



Physiological and environmental factors affecting biofilm formation and activity in vapor phase bioreactors
by Raj Mirpuri

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in
Chemical Engineering in
Montana State University
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Abstract:

Vapor phase bioreactors (VPBRs) are being used increasingly to remediate vapor streams contaminated with volatile organic compounds (VOCs). VPBRs are packed columns of inert materials which support growth of biofilms. Physiological and environmental factors influence biofilm formation and activity significantly within VPBRs and these factors were investigated in the present dissertation. Throughout this investigation, monocultures of *P. putida* 54G were grown on toluene as the sole exogenous source of carbon and energy in planktonic and in biofilm cultures.

Kinetics of toluene degradation by suspended and biofilm cells were similar when specific activity (mg toluene degraded/unit biomass quantity-hr) was based on numbers of uninjured cells rather than total biomass. When based on total biomass, specific activity was lower for biofilm than for suspended cells, and may have been a result of prolonged toluene exposure.

Planktonic culture studies indicated that physiological stress and cell injury affected cell growth and bacterial respiration rates. Bacterial injury increased with increase in toluene concentration and duration of toluene exposure and coincided with formation of unconverted intermediates. Rate expressions for injury and irreversible loss of toluene degradation pathway were computed by fitting a theoretical injury model to experimental results.

In a flat plate VPBR, results from oxygen concentration profiles, microfluorimetry and cryosectioning combined with fluorescent cell staining indicated that stratified layers of respiratory activity formed in toluene-degrading biofilms with considerable activity observed at the base of the biofilm. This observation was in contrast to conventional biofilm models which indicate that the majority of the respiratory activity is located at the biofilm-liquid interface in thick biofilms.

Microscale parameters determined from planktonic cell studies and measured physical and chemical characteristics were used to calibrate a mathematical process model. The process model accurately predicted flat plate and column VPBR performance. A sensitivity analysis conducted on predicted results indicated that parameters adjusted to fit the model (biomass density and death and endogenous decay rates) showed a negligible effect on toluene degradation while Henry's law constant for toluene, biotransformation kinetics and reactor surface area affected toluene degradation significantly. Since these parameters were estimated accurately, confidence in predicting VPBR performance increased considerably.

PHYSIOLOGICAL AND ENVIRONMENTAL FACTORS AFFECTING BIOFILM
FORMATION AND ACTIVITY IN VAPOR PHASE BIOREACTORS

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies

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ABSTRACT

Vapor phase bioreactors (VPBRs) are being used increasingly to remediate vapor streams contaminated with volatile organic compounds (VOCs). VPBRs are packed columns of inert materials which support growth of biofilms. Physiological and environmental factors influence biofilm formation and activity significantly within VPBRs and these factors were investigated in the present dissertation. Throughout this investigation, monocultures of *P. putida* 54G were grown on toluene as the sole exogenous source of carbon and energy in planktonic and in biofilm cultures.

Kinetics of toluene degradation by suspended and biofilm cells were similar when specific activity (mg toluene degraded/unit biomass quantity-hr) was based on numbers of uninjured cells rather than total biomass. When based on total biomass, specific activity was lower for biofilm than for suspended cells, and may have been a result of prolonged toluene exposure.

Planktonic culture studies indicated that physiological stress and cell injury affected cell growth and bacterial respiration rates. Bacterial injury increased with increase in toluene concentration and duration of toluene exposure and coincided with formation of unconverted intermediates. Rate expressions for injury and irreversible loss of toluene degradation pathway were computed by fitting a theoretical injury model to experimental results.

In a flat plate VPBR, results from oxygen concentration profiles, microfluorimetry and cryosectioning combined with fluorescent cell staining indicated that stratified layers of respiratory activity formed in toluene-degrading biofilms with considerable activity observed at the base of the biofilm. This observation was in contrast to conventional biofilm models which indicate that the majority of the respiratory activity is located at the biofilm-liquid interface in thick biofilms.

Microscale parameters determined from planktonic cell studies and measured physical and chemical characteristics were used to calibrate a mathematical process model. The process model accurately predicted flat plate and column VPBR performance. A sensitivity analysis conducted on predicted results indicated that parameters adjusted to fit the model (biomass density and death and endogenous decay rates) showed a negligible effect on toluene degradation while Henry's law constant for toluene, biotransformation kinetics and reactor surface area affected toluene degradation significantly. Since these parameters were estimated accurately, confidence in predicting VPBR performance increased considerably.

CHAPTER 1

GOAL AND OBJECTIVES

Motivation: Vapor phase bioreactors (VPBRs), or biofilters as they are more commonly known, have been in use for more than twenty five years in Europe while their use as an alternate air pollution control technology is at an early phase of development in the U.S. Success in applying VPBR technology to controlling vapor phase volatile organic compounds (VOCs), such as TCE and toluene, depends on combining laboratory-scale studies with VPBR mathematical models which incorporate microscale and mesoscale phenomena for predictive purposes. At the microscale, it is important to study the effect of VOC degradation on the physiological and genetic response of bacteria and to investigate the kinetics of VOC degradation by biofilm-bound cells. Measurement of such processes can significantly increase confidence in the predictive capabilities of VPBR models, and can aid in identifying those parameters which most affect reactor performance.

Goal: Quantify microscale process dynamics of the progression of physiological state and activity during the biodegradation of toluene by *Pseudomonas putida* 54G in a vapor phase bioreactor.

Objectives: The objectives of the dissertation are detailed below:

- Determine the physiological response of suspended and biofilm cultures of *Pseudomonas putida* 54G during toluene degradation.
- Compare suspended and biofilm cell kinetics for toluene degradation by *P. putida* 54G to determine if suspended cell kinetics can be used to model bacterial activity in VPBRs.
- Determine stratification in respiratory activity (by using microscopy combined with cryosectioning) and oxygen concentration (by oxygen microsensors) in *P. putida* 54G biofilms during toluene degradation in a flat plate vapor phase bioreactor.
- Incorporate kinetic, stoichiometric, injury and genetic loss coefficients determined from suspended cell studies and measured values of Henry's law constant for toluene and gas-liquid and liquid-biofilm interfacial thicknesses into a predictive VPBR model.
- Assess the capabilities of the VPBR model for predicting performance of VPBRs under different loading rates during toluene degradation by *P. putida* 54G and determine which parameters affect reactor performance.

CHAPTER 2

INTRODUCTION

We live in a world in which the existence of carcinogens, mutagens and teratogens is taken for granted. Waste materials released from industry and agriculture are responsible for considerable contamination of soil, water and air in the United States. Groundwater and soils in many regions of the U.S. contain various volatile organic compounds (VOCs), rendering the water unsuitable for human consumption. Stringent air emission standards in the U.S. have mandated the need to develop innovative and cost efficient technologies to remediate vapor streams contaminated with volatile organic compounds. Such streams are often produced as a result of remediation activities for treatment of gasoline and solvent spills in groundwater aquifers. Several treatment alternatives are available to remove VOCs from groundwater and soils, including soil excavation and subsequent disposal in a hazardous waste landfill and either *in situ* or above-ground volatilization/stripping followed by physico-chemical treatment.

There are many physico-chemical methods available for VOC elimination from the vapor phase. Adsorption on activated carbon, condensation, incineration, and liquid scrubbing are methods that have been used in the chemical industry for recovery or

destruction of contaminants (8). Activated carbon is frequently used as an appropriate adsorbent for VOCs. A major disadvantage of this process is the saturation of the adsorbent which necessitates carbon regeneration. Routine disposal of the spent activated carbon adds a significant expense to remediation costs. Organic compounds can be incinerated at sufficiently high temperatures which also entail high energy costs. The combustion temperature can be considerably reduced if a catalyst is used, the choice of which determines the cost (35). Typical operating conditions that have been used to ensure 95 - 99% removal of VOCs from waste gases require a retention time of 0.3 - 1.0 seconds and temperature of 1000°C - 1400 °C (18). Condensation and liquid scrubbing, as the names suggest, are methods which result in a phase transfer of the contaminants to the liquid phase rather than elimination.

Biological treatment of contaminated vapor streams is an attractive alternative because it offers the potential to: (1) permanently eliminate contaminants through biochemical transformation or mineralization, (2) provide an environmentally friendly solution by avoiding harsh chemical and physical treatments and intensive energy use, (3) be cost-effective and (4) provide an easily operable technique (45). In recent years, many bacteria have been isolated which show a high specificity, high activity and tolerance towards volatile organic compounds (34). Use of microorganisms to remove pollutants is well established in the area of wastewater treatment. However, not until recently have biological technologies been seriously considered in the U.S. for removal of pollutants from anything other than aqueous streams (28). The concept of using microorganisms for the removal of toxic compounds such as petroleum hydrocarbon and odorous compounds

in the vapor phase is well established in the Netherlands and Germany (9). Biofiltration is a standard air pollution control technology in Europe but is not well recognized in the U.S. because of limited governmental support for research and development, lack of regulatory programs, lack of descriptions written in the English language and a lack of full-scale demonstration projects in specific industries (28, 30).

Biological treatment can eliminate hazardous compounds by biotransforming them into innocuous forms, degrading them by mineralization, or anaerobically transforming them to carbon dioxide and methane (22). Biological processes have the additional advantage of being carried out at or near ambient temperature and pressure (36). Some volatile components sufficiently resemble biogenic structures to be eliminated biologically. VOCs are eliminated by microbial activity because they serve as energy and/or carbon source for microbial metabolism. Other xenobiotics, termed recalcitrant or persistent compounds, possess such unnatural chemical structures that their biological degradation is usually too slow to be of practical significance. Extensive studies on microorganisms that degrade recalcitrant and xenobiotic compounds have been carried out and literature in this field is ever expanding. New metabolic pathways continue to evolve by mutations and exchange of genetic properties. Such naturally transmitted changes may lead to the development of enzymes which are capable of degrading xenobiotics and other refractory compounds. Once a bacterial strain or a mixed microbial culture capable of degrading a specific compound has been isolated, its practical application may generally be extended to the field of biodegradation in biofilters, bioscrubbers and trickling filters. The microbial population can either be freely

dispersed in the water phase or immobilized on an inert support. The former is carried out in bioscrubbers, the latter in biofilters and biological trickling filters.

Biofilters

A biofilter (Figure 2.1) can eliminate a broad range of inorganic and organic pollutants. In Europe, biofiltration has been successfully used to control odors and organic and inorganic air pollutants from a variety of industrial and public sources. A biofilter consists of a simply structured bed in which the flowing gas is contacted with an immobilized microbial flora which provides a high biological surface area. After sufficient time, a biofilm is formed on the filter material at the expense of the contaminant. Aerobic degradation of the contaminants in the filter reduces them to carbon dioxide, water and biomass. Continuous availability of water and transfer of oxygen from the vapor to the liquid phase are critical factors in the design of a biofilter. Maintaining the porosity of the compost by turning it over, and/or replacing it entirely, once spent, are the two major maintenance requirements for biofilters with compost-based filter materials (36).

Since inorganic nutrients are also required, natural materials such as compost, peat and bark are added. This type of a system provides a high biodegradation efficiency, up to 90% in some cases, with low operating costs. Carrier materials have been used for as long as five years before being discarded (8). Since the microorganisms cannot survive extreme temperatures commonly encountered in waste gas emissions, the gas temperature

is usually controlled before it is passed through the biofilter. Since the packing must be constantly wetted for continuous growth of biofilm, water quenching is used to achieve both objectives. Due to high porosity and low pressure drop of packing materials as well as an increased biological activity, high gas flow rates and high organic loads may be realized. Typical pressure drops in the region of 500 Pa/m are common for biofilters of mixtures of compost, peat and wood chips (8).

Until recently, most commercial biofilters have been built as single-bed systems that are open to the atmosphere. In open filters, the bed is exposed to fluctuations in weather conditions such as rain, sunshine and temperature which may directly influence the biological process resulting in higher pressure drop and channeling. However, there has been a shift towards closed systems because of their lower susceptibility to changing climatic conditions and the possibility of continuous off-gas monitoring. The increasing use of closed system biofilters has resulted in better control of humidity and temperature which are continuously monitored and recorded (8). The type of construction and installation of a biofilter for a given application usually depends on availability of space relative to the required filter volume. Biofilters can be operated in both a continuous and intermittent mode, based on the intended application. Pre-conditioning of the influent gas followed by transport to and distribution in the filter bed are other important components of a biofilter system. Table 2.1 lists some full scale applications of biofilters.

Bioscrubbers

Bioscrubbers (Figure 2.2) are mainly suited for the treatment of waste air streams of medium concentration with components of a high or moderate water solubility. The gas is contacted with water in a spraying tower containing inert packing, resulting in absorption of waste gas components into the water phase. Microorganisms are suspended in an aqueous solution. In contrast to biofilters the liquid phase in bioscrubbers is mobile, which allows a better control of reaction conditions. Nutrients and buffers can be added and the liquid can be replenished and discharged in order to remove undesired products. Better control with reference to temperature, pH and ionic strength can be achieved. Other advantages compared to filters include a rapid adaptability to changing crude gas compositions, the possibility of discharging reactants and an easier heat dissipation. In comparison to biofilters, they are more expensive, complicated to use, inappropriate for low water-solubility contaminants and provide a lower specific gas/liquid surface area (5).

The bioscrubber process can be divided into two steps - adsorption and regeneration. In the absorption phase, water soluble components of waste gases are transferred to the liquid phase by continuous contact in a contacting apparatus like a spray tower. At steady state, the mass transfer rate is equal to the elimination rate of VOCs which can be estimated by the product of an overall mass transfer coefficient, total specific contact area between the liquid and the gas phase and the concentration gradient. The overall mass transfer coefficient depends on the transfer resistance in the continuous

(gas) phase as well as the dispersed (liquid) phase. For an efficient absorber performance, the concentration of the compound in the liquid phase must be as low as possible (11).

In the regeneration phase, microbial oxidation of the absorbed compounds leads to their elimination from the liquid phase in an activated sludge reactor. The sludge tank is usually aerated by diffused air or mechanical stirring which also helps in keeping the sludge in suspension and increases contact between the liquid phase and the sludge.

Biological Trickling Filters

When biodegradability of contaminants is assured, the application of biofilters for treating contaminated vapor streams appears unlimited. However, problems may arise if acid metabolites are produced during the biological degradation of vapor phase contaminants. When the pH buffering of the packing material is effective for only a relatively short period of operation, the presence of a continuously flowing liquid phase is required for removal of the inhibiting metabolites or end products which may otherwise accumulate. This type of a situation is mainly encountered in the degradation of chlorinated hydrocarbons or reduced sulfur compounds.

In biological trickling filters, shown in Figure 2.3, the waste gas is forced to rise countercurrently through a column containing inert packing materials in order to obtain the greatest rate of absorption (21). Water soluble compounds are transferred to the liquid, from which they diffuse into the biofilm, growing on the packings, where they are eliminated by the constituent microorganisms. Inert materials used as a support media

include glass, plastics, ceramic and activated carbon. Their interfacial area/volume of reactor results in large void volume for gas flow which further leads to a reduced pressure drop. The flowing liquid allows for continuous control and adjustment of environmental physiological conditions which are necessary for optimal microbial activity (i.e. temperature, pH, and nutrients) as well as removal of acid metabolites.

A trickling filter is more adaptable for modeling purposes because inert manufactured packing material of distinct size and shape are used as support medium instead of natural materials that cannot be easily quantified. This is important for scale-up purposes especially when the results of a lab scale experiment are to be used in the design of a pilot plant. Trickling filters (packed column) also have the obvious advantage of maximizing bioavailability and limiting availability of waste products. The system can be modeled to quantify mass transfer characteristics independent of kinetics.

Literature Review on Biofilters and Biological Trickling Filters

Biofilters and biological trickling filters have been used for many different applications including odor control problems at wastewater treatment plants, ammonia and sulfur odors and VOC control for methanol, phenol, formaldehyde, ethanol, toluene, and benzene. Kirchner et al. (20) used trickle-bed filters to biodegrade important solvents and substances such as aldehydes, methyl ethyl ketone and ethyl acetate at an influent concentration ranging from 5 - 40 ppm. Using suitable bacterial strains they obtained conversions of 90% at space velocities of 1500 hr^{-1} and rate-determining parameters for

the biodegradation process were identified. Kirchner et al (21) obtained conversion rates of between 68 and 96% for propionaldehyde in trickle-bed biofilters. Ottengraf and van den Oever (33) and Ottengraf et al (35) investigated the removal of volatile organic compounds in biofilters and identified bacterial strains that could be used to convert many organic and inorganic compounds to mineral end-products. Kinetic models for the biodegradation process were developed to predict the elimination capacity of the biofilter bed. Diks and Ottengraf (7, 8) demonstrated the use of biological trickling filters for degrading dichloromethane from waste gases in concentration ranges of 0 - 10,000 ppm. They operated the filters in co- and counter-current mode and determined that flow direction did not significantly affect the elimination capacity of the bed. A simplified model, "Uniform-Concentration-Model", was developed and used to predict filter performance. Maximum dichloromethane elimination capacities of 157 g/(m³-hr) were obtained.

Apel et al. (2) applied gas phase bioreactors to treat vapor streams contaminated with methane, trichloroethylene (TCE) and p-xylene. Methanotrophic bacteria were used to process methane and TCE while a xylene resistant strain of *P. putida* was used to process p-xylene. For methane, the gas phase bioreactors demonstrated better efficiencies (almost twice) compared to sparged liquid phase bioreactors and conventional shaken cultures. At a feed rate of 140 µg of xylene min⁻¹, approximately 46 % of the xylene was mineralized to carbon dioxide in a single pass through a column scale gas phase bioreactor. Zilli et al (50) investigated the feasibility of biologically removing phenol from waste gases using a biofilter. During a one-year operation of a laboratory scale

biofilter, packed with peat and glass beads, degradation efficiencies of 93 - 99.6% were obtained for inlet phenol concentrations of 50 - 2000 mg m⁻³ with a residence time of 54 s.

Shareefdeen et al (41) used laboratory columns containing bacteria supported on mixtures of peat and perlite to biodegrade methanol vapors. Rates of up to 112.8 g-hr⁻¹ m⁻³ were measured in the columns. Mathematical models were developed to validate the experimental results and based on experimental data and model predictions, the methanol biofiltration process was shown to be limited by oxygen diffusion and methanol degradation kinetics. Baltzis and Shareefdeen (4) developed a model for predictive and scale-up calculations of a methanol vapor degrading biofilter. The model indicates that oxygen availability can be the rate limiting factor for the biodegradation process and can be used to predict the performance of an existing biofilter or in preliminary design of a new unit.

Shareefdeen and Baltzis (42) used the same model to predict steady and unsteady state toluene degradation in a peat/perlite biofilter. By incorporating kinetics for toluene degradation in suspended cell cultures and other experimentally determined parameters into a biofilter model, they satisfactorily predicted effluent toluene concentrations. Based on a sensitivity analysis conducted on predicted results, they concluded that an accurate estimate of biolayer surface area per unit volume was critical in modeling a biofilter.

A modular column vapor phase bioreactor has been in operation for three years at the Biotechnology Research Department at Orange County Water District (37). In the reactor a consortia of hydrocarbon degrading bacteria derived from a shallow gasoline-contaminated aquifer was established on the packing and grown on benzene, toluene, p-

xylene, hexane, cyclohexane, 2,2,4, trimethylpentane, octane and an inorganic minimal media. The maximum removal of contaminants occurred when conditions for unlimited microbial growth were present, and starvation for nitrogen (one of the components of the inorganic medium) significantly diminished contaminant removal. Groestijn and Hesselink (11) and Leson and Winer (28) have presented excellent reviews on applications of biofilters for industrial and domestic use.

Scope of the Dissertation

Despite a rapidly expanding literature in the field of biofilters during the last five years, this technology is still far from being applied commercially as an alternate air pollution control technology. Relatively few studies address interactions among different scales of biofilter operation nor do they consider how phenomenological effects can be integrated to make predictions of field-scale process behavior. The relative absence of practitioner-oriented tools for decision making suggests that a process engineering approach is necessary to improve the state-of-the-art of biofilter practice. Process engineering, in the context of biofilters, involves the integration of chemical and microbiological characteristics and laboratory and field data, in order to make predictions and design decisions. To increase the applicability of biofilters in the field, a scale-up approach is essential to understanding and interpreting complicated biofilm processes within a biofilter. A possible biofilter scale-up scenario would appear in this fashion:

- 1) investigate microbiological and engineering phenomena at the microscale

using ideal laboratory reactors such as batch, continuous stirred tank and flat plate reactors or laboratory-scale biofilters,

- 2) calibrate a computer model that describes biofilter performance at the microscale, utilizing parameters determined from microscale studies and predict toluene degradation at this scale,
- 3) conduct mesoscale biofilter performance experiments using column laboratory reactors,
- 4) predict mesoscale reactor performance using the biofilter model and determine parameters which affect biofilter performance significantly and
- 5) utilize information from mesoscale reactors and the predictive biofilter model to design a pilot-scale system.

Figure 2.4 shows the focus of this dissertation designated as VPBR project, which attempts to address questions 1 through 4. Throughout this dissertation the term vapor phase bioreactors (VPBRs) is used to describe a biofilter containing inert manufactured packing material which support biomass growth and promote VOC degradation. The integration of different phenomena during scale-up are explained in the following.

Planktonic and Biofilm Cell Kinetics: Scale-up of VPBRs can best be achieved through use of predictive models, the success of which depends on accurate estimates of kinetic and stoichiometric coefficients. Most bioreactor models that are developed for predictive purposes incorporate planktonic cell kinetics to model substrate degradation and biofilm growth. No clear consensus is available

relating biofilm and suspended cell kinetics. Fletcher and Marshall (10), van Loosdrecht et al (47), Karel et al (17), Hamilton and Characklis (13) and Hamilton (12) have published extensive review articles that have shown both increases and decreases in activity for attached cells in comparison to freely suspended cells. Cellular processes involved during VOC removal by free and attached bacteria could be different from degradation of benign substrates such as glucose or acetate. LaMotta (25), Harremoës (14), Rittmann and McCarty (40), Skowlund and Kirmse (43) and Skowlund (44) are among many researchers that have developed mathematical models to evaluate steady-state biofilm kinetics but, in contrast very few studies on biofilm-mediated degradation kinetics of VOCs exist. Alvarez et al (1) and Arcangeli and Arvin (3) have presented information on toluene degradation kinetics without relating suspended and biofilm cell kinetics. With such conflicting information on kinetics and a need to understand degradation kinetics of VOCs such as toluene, the relation between kinetics of free and biofilm cells is important to understand. Chapter 3 titled "A Comparison of Toluene Degradation Kinetics by Planktonic and Biofilm Cells of *P. putida* 54G" details a comparative study for suspended and biofilm cells of *P. putida* 54G during toluene degradation.

Physiological Changes: Injury has been defined as the sub-lethal physiological structural consequence(s) resulting from exposure to injurious factors within aquatic environments (31, 32). This is reflected by the inability of

injured cells to reproduce under selective or restrictive conditions that are tolerated by uninjured cells. Although there is overwhelming evidence to indicate presence of stressed or injured bacteria in most environments, very few studies exist on the topic of hydrocarbon-related injury.

Tebbe et al (46) have determined that a majority of naphthalene- degrading microbes that they investigated only expressed their genotype after non-selective isolation while some organisms did not express their genotype at all. Love and Grady (29) determined that when continuous cultures of *P. putida* were grown on benzoate and m-toluate and transferred to plates containing the same medium, they lost culturability in comparison to continuous cultures grown on glucose. Ridgway (39) determined a similar phenomena wherein a stressed subpopulation of viable hydrocarbon-degrading bacteria from a gasoline-contaminated aquifer led to an underestimation of true viable-hydrocarbon degrading bacteria. Leddy et al (27) determined that *P. putida* 54G cells, when grown on vapor phase toluene for 10 - 15 days, formed Tol- mutants that showed a selective loss of catabolic functions. These mutants could not degrade toluene but continued to grow in the presence of toluene, metabolizing other carbon sources such as organic compounds leaking from wild-type cells.

In this context, it is important to determine how stress response can be related to the ability/efficiency of a microorganism to degrade hydrocarbon vapors. This information when obtained in a well defined system could be utilized to formulate rate expressions based on growth of stressed and injured

cells. Establishment of quantitative rate relationships would permit incorporation of stress response into process models, leading to a superior ability to design and control bioremediation systems. Chapter 4 titled "Physiological Stress and Injury in Batch Cultures of *P. putida* 54G during Toluene Degradation" covers the topic of stress and injury related to toluene degradation by *P. putida* 54G.

Stratification of Respiring Cells: Quantitative information on stratification of cell layers in biofilm reactors used in the degradation of volatile, potentially toxic, organic compounds such as toluene is non-existent. For over 25 years, researchers have presumed that the entire mass of attached microorganisms in fixed-film reactors was not uniformly active but different layers of activity developed within thick biofilms (15, 23). Many different techniques have been used to study the spatial and temporal variations of activity in biofilms (16, 19, 24, 26, 48, 49) but information on stratification of respiratory activity in toluene degrading biofilms has never been published. Toluene might cause injury to the layer of biofilm cells that are at the fluid/biofilm interface with active cells respiring in the depths of the biofilm. Clearly such a phenomena could exist in VPBRs degrading toluene and could effect the predictive capabilities of VPBR models if they are not accounted for. This study was carried out in a flat plate VPBR as detailed in Chapter 5 titled "Spatial Distribution of Respiratory Activity in *P. putida* 54G Biofilms during Toluene Degradation". In this study, oxygen profiles in the vapor, liquid and biofilm phases were measured using microsensors

and coupled with cryosectioning and fluorescent microscopy results to assess stratification of respiratory activity in *P. putida* 54G biofilms.

Process Model: Results obtained from the microscale experiments were used to calibrate a VPBR model which was developed by Peter Reichert at EAWAG, Switzerland (38). Measured values of kinetic and stoichiometric coefficients (Chapter 3), injury and irreversible loss coefficients (Chapter 4), were combined with Henry's law constant for toluene and boundary-layer thickness at the gas/liquid and liquid/biofilm interface. The model is used to predict toluene degradation along the length of the reactor and overall toluene degradation in a flat plate VPBR. Only three parameters are adjusted to fit the experimental results: biomass density, death and endogenous decay rates. A sensitivity analysis was conducted on predicted results to determine which parameters affected toluene degradation in the flat plate VPBR. This modeling approach provided a rational design for predicting an upper limit of toluene degradation in a VPBR. Chapter 6 titled "A Predictive Model for Toluene Degradation in a Flat Plate Vapor Phase Bioreactor", details the calibration of the process model from microscale experiments and the oxygen profiles from the flat plate VPBR experiment. Model results are compared with actual degradation measured in a flat plate VPBR operated under two different influent vapor phase toluene concentrations of 150 and 750 ppm. A section which includes the verification of column VPBR performance operated under the same conditions as the flat plate

VPBR except for a significantly higher flow rate and surface area has been added to Chapter 6. This chapter includes a detailed description on a sensitivity analysis conducted on parameters that affect column performance and a comparison of parameters that affect toluene degradation significantly at the micro- and mesoscale.

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Table 2.1. Biofilter applications for off-gas treatment in European industry (Adapted from van Groenestijn and Hesselink, 1993)

Compound	Industry type	Elimination efficiency(%)	Flow rate (m ³ h ⁻¹)	Volumetric load (h ⁻¹)
Odor	Animal rendering	94-99	214,000	66
Odor	Vegetable oil	97	39,000	120
Odor	Cocoa roasting	>99	4,000	73
Odor	Wastewater treatment	80-90	5,000	7
VOCs	Storage tanks	90	2,000	8
VOCs	Industrial wastewater treat.	70-90	65,000	31
VOCs	Fish processing	95	6,300	105
VOCs	Fish processing	85	10,300	184
H ₂ S	Landfill gas	>99	300	17
Alcohols	Foundry	>99	30,000	150
Aromatics	Foundry	80	40,000	120
Styrene	Resins processing	65	pilot plant	100
Phenol	Phenol resins	97	pilot plant	200

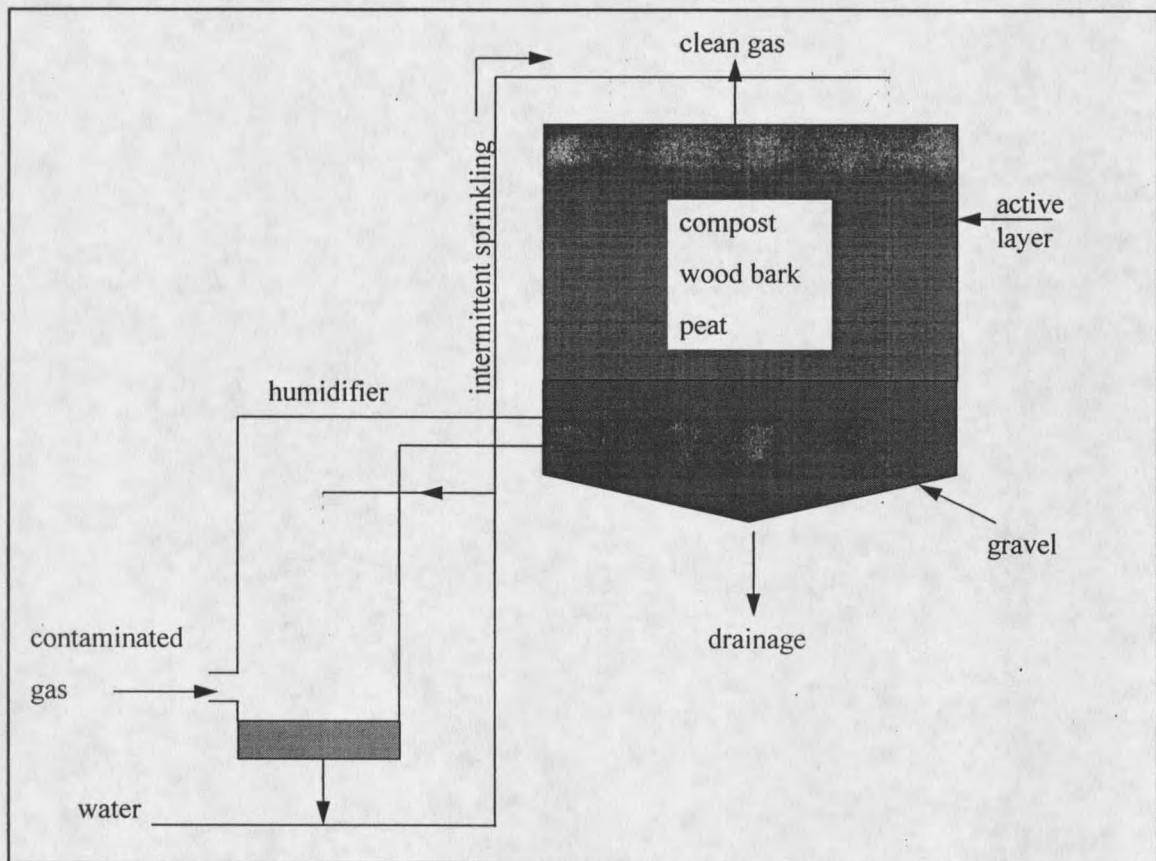


Figure 2.1. Schematic of a biofilter.

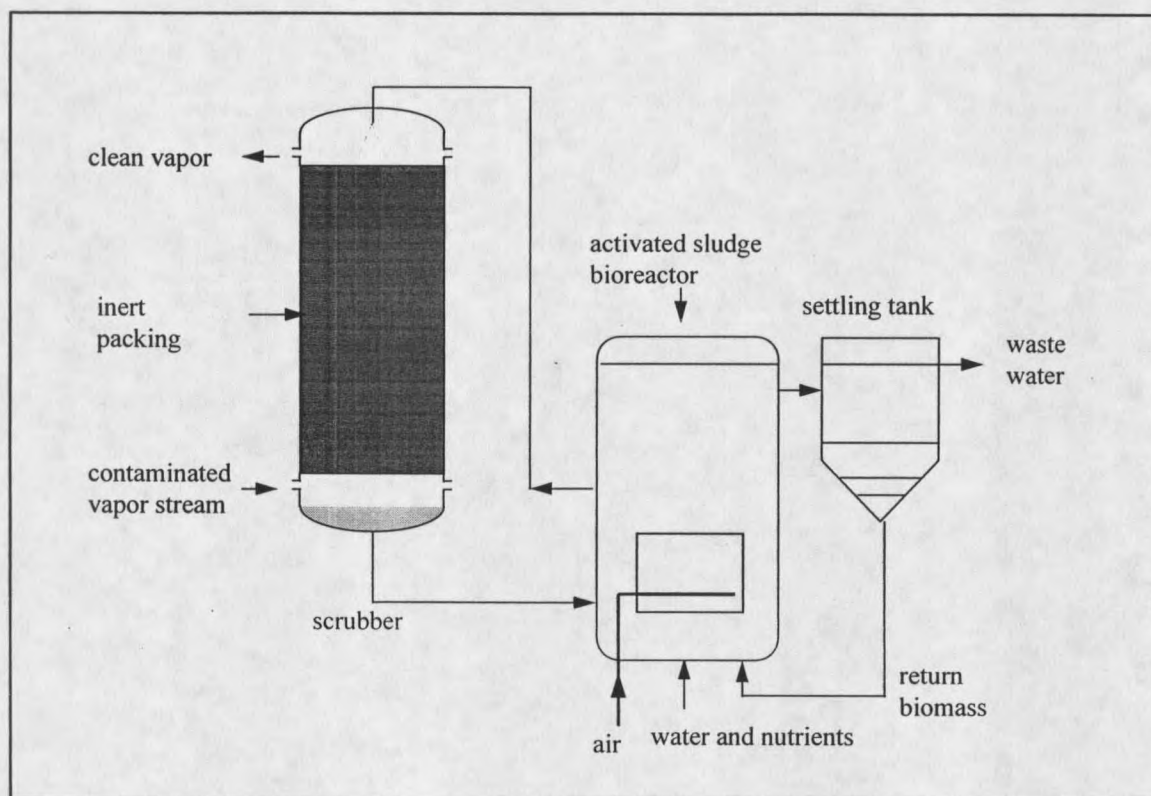


Figure 2.2. Schematic of a bioscrubber

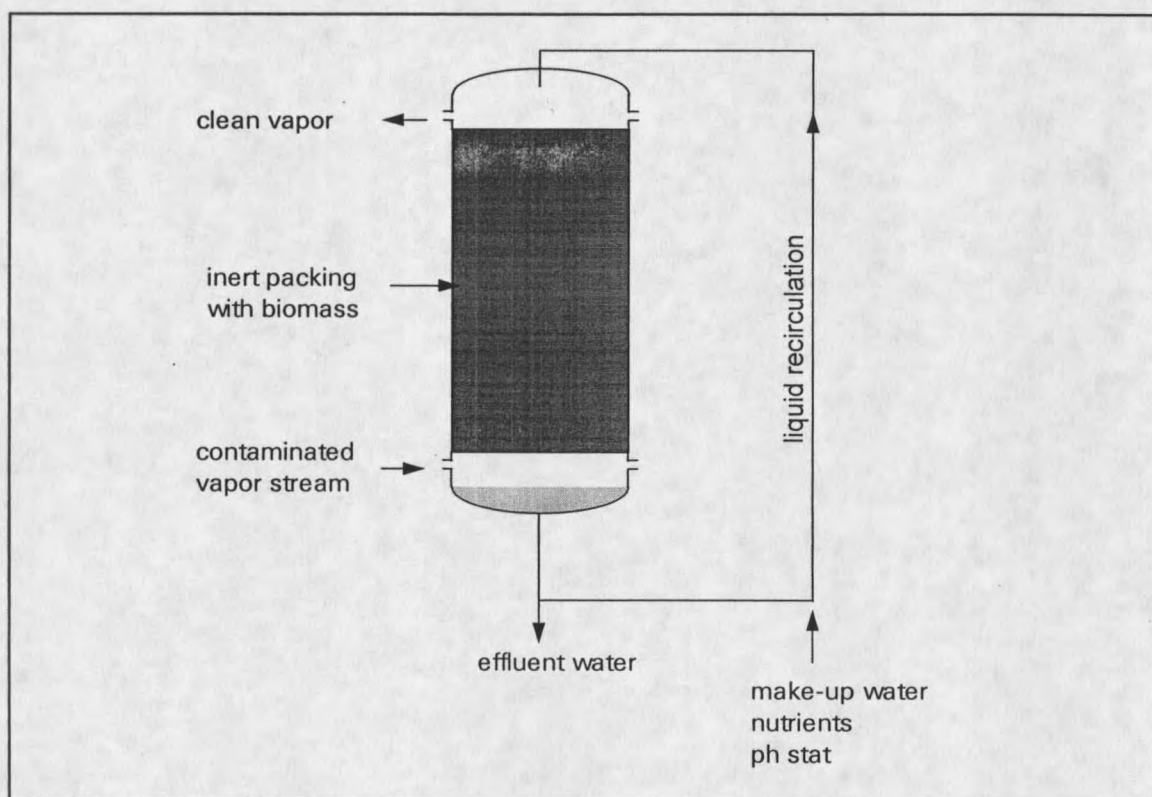


Figure 2.3. Schematic of a biological trickling filter.

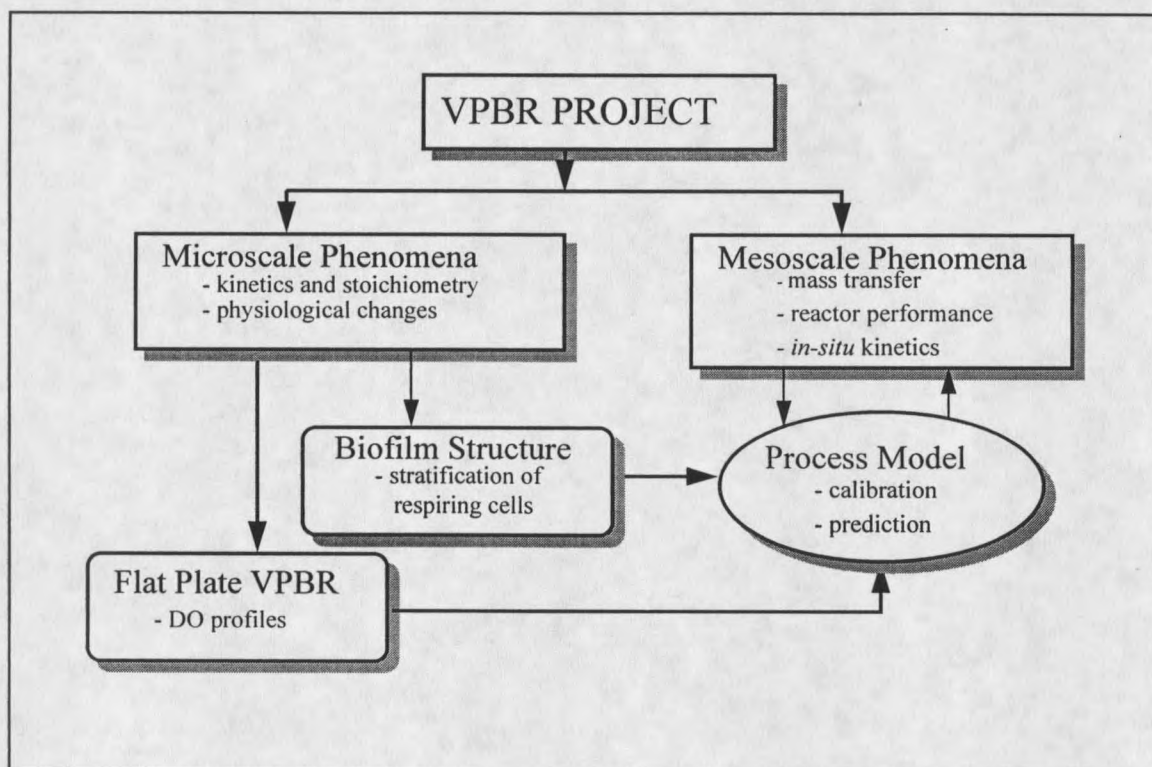


Figure 2.4. Schematic of the VPBR project.

CHAPTER 3

A COMPARISON OF TOLUENE DEGRADATION KINETICS BY
PLANKTONIC AND BIOFILM CELLS OF *P. putida* 54GIntroduction

Air emissions of volatile organic compounds (VOCs) have been the subject of stringent environmental regulations during the last decade. Applications of vapor phase bioreactors (VPBRs) to treat contaminated vapor streams have increased considerably because they offer a low-cost and a low maintenance alternative to other air pollution control technologies. VPBRs use biofilms (attached bacteria) to degrade volatile contaminants, and performance of these reactor systems is the ultimate result of a complex interaction between transport of rate-limiting nutrients to bacteria within the biofilm and intrinsic reactions within the biofilm. In contrast to planktonic (free) cells, biofilms attach to surfaces producing extracellular polymers and metabolizing nutrients that are transported to the biofilm. Results from laboratory-scale VPBRs have been published for the removal of VOCs such as methanol (38), phenol (42), benzene (6), toluene (6, 22), dichloromethane (10, 11), propionaldehyde (22), and trimethylamine (30).

Scale-up of VPBRs can best be achieved by use of predictive models which depend on accurate estimates of kinetic and stoichiometric coefficients. A majority of VPBR models that have been reported in the literature use planktonic cell kinetics to predict biofilm growth rates under the assumption that kinetic values are valid for biofilm cells (6, 10, 11, 30, 38). Effects of surface interaction and EPS production might lead to differences in free and attached cell kinetics.

Hamilton and Characklis (16) have reported that activities of cells in biofilms were likely to differ qualitatively from those of planktonic bacteria, and, in assessing relative activities, it is necessary to identify and evaluate important cellular processes involved in bacterial growth. For an accurate understanding of molecular mechanisms associated with the analysis of kinetic and stoichiometric coefficients, they suggested that the following should be considered:

- (1) role of EPS associated physicochemical and biological variables within the biofilm and
- (2) *in-situ* measurements of fundamental cellular processes supported by assays of relevant metabolic activities

Whether the activity of biofilm and planktonic cells are different is debatable. This topic has been considered in some detail by Fletcher and Marshall (12) who concluded that both increases and decreases in activity were observed for surface-attached cells relative to planktonic cells. Karel et al (20) reported that under certain conditions, metabolic activity of adhered cells was different from planktonic cells but there was no consistent method of predicting how the behavior would be different. Since in many

cases no difference in activity was observed, they suggested that activity data obtained for planktonic bacteria could be used to model the behavior of attached cells.

Hamilton (15) reported that attached cells were generally assumed to be at an advantage, but experimental evidence for this advantage or a related increase in metabolic activity was inconclusive. In an exhaustive review on the effect of interfaces on microbial activity, van Loosdrecht et al (41) similarly concluded that while a qualitative consensus existed to support the theory that attachment to surfaces influenced bacterial metabolism, experimental observations had been inconsistent. They observed that bacterial growth and substrate metabolism depended on the nature of the organism and the substratum, and concentration of substrate. Any resulting differences between adhered and free cells could be attributed to a modification of the surroundings of the cells due to the presence of surfaces.

Bakke et al (4) studied the activity of biofilm cells of a monoculture of *P. aeruginosa* and compared kinetic parameters with those obtained by Robinson et al (37) for planktonic cells of the same bacterium. They concluded that kinetics derived for planktonic cells closely represented biofilm cells. Keen and Prosser (21) reported that attachment of *Nitrobacter* cells to surfaces increased their growth rates by 25%. McFeters et al (27) concluded that sand-associated biofilm bacteria adapted more quickly and had a greater degradative activity for nitrilotriacetate than planktonic cells. Jefferey and Paul (19) reported that specific growth rates decreased when bacteria attached onto surfaces because of loss of cell surface available for substrate uptake. Harms and Zehnder (17) concluded that free and attached-cells of *Sphingomonas* sp. Strain HH19k

exhibited different activities during dibenzofuran uptake. Deretic et al (8, 9) and Martin et al (25) have recently hypothesized that planktonic-biofilm transformation is controlled by a σ factor, akin to control of sporulation in gram-positive bacteria, which might lead to a phenotypic change in a large cassette of genes for biofilm bacteria, that would result in a phenotypically distinct expression of the genome.

Based on these studies, differences in substrate utilization kinetics of free and attached bacteria seem to be system (system and substrate) dependent. Although mathematical models to evaluate steady-state biofilm kinetics have been documented (18, 23, 35, 39, 40), very few studies on biofilm-mediated degradation kinetics of VOCs exist. Arcangeli and Arvin (3) studied the biodegradation of toluene (sole carbon and energy source) by biofilm cells. Cells were grown in annular reactors which provided ideal conditions for biofilm growth. They determined that maximal toluene removal in biofilms occurred within 20 - 30 hours of attachment to the substratum. Concomitant with a reduction in toluene removal with time, they estimated that the active biomass was only about 5% of the biofilm biomass. Similar results were obtained by Diks and Ottengraf (11) for the degradation of dichloromethane in a VPBR. In both studies, mass transfer limitation was not a factor.

With such conflicting information available, we need to understand the relation between VOC degradation by free and attached cells. Clearly, cellular processes involved during VOC removal by free and attached bacteria could be different from the degradation of benign substrates such as glucose or acetate. Bacterial injury, defined as a physiological or genetic response to a sublethal environmental effect (26), due to toluene

degradation has been observed by Ridgway (33) by *P. putida* 54G. Leddy et al (24) have reported the formation of TOL- mutants of *P. putida* 54G, which lose their toluene degradation capability on exposure to toluene.

Before kinetics of toluene degradation by free and attached cells are compared, a basis for this comparison which would be absolute (normalized) from a practical standpoint should be chosen. Specific activity, defined as the rate of substrate removal per biomass or cells, provides an absolute scale for evaluation of substrate uptake rates of free and attached cells. Also, the use of a pure culture biofilm provides the opportunity to elucidate the fundamental processes leading to accumulation and growth without the confounding factors of population dynamics and species interaction.

We hypothesize that suspended and biofilm cells of *P. putida* 54G exhibit similar toluene degradative capabilities, when kinetic parameters are expressed on the appropriate cellular basis. In addition, the specific activity for biofilm and planktonic cells decreases during long term toluene exposure. The increased exposure to VOCs injures *P. putida* 54G which in turn increases the inactive biomass fraction. In the present investigation, kinetics of toluene degradation by planktonic and biofilm cells of *P. putida* grown on toluene as a sole source of carbon and energy are compared.

Materials and Methods

Bacterial Strain: *Pseudomonas putida* 54G, a toluene-degrading bacterium, was isolated from a gasoline contaminated aquifer at Seal Beach, California. The isolate was capable of growth on HCMM2 mineral salts media (containing only inorganic compounds) in the presence of vapor phase toluene and on complex carbon sources such as R2A medium. HCMM2 mineral salts medium was used as the liquid inorganic nutrient medium as described elsewhere (34).

Enumeration of Viable Cells: Growth media employed in this investigation were:

- (1) R2A plates
- (2) toluene vapors supplied continuously to cells on HCMM2 plates in sealed containers incubated at room temperatures, designated as HT plates.

P. putida 54G cells grown on toluene as the sole source of carbon and energy were enumerated for viable cells on R2A and HT plates. The difference between R2A (non-selective) and HT (selective) plate counts was used to determine injured cells according to (26):

$$\text{Injury \%} = \left(\frac{\text{Non-Selective Counts} - \text{Selective Counts}}{\text{Non-Selective Counts}} \right) \times 100 \quad (1)$$

Protein Assay: Levels of suspended cell biomass were quantified using the BCA Protein Assay Reagent (31). Bovine-serum albumin served as a standard. Protein

amounts were determined in units of $\mu\text{g/ml}$ and converted to biomass by using a multiplication factor of 2 (28).

Toluene Sampling: Gas samples were analyzed for toluene using a HP 5890 Series II GC equipped with a FID detector and an Alltech 0.1% A1-1000 Graphpac 80/100, 6' x 1/8" x 0.085", S.S. column. The column was operated at a constant temperature of 140°C. A sample (250 μl) of headspace was withdrawn and injected into the GC. Toluene concentration in the liquid was determined by combining 1 ml of the aqueous samples with 0.5 ml of hexane and injecting 2 μl of the hexane phase into a HP 5890 Series II GC equipped with a PID detector and a DB-624 J & W Scientific megabore capillary column, 30 m x 0.53 mm i.d and temperature ramped from 140°C - 200 °C. Helium was used as the carrier gas in both columns at a flow rate of 30 ml/min.

Ion Chromatography: Nitrate, phosphate and sulfate ion concentrations in HCMM2 medium were determined using an ion chromatograph. Aqueous samples were filtered and diluted in autoclaved water (double-distilled). Aliquots (15 μl) of the diluted sample was injected into a Dionex ion chromatograph (model AI-450; Danes Co., San Francisco, CA) equipped with a pulse electrochemical detector (model DX-300) and a Dionex Ionpac AS4A-SC column (2mm, column temperature = ambient).

Biofilm Thickness Measurement: Biofilm thickness was measured using a BIO-RAD MRC600 confocal laser (Kr/Ar) system with an Olympus BH2 microscope and a

MS plan 10X objective. Ceramic rings were mounted on a sample holder which was filled with liquid HCMM2 medium previously stained with 10 mg/l propidium iodide.

Kinetic Measurements: Three different studies were conducted. The relevant information including physiological state of the bacteria, parameters analyzed and duration of study are summarized in Table 3.1.

a) Planktonic cell kinetics. In the planktonic cell study, liquid HCMM2 medium was combined with ^{14}C toluene (56.2 mCurie/mmole, Sigma Chemical Company, St. Louis, MO) and *P. putida* 54G cells, previously exposed to toluene vapors, in 26 ml Supelco glass vials. Vials were sealed using Teflon-lined rubber septa and an aluminum cap. Total liquid in each vial was 14 ml allowing sufficient headspace such that oxygen was not stoichiometrically limiting. Labeled toluene was dissolved in autoclaved water (double-distilled) and combined with HPLC-grade toluene (Fisher Scientific Company, Fair Lawn, NJ) in the glass vials to final concentrations ranging from 1 - 50 mg/l. Vials were incubated in a water bath at 24°C with stirring. Controls were prepared without bacteria to determine abiotic losses and were used to determine actual toluene removal.

At timed intervals three biotic samples and one abiotic control were removed from the water bath and transferred to a chemical hood to determine rate of ^{14}C toluene degradation and rates of ^{14}C CO_2 and ^{14}C biomass production. For each biotic sample, three scintillation vials were labeled A, B and C, respectively. One ml of NaOH (16 M) was introduced into vials A and B and 1 ml of HCL (6 N) acid was introduced into vial C. An aliquot of 0.5 ml of aqueous sample was withdrawn from a biotic vial, introduced into

scintillation vial A, and combined with 6 ml of scintillation cocktail (Scintiverse II, Fisher Scientific Company, Fair Lawn, NJ). A 0.5 ml aliquot of the same sample was placed in vial B and air-stripped for 5 minutes. The resulting solution was combined with 6 ml of scintillation cocktail. Similarly, 0.5 ml of the same sample was placed in vial C, air stripped for 5 minutes and combined with scintillation cocktail. This procedure was repeated for the other two biotic samples while only vials A and B were used for the abiotic controls. Radioactivity (^{14}C) in the resulting 11 (9 biotic and 2 abiotic) vials for each sample time were analyzed using a 2200CA Tri-Carb liquid scintillation analyzer (Packard Instrument Company, Laguna Hills, CA).

Quenched standards were run weekly to produce a standard curve used to correct for quenching. HCMM2 minimal medium in scintillation cocktail served as a blank to determine any background activity which was subtracted from the results of all other measurements. Radioactivity (^{14}C) obtained as disintegrations per minute (DPMs - measured by the scintillation counter) was converted to mg/l of carbon by determining DPMs for known concentration of labeled toluene. Mass balance on ^{14}C activity resulted in the following equation: total ^{14}C toluene introduced = ^{14}C toluene unreacted + ^{14}C CO_2 produced + ^{14}C biomass produced. The results obtained from scintillation vials were analyzed according to (all activity terms denote radioactivity due to ^{14}C):

- (a) ^{14}C activity in vial A = activity due to ^{14}C toluene + ^{14}C CO_2 + ^{14}C biomass.
- (b) ^{14}C activity in vial B = activity due to ^{14}C CO_2 + ^{14}C biomass.
- (c) ^{14}C activity in vial C = activity due to ^{14}C biomass.

The difference in radioactivity between (a) and (b) was the amount of toluene degraded,

while the difference between (b) and (c) was the amount of CO₂ produced and (c) was the amount of biomass produced. For abiotic controls, no CO₂ or biomass production was expected and the difference in vials A and B was calculated as the amount of unconverted toluene. The abiotic controls showed a decrease of less than 10% of the initial toluene concentration, at the end of the experiment.

b) Biofilm Kinetics. Biofilm kinetics were investigated by supplying toluene to three packed-bed VPBRs in a counter-current mode of operation (Figure 3.1). House air was split into two streams with one stream sparged through a carboy containing water (to maintain humidity) and the other stream sparged through a liquid toluene reservoir. The two streams were mixed together forming a feed into the bottom of all three VPBRs. Liquid HCMM2 medium flowed downward through the VPBRs, providing a source of inorganic nutrients for biofilm growth. Physical parameters and packing characteristics involved in the biofilm kinetic study are shown in Table 3.2. Two different experimental conditions were used, with influent toluene concentrations of 150 and 300 ppm. These experimental conditions are hereby designated as 150T and 300T, respectively. Exactly 250 ceramic Raschig® rings were introduced into each reactor and the entire system was sterilized by autoclaving. After sterilizing, toluene vapor was fed into the column continuously until steady state abiotic losses of approximately 10 -15% were measured (approximately 2 days). A bacterial inoculum of *P. putida* 54G cells (5 ml) previously grown on toluene vapors, was introduced into the columns and the reactor liquid contents recycled for 6 hours allowing the bacteria to attach to the ceramic rings. Following this,

the VPBRs were switched to a once-through mode using solely mineral salts media.

Vapor phase toluene concentration in the influent and effluent gas ports and liquid phase toluene concentration in the effluent were measured daily to determine reactor performance. Abiotic toluene losses were subtracted from observed reactor performance to obtain actual biological removal efficiencies.

After one week of operation, one VPBR was destructively sampled (Week 1) by transferring the ceramic rings from the top, middle and bottom of the column aseptically to three sterile petri dishes. For each column position, packing material was subjected to the following analysis:

- Six ceramic rings were combined with 6 ml of HCMM2 medium, vortexed and sonicated for three minutes. An aliquot (0.1 ml) of the resulting aqueous sample was used to enumerate cell numbers by diluting and spread plating on R2A and HT plates. Base (NaOH) was added to the remainder of the suspension containing the packing to a final concentration of 1 N and the suspension was boiled in a 120°C incubator for 1.5 hours to disrupt all the cells. The suspension was then vortexed and protein concentration of the suspension determined as discussed previously and then expressed as g of biomass/m² of packing.
- Biofilm thickness was measured on three ceramic rings using confocal laser microscopy as described in the next section. For each ceramic ring, nine measurements of thickness were determined and averaged. Since three ceramic rings were sampled at each column position, a statistically significant value was determined by evaluating a standard error.

- Six ceramic rings were combined with liquid HCMM2 medium in 26 ml Supelco glass vials, and vortexed to suspend the cells. Initial-rate ^{14}C toluene degradation studies (maximum time = 2.25 hours) were conducted as discussed previously. Since bacteria were suspended, mass transfer limitations were assumed to be negligible. Toluene concentrations used in the glass vials were similar to the concentration that the bacteria had been previously exposed to in the VPBRs. For example, 300 ppm vapor phase toluene concentration is equivalent to approximately 3 mg/l liquid phase toluene concentration. Packing material was left in the glass vials during the degradation studies. Comparable controls with the packing material removed from the vials did not show any appreciable differences in toluene degradation rates.

This procedure was repeated for another VPBR at two weeks from the start of the study (Week 2). For the third VPBR, toluene concentrations were measured until constant removal efficiencies were obtained. This indicated that steady state conditions, with respect to toluene degradation, prevailed in the VPBR. At this point (usually between 22-25 days from the start of the study) the third VPBR was destructively sampled (Week 3).

c) Long-term toluene exposure study. This study was designed to investigate the effects of long-term toluene exposure on planktonic cells of *P. putida* 54G. Three 400 ml Erlenmeyer flasks were used in the VPBR system shown in Figure 3.1, replacing the reactors. Toluene-contaminated air was bubbled through 250 ml of liquid HCMM2

medium in each flask at a vapor concentration of 150 ppm and a vapor flow rate of 50 ml/min. Medium in the flasks was continuously stirred to provide sufficient aeration. At the start of the study, suspended cells of *P. putida* 54G pregrown on toluene vapors were inoculated into two flasks (biotic flasks). An abiotic control flask was operated in a similar fashion to the biotic flask except for the absence of cells. Toluene concentration was measured in the influent and effluent ports of the flasks. Liquid samples were withdrawn from the flasks daily for (1) cell enumeration on R2A and HT plates, (2) measuring concentrations of nitrate, sulfate and phosphate ions in solution using ion chromatography, (3) toluene concentration, and (4) biomass amount. Liquid and gas samples were measured at timed intervals until the end of the study (29 days). Liquid volume in the flasks was reduced by 20 ml (< 10%) due to sampling at the end of this study.

Data Analysis: For planktonic cells, substrate degradation (dS/dt) information was converted to specific growth rate (μ) using the following (13):

$$\mu = -\frac{Y_{x/s}}{X} \frac{dS}{dt} \quad (2)$$

where X is biomass concentration and $Y_{x/s}$ is the yield. Growth rate, μ , information was correlated to substrate concentration using the Andrews substrate inhibition model (2):

$$\mu = \frac{\mu_{\max} S}{(K_S + S + \frac{S^2}{K_I})} \quad (3)$$

where $\mu_{\max}$ [day^{-1}] is the maximum specific growth rate, K_S [mg/l] is a half saturation constant and K_I [mg/l] is a substrate inhibition constant. The kinetic coefficients $\mu_{\max}$, K_S and K_I were determined by using a non-linear regression fit of the Andrews inhibition model to the experimental results of μ vs S . Specific activity was used to compare biofilm and suspended cell kinetics, as defined in two different units :

- 1) mg of toluene degraded per mg of biomass produced per hr, designated as SAB
- 2) mg of toluene degraded per the number of cells grown on HT plates; expressed in CFUs/ml per hr, designated as SAH.

Results and Discussion

Planktonic Cell Kinetics: Toluene degradation experiments were carried out for toluene concentrations ranging from 1- 50 mg/l. Toluene degradation profiles are shown in Figure 3.2. All the profiles show a similar behavior: a lag phase, indicated by little or no change in toluene concentration, followed by a growth phase where all the toluene is degraded. The time of the lag phase decreased with decreasing toluene concentration which was consistent with an induction period necessary for acclimatization of the bacteria to toluene concentrations used (36). Cell numbers on R2A and HT plates were initially 5×10^4 CFUs/ml and changed by a maximum of four orders of magnitude (data not shown) after complete mineralization of toluene. At toluene concentration of 20 mg/l and below, lag phases were shorter and suggested that higher toluene concentrations could inhibit growth of *P. putida* 54G cells. This observation is consistent with results shown in Figure 3.3, where growth rate decreased as toluene concentration increased above 20 mg/l. Growth rates for planktonic cells of *P. putida* 54G correlated well with the Andrews substrate inhibition model ($r^2 = 0.93$) of Equation (4). Values of yield ($Y_{x/s}$) were calculated to be 0.90 ± 0.13 mg of ^{14}C biomass produced per mg of ^{14}C toluene degraded. Alvarez et al (1) have presented kinetics of toluene and benzene degradation in sandy aquifer material and detailed a survey of Monod coefficients for toluene and benzene degradation found by different researchers. More recently Chang et al (7) and Oh et al (28) presented results on kinetic parameters estimated for aerobic growth of pseudomonads using toluene. Results of their studies in comparison to the present

investigation are detailed in Table 3.3. While Chang et al (7) used the Monod model to fit their data, Oh et al (28) determined that the Andrews inhibition model fit their data. We obtained K_s values lower than those obtained by Oh et al (28) while μ_{max} and K_i values were comparable. The lower K_s values probably resulted from different nutritional conditions and differences in the strains of *P. putida* used. Bacterial injury levels during the planktonic cell studies, as calculated from equation 1, were never higher than 10%.

Biofilm Kinetics: Reactors in the VPBR set-up were destructively sampled every week as detailed previously. Biomass was estimated by determining levels of protein on the ceramic rings. Biofilm thickness on the rings was determined by scanning confocal laser microscopy. Figure 3.4 shows biomass in g/m^2 packing and biofilm thickness in μm for a Week 2 sample during the 300T study. Biomass amounts and biofilm thicknesses were highest at the bottom (near the gas inlet) and decreased towards the top of the column. Similar results were obtained from each column for both experimental conditions. The difference in biofilm thicknesses between the top and bottom of the column decreased with time while toluene removal efficiency of the column increased (data not shown), which is consistent with unsteady-state operation of the VPBRs.

Toluene (^{14}C) degradation experiments were conducted on biofilm cells to evaluate specific activity. Representative results are shown in Figure 3.5. Biofilm cells at the bottom of the column were more active in degrading toluene in comparison to bacteria at the top of the column. In approximately 1.25 hours all the toluene in the test

vials had been degraded. In comparison, the doubling time for *P. putida* 54G at 3 mg/l aqueous toluene concentration was about 5.8 hours, so that biomass growth in the specific activity assay could be neglected.

Similar toluene degradation experiments were conducted for all VPBRs during 150T and 300T studies. Toluene degradation rate measured in mg/l-hr was divided by biomass measurements to evaluate performance as specific activity (SAB). Figure 3.6 shows specific activity during both experimental conditions for biofilm cells at the top and bottom of the column. Also shown in Figure 3.6 is the specific activity for free cells (batch) evaluated as $\mu_{\max} / Y_{x/s}$. Specific activity for free cells was computed at a substrate concentration of 3 mg/l, since this value was approximately equivalent to the gas phase toluene concentration to which the biofilm cells were exposed. Comparison of SABs for the VPBR experiments suggested higher activity for biofilm cells exposed to the lower toluene concentration. When toluene concentration was increased from 150 to 300 ppm, SAB decreased by a factor of 2 on the average. Biomass contents measured during the 300T study were consistently higher than those obtained during the 150T experiment (data not shown). This suggested that although an increase in toluene concentration produced an increase in biomass concentration, this increase was probably offset by a loss of biomass activity at the higher toluene concentration. Since mass transport limitation was not a factor (the biofilm had been resuspended), we consider that the biofilm was fully penetrated. Thus, we conclude that large amounts of inactive biomass were being formed during the 300T experiment. Specific activity (SAB) decreased from Week 1 to Week 3 samples for both the 150T and 300T studies indicating that toluene degrading

capability of the biofilm decreased with time.

Specific activity (SAB) for the biofilm experiments was between five and ten times lower than that observed with planktonic cells. This implied that inactive biomass formed during both 150T and 300T experiments and that the inactive biomass fraction increased with toluene concentration. Since the planktonic cell studies were only conducted for a day, it was possible that this reduction in specific activity could be attributed to effects caused due to long-term toluene exposure. If this hypothesis were true, then specific activity based on toluene degrading cells (SAH mg toluene degraded/CFUs/ml toluene degrading cells - hr) would remain constant for both biofilm and planktonic cells.

Figure 3.7 shows that the specific activity (SAH) for biofilm and planktonic cells of *P. putida* 54G was approximately the same, 2.5×10^{-10} mg toluene degraded/CFUs (HT)-hr/ml. The observed values for SAH are consistent with our hypothesis that long term toluene exposure produced large numbers of inactive cells, thus reducing the specific activities (SAB) for biofilm cells.

Diks and Ottengraf (11) obtained similar results for a dichloromethane degrading biofilm in a biofilter. They observed that specific activity for a *Hyphomicrobium* spp. computed from batch dichloromethane degradation experiments was 0.64 g/g-hr. When biofilm cells of *Hyphomicrobium* spp. were removed from the biofilter and resuspended in batch cultures containing dichloromethane, specific activity was found to be 0.08 g/g-hr. Based on these results, they suggested that only 12% of the biomass was active in degrading dichloromethane. In a related study, Okabe (29) observed that maximal growth

rate for *D. desulfuricans* biofilms on lactate and sulfate decreased from 0.37 hr^{-1} to 0.1 hr^{-1} , whereas the growth rate for planktonic cells was measured at 0.37 hr^{-1} . During his study the biofilm was not nutrient limited. He speculated that this decrease in specific cellular growth rates could be attributed to an increase in maintenance energy requirement or metabolic product inhibition by sulfide and acetate, suggesting that attachment to surfaces led to changes in cell physiology.

Since mass transport limitations did not affect kinetics of toluene degradation by free and attached cells, other mechanisms are necessary to explain this difference. Reports of injury and/or toxicity due to toluene exposure have not been documented and to better understand these observations, we conducted long term toluene exposure experiments. Our objective was to determine if the specific activities (SAB) for planktonic cells exhibited the same reduction as biofilm cells on exposure to toluene.

Long Term Toluene Exposure Study: The results shown in Figure 3.8 are from an experiment where cells that were withdrawn from flasks after timed exposure to toluene and enumerated on R2A and HT plates. After the first day, cells that formed colonies on R2A plates remained constant at approximately 4×10^8 CFUs/ml while cells on HT plates decreased continuously during the same time period from 3.2×10^8 CFUs/ml to 5.1×10^7 CFUs/ml. The difference in the cell numbers on R2A and HT plates is interpreted as a measure of toluene injury. Injury increased continuously for the duration of the study to 86% and suggests that long-term exposure to toluene caused significant levels of bacterial injury. Dissolved nitrate, phosphate and sulfate ion

concentrations decreased to approximately 50% of their initial concentration, indicating that these ions were not rate limiting (data not shown). Specific activity for the planktonic cells was determined by exposing the cells to a 1.5 mg/l liquid phase concentration of ^{14}C -toluene, as explained previously. This procedure was repeated at timed intervals and, combined with biomass data, produced the specific activities shown in Figure 3.9.

Values of specific activities (SAB) decreased from 0.25 to about 0.025 mg/mg-hr at the end of the experiment, while SAH decreased initially to 2.4×10^{-10} CFUs/ml in 1.5 days and then decreased slightly to about 2.25×10^{-10} CFUs/ml at the end of the experiment; 26 days. The initial drop in SAH is characteristic of a stationary phase decline in activity, while cell numbers on HT plates remained constant. The onset of injury in this experiment is at approximately 1 day, which correlates with transition of the cells from a log-phase culture to a stationary-phase culture. It is noteworthy that the trend and values for both SAB and SAH were similar for biofilm and planktonic cultures.

Results of the present investigation indicate that long term exposure to toluene produces a toxic effect on the bacterial biomass which reduces the specific activity. Cell wall damage due to toluene exposure might lead to a large fraction of the biomass becoming inactive. Toluene-degrading cells decreased with time showing that the active biomass fraction was decreasing. These phenomena can have a dramatic effect on VPBR performance.

Leddy et al (24) have studied the growth of planktonic cells of *P. putida* 54G on toluene in batch cultures and observed that TOL- mutants of the wild type cells were produced which increased in concentration with duration of toluene exposure. These

TOL- mutants possibly grew on organic compounds leaking from injured cells or intermediates formed due to incomplete toluene degradation. They have also shown that benzyl alcohol, a degradation product, mediates formation of TOL- mutants. We have completed similar studies that support these findings.

Clearly, traditional bioreactor design strategies that have been used to model VOC degradation may be improved by incorporating effects of injury or toxicity caused by VOCs. Without coupling the processes of growth and injury or toxicity into a phenomenological model, bacterial activity could be grossly overestimated, compromising the predictive capabilities of such models.

Conclusions

In the present investigation, toluene degradation by biofilm and planktonic cells of *P. putida* was studied. We observed that:

- 1) Specific activity (mg toluene degraded/mg biomass produce-hr and mg toluene degraded per CFUs/ml toluene degrading cells per hr) for biofilm and planktonic cells were similar.
- 2) Long-term toluene exposure and increase in toluene concentrations rather than surface attachment caused a difference in activity for free and attached cells of *P. putida* 54G. Exposure to toluene caused bacterial injury which increased with duration of toluene exposure.
- 3) During logarithmic growth, toluene degradation kinetics followed the Andrews

inhibition model, with substrate inhibition being notable above 20 mg/l toluene.

This value is much higher than the levels to which cells were exposed (1-3 mg/l) when injury was observed.

- 4) Caution should be used when applying suspended cell kinetics to model bioreactor kinetics and kinetics for biofilm-mediated degradation of xenobiotic compounds should be investigated for each type of system.

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Table 3.1. List of parameters analyzed, duration of study and physiological state of the bacteria during the biofilm cell kinetics, suspended cell kinetics and long term toluene exposure studies.

Experiment	Maximum duration of toluene exposure	Physiological state	Analyzed for:
Planktonic cell kinetics study	1 day	Planktonic cells	1) ^{14}C toluene degradation 2) Cell #s on R2A and HT plates 3) Biomass = ^{14}C biomass produced
Biofilm cell kinetics study	22 - 25 days	Biofilm cells	1) ^{14}C toluene degradation 2) Cell #s on R2A and HT plates 3) Biomass = protein analysis x 2 4) Biofilm thickness by SCLM*
Long term toluene exposure study	29 days	Planktonic cells	1) ^{14}C toluene degradation 2) Cell #s on R2A and HT plates 3) Biomass = protein analysis x 2

* = Scanning Confocal Laser Microscopy

Table 3.2. Physical parameters and packing characteristics for packed-bed VPBRs used in studying biofilm kinetics

Physical Parameters	
Vapor flow rate =	50 ml/min
Liquid flow rate =	1 ml/min
Toluene concentration =	150 & 300 ppm
Inoculation recycle time =	6 hours
Column description =	8" tall & 1" i.d.
Packing Characteristics	
Type =	Ceramic Raschig Rings
Description =	1/4" o.d. & 1/4" long

Table 3.3. A comparison of kinetic parameters for bacterial growth on toluene by *Pseudomonads*.

Bacterial strain	$\mu_{\max}$ (day ⁻¹)	K_s (mg/l)	K_i (mg/l)	$Y_{x/s}$ (mg/mg)	Reference
<i>Pseudomonas</i> B1	13.03 $\pm$ 1.83	1.96 $\pm$ 0.91	--	1.22 $\pm$ 0.10	Chang et al, 1993
<i>Pseudomonas</i> X1	10.84 $\pm$ 2.77	1.88 $\pm$ 1.26	--	0.99 $\pm$ 1.26	
<i>Pseudomonas putida</i>	17.28	15.07	44.43	0.64	Oh et al, 1994
<i>Pseudomonas putida</i> 54G	10.08 $\pm$ 1.2	3.98 $\pm$ 0.78	42.78 $\pm$ 3.87	0.90 $\pm$ 0.13	Present Investigation

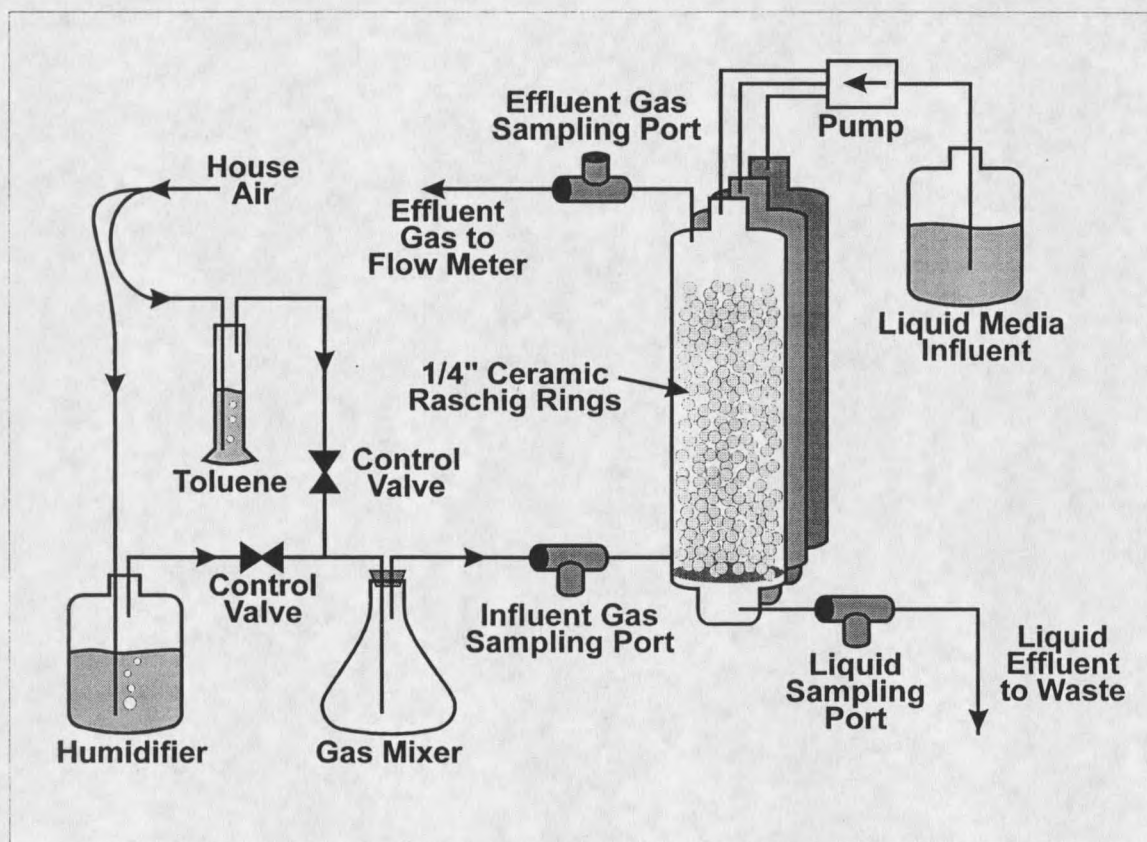


Figure 3.1. Schematic of vapor phase bioreactor used to determine biofilm kinetics.

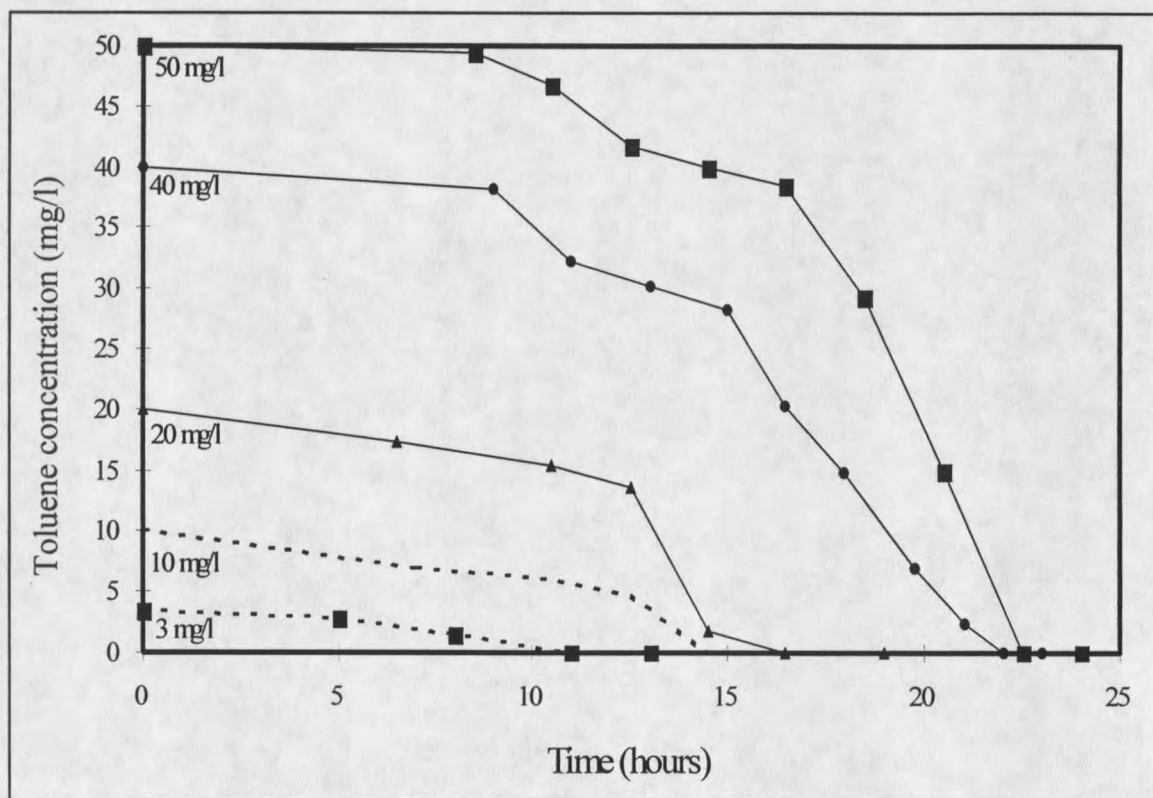


Figure 3.2. Degradation profiles (1 - 50 mg/l) during ^{14}C toluene metabolism by planktonic cells of *P. putida* 54G in batch cultures. Profiles at 1, 6 and 28 mg/l are not shown on this graph, since they exhibit similar trends.

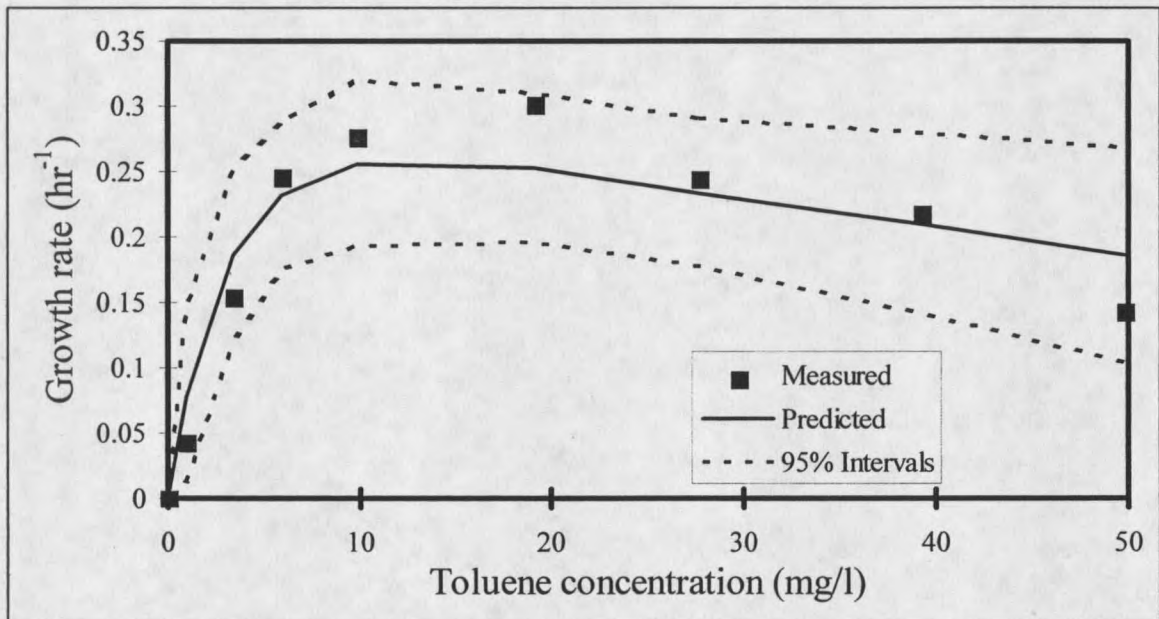


Figure 3.3. Specific growth rate for planktonic cells of *P. putida* 54G on toluene.

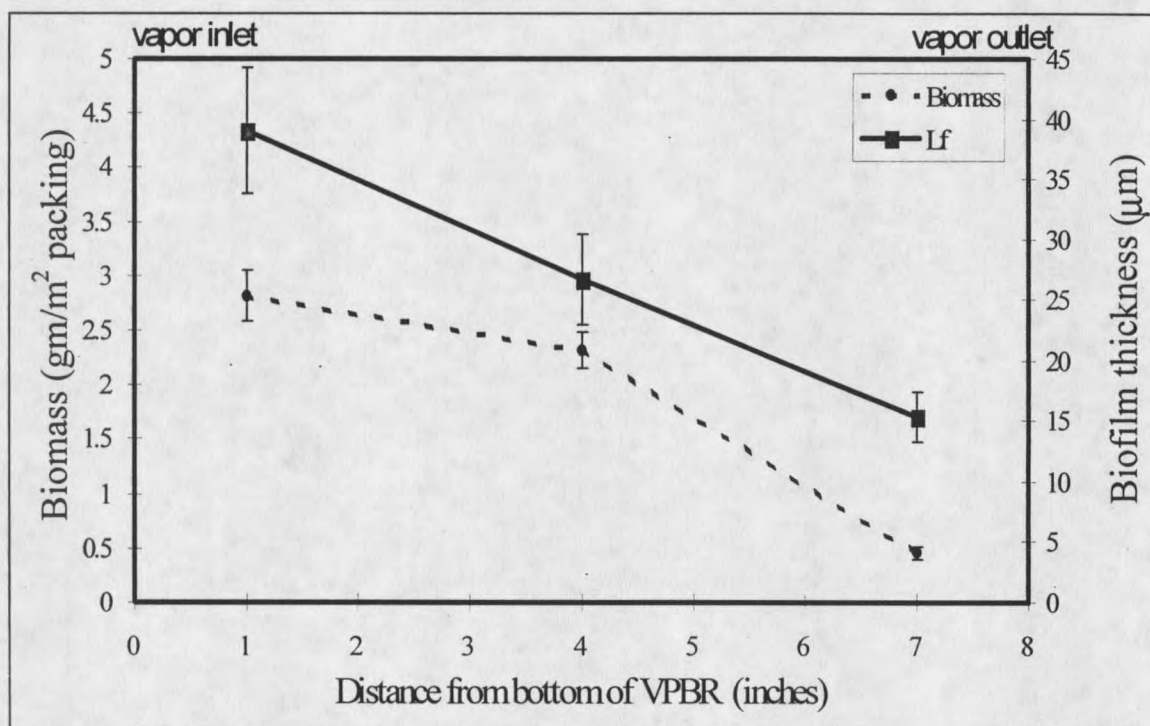


Figure 3.4. Biomass (g/m² of packing) and biofilm thickness (μm) for a Week 2 VPBR sample during the 300T experiment at three different reactor positions. The bottom of the reactor, 0 on the abscissa, is the gas inlet position.

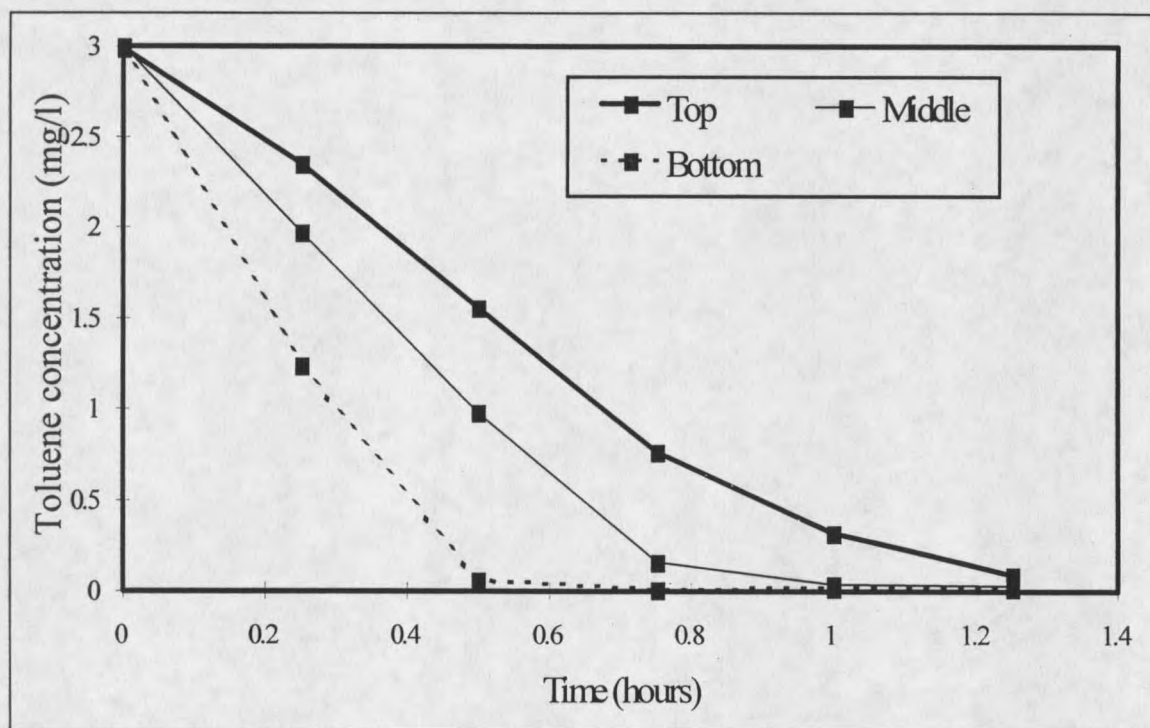


Figure 3.5. ^{14}C toluene degradation profiles for the sample from Figure 3.4.

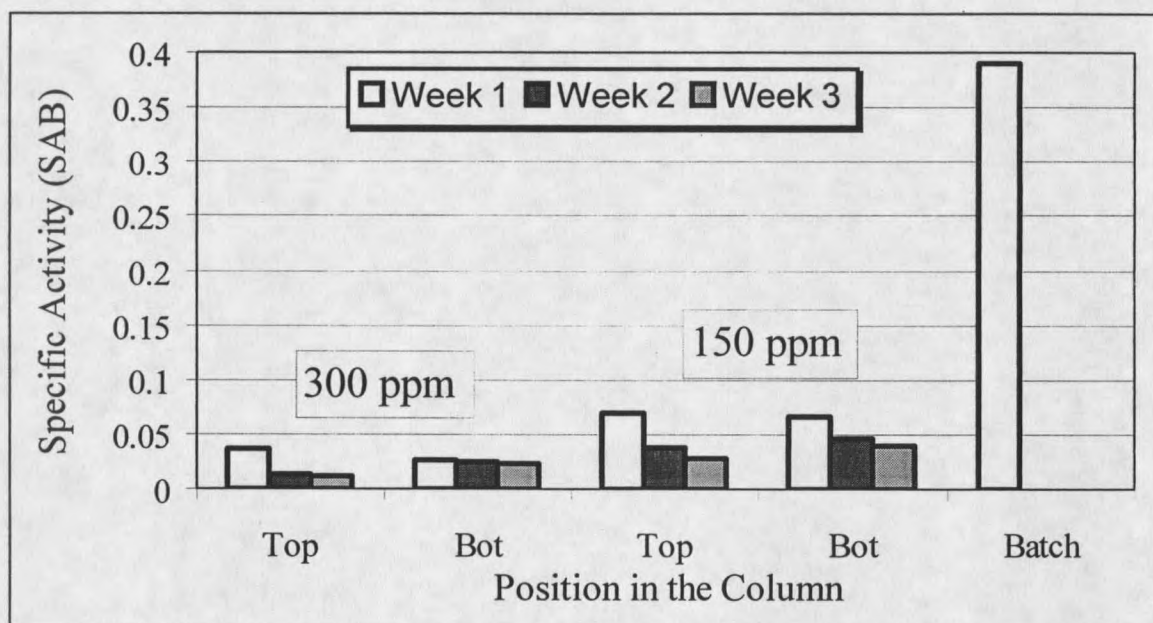


Figure 3.6. Specific activity (SAB) in units of mg toluene degraded/mg biomass produced-hr for planktonic and biofilm cells. Planktonic cells are shown as batch while biofilm cells are shown for two different experimental conditions, influent vapor phase concentration of 150 and 300 ppm.

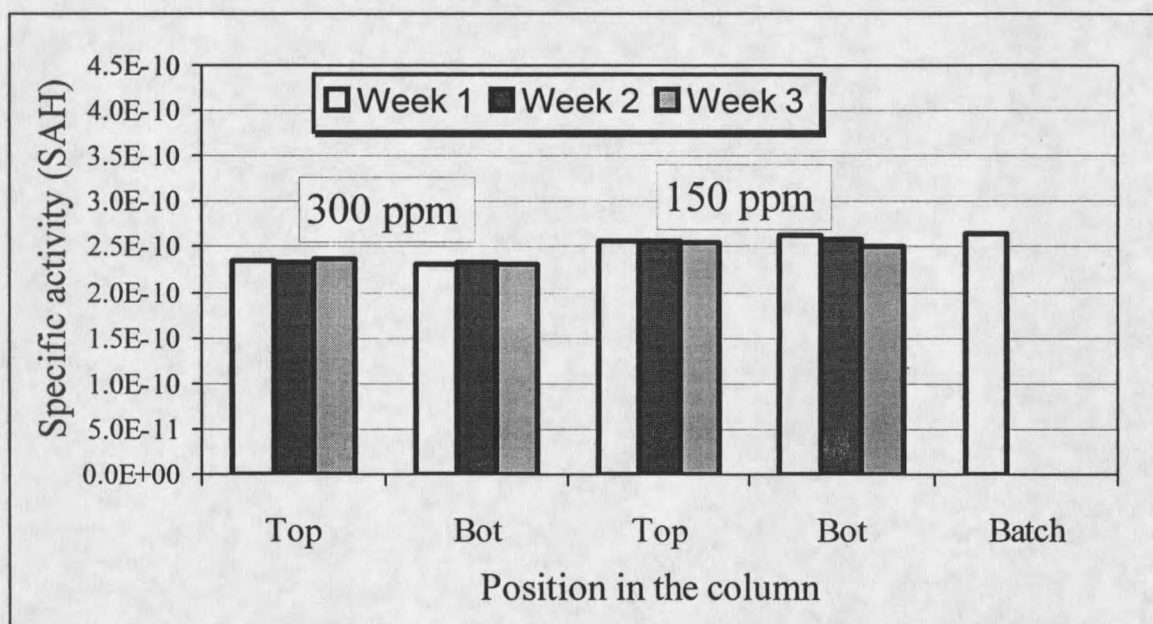


Figure 3.7. Specific activity (SAH) in units of mg toluene degraded/mg total biomass produced-hr for planktonic and biofilm cells. Planktonic cells are shown as batch while biofilm cells are shown for two different experimental conditions, influent vapor phase concentration of 150 and 300 ppm.

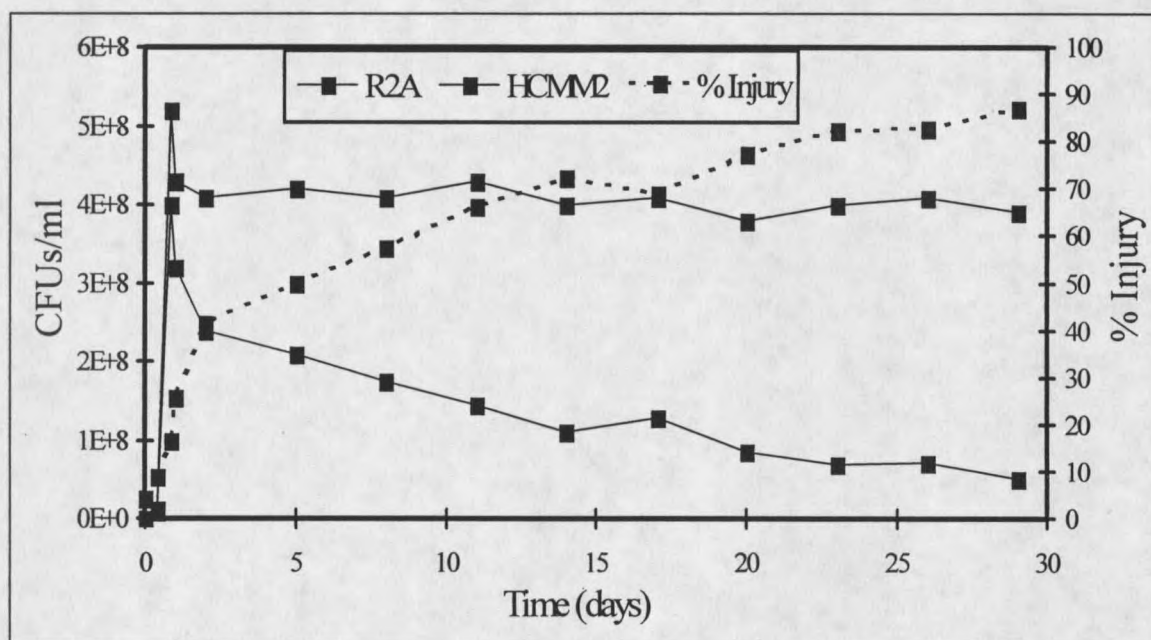


Figure 3.8. Cell numbers on R2A and HT plates during the long term exposure study for suspended cells. Dashed line with markers shows injury % on the right ordinate.

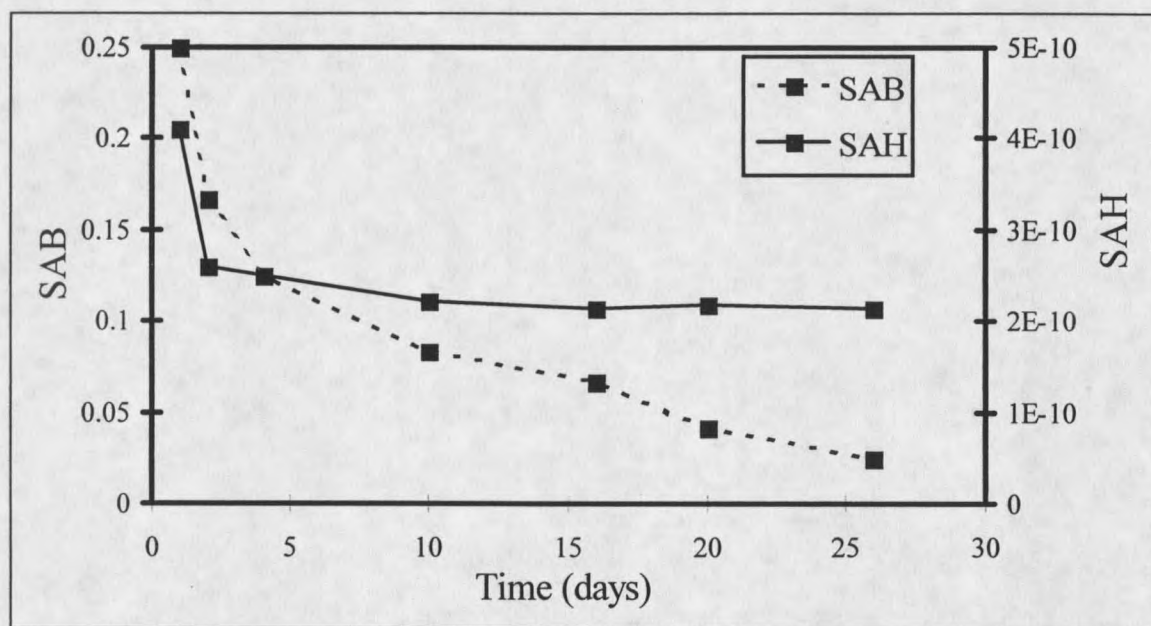


Figure 3.9. Specific activity in mg/mg-hr (SAB) and mg/CFUs - hr/ml (SAH) evaluated for planktonic cells from Figure 8. SAB is on the left and SAH is on the right ordinate.

CHAPTER 4

PHYSIOLOGICAL STRESS AND INJURY IN BATCH
CULTURES OF *P. putida* 54G DURING TOLUENE DEGRADATIONIntroduction

The biodegradation of volatile organic compounds (VOCs) such as benzene, toluene and trichloroethylene has been studied extensively for many years (8). Bioremediation processes for VOCs that are accompanied by addition of limiting nutrients often use recovery and enumeration of environmental isolates as a tool to assess biodegradation potential and evaluate bacterial population dynamics. Enumeration of isolates derived from contaminated aquifers or soils on minimal media enriched with the VOC of choice is commonly employed to determine viability of hydrocarbon-degrading bacteria. Visible colonies that form on such a medium are assumed to indicate the population of viable hydrocarbon-degrading cells. Although these investigations have shown the presence of environmental bacteria that degrade these compounds, less attention has been focused on understanding the physiological effects of isolation and growth on VOCs.

Physiological stress or injury has been defined as the physiological, and structural consequence(s) resulting from exposure to sublethal injurious environmental conditions

and/or chemical agents (15). This is reflected by the inability of injured cells to reproduce under selective or restrictive conditions that are tolerated by uninjured cells. The definition and concept of injury includes the capability of debilitated cells to repair any cellular damage or other physiological effects and regain tolerance for growth under selective conditions (15). Consequently, nonselective media must be used to accurately estimate viable cell numbers.

The concept of bacterial injury has been studied for over 20 years in the food industry and over 10 years in aquatic environments (16). An extensive body of experimental evidence is available indicating that a significant proportion of bacteria exist in a physiologically compromised state variously referred to as "stressed" (1, 4) or "injured" (3, 5). Chemical stressors such as disinfectants and biocides (3, 11, 14, 20, 27) and metals such as copper (5, 26) have been shown to cause bacterial stress. Physical factors including sunlight (7), UV radiation (18), acidic pH (6, 9), thermal stress (10, 29) and related environmental factors (1) can significantly contribute to bacterial stress and injury in aqueous systems.

Differential recovery on selective and non-selective growth media has been used to detect and quantify the occurrence of stressed bacteria (1, 2, 11, 19, 21). In studies conducted on indicator bacteria in drinking water distribution systems, the presence of stressed sub-populations have often led to an underestimation of the total viable cells (15). Injured cells become sensitive to inhibitory agents in specific selective media and are unable to grow and produce colonies (1). Resuscitation on rich medium before enumeration on selective medium provides a better indication of the total viable cell

population.

Very few studies exist on the topic of hydrocarbon-related stress and injury. Tebbe et al (28) used gene probes to differentiate between different categories of operon expression for naphthalene-degrading bacteria and determined that: (1) some organisms expressed their genotype upon selective isolation, (2) a majority of the organisms only expressed their genotype after initial non-selective isolation and (3) some organisms did not express their genotype at all. They suggested that the proportion of organisms that immediately expressed their genotype depended on the *in-situ* selective pressure imposed on the cells and on their ability to adapt to different nutrient conditions. Love and Grady (13) determined that the continuous culture growth of *P. putida* in the presence of benzoate and *m*-toluate caused cells to lose culturability on benzoate and *m*-toluate plates in comparison to culturability on glucose plates. They suggested that a reversible physiological response could have caused the loss of culturability rather than an irreversible mutation. Increased substrate uptake rates resulted when cells were transferred from hydrocarbon-limited conditions to hydrocarbon-sufficient conditions. These increased rates led to inhibitory levels of substrate and/or intermediates that were hypothesized to be responsible for loss in culturability.

Ridgway (23) has studied growth of hydrocarbon-degrading bacteria from a gasoline-contaminated aquifer and concluded that stressed subpopulations could result in an underestimation of the true number of viable hydrocarbon-degrading bacteria in the aquifer. In a related investigation, Leddy et al (12) determined that after 10 - 15 days of growth on vapor phase toluene, *P. putida* 54G formed Tol- mutants with a selective loss

of catabolic functions. The mutants could not degrade toluene but continued to grow in the presence of toluene, metabolizing other carbon sources such as organic compounds leaking from wild-type cells or toluene degradation intermediates. They also observed that benzyl alcohol mediated irreversible defects in both a plasmid-associated and chromosomal-associated pathway utilized by *P. putida* 54G for toluene degradation.

Clearly, it is important to determine how stress response can be related to the ability/efficiency of a microorganism to degrade hydrocarbon vapors since these effects might lead to reduced hydrocarbon-degradative activity in bioreactors. The effect of physical and chemical parameters such as hydrocarbon concentration and duration of hydrocarbon exposure on degradation processes can provide critical information on hydrocarbon-related physiological stress and injury. This information, when obtained in a well defined system, could be utilized to formulate rate expressions based on growth of stressed and uninjured cells. Establishment of quantitative rate relationships would permit incorporation of stress response into process models, leading to an improved ability to design and control bioremediation systems.

We hypothesize that toluene causes injury in *P. putida* 54G cells, with a majority of the injured cells able to revert to their uninjured state after passage on rich non-selective medium. A small fraction of the injured cells suffer irreversible loss of toluene degradative capability. Toluene concentration, duration of toluene exposure and formation of intermediates as a result of incomplete toluene degradation increase bacterial injury. In this investigation, *P. putida* 54G cells were grown in suspended cultures using toluene as a sole carbon and energy source. Total viable and injured cells were

determined for influent vapor phase toluene concentrations of 150 and 750 ppm, respectively. The results of the batch experiments were used to formulate rate expressions for injured and uninjured *P. putida* 54G cells.

Materials and Methods

Bacterial Strain: *Pseudomonas putida* 54G, a toluene-degrading bacterium, was isolated from a gasoline contaminated aquifer at Seal Beach, California. The isolate was capable of growth on HCMM2 mineral salts media (containing only inorganic compounds) in the presence of vapor phase toluene and on complex carbon sources such as R2A medium (24). *P. putida* 54G has been shown to exhibit catechol-2,3-dioxygenase activity indicating a *meta* pathway when grown on mineral salts with toluene (12).

Media and Enumeration of Viable Cells: HCMM2 mineral salts medium was used as the liquid inorganic nutrient medium as described elsewhere (24). Plate counts on the following solid medium were employed in this investigation.

- (1) **R2A medium** (22), which has been used as a non-selective medium for *P. putida* 54G (24),
- (2) toluene vapors supplied continuously to cells on HCMM2 plates (selective medium) in sealed containers incubated at room temperatures, designated as **HT** plates and
- (3) 500 mg/l glucose added to HCMM2 plates, designated as **HG** plates, were also used as a putative non-selective medium.

P. putida 54G cells grown on toluene as the sole source of carbon and energy were enumerated for viable cells on R2A, HT (Table 4.1) and HG. The difference between selective and non-selective media was used to determine the level of injury (15) according to

$$\text{Injury \%} = \left(\frac{\text{Non-Selective Counts} - \text{Selective Counts}}{\text{Non-Selective Counts}} \right) \times 100 \quad (1)$$

Colonies that grew on the non-selective medium were transferred aseptically onto the selective medium. Colonies that did not grow on the selective medium after transfer were termed as injured non-recoverable and colonies that grew after transfer were termed as injured recoverable (Table 4.1).

Microscopy: Total and actively respiring bacteria (Table 4.1) were determined by direct epifluorescent microscopy. Samples were counterstained by the DNA-binding fluorochrome 2,4-diamidino-2-phenylindole (DAPI) following reaction with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) based on a procedure outlined by Rodriguez et al (25) with minor modifications at final concentrations of 1 mg/l and 1520 mg/l, respectively. Aqueous samples withdrawn from the reactor system were diluted in HCMM2 medium, vortexed and homogenized (Model TR-10, 60 % of maximum power, Tekmar Co., Cincinnati, OH). Suspensions were then stained with CTC on 0.2 µm pore-size black polycarbonate membrane filters (Poretics Corp., Livermore, CA) and incubated at room temperature with stirring for two hours. Samples were then washed and stained

with DAPI on the filter for 40 minutes, followed by two more washing steps. Filters were air dried and placed on glass slides for microscopic examination using an Olympus BH2-RFCA epifluorescent light microscope (Scientific Instrument Company, UT) with a 100X (refractive index=1.3) oil immersion lens. Cells that stained with CTC appeared red indicating actively respiring cells while inactive cells appeared green and provided a measure of total (respiring + inactive) cells.

Toluene Sampling: Gas and liquid samples were analyzed for toluene using gas chromatography (GC). In the headspace, toluene was measured using a HP 5890 Series II GC equipped with a flame-ionization detector and an Alltech 0.1% A1-1000 Graphpac GC 80/100, 6' x 1/8" x 0.085", S.S. column at a constant column temperature of 140°C. Headspace samples (250 μ l) were withdrawn and injected into the GC. Toluene concentration in the liquid were determined by combining 1 ml of the aqueous samples with 0.5 ml of hexane and injecting 2 μ l of the hexane phase into a HP 5890 Series II GC equipped with a photo-ionization detector and a DB-624 Column, 30 m x 0.53 mm i.d which was temperature ramped from 140°C - 200°C. Helium was used as a carrier gas in both columns at a flow rate of 30 ml/min.

Measurement of Non-Volatile Soluble Intermediates: Liquid samples were withdrawn from the system and combined with methylene chloride. Aliquots (2 μ l) of the methylene chloride phase were injected into a HP 5890 Series II Plus GC combined in series with a VG Analytical GC-MS 70E-HF Double Focusing Mass Spectrometer (MS).

A HP-5 capillary column with dimensions of 30 m x 0.25 mm i.d. and a film thickness of 0.25 μm was used in the GC. The entire sample was transported to the MS using helium as a carrier gas at a flow velocity of 30 cm/s.

Manifold System: A manifold system (Figure 4.1) was designed and operated to measure bacterial injury during toluene degradation at constant exposure levels of toluene. The reactor system allowed for *in-situ* measurement of toluene in the gas and liquid phases. Liquid batch cultures of *P. putida* 54G were grown with toluene as sole carbon and energy source under three different constant experimental conditions:

Experiment 1: An arbitrary vapor phase toluene concentration of 300 ppm was used with viable cell numbers enumerated on HG and HT plates. R2A plates were used as non-selective medium. However, we wanted to determine if a defined medium such as HG could be used as a satisfactory non-selective medium. If the difference in cell numbers on HG were not significantly higher than numbers on HT plates, HG plates were insufficient for resuscitation of injured cells, and only R2A plates would be used as a non-selective medium.

Experiment 2: Vapor phase toluene concentration of **150 ppm** (designated as 150 STUDY) with viable cell numbers enumerated on HT and R2A plates and total and active cell numbers enumerated by staining with DAPI and CTC as explained previously.

Experiment 3: Vapor phase toluene concentration of **750 ppm** (designated as 750 STUDY) with cells enumerated as in experiment 2.

Air flow was separated into two streams with one stream flowing into a

humidifier and the other flowing into an Erlenmeyer flask containing liquid toluene. For the 750 STUDY, oxygen was used as a carrier gas for toluene to ensure that oxygen was not stoichiometrically limiting (flasks were operated abiotically for 5 days and oxygen and toluene concentrations were measured). The two streams were mixed and flowed into 400 ml flasks where they bubbled through 250 ml of HCMM2 medium. Gas effluent from the flasks were vented into a chemical hood. Flasks equipped with sampling ports containing Teflon lined septa were stirred continuously to ensure sufficient aeration. At the start of the experiment, cells of *P. putida* 54G, pregrown on toluene vapors, were inoculated into the flasks. At timed intervals, toluene concentration in the gas and liquid phase as well as cell numbers plus non-volatile intermediates were measured by sampling the flasks.

Results

Test for HG as a Non-Selective Medium: An arbitrary influent toluene vapor phase concentration of 300 ppm was chosen for this study. At timed intervals, liquid samples were withdrawn from the flasks and enumerated on HT and HG plates (Figure 4.2). Cell numbers on both media show similar patterns, wherein there was an initial increase in viable cell numbers during log growth phase until the end of day 1. During this period cell numbers increased by four orders of magnitude from 7×10^4 to 8.4×10^8 colony forming cell numbers (CFUs/ml) for HT counts. This was followed by a decrease in cell numbers indicative of a stationary phase decline from 8.4×10^8 to 7.83×10^7 CFUs/ml, which continued until the end of the experiment (27 days). Cells on HG plates showed a continuous decrease from 9.1×10^8 to 9.8×10^7 CFUs/ml. No significant difference was measured between cell numbers based on growth on HT and HG plates. This suggested that toluene exposure caused cells to lose culturability on glucose plates and hence HG plates could not be used as a non-selective growth source. We also tested HCMM2 + glutamate as a potential non-selective medium and obtained the same result (data not shown). At this point, we decided to use R2A plates as a non-selective medium and HT plates as selective medium for all subsequent injury experiments.

Injury during 150 STUDY: Liquid samples were extracted and enumerated for viable cells on HT and R2A plates. Total cell numbers and actively respiring cells were estimated using microscopic techniques combined with DAPI and CTC staining,

respectively. The progression of cell numbers (viable and total) in the manifold system are shown in Figure 4.3. Each data point in the 150 and 750 STUDY represents an average of three values with each experiment performed in duplicate. The maximum standard error for these values was approximately 12%, but averaged 5%. There was an initial rise in cell numbers during the log phase followed by a stationary phase characterized by a relatively constant number of cells. This phenomena was true for the DAPI and CTC stained cells as well as colony-forming cell numbers on R2A plates. Cell numbers on the HT plates showed a steady decline from $(4 \pm 0.43) \times 10^8$ CFUs/ml at 0.833 days to $(5.1 \pm 0.17) \times 10^7$ CFUs/ml at 29 days. CTC stained cells and viable cells on R2A plates were approximately the same in number. Since CTC-stained cells represented actively respiring cells while cells on R2A plates indicated total viable cells, these results suggest that almost all viable cells were actively respiring.

The difference in cell numbers on R2A and HT plates was used as a measure of injury that increased from 3.5 % at the start of the experiment to 86.95% after 29 days (Figure 4.4(a)). Based on this study it was apparent that injury was a strong function of duration of toluene exposure. Colonies that formed on non-selective R2A medium were transferred aseptically onto selective medium, HT plates. Colonies that grew on the selective medium were termed recoverable injury and the fraction that did not grow on the selective medium were termed non-recoverable injury. Figure 4.4(a) shows that the fraction of non-recoverable injury increases to 12.6% after 29 days of toluene exposure suggesting that these cells irreversibly lost their ability to degrade toluene. The recoverable injury fraction accounted for 74.39 % of the cells implying that resuscitation

on rich non-selective medium allowed the cells to repair any damage that developed due to toluene exposure.

Respiration % was calculated as the ratio of CTC to DAPI stained cells and indicated the fraction of cells that were respiring. Respiring cells decreased from 100% at the start of the experiment to about 35% at the end of the experiment, at day 29. Respiration could be correlated with injury because as injury increased, the number of respiring cells decreased (Figure 4.4(a)). This trend was consistent throughout the duration of the experiment.

Injury during 750 STUDY: The manifold system was operated at an influent vapor phase toluene concentration of 750 ppm with the same enumeration procedures as in the 150 STUDY. For a 14 day experimental run, cell numbers on HT plates increased to $(1.94 \pm 0.22) \times 10^{10}$ CFUs/ml during log-growth phase and then decreased continuously to $(7.8 \pm 0.59) \times 10^7$ at day 14 (data not shown). Injury increased continuously from 1.8% at the start of the experiment to 99.6% after day 14 (Figure 4.4(b)). The non-recoverable fraction increased from 0 to 15.48% by day 14, while the injured recoverable fraction increased from 1.8 % to 84.1 % concurrently. Respiring cells decreased from 95% to about 19% at the end of the 14-day experiment. Similar conclusions from the 150 STUDY can be drawn relating injury and respiratory activity during the 750 study.

During the 750 STUDY, liquid samples were withdrawn for identification of soluble non-volatile metabolites. Three principal compounds were identified: benzyl

alcohol, benzaldehyde and benzoate (Figure 4.5). Benzyl alcohol increased initially and then decreased to a constant value of approximately 1.5 mg/l. Benzaldehyde and benzoate increased initially and then decreased gradually to less than 0.5 mg/l. All three compounds are associated with a plasmid-associated toluene degradation pathway for *P. putida* 54G (12). We also noticed accumulation of *o*-cresol (data not shown) during the experiment which could not be associated with known toluene degradation pathways of *P. putida* 54G. Abiotic controls indicated that no trace contaminants had accumulated in the liquid medium with toluene being the only measurable hydrocarbon.

Rate Expressions for Injured and Uninjured Cells: Rate expressions for uninjured cells (X^{++}), injured recoverable cells (X^{+-}) and cells that have irreversibly lost their toluene degrading capability (X^{-}) were determined by formulating mass balances for each cell type in a batch reactor system. For example, a mass balance on X^{++} cells included growth (μ^{++}), injury (K_I), irreversible loss of toluene degradative capability (K_{GL}) and decay (K_D^{++}) of X^{++} cells. Shown in equation 2 through 4 are mass balances on the three cell phenotypes:

$$\frac{dX^{++}}{dt} = [\mu^{++} - K_I - K_{GL} - K_D^{++}] X^{++} \quad (2)$$

$$\frac{dX^{+-}}{dt} = \mu^{+-} X^{+-} + K_I X^{++} - K_D^{+-} X^{+-} \quad (3)$$

$$\frac{dX^{-}}{dt} = \mu^{-} X^{-} + K_{GL} X^{++} - K_D^{-} X^{-} \quad (4)$$

where μ^{+-} and K_D^{+-} are growth and decay of X^{+-} cells and μ^- and K_D^- are growth and decay of X^- cells, respectively.

These equations were solved by using the following set of assumptions:

- 1) Growth of X^{+-} cells was negligible because they had to be resuscitated on rich mineral medium prior to degrading toluene. On the same basis, decay of X^{+-} cells was related to toluene degradation and hence negligible.
- 2) The difference in the growth and decay of X^{++} cells was negligible in comparison to other processes that the uninjured cells undergo such as irreversible loss and injury (this can be established by the ratio of uninjured cells at day 1 in comparison to the cell numbers at day 29, a decrease by a factor of 10).
- 3) The difference in growth rate and decay of X^- cells could be combined into one variable, μ_{NET} , which expresses the preference of the X^- cells for growth over decay.

Based on these assumptions, mass balances for the different cell type were simplified using equation 5:

$$\frac{d(R2A)}{dt} = \frac{dX^{++}}{dt} + \frac{dX^{+-}}{dt} + \frac{dX^-}{dt} = 0 \quad (5)$$

where R2A denotes cells that grow on R2A plates. The rate of change of the cells on R2A plated is zero as shown in Figure 4.3 (from day 1.5). Equations 2 - 5 were combined and solved analytically using the initial condition that at time $t = 0$, the number of X^{+-} and X^- cells were zero and X^{++} cell numbers = X_0 to yield the following solution set:

$$X^{++} = X_0 \exp[-(K_I + K_{GL})t] \quad (6)$$

$$X_{+-} = \frac{K_I}{K_I + K_{GL}} X_o (1 - \exp[-(K_I + K_{GL})t]) \quad (7)$$

$$X_- = \frac{K_{GL} X_o \exp[\mu_{NET} t]}{(K_I + K_{GL} + \mu_{NET})} (1 - \exp[-(K_I + K_{GL} + \mu_{NET})t]) \quad (8)$$

In equations 6, 7 and 8, X_o is the maximum number of uninjured cells formed at the end of the logarithmic growth phase. The time scale was reduced by a day using the assumption that injury started at the end of the log-growth phase. The equations were calibrated (Figures 4.6(a) and 4.6(b)) to results from the 150 STUDY and 750 STUDY to obtain numerical values for the three parameters, K_I , K_{GL} and μ_{NET} (Table 4.2). As seen in Table 4.2, K_I and K_{GL} increased as influent vapor phase toluene concentration was increased. The ratios of K_I and K_{GL} for the 750 STUDY to their values in 150 STUDY were approximately 6 and 10, respectively. This indicated, that following a five fold increase in toluene concentration, the rate of injury increased by a factor of 6 and irreversible loss of toluene degradative capability increased by a factor of 10. μ_{NET} dropped by a factor of 2 when toluene concentration increased suggesting that an increase in decay could be expected during the 750 STUDY (μ_{NET} = growth of X_- cells - decay of X_- cells).

Discussion

This investigation demonstrates that hydrocarbon-degrading bacteria undergo a physiological stress response that can be quantified via differential plating on selective minimal and rich non-selective media. Growth on selective medium does not necessarily represent a true estimate of the viable cell numbers and this discrepancy can be explained on the basis of a stress response of the bacteria to sublethal physiological and selective pressure. Two different types of stressed cells were identified, injured recoverable and injured non-recoverable cells. The injured recoverable cells suffered a repairable stress-related defect wherein they reverted back to their original state by passage on rich R2A medium. The injured non-recoverable cells suffer irreversible loss of toluene degrading capability. Leddy et al (12) have described this phenomena as the formation of Tol-mutants of *P. putida* 54G.

Choice of non-selective medium is critical when determining bacterial injury. In this study, both glucose and glutamate proved unsuitable as non-selective nutrients because *P. putida* 54G showed lower recovery on both media than on R2A. Love and Grady (13) have determined that glucose grown cells did not suffer a loss of culturability on benzoate solid medium. In this context, appropriate non-selective media should be evaluated for each bacterium before bacterial injury experiments are carried out.

Irreversible loss of toluene degradative pathway did not seem to inhibit the growth of these cells because other substrates were available. Some of the nutrients could have been organic compounds leaking from death and lysis of other cells and intermediates

formed due to incomplete toluene degradation by injured cells (12). However, the calculated growth rate of the irreversibly injured cells (μ_{NET}) decreased with increasing toluene concentration, suggesting that some of the intermediates were not conducive to growth (Table 4.2). The formation of intermediates such as benzyl alcohol have been shown to promote formation of Tol- mutants (12). In the present investigation, three principal intermediates were formed: benzyl alcohol, benzaldehyde and benzoate. This suggested that while the intermediates were being consumed by the uninjured cells, irreversibly injured cells were being formed at regular frequencies and other growth substrates were being used to support the growth of the mutants. The increase of benzyl alcohol at day 2 coincides with an increase in injury (Figure 4.4). We noticed a steady state accumulation of approximately 1.5 mg/l benzyl alcohol. It is quite possible that the injured cells degrade toluene incompletely leading to an increase in the formation of the intermediates. Formation of *o*-cresol seems to contradict known pathways of toluene degradation by *P. putida* 54G and this observation is being presently investigated.

The level of injury observed in batch cultures correlated with the fraction of respiring cells. Since irreversibly injured cells were actively respiring, respiratory activity could be used as a measure of injured recoverable cells. This observation makes intuitive sense because as the number of injured recoverable cells increased, respiring cells decreased indicating that cells that did not metabolize substrate were incapable of respiration. We have measured stratified layers of activity in *P. putida* 54G biofilms during toluene degradation (17). These stratified layers are regions of injured recoverable cells, uninjured cells and mutants which stratify depending on the presence of substrates

that they can metabolize. Figure 4.7 shows a schematic of a possible scenario wherein different cell phenotypes of *P. putida* 54G are formed as a result of sublethal stress. The injured recoverable cells are shown as cells that might have suffered some repairable cell-wall damage, while the irreversibly injured cells are shown without the Tol plasmid to exaggerate the fact that these cells have lost their toluene degradative pathway. Toluene, oxygen and intermediates (including incomplete toluene degradation products and organic compounds leaking from injured cells) act as primary substrates in this hypothetical cartoon.

Rate expressions evaluated from calibrating a theoretical injury model to experimental results yielded values of injury and irreversible loss coefficients. As toluene concentration was increased five times, injury coefficient, K_I , increased by a factor of six and irreversible loss coefficient, K_{GL} , increased by a factor of ten. These rate expressions suggest that increasing toluene concentration increased bacterial injury substantially alluding to inhibitory conditions in the batch cultures. Increased duration of toluene exposure leads to increases in the amount of injury and irreversible loss which eventually negates the growth of uninjured cells.

There are a number of justifications for the enumeration of injured bacteria in environmental systems related to groundwater pollution and remediation. Most isolation techniques involving selective media frequently underestimate numbers of viable hydrocarbon-degrading bacteria. In general, actual environmental conditions provide sub-optimal growth conditions. It is imperative to determine if injury in biofilm systems is different from injury caused to planktonic bacteria. The ramification of such a stress

response are numerous and need to be accounted for to improve the confidence in designing remediation systems and assessing bioremediation potential.

In conclusion, we have observed that (1) toluene degradation causes injury in *P. putida* 54G cells, (2) injury increased with duration of toluene exposure, concentration of toluene and coincided with the formation of toluene degradation intermediates, and (3) two different phenotypes of *P. putida* 54G were formed, cells that were reversibly injured and cells that irreversibly lost their toluene degradative pathway.

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Table 4.1. Description of cell enumeration using different microbiological techniques.

Description	Enumeration Method	Procedure
Total Cells	DAPI	Direct microscopic counts of filtered DAPI-stained bacteria
Respiring Cells	CTC	Direct microscopic count of filtered CTC-stained bacteria
Viable Cells	R2A	Plate counts on non-selective medium
Uninjured Cells	HCMM2 + Toluene (T)	Plate counts on selective medium
Injured Recoverable Cells	R2A $\Rightarrow$ HCMM2 + T	Growth on selective medium after transfer from non-selective medium
Injured Non-Recoverable Cells	R2A $\Rightarrow$ HCMM2 + T	No growth on selective medium after transfer from non-selective medium.

Table 4.2. Parameters obtained by fitting theoretical injury model to results from the 150 and 750 STUDY.

Parameter	150 ppm	750 ppm
X_o , CFUs/ml	3.2×10^8	1.94×10^{10}
K_I , day ⁻¹	0.0678	0.4269
K_{GL} , day ⁻¹	0.0065	0.0655
μ_{NET} , day ⁻¹	0.0255	0.0118

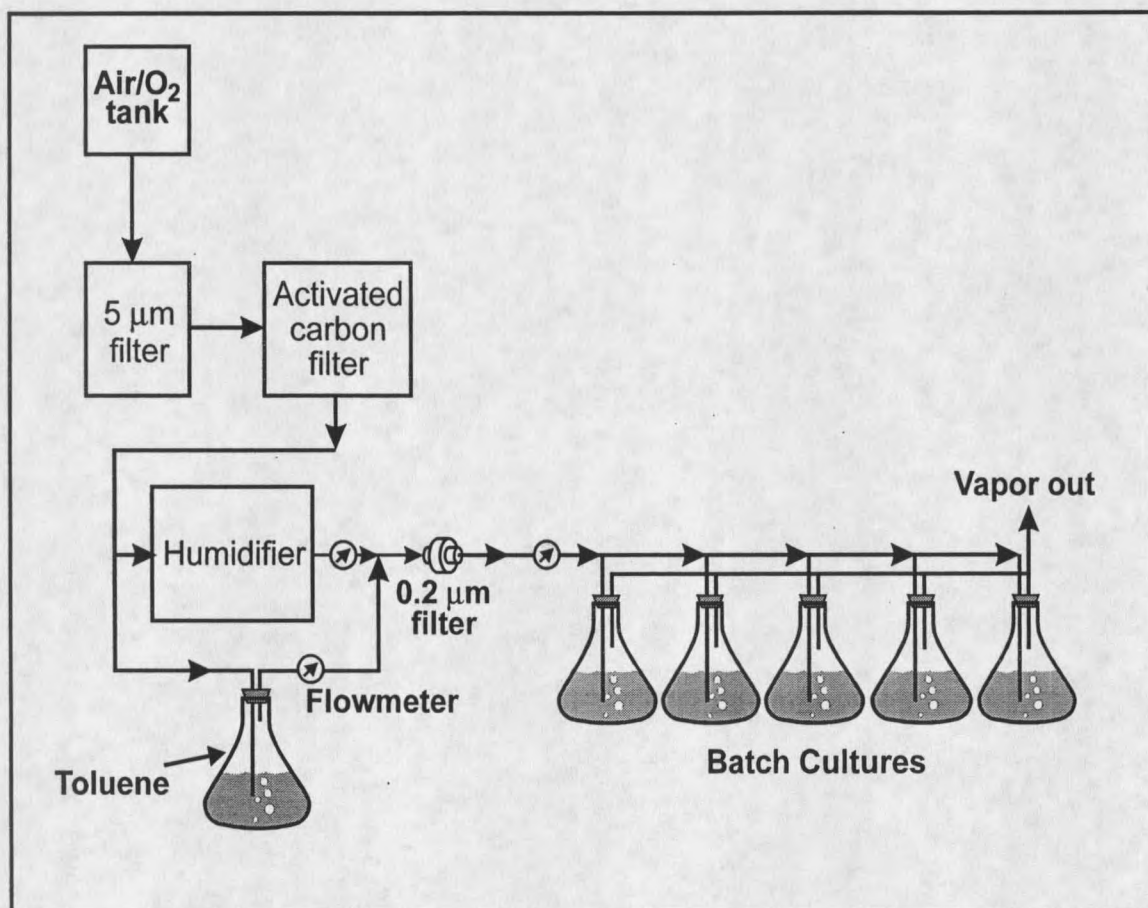


Figure 4.1. Manifold system to assess bacterial injury.

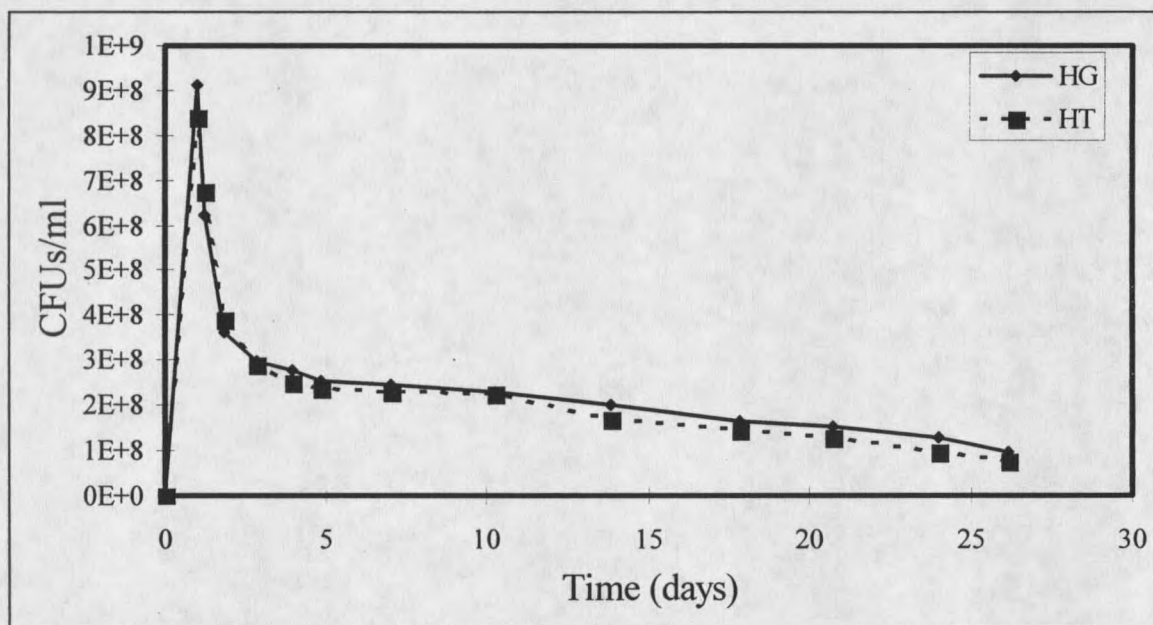


Figure 4.2. Cell numbers on HCMM2 plates combined with glucose (HG) and HCMM2 plates incubated in the presence of vapor phase toluene (HT).

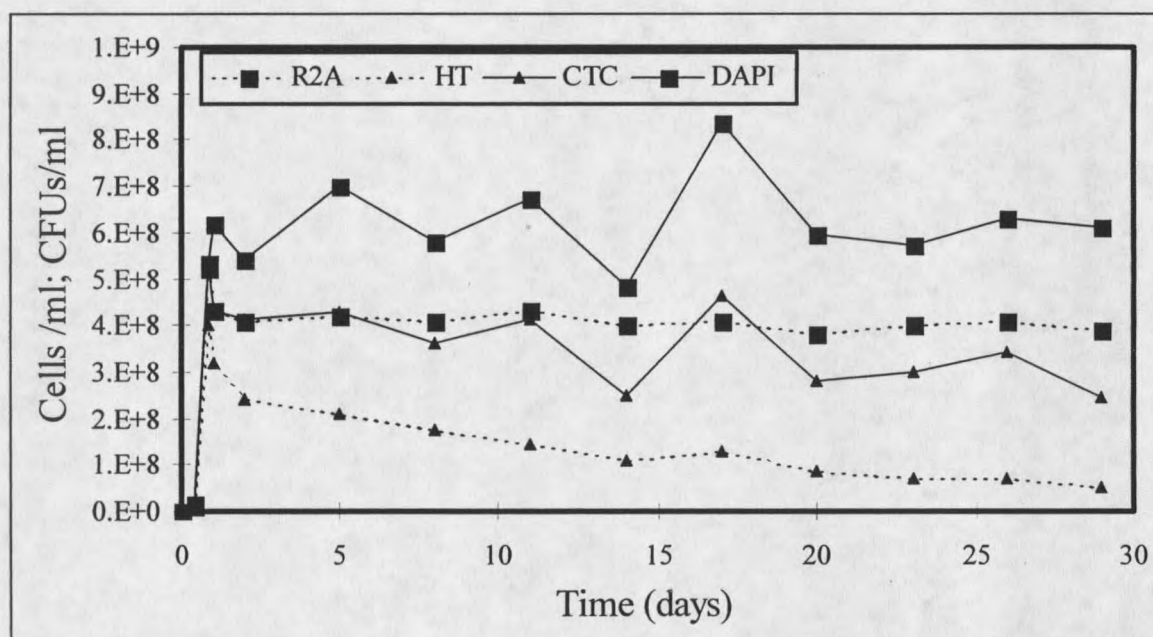


Figure 4.3. Progression of cell numbers, during the 150 STUDY, determined by staining with CTC and DAPI in cells/ml. Also shown are viable cell numbers in CFUs/ml obtained on R2A plates and HCMM2 plates incubated in the presence of vapor phase toluene (HT).

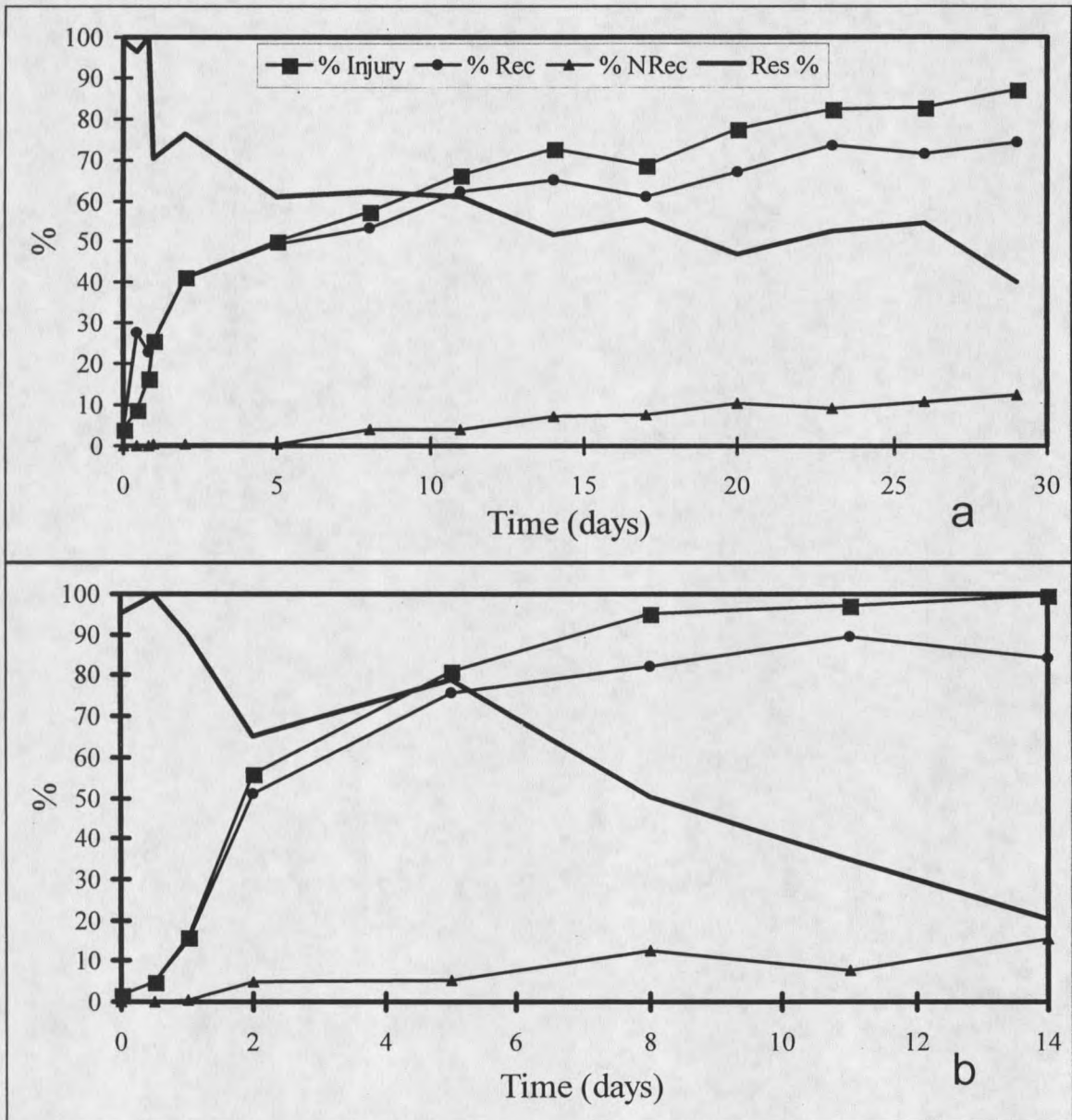


Figure 4.4. Percentage of injured cells (% Inj), injured recoverable cells (% Rec), injured non-recoverable (% NRec) and respiring cells (Res %) during the (a) 150 STUDY and (b) 750 STUDY.

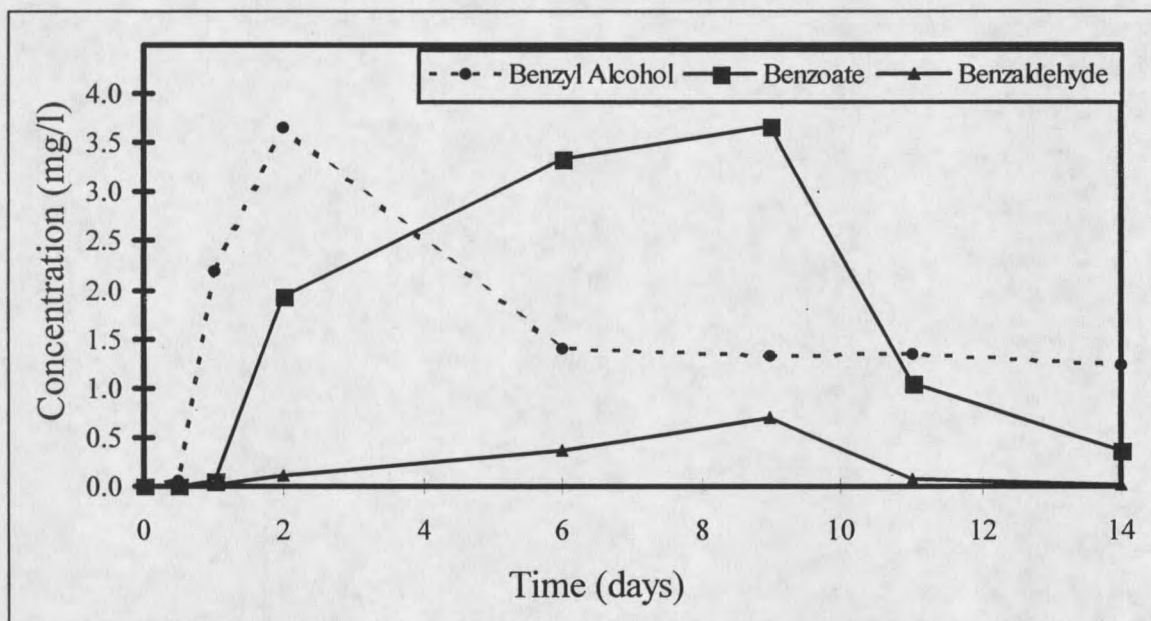


Figure 4.5. Accumulation of intermediates due to incomplete toluene degradation during the 750 STUDY.

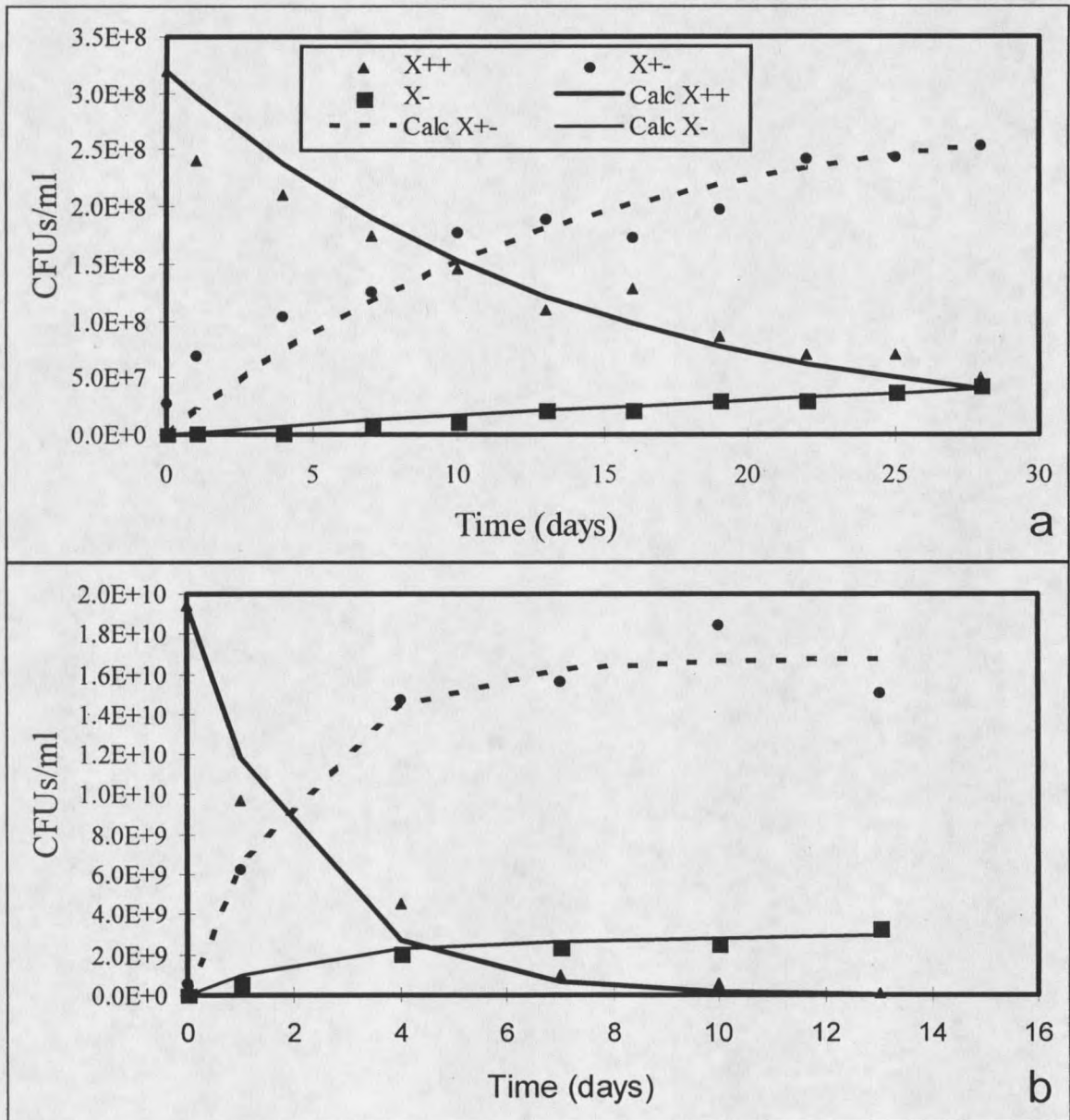


Figure 4.6. Injury model fitted to results obtained from the (a) 150 STUDY and (b) 750 STUDY. Markers indicate experimental results and lines represent predicted results.

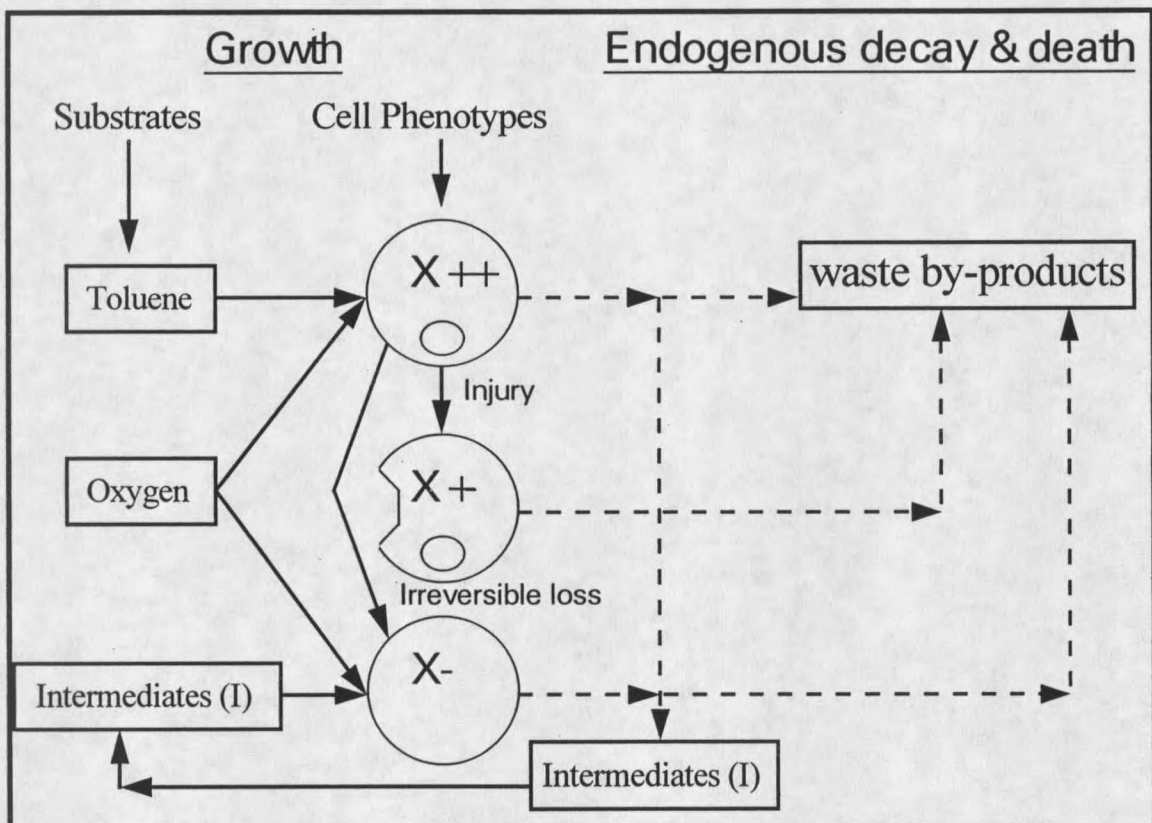


Figure 4.7. Formation of injured recoverable cells (X+-) and cells that have undergone irreversible loss of toluene degrading capability (X-) from wild type cells of *P. putida* 54G (X++). Toluene, oxygen and intermediates produced due to incomplete toluene degradation as well as from lysed products are substrates used by the three different phenotypes.

CHAPTER 5

SPATIAL DISTRIBUTION OF RESPIRATORY ACTIVITY IN
P. putida 54G BIOFILMS DURING TOLUENE DEGRADATIONIntroduction

Air emissions containing volatile organic compounds (VOCs) from industrial and agricultural processes, from end-of-pipe treatment and from soil-vapor extraction at contaminated aquifers pose a serious air pollution problem (7, 11, 36, 47). Vapor phase bioreactors (VPBRs), or biofilters as they are more commonly known, are being applied increasingly to remediate vapor streams contaminated with volatile hydrocarbons such as methanol, phenol, benzene, toluene and dichloromethane (1, 7, 8, 9, 11, 25, 36, 47). Most modeling efforts to describe the reaction-diffusion system of VPBRs are thwarted by a lack of fundamental kinetic and mass transfer rate information. An understanding of the spatial distribution of respiratory activity in relation to key components such as microbial species and dissolved as well as particulate substrates in biofilms is critical to bioreactor design and operation (41). For 25 years, researchers (12, 19, 40) had presumed that the mass of attached microorganisms in fixed-film reactors was not uniformly active. As dissolved electron donors and acceptors were metabolized, different layers of

microbial activity developed within thick biofilms. Qualitative information to support this observation was reported for the degradation of dichloromethane (7, 8, 9) and toluene (2). Bioreactor mathematical models exist that predict the development of stratified biofilms, with active layers at the biofilm/fluid interface and inactive or dead cells existing closer to the biofilm/substratum interface (37, 38). Such observations are consistent with reaction-diffusion models which predict mass transfer limitations of limiting substrate(s) in thick biofilms, where substrate utilization rate increases with increasing substrate concentration.

A variety of techniques have been used to quantify spatial distribution of cells in biofilms: microelectrodes (29), electron microscopy (14), autoradiography (17, 30, 39), microfluorimetry (20), micro-slicing technique followed by phospholipid analysis (45, 46) or dual radiolabeling (4), confocal scanning laser microscopy (21), distribution of adenylate concentrations and adenylate energy charge (18), and cytochemical staining techniques combined with microscopy (6, 26, 33, 35). Huang et al (13) used cryosectioning techniques in combination with staining to demonstrate that gradients of respiratory activity in biofilms could occur when a biocide (monochloroamine) treatment was applied. Due to mass transfer limitations of monochloroamine in 140 μm thick biofilms, dead cells stratified at the biofilm/liquid interface in comparison to respiring cells accumulating at the substratum.

Wimpenny and Kinniment (42) recently identified key "enabling" technologies that could significantly contribute to the investigation of heterogeneity within biofilms; including (1) deployment of microelectrodes coupled with mathematical simulations for

calculating rate data, (2) cryosectioning combined with the use of "viable" cell assays and (3) cryosectioning coupled with microscopy. Instead of using a single analytical tool, combining two or more techniques can aid in interpreting results (13).

Quantitative information on stratification of cell layers in biofilm reactors used in the degradation of volatile, potentially toxic, organic compounds such as toluene is non-existent. Toluene degradation by *P. putida* 54G has been studied and it has been shown that the hydrocarbon causes bacterial injury (27, 31). While toluene is not as toxic as a biocide, the hydrocarbon might cause similar effects when exposure time increases.

We contend that gradients in respiratory activity exist in toluene degrading biofilms and these gradients can be related to the presence of other substrates that are formed due to toluene degradation. In this study, biofilms of a mononculture, *P. putida* 54G, were used to degrade toluene in a flat plate VPBR. Cryosectioning with total cell and respiratory-activity staining was coupled with microsensor measurements of local oxygen concentrations in the liquid and biofilm phases to assess stratification of respiratory activity.

Materials and Methods

Flat Plate Bioreactor: The flat plate reactor (Figure 5.1) was made of polycarbonate with dimensions of 61 cm long and 5 cm wide, with a 12 cm headspace above the substratum. The reactor was equipped with ports on each end for gas and

liquid sampling at an influent and effluent sampling position. The bottom of the reactor was made of glass to facilitate viewing using an inverted microscope. The reactor lid was fastened to the reactor using screws and a rubber gasket. Nine ports of 2 cm diameter each on top of the lid allowed sampling of the liquid and biofilm on the glass bottom. Latex glove "fingers" were fastened onto the sample ports using rubber o-rings. The "fingers" allowed the introduction of oxygen probes into the reactor and provided flexibility in moving the probe in different directions while maintaining gas tight conditions. Compressed air was bubbled through a humidifier and an Erlenmeyer flask containing liquid toluene. Air streams were combined together and delivered through a 0.2 μm Teflon filter into the VPBR at a flow rate of 50 ml/min and toluene vapor concentration of 150 ppm at the influent gas port. Peristaltic pumps were used to provide sterile HCMM2 mineral salts medium (32) to the reactor at a constant flow rate of 1 ml/min in a countercurrent mode to the vapor phase. HCMM2 medium did not contain any organic compounds; the sole exogenous carbon and energy source during bacterial degradation was toluene. Liquid medium filled an influent well, flowed through the reactor under laminar flow conditions and was pumped out through an effluent well to a drain. The laminar liquid layer achieved a maximum thickness of 4 mm. Glass coupons, 6 mm x 6 mm, were placed on the bottom to allow for destructive sampling of the biofilm. The thickness of the coupons was around 150 μm and did not significantly affect the hydrodynamics of flow.

During start-up, suspended cells of *P. putida* 54G (5 ml at 1.3×10^8 CFUs/ml), pre-grown on toluene vapors, were introduced into the reactor and recycled for 12 hours,

after which the reactor was operated continuously in a once-through mode. At this point, intensive sampling of reactor fluids and biomass was combined with microelectrode measurements to obtain a comprehensive picture of reactor behavior. Gas and liquid samples were taken at the influent and effluent ends of the reactor, and through the sampling ports at several points along the reactor length, and analyzed for toluene concentration. Liquid effluent samples were analyzed for total and actively respiring cells. Polarographic oxygen probes were then introduced through the sampling ports, and oxygen profiles were obtained starting at the air/liquid interface and ending just above the substratum. An inverted microscope allowed viewing of the tip of the probe, and was used to position the probe tip within the biofilm. Once oxygen profiles had been collected, and cell numbers had been determined, the reactor flow was shut off, and the lid removed. Glass coupons were then withdrawn from the bottom of the reactor for cryosectioning and staining.

Bacterial Strain and Liquid Media: *Pseudomonas putida* 54G, a toluene-degrading bacterium, was acquired from Dr. Harry Ridgway at the Biotechnology Department in Orange County Water District. *P. putida* 54G was isolated from a gasoline contaminated aquifer at Seal Beach, California. The isolate was capable of growth on HCMM2 mineral salts medium in the presence of vapor phase toluene. HCMM2 mineral salts medium was used as a source of inorganic nutrients in the VPBR with composition described elsewhere (32).

Oxygen Probes: Oxygen probes were fabricated as explained previously (15, 24, 28, 29). Tips of the oxygen probes were 10 μm which allowed for non-invasive measurements of oxygen concentration in the biofilm. Probes were mounted on micro-manipulators and used for *in-situ* measurement of oxygen in the liquid and biofilm phases through the sampling ports provided. Probes were calibrated in air-saturated water and nitrogen-saturated water. Effect of stirring on the microsensors was less than 5% of the response.

Microscopy: Total and actively respiring bacteria were determined by direct epifluorescent microscopy. Samples were counterstained by the DNA-binding fluorochrome 2,4-diamidino-2-phenylindole (DAPI) and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) based on a procedure outlined by Rodriguez et al (33) with minor modifications at final concentrations of 1 mg/l and 1520 mg/l, respectively. Biofilm and liquid samples withdrawn from the reactor system were diluted in HCMM2 medium, vortexed and homogenized. Suspensions were then stained with CTC on 0.2 μm pore-size black polycarbonate membrane filters and incubated at room temperature with stirring for two hours (samples amended with 0, 0.1 $\mu\text{g/ml}$ and 1 $\mu\text{g/ml}$ Yeast extract + Casamino acid solutions were tested, and a negligible difference in cell numbers was obtained). Samples were then washed and stained with DAPI on the filter for 40 minutes, followed by two more washing steps. Filters were air dried and placed on glass slides for microscopic examination using an Olympus BH2-RFCA epifluorescent light microscope (Scientific Instrument Company, UT) with a 100X oil immersion lens.

Cryosectioning: Coupons taken from the VPBR were immersed in separate beakers containing CTC and DAPI separately using similar concentrations as outlined above. After staining, coupons were cryoembedded using the techniques of Yu et al (43, 44). The stained samples were placed on dry ice with the biofilm facing up, and a thick layer of Tissue-Tek® OCT (Miles Incorporated, Elkhart, IN) was dispensed on top of the biofilm. The embedded biofilm was allowed to rapidly freeze until the sample turned opaque white. The biofilm sample was then transferred to a -19° C incubator and allowed to equilibrate. The substratum was then gently separated from the biofilm and more OCT was placed on the substratum side of the biofilm. The sample was then stored at -70° C in aluminum foil until cryosectioning (43). 5 μ m thick frozen sections were collected on glass slides for visual examination using an Olympus BH2-RFCA epifluorescent light microscope (Scientific Instrument Company, UT) with a 100X lens.

Toluene Sampling: Gas and liquid samples were analyzed for toluene using gas chromatography (GC). In the headspace, toluene was measured using a HP 5890 Series II GC equipped with a flame-ionization detector and an Alltech 0.1% A1-1000 Graphpac GC 80/100 column at a constant column temperature of 140°C. Headspace samples (250 μ l) were withdrawn and injected into the GC. Toluene concentrations in the liquid were determined by combining 1 ml of aqueous samples with 0.5 ml of hexane and injecting 2 μ l of the hexane phase into a HP 5890 Series II GC equipped with a photo-ionization detector and a DB-624 column. The column temperature was programmed to ramp from 140°C-200°C. Helium was used as a carrier gas in both columns at a 30 ml/min flow rate.

Results

Oxygen Profiles: The flat plate VPBR was operated for approximately 50 days at an influent toluene vapor phase concentration of 150 ppm, and influent and effluent gas samples were taken periodically to determine the amount of toluene degraded. Figure 5.2 shows oxygen concentration profiles measured in the flat plat VPBR as a function of distance from substratum. The lower curve represents oxygen concentration in the liquid and biofilm measured through the second sampling port (designated as Port 2) from the gas influent side of the VPBR. These profiles were taken after a constant effluent toluene concentration of 50 ppm had been measured continuously for a week. This indicated that steady state conditions with respect to toluene existed in the reactor. The oxygen concentration decreased from about 6.5 mg/l to 6.1 mg/l at a distance of 2 mm from the substratum. Between 1.4 and 2 mm from the substratum, a transition region can be seen indicating the biofilm/liquid interface (confirmed by observation of the probe tip using the inverted microscope). In the biofilm, the oxygen concentration was reduced from 5.8 mg/l (1.4 mm) to 2.6 mg/l at the substratum. The liquid phase toluene concentration at this point was measured at 0.72 mg/l.

The upper curve in Figure 5.2 is an oxygen profile at the same location as the lower curve except that the reactor was tilted to slough loosely attached biofilm before the profile was taken. The transition region of the biofilm/liquid interface lies at about 0.45 mm from the substratum with oxygen concentration reducing from 6.6 mg/l at 2.9 mm to 4.2 mg/l at the substratum. Based on the two curves, it can be established that

approximately 1.25 mm of biofilm was lost during the sloughing event. Biofilm thickness after sloughing was measured using confocal laser microscopy to be between 0.1 - 0.15 mm (data not shown). A section of the lower curve in Figure 5.2 from 0 - 1.4 mm was expanded and shown in Figure 5.3. This section of the profile provides information on oxygen consumption through the biofilm. Based on the continuity equation, the slope of the oxygen profile is proportional to the rate of substrate uptake (5). The slope of the oxygen profile from 0 - 0.125 mm is $65.7 \text{ mg O}_2 \text{ l}^{-1} \text{ cm}^{-1}$ and from 0.125 - 1.4 mm is $19.2 \text{ mg O}_2 \text{ l}^{-1} \text{ cm}^{-1}$. This indicates that the biofilm immediately adjacent to the substratum ($< 0.125 \text{ mm}$) was 3.4 times more active than the remainder of the biofilm (0.125 - 1.4 mm).

The toluene concentration in the liquid dropped from 0.72 mg/l at Port 2 to 0.23 mg/l at Port 8. The oxygen profile taken in the liquid and biofilm phases through Port 8 is shown in Figure 5.4. In this profile, oxygen concentration at 2.9 mm is 6.8 mg/l and drops to 6.7 mg/l at 1.7 mm. A transition region can be observed at this position the liquid/biofilm interface. At the substratum the oxygen concentration is 6.29 mg/l in comparison to 2.6 mg/l at Port 2. The cells at the substratum (0 - 0.125 mm) are more reactive than the cells in the remainder of the biofilm based on the slope of the oxygen profile. Other oxygen profiles taken at different positions in the liquid and biofilm phase are consistent with profiles shown in Figures 5.2, 5.3 and 5.4. At every sampling position, the cells in the biofilm attached to the substratum (basal film) were more active than the cells in the remainder of the biofilm. Further, the sloughing event induced by tilting the reactor was observed throughout the reactor, and oxygen profiles taken after

sloughing were similar to that shown in Figure 5.3.

Respiratory Staining: CTC and DAPI were used to determine the respiratory spatial activity of the bacteria within the biofilm. Cells that stain with CTC appear red and indicate respiring bacteria while DAPI stained cells appear green and measure total (respiring + dead) cells (49). Samples were taken in the biofilm at Port 2 and the effluent sampling well. The reactor was tilted to initiate sloughing and the sampling procedure was repeated. Cell numbers in the sloughed (upper) layers of the biofilm are computed by sampling the effluent well before and after sloughing the upper layer into the well, calculating the difference and normalizing to the biofilm area. Table 5.1 shows numbers of actively respiring and total cells in the basal film and upper layer of the biofilm and in the whole biofilm.

Based on the results reported in Table 5.1, it can be seen that the sum of the cell numbers in the upper and basal layers approximately correspond to 84 % of the total cell numbers and that CTC positive cells in the upper basal layers account for 43 % of the total cells in the whole biofilm. The ratio of CTC to DAPI stained cells (respiration % in Table 5.1) is a measure of the respiratory state of the bacterial population. CTC stained cells in the upper layers of the biofilm number 2.2×10^7 cells/cm² of reactor surface while total cells based on DAPI staining were measured at 2.9×10^8 cells/cm². 7.7 % of the cells were actively respiring in the upper layer of the biofilm. CTC and DAPI stained cells numbered 1.1×10^7 and 1.6×10^7 cells/cm², respectively, in the basal layer retained after sloughing. The ratio of respiring to total cells in the basal layer was 71 %. Prior to

sloughing, the ratio of CTC and DAPI stained cells in the biofilm was 21.3 % (Table 5.1).

Cryosectioning: Cryosectioning coupled with staining can be used to show stratification of activity in biofilm samples. In each photomicrograph in Figure 5.5, the substratum side of the sample is at the bottom, with cells towards the biofilm/liquid interface in the upper part. Figures 5.5(A) and 5.5(B) are examples of cryosectioning and staining *P. putida* 54G biofilms on glass coupons. Since the reactor was destructively sampled to remove the glass coupons from the glass bottom, further sloughing occurred. Biofilm attached to the coupons was between 0.1 - 0.2 mm thick and consisted of a basal film with loosely attached bacteria at the top of the film. Each photomicrograph represents a 5 μm thick section (depth into the page). Red cells signify respiring bacteria based on CTC reduction while green cells are a measure of total (respiring + dead) cell based on reaction with DNA-binding DAPI stain. Since the CTC stain is extremely fluorescent, only non-respiring cells appear green. Figure 5.5(A) shows a profile of biofilm sample taken from a glass coupon at Port 2. Red cells are observed adjacent to the substratum and the green cells appear towards the liquid/biofilm interface. As indicated above, toluene concentration measured in the liquid at Port 2 was 0.72 mg/l. Figure 5.5(B) shows a profile of biofilm sample on a glass coupon at Port 8 with a liquid phase toluene concentration of 0.23 mg/l. While the red cells are lower in number in comparison to Figure 5.5(A), the stratification of the CTC and DAPI stained cells shows a similar pattern. Additional photomicrographs taken along the reactor exhibit similar trends of stratification with respiring cells appearing at or closer to the substratum and

non-respiring cells away from the substratum. Qualitatively, cryosectioning results support the information from previous sections, showing that the majority of respiratory activity occurred near the substratum in this system.

Discussion

In immobilized cell systems, the kinetics of substrate consumption and product formation is linked to the spatial and temporal dynamics of cell populations (10, 16, 17). Wimpenny and Kinniment (42) suggests that deeper cell layers in biofilms could become diffusion limited for essential growth limiting nutrients. They studied *P. aeruginosa* biofilms exposed to low-nutrient concentrations and determined that a large number of dead cells could be found at the base while a large number of viable cells were present in the upper layers of biofilm close to the surface. Kuhn et al (20) evaluated temporal and spatial patterns of cell loading for *E. coli* entrapped in Sr-alginate beads and concluded that mass transfer limitation in the immobilized beads resulted in only 10% of the carrier being occupied, at the outer radial positions, by actively growing cells. Sayles and Ollis (34) predicted similar results quantitatively using a mathematical model.

Oxygen concentration profiles (Figures 5.2, 5.3 and 5.4) demonstrate that respiratory activity of a toluene degrading biofilm decreases towards the biofilm/liquid interface. The microscopy results (Table 5.1) provide evidence that the outer sloughed biofilm layer consisted of a large number of inactive cells with only a fraction of the

biofilm active in degrading toluene. Results from the cryosectioning experiment (Figures 5.5(A) and 5.5(B)) suggest that the basal layer is very active compared to the biofilm cells away from the substratum. These observations are not in agreement with the results obtained by previous researchers. The experimental system used in the present study is inherently different from the systems described above based on the choice of growth substrate. Leddy et al (22) have shown that Tol- mutants of *P. putida* 54G are formed due to long term toluene exposure. The numbers of mutants increased with time and grew in the presence of toluene, without degrading toluene, while expressing higher growth rates, in comparison to the wild type cells, as the age of culture increased. Conversion from Tol+ to Tol- was mediated by metabolic intermediates formed during toluene degradation. They suggested that growth of the Tol- mutants in the presence of toluene was possible because substrates such as organic compounds leaking from injured cells were available. Banks and Bryers (3) have demonstrated cryptic growth rates for *P. putida* and *Hyphomicrobium* sp. on their own soluble lysis products. We have measured endogenous rates of approximately 50% of the total respiration rate, based on O₂ consumption in the absence of toluene, in the present system (data not shown).

The biofilm thickness in the flat plate VPBR was approximately 1400 μm , leading to potential mass transfer limitation within the deeper layers of the biofilm. This suggests that cells adjacent to the substratum could become substrate limited. At the biofilm/liquid interface, long-term exposure to toluene causes significant stress and injury (27). We have determined that injured cells oxidize toluene incompletely to soluble non-volatile intermediates (27). Injury could also lead to release of organic products from

leaking cells (22) or cell lysis products formed as a result of toluene degradation. These substrates could diffuse to the substratum. In the presence of other growth substrates, cells which would normally be substrate limited by mass transfer of the primary substrate were respiring at the substratum. Increased toluene concentration at the substratum would also be expected to result in decreased rates of injury and Tol- mutant formation. The oxygen profiles, microscopy and cryosectioning results support the observation that cells in the basal film at the substratum were the most actively respiring.

The results of this study have shown that, during growth on an injurious and mutagenic substrate, biofilm structure and function differs significantly from that of a biofilm growing on a "benign" carbon source such as glucose. Clearly, such an extrapolation from a single organism and substrate needs to be justified by further study of other strains and mixed cultures and alternative substrates. Nevertheless, the phenomena of diffusion resistance, cell injury and mutation appear to have combined to produce a biofilm with the highest activity furthest away from the source of the primary carbon and energy source. This result suggests that biofilm models applied to bioreactor systems degrading hazardous organics may need to incorporate more physiological phenomena in order to accurately reflect the activity and performance of the reactor systems. It also suggests that alternatives to traditional approaches to design and operation of these systems may be appropriate. Such alternatives might include recycle of reactor effluent to dilute the concentration of the hazardous organic below some threshold limit to avoid injury, or strategies to maintain a thick biofilm, thereby providing a diffusional resistance necessary to continued activity of the basal layer.

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Table 5.1. Total cell counts in the upper and basal layers in the biofilm. Ratio of CTC to DAPI cells is denoted as Resp. %.

Sample	Cell numbers measured by microscopy at 150 ppm		
	CTC (cells/cm ²)	DAPI (cells/cm ²)	Resp. %
Upper (Sloughed) layer	$(2.24 \pm 0.47) \times 10^7$	$(2.92 \pm 0.41) \times 10^8$	7.67
Biofilm - Port 2 (Post-Sloughing)	$(1.13 \pm 0.20) \times 10^7$	$(1.59 \pm 0.32) \times 10^7$	71.02
Biofilm - Port 2 (Pre-Sloughing)	$(7.83 \pm 0.62) \times 10^7$	$(3.68 \pm 0.69) \times 10^8$	21.31

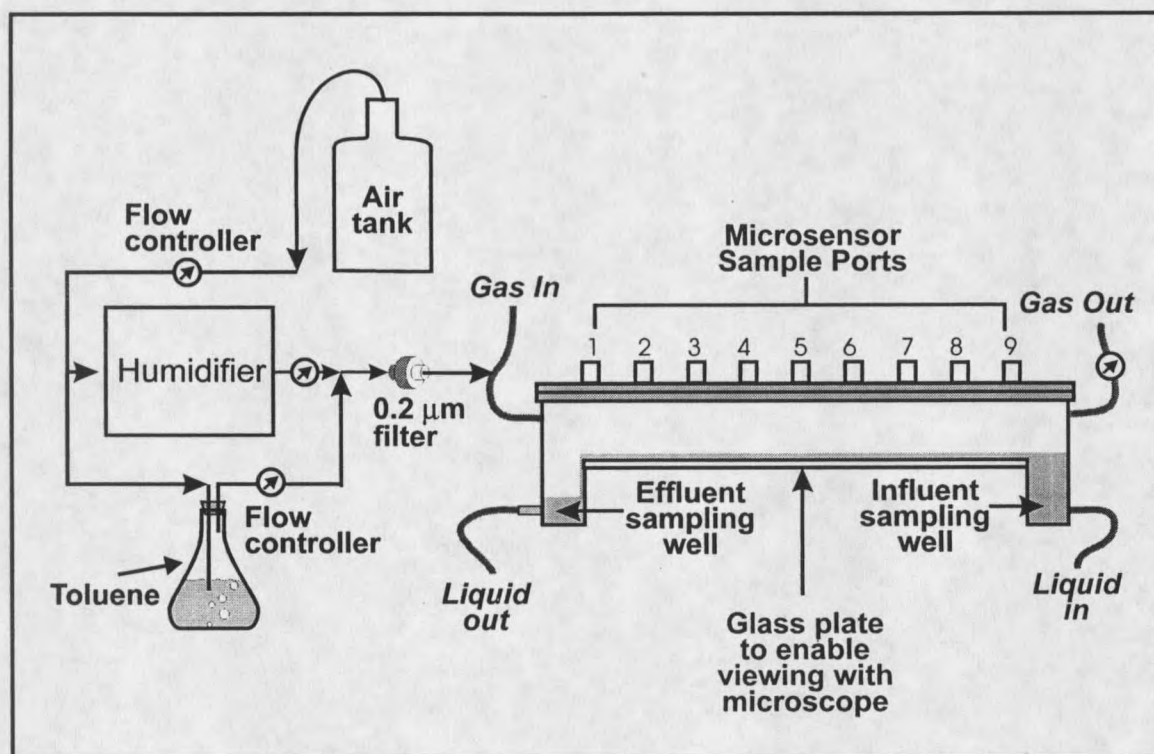


Figure 5.1. Schematic of a flat plate vapor phase bioreactor used to study spatial patterns of respiratory activity in *P. putida* 54G biofilms during toluene degradation.

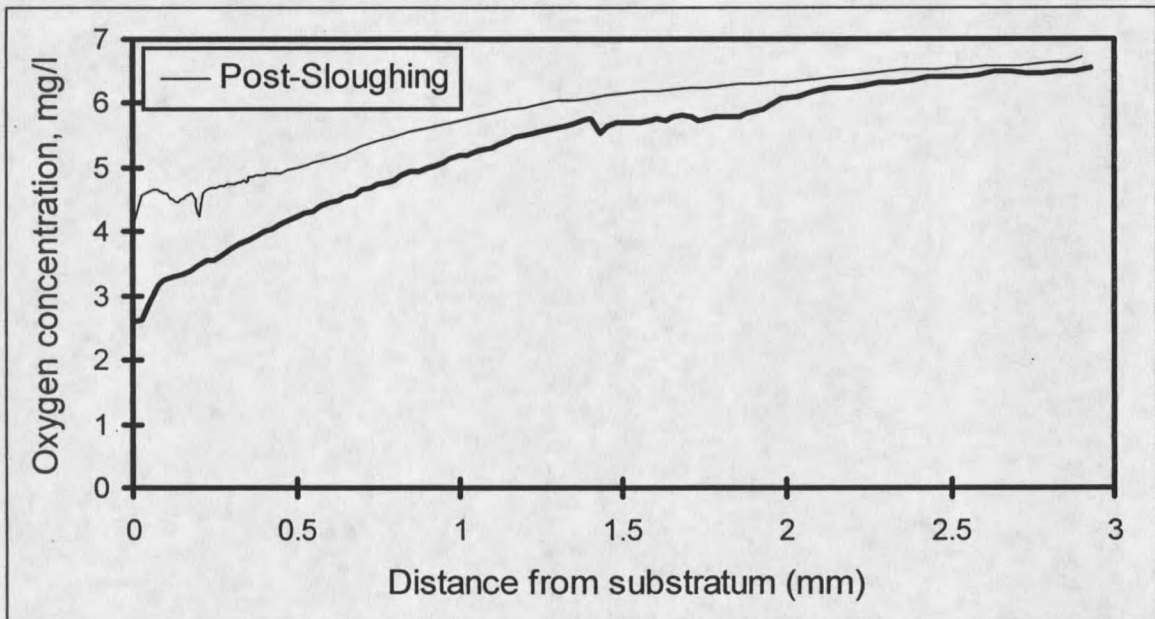


Figure 5.2. Oxygen profile through the second sampling port from the gas influent side (Port 2). The lower curve (heavy line) represents oxygen through the liquid and biofilm phases. The upper curve (thin line) represents oxygen profile at the same location after sloughing the upper layers of the biofilm.

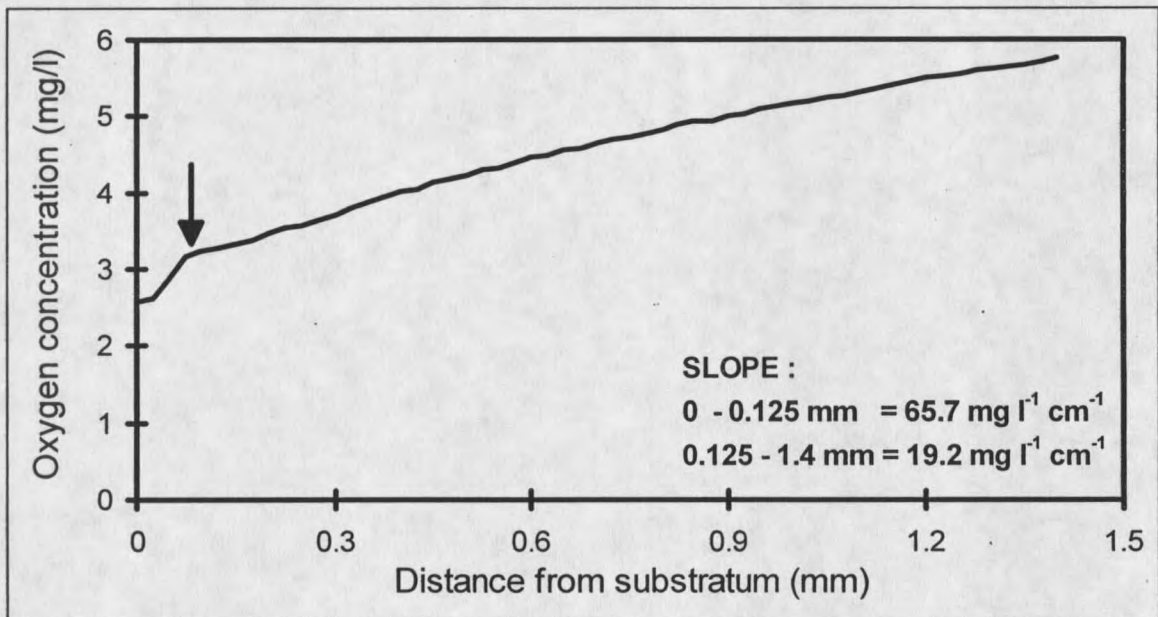


Figure 5.3. Oxygen profile of a section (0 - 1.4 mm) of the lower curve from Figure 5.2.

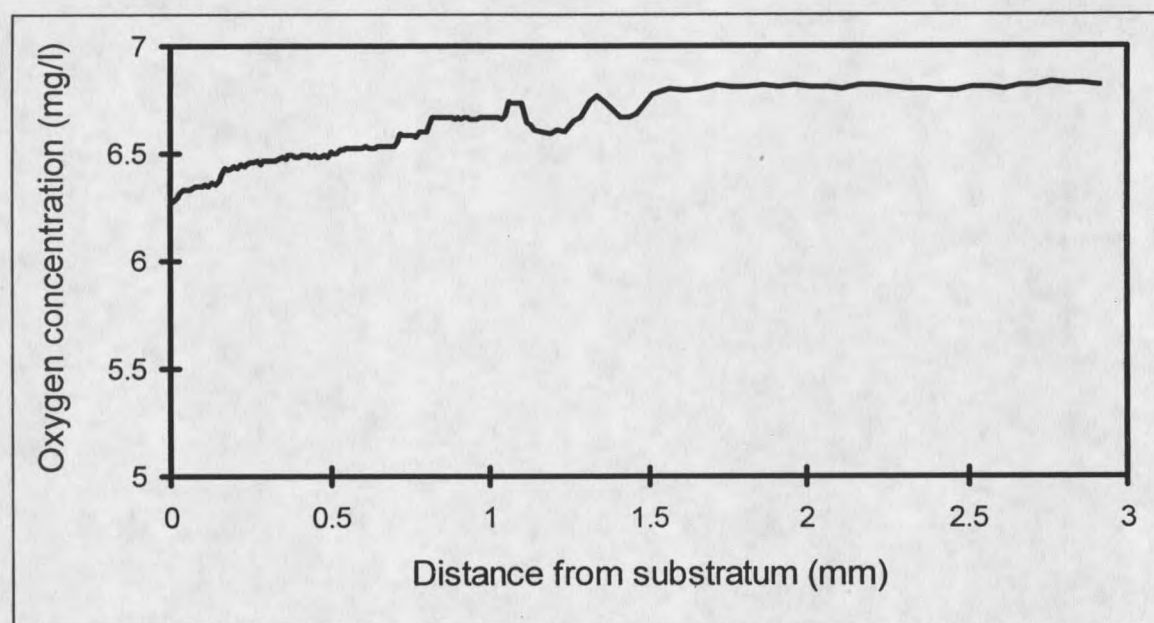


Figure 5.4. Oxygen profile in the liquid and biofilm phases through the eight sampling port from the gas influent side (Port 8).

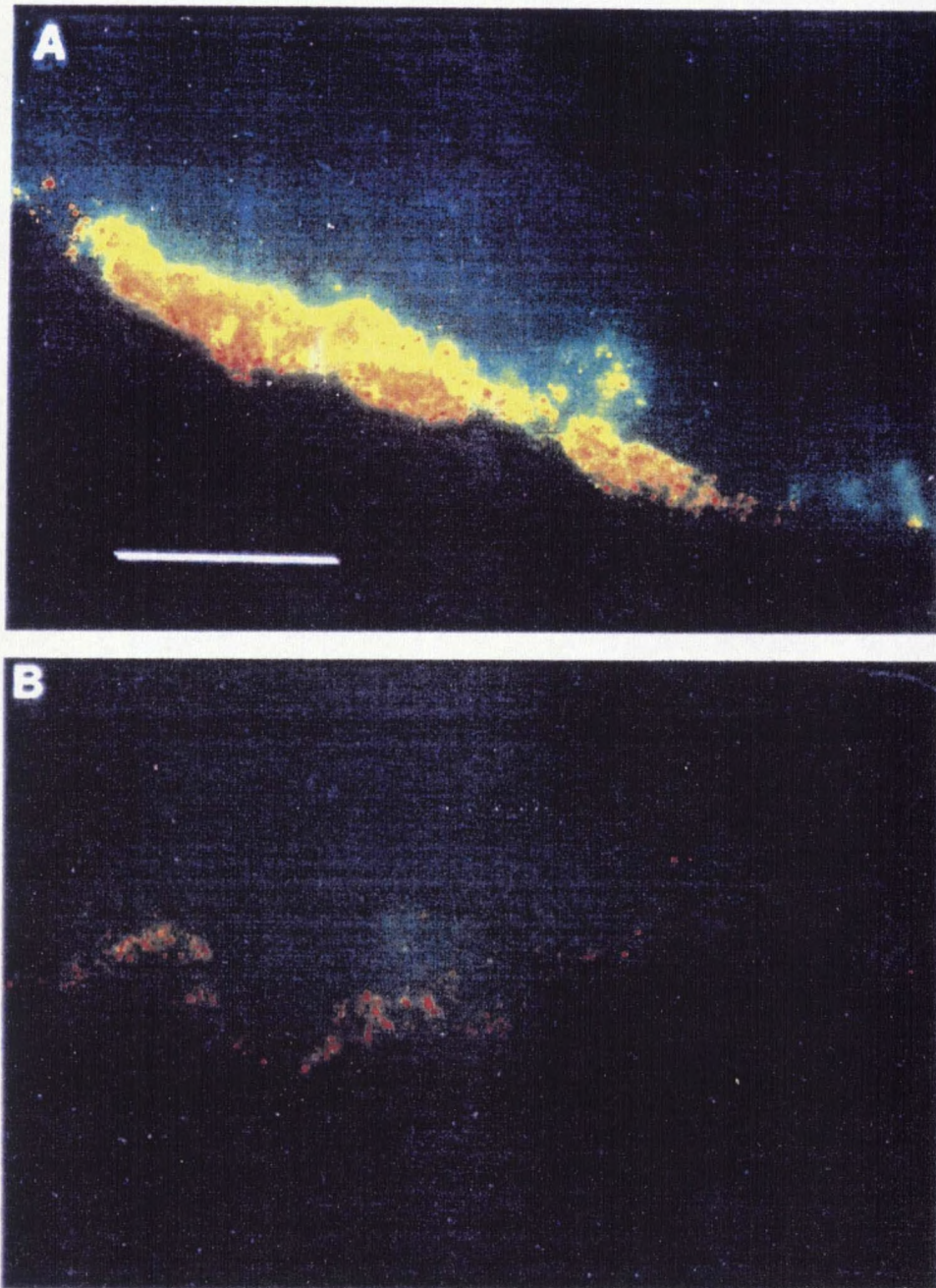


Figure 5.5. Photomicrographs showing cryosectioning results on glass coupons withdrawn at Port 2 (Figure A) and Port 8 (Figure B). The lower part of the micrographs represents cells at the substratum and the upper part represents cells towards the biofilm/liquid interface. The figures represent respiring and non-respiring cells on the glass coupons. Bar = 35 μm .

CHAPTER 6

A PREDICTIVE MODEL FOR TOLUENE DEGRADATION IN A FLAT PLATE VAPOR PHASE BIOREACTOR

Introduction

Vapor phase bioreactors, or biofilters as they are commonly known, have been used to treat relatively low concentrations of highly odorous compounds and potential air pollutants such as volatile organic compounds produced by industrial processes, waste treatment and domestic activities (4). Recent amendments to the Clean Air Act have increased the level of interest in treating contaminated air streams using biofilters because they offer a low-cost and low-maintenance alternative to other air pollution control technologies. The traditional approach of treating VPBRs as a black-box for remediation of contaminated gases has produced promising results in some cases but from a process control stand-point, this technology is still in the early phase of development. Design and control strategies in reference to packing characteristics, biofilm processes, loading rates, reactor plugging, medium pH, pressure drop and oxygen content are necessary. Traditional biofilter design and operation have been carried out without detailed studies on biofilm processes within VPBRs. Biofilm processes leading to substrate degradation and subsequent cell growth are critical to the performance of VPBRs. Processes of

reaction and diffusion in VPBRs are complicated because they involve diffusion from the bulk gas layer through a stagnant liquid layer into the biofilm where substrate degradation occurs. As biofilm thickness increases, mass transfer limitations on both electron acceptor and electron donor may occur, leading to the formation of anaerobic zones and/or dead cells within the biofilm. Other processes such as bacterial injury and toxicity due to degradation of volatile organic compounds are not well understood. These processes are extremely important for assessing steady state performance of VPBRs. Scale-up of VPBRs can best be achieved through use of predictive models, the success of which depend on accurate estimates of kinetic, stoichiometric and mass transfer coefficients.

In the present investigation, a VPBR model is developed which incorporates measured values of kinetic, stoichiometric, injury and genetic loss coefficients for toluene degradation from suspended cell studies of *P. putida* and physical parameters such as Henry's law constants, biofilm surface area and biofilm-liquid interface thickness into a predictive VPBR model. The model is executed by adjusting only three parameters: intrinsic biomass density, death rate and endogenous decay rate. Model results are compared with measured toluene degradation profiles in a flat plate VPBR at two different loading rates, 152 and 767 influent ppm vapor phase toluene concentration. Sensitivity analysis is conducted to determine those parameters that most affect the model results and this analysis provides a practical design tool.

Literature Review

Most models developed for predicting VPBR performance have used simplifying assumptions that degradation kinetics are zero-order or that no external mass transfer resistance limitations exist (1, 2). For most situations, these assumptions yield satisfactory predictions of VPBR performance. Nevertheless these assumptions should be based on experimental verification rather than theoretical considerations. Diks and Ottengraf (2) developed a simplified model to describe the removal of dichloromethane from a biological trickling filter. They concluded that biological processes within the filter rather than transport processes were rate-limiting. They assumed that biological reaction kinetics inside the biofilm were of zero order in substrate concentration. Baltzis and Shareefdeen (1) and Shareefdeen and Baltzis (11) modeled the degradation of single and mixed pollutants (methanol, benzene, toluene) under steady-state conditions in a peat/ perlite biofilter. They assumed that the contaminants and oxygen were depleted in a fraction of the actual biolayer, termed an effective biolayer. In all three studies reasonable fits to experimental data were obtained by adjusting rate-limiting parameters such as mass transfer coefficients or biofilm surface area. None of these studies have incorporated the physiological and genetic response of the bacteria to hydrocarbon degradation-related stress. This stress response is critical in assessing VPBR performance (Chapters 3 and 4).

Suspended Culture and Flat Plate VPBR Studies

We conducted suspended culture studies to determine injury for *P. putida* 54G during toluene degradation. Physiological stress or injury has been defined by McFeters (6) as the physiological, genetic and structural consequence(s) resulting from exposure to sublethal injurious environmental conditions and/or chemical agents. We have determined that toluene degradation causes significant injury on *P. putida* 54G cells with an increase in injury due to duration of toluene exposure and increased toluene concentrations (Chapter 4). Results were consistent qualitatively with research carried out by Leddy et al (3) who determined that Tol- mutants of *P. putida* 54G were formed during toluene degradation. They suggested that the mutants grew on other carbon sources such as organics leaking from injured cells, and mutant cells continued to increase in numbers in the presence of toluene. Based on the injury studies (Chapter 4), we concluded that three different cell types were found in suspended monocultures of *P. putida* 54G during toluene degradation:

- 1) wild-type cells that degraded toluene and were uninjured - type 1.
- 2) cells that were reversibly injured and could grow on toluene only after resuscitation on rich medium - type 2.
- 3) cells that irreversibly lost their toluene degrading capability - type 3.

Rates of formation of these cell types were determined by correlating a theoretical injury model to observed suspended culture results (Chapter 4) and we evaluated three

parameters, an injury coefficient (K_{INJ}) based on conversion of cell type 1 to cell type 2, an irreversible loss coefficient (K_{GL}) based on conversion of cell type 1 to cell type 3 and finally a net growth rate for type 3 cells (μ_{net}). Values for these parameters are shown in Table 6.1. Henry's law constant for toluene was measured in batch reactors as 0.19 g m^{-3} toluene in air / g m^{-3} toluene in water in comparison to a value of 0.271 (same units) published by Mackay et al (5).

Short-term toluene degradation studies by *P. putida* 54G were conducted in batch cultures (Chapter 3) by varying toluene concentration from 1 - 50 mg/l. Toluene and oxygen degradation (Monod relationship) kinetics were combined as shown below:

$$\mu = \frac{\mu_{\max} T}{(K_{ST} + T + \frac{T^2}{K_I})} \frac{O}{(K_{SO} + O)} \quad (1)$$

where μ = specific growth rate [day^{-1}]; $\mu_{\max}$ = maximum specific growth rate [day^{-1}]; T = toluene concentration [g m^{-3}]; K_{ST} = toluene half-saturation constant [g m^{-3}]; K_I = inhibition constant [g m^{-3}]; O = oxygen concentration [g m^{-3}] and K_{SO} = oxygen half-saturation constant [g m^{-3}]. Values of cell yield were also determined from this data set. Values for all parameters used are shown in Table 6.1.

A flat plate VPBR was set-up as shown in Figure 6.1. The advantage of using a flat plate VPBR instead of a conventional biofilter with packing material is that it provides the opportunity to study a well defined system wherein parameters such as biofilm surface area, liquid-biofilm interface thickness and other parameters can be

determined accurately. Humidified toluene vapors were supplied at a flow rate of 50 ml/min in counter-current mode to liquid media flow at 1 ml/min. Liquid HCMM2 which contained no organic compounds was used as a source of inorganic nutrients with composition described by Ridgway et al (10). *P. putida* 54G biofilms grew on the bottom of the reactor (glass bottom) with toluene vapors being the only carbon and energy source. Two different experimental conditions were operated with influent vapor phase toluene concentration of 152 and 767 ppm, respectively. At the higher toluene concentration, oxygen was stoichiometrically limiting, while toluene was limiting at the lower concentration.

Sample ports at an influent and effluent position and along the lid of the reactor were provided as shown in Figure 6.1. Toluene concentration in the gas phase at the influent, effluent and intermediate sample ports (2, 5 and 8) were determined at timed intervals using gas chromatography. A sample (250 μ l) of headspace vapor was injected into a HP 5890 Series II gas chromatograph equipped with an FID detector and an Alltech 0.1% A1-1000 Graphpac GC 80/100, 6' x 1/8" x 0.085", S.S. column (constant temperature of 140°C). Oxygen microsensors with tips < 10 μ m were inserted through sample ports 2, 5 and 8 to measure oxygen concentration profiles in the vapor, liquid and biofilm phases. Oxygen concentration profiles were used to estimate the gas-liquid, liquid-biofilm interface thickness and biofilm thickness. Oxygen concentration profiles in the presence and absence of toluene were used to evaluate oxygen consumption due to endogenous growth of *P. putida* 54G cells. Based on those results, an endogenous yield on oxygen was computed (Table 6.1).

A column reactor was operated as shown in Figure 6.2. The VPBR contained 2 1-ft sections containing ceramic Raschig rings which were 1/4" long and 1/4" in o.d. House air was split into two streams with one stream sparged through a carboy containing water and the other stream sparged through a liquid toluene reservoir. The two streams were mixed together forming a feed into the bottom of the VPBR. Toluene feed was humidified to avoid drying packing material in the VPBRs. Liquid HCMM2 media flowed downward through the VPBRs providing a source of inorganic nutrients for biofilm growth. Sample ports were provided at an influent and effluent position and between the upper and lower section (designated as "middle" sampling port). The sampling ports allowed for the measurement of toluene concentrations in the gas phase. The column bioreactor was operated under the same conditions as the flat plate VPBR except that the vapor flow rate was increased to 1000 ml/min. Kinetic and physical parameters for the column VPBR were similar to those shown in Table 6.1, except that surface area was 0.65 m^2 , the total volume was 0.005 m^3 .

Model Description

VPBRs were simulated using AQUASIM, a biofilm process simulation package capable of modeling any combination of mixed fluid reactors and mixed biofilm reactors (7, 8, 9). AQUASIM is also capable of modeling attachment, detachment and advective transport of bacteria and substrates in liquid and biofilm phases. The flat plate VPBR was modeled using nine mixed reactors for the gas phase which were combined with nine biofilm reactors through diffusive links (Figure 6.3). Reactors of the same type i.e., gas or biofilm, were combined through advective links. Additional information on the model is available in Reichert (7, 8, 9). We conducted liquid phase residence time distribution studies using LiBr as a tracer and determined that the liquid phase in the column VPBRs essentially behaved as a mixed reactor. Based on these results we reduced the number of reactors used in AQUASIM to model column VPBRs to a combination of 4 mixed vapor and 4 mixed biofilm reactors in parallel.

In the model, contaminant diffuses from the gas phase in a mixed vapor reactor (flowing countercurrent to the liquid phase) through a stagnant liquid layer into the biofilm phase where biological reaction occurred (denoted as a biofilm reactor). The following assumptions were made to simplify the model:

- 1) growth and injury kinetics determined for suspended cells of *P. putida* 54 were valid for biofilm cells of the same bacterium,
- 2) water volume fraction in the biolayer was constant, and only growth resulted in an increase in biofilm thickness,

- 3) published values of Henry's law and half-saturation coefficients for oxygen were valid for the present system,
- 4) biofilm density was constant throughout the reactor,
- 5) planar geometry,
- 6) well-mixed bulk phase and
- 7) diffusivity in biofilm was constant ($= 0.8 \times$ diffusivity in water).

The model was used to evaluate steady state toluene degradation and oxygen consumption within the biofilm as well as biofilm thickness values at different positions in the flat plate. For the column VPBR, toluene degradation profiles and total toluene degraded in g/day were modeled. Values of parameters (Table 6.1) along with an influent vapor phase toluene concentration (152 or 767 ppm) were input into the model.

Sensitivity Analysis

Sensitivity analyses using the computational model are of critical importance to design of any experimental program and for determination of rate-limiting steps and process parameters which may be important to scale-up. AQUASIM uses the following expression, described in Reichert (8), to evaluate the sensitivity of a function, such as toluene degradation rate in our case, to different parameters:

$$\delta_{f,p} = \frac{f}{p} \frac{\partial p}{\partial f} \quad (2)$$

where sensitivity index, $\delta_{f,p}$, is the relative change in function p for a relative change in

parameter f calculated as a linear approximation. Because the units of the sensitivity index do not depend on the units of the parameter used, comparison of sensitivity of a function can be made on a normalized basis. The advantage of using such a function is that sensitivity of toluene degradation rates to both experimentally and theoretically determined parameters can be compared and used to identify those parameters whose values must be determined most accurately.

Results and Discussion

Table 6.2 shows a comparison of toluene degradation profiles experimentally determined in the flat plate VPBR and results calculated using AQUASIM. The model results match the experimental values of toluene degradation at different points along the length of the reactor as well as total toluene degradation calculated in g day^{-1} . The experimental results show a plug flow operating regime for the VPBR which is very closely approximated using nine reactors in series in AQUASIM. Increasing the toluene concentration by a factor of five leads to approximately five fold increase in toluene degradation. Due to the reactor geometry, we expect that the flat plate VPBR would offer a well-described system with a lower performance for vapor phase toluene degradation in contrast to a conventional biofilter. The laminar liquid flow supports the formation of a "fluffy biofilm" which is over 1 mm thick (Chapter 5). Mass transport limitations as a result of the thick biofilm might arise. Nevertheless, toluene degradation efficiencies were determined to be about 62% and 53%, at 152 and 767 ppm influent toluene

concentration, respectively.

Table 6.3 shows results of sensitivity analyses conducted on toluene degradation rates [g day^{-1}] based on physical and kinetic parameters for both experimental conditions. The negative sign associated with the sensitivity index indicates that as the parameter under consideration increases, toluene degradation decreases. Clearly, Henry's law constant for toluene (H_T), biomass yield on toluene (Y_T) and biofilm surface area (A) are the most important parameters affecting predictions of toluene degradation. All three of these parameters were determined independently from experiments conducted in our laboratories. Based on the fit, predicted values of both death and endogenous decay rate increased with toluene concentration.

In comparison to the 152 ppm experiment, sensitivity analyses during the 767 ppm experiment suggests that accurate estimates of yield on toluene is necessary. Model predictions from the higher toluene concentration also suggest an increasing dependency on oxygen-related parameters (Y_O , Y_{Ob} and H_O). Quantitatively, at any vapor phase toluene concentration of 450 ppm or higher (based on the measured Henry's law constant), oxygen becomes stoichiometrically limiting. The other important parameters that show affect on toluene degradation are the kinetic coefficients, $\mu_{\max}$ and K_{ST} . This suggests that the system is reaction rate limited rather than diffusion rate-limited. Baltzis and Shareefdeen (1) similarly concluded that the biolayer surface area per unit volume of reactor was the most sensitive parameter in their model. Effect of surface area was more pronounced at the higher toluene concentration. Parameters such as K_I , μ_{net} , λ_{GL} showed a negligible effect on toluene degradation. Surprisingly all three parameters that were

adjusted, arbitrarily, to fit the VPBR model to experimental results (ρ_x , b_x and d_x) showed a weak effect on toluene degradation. This increases the confidence of our model substantially.

Figure 6.4 shows experimental and predicted results for toluene degradation in the column VPBR under steady state conditions at an influent vapor phase toluene concentration of 150 ppm. The model predictions at the middle and effluent positions closely match experimental results. Figure 6.5 shows model predictions and experimental results for total toluene degradation under the same conditions from Figure 6.4. Again, AQUASIM provided a good fit to observed values. Figure 6.6 and 6.7 show similar results for the higher toluene concentration case and based on these figures, AQUASIM satisfactorily predicts toluene degradation in a column VPBR under electron acceptor and electron donor conditions. Values of toluene degradation flux were approximately $1 \text{ g m}^{-2}\text{-day}^{-1}$ and $4.6 \text{ g m}^{-2}\text{-day}^{-1}$ for the 150 and 750 influent vapor phase toluene concentration cases, respectively. Surprisingly these values were similar for the flat plate VPBR. Shareefdeen and Baltzis (11) calculated toluene removal rates in a peat/pearlite biofilter of $4.8 - 26.8 \text{ g m}^{-3}\text{-day}^{-1}$ depending on the residence time of the vapor phase. Based on the value of surface area of biolayer per unit volume of reactor used in their model (40 m^{-1}), comparable removal rates of toluene can be calculated as approximately $2.88 - 16.08 \text{ g m}^{-2}\text{-day}$.

Table 6.4 shows results of sensitivity analyses conducted on toluene degradation rates [g day^{-1}] based on physical and kinetic parameters for the column VPBR under both experimental conditions. Small changes (1%) in kinetic parameters (μ_{max} and K_{ST}),

Henry's law constant for toluene, and death and endogenous decay rates affect toluene degradation rates significantly. In contrast to the flat plate VPBR case (Table 6.3), changes in surface area produce small changes in toluene degradation rates. This result provides crucial information in our modeling effort because in the column VPBR, surface area is a difficult parameter to estimate accurately. Since values for kinetic parameters and Henry's law constant for toluene have been estimated in our laboratories, the only uncertainties in the model arise from adjusted values of death and endogenous decay rates. These rates are difficult to measure accurately and prove a shortcoming of this modeling approach. Since death and endogenous decay control the formation of an effective biolayer which is solely responsible for toluene degradation, these parameters exhibit an enhanced effect on toluene degradation in the column VPBR in contrast to the flat plate VPBR. These differences in sensitivity analysis for the different reactor types could arise due to a variation in flow rates and hence residence time for the vapor phase.

Surprisingly, results of the sensitivity analysis show that increasing toluene concentration does not cause oxygen related parameters to show an effect on toluene degradation in the column VPBR. A logical explanation for this result may be the increasing dependence of column reactor performance on kinetics of toluene biotransformation, which overshadows most of the other processes. While these results do not completely provide all the answers, AQUASIM does prove a satisfactory tool for modeling and scale-up of VPBRs and more importantly provides a framework for increasing confidence of modeling capability which eventually would lead to improved design of VPBRs. This methodology clearly demonstrates that satisfactory measurements

of a few parameters in the laboratory could help substantially in increasing confidence in modeling VPBRs not only at the laboratory scale but might prove a satisfactory tool for scale-up.

Conclusions

In this study, toluene degradation in a flat plate VPBR was modeled using AQUASIM and model predictions were compared with experimental observations.

Based on this study:

- 1) A good correlation was attained between experimental observations and model predictions for toluene uptake rate and toluene concentration profiles in a flat plate and in a column VPBR,
- 2) In the flat plate VPBR, values for biofilm surface area, Henry's law constant for toluene and biomass yield on toluene significantly affected toluene degradation for both experimental conditions and further,
- 3) When toluene concentration was increased, toluene degradation was also affected by oxygen-related parameters such as endogenous yield on oxygen, Henry's law constant for oxygen and yield of biomass on oxygen,
- 4) In the column VPBR, kinetics of biotransformation, Henry's law constant for toluene, and death and endogenous rates affect toluene degradation significantly, but importance of surface area was considerably reduced.
- 5) The differences in the sensitivity analysis conducted on the two reactor types arise from the different geometry and physical operating characteristics.
- 6) AQUASIM proved a useful tool to scale-up from a flat plate VPBR to a column VPBR provided that accurate estimates of rate-determining parameters are available.

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Notation

A	= biofilm surface area [m^2]
A_{GL}	= gas-liquid interfacial area [m^2]
b_x	= endogenous decay rate [day^{-1}]
d_x	= death rate [day^{-1}]
f	= parameter on which sensitivity analysis is based
H_{O}	= Henry's law constant for oxygen [g m^{-3} air / g m^{-3} water]
H_{T}	= Henry's law constant for toluene [g m^{-3} air / g m^{-3} water]
K_{GL}	= genetic loss rate [day^{-1}]
K_{i}	= inhibition constant [g m^{-3}]
K_{INJ}	= injury rate [day^{-1}]
K_{SO}	= oxygen half-saturation constant [g m^{-3}]
K_{ST}	= toluene half-saturation constant [g m^{-3}]
p	= function used in AQUASIM for sensitivity analysis
Y_{O}	= yield of biomass on oxygen [$\text{g biomass} / \text{g oxygen}$]
Y_{Ob}	= endogenous yield on oxygen [$\text{g biomass} / \text{g oxygen}$]
Y_{T}	= yield of biomass on toluene [$\text{g biomass} / \text{g toluene}$]
$\delta_{f,p}$	= sensitivity index on a normalized scale used in AQUASIM
λ	= biofilm-liquid interface thickness [m]
λ_{GL}	= gas-liquid interface thickness [m]
μ	= specific growth rate [day^{-1}]
μ_{max}	= maximum specific growth rate [day^{-1}]
μ_{net}	= net growth rate for X_n cells [day^{-1}]
ρ_x	= biomass density [g m^{-3}]

Table 6.1. Physical and kinetic parameters used in Aquasim to model a flat plate and a bench-scale VPBR at 152 and 767 influent toluene vapor concentration. Parameters in bold represent coefficients adjusted to obtain best fit to total toluene degradation.

Description	Units	152 ppm	767 ppm
<i>Physical parameters</i>			
Gas Volume (V_G)	m^3	7.20E-04	
Liquid Volume (V_L)	m^3	8.00E-05	
Total Reactor Volume (V)	m^3	8.00E-04	
Surface Area (A)	m^2	0.02	
Gas-liquid interfacial area (A_{GL})	m^2	0.02	
Gas-liquid interface thickness (λ_{GL})	m	1.00E-05	
Liquid-biofilm interface thickness (λ)	m	2.50E-05	
Henry's law constant for toluene (H_T)	$g\ m^{-3}/g\ m^{-3}$	0.19	
Henry's law constant for oxygen (H_O)	$g\ m^{-3}/g\ m^{-3}$	43.0	
Density of biomass (ρ_X)	$g\ m^{-3}$	5.00E+04	
<i>Kinetic parameters</i>			
Maximum specific growth rate (μ_{max})	day^{-1}	10.08	
Toluene half-saturation constant (K_{ST})	$g\ m^{-3}$	3.98	
Oxygen half-saturation constant (K_{SO})	$g\ m^{-3}$	0.025	
Inhibition constant (K_I)	$g\ m^{-3}$	42.78	
Yield of biomass on toluene (Y_T)	g / g	0.86	
Yield of biomass on oxygen (Y_O)	g / g	0.55	
Endogenous yield on oxygen (Y_{Ob})	g / g	0.55	
Injury rate (K_{INJ})	day^{-1}	.06783	0.42696
Genetic loss rate (K_{GL})	day^{-1}	0.00655	0.06551
Net growth rate for X_n cells (μ_{net})	day^{-1}	0.02551	0.01182
Endogenous decay rate (b_X)	day^{-1}	0.65	1.5
Death rate (d_X)	day^{-1}	0.10	0.5

Table 6.2. Results of the comparative study of experimentally determined toluene degradation in a flat plate VPBR and results calculated from a VPBR model.

Experiment Conducted	Experimental	Calculated	Units
152 ppm experiment			
Influent	152.00 $\pm$ 5.6	152.00	ppm
Port 2	123.00 $\pm$ 5.2	125.90	ppm
Port 5	94.00 $\pm$ 4.7	94.07	ppm
Port 8	74.00 $\pm$ 3.9	69.39	ppm
Effluent	59.00 $\pm$ 4.2	57.86	ppm
Toluene degradation rate	0.0212	0.0214	g day ⁻¹
767 ppm experiment			
Influent	767.00 $\pm$ 32.2	767.00	ppm
Port 2	642.00 $\pm$ 39.3	683.20	ppm
Port 5	475.00 $\pm$ 29.5	564.80	ppm
Port 8	425.00 $\pm$ 38.2	451.90	ppm
Effluent	363.00 $\pm$ 29.2	383.00	ppm
Toluene degradation rate	0.0920	0.0874	g day ⁻¹

Table 6.3. Sensitivity analysis conducted on select parameters using Aquasim for modeling a flat plate VPBR. Sensitivity index - S. I. (%), evaluated in the table is described in the sensitivity analysis section and denotes the % change in toluene degradation (g day^{-1}) when a particular parameter is changed by 1%. '—' represents a negligible effect of that parameter on toluene degradation.

Flat plate VPBR	152 ppm		767 ppm	
Description	S. I. (%)	Rank	S. I. (%)	Rank
Surface Area (A)	0.500	2	1.634	1
Gas-liquid interfacial area (A_{GL})	—	—	—	—
Gas-liquid interface thickness (λ_{GL})	—	—	—	—
Liquid-biofilm interface thickness (λ)	- 0.097	6	- 0.063	9
Henry's law constant for toluene (H_T)	- 0.577	1	- 0.459	3
Henry's law constant for oxygen (H_O)	—	—	- 0.167	4
Density of biomass (ρ_X)	—	—	—	—
Maximum specific growth rate (μ_{max})	0.197	4	0.144	5
Toluene half-saturation constant (K_{ST})	- 0.167	5	- 0.085	7
Oxygen half-saturation constant (K_{SO})	—	—	—	—
Inhibition constant (K_I)	—	—	—	—
Yield of biomass on toluene (Y_T)	- 0.203	3	- 0.462	2
Yield of biomass on oxygen (Y_O)	—	—	0.097	6
Endogenous yield on oxygen (Y_{Ob})	—	—	0.059	10
Injury rate (K_{INJ})	- 0.026	7	- 0.026	12
Genetic loss rate (K_{GL})	—	—	—	—
Net growth rate for X_n cells (μ_{net})	—	—	—	—
Endogenous decay rate (b_X)	0.0146	9	0.050	11
Death rate (b_X)	0.0149	8	0.069	8

Table 6.4. Sensitivity analysis conducted on select parameters using Aquasim for modeling a bench-scale VPBR. Sensitivity index - S. I. (%), evaluated in the table is described in the sensitivity analysis section and denotes the % change in toluene degradation (g day^{-1}) when a particular parameter is changed by 1%. '—' represents a negligible effect of that parameter on toluene degradation.

Bench-scale VPBR Description	152 ppm		767 ppm	
	S. I. (%)	Rank	S. I. (%)	Rank
Surface Area (A)	0.013	6	0.017	7
Gas-liquid interfacial area (A_{GL})	—	—	—	—
Gas-liquid interface thickness (λ_{GL})	—	—	—	—
Liquid-biofilm interface thickness (λ)	—	—	—	—
Henry's law constant for toluene (H_T)	- 0.150	1	- 0.423	4
Henry's law constant for oxygen (H_O)	—	—	—	—
Density of biomass (ρ_X)	—	—	—	—
Maximum specific growth rate (μ_{max})	- 0.149	2	0.945	1
Toluene half-saturation constant (K_{ST})	- 0.140	3	- 0.487	3
Oxygen half-saturation constant (K_{SO})	—	—	—	—
Inhibition constant (K_I)	—	—	0.038	6
Yield of biomass on toluene (Y_T)	—	—	—	—
Yield of biomass on oxygen (Y_O)	—	—	—	—
Endogenous yield on oxygen (Y_{Ob})	—	—	—	—
Injury rate (K_{INJ})	—	—	—	—
Genetic loss rate (K_{GL})	—	—	—	—
Net growth rate for X_n cells (μ_{net})	—	—	—	—
Endogenous decay rate (b_X)	- 0.138	4	- 0.525	2
Death rate (b_X)	- 0.021	5	- 0.421	5

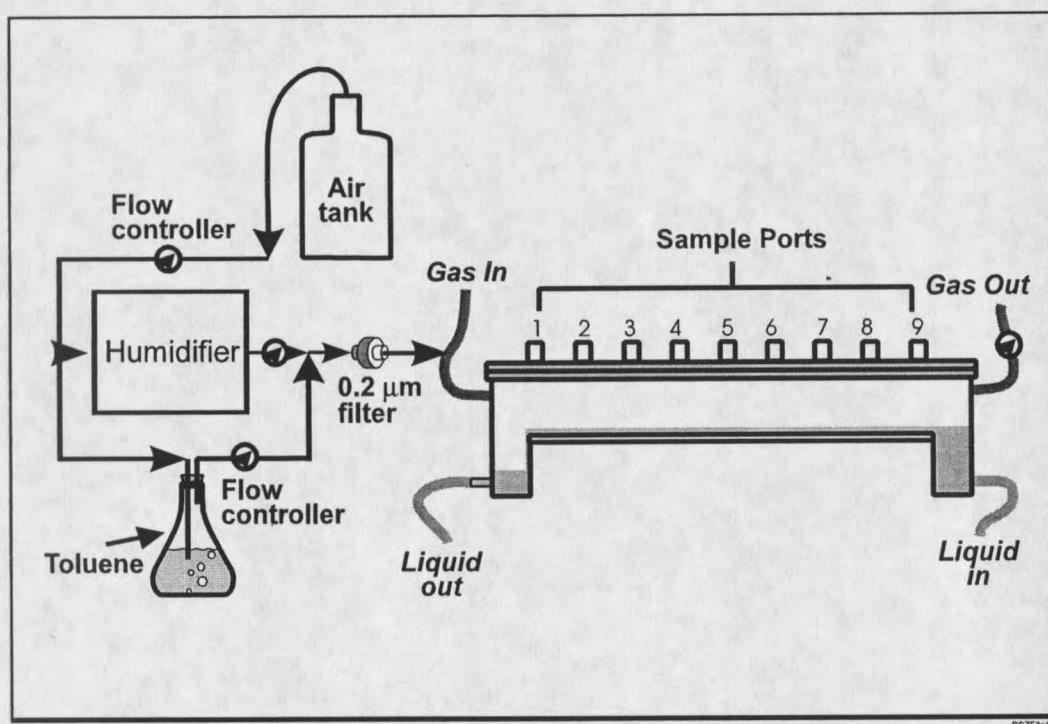


Figure 6.1. Schematic of a flat plate vapor phase bioreactor for toluene degradation by *P. putida* 54G biofilms. Toluene concentration in the vapor phase was measured in the influent, effluent and sampling ports 2, 5 and 8.

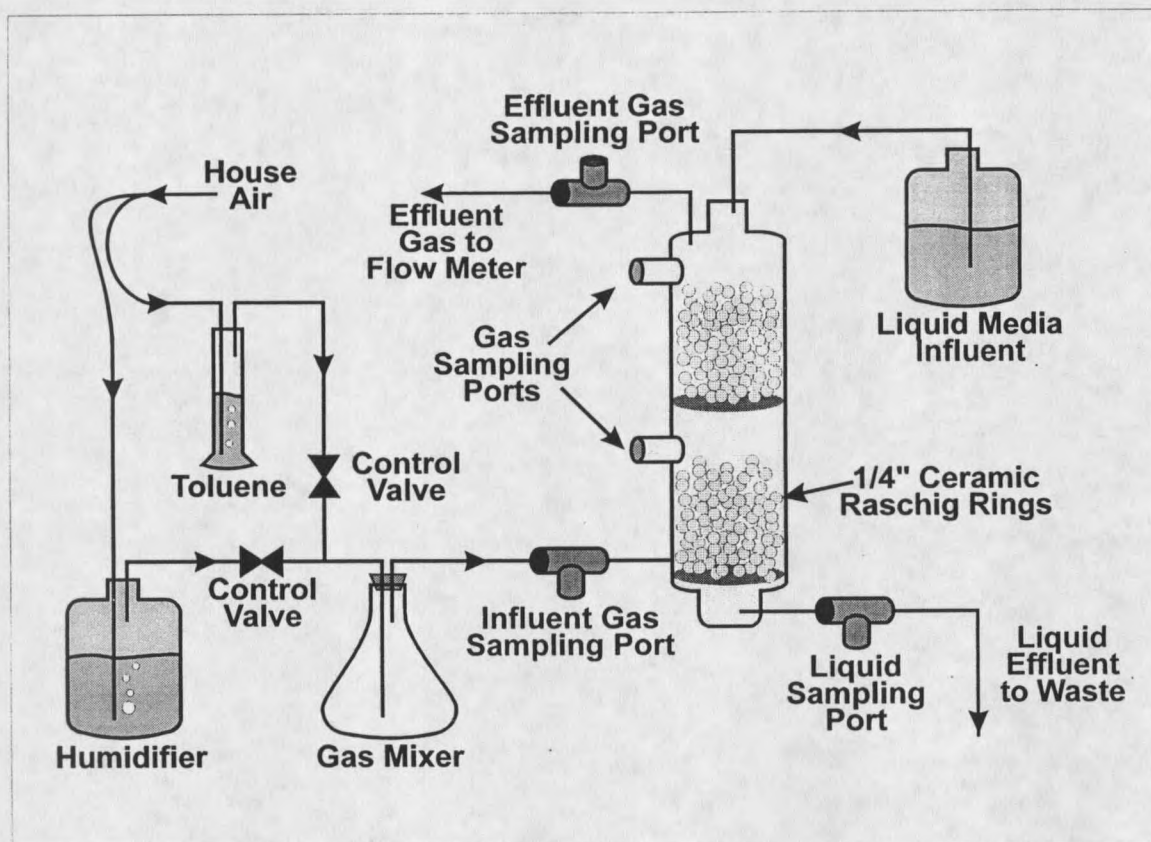


Figure 6.2. Schematic of a bench-scale vapor phase bioreactor used to study toluene degradation by *P. putida* 54G biofilms immobilized on ceramic rings.

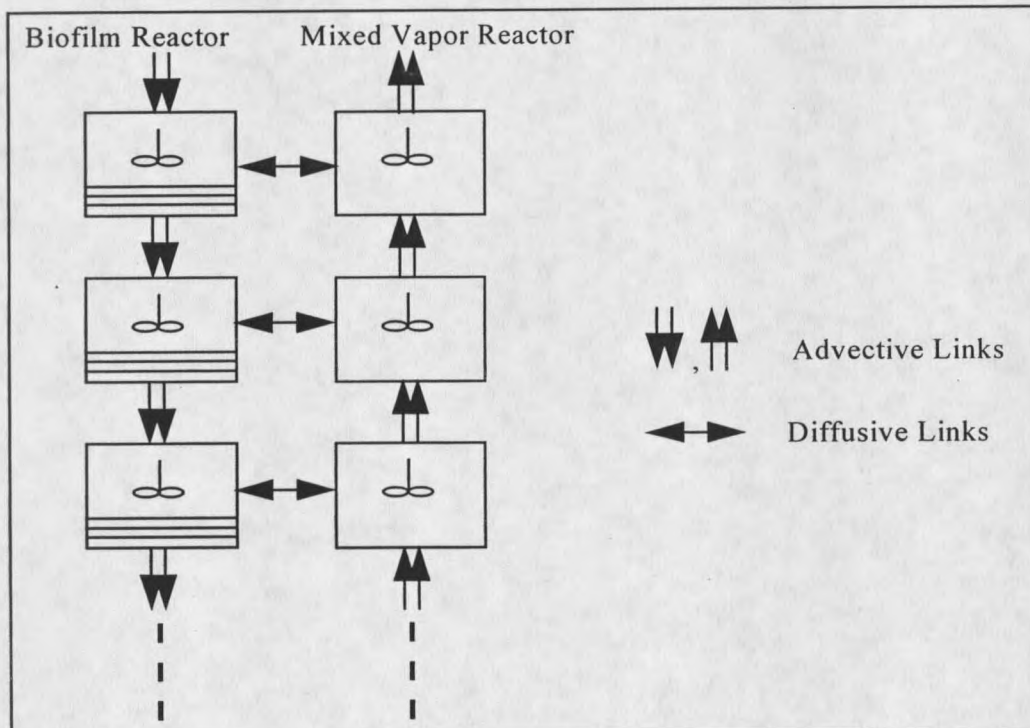


Figure 6.3. Schematic of biofilm and vapor reactors used in Aquasim to model vapor phase bioreactors.

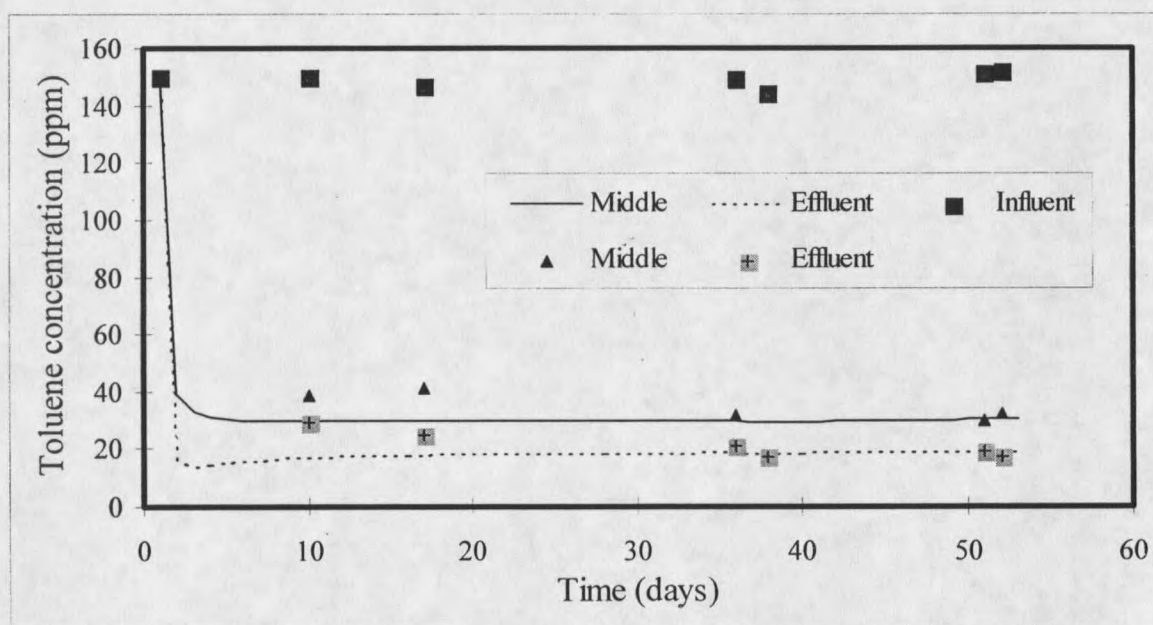


Figure 6.4. Steady state performance of a bench-scale VPBR operated at an approximate influent concentration of 150 ppm. The markers indicate experimental results and lines represent predicted results.

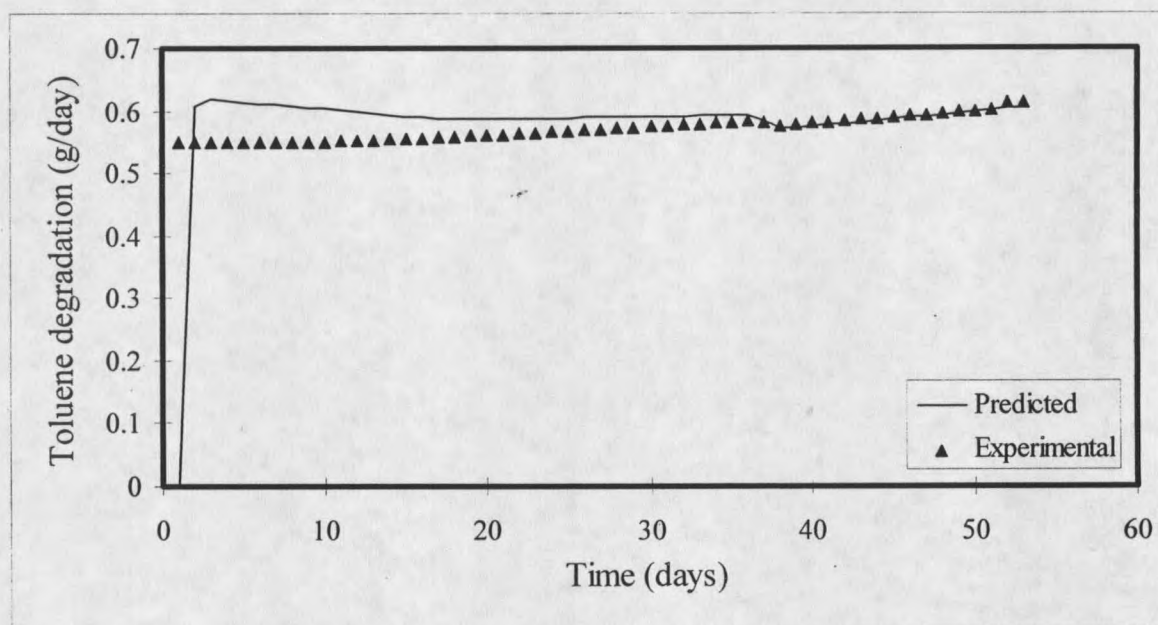


Figure 6.5. Experimental and model predictions of toluene degradation measured in g/day for experimental conditions shown in Figure 6.4.

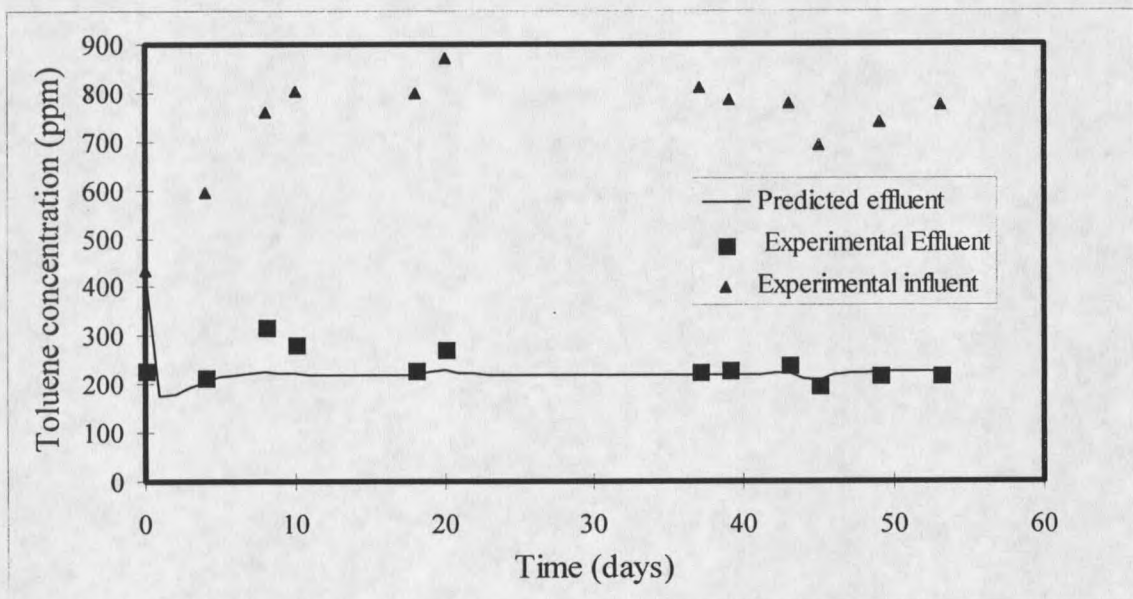


Figure 6.6. Steady state performance of bench-scale VPBR at an approximate vapor phase toluene concentration of 750 ppm. The markers indicate experimental results and lines represent predicted results.

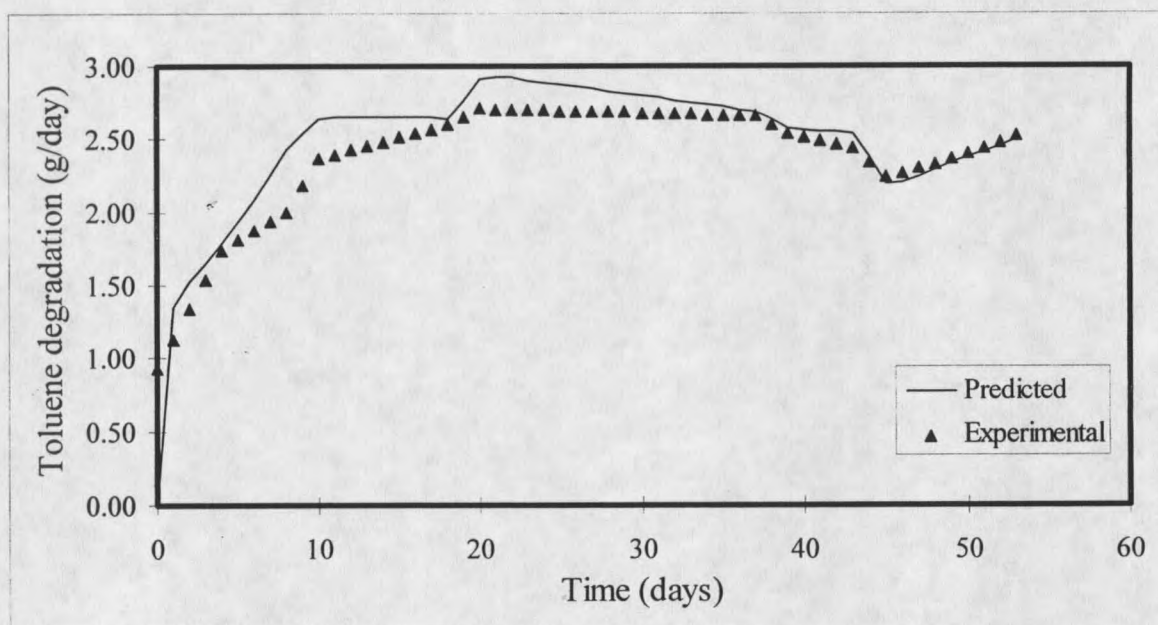


Figure 6.7. Experimental and model predictions of toluene degradation measured in g/day for experimental conditions shown in Figure 6.6.

CHAPTER 7

SUMMARY AND CONCLUSIONS

Vapor phase bioreactors (VPBRs) are being used increasingly to remediate vapor streams contaminated with volatile organic compounds such as benzene, toluene, ethylbenzene, o- and p-xylene and trichloroethylene. While emphasis is being placed on investigating innovative designs for support media used to immobilize bacteria, there is limited literature available on biofilm processes within the bioreactors. These processes are complicated and a better understanding of such processes could lead to improvements in design and performance of VPBRs. Most often, practitioners implement field-scale VPBRs without considering the benefits of operating lab-scale feasibility studies.

Success at applying VPBRs to remediate contaminated vapor streams in the field can best be achieved through use of a modeling approach as was presented in Figure 2.4.

Information from laboratory scale reactors in combination with predictive process models could yield substantial information that result in considerable savings in time and money at the macroscale. The impetus of the present investigation was two-fold:

- (1) measure micro- and mesoscale parameters such as kinetic, stoichiometric, injury and irreversible loss coefficients, Henry's law constant for toluene, and gas-liquid and liquid-biofilm thicknesses using laboratory scale reactors and

- (2) incorporate these parameters into a phenomenological process model to predict performance of a flat plate and a column VPBR.

Planktonic and biofilm cell kinetics were compared to determine if planktonic cells kinetics could be used to evaluate biofilm growth and activity in bioreactors. Results of this investigation suggest that specific activity during toluene degradation (based on total biomass and number of injured cells) were similar for biofilm and planktonic cells. Long term toluene exposure reduced specific activities (based on total biomass) for biofilm and planktonic cells. Based on these findings, it is likely that injured cells were inactive in degrading toluene whether in biofilm or planktonic states.

Planktonic cell injury studies were conducted to assess the potential affects of toluene degradation on cell growth and respiration rates. Results from these experiments suggest that toluene degradation caused significant injury to wild-type *P. putida* 54G cells. As a result of bacterial injury, two different types of stressed cells were formed, injured recoverable and injured non-recoverable. The injured recoverable cells suffered a repairable stress-related defect wherein they could revert back to their original state by passage on rich medium. The injured non-recoverable cells suffered an irreversible loss of toluene degrading capability. Cells that suffered irreversible loss of toluene degradative pathway continued to grow on other growth substrates which could include organic compounds leaking from wild-type cells and on intermediates formed due to incomplete toluene degradation. Respiration rates were inversely correlated with injury rates, with higher toluene concentration leading to higher injury rates and lower respiration rates.

In the flat plate VPBR experiment, oxygen concentration profiles, light microscopy and cryosectioning combined with fluorescent staining indicated that actively respiring cells were stratified at the base of the biofilm. These results are contrary to traditional biofilm substrate uptake models, which predict that almost all of the respiratory activity was relegated to the biofilm-liquid interface in thick biofilms. Instead, stratification of the biofilm during toluene degradation more resembles that of a biocide-treated biofilm. Results of injury studies have indicated that other growth substrates may be available at the base of the biofilm. During growth in suspension, intermediates were formed as a result of incomplete toluene degradation. The increase in numbers of cells that had undergone irreversible loss of toluene degradative pathway suggested that the mutants could possibly stratify at the base of the biofilm growing on unconverted intermediates and on organics leaking from injured cells. Since the basal film represented a region where toluene concentration was at a minimum, this film represented optimal conditions to support growth of mutants.

Rate expressions for injury and irreversible loss were determined by fitting a theoretical injury model to experimental results at influent vapor phase toluene concentrations of 150 and 750 ppm. These parameters, along with kinetic and stoichiometric coefficients, measured value of Henry's law constant for toluene and gas-liquid and liquid-biofilm interface thicknesses were incorporated into a process model for calibrative purposes. Simulations were conducted to determine toluene degrading performance of a flat plate and a column VPBR. Kinetic rate expressions and the measured value of Henry's law for toluene contributed significantly to the success of the

modeling effort. Vapor phase toluene concentration and total toluene degradation rates were accurately predicted in both reactor types. In the absence of measured kinetic parameters, the model failed to predict VPBR performance satisfactorily.

The importance of a scale-up approach to modeling and designing VPBRs is evident in the results of a sensitivity analysis conducted on model predictions for the flat plate and column VPBRs. The analysis indicates that parameters which were adjusted during calibration of the model to experimental data (biomass density, and death and endogenous decay rates) showed a negligible effect on toluene degradation in the flat plate VPBR. The parameters that had a significant effect on toluene degradation were Henry's law constant for toluene, surface area and the kinetics of biotransformation, all of which were experimentally measured separately. Since all the important parameters were determined accurately in laboratory scale reactors, two important conclusions follow:

- (1) the model indicates the applicability of planktonic cell parameters to model biofilm activity in VPBRs and
- (2) the model provides an increased level of confidence in understanding and predicting VPBR performance.

A similar analysis on model predictions for the column VPBR indicates that Henry's law constant for toluene, kinetics of biotransformation and death and endogenous rates affect toluene degradation. Surface area, a parameter which cannot be accurately measured in column VPBRs, only marginally affected toluene degradation. More importantly, both death and endogenous decay rates affected toluene degradation. Since these two parameters were adjusted to ensure an agreement to the experimental results,

the model provides crucial information on which parameters need to be accurately measured.

This dissertation provides a framework (Figure 2.4) for scale-up of vapor phase bioreactors that has potential applications in other bioreactor systems, but more research needs to be carried out. Some of the future research goals that need to be addressed to better understand VPBRs are listed below:

- (1) Investigate microbial degradation of mixed contaminants and multispecies biofilms in vapor phase bioreactors,
- (2) Determine if observed stratification of cell phenotypes in biofilms can be predicted by the process model,
- (3) Determine the application of the process model to describe transient conditions within VPBRs,
- (4) Investigate the ramifications of stress response on respiration rates and biofilm activity in VPBRs and
- (5) Study the mechanism of the stress response to understand how injury is caused in bacterial cells.

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