The data indicated that the removal rate increased with the inlet concentration with a tendency to reach a maximum value at an intermediate inlet methanol concentration (6-7 g/m^3). The data also indicated that at constant inlet concentrations removal rates remained essentially unchanged as the superficial air velocity increased except for a drop at the highest flow rate possibly due to a toxicity effect. This was indicative of reaction rather than mass transfer limitation for the process. Oh's (1991) study used the value of the kinetic constants derived from batch experiments in a model for the biofilter. The data from the suspensed system showed a drop in specific growth rate at high concentrations of methanol indicating inhibition kinetics. Oh assumed that the biomass in the biofilter remained essentially constant over considerable time periods which he justified by the low yield coefficient value (0.28) and the low methanol concentrations used.

One of the most extensive studies on vapor phase bioreactors was conducted in the Netherlands by Diks (1992). He achieved stable dichloromethane (DCM) elimination over two years of operation with feed concentrations ranging from 0-10000 ppm using the strain Hyphomicrobium sp GJ21. Even though DCM is poorly water-soluble, Diks showed that high

degrees of conversion could be reached. The maximum elimination capacity remained constant with a constant average organic load which Diks explained by the existence of a balance between biomass accumulation by growth and biomass removal by decay and sloughing, resulting in a constant holdup of active biomass. Biofilm was unaffected by short-term fluctuations in the organic load, which was important in view of the dynamic behavior of the filter system at briefly changing process conditions. He concluded that the assumption of steady-state was valid in experiments which required a changing organic load, if the total period of time consumed only amounted to several days. On a longer term, steady-state existed if a constant average organic load was maintained.

Experimental and theoretical results found that the gas-liquid mass-transfer resistance in the trickling filter bed was negligible and that the gas and liquid phases were close to equilibrium. The biological process inside the biofilm was the rate limiting step. The specific activity of the biomass was determined to be $R_s = 0.08$ g DCM/g TSS/h. This value was one eighth the specific activity of the suspended pure culture. This indicated that only about 12% of the biomass present in the bioreactor actually degraded dichloromethane which led Diks to question the reliability of applying intrinsic kinetic growth parameters calculated from suspended systems to fixed-cell system.

Diks evaluated the effects of both oxygen limitation and temperature

fluctuations on system performance. By comparing the substrate availability and the rate of reaction for oxygen and dichloromethane theoretically, Diks found that no oxygen limitation occurred throughout the substrate concentration range indicated. To investigate the influence of temperature, experiments were performed at 20°C. and 30°C. It is well known that temperature can affect a biological reaction rate by increasing the growth rate of the organism and the diffusion rate of the substrate. At the same time Henry's constant increases which reduces the concentration, and therefore availability, of the substrate in the liquid phase. Diks found that the elimination capacity of the system in the diffusion limited range was not affected by a temperature rise. He concluded that the increase of the reaction rate in the biofilm was offset by the decrease in the mass transfer rate from the gas to the liquid phase. The trickling filter system was operated co-currently and counter-currently. Diks expected co-current flow to give better results because of the lack of stripping effects but only small differences existed throughout the parameter ranges indicated. The phenomenon was explained by the smoothing effect the recirculation of the liquid phase had on the axial concentration profile. Although the overall filter efficiency depended on the local liquid concentration it was unaffected by the relative flow direction of the mobile phases.

Gas velocity was measured because of its effect on the system's mean residence time. The elimination capacity increased at higher superficial gas

velocities for inlet concentrations smaller than 11 g/m^3 (diffusion limited range) because the average gas phase concentration in the system increased. At higher inlet concentrations, the biofilm was fully penetrated with substrate and filter performance was limited only by reaction rate with no effect from the increased average gas phase concentration. Diks concluded that the degree of conversion was determined by the superficial gas contact time rather than superficial gas velocity or reactor height.

Canter et al.(1989) studied cometabolic removal of TCE, TCA, and butane with two glass bioreactors packed with either celite biocatalyst carrier R635 (.27 m^2/g) or R630 (1.3 m^2/g) by Mannville. Up to 94% degradation of TCE was achieved with the R635 reactor. Removal efficiency of all three compounds was greater in the R635 reactor than in the R630 reactor. Four pairs of butane sampling ports were located along each column. Butane removal was evident at each port indicating that the celite biocatalyst carriers allowed microbial growth throughout the entire length of the bioreactor.

The Idaho National Engineering Laboratory conducted a limited study assessing vapor phase bioreactors for their potential in bioprocessing methane, TCE, and *p*-xylene. Rates of methane removal were 2.1 and 1.6 fold greater than those exhibited by batch and chemostat reactors respectively. Cometabolism of TCE using methanotrophs removed 9 micrograms TCE/day per bioreactor. At a feed rate of 139.9 micrograms xylene carbon/min, 46% of the xylene was mineralized to carbon dioxide in a bench scale gas phase reactor

inoculated with *Pseudomonas putida* Idaho (Apel,1991).

Aromatic Hydrocarbons

The above vapor phase reactor study and this project evolved from work done at INEL involving isolation of microorganisms with the ability to use methylated aromatics, such as xylene and toluene, as their sole carbon source. The ability to utilize naturally occurring hydrocarbons is widely distributed among diverse microbial populations. When natural populations are contaminated with petroleum hydrocarbons, the indigenous microbial communities are likely to contain populations of differing taxonomic relationships which are capable of degrading the contaminating hydrocarbon (Atlas, 1981). Adaptation can occur by three interrelated mechanisms, namely; induction and or depression of specific enzymes, genetic changes which result in new metabolic capabilities, and selective enrichment of organisms able to transform the compound or compounds of interest. Selective enrichment has been widely observed in studies of hydrocarbon and petroleum degradation in the environment (Leahy and Colwell, 1990). Several reports by Colwell and Walker (1977), Atlas (1981), Floodgate (1984), and Cooney (1984) have shown that the numbers of hydrocarbon-degrading microorganisms and their proportion in the heterotrophic community increase upon exposure to petroleum or other hydrocarbon pollutants and that the levels of hydrocarbon-utilizing microorganisms generally reflect the degree of contamination of the ecosystem.

Petroleum is a complex mixture of hydrocarbons. Several classes, based on related structures, are recognized from the hundreds of individual components. The petroleum mixture can be fractioned by silica gel chromatography into a saturate or aliphatic fraction, an aromatic fraction, and an asphaltic or polar fraction (Atlas, 1981). Aromatic hydrocarbons constitute a major fraction of gasoline. These compounds are more water soluble and less volatile than many aliphatic constituents which favors the prevalence of aromatic hydrocarbons in groundwater contaminated by gasoline (Ridgway, 1990). Ridgway et al. (1990) isolated 297 gasoline-degrading bacteria from well water and core material from a shallow coastal aquifer contaminated with unleaded gasoline. Their responses on 15 gasoline hydrocarbons were evaluated on the basis of aerobic growth. Toluene, *p*-xylene, ethylbenzene, and 1,2,4-trimethylbenzene were most frequently utilized as growth substrates, whereas cyclic and branched alkanes were least utilized.

INEL Studies

Pseudomonas putida Idaho Isolation

Identification of 244 of the isolates in Ridgway's study revealed four genera: *Pseudomonas, Alcaligenes, Nocardia,* and *Micrococcus,* with pseudomonads making up 86.9% of bacteria identified. Jensen studied the bacterial flora of soil after application of oily waste and found that the predominant species of oil degraders belonged to the genera *Arthrobacter* and

Pseudomonas (Atlas, 1981).

INEL collected and screened several petroleum-contaminated soil and water samples for organisms that could degrade toluene, xylenes, and pseudocumene. After several transfers were done on a basal agar medium and incubated in a dessicator with an open beaker of *p*-xylene or toluene, the pure colonies that grew on these plates were introduced into a liquid broth with 1-10 ppm of the aromatic compound. Growth was determined by turbidity. A chemostat was set up and inoculated to control the essential parameters of aeration and pH. Initially toluene or *p*-xylene was introduced via vaporization. Cells grew to a density of 10^8-10^9 cells/ml and survived under continuous feed conditions for three years. One isolate was not only tolerant to high concentrations of toluene and *p*-xylene but utilized these compounds as its sole carbon source and grew under a layer of liquid *p*-xylene on basal salts agar media. The isolate was determined to be a strain of *Pseudomonas putida* (Rogers et al.,1990).

Cruden et al. (1992) analyzed some physiological properties of the INEL isolate named *Pseudomonas putida* Idaho. Aromatic compounds that can serve as growth substrates for *P. putida* Idaho include toluene, *m*-xylene, *p*-xylene, 1,2,4-trimethylbenzene, 3-ethyltoluene, benzylalcohol, benzoic acid, *m*-toluic acid, *p*-toluic acid, *p*-hydroxybenzyl alcohol, *m*-cresol, and *p*-cresol. Growth was also observed on solid media when the surface of the agar was overlaid with 5.0 ml of toluene, *m*-xylene, *p*-xylene, 1,2,4-trimethylbenzene, or 3-

Table 1. Growth of selected pseudomonas strains in the presence of organic solvents[a] (Cruden, 1992).

Solvent	Log P_{oct}[c]	Growth[b] *P.putida* Idaho	*P.mendocina* KR1	*P.putida* mt-2	*P.putida* F1
Decane	5.6	+ + +	+ + +	+ + +	+ + +
Hexane	3.5	+ + +	+ + +	+ + +	+ + +
Cyclohexane	3.2	+ + +	+ + +	+ + +	+ + +
Pentane	3.0	+ + +	+ + +	+ + +	+ + +
p-Xylene	3.1	+ + +	-	-	+ + +
Cyclopentane	2.5	+ + +	-	-	+ + +
Toluene	2.5	+ + +	-	-	-
1-Heptanol	2.4	+ + +	-	-	-
Benzene	2.0	-	-	-	-

[a]Cells were grown in LB medium in the presence of 25%(vol/vol) solvent.
[b]Symbols indicating turbidity values at 600 nm: + + +, >1 after 24 hr; + + 0.7 to 1.0 after 48 hr; -, <0.2 after 48 hrs.
[c]Logarithm of the octanol-water partition coefficient.

ethyltoluene. Three other species of *Pseudomonas* which can metabolize toluene were examined for their tolerance of selected organic solvents(Table 1). None of the strains tested was able to tolerate organic solvents to the same extent as *P. putida* Idaho. Results suggested that this isolate utilizes the same metabolic pathway as *P. putida* mt-2 which contains the TOL plasmid pWWO for the degradation of alkyl-substituted aromatic hydrocarbons. Attempts to detect the presence of a catabolic plasmid in *P. putida* Idaho were not successful. Southern hybridization experiments indicate significant homology

between fragments of *P. putida* Idaho DNA and a fragment of *P. putida* mt-2 DNA. The ability of this isolate to degrade toluene and *p*-xylene is stable through numerous transfers on nonselective media, as is the resistance to solvents. It is possible that the chromosomal location of the genes may make its ability to degrade aromatic hydrocarbons more stable.

Chemostat Studies

Commercial use of *Pseudomonas putida* Idaho required development of a bioprocess system capable of continuously degrading aromatic compounds. INEL modified their chemostat to allow mass balances to be taken. They concentrated on observing the response of the system to very high xylene loading rates and to changes in the dilution rate within the system. The studies were useful in determining the operating parameters for a system under extreme loadings but they could not be used for estimation of the fundamental kinetic parameters of the organism due to the extreme heterogeneity of the reactor. Experiments are in progress at Montana State University to determine the kinetic parameters for the organism's growth at significantly lower loading rates. The resulting data will be analyzed to evaluate μ_m, K_s, and the stoichiometry of xylene utilization (growth yield) for *Pseudomonas putida* Idaho.

VPBR Design Criteria

Because aromatics are highly volatile, traditional aeration procedures used in INEL's chemostat studies produced a significant xylene-saturated gas

stream leaving the system. This project was initiated to concentrate on biodegradation of this vapor phase xylene. Two gas phase reactors were designed and constructed to be operated concurrently. Inert, manufactured packings were used because of the modelling advantages. One column was packed with glass spheres and the other with diatomaceous earth pellets of a comparable external surface area with both resembling natural surfaces (in contrast to plastics) that appear to be readily colonized (Diks, 1991). A potential benefit of the porous pellets is a degree of protection for interior colonized organisms from both surface shear conditions, microbial competition, and shock loadings. The nominal pore size of 20 micrometers provides a high surface area but does not seriously impede movement and colonization of all pellet interior surfaces (Sturman, 1991). Gas and liquid loading rates are important process parameters due to their effect on mass transfer and interfacial area (Gossett, 1985). The system was designed so that the gas and liquid flow rates could be varied independently to measure their effect on column performance. No chemical engineering correlations describing mass transfer for the pellets were available. This and the fact that actual performance can differ significantly from general correlations prompted separate abiotic studies to determine mass transfer characteristics independent of kinetics. A countercurrent flow regime offered the advantage of a consistent concentration gradient for mass transfer between the gas and liquid phases for the entire column even in the absence of biodegradative activity. Once

inoculated, however, Diks and Kirchner both concluded that the overall reactor efficiency was not dependent on the flow regime. A flow rate sufficient to merely wet the biofilm surface was chosen to minimize the liquid waste effluent. Oxygen was delivered to the system via the air stream carrying the xylene vapor. Although oxygen is required for biological reaction, the Monod constants are generally extremely low (10^{-3}-10^{-1} mg/l) and so reaction kinetics are regarded as zero order in oxygen concentration (Kampbell et al., 1987). *Pseudomonas putida* is a mesophylic bacteria which grows at a temperature of about 20-45°C.(Kampbell et al., 1987). The reactor temperature was maintained between 20°C. and 30°C.

METHODS AND MATERIALS

The experimental approach can be divided into the following four parts: 1) Growth and preliminary chemostat studies on *Pseudomonas putida* Idaho. 2) Abiotic reactor studies. 3) Biotic reactor studies. 4) Biomass determination studies. All experiments were conducted with Baker Analyzed Reagent (J.T. Baker Chemical Co.) *p*-xylene and *Pseudomonas putida* Idaho.

Growth Studies

Batch Studies

Batch growth studies were conducted at MSU to determine whether

Table 2. Basal salts media.

Compound	Concentration (mg/l)
KH_2PO_4	700
K_2HPO_4	700
MgCl2	300
$(NH_4)_2SO_4$	500
Trace Compounds*	
$FeSO_4*7H_2O$	1.0
$MnSO_4*H_2O$	1.0
$NaMoO_4*7H_2O$	1.0
$ZnSO_4*7H_2O$	1.0

*Trace compounds added to M.S. media for chemostat and reactor studies.

Note: 10 liters autoclaved 90 minutes at 121°C.
20 " " 4 hours at 121°C.

certain trace minerals and metals enhance or inhibit *Pseudomonas putida* growth on *p*-xylene. All studies were carried out in closed flasks containing 100 ml aliquots of sterile basal salts media (Table 2), 1 ml of xylene, and 1 ml of cells from a batch culture in log phase of growth. One ppm of Fe, Mo, Co, Mn, Zn and 10 ppm of Ca were added to the first culture (G-1). The second culture included 0.1 ppm of Cu (G-2). The third culture contained 10 ppm of Fe and Mo, 1 ppm Co,Mn, and Zn, and 100 ppm Ca (G-3). The fourth culture contained 1ppm of Fe, Mo, Co, Zn, Mn, and Cu, and 10 ppm Ca (G-4). Incubations were performed at 22°C. on a gyratory shaker. Cell growth was demonstrated by measuring increases in optical density on a Varian DMS 90 UV Visible Spectrophotometer set at a wavelength of 600 nm. Growth rates were determined by cell enumeration done at 2-3 hour intervals for 25-30 hours. Each culture was diluted and spread in duplicate on Difco (Detroit, MI) nutrient agar plates. The plates were incubated in a xylene and water saturated atmosphere at room temperature for three days. Colonies were counted after incubation. The arithmetic mean of the observations was used as a colony forming unit (cfu) count. When possible, the dilution counted contained between 30 and 300 cfu per plate.

Chemostat Studies

A New Brunswick Bioflo III computer controlled reactor was used to conduct preliminary kinetic studies. Chemostat experiments were carried out

at a temperature of 30°C., an agitation rate of 150 rpm, a oxygen flow rate of 30 ml/min, and a pH no less than 5.2. Mineral salts solution saturated with xylene was fed to the chemostat. Masterflex (Cole Parmer, Chicago, IL.) peristaltic pump systems were used for influent and effluent streams. Samples were removed with disposable hypodermic syringes through a mininert valve, injected into glass vials, and sealed with teflon-lined crimped lids. Xylene utilization (growth yield) was quantified at dilution rates of .012, .024, and .036 per hour by using a carbon analyzer for influent xylene concentrations and cell carbon determinations.

Abiotic Studies

The abiotic and biotic studies used the same system configuration (Figure 2). The bioreactors were constructed with glass process pipe columns (86 cm x 7.55 cm) with two glass ports at the base, three along the column length, and three on the domed top. Teflon tubing (.4 cm od) and fittings and viton tubing were used for all streams containing xylene. One column (BR1) was packed to a height of 58 cm with 10 mm glass spheres. The second column (BR2) was filled to a height of 56.5 cm with Manville-R635 (Lompoc, CA) diatomaceous earth pellets, irregular cylinders approximately 6 mm in diameter and varying in length from 2-10 mm. Pellet chemical

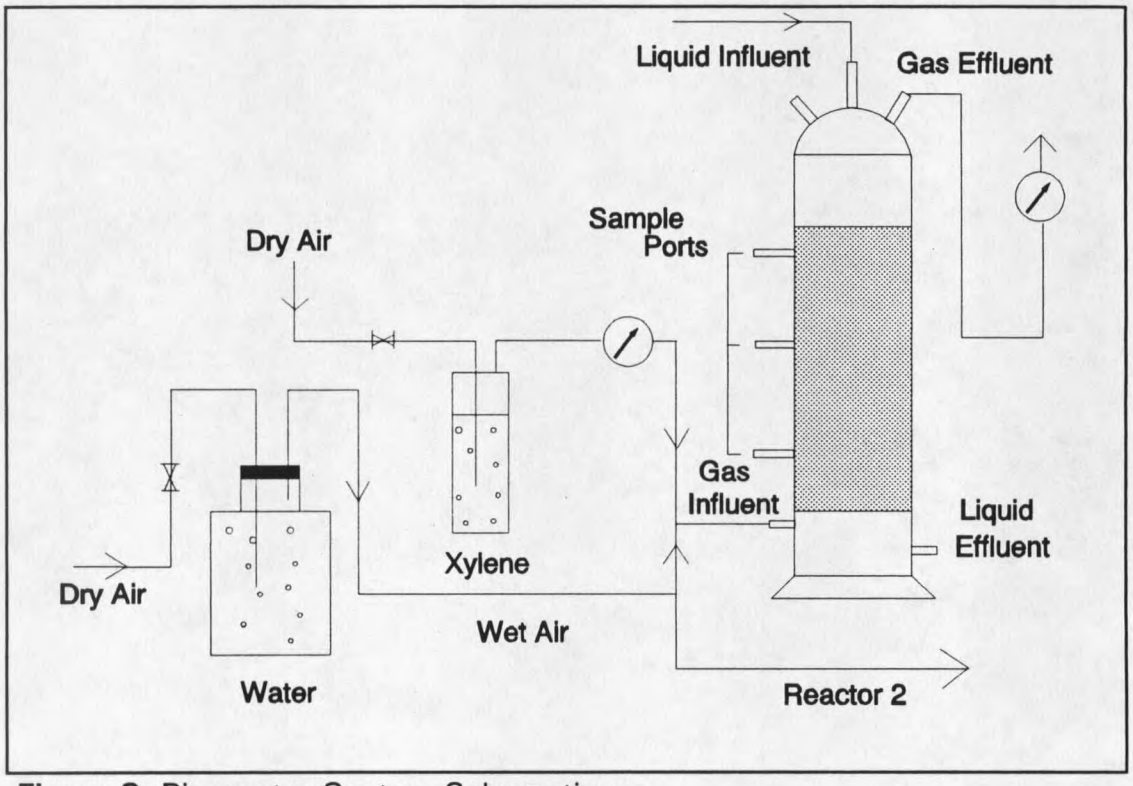

Figure 2. Bioreactor System Schematic

Table 3. Diatomaceous earth pellets: Chemical analysis.

Compound	% by Weight
SiO	82.3
Al_2O	7.2
CaO	2.6
MgO	1.2
Fe_2O_3	1.9
Na_2O	3.3
K_2O	0.9
P_2O_5	0.4
TiO_2	0.2

Table 4. Diatomaceous earth pellets: Physical properties.

Property	
Average Size	0.64 cm D X 0.5-1.25 cm L
Mean pore diameter	20 μm
Surface area	0.27 m^2/g
Total pore volume	0.61 cm^3/g
volume fraction 1-10μm	12.5%
" 10-20 "	35.8%
" 20-30 "	39.0%
" 30-40 "	8.1%
Compacted bed density	513 kg/m^3

composition (Table 2) and physical properties (Table 3) were provided by the manufacturer.

Air was sparged through a *p*-xylene reservoir maintained at a constant temperature of 30°C at flowrates controlled by a peristaltic pump. Influent gas flow rate and concentration were controlled by metering the xylene-saturated vapor stream into an air stream from the house supply that was filtered and humidified. The influent gas was fed into a port at the base of the column. A peristaltic pump distributed mineral salts media (Table 1) countercurrently from a 20 liter carboy through size #27 hypodermic needles. Liquid effluent was removed from the bottom of the reactors by a pump. All pumps used were Masterflex. Total gas and xylene flow rates were measured independently with Gilmont flowmeters. Liquid volumetric flow rates were determined manually.

The operating variables in the abiotic studies were gas and liquid flow rates. Xylene breakthrough was measured at air flow rates of 50, 75, 100, 150, and 200 ml/min and a liquid flow rate of 10 ml/min. At a liquid flow rate of 5 ml/min, the air flow rates measured were 50 and 200 ml/min. All the abiotic studies were conducted at a constant influent xylene concentration of 150 ppm (v/v).

Biotic Studies

Following the abiotic studies, the columns and feed tubing were autoclaved for two hours at 121°C. *Pseudomonas putida* Idaho was cultured in a flask with sterile basal salts media to a density of 10^7 cfu/ml. The bacterial suspension was recirculated for three days through the column. Clean air was

bubbled through the tower at a low flow rate (<10ml/min) to keep the system aerobic. On the fourth day, the reactor was drained and xylene was introduced at 150 ppm (v/v) at a air flow rate of 100 ml/min to insure growth occurred along the entire column length. Liquid media (Table 1) was distributed at a rate of 5 ml/min. The system was operated under these conditions for two weeks.

The process parameters analyzed for the biotic studies were gas and liquid flow rates and influent xylene concentrations. Degradation in the glass sphere column was measured beginning with a gas flow rate of 50 ml/min, increasing in 50 ml/min increments until breakthrough occurred. Influent and effluent xylene concentrations were measured at influent gas flow rates of 600, 1000, 1200, 1700, and 2000 ml/min. Breakthrough in the pellet reactor was measured at influent gas flow rates of 3000, 4000, 5000, and 6000 ml/min. Xylene removal was also measured at the sample ports along the column length. Both reactors were analyzed at liquid flow rates of 5 or 10 ml/min and xylene concentrations of 150 ppm (v/v). For studies using high xylene concentrations (1500 ppm (v/v)), the air flow rates were reduced to 100 and 200 ml/min in the glass sphere reactor and 400 and 600 ml/min in the pellet reactor for analysis at loading rates comparable to the first runs.

Biomass Analyses

Cell biomass was determined by protein and total organic carbon analyses and viable cell plate counts. Biofilm samples were prepared by removing packing pieces from the top, middle, and bottom of the reactors. In the first reactor, one glass sphere from each level was placed in 10 ml of mineral salts solution. The sample was sonicated for 3 minutes with a Bransonic 220 to remove any attached biofilm. Biomass volume was quantified by measuring meniscus displacement with a micromanipulator before and after sonication. The biomass suspension was homogenized for 30 seconds with a Tekmar tissuemizer at 20,000 rpm. Three pellets from each level of the second reactor were placed in 20 ml of MS media. Pellets were broken up with a mortar and pestle and the suspension homogenized for 1 minute. The prepared samples were divided evenly into teflon-capped glass vials for the biomass analyses described below.

One ml from each biomass sample was diluted in series and spread on Difco R2A agar plates for cell enumeration by the method described in the batch growth studies. Plate counts were also used to differentiate between percentage of total cells growing on either the interior or exterior pellet surface. Pellet sectioning was done with a razor knife by slicing and removing the ends and sides of the cylinder. Pellet interior and exterior volumes were quantified by suspending each section in 10 ml M.S. media and measuring the displacement with a micromanipulator.

Total cellular protein determination was made using Bio-Rad's Protein Assay, a proprietary modification of the traditional Lowry method. The assay is based on an observed shift in absorbance maximum when protein reacts with the reagent. The standard curve was prepared using dilutions of a 250 mg/l bovine serum albumin standard solution. Biomass samples were boiled for 1 minute. A 0.4 ml suspension from the first bioreactor was added to 2.4 ml of MS media and 0.6 ml of dye reagent. A 0.1 ml suspension was used from the second bioreactor. Samples were read on a Varian DMS 90 Spectrophotometer at 595 nm after at least five minutes and before one hour after addition of dye.

Biofilm samples for carbon analysis were acidified and then frozen. After thawing, samples were sonicated for 5 minutes and sparged with oxygen for 6 minutes to remove any xylene or CO_2. Biomass carbon concentration was determined by averaging at least three readings for each sample.

Analytical Methods

Xylene concentrations in all vapor streams were measured with a Hewlett Packard 5890 Series II gas chromatograph equipped with a flame ionization detector and an AllTech Super Q preconditioned column. The carrier gas was helium flowing at a rate of 28 ml/min. The oven temperature was 220°C. and the injector temperature was 250°C. Samples were removed through teflon septa with a Hamilton 500 microliter gas-tight syringe and injected into the chromatograph. The syringe was purged with nitrogen gas

between samples. Xylene concentrations in the liquid effluent were determined by removing approximately 5 ml of effluent into a 10 ml teflon-capped glass vial containing 1 ml of methylene chloride. Exact effluent volume was determined by weight. Samples were inverted and equilibrated. A 1 microliter Hamilton liquid syringe was used to extract the sample from the methylene chloride layer and to inject it into the gas chromatograph.

A Dohrmann DC 80 Carbon Analyzer standardized at 400 ppm was used for xylene carbon concentration and suspended cell carbon determination in the chemostat studies. Total organic carbon for the packed bed reactors were measured with a DC-190 High-Temperature TOC Analyzer calibrated with a 500 ppm standard using a 250 microliter syringe.

RESULTS

Batch culture experiments were conducted to isolate factors that might influence the growth rate of *Pseudomonas putida* Idaho. A chemostat was used to determine the intrinsic kinetic parameters of *P. putida* in a suspended cell system. Raw data for these experiments is given in Appendix A.

The reactor systems were initially evaluated abiotically to provide information on the mass transfer of xylene from the gas to the liquid phase. Stable operation of the abiotic system with respect to regulating gas inlet concentrations and gas and liquid flow was important to insure that viability of the biofilm could be maintained after inoculation.

The biotic studies evaluated the degradative capacity of the reactors as a result of changing process parameters; namely, the gas and liquid flow rates and xylene inlet concentration.

Quantifying substrate removal as a result of reaction within a biofilm requires some measure of biomass. Biomass concentration in both reactors was determined by three different analyses. Raw data is shown in Appendix B.

Batch Growth Studies

Viable cell plate counts were used to quantify the effect of increased trace mineral concentrations in the culture media on the growth rate of *Pseudomonas putida* Idaho (Figure 3). Growth rates were determined by

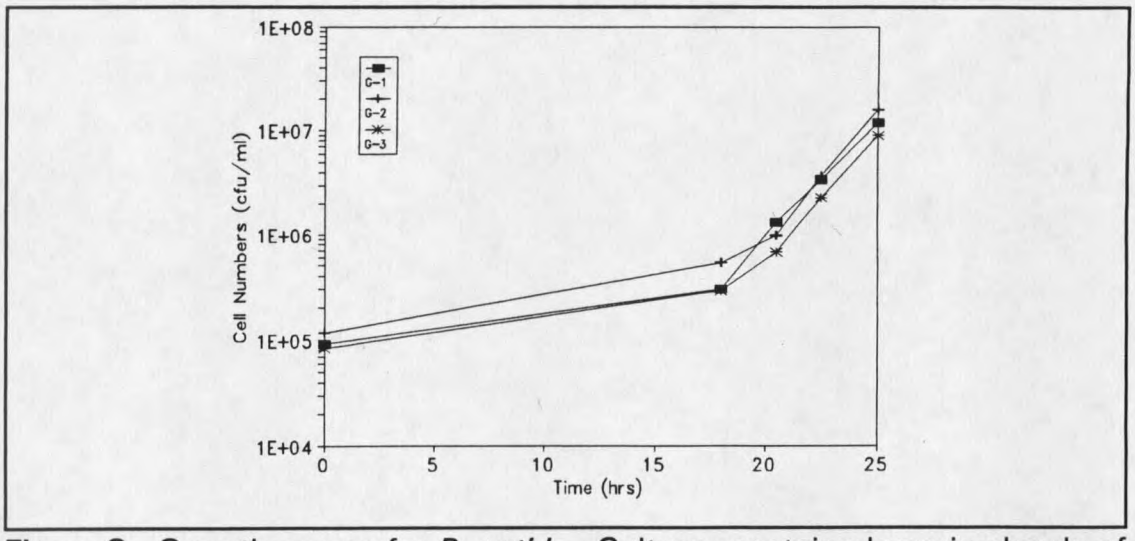

Figure 3. Growth curves for *P. putida*. Cultures contained varying levels of trace compounds in M.S. media.

calculating the ratio of the natural log of cell numbers versus time. Maximum growth rates (μ_m) for G-1 (1 ppm trace compounds, no Cu), G-2 (1 ppm trace compounds, 0.1 ppm Cu), and G-3 (10 ppm trace compounds, no Cu) were .587, .668, and .608/hr, respectively. The culture containing 1 ppm Cu (G-4) exhibited no growth. The increase in turbidity with time indicating cell growth was measured by absorbance at 600 nm (Figure 4). The steepest rise occurred during the log phase of growth (17.5 to 22.5 hrs).

Chemostat Studies

Because effluent xylene levels were below detection limits (<1mg/l), xylene utilization (growth yield) in the chemostat was determined from the ratio of cell carbon (mg/l) in suspended cells to carbon (mg/l) in influent xylene. At dilution rates of .012, .024, and .036/hr the respective yields (mean ± S.E.)

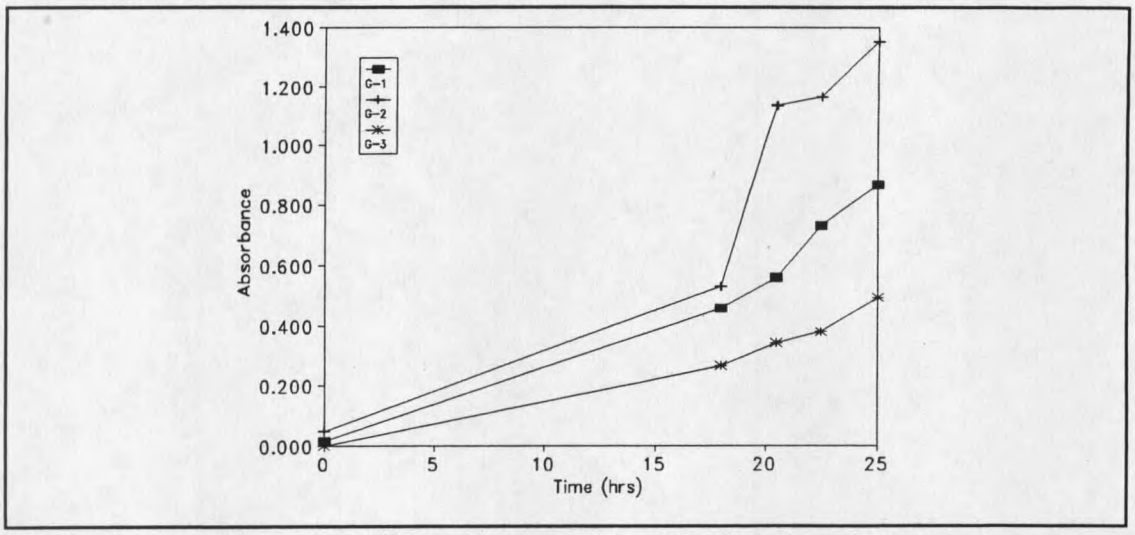

Figure 4. Increases in cell growth as measured by absorbance.

were .263 ± .008, .255 ± .01, and .261 ± .016 mg C_{cells} /mg $C_{substrate}$ for an overall average of .260 ± .008 mg C_{cells}/mg $C_{substrate}$.

Abiotic Studies

Percent breakthrough in the abiotic reactors was determined by measuring the xylene concentration in the effluent gas stream with respect to the influent xylene concentration (150 ppm (v/v). Incremental increases in the gas flow rate resulted in increased breakthrough (Figures 5 and 6). Xylene removal from the effluent gas stream was greater in BR2 than BR1 at all gas flow rates except 200 ml/min. Percent breakthrough at 50 ml/min was 3 to 5 times higher at a liquid flow rate of 5 ml/min than at 10 ml/min for both systems. Breakthrough at the highest flow rates for both systems appeared to level off at 72%.

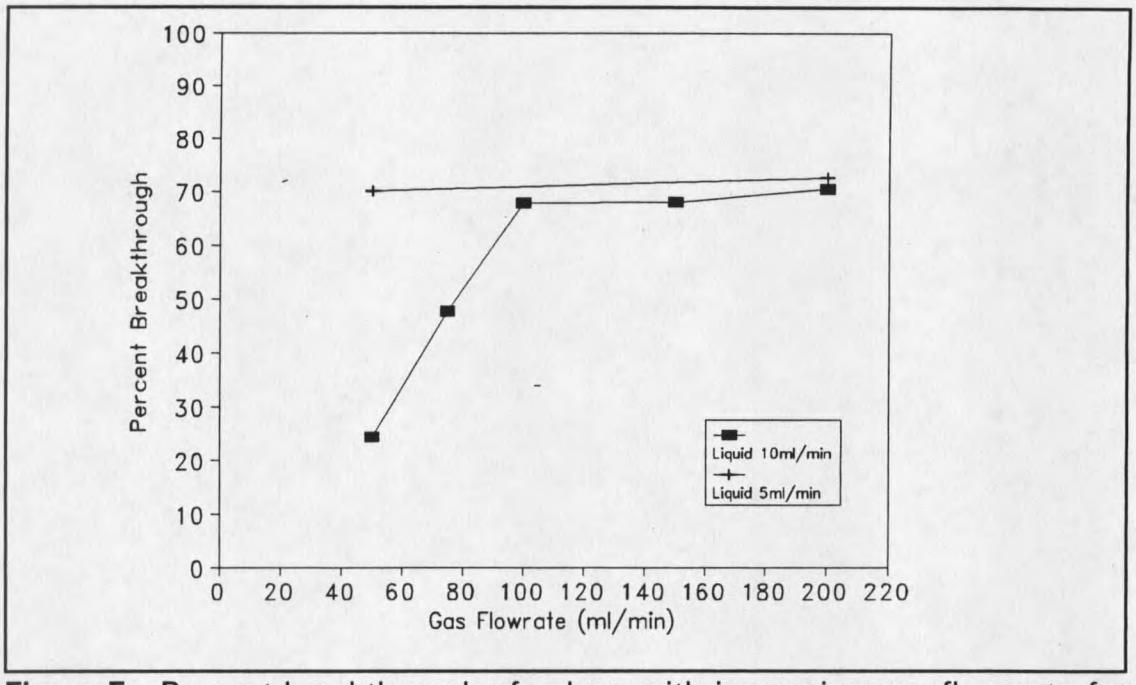

Figure 5. Percent breakthrough of xylene with increasing gas flow rate for BR1 without biofilm.

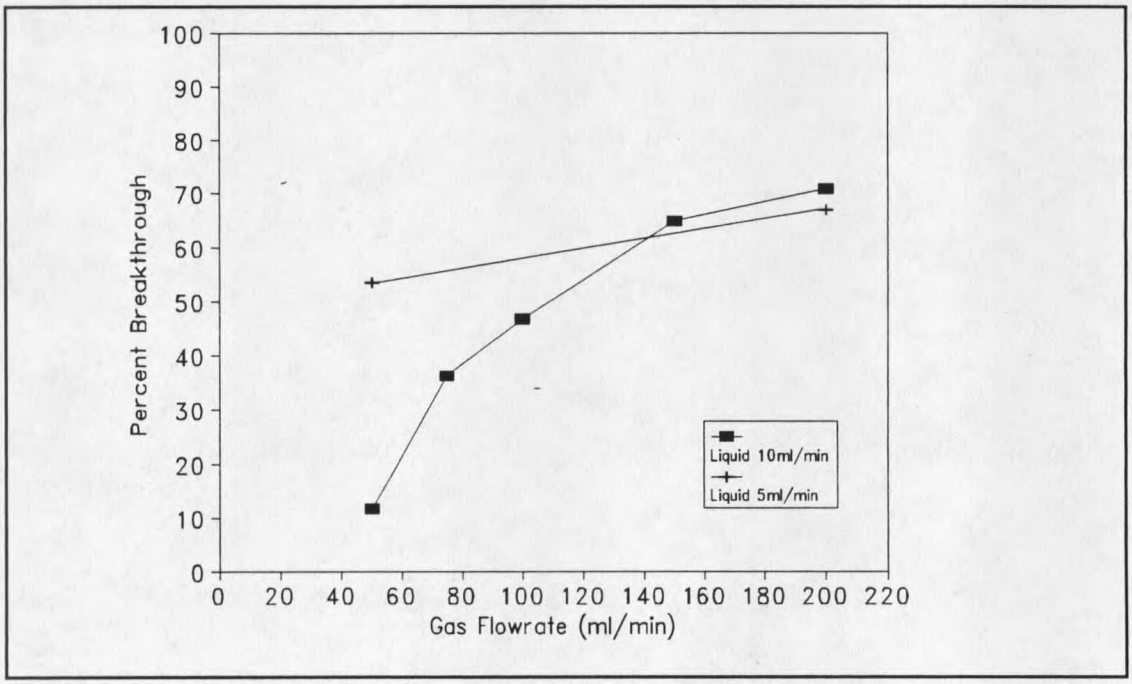

Figure 6. Percent breakthrough of xylene with increasing gas flow rate for BR2 without biofilm.

Biotic Studies

Once biofilm had developed on the packing throughout both columns (three weeks after inoculation), the xylene concentration in the effluent gas

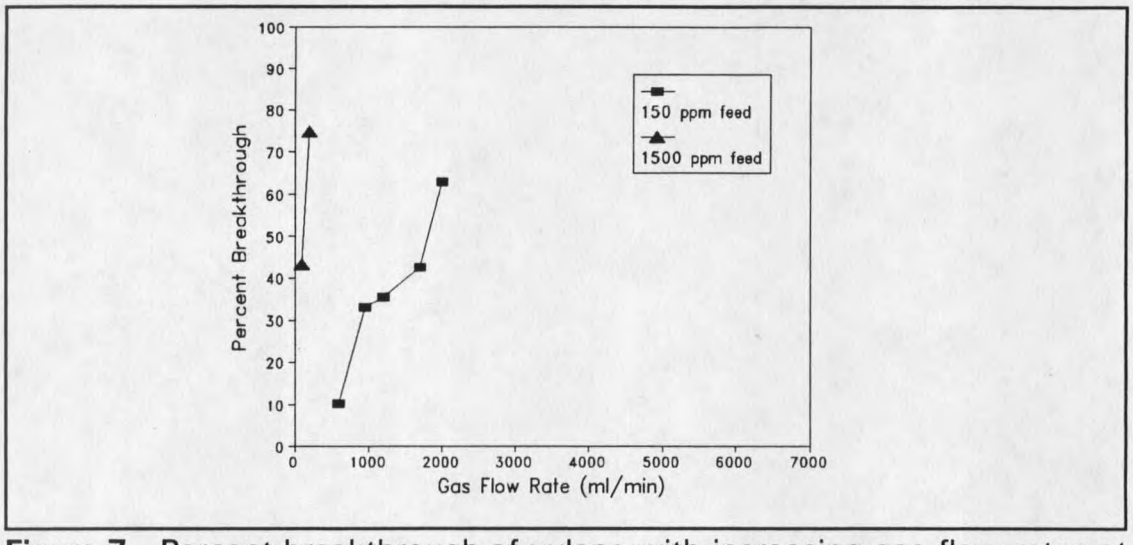

Figure 7. Percent breakthrough of xylene with increasing gas flow rates at feeds of 150 and 1500 ppm in BR1 at a liquid flow rate of 5 ml/min.

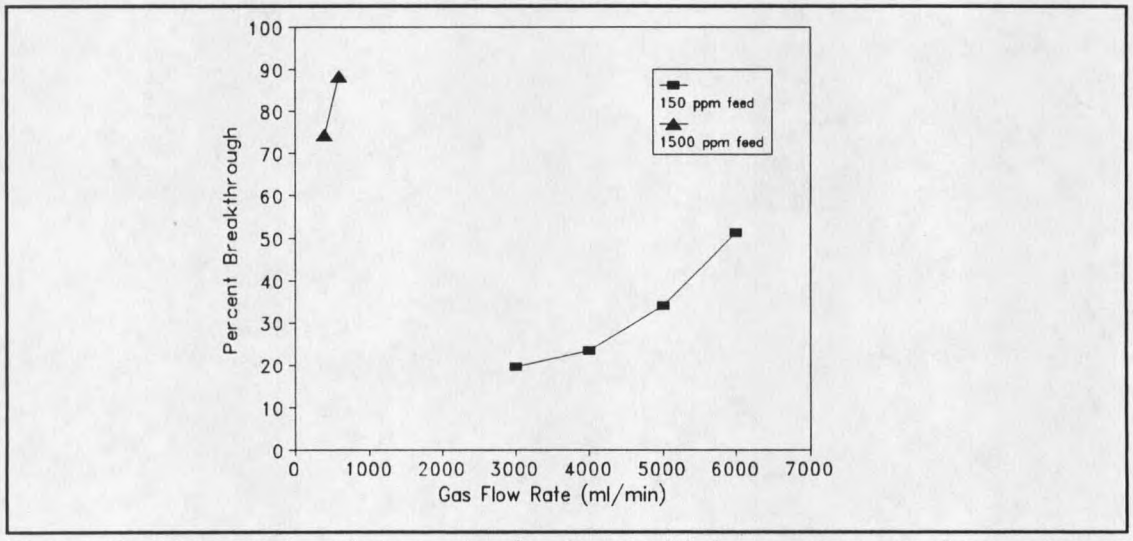

Figure 8. Percent breakthrough of xylene with increasing gas flow rates at feeds of 150 and 1500 ppm in BR2 at a liquid flow rate of 5 ml/min.

stream was measured with increasing gas and liquid flow rates and inlet xylene concentrations. No xylene was measured in the effluent gas stream for either reactor at flow rates less than 300 ml/min. Breakthrough occurred in BR2 at gas flow rates greater than 2500 ml/min. Percent xylene breakthrough with respect to inlet concentration was determined with increasing gas flow rates and inlet feed concentrations (Figures 7 and 8) and increasing liquid flow rates (Figures 9 and 10).

The xylene concentration in the liquid effluent was measured at each change in process parameters for both reactors (Table 5). The concentration

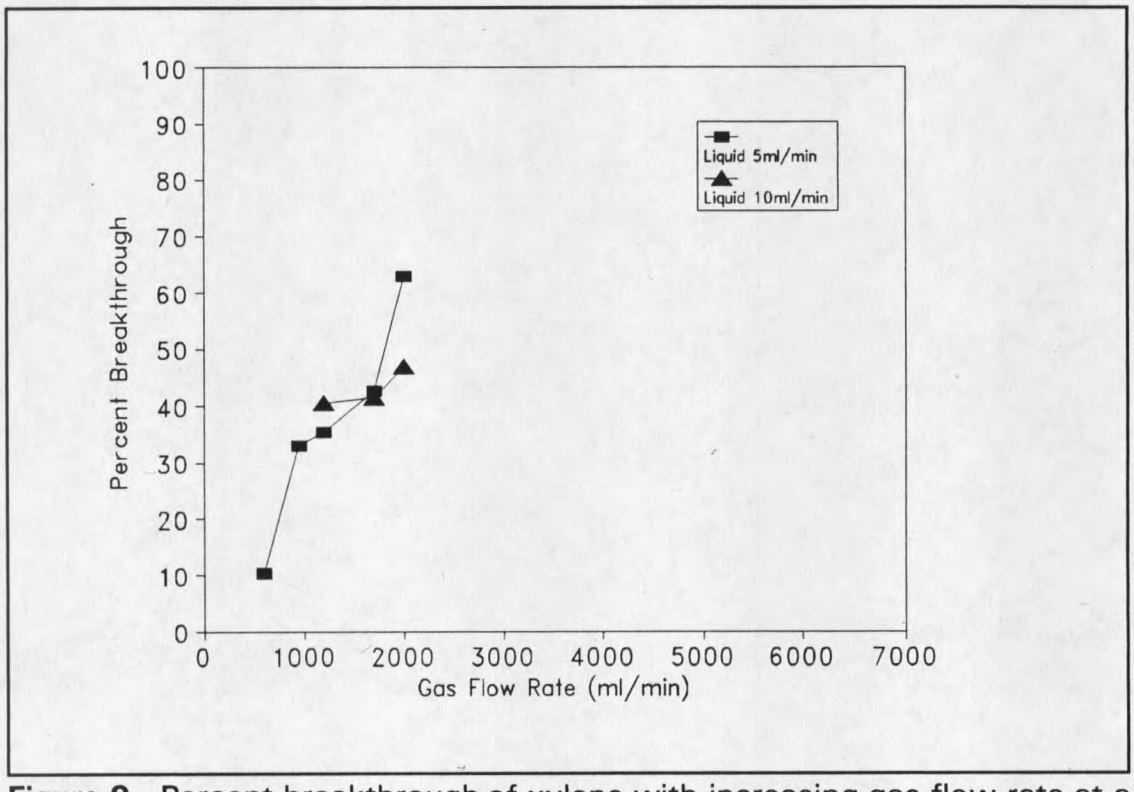

Figure 9. Percent breakthrough of xylene with increasing gas flow rate at a liquid flow rate of 5 and 10 ml/min in BR1 at a gas feed of 150 ppm.

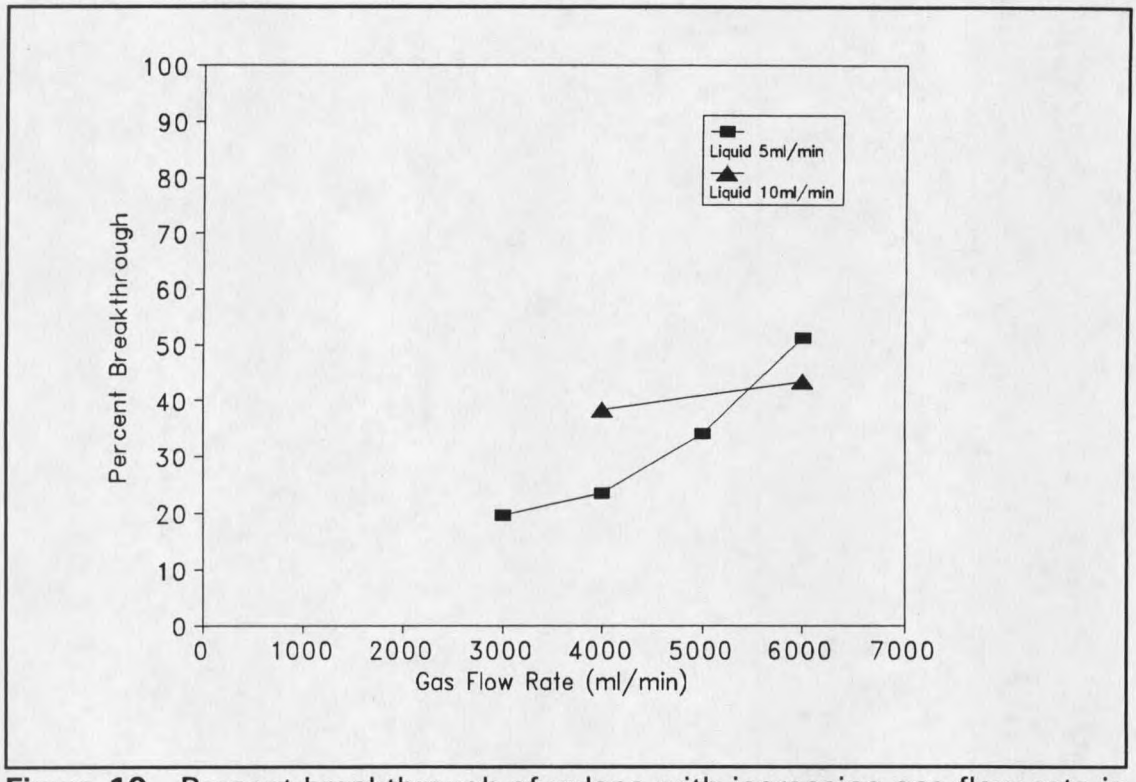

Figure 10. Percent breakthrough of xylene with increasing gas flow rate in BR2 at a liquid flow rate of 5 and 10 ml/min at a gas feed of 150 ppm.

in the liquid effluent for experimental runs done at an inlet feed of 150 ppm went from a low of .756 mg/l in BR1 at 1200 ml/min to a high of 3.63 mg/l in BR2 at 6000 ml/min. For the runs done at a feed of 1500 ppm, the liquid effluent concentrations were at least an order of magnitude higher. In BR1, the respective concentrations at 100 ml/min and 200 ml/min were 39.0 mg/l and 48.2 mg/l. In BR2, the respective concentrations at 400 ml/min and 600 ml/min were 96.4 mg/l and 36.7 mg/l. The measurement at 400 ml/min for BR2 was an outlier and was likely due to sampling error.

Table 5. Xylene levels measured in mg/min in liquid effluent for BR1 and BR2.

BR1				BR2			
GFR	LFR	PPM	mg/min	GFR	LFR	PPM	mg/min
600	5	150	.00673	3000	5	150	.00496
950	5	150	.00465	4000	5	150	BLD
1200	5	150	.00378	5000	5	150	BLD
1700	5	150	.00600	6000	5	150	.01900
1300	10	150	.00398	4000	10	150	BLD
1700	10	150	BLD	6000	10	150	.01820
2100	10	150	.00588	400	5	1500	.47000
100	5	1500	.19700	600	5	1500	.18000
200	5	1500	.24000				

GFR-Gas Flow Rate
LFR-Liquid Flow Rate
PPM-Influent feed concentration
BLD-Below Limits of Detection

A mass balance was used to determine degradation rate by difference utilizing gas-phase influent and effluent concentrations and liquid-phase effluent concentration. The results are expressed on the basis of total mass rates (Figures 11 and 12). There was 100% xylene degradation at loading rates up to 0.314 mg/min in BR1 and up to 1.72 mg/min in BR2. Both loading and degradation rates were significantly higher in the DE pellet reactor than the glass sphere reactor.

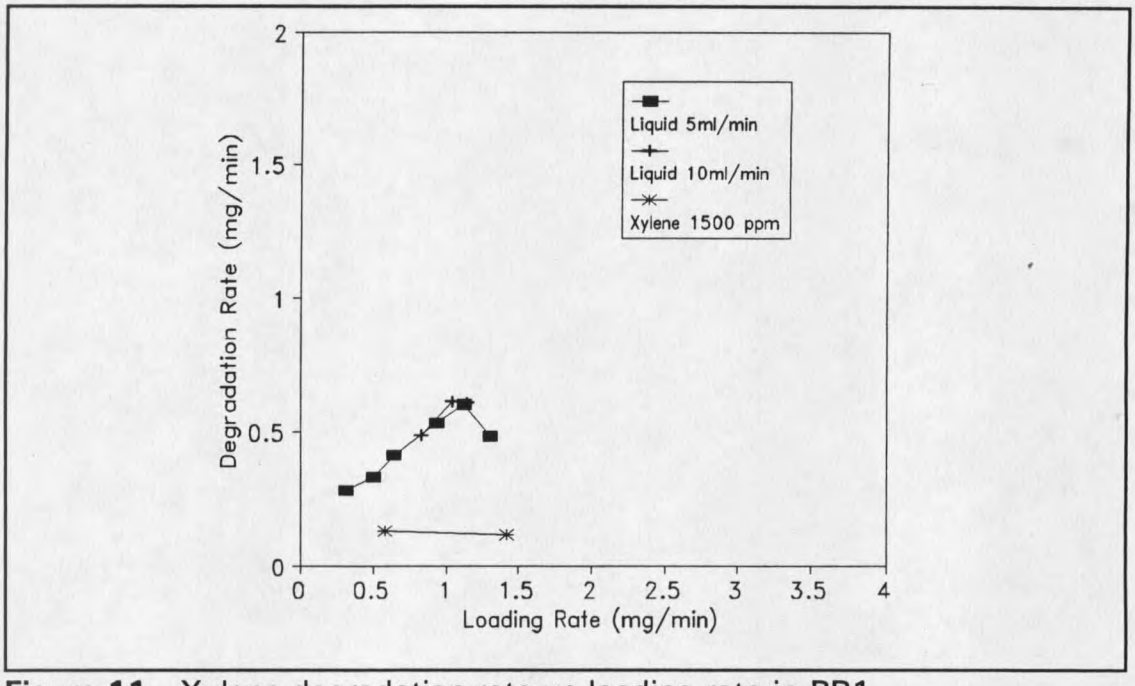

Figure 11. Xylene degradation rate vs loading rate in BR1.

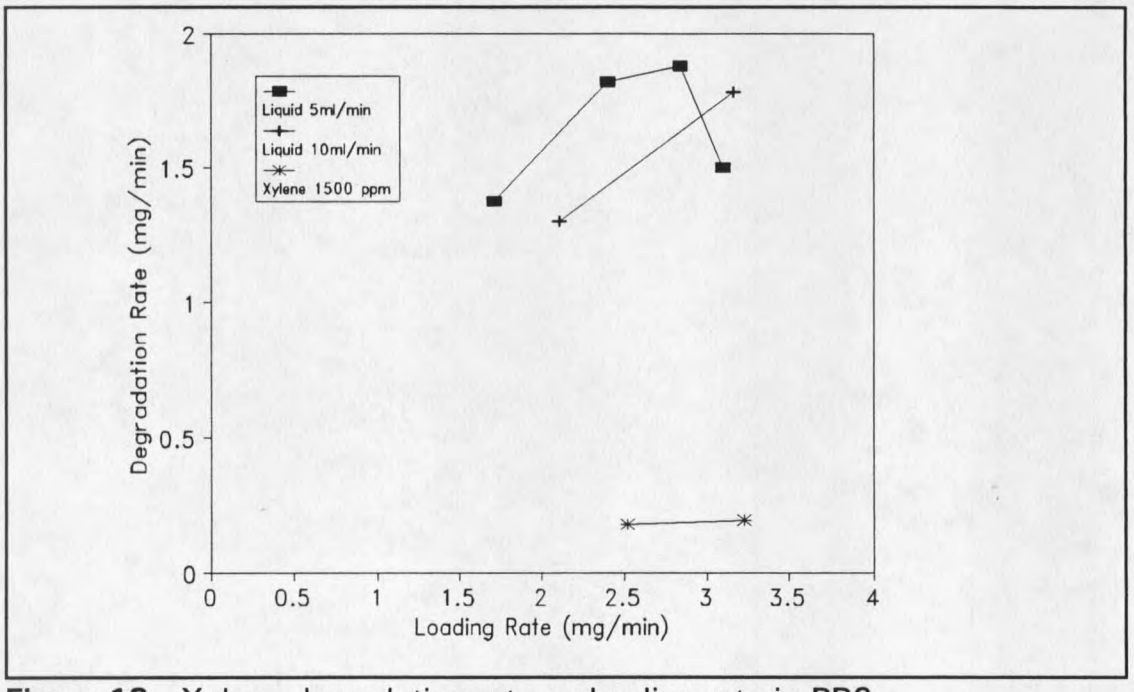

Figure 12. Xylene degradation rate vs loading rate in BR2.

Xylene removal relative to column position was determined by measuring xylene breakthrough in the air stream at ports 1, 2, and 3 (bottom to top) at feed concentrations of 150 and 1500 ppm (Figures 13 and 14).

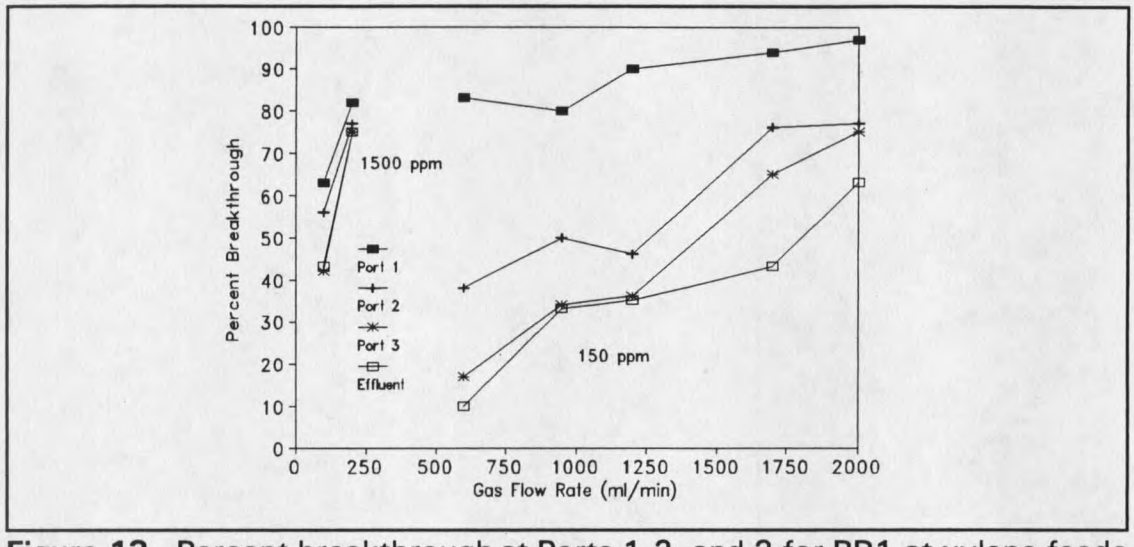

Figure 13. Percent breakthrough at Ports 1,2, and 3 for BR1 at xylene feeds of 150 and 1500 ppm and a liquid flow rate of 5 ml/min.

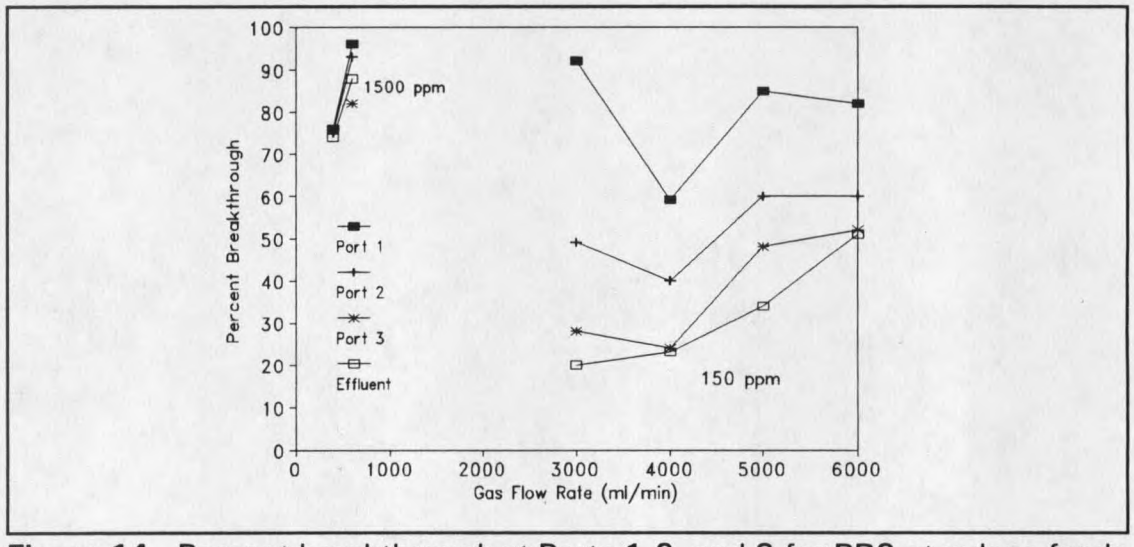

Figure 14. Percent breakthrough at Ports 1,2, and 3 for BR2 at xylene feeds of 150 and 1500 ppm and a liquid flow rate of 5 ml/min.

After completing the biotic studies, xylene breakthrough was again evaluated at fixed gas and liquid flow rates to investigate the reproducibility of the measurement techniques. Multiple points represent measurements taken under the same conditions described above but separated from the first data set by either several days or several weeks (Figure 15 and Figure 16).

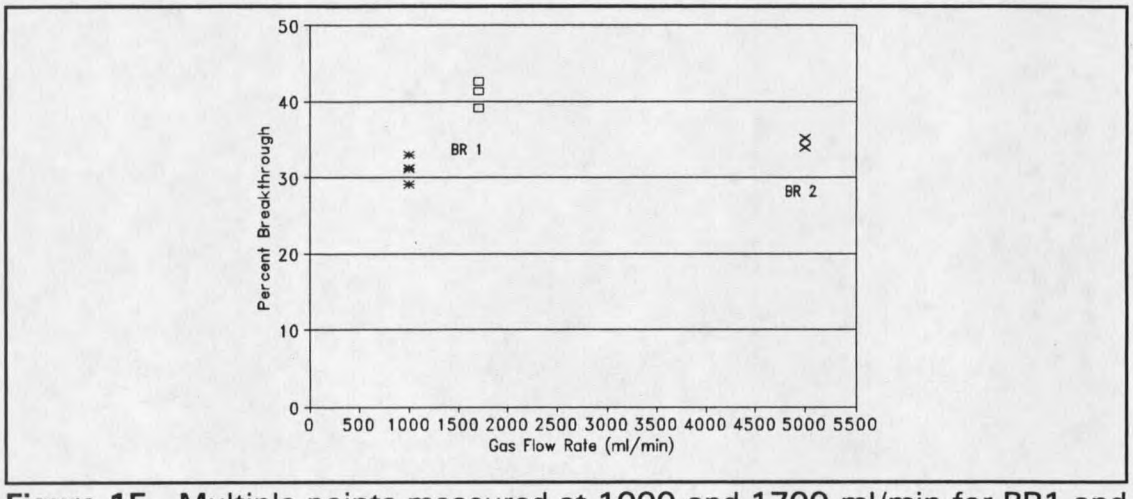

Figure 15. Multiple points measured at 1000 and 1700 ml/min for BR1 and 5000 ml/min for BR2 at 150 ppm.

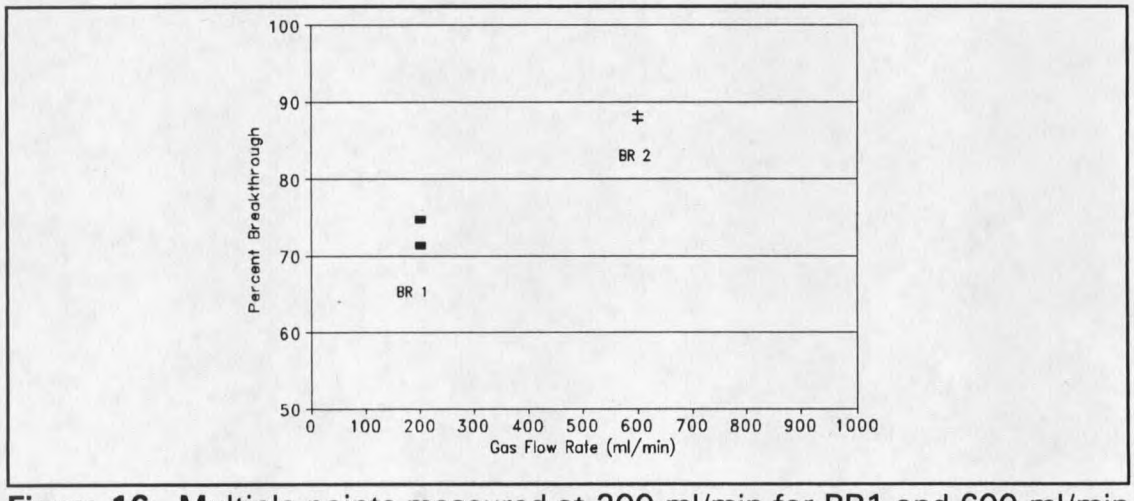

Figure 16. Multiple points measured at 200 ml/min for BR1 and 600 ml/min for BR2 at 1500 ppm.

An important parameter for industrial operation of a tower is the pressure loss across the packed bed. Pressure drop was measured in BR2 with biofilm after two months of operation at 4000, 5000, and 6000 ml/min. The respective pressure drops were .336 mm Hg, .504 mm Hg, and .672 mm Hg.

Biomass Analyses

Viable cell plate counts, total cellular protein, and total organic carbon were used to determine cell biomass. Biofilm was removed from packing pieces taken from the top, middle, and bottom of both reactors. Results for plate counts are expressed per unit external surface area of each packing piece (cfu/cm^2, Figure 16). Protein measurements are given in μg/cm^2 (Figure 17)

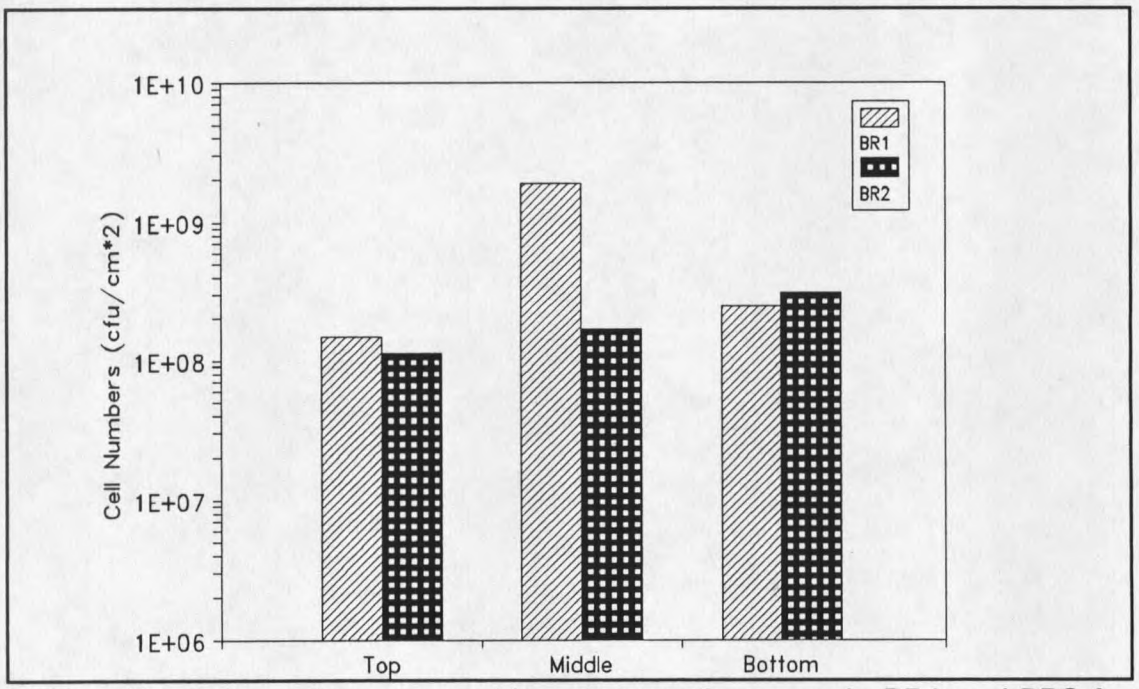

Figure 17. Cell numbers per packing piece surface area in BR1 and BR2 for top, middle, and bottom of each reactor.

and organic carbon measurements are given in mg/cm^2 (Figure 18).

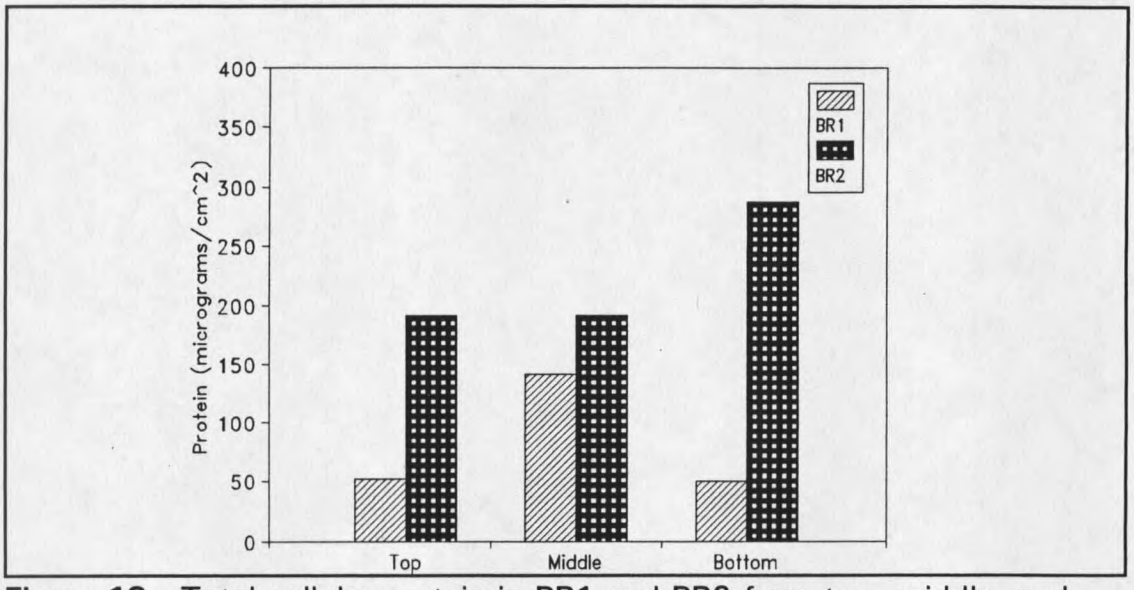

Figure 18. Total cellular protein in BR1 and BR2 from top, middle, and bottom of each reactor.

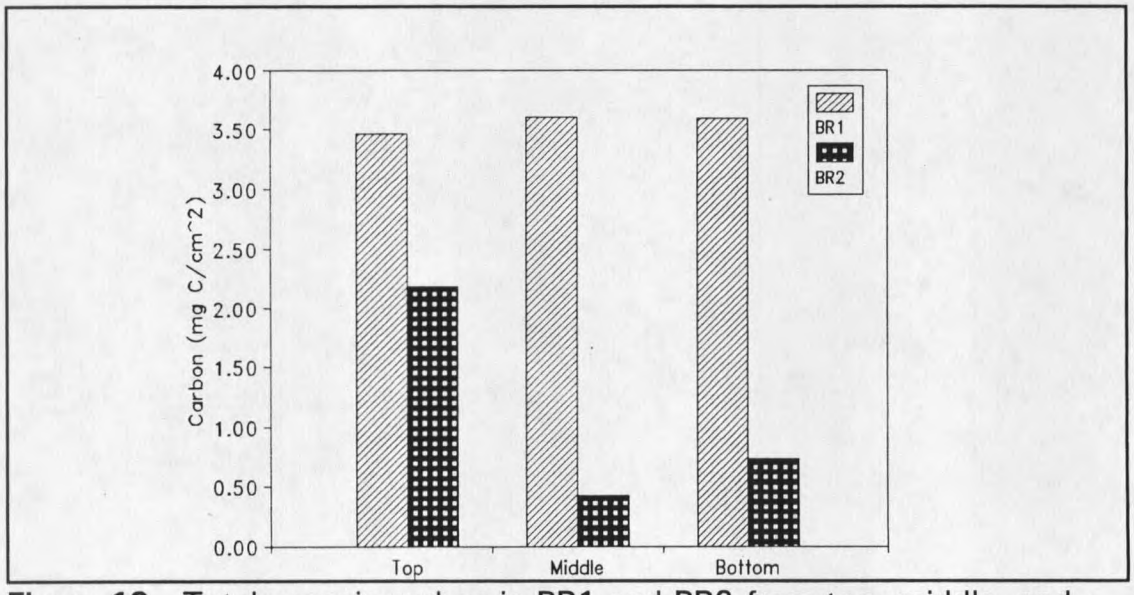

Figure 19. Total organic carbon in BR1 and BR2 from top, middle, and bottom of each reactor.

Sampling procedures in BR1 resulted in sloughing of the biofilm from the glass spheres making it difficult to obtain a representative biofilm sample. This problem was reflected in the biomass measurements of the middle sample from BR1 which were unjustifiably high.

The outer section of a pellet from BR2 was volumetrically determined to be between 4 and 14% of the entire pellet volume. Colony forming units were counted per milliliter of pellet volume of the exterior and interior sections (Figure 20).

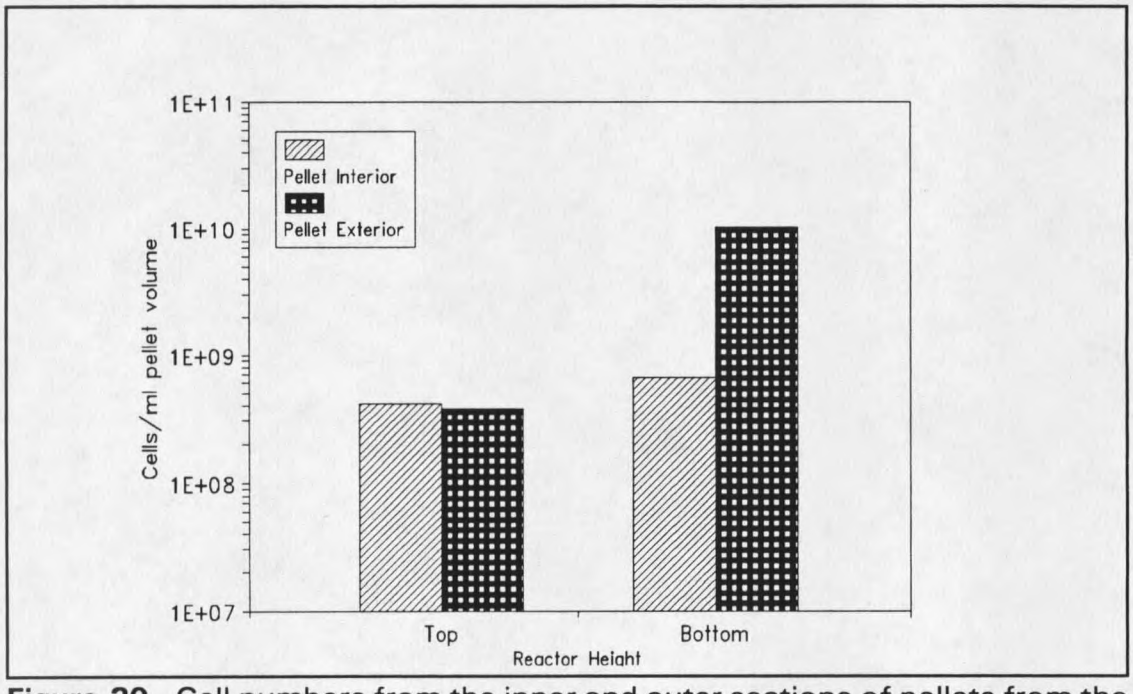

Figure 20. Cell numbers from the inner and outer sections of pellets from the top and bottom of BR2.

DISCUSSION

Batch Growth Studies

Results from the batch experiments indicated no significant growth factor requirement for the cultures studied. Copper was found to be toxic to the organism at 1 ppm but not at 0.1 ppm. Cruden et al. (1992) reported that turbidity (measured as absorbance) and cell dry weight measurements of *P. putida* Idaho paralleled the increase in viable cells during the lag and exponential phases of growth. Cruden noted that after the cells reached stationary phase, turbidity and dry weight continued to increase even though the number of viable cells decreased dramatically.

Reactor Performance Studies

The performance of a trickling filter is governed, in part, by transport phenomena as well as the intrinsic degradation kinetics of the biofilm. This includes (conceptually) at least the following three steps: 1) mass transfer from the gas to liquid phase 2) mass transport through the liquid and into the biofilm 3) degradation of the substrate by reaction within the immobilized biomass. If the depth of penetration of substrate is smaller than the biofilm thickness, the conversion rate will be controlled by the rate of diffusion into the biofilm. This situation is referred to as diffusion limitation. If there is no diffusion limitation, the wetted biofilm is fully penetrated with substrate and the

conversion rate is only controlled by the reaction rate. This is defined as reaction limitation (Ottengraf, 1983). Finally, if mass transfer from the gas to the liquid phase cannot keep pace with diffusion and reaction, the system may be termed externally mass transfer limited.

In the abiotic systems, xylene removal was greater in the D.E. pellet reactor. The pellets have a higher total surface area which may contribute to increased mass transfer from the gas to liquid phase. Xylene concentrations decreased in the effluent gas stream at higher liquid flow rates possibly the result of an increased "wetted" surface area which enhanced mass transfer. The abiotic systems operated at much lower loading rates and demonstrated xylene removal several orders of magnitude less than that shown in the biotic systems.

After inoculation, both reactors demonstrated 100% xylene removal beginning at a flow rate of 50 ml/min with an inlet xylene concentration of 150 ppm (v/v). After initial xylene breakthrough, additional increases in gas flow rates resulted in an increase in percent breakthrough for both columns. Both Kirchner's studies (1989 and 1991) demonstrated this trend.

Mass loading is the product of gas volumetric flow rate and inlet substrate concentration. The initial reactor studies were conducted at a constant inlet concentration of 150 ppm (v/v) and increasing gas flow rates. The increase in mass loading produced a corresponding increase in degradation rate for both systems except at the highest loading rate. This suggested a

diffusion limited system. Kirchner et al. (1989) reported a linear increase in removal with increasing mass flow except at the higher mass flow rates studied. The data presented in Oh et al. (1992) indicated that removal rate at a constant flow rate increased with the inlet concentration, with a tendency to reach a maximum value at an intermediate inlet concentration. At the highest inlet concentration, a substantial drop in removal rate was noted. For experiments at a constant inlet concentration and increasing gas flow rates, the removal rate remained essentially unchanged. Oh concluded that this was indicative of reaction rather than mass transfer (diffusion) limitation for the process and that as the flow rate increased, the change in the average methanol concentration practically offset the change in space time. Ottengraf's (1983) work with biofilters showed that the elimination capacity was constant above a critical value of the gas phase concentration, marking a change from diffusion limitation in the biolayer to reaction limitation. Diks (1992) found that the elimination capacity increased with increasing superficial gas velocities for inlet concentrations less than the critical inlet gas phase concentration, as defined by Ottengraf. At inlet concentrations above the critical inlet concentration, increases in gas velocity or concentration did not change the elimination capacity. Once the biofilm was fully penetrated (reaction limited) the system operated at a constant maximum elimination capacity.

BR1 and BR2 approached a maximum degradative capacity at an intermediate mass loading rate but did not level off at a constant value. At the

highest flow rates, there was a significant drop in degradation rate which may be attributed to insufficient retention times or to inhibitory effects. An even more dramatic decrease in reactor performance was recorded for the experiments conducted at inlet xylene feed concentrations of 1500 ppm (v/v). At comparable loadings, the degradation rate decreased in BR2 from 1.88 mg/min at 150 ppm to 0.19 mg/min at 1500 ppm. There was a corresponding increase in the xylene concentration of the liquid effluent from below the limits of detection (<0.6 mg/l) at 150 ppm to 37 mg/l at 1500 ppm. This result may be explained by either inhibitory kinetics or toxicity effects. Oh et al. (1992), Kirchner et al. (1991), and Canter et al. (1989) all documented apparent increasing inhibition of the biological degradation reaction at high pollutant loads. It is important to note that both systems quickly recovered their maximum elimination capacity following two weeks of operation under conditions that had significantly reduced degradation rates. This is evidence of inhibition (which is reversible) rather than toxicity (which is not). Diks (1991) noted that after a significant decrease in EC_{max}, the elimination capacity totally recovered within three weeks of restoring optimum conditions. He concluded that the biology of his system was unaffected overall by short-term fluctuations in the organic load.

Although the range of liquid flow rates studied was relatively narrow, it appeared that an increase from 5 ml/min to 10 ml/min improved reactor performance at the highest gas flow rates. Kirchner et al. (1991) noted that

the percentage of conversion in his system rose with each increase in liquid flow rate (termed trickle density). Diks et al. (1992) showed that an increase in liquid flow rate was quickly followed by a rise in elimination capacity. Diks explained this by the existence of a "Randomly Wetted Area." He expected an active biofilm only on the continuously wetted area in the filter bed which received both a continuous supply of substrate and simultaneous removal of acids or other metabolites produced. In Diks' system, hydrochloric acid was produced and accumulated in the biofilm in the flow direction of the liquid phase. He determined in batch investigations on filter biomass that the rate of the biological reaction depended on the local pH value. Because the local pH depended on the wetting frequency, the randomly wetted areas of the biofilm contained biomass with a potential activity that became involved in elimination at higher liquid flow rates when the optimal pH was restored.

This system did not exhibit a measurable change in pH but there was evidence from the batch and chemostat studies that biological metabolites were formed in the oxidation of xylene that potentially affect biofilm growth. Increased liquid flow rates may have facilitated removal of these compounds and restored optimum physiological conditions in the biofilm. Higher liquid flow rates may also increase the wetted surface area on the packing resulting in increased mass transfer from the gas to liquid phase and enhanced degradation. Improved reactor performance with increased liquid flow rate was particularly evident at the highest gas flow rates. This may be the result of a reduction in

the local xylene concentration in the liquid layer surrounding the biofilm thereby offsetting the effects of inhibition.

Although similarities exist between the vapor phase reactor studies referenced above, direct comparisons cannot be made due to differences in populations, substrates (solubility, toxicity, etc.), packings, and (most important) reactor sizes. Residence times (volume/gas flow rate) were calculated for each system to correlate reactor sizes (based on total reactor volume) to the gas flow rates evaluated. Residence times are a measure of the efficiency of a system (ie. greater pollutant volumes degraded in shorter times reduces costs). Volumetric rates per cross-sectional area of the reactor were used to compare liquid flow rates.

Kirchner operated his system at a residence time that began at 0.06 minutes and decreased thereafter. By contrast, the residence time for BR2 was 0.633 minutes at the highest flow rate. The majority of Diks' experiments were conducted with a residence time of 1.5 minutes. The residence time of Oh's reactor at the highest flow rate evaluated was 4.3 minutes.

The difference in liquid flow rates between this system and the others studied was significant. The liquid range in Kirchner's system was 1.11 to 20.4 m^3/m^2hr. Diks evaluated liquid flow rates ranging from 1.8 to 11 m^3/m^2h. Both systems used recycle. The liquid flow rate in BR1 and BR2 ranged from .067 m^3/m^2hr to .134 m^3/m^2hr. Low liquid flow rates were used to minimize liquid waste volume as mentioned previously. Oh's reactors did not have a

continuous liquid flow.

INEL's study (described previously) used a glass reactor the same size as BR1 and BR2. *P.putida* Idaho was immobilized on 5/8" polypropylene Pall Rings and *p*-xylene was introduced countercurrently. At a residence time of 219 minutes and a liquid flow rate of .0225 m^3/m^2 h, the degradation rate was .0639 mg/min. Peak removal rates in BR2 were thirty times higher with a residence time less than 1% of INEL's system.

In order to compare the elimination capacity of each reactor, substrate loading was normalized to the total amount of external surface area provided by the individual packings (Figure 21). Although loading rates were comparable, the maximum degradation rate for the DE pellet reactor was 66 mg/m^2 h versus 45 mg/m^2 h in the glass sphere reactor. This suggests a

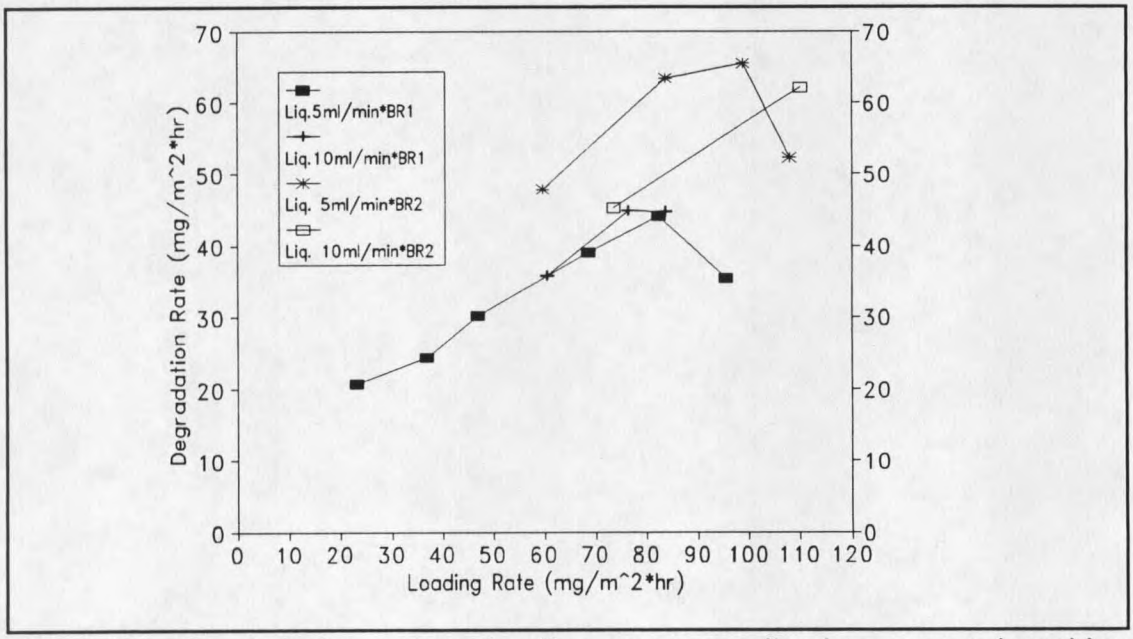

Figure 21. Degradation rate vs loading rate normalized to external packing surface area for BR1 and BR2

contribution from the external and intrapellet pore surfaces to increased biofilm development above that of the smooth glass surface, resulting in enhanced xylene biodegradation. Alternatively, the higher tortuosity within the pellet reactor could have increased external mass transfer.

Biomass Analyses

A biomass gradient was visibly evident in both systems increasing from the top to the bottom of the reactors. Cellular protein determination in BR2 appeared to substantiate this observation. Protein levels increased one and a half times from the top half of the column to the bottom half. CFU/cm^2 were almost 3 times higher on pellets from the base of the column than from the top. The breakthrough percentages recorded along the column length demonstrated, in most cases, twice as much xylene removal in the bottom half of the columns than the top half. Protein measurements in BR2 were 3 to 4 times greater than BR1, except for the middle BR1 sample. This correlated with the results described above indicating increased degradation rates in the D.E. pellet reactor. Cell numbers were slightly higher in BR2 than BR1 but not significantly different.

The results of total organic carbon measurements revealed an opposite trend from protein measurements or plate counts. Carbon levels from biofilm samples immobilized on the glass spheres were up to 9 times higher than the samples from DE pellets taken from the middle and bottom of the second

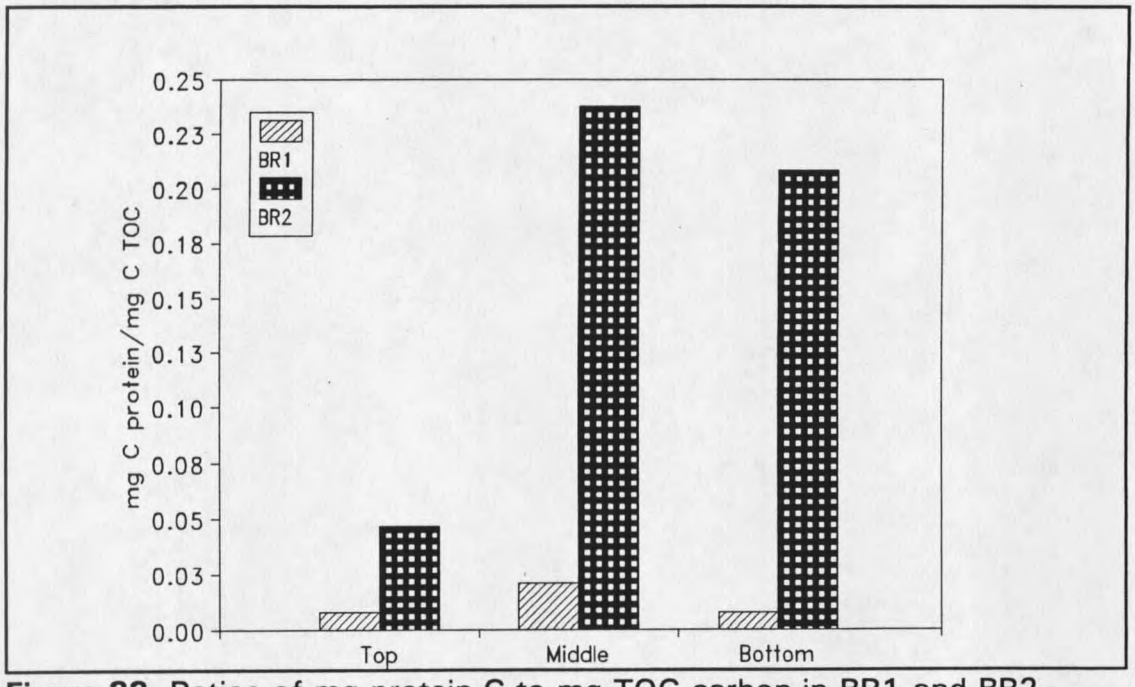

Figure 22. Ratios of mg protein C to mg TOC carbon in BR1 and BR2.

reactor. TOC measurements in BR2 were three times greater for the top sample which had visibly less biofilm. This may indicate greater extra cellular polymer (EPS) production in these samples. EPS matrix can account for as much as 50 to 90% of the biofilm organic carbon (Bakke et al., 1984). The presence of capsules or a slime layer (EPS) may significantly influence the cellular composition determined by traditional techniques. Since both capsules and EPS are mainly polysaccharides, they may lead to an overestimate of the carbon per cell even though the amount of nitrogen and phosphorus per cell is unaffected (Characklis, 1990). The ratio of milligrams of protein carbon to milligrams of total carbon was calculated (Figure 22). The D.E. pellet reactor (particularly the bottom half) had a much higher proportion of protein carbon.

Conversely, BR1 had a higher ratio of non-protein carbon.

Colony counts from the exterior and interior pellet sections normalized to pellet volume were approximately the same for pellets taken from the top of the column. In contrast, cell numbers from the exterior section of pellets from the bottom of the reactor were more than an order of magnitude above that of the interior section. Sturman (1991) found that interior colonized chlorobenzene degraders immobilized on DE pellets were at a relative disadvantage due to substrate or electron acceptor conditions. He concluded that both motile and non-motile microorganisms colonize exterior pellet surfaces to a much greater extent than interior surfaces. This suggests that once the biofilm is fully developed on the exterior, the inner pore spaces become diffusion limited. It is important to note that although cell growth was higher on the exterior surfaces, viable cells on the interior surfaces were not completely washed out.

Bratbak (1985) estimated that the dry weight of one bacterial cell is approximately 1×10^{-13} g. By assuming that 40% of a cell is carbon, cell densities were calculated based on the protein (53% C) and TOC measurements. Calculated cell counts based on TOC were 1 to 2 orders of magnitude higher than the viable cell counts actually reported. This implied that the entire biofilm was not actively involved in xylene degradation. Diks (1991) removed biomass from his trickling filter system and determined its specific activity defined as the amount of substrate degraded per unit of

biomass and time (gDCM/gTSS*h). He measured an 88% decrease in activity compared to a suspended pure culture system. Diks concluded that only about 12% of the biomass present in the filter actually degraded DCM and that the major part of the biofilm consisted of inactive material and secondary organisms. Immobilized cells from BR1 were streaked for isolation on R2A agar. The colonies were then restreaked on *Pseudomonas* isolation agar (PIA). One of the dominant isolates from R2A medium failed to grow on PIA indicating that the biofilm was no longer a pure culture. The secondary organisms, though xylene tolerant, may not have contributed to xylene degradation but would be measured as protein or TOC. It is important to point out that even after contamination occurred, the system continued to maintain its maximum degradative capacity.

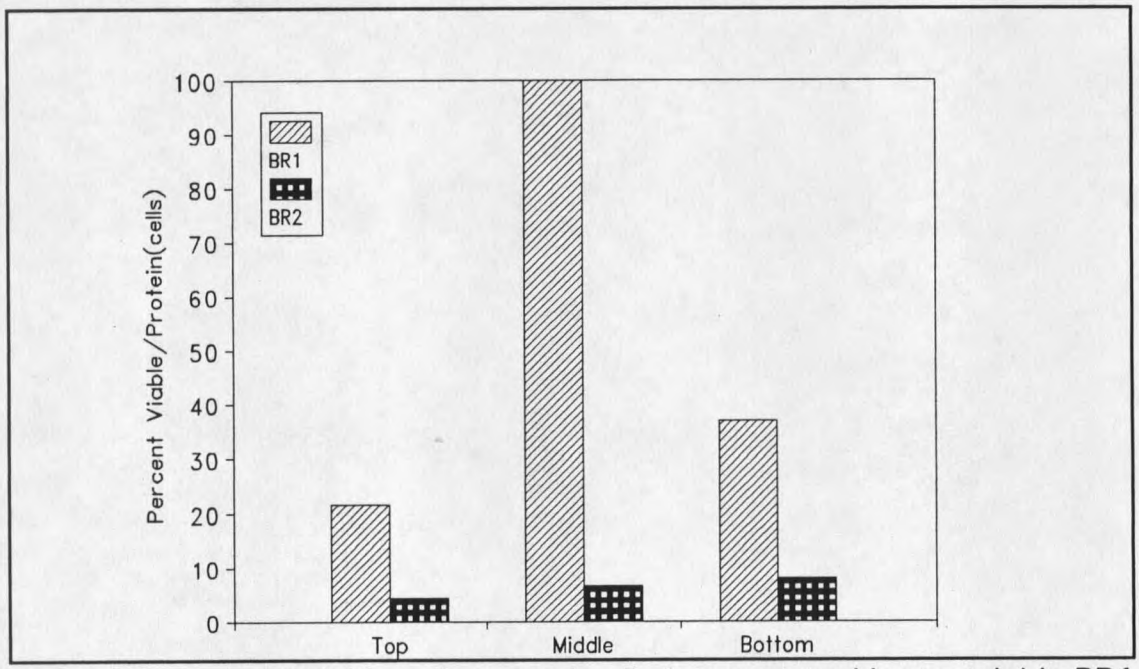

Figure 23. Percent viable cells to total cells (as measured by protein) in BR1 and BR2.

The percent viability of total cells was estimated from the ratio of plate counts to cell counts extrapolated from protein measurements (Figure 23). It appeared that more of the protein in BR1 was from active cells while only a small percentage of that measured in BR2 represented viable cells. In the culture studies using *Pseudomonas putida* Idaho, Cruden et al. (1992) noted a discrepancy between the total amount of biomass present and viable cells. There were striking differences in turbidity, cell dry weight, and numbers of viable cells. After 48 hours, turbidity and dry cell weight measurements for cells grown with 20% (v/v) *p*-xylene were almost twice the values observed with benzoate. In contrast, the number of viable cells present in cultures grown with *p*-xylene was 2.2×10^6 cells per ml compared with 4.8×10^9 cells per ml for cells grown with benzoate. The number of viable cells present during exponential growth with *p*-xylene gave values in the range of 5×10^5 to 5×10^8 cells per ml. Cell viability decreased after cells entered the stationary phase even though cell mass continued to increase. The decrease in cell viability occurred 4 to 5 hours after the cells entered the stationary phase of growth. Transmission electron micrographs prepared from cells grown with *p*-xylene showed profound damage to the membranes and increased membrane synthesis. The increased dry weights of cultures relative to viable counts may be due to the membranous material shed into the culture fluid as well as to continued growth of a small proportion of the cells after the majority of the population enters stationary phase or is killed by the solvent (Cruden et al.,

1992). The low number of viable cells relative to protein measurements in BR2 may be an indication of biomass that had been active in xylene degradation but, due to injury or death, was no longer viable. The resistance of *P. putida* to solvents may be due to an ability to synthesize membranes rapidly to compensate for those damaged by the solvent or to some biochemical difference in the cytoplasmic membrane which makes it more stable in the presence of solvent (Cruden et al, 1992).

CONCLUSIONS

The experiments described in this thesis were conducted at the Engineering Research Center. Two vapor phase reactors were designed for the degradation of *p*-xylene using *Pseudomonas putida* Idaho as the inoculum. Realizing the inherent complexity and variability of biological systems, these conclusions pertain specifically to the foregoing experimental design.

1. Total mass degradation rates were significantly higher in the D.E. pellet column than in the glass sphere column. Both bioreactors maintained maximum degradation rates even after the loss of pure culture.

2. When normalized to total exterior packing surface area, the D.E. pellet reactor continued to support a higher substrate flux than the glass sphere reactor at comparable loading rates and at less than 2 mm Hg of pressure drop.

3. Protein levels per unit surface area were significantly higher in the D.E. pellet column. Cell counts were comparable on the two substrata. TOC levels were significantly higher in the glass sphere column.

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APPENDICES

APPENDIX A

Growth Studies

Table 6. Growth Study. Raw Data.

Time (hrs)	G-1(cells/ml)	G-2(cells/ml)	G-3(cells/ml)
0	9.17E+4	1.18E+5	8.38E+4
18	3.0E+5	5.37E+5	2.9E+5
20.5	1.3E+6	9.7E+5	6.7E+5
22.5	3.36E+6	3.69E+6	2.26E+6
25.5	1.18E+7	1.58E+7	8.9E+6

Table 7. Chemostat Study. Raw Data.

TC-mg/l C (feed)	TOC-mg/l C (cells)	Yield (X/S)
Dilution Rate = .012 hr		
71.7	19.6	.274
68.2	16.8	.246
65.7	16.6	.253
62.7	17.4	.278
Dilution Rate = .024/hr		
65.1	16.5	.254
66.2	16.1	.244
66.8	19.2	.287
73.6	17.2	.233
Dilution Rate = .036/hr		
72.1	22.1	.307
83.9	25.5	.304
75.0	19.7	.263
88.7	17.5	.197
75.6	17.9	.236
71.3	18.6	.261

APPENDIX B

Reactor Biomass Analyses

Table 8. Carbon analysis for BR1. Raw Data.

# pieces	susp. (ml)	TOC (mg/l)
Top		
1	10	1151
1	10	1066
1	10	1054
Middle		
1	10	1123
1	10	1081
1	10	1191
Bottom		
1	10	1116
1	10	1139
1	10	1132

Table 9. Carbon analysis for BR2 (T-top,M-middle,B-bottom). Raw Data.

# pieces	susp. (ml)	TOC mg/l
T-3	20	642
T-3	20	630
T-3	20	676
T-3	20	648
T-3	20	638
M-3	20	107
M-3	20	118
M-3	20	102
B-3	20	190
B-3	20	190
B-3	20	179
T-3	30	368
T-3	30	369
T-3	30	359
M-3	30	55
M-3	30	50
M-3	30	93
M-3	30	80
M-3	30	85
B-3	30	140
B-3	30	138
B-3	30	145

Table 10. Viable cell plate counts. Raw Data.

Height	# pieces	ml susp	Dilution			
			10e-4	10e-5	10e-6	10-e-7
BR1						
Top	1	10		125/140	16/86/107	
Middle	1	10				58/84/36
Bottom	1	10			34/120	
BR2						
Top	3	20		159/193	30/46/43	
Middle	3	20		186/175	19/18/21	16/7
Bottom	3	20			65/56	15/11/6
Pellet Section						
Top-Inner		10	139	92	23	
Top-Outer		10	165			
Bot-Inner		10		178		
Bot-Outer		10		448		

Table 11. Protein analysis. Raw Data.

Height	#pieces	susp (ml)	μg/piece
BR1			
Top	1	10	164
Middle	1	10	443
Bottom	1	10	158
BR2			
Top	3	30	340
Middle	3	30	340
Bottom	3	30	523
BR2			
Top	3	20	349
Middle	3	20	349
Bottom	3	20	523