Physiological and Chemical Gradients in a *Pseudomonas putida* 54G Biofilm Degrading Toluene in a Flat Plate Vapor Phase Bioreactor

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Received 8 August 1996; accepted 27 March 1997

Abstract: A *Pseudomonas putida* 54G biofilm was grown on toluene vapor supplied as the sole external carbon and energy source in a flat plate biofilm reactor. Enumerations of cells in the biofilm were made using culture techniques (selective and nonselective for toluene) and microscopic techniques (total and respiring cells), and an analysis of the progression of the state of the culture was made by examination of various fractions of the populations. Long-term exposure to higher levels of toluene produced the following trends: (i) lower fraction of total cells that respired; (ii) lower fraction of culturable cells that also grew on toluene; (iii) higher fraction of respiring cells that could not grow on toluene plates; and (iv) a relatively constant fraction of total cells that could not be cultured on toluene. Respiration rate was determined using oxygen microsensors, and the fraction of the total respiration that was not associated with toluene uptake increased with higher toluene exposure. A combination of cryosectioning and respiration rate data was used to demonstrate that more respiring cells and a higher respiration rate both occurred at the base of the film, suggesting a deterioration in physiological state with continued exposure to toluene. *Biotechnol Bioeng* **56:** 361–371, 1997.
Keywords: biofilms; bioremediation; toluene; vapor phase bioreactors

INTRODUCTION

Significant advances have been made in the design, scale-up, and modeling of biological processes for VOC destruction. However, the processes that govern the biodegradation of xenobiotic compounds are not thoroughly understood. A number of anomalous phenomena observed during the biodegradation of volatile organic compounds (VOCs) include the following: (1) the fraction of active biomass contained in VOC degrading biofilms is small (Arcangeli and Arvin, 1992); (2) reported VOC uptake rates are different for suspended and biofilm cultures (Mirpuri, 1996); (3) elevated levels of endogenous metabolism are found in fungal cultures (Weber et al., 1995) and mixed biofilms (Arcangeli and Arvin, 1992) growing on toluene; (4) respiratory activity is spatially stratified in biofilms growing on toluene (Mirpuri, 1996); and (5) an absence of clogging is observed in biofilters degrading VOCs after long operation periods (Hill et al., 1991; Leson and Winer, 1991), despite reports of high biomass yield for cultures growing on VOCs (Arcangeli and Arvin, 1992; Vecht et al., 1988).

Differences in VOC-uptake rates reported for pure and mixed cultures, as well as the differences in adaptation of natural microbial communities in VOC degradation processes (Spain et al., 1980; Spain and van Veld, 1983) can be explained based on the different nature and activities of the cultures, and the microbial interactions between the species forming the community (Fredrickson et al., 1991; Mikesell et al., 1993; Swindoll et al., 1988). However, the effects of the toxic substrate on the physiology and genetics of VOC-utilizing microorganisms, and their consequences on the overall degradative process, are unknown.

Beveridge and Hugo (1964) reported the ability of members of the suborder *Pseudomonadinae* to degrade aromatic compounds, which led to extensive investigations of the biochemistry, physiology, and genetics of VOC-utilizing bacteria. Microbial catabolic pathways of many hydrocarbons have been described (Foght and Westlake, 1988; Shields et al., 1989, 1991; Whited and Gibson, 1991; Worsey and Williams, 1975). Genetic information necessary for hydrocarbon degradation has been carried on both transmissible plasmids and on the chromosome (Burlage et al., 1989; Holloway and Morgan, 1986; Polissi et al., 1990; Shields et al., 1995; Zylstra et al., 1988).

Despite this information the consequences of the physiological changes resulting from exposure to toxic compounds on the overall VOC-degradative process has not been quantified. The significance of these effects is prompting a search for valuable parameters to control VOC degradation processes.

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Contract grant sponsor: Orange County Water District
Contract grant sponsor: National Water Research Institute
Contract grant sponsor: Center for Biofilm Engineering
Contract grant sponsor: National Science Foundation
Contract grant number: NSF cooperative agreement EEC-8907039

 CCC 0006-3592/97/040361-11

Physiological stress or ''injury'' has been defined as the physiological, genetic, and structural consequences resulting from exposure to sublethal injurious conditions and/or chemical agents. Injured cells do not reproduce under selective conditions that are tolerated by uninjured cells (McFeters, 1990). The concept of injury has been used in the food industry and drinking water fields for many years as a quality indicator (Hurst, 1967; McFeters, 1989), wherein survival of injured microbes in foods (Busta, 1976) and drinking water (McFeters, 1990) has been reported. In the VOC-degradation field, Love and Grady (1995) determined the loss of culturability of *Pseudomonas putida* growing on benzoate and m-toluate plates, and Leddy et al. (1995) reported the formation of *Tol*-mutants on *Pseudomonas putida* cultures growing on toluene. These mutants could not degrade toluene, but continued to grow in the presence of toluene, metabolizing other carbon sources such as organics resulting from leaking and lysis of wild-type cells or toluene degradation intermediates. Long-term exposure to toluene initiated the formation of mutants and loss of culturability on the toxic substrate (Leddy et al., 1995), either of which could severely affect the toluene utilization rate. These observations suggest that the nonculturability on the toxic substrate can be used as an indicator of loss of efficiency in the VOC-degradation process.

The goal of this work is to quantify the nonculturability of *P. putida* 54G in a toluene-degrading biofilm and to correlate the active cell fraction, the spatial distribution of the respiratory activity, and the oxygen uptake rates within the biofilm with measurable subpopulations such as number of toluene-culturable cells, respiring cells, and respiring cells no longer able to grow on toluene. A pure biofilm culture of *Pseudomonas putida* 54G was selected for this study as an example of a well-investigated microorganism for this topic.

MATERIALS AND METHODS

Bacterial Strain and Liquid Media

Pseudomonas putida 54G, a toluene-degrading bacterium isolated from a gasoline-contaminated aquifer at Seal Beach, California, was supplied by Dr. Harry Ridgway (Biotechnology Department in Orange County Water District, Los Angeles, CA). Cells used for inoculation were grown on HCMM2 mineral salts medium in the presence of toluene vapors until a stationary phase was reached. The HCMM2 mineral salts medium composition has been described elsewhere (Ridgway et al., 1990).

Flat Plate Vapor Phase Biological Reactor (VPBR)

A flat plate reactor (Fig. 1) was made mostly of polycarbonate and had dimensions 50 cm long, 5 cm wide, and 12 cm tall. A flat plate, 40 cm long and 5 cm wide, served as substratum for the biofilm growth, and was made of glass to facilitate biofilm monitoring using an inverted microscope. Several glass coupons (12 × 12 × 0.1 mm) were positioned on the glass plate for biofilm sampling. The reactor lid was fastened to the reactor to form a gas-tight seal. Nine ports, each 2 cm in diameter, were distributed along the lid and allowed for sampling of the liquid and biofilm on the glass plate. The center of the first port (Fig. 1) was located downstream at 4 cm from the edge of the glass plate (9 cm from the gas inlet), the ninth was placed upstream at 4 cm from the other edge of the plate (9 cm from the gas exit), and the other seven ports were placed every 4 cm between the first and ninth ports. Latex glove ''fingers'' were fastened onto the nine sampling ports using rubber O-rings, which served to maintain gas-tight conditions between sampling. Ports on each end, each with Teflon septa, permitted gas and liquid sampling from the influent and effluent. Gas feed was delivered by two lines, one comprised compressed air bubbled through a humidifier, and the second stream passed through an Erlenmeyer flask containing liquid toluene. Gas streams were combined and delivered through a 0.2-μm Teflon filter into the VPBR and out to the atmosphere in a safety hood at a flow rate of 50 mL/min. Peristaltic pumps were used to provide sterile HCMM2 medium to the reactor at a constant flow rate of 1 mL/min. HCMM2 medium contained less than 0.5 mg/L of TOC (influent and effluent). Medium was adjusted to pH 7.2 with 1N NaOH and delivered through the reactor and out through the effluent well. The sole external carbon and energy source during bacterial degradation was the toluene introduced in the vapor phase. The superficial flow velocity was about 1 cm/s. The liquid layer had a maximum thickness of 4 mm under laminar flow conditions (Reynolds number = 0.25). The linear shape of the concentration profiles in the liquid layer also lends credence to the assumption that molecular diffusion was the dominant mechanism of mass transport. The reactor operated in the countercurrent mode, and for all experiments the toluene concentration in the gas phase, at steady state, decreased toward the gas exit, and the toluene concentration in the liquid increased toward the effluent well due to the absorption of toluene vapors into the liquid phase. The flow conditions for gas and liquid phases are shown in Table I.

Experiments were run at room temperature of 25°C. During reactor start-up, suspended cells of *P. putida* 54G, pregrown on toluene vapors, were introduced into the reactor and recycled for 12 h, after which the reactor was operated continuously without recycling. The biofilm development was monitored daily until a steady state in substrate removal rate, biofilm thickness, and density was observed, which occurred after 1 month of operation.

Experimental Design

The reactor operated at two different toluene concentrations in the gas phase influent for a total period of 6 months. During the first 3 months, the toluene concentration in the gas phase influent was set at 150 ppm (0.474 g m^{-3}). Under these conditions, we assumed the toluene was the limiting substrate and oxygen was present in excess, which was verified by measuring oxygen concentration profiles

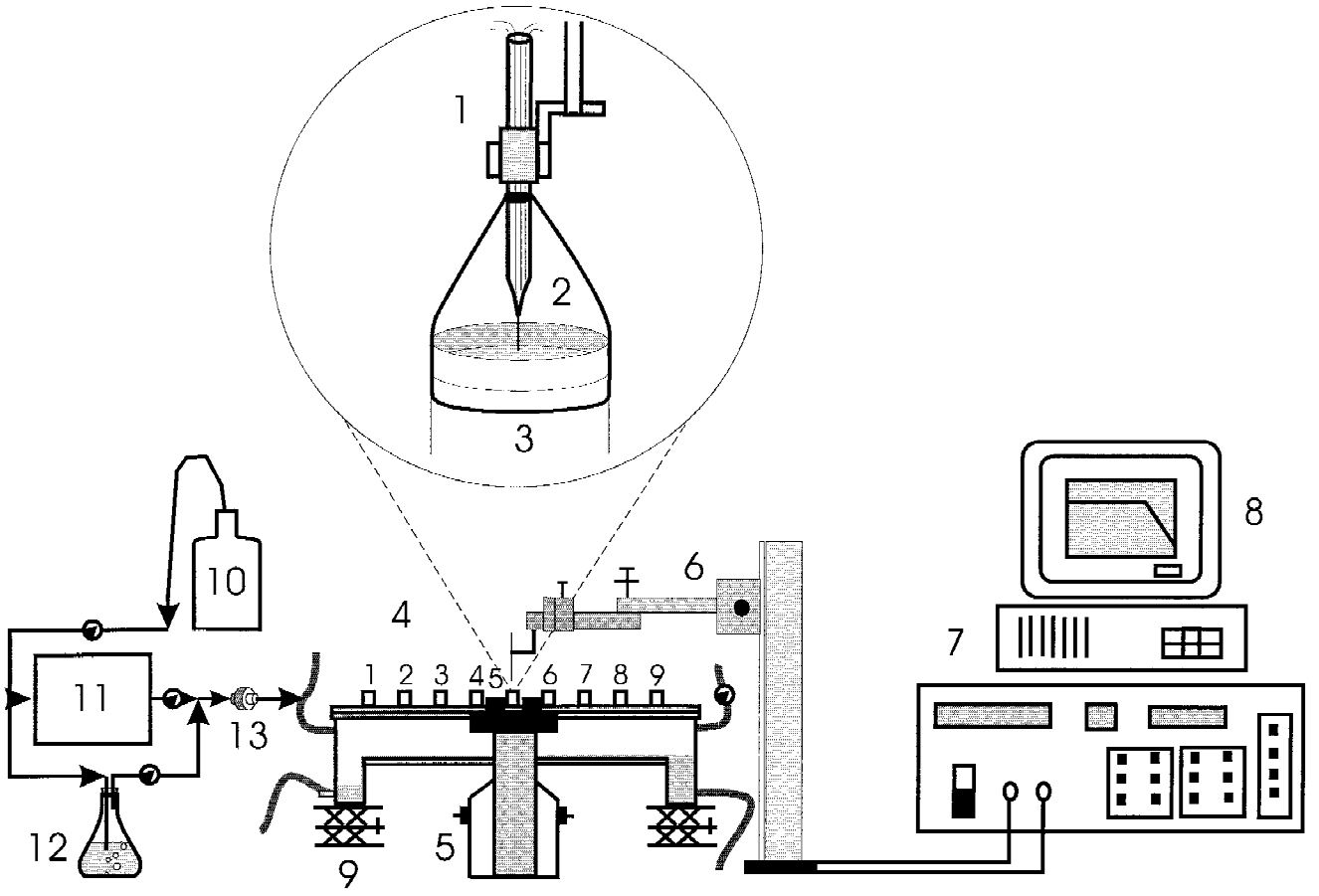

Figure 1. Schematic of the flat plate vapor phase bioreactor and the devices used to measure the oxygen concentration profiles: 1—microelectrode; 2—latex glove finger; 3—sampling port; 4—flat plate VPBR (with nine sampling ports); 5—inverted microscope; 6—micromanipulator; 7—picoamperometer and voltage source; 8—PC; 9—lab jacks; 10—air tank; 11—humidifier; 12—toluene source; 13—gas filter (0.2 μm).

through the liquid and biofilm phases with oxygen microelectrodes. During the last 3 months of operation the gas phase influent concentration of toluene was 750 ppm (2.37 g m^{-3}), making oxygen the limiting substrate within the biofilm, which was also confirmed by oxygen microelectrode measurements. The C/N/P ratio in the liquid phase in the reactor was 1/26.4/28.7 (on mass basis) for the first period and 1/5.3/5.7 for the second. Once steady state was reached, to quantify the respiration rate within the biofilm, oxygen concentration profiles were taken at different axial locations through the different ports along the reactor (see Fig. 1). Biofilm samples were also taken at the same locations by removing the glass coupons from the bottom of the reactor, and analyzed with the spread plating and staining techniques described below.

Table I. Operation conditions for the flat plate vapor phase bioreactor. Flow conditions for gas and liquid phases were constant through all of the experimentation period.

Phase	Gas	Liquid
Flow rate (L/h)	3.0	0.06
Fluid superficial velocity (m/h)	1.87	0.37
Residence time over the glass plate (h)	0.21	1.07
Direction of flow	From first to ninth port	From ninth to first port
Toluene concentration gradient in direction of flow	Decreasing	Increasing

Two different measurement approaches were used when taking oxygen concentration profiles. First, to determine the distribution of respiratory activity within the steady state biofilm, the concentration profiles were taken through the gas, liquid, and biofilm phases. The oxygen uptake rate can be evaluated from the diffusional flux of oxygen at the biofilm–liquid interface obtained from each profile. Use of an inverted microscope allowed us to locate the biofilm–liquid interface. Three or more concentration profiles were taken at different locations within a biofilm coupon, which was then sampled by removing the glass coupon from the bottom of the reactor for analysis by epifluorescent microscopy, plate counts, and cryosectioning. Because several microelectrode measurements were made, and the results were averaged, we assumed that this average was representative for the entire coupon.

Second, an evaluation was made of the amount of oxygen consumed by oxidation of intracellular material (endogenous metabolism) and lysis products (cryptic growth). Fol-

lowing the taking of a first oxygen concentration profile at steady state, a second profile was taken at the same axial location in the absence of external carbon source (Larsen and Harremoes, 1994). In this second approach both oxygen concentration profiles were taken only through the vapor and liquid phases to avoid any change in the biofilm structure. After taking the first profile the toluene feed was shut off leaving only the air stream, and liquid and biofilm samples were collected and analyzed until no additional toluene could be detected. At least five liquid phase residence times elapsed between shutting off the toluene feed and taking of the second profile. The toluene concentration at this point was below the detection limit (<20 μg/L), and effluent TOC was <0.5 mg/L. It was assumed that any intermediates of toluene metabolism (Leddy et al., 1995; Mirpuri, 1996) were washed from the system. Following the taking of the second profile, biofilm samples were taken by removing the glass coupons from the bottom of the reactor, and analyzed by epifluorescent microscopy, and plate counts.

The oxygen uptake rate can be evaluated from the diffusional flux of oxygen in the liquid phase obtained from each profile. The small number of data points obtained in the liquid side of the gas–liquid interface made it difficult to obtain an accurate calculation of the oxygen flux at that position. Consequently, the diffusional flux of oxygen (milligrams O_2 cm^{-2} s^{-1}) was calculated at the liquid–biofilm surface from the gradient of oxygen concentration in the liquid with depth (milligrams O_2 cm^{-4}), and the diffusivity of oxygen in water (2.5E-5 cm^2 s^{-1} at 25°C). Profile slopes at the biofilm–liquid interface were calculated using the last 20 points of each profile above the biofilm surface.

Oxygen Microsensors

Oxygen probes were fabricated as described elsewhere (Revsbech, 1989; Revsbech and Jorgenson, 1986). Tips of the oxygen probes were about 10 μm in diameter. Probes were calibrated in the air- or nitrogen-saturated water at 25°C. Effect of stirring on the microsensors was less than 5% of the response and the response time less than 1 s.

Probes were mounted on motorized micromanipulators, inserted through the sampling ports, and used for in situ measurement of oxygen in the liquid and biofilm phases. Step sizes and reading times for the probes were controlled by a computer, allowing changes in both while taking profiles of oxygen concentration in the direction normal to the glass substratum. The latex finger glove provided flexibility in manipulating the probe directions while maintaining gastight conditions (Fig. 1). An inverted microscope was used to monitor the position of the tip of the probe and the biofilm–liquid interface.

Plate Counts

Biofilm samples, taken at several axial points along the reactor, were analyzed for viable and toluene-culturable cells after appropriate dilution and spread plating. The number of total viable cells were evaluated from total cell counts on nonselective medium and the number of toluene-culturable cells from total cell counts on toluene selective medium. Growth media employed in this investigation were: (1) R2A plates (Reasoner and Geldreich, 1985), which have been used as nonselective medium for *Pseudomonas putida* 54G (Mirpuri, 1996; Ridgway et al., 1990); and (2) toluene vapors supplied continuously to cells on HCMM2 plates (selective medium) in sealed containers incubated at room temperature (Mirpuri, 1996; Ridgway et al., 1990). Biofilm organism numbers per milliliter of film were calculated by determining biofilm thickness (microscopy and microelectrode) and normalizing counts to the total biofilm volume sampled.

Staining Techniques

Biofilm samples taken at several locations along the reactor were analyzed for total and actively respiring cells by direct epifluorescent microscopy as per Rodriguez et al. (1992). Biofilm samples withdrawn from the reactor were diluted in HCMM2 medium, vortexed and homogenized. Suspensions were then stained with 5-cyano-2,3-ditolyl-tetrazolium chloride (CTC) at a final concentration of 1520 mg of CTC/L on 0.2-μm pore-size black polycarbonate membrane filters (Poretics Corp., Livermore, CA) and incubated at room temperature for 2 h. Samples were then washed three times and stained with DNA-binding fluorochrome 2,4-diamidino-2-phenylindole (DAPI) at a final concentration of 1 mg/L on the filter for 40 min, 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 Co.) with a UV100X (refractive index = 1.3) oil immersion lens. Only respiring cells were able to reduce CTC to the insoluble fluorescent formazan (CTF) product, and appeared as red-fluorescing objects (Yu and McFeters, 1994). An Olympus G cubic filter unit, with an excitation filter (BP-545), a dichroic mirror (DM-570), and a barrier filter (0590), was used to visualize the CTC-formazan crystals. All DNA-containing cells reacted with DAPI, appearing blue-green under epifluorescent microscopy light using an Olympus U cubic filter unit with an excitation filter (UG-1), a dichroic mirror (DM-400), and a barrier filter (L-420). Several samples amended with 0, 0.1, and 1 μg/mL of yeast extract + casamino acid solutions were stained with CTC and enumerated for actively respiring cells. The difference in cell numbers obtained for the amended versus nonamended samples (data not shown) was negligible, therefore further amending was no longer used.

Cell Numbers and Cell Fractions

Besides the direct enumeration of total, respiring, viable, and toluene-culturable cell number, valuable information can be extracted from other numbers and fractions as a

result of their combination. It is assumed that, eventually, all the colonies formed on both selective and nonselective media come from single cells, and their number can be compared with cell numbers obtained via microscopy.

The difference between nonselective and selective media counts has been defined in drinking water microbiology as injured cells (McFeters, 1990) and refers to viable cells no longer culturable on the selective medium. However, mutants of wild-type cells, which are able to grow on nonselective medium but not on selective medium, have been reported in toluene-degrading cultures (Leddy et al., 1995). Also, enumeration of viable cells by plate counts on nonselective medium may not include all injured cells (Yu and McFeters, 1994). Consequently, the difference between nonselective and selective media counts is only a relative number. However, it can provide valuable information as a stress indicator and we will refer to this group as viable stressed cells, in which mutants and most of the injured cells are included.

Cell densities obtained from CTC counts (respiring cells) are typically higher than those on selective media. It is not clear whether bacteria that are physiologically stressed can reduce CTC (Schaule et al., 1993); however, the difference between CTC and selective medium counts results in a positive number of respiring cells that are not viable on selective medium. We will refer to them as respiring non-toluene-culturable cells.

Toluene-culturable and non-toluene-culturable cells can be related to total and respiring cells through the evaluation of different fractions. Fractions were expressed on the basis of either total or respiring cells and were differentiated by adding ''total'' or ''respiring'' to their names, respectively. For example, the fraction of total respiring cells was evaluated as the ratio of CTC and DAPI counts. All cell numbers and fractions of interest for this investigation are presented in Table II with the numerical equations used for their calculation. The fraction of total toluene-culturable cells represents the fraction of total cells that can reproduce on selective medium. The fraction of total non-toluene-culturable cells is the fraction of total cells that cannot grow on toluene, and the respiring non-toluene-culturable cell fraction refers to the fraction of respiring cells that cannot grow on toluene.

Biofilm Cryogenic Sectioning

Glass coupons taken from the substratum of the flat plate VPBR were immersed in separate beakers containing CTC and DAPI separately using similar concentrations as outlined above. After staining, coupons were cryoembedded using techniques reported by Yu and McFeters (1994). The stained samples were placed on dry ice with the biofilm facing up, and a thick layer of Tissue-Tek® OCT (Miles Inc., Elkhart, IN) was dispensed on the top of the biofilm. The embedded biofilm was allowed to freeze until the sample turned opaque white. The biofilm sample was then transferred to a −19°C incubator and allowed to equilibrate. The glass coupon was gently separated from the biofilm and more OCT was placed on the substratum side of the biofilm. The specimen was wrapped in aluminum foil and stored at −70°C in aluminum foil until cryotomy (Yu and McFeters, 1994). Frozen sections were cut with a cryostat (Reichert-Jung Cryocut 1800; Leica) operated at −19°C. Frozen sections (5 μm thick) were collected on glass slides for visual examination using an Olympus BH2-RFCA microscope (Scientific Instrument Co.) with epifluorescent light (100-W mercury lamp) with a 100× lens. An Olympus B cubic filter unit with an excitation filter (BP-490), a dichroic mirror (DM-500), and a barrier filter (AFC + O515) was used to visualize simultaneously the CTC-formazan and DAPI fluorescence within the biofilm sections. The nonrespiring bacteria appeared green when stained with DAPI, whereas the respiring cells contained red crystals of CTC-formazan.

Toluene Sampling

Gas and liquid samples were taken at the influent and effluent ends of the reactor, and from the sampling ports at several axial locations along the reactor, to analyze for toluene concentration. Because a toluene concentration gradient is anticipated with depth in the liquid layer, reported values are linear averages through the liquid layer at each axial

Table II. Numbers and fractions of cells evaluated within the *P. putida* 54G biofilm. Cell numbers are expressed per milliliter of biofilm sample and cell fractions are dimensionless.

Number and fractions of cells	Calculation and units
Number of toluene-culturable cells	Selective medium counts (SM), CFUs/mL
Number of total viable cells	Nonselective medium counts (NSM), CFUs/mL
Number of respiring cells	CTC staining (CTC), cells/mL
Number of total cells	DAPI staining (DAPI), cells/mL
Number of nonrespiring cells	(DAPI) − (CTC), cells/mL
Number of non-toluene-culturable cells	(CTC) − (SM), cells/mL
Number of viable stressed cells	(NSM) − (SM), CFUs/mL
Fraction of total cells respiring	(CTC)/(DAPI)
Fraction of total cells toluene-culturable	(SM)/(DAPI)
Fraction of total cells non-toluene-culturable	[(CTC) − (SM)]/(DAPI)
Fraction of respiring cells non-toluene-culturable	[(CTC) − (SM)]/(CTC)

Table III. Total cell densities for viable cells (cell counts on R2A medium; CFUs/mL), toluene-culturable cells (cell counts on selective medium, HCMM2 + toluene vapors; CFUs/mL), total cells (DAPI-stained cells counts; cells/mL), and respiring cells (CTC-stained cell counts; cells/mL) for a milliliter sample of *P. putida* 54G biofilm at different toluene concentrations.[a]

Toluene (mg/L)	Port	Viable cells	Toluene-culturable cells	Total cells	Respiring cells
0.24	8th	$1.70 \cdot 10^8$	$7.70 \cdot 10^7$	$2.51 \cdot 10^8$	$1.74 \cdot 10^8$
0.43	5th	$1.90 \cdot 10^8$	$8.34 \cdot 10^7$	$2.46 \cdot 10^8$	$1.65 \cdot 10^8$
0.72	2nd	$3.90 \cdot 10^8$	$1.12 \cdot 10^8$	$7.48 \cdot 10^8$	$3.92 \cdot 10^8$
2.35	8th	$5.34 \cdot 10^8$	$1.08 \cdot 10^8$	$1.75 \cdot 10^9$	$9.07 \cdot 10^8$
4.51	5th	$4.27 \cdot 10^9$	$5.43 \cdot 10^8$	$1.12 \cdot 10^{10}$	$5.07 \cdot 10^9$
6.59	2nd	$1.07 \cdot 10^{10}$	$1.05 \cdot 10^9$	$5.23 \cdot 10^{10}$	$1.94 \cdot 10^{10}$

[a]Values were obtained from the steady-state biofilm when the reactor operated with an influent toluene gas concentration of 150 ppm (for the first three rows of data) and 750 ppm (for the second three rows of data). The standard deviation was inferior to 5% of reported values for all cell numbers.

point. Gas and liquid samples were analyzed using gas chromatography (GC). Toluene in the gas phase was measured using an HP 5890 Series II GC equipped with a FID and an Alltech 0.1% A1-1000 Graphpac GC 80/100 (6′ × 1/8″ × 0.085″) S.S. column at a constant column temperature of 140°C. The injector and detector temperatures were both 200°C. Two hundred fifty microliters of reactor headspace was withdrawn and injected into the GC. Toluene concentrations in the liquid were determined by combining 1 mL of the aqueous samples with 0.5 mL of hexane, and injecting 2 μL of the hexane phase into an HP 5890 Series II GC equipped with a PID and a DB-624 column (30 m × 0.53 mm i.d.). The column temperature was programmed to ramp from 140° to 200°C. Helium was used as a carrier gas in both columns at a flow rate of 30 mL/min.

RESULTS AND DISCUSSION

Biofilm Cultivation on Toluene and Respiratory Activity Within the Biofilm

At steady state, biofilm was sampled at various locations in the reactor and samples analyzed for total (DAPI-stained cell counts) and actively respiring (CTC-stained cell counts) cells and viable (R2A plate counts) and toluene-culturable cells (''HCMM2 + toluene vapors'' plate counts). Cell numbers obtained from CTC counts were typically higher than those obtained from R2A plate counts which concurs with observations reported by other investigators (Schaule et al., 1993; Yu and McFeters, 1994). In the plate counts method, cell viability is defined as the ability of a cell to grow and form a visible colony, whereas CTC reduction reflects the presence of a functional electron transport system or certain active dehydrogenases. However, Mason et al. (1986) suggest the difference between CTC counts (respiring cells) and nonselective media counts (viable cells) is that some of the cells that can reduce CTC may have lost their ability to reproduce although they still can carry out substrate transformations.

Table III shows these cell densities for different liquid toluene concentrations at different ports. A 25-fold increase in toluene is seen to result in cell number increases from 13-fold (toluene-culturable cells) to over 200-fold (total cells). All cell numbers increased with the toluene concentration in the liquid regardless of the axial position of the reactor where the biofilm was sampled. Consequently, changes in cell densities can be correlated with the toluene liquid concentration, and the effect of detachment can be neglected. This assumption was confirmed by direct microscope observation of the biofilm surface at different axial locations before, during, and after microelectrode measurements for periods of at least 2 h, from which no observable sloughing was monitored.

Accumulation of viable stressed cells and nonrespiring cells increased tremendously as the toluene concentration increased. Figure 2 shows the numbers of toluene-culturable cells, viable stressed cells, and nonrespiring cells versus toluene liquid concentration. Although all three cell counts increased with increasing toluene concentration, the nonrespiring and viable stressed cell numbers increased faster, leading to their domination of the biofilm.

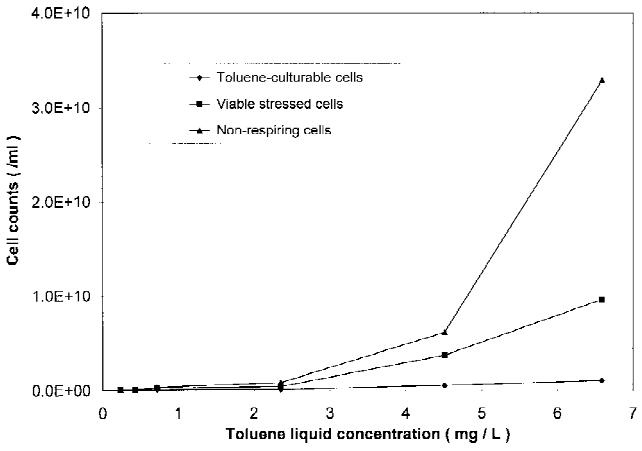

Figure 2. Numbers of toluene-culturable cells (selective medium counts), viable stressed cells (nonselective − selective media counts), and nonrespiring cells (DAPI − CTC counts) versus toluene concentration in the liquid.

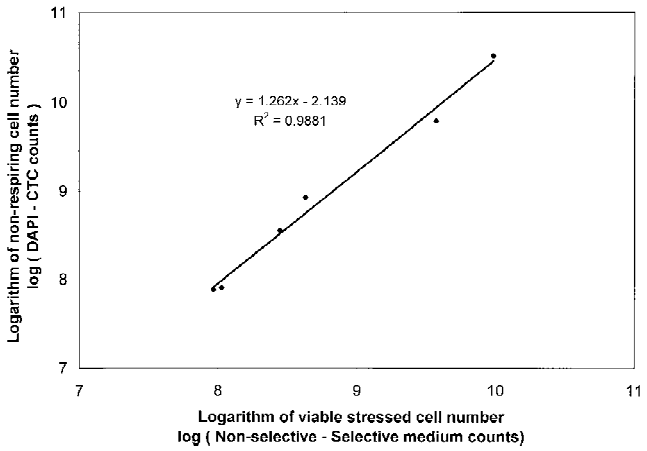

Figure 3. Logarithm of nonrespiring cell number (DAPI − CTC counts) versus logarithm of viable stressed cell number (nonselective − selective media counts).

Specific activity of VOC-degrading biofilms is commonly reported on the basis of total biomass (Arcangeli and Arvin, 1992; Diks and Ottengraf, 1991a, b). However, based on the aforementioned observations, the specific activity of biofilms degrading VOCs will differ, depending on whether activity is reported on the basis of total, respiring, viable or VOC-culturable cells. This observation is further supported by Mirpuri (1996) who reported that the specific activity of suspended and biofilm cultures of *P. putida* 54G growing on toluene remained constant for different toluene concentrations and culture ages when expressed on the basis of toluene-culturable cells, but decreased when expressed on the basis of viable cells and total biomass.

Loss of respiratory activity within the biofilm was compared to the loss of culturability on selective medium. Figure 3 shows the logarithm of nonrespiring cell number versus the logarithm of viable stressed cell number. The results demonstrate an excellent correlation between the number of inactive cells and the number of viable stressed cells within the biofilm. The number of viable stressed cells within the steady-state biofilm, increased from 9.20×10^7/mL for toluene liquid concentration of 0.237 mg/L (at port 8 in the experiment done with an influent toluene gas concentration of 150 ppm), to 9.65×10^9/mL for toluene liquid concentration of 6.587 mg/L (at port 2 in the experiment done with an influent toluene gas concentration of 750 ppm). Consequently, as the toluene concentration increased both the number of viable stressed cells and the number of nonrespiring cells increased.

Biofilm Cultivation on Toluene and Oxygen Consumption within the Biofilm

Respiring non-toluene-culturable cells actively respire within the biofilm and grow at the expense of leakage and lysis products and/or intermediates of toluene degradation (Leddy et al., 1995). For those microorganisms that cannot decompose the target compound, cryptic growth might be the only chance for survival. Cryptic growth refers to the growth process taking place on lysis and leakage products (Ryan, 1959), and has been mathematically modeled with death, lysis, and particulate solubilization as endogenous metabolism (Mason and Hamer, 1987). For further discussion, we will refer to non-toluene-associated respiration as the oxygen consumed within the biofilm by endogenous respiration and cryptic growth.

Additional insight into population structure may also be gained by examining the fractional composition of the film (Table IV). The fractions of the total cells that actively respired (CTC/DAPI) and that were culturable on toluene (SM/DAPI) consistently decreased with increasing toluene concentration. The difference between these two fractions remained relatively constant, however, and was consistently higher than the total toluene-culturable cell fraction. Thus, while the total cell number increased with increasing toluene concentration, the number of cells that respired but did not actively degrade toluene increased proportionally. Conversely, even though the number of respiring and toluene-culturable cells increased with toluene concentration, the decrease in their fraction of the total points out a possible deleterious effect of increased toluene. Finally, the fraction of the respiring cells not culturable on toluene increased dramatically with increased toluene concentration. This result implies that respiration in the biofilm came to rely decreasingly on direct toluene metabolism and more on alternative substrates as the toluene concentration increased.

Table IV. Fractions of total respiring cells, total toluene-culturable cells, total non-toluene-culturable cells, and respiring non-toluene-culturable cells within the *P. putida* 54G biofilm for different toluene concentrations in the liquid. It should be noted that the first column must be equal to the second plus third columns.

Toluene (mg/L)	Total respiring cell fraction	Total toluene-culturable cell fraction	Total non-toluene-culturable cell fraction	Respiring non-toluene-culturable cell fraction
0.24	0.69	0.31	0.38	0.56
0.43	0.67	0.33	0.34	0.49
0.72	0.52	0.15	0.37	0.71
2.35	0.52	0.06	0.46	0.88
4.51	0.45	0.05	0.40	0.89
6.59	0.37	0.02	0.35	0.95

Dissolved oxygen profiles in the gas and liquid phases above the biofilm were used to evaluate endogenous respiration (Fig. 4). The upper profile was taken during steady-state operation, while the liquid phase toluene concentration was 0.237 mg/L. The lower profile was taken after the toluene source had been removed, and the liquid phase toluene concentration had dropped below the detection limit (20 μg/L). Using the diffusivity of oxygen in water at 20°C (9.0×10^{-6} m^2 h^{-1}), fluxes of oxygen into the biofilm were 9.32×10^{-8} and 4.66×10^{-8} mg cm^{-2} s^{-1} in the presence and absence of toluene, respectively. Thus, the fraction of respiratory activity not attributable to toluene degradation at this concentration was about 50%. As the toluene concentration increased, both the total and non-toluene-associated respiration rates increased; at a toluene concentration of 6.6 mg/L, the fluxes were 3.0×10^{-7} and 2.9×10^{-7} mg cm^{-2} s^{-1}, respectively, so that the non-toluene-associated flux was 97% of the total. This result is consistent with the population fraction information (Table IV), where the fraction of respiring cells that could not be cultured on toluene also increased with toluene concentration. However, no correlation could be found between the toluene-associated respiration rate and any of the appropriate parameters that we measured. Therefore, we do not have ready explanation for the unusually low ratio of substrate-associated respiration to endogenous respiration rates. It is possible that the measured endogenous rates were inflated, because toluene feed had to be switched off to measure them, and previously toluene-degrading cells were able to switch from toluene to endogenous substrates during this period.

In Figure 5, the ratio of non-toluene-associated respiration to total respiration rate is plotted against the fraction of respiring non-toluene-culturable cells. The extraordinary correlation between these fractions lends credence to the hypothesis of cryptic growth of the non-toluene-culturable cells. The hypothesis is also supported by the following

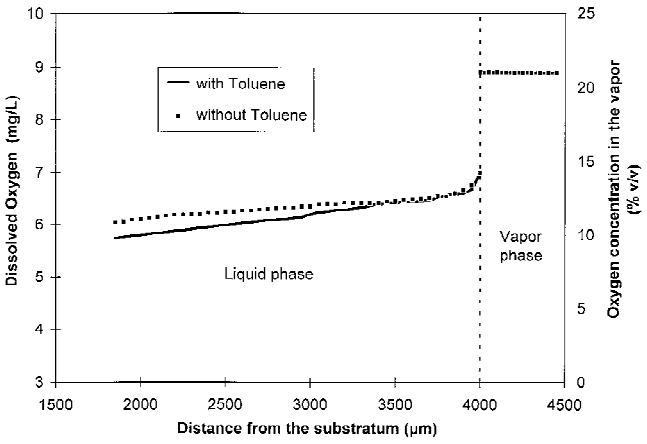

Figure 4. Oxygen concentration profiles in the vapor and liquid phases taken at the same point through the eighth port in conditions of: 0.237 mg toluene/L in the liquid (continuous lines) and absence of toluene (dashed lines).

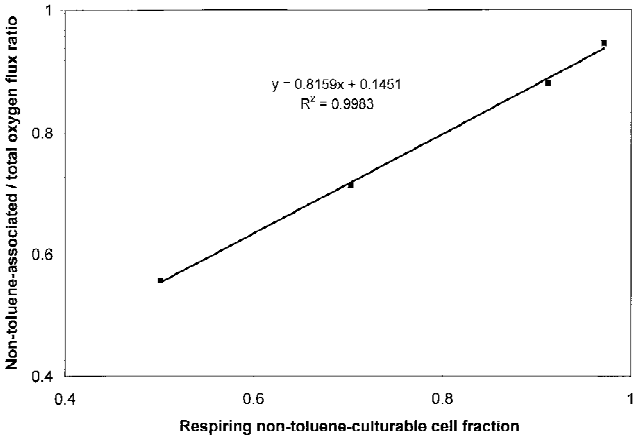

Figure 5. Oxygen flux fraction due to non-toluene-associated respiration (non-toluene-associated/total oxygen flux ratio) versus the fraction of respiring non-toluene-culturable cells.

three considerations: (1) Cell wall turnover, lysis of dead cells, and leakage of intracellular constituents can produce significant concentrations of intracellular products in the cell environment (Hugo, 1967; Mason et al., 1986). (2) It has been reported that bacteria are capable of growth on soluble lysis products at near maximal rates with a high ''cryptic'' yield coefficient near 0.5 mg cell C/mg substrate C (Banks and Bryers, 1990; Mason et al., 1986). (3) It has been reported that both wild-type cells (which are able to grow on the toxic substrate) and mutants (which cannot degrade the toxic substrate) can grow on leakage and cell lysis products (Herman and Costerton, 1993; Leddy et al., 1995). In a recent publication, Weber and Hartmans (1996) reported that the toluene removal rate in a trickling filter degrading toluene decreased with time when no biomass was removed from the system, but decreased at a much lower rate when NaOH wash was applied to the filter every 2 weeks. In both cases, however, the production rate (on carbon basis) of carbon dioxide remained constant, and eventually exceeded the toluene removal rate (on carbon basis) at the end of the reported period. This clearly supports the possibility of a parallel metabolic process in the filters, probably involving lysis products or toluene-degradation intermediates.

The growth of non-toluene-culturable cells at the expense of leakage and lysis products can be seen as a biomass recycling process which may limit clogging in some VOC biofiltration processes after months of continuous operation (Shareefdeen and Baltzis, 1993, 1994). Some practical implications of the non-substrate-associated respiration on the overall biodegradation process of toxic substrates might be the increase of the oxygen requirements, the mineralization of the inactive biomass, and the recycling of nutrients. These observations suggest that non-toxic-associated respiration may profoundly affect biofilm composition and structure, thus affecting the overall degradation process of xenobiotic compounds.

Respiratory Activity Stratification Within the Biofilm

Figure 6 presents two oxygen concentration profiles taken at the second port through the biofilm at steady state for the two experimentation periods: profile A, under toluene-limiting conditions (influent toluene gas concentration of 150 ppm); and profile B, under oxygen-limiting conditions (influent toluene gas concentration of 750 ppm). Toluene concentration in the vapor was 123 ppm (0.39 g m^{-3}) when profile A was taken, and 475 ppm (1.50 g m^{-3}) for profile B. Toluene concentrations in the liquid phase were 0.72 mg/L and 4.51 mg/L, respectively. The biofilm thicknesses at the point where the profiles were taken were 1.8 mm for profile A and 2.2 mm for profile B. With profile A, the oxygen concentration gradient was 0.51 mg/L·mm in the top 1.5 mm, and increased to 1.3 mg/L·mm in the last 300 μm. The ratio between these slopes is 2.55, which means that the innermost 300 μm are more than 2.5 more active than the rest of the biofilm.

This unusual behavior was also observed in profile B. The oxygen concentration gradient was 2.24 mg/L·mm in the top 1.85 mm, and increased to 3.4 mg/L·mm in the next 150 μm (in the last 150 μm the oxygen concentration dropped under the detection limit of 0.02 mg/L). The ratio between the concentration gradients at the base and upper films is 1.52, which means that the base film is about 52% more active that the rest of the biofilm.

This stratification was further confirmed when the reactor was tilted at the end of the first (150 ppm) experiment, causing most of the top of the biofilm to slough. Biofilm and liquid effluent samples were taken through the second port before and after the sloughing event and counterstained with CTC and DAPI to measure for respiring and total cell numbers (respectively) in the overall film and base film. The overall percentage of respiring cells was 7.67% in the sloughed biomass and 71.02% for the retained base film, which demonstrates the potential for much higher respiratory rates at the biofilm base. The lack of significant respiratory activity in the overlaying layer of accumulated cells is confirmed by the linear shape of the oxygen concentration profile in this upper layer of the biofilm.

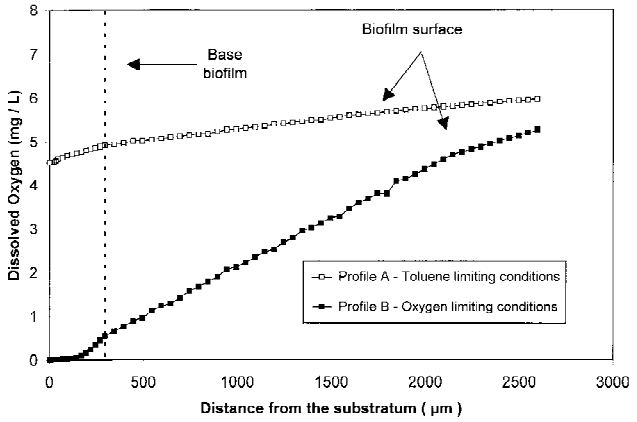

Figure 6. Oxygen concentration profiles taken through the second port of the reactor under toluene-limiting conditions (profile A), and oxygen-limiting conditions (profile B). The toluene concentration in the liquid phase was 0.72 mg/L and 4.51 mg/L, respectively.

Although tilting the reactor gave valuable insights, it is not a controlled technique to study the stratification of the respiratory activity of the biofilm. Consequently, cryosectioning of the base film was carried out to verify the above observations (Huang et al., 1995; Wimpenny and Kinniment, 1995). Glass coupons were sampled from the second port of the reactor followed by biofilm staining with CTC and DAPI. Biofilm attached to the glass coupons was about 200 μm thick, encompassing the base film to which we referred in Figure 6. Photomicrographs of cryosectioned samples were then examined. Care was taken to identify the top and bottom of the film during preparation. Cell density was always highest closest to the substratum. Furthermore, the relative numbers of CTC-positive cells was also highest adjacent to the substratum. While minor differences existed between samples from high and low toluene concentration environments, the stratification phenomenon was universal.

This stratification phenomenon may infer a higher resistance to transport of substrates within the biofilm than what has been predicted by traditional modeling results (Diks and Ottengraf, 1991; Oh and Bartha, 1994; Shareefdeen and Baltzis, 1993, 1994) where an active layer is assumed to be located above an inactive one. The additional mass transfer resistance may protect against the harmful effects of the toxic substrate. This hypothesis was indirectly suggested by Keweloh et al. (1989) who investigated the tolerance for phenol of free and immobilized cells. Their results highlighted a higher resistance for immobilized cells, which is increased with cell colony size. Overall, the importance and practical consequences of this unique stratification phenomenon will be system-specific where, for instance, different hydraulics may alter or even remove much of the inactive layer.

CONCLUSIONS

The concept of nonculturability on a toxic substrate has been described in a *Pseudomonas putida* 54G biofilm growing on toluene as sole external carbon source in a flat plate VPBR. To study the phenomenon and its consequences, four cell fractions were defined. The total respiring cell fraction (1) and total toluene-culturable cell fraction (2) decreased as the liquid toluene concentration increased across the reactor. The fraction of total non-toluene-culturable cells (3) remained essentially constant with toluene concentration, and the respiring non-toluene-culturable cell fraction (4) increased with increasing the liquid toluene concentration.

The oxygen utilization rate for the respiring non-toluene-culturable cells, referred to as non-toluene-associated respiration within the biofilm, was higher than the toluene-associated respiration rate. When the toluene concentration increased the non-toluene-associated respiration rate in-

creased and so did the fraction of respiring non-toluene-culturable cells. This indicated the existence of cryptic growth within the biofilm.

Stratification of the respiratory activity was observed within the biofilm by using oxygen microsensors and cryosectioning techniques. The spatial pattern of the respiratory activity within the biofilm can be described as a two-layer biofilm, with a thick, quite inactive upper film and a thin, active base film. The activity of the base film was positively correlated with the respiring cell fraction. This can be a consequence of the loss of culturability on the toxic substrate by the accumulated cells.

We conclude that, in biofilms exposed to toxic materials, cells at the surface are inactivated. Cells at the base of the biofilm continue to grow, so that the surface layer of inactive cells becomes thicker and thicker. This continues until it becomes a diffusive barrier sufficient to reduce concentrations of the substrate in the base film to nontoxic levels. Because a thicker, diffusive barrier is needed if aqueous substrate concentrations are high, the fraction of cells that are inactive will be greater where the substrate concentration is higher.

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