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Toluene Diffusion and Reaction in Unsaturated *Pseudomonas putida* Biofilms

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Abstract

Biofilms are frequently studied in the context of submerged or aquatic systems. However, much less is known about biofilms in unsaturated systems, despite their importance to such processes as food spoilage, terrestrial nutrient cycling, and biodegradation of environmental pollutants in soils. Using modeling and experimentation, we have described the biodegradation of toluene in unsaturated media by bacterial biofilms as a function of matric water potential, a dominant variable in unsaturated systems. We experimentally determined diffusion and kinetic parameters for *Pseudomonas putida* biofilms, then predicted biodegradation rates over a range of matric water potentials. For validation, we measured the rate of toluene depletion by intact biofilms and found the results to reasonably follow the model predictions. The diffusion coefficient for toluene through unsaturated *P. putida* biofilm averaged 1.3×10^{-7} cm²/s, which is approximately two orders of magnitude lower than toluene diffusivity in water. Our studies show that, at the scale of the microbial biofilm, the diffusion of toluene to biodegrading bacteria can limit the overall rate of biological toluene depletion in unsaturated systems.

Keywords

unsaturated biofilm; diffusion; substrate utilization kinetics; matric water potential; toluene

Introduction

Volatile hydrocarbon pollutants are prevalent in the unsaturated (vadose) zones of contaminated U.S. Department of Energy (DOE) sites (Lenhard et al., 1995), U.S. Air Force sites (Downey and Elliot, 1990), and commercial sites throughout the world where refined petroleum has been spilled. Remediation systems are engineered to reduce subsurface contamination near the source (e.g., underground fuel tanks) (Newton, 1990). In the vadose zone, residual fuels in the form of non-aqueous phase liquids (NAPLs) and dissolved constituents can be mobilized into the soil gas and swept with advecting air to above-ground treatment systems by vapor extraction or vapor venting (Sims et al., 1993). Transport models

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used in predicting vapor extraction rates may not include sink terms for microbial degradation of volatile hydrocarbons (Armstrong et al., 1994). However, soil gas profiles (Ostendorf and Kampbell, 1991) and laboratory bench studies (English and Loehr, 1991) show that surface and subsurface soils biologically “filter” some volatile hydrocarbons from soils. The recognition that microbes act as a sink for many volatile hydrocarbons has led the U.S. Air Force, for example, to focus on “bioventing” as an important remediation strategy for JP4 (aviation fuel) contaminated sites (Sayles et al., 1992). Bioventing is a remediation technology that optimizes the operation of vapor venting systems to maximize in situ biodegradation of volatile pollutants (Hoeppe et al., 1991). The ex situ analogy to bioventing is biofiltration, whereby toxic or malodorous compounds are scrubbed from waste gas streams by microbes adherent to biofilter media.

Microbes are central to successful bioremediation; nevertheless, macropore vapor transport rates are often used as central criteria for bioventing or biofiltration designs (Coffin and Glasgow, 1992; Johnson et al., 1990). De-emphasizing the role of soil microbes in biological processes is problematic. This is especially so in systems like bioventing or biofiltration where advective transport ensures rapid resupply of volatile carbon to biodegrading microbes. In such systems, microbial catalytic rates may limit net hydrocarbon depletion per volume of advected soil gas (Kampbell and Wilson, 1991). Thus, mass transfer and reaction rates that occur at the scale of biofilms may limit the overall effectiveness of remediation efforts in unsaturated systems.

The design and operation of hydrocarbon pollution remediation systems, such as in situ bioventing or ex situ biofiltration, currently lack as input, a mechanistic understanding of the biofilm diffusion-reaction processes that govern the rate of hydrocarbon biodegradation. An understanding of both vapor transport and biodegradation will allow engineered systems to be optimized with the rate-limiting processes in mind. In this study, we present mathematical modeling and experimental determinations of internal mass transfer and microbial kinetic parameters for an unsaturated biofilm utilizing toluene as a carbon and energy source. In our work, we define unsaturated biofilms as those that have a continuous air-biofilm interface, without a significant layer of free water exterior to the biofilm. The focus of our mathematical model, i.e., the scale of the microbial habitat in the unsaturated zone, is most appropriate for either in situ bioventing or ex situ biofiltration reactor systems, where volatilized hydrocarbon transport through macropores is rapid. Our model and measured parameters are evaluated to reveal the relative importance of diffusion and microbial reaction to the biodegradation of toluene over a range of environmentally-relevant matric water potentials. The validity of our model and the measured parameters is assessed by comparing the modeled rate of toluene depletion to experimentally-determined overall rates of toluene depletion as a function of matric water potential.

Unsaturated Biofilm Model

Microorganisms in the unsaturated zone (including surface soil) are primarily attached to solid surfaces, embedded in a microbially-derived, hydrated matrix of exopolymeric substances (EPS) (Gray et al., 1968) (Fig. 1). This growth habit is similar to that of a biofilm, a term whose definition is broadly applicable to both saturated and unsaturated systems (Characklis and Marshall, 1990). For the highly volatile constituents of petroleum hydrocarbon pollution, high concentrations in the gas phase are favored where diffusion through soil macropores is rapid. The availability of the contaminant to biodegrading populations may thus be limited by solubilization into biofilms and subsequent liquid-phase diffusion. Assuming that volatile organic compound (VOC) transport through soil macropores is non-limiting, a mass balance for VOC in a control volume containing a non-growing, VOC-metabolizing biofilm, yields:

$$D_e \frac{\partial^2 C_{b,z}}{\partial z^2} - r \rho_m p_c = \frac{\partial C_{b,z}}{\partial t} \quad (1)$$

If VOC is not accumulating measurably within the biofilm, the condition of quasi-steady state can be assumed, and Equation (1) simplifies to:

$$D_e \frac{\partial^2 C_b}{\partial z^2} - r \rho_m p_c = 0 \quad (2)$$

Assuming that the microbial reaction rate expression is Michaelis-Menten and that cells are non-growing, the rate term is replaced in Equation (2) to yield:

$$D_e \frac{\partial^2 C_{b,z}}{\partial z^2} = \frac{q_m C_{b,z}}{(K_s + C_{b,z})} \rho_m p_c \quad (3)$$

Our assumption of no growth is applicable to short time frames, and is an appropriate simplification to analyze diffusion and reaction processes. The boundary conditions for Equation (3) are:

$$C_{b,z} = \frac{C_g}{H_c} \text{ at } z = L_b, \quad \frac{\partial C_{b,z}}{\partial z} = 0 \text{ at } z = 0,$$

where L_b is the full biofilm depth as shown in Figure 1. A single integration of Equation (3) yields:

$$\begin{aligned} D_e \frac{\partial C_{b,z}}{\partial z} \Big|_{z=L_b} \\ = \int_{z=0}^{z=L_b} \frac{q_m C_{b,z}}{(K_s + C_{b,z})} \rho_m p_c dz \end{aligned} \quad (4)$$

We can define η_i , the internal effectiveness factor, as follows:

$$\eta_i \frac{q_m \frac{C_g}{H_c}}{\left(K_s + \frac{C_g}{H_c}\right)} \rho_m p_c = \int_{z=0}^{z=L_b} \frac{q_m C_{b,z}}{(K_s + C_{b,z})} \rho_m p_c dz \quad (5)$$

The effectiveness factor, η_i , is the ratio of the mass transfer-limited biofilm transformation rate to the intrinsic uptake rate (Bailey and Ollis, 1986). In the absence of diffusional mass transfer limitations, the VOC would be available for microbial uptake at a concentration of C_g/H_c throughout the biofilm and η_i is equal to 1. However, observed microbial kinetics are dependent on the rate of VOC diffusion through the biofilm. When the rate of substrate depletion is limited by diffusional resupply, η_i is less than 1.

Equation (5) can be expressed in terms of the following dimensionless parameters (Bailey and Ollis, 1986):

$$\begin{aligned} C &= \frac{C_{b,z}}{\left(\frac{C_g}{H_c}\right)} \\ Z &= \frac{z}{L_b} \\ \kappa &= \frac{K_s}{\left(\frac{C_g}{H_c}\right)} \end{aligned} \quad (6)$$

$$\phi^2 = \frac{\rho_m p_c q_m L_b^2}{K_s D_e} \quad (7)$$

where ϕ^2 is the square of the Thiele modulus. The Thiele modulus is a dimensionless ratio of the intrinsic microbial reaction rate to the rate of diffusion (Bailey and Ollis, 1986).

The transformation of Equation (5) by nondimensionalization yields:

$$\eta_i = \frac{dC}{dZ} \Big|_{z=1} \frac{(1+\kappa)}{\phi^2} \quad (8)$$

Equation (8) can be solved numerically given a gradient of $C_{b,z}$ through the biofilm. For a biofilm metabolizing a contaminant in soil, knowing the values of parameters in Equations (6) and (7) enables the determination of ϕ^2 , which in turn enables the determination of η_i .

In lieu of solving η_i numerically, we used an analytical solution provided by Atkinson and Davies (1974) for the case of slab geometry, which is applicable to a biofilm continuum:

$$\eta_i = \begin{cases} 1 - \frac{\tanh \varphi}{\varphi} \left(\frac{1/\eta_d}{\tanh 1/\eta_d} - 1 \right) & \text{for } 1/\eta_d \leq 1 \\ \eta_d - \frac{\tanh \varphi}{\varphi} \left(\frac{1}{\tanh 1/\eta_d} - 1 \right) & \text{for } 1/\eta_d \geq 1 \end{cases} \quad (9)$$

In Equation (9), η_d , the asymptotic effectiveness factor (Atkinson and Davies, 1974; Bailey and Ollis, 1986) for the case of significantly large ϕ and Michaelis-Menten kinetics, is calculated as:

$$\eta_d = \frac{1}{\varphi} \frac{1+\kappa}{\kappa} \sqrt{2[\kappa - \ln(1+\kappa)]}^{1/2} \quad (10)$$

To determine the overall reaction rate from our modeling approach, the values of the parameters in Equation (7) must first be experimentally determined, then ϕ is calculated. Next, η_i can be solved from Equations (6), (9), and (10), and an overall rate of reaction can be predicted by $(\eta_i \cdot r)$. Thus, the important parameters governing biodegradation of VOCs in soil biofilms would be:

- D_e , diffusivity of contaminant through the biofilm

- q_m and K_s , the bacterial intrinsic kinetic parameters
- H_c , the Henry's Law constant for a VOC in biofilm
- L_b , total film thickness
- ρ_m and p_c , cell number density and cellular protein.

Soil Water Potential Effects on VOC Biodegradation

The diffusion (L_b , D_e) and reaction (K_s , q_m , ρ_m , p_c) parameters may vary depending on environmental conditions. Several environmental factors have been identified as potentially important constraints to microbial catalysis in unsaturated zone bioremediation: pH, temperature, inorganic nutrients, oxygen, redox potential, and soil water (Sims et al., 1993). Soil water is an integrating factor in that it can affect external mass transfer of solutes and gases (in soil macropores), internal mass transfer through biofilms and water films, and intrinsic microbial physiology. Generally in the unsaturated zone, the average free energy per unit volume of water, i.e., water potential, is negative. Hence, biofilms in equilibrium with surrounding water in the vadose zone reflect the negative water potential characteristic of the vadose zone.

Our concept for how the rate of VOC biodegradation in soil depends on matric water potential (ψ_m , -MPa) is depicted in Figure 2. The conceptual relationships in Figure 2 were synthesized from literature in soil microbiology and soil physics (eg., Harris, 1981; Jury et al., 1991; Papendick and Campbell, 1981; Stark and Firestone, 1995). Matric water potential is the reduction of potential energy of soil water per unit volume of water that is due to the adsorption of soil water onto solid surfaces. Although both osmotic and matric water potential contribute to total water potential, matric water potential is the dominant component of water potential in nonsaline soils (Papendick and Campbell, 1981). In Figure 2, progressively dryer conditions (Region 1 to Region 4) change the VOC removal rate; the changes in VOC removal rate are due to the effects that drying has on mass transfer and reaction processes. In Region 1 (Fig. 2), as drying occurs and the matric potential decreases from 0 to slightly negative, the overall VOC removal rate will improve due to improved gas phase mass transfer through soil pores with decreasing water content. Over a range of slightly negative water potentials (Region 1 and Region 2), improved gas-phase mass transfer will result in maximal VOC removal rates. However, as matric water potential progressively decreases (Region 3), the improvement on overall VOC removal rate by increased gas-phase mass transfer will not be great enough to overcome the negative effects of low water potential on intrinsic microbial physiology (Harris, 1981; Stark and Firestone, 1995) and on resupply of solution-phase nutrients to biodegrading microbes (Soroker, 1990; Stark and Firestone, 1995). Therefore, overall VOC biodegradation rates will be decreased. At very negative matric water potentials (Region 4), microbial physiology and solution phase mass transfer of ionic nutrients may severely impair the VOC removal rate. Also, the transport properties of the biofilm may be significantly altered. For example, scanning electron photomicrographs of a mono-species biofilm grown at various water potentials revealed increased exopolymer production by biofilm microbes in response to desiccation (Roberson and Firestone, 1992). Changes in exopolymer production may, at the biofilm scale, alter biofilm matrix thickness, porosity, and tortuosity. Hydration and dehydration of non-submerged biofilms will also alter porosity if the model for biofilm is that of structural collapse during drying and expansion during wetting (Holden, 1995) (Fig. 3). Finally, it is possible that biofilm drying could cause preferential flow paths (cracks) to form in the biofilm (Roberson and Firestone, 1992). This could lead to improved mass transfer characteristics under dry conditions and possibly improved overall VOC removal rates (Fig. 2, Region 4, broken line). Thus, the effect of water potential on microbial biofilms may be partly architectural (i.e., cell packing and EPS content) with an implication for altered internal (biofilm) mass transfer characteristics. In summary, our conceptual model in Figure 2 implies

that, through its effects on microbial reaction and substrate diffusion, matric water potential could be a critical environmental determinant of VOC biodegradation rates by unsaturated biofilms. Thus, Figure 2 summarizes our conceptual justification for including matric water potential as the primary variable in our studies of diffusion and reaction in unsaturated biofilms.

Material and Methods

Chemicals and Microbiological Media

Analytical grade toluene, benzene, xylene and D-glucose were obtained from Mallinckrodt, Inc. (Paris, KY) Polyethylene glycol, average molecular weight 8000 (PEG-8000), was obtained from Sigma Chemical Company (St. Louis, MO). All other chemicals were analytical grade or better.

The liquid basal medium consisted of 0.5 g NH_4Cl ; 1.725 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$; 1.38 g KH_2PO_4 ; and 10 mL half-strength Hutner's Mineral Base (Smibert and Krieg, 1994) per liter of Milli-Q water. The water potential of the basal medium was -0.15 MPa. To simulate the matric component of total water potential, the basal medium was amended with PEG-8000 to achieve respective matric potentials as follows: 100 g/L for -0.25 MPa, 150 g/L for -0.5 MPa, 262 g/L for -1.0 MPa, and 330 g/L for -1.5 MPa. PEG-8000 does not permeate cell walls and had been previously determined to not support growth of *Pseudomonas putida* mt-2 (Holden et al., to appear). The water potential of media was verified by thermocouple psychrometry. All media was filter sterilized ($0.2 \mu\text{m}$) prior to use.

Liquid Culturing

We cultured *P. putida* in liquid culture to provide the inocula for other liquid culture studies and for biofilm studies. The inoculum for liquid and biofilm cultures was prepared as follows. *Pseudomonas putida* mt-2 cells were maintained at -80°C and were grown on luria agar for 48 h prior to use. The plates were checked for purity and several fresh colonies were inoculated into a 500 mL triple-baffle side arm flask (Bellco Biotechnology, Vineland, NJ) that contained 100 mL of basal medium. The flasks were made gas tight by wrapping threads with Teflon tape and capping the top with a 38 mm Teflon-lined cap and the side with an 18 mm mininert cap (Alltech Associates, Waukegan, WI). Toluene was provided as the carbon source at an initial liquid concentration of 150 mg/L. We had previously determined that toluene was not toxic to *P. putida* mt-2 up to 300 mg/L (Holden, 1995). Cultures were grown at 27°C and shaken at 150 rpm. During mid-exponential phase (approximately 24 h) cells were separated from culture medium by centrifugation (30,000 g; 10 min; 5°C), washed once, and resuspended in approximately 1 mL basal medium.

Biofilm Culturing

Biofilms were cultured for several purposes: for determining biofilm mass transfer characteristics (L_b and D_e), for visualizing biofilm architecture and for determining the toluene depletion rate by intact biofilms. Biofilm cultures were grown in two-compartment glass biofilm reactors (Fig. 4) designed for growing biofilms under controlled matric water potential conditions. The upper compartment, containing PEG-amended media, was separated from a lower compartment by a 3000 MW (cutoff size) ultrafiltration membrane (Millipore, Inc., Beverly, MA). The membrane consists of a 600 nm thickness, ultrafiltration “skin” supported by a polypropylene backing ranging in thickness from 175 to 225 μm . The membrane served as the substratum for biofilm growth and excluded PEG-8000 from the biofilm growth environment while allowing free passage of water and nutrients (Soroker, 1990). Reactors were autoclaved then re-assembled under sterile conditions. The *P. putida* liquid inoculum (20 μL) was applied with a pipette onto the membrane. Either glucose (39 mg) or toluene (8.6 mg) was provided as the carbon source. Glucose was incorporated into the liquid media (described for

liquid culturing) during media preparation; neat toluene was added to the lower compartment of the reactor following assembly. Reactors were operated in a 27°C environment. Toluene utilization by biofilm cultures during biofilm cultivation was monitored by gas chromatographic (GC) analysis of the lower compartment over time. Glucose concentrations were not monitored.

Biofilm Microscopy and Determination of Tortuosity, Porosity, L_b and ρ_m

Biofilms were cultured for transmission electron microscopy (TEM) as before, except that several sterile 1/8-inch diameter, 23 μm thick cigarette paper disks were overlain on the membrane during reactor assembly and prior to inoculation. After substrate depletion (approximately 5 d), the membrane with disks was removed and placed with the biofilm up in a sterile petri plate containing several milliliters of media at the water potential of growth. This measure was taken to ensure that biofilm hydration did not change prior to cryopreservation of the paper disks with an RMC, Inc. (Tucson, AZ) MF 7200 propane jet freezer. Cryopreserved samples were maintained in liquid nitrogen until freeze substitution at -78°C for 72 h with 1% osmium and 0.1% uranyl acetate in an anhydrous acetone carrier, then gradually warmed to room temperature over a period of 3 h prior to embedment. Sectioned samples were post-stained first with 1% uranyl acetate in 70% methanol for 10 min, then Reynold's lead citrate for 5 min. Sections were examined on a JEOL 100C electron microscope operating at 80 kV.

Biofilm tortuosity and porosity were estimated from electron micrographs that were scanned into a computer graphics software program. Five measurements of tortuosity were made for each micrograph (e.g., each matric water potential for each carbon source). Tortuosity was measured as the ratio of around-cells to straight-line (perpendicular) distance that extended from the biofilm-air interface to the substratum. Porosity was estimated for each micrograph by calculating the pore area and dividing this area by the total biofilm area. Cell number density of biofilms (ρ_m) was calculated from transmission electron micrographs of toluene-grown biofilms by dividing the total cell number by the total biofilm area. This estimation of ρ_m was based on the observation that cell number density was constant across the biofilm and directionally independent.

Determination of Biofilm Thickness (L_b) and Toluene Diffusivity ($D_{t,e}$) Through Biofilm

Experimental Measurements—Biofilm thicknesses (L_b) when toluene was the carbon source were estimated as follows: (1) the thickness of the basal medium-grown (0.0 MPa) biofilm was scaled from a 5000 $\times$ magnification electron micrograph, and this thickness was designated as the “base” thickness; (2) a ratio was calculated for each matric potential by dividing the associated porosity into the porosity for the 0.0 MPa condition; and (3) this porosity ratio was multiplied by the base biofilm thickness. This method of estimating porosity assumes that porosity changes by water loss and is supported by our previous finding that cell yield was constant in liquid culture across the range of matric water potentials studied here (Holden et al., to appear).

We performed mass transfer studies to determine the diffusivity of toluene ($D_{t,e}$) through mature biofilms. For mass transfer studies, a pair of side-by-side reactors (uninoculated control and inoculated test) was operated for each matric water potential condition: 0.0, -0.25 , -0.5 , -1.0 , and -1.5 MPa. The biofilm cultures were first grown for 5 days using toluene as the carbon source at an initial liquid phase concentration of 150 mg/L. The toluene was depleted from the reactors at the end of this growth period. Benzene was then used as the diffusing substance, because it is structurally and physicochemically similar to toluene (Table I) and thus is expected to have mass transfer characteristics that are similar for toluene. Benzene had been determined previously through shake flask studies to not support growth of *P. putida* mt-2 (Holden, 1995). The mass transfer of benzene through the reactor (from the lower compartment

to the headspace in the liquid compartment) was measured after first adding approximately 1 mL of liquid benzene to the lower compartment of each reactor and then by analyzing for the concentration of benzene (by GC) as it accumulated in the upper compartment headspace over time. The mass of benzene initially added was adequate to ensure that neat benzene would exist throughout the experiment.

Modeling of Biofilm Mass Transfer Parameters—Mathematical models were required to: (1) calculate mass transfer coefficients for benzene through the biofilm from the measurements of benzene concentration vs. time in the biofilm reactor headspace, and (2) calculate toluene diffusivity from benzene mass transfer coefficients. Figure 5 depicts a reactor, oriented horizontally, with mass transfer regions indicated. The mass balance-based model describing the accumulation of benzene in the upper compartments (liquid and headspace regions, Fig. 5) over time is:

$$K[C_0 - C_{(t)}] = \left[\frac{L_w}{H_c} + L_a \right] \frac{dC_{(t)}}{dt} \quad (11)$$

The assumptions made in Equation (11) were: (1) the lower compartment was at a saturated benzene concentration of C_0 , (2) the upper compartment achieved equilibrium between liquid and gas phases with a gaseous concentration of $C_{(t)}$, and (3) the concentration profiles through the biofilms and membranes were linear and quasi-steady. Because benzene was not biodegraded by *P. putida*, Equation (11) specifically excludes a term for benzene depletion in the reactor. The integrated form of Equation (11), assuming an initial condition of $C_{(t=0)} = 0$ is:

$$\ln [C_0 - C_{(t)}] = \frac{K \cdot t}{\frac{L_w}{H_c} + L_a} + \ln C_0 \quad (12)$$

When the left side of Equation (12) is plotted vs. time, the slope of the line is equal to

$$\frac{K}{\frac{L_w}{H_c} + L_a}$$

from which K is obtained.

The overall mass transfer coefficient, K , is comprised of individual mass transfer coefficients for the various phases, although the dominant resistances are from the biofilm and the membrane plus associated interfaces (Bailey and Ollis, 1986):

$$\frac{1}{K} = \frac{H_c}{k_b} + \frac{H_c}{k_m} \quad (13)$$

For the uninoculated control reactors, Equation (13) does not include the term H_c/k_b . For each pair of reactors at a given matric potential, the values of H_c/k_m are equal. Thus, the resistance

due to biofilm growth for a given matric potential can be determined by subtracting the overall resistance for the control reactor from the overall resistance for the biofilm reactor.

The Henry's Law constant, H_c , for benzene may change as a function of PEG-8000 concentration as has been shown for toluene (Holden et al., to appear). However, a cosolvent effect of PEG-8000 on benzene will be equal for the biofilm and control reactors for a given matric potential. For this analysis, H_c for benzene is assumed constant at 0.22 (gas/liquid dimensionless) (Schwarzenbach et al., 1993).

The effective diffusion coefficient for benzene through the biofilm may be estimated, given the biofilm thickness, by:

$$D_{b,e} = k_b L_b \quad (14)$$

Finally, the effective diffusion coefficient for toluene is calculated from the coefficient for benzene according to the Wilke-Chang correlation (Welty et al., 1984):

$$D_{t,e} = D_{b,e} \frac{V_b^{0.6}}{V_t^{0.6}} \quad (15)$$

Kinetic Coefficients K_s , q_m , p_c and k_{bio} —We measured kinetic coefficients (K_s , q_m) for toluene depletion by resting cells, and we report kinetic parameters (k_{bio}) for toluene depletion by rapidly growing cells. These two sets of kinetic data were used to evaluate toluene reaction kinetics for the two metabolic extremes that commonly occur in soil: starvation and rapid growth (Paul and Clark, 1996).

K_s and q_m Determinations, and Reported p_c —The Michaelis-Menten parameters of toluene depletion kinetics, K_s and q_m , were measured in liquid culture using *P. putida* resting cells. As for other liquid culturing, PEG-8000 amended basal media were used to simulate reduced matric water potential in resting cell studies. Duplicate flasks for the five matric potential conditions (basal through -1.5 MPa) were initially inoculated with 100 μ L inoculum (as before) and cultures were grown to late exponential phase with toluene (150 mg/L initial liquid phase concentration) as the carbon source. Growing cells were centrifugally separated and resuspended in 120 mL of fresh medium at the matric potential of growth. Cultures were incubated at 27°C and shaken at 150 rpm for 12 to 20 h without exogenous carbon to establish resting cell characteristics. Starvation studies (not shown) had previously indicated that culture protein content and total population size (by culturable counts) decreased significantly during the first 12 h of starvation, but remained stable for up to 12 h thereafter (Holden, 1995). Ten 40 mL glass vials, each with a 24 mm mininert cap, were prepared for each matric potential by adding 10 mL of rested culture to each vial. Saturated toluene vapor was added to each vial such that the initial concentration of toluene in culture liquid evenly spanned the range 1 to 250 mg/L. Toluene vapor was added to one vial at a time and toluene headspace analysis by GC immediately followed; addition and analysis was staged in this manner so that each vial was continuously sampled during the first 10 min following toluene addition. Vial contents were mixed by swirling before and after headspace analysis to equilibrate gas and liquid phases. Following the final GC measurement (within 2 h), the vial liquid was sampled for culturable cell numbers (CFUs per milliliter of resting culture). The protein content per cell, p_c , had been measured previously (Holden et al., to appear) for early stationary phase, toluene-grown *P. putida* cultures using Bradford's method (Bradford, 1976). Protein concentration per milliliter

of resting culture was calculated by multiplying CFU/mL by p_c . Based on the slow growth rates found previously for *P. putida* mt-2 (Holden et al., to appear), where growth rates were measured for identical matric potential conditions and with similar substrate concentrations to resting cell studies herein, the culturable counts and protein concentrations of resting cultures were not expected to change significantly during the course of headspace sampling for toluene. Oxygen was not expected to limit toluene depletion during resting cell studies as greater than stoichiometric quantities of oxygen were provided in the vials.

Headspace concentrations of toluene were converted to liquid concentrations using appropriate Henry's Law constants which varied slightly for each matric potential (Table II) (Holden et al., to appear). The initial rate of substrate depletion for each initial substrate concentration was calculated by the slope of liquid phase toluene concentration vs. time. This slope was calculated by linear regression (95% CI) performed over the first and most linear (highest R^2) period of substrate decay. The values of initial rates were normalized to culture protein content. Normalized rates were plotted vs. initial substrate concentration for each matric water potential condition. Kinetic plots consistently indicated multiphasic kinetic relationships. Multiphasic kinetic relationships occurring over a broad range of initial substrate concentrations have been previously reported for other pure cultures (Ishida et al., 1982; Lewis et al., 1985). We chose to evaluate the Michaelis-Menten parameters q_m and K_s , over the range of initial substrate concentrations below 50 mg/L (liquid phase) because this concentration range is the same magnitude as the average equilibrium aqueous phase concentration of toluene (25 mg/L) found in gasoline-contaminated subsurface environments (Cline et al., 1991). The Michaelis-Menten parameters of maximum rate (q_m) and half saturation constant (K_s) were computed by the method of double reciprocal plots (Bailey and Ollis, 1986).

Reported Values for k_{bio} —We used the toluene depletion rates per rapidly growing cell (k_{bio}) as a measure of the maximum toluene depletion rate constants that would likely be expressed by soil bacteria. The details of the methods used to make k_{bio} measurements and the values are reported elsewhere (Holden et al., to appear). Generally, we cultured *P. putida* in batch liquid culture as described previously, with the exception that the initial toluene concentration in the liquid media was 250 mg/L. We measured toluene concentration in the flask headspace by GC analysis and monitored cell mass by optical density at regular intervals throughout culture growth. Our parameter k_{bio} differs from q_m in two distinct ways. First, k_{bio} is based on substrate depletion by *growing* cells where substrate depletion is first-order with respect to cells, but zero-order with respect to substrate concentration (Bailey and Ollis, 1986). Thus, whereas rested cells were metabolically-inactive just prior to the toluene depletion studies, cells used in making k_{bio} measurements were rapidly growing. Second, the initial liquid phase toluene concentration during k_{bio} measurements was 250 mg/L. The Michaelis-Menten intrinsic rate coefficient, q_m , was evaluated by conducting toluene depletion experiments using the range of substrate concentrations up to 50 mg/L (as described above). Thus, these differences in experimental methodology (initially inactive vs. initially active cells) and data analysis (evaluation over low substrate concentration range vs. evaluation at high initial substrate concentration) can be expected to result in k_{bio} values that are greater than q_m values.

Prediction of Toluene Depletion Rate Using Model and Measured Parameters—

Rates of toluene depletion by our *P. putida* mt-2 biofilms were predicted as a function of matric water potential using our mass balance model [Eq. (3)] as the basis for the predictions. The predicted rates were made by first calculating the Thiele modulus, ϕ , by Equation (7) using measured parameter values. Thiele moduli were calculated in two ways for each matric water potential: using either q_m or k_{bio} as the intrinsic maximum rate coefficient. Next, κ was calculated [Eq. (6)] using experimentally determined values of K_s and assuming an aqueous (biofilm) toluene concentration (C_g/H_c) of 25 mg/L at the biofilm-air interface. The internal effectiveness factor, η_i , was then calculated using Equations (9) and (10). Values of η_i were

calculated for both sets of Thiele moduli (for the two parameters, q_m and k_{bio} , and across the range of matric potential conditions). Finally, the predicted rates of toluene depletion were calculated by $(\eta_i \cdot r)$. Thus, two predicted relationships between toluene depletion rate and matric water potential were generated. Statistical analyses, either for comparing the effect of water potential on predicted rate or for comparing the predicted rates to one another, were not performed because (1) the lack of replication in most parameters did not support these analyses, and (2) propagating the experimental error (associated with each parameter) through the calculation of η_i was deemed overly complex, and not especially useful given that a reasonable relationship between rate and matric water potential could be revealed through graphical representation (see Results).

Composite Toluene Depletion Rate by Intact Biofilms—For comparison to model predictions, we measured the rate of toluene depletion by intact biofilms as a function of matric water potential. The initial rates of toluene depletion were determined in three independent experiments for toluene-grown, *P. putida* biofilms. For each experiment, biofilms were cultured with toluene as the carbon source, as described previously. After a 5-day culture period, each membrane was removed from its associated reactor and positioned, biofilm up, on top of 1 mL of sterile media (equivalent to the water potential of growth) in a 40 mL sterile glass vial with 24 mm mininert cap. The purpose of the underlying media was to maintain membrane, and hence biofilm, hydration identically to what was established during biofilm growth in the reactor. Vapor phase toluene was added to achieve an initial concentration in the biofilm of approximately 25 mg/L. For this initial toluene concentration, greater than stoichiometric quantities of oxygen were available for toluene mineralization in the vials. After toluene addition, the vapor phase toluene concentration was determined over time by GC analysis. The total period of toluene headspace measurements lasted up to several hours. However, only initial depletion rates were calculated. The onset of depletion was immediate in most vials, but, where it was delayed, the initial rate was determined by performing a linear regression analysis on the series of three or more points where the first point marked the beginning of toluene depletion. Sampling headspace for GC analysis minimally changed the headspace toluene concentration because each sample volume (60 μ L) was less than 0.2% of the total volume in the 40 mL vials. Weighted averages of the rates were calculated for each matric water potential and the uncertainty associated with each weighted mean was calculated using standard principles of error propagation (Taylor, 1982).

Analytical Methods

The water potential of PEG-amended media was verified before use with a thermocouple psychrometer and nanovoltmeter/thermometer (Models SC10 and NT-3, Decagon Devices, Inc., Pullman, WA). Known concentrations of NaCl in deionized, distilled water were used for standards. The water potentials of the standards were calculated using the Van't Hoff relationship (Papendick and Campbell, 1981). All measurements were made at a constant temperature (25°C).

Toluene and benzene concentrations were determined by GC analysis of headspace samples (60 μ L) using a Hewlett-Packard 5890A gas chromatograph with flame ionization detector (FID) (modified EPA Method 3810, SW 846). Carrier gas through the packed column was ultra high purity nitrogen (UHP, Matheson Gas Products, Newark, CA) at a flow rate of 30 mL/min. Air and hydrogen flow rates were 400 mL/min and 30 mL/min, respectively. Other operating conditions were: oven temperature-105°C; injector temperature-125°C; detector temperature-105°C. Toluene or benzene standards that were less than 1 mg/L were made by injecting known amounts of saturated vapor into 2-L static dilution bottles with 24 mm mininert caps (Tekmar Co., Rosemount Analytical, Inc., Cincinnati, OH). Various volumes of liquid toluene and xylene were combined in gas-tight vials to provide a range of gas-phase toluene

standards above 1 mg/L and Raoult's Law was used to calculate the resulting headspace concentrations in these mixtures. All standards and samples were in thermal equilibrium prior to GC analysis.

For protein measurement of resting cultures, cells were separated by centrifugation (30,000 g, 20 min, 5°C) then solubilized at 90°C for 10 min in 1 N NaOH. Protein content was determined by Bradford's method (Bradford, 1976). For the protein content of biofilms, whole membranes with attached biofilms were washed in 5 mL of sterile 1% SDS by vortexing for 10 sec followed by a 1 min sonication. Solubilized biofilm was removed by pipette and protein content was determined by Bradford's method, as above, using detergent-compatible reagents (BioRad, Hercules, CA).

Results

Biofilm Architecture: Porosity, Tortuosity, and ρ_m

For both toluene and glucose, biofilm continuously, and nearly uniformly, covered the area of membrane exposed to the lower reactor compartment. As shown in Figure 6, electron micrographs revealed a very smooth biofilm/air interface (BAI). This was similar for both carbon sources. When glucose was the carbon source (Fig. 6a–c), biofilm cell morphology and cell packing changed with decreasing matric potential. Denser cell packing was reflected by decreasing porosity with decreasing matric potential (Table III). The porosity of biofilms grown with toluene as the carbon source (Table III) did not change with matric water potential in any discernible pattern.

The tortuosity of biofilms did not vary with matric water potential [$P = 0.05$, Tukey's HSD (Daniel, 1991)] when glucose was the carbon source (Table III); however, when toluene was the carbon source, the mean tortuosity at -1.5 MPa was significantly higher than the tortuosity at either -0.5 or -1.0 MPa.

The cell number density of biofilm (ρ_m), as measured from electron micrographs when toluene was the carbon source, are tabulated in Table III; densities do not appear to vary with matric potential of growth.

Biofilm Thickness, L_b , and Toluene Diffusivity, $D_{t,e}$

The thicknesses of toluene-grown biofilms, L_b , were similar at all matric potentials with the exception of the -0.25 MPa condition where the biofilm thickness was lower (Table IV).

During the biofilm mass transfer studies, the biofilm offered additional resistance to benzene transport (Fig. 7). The effective diffusion coefficients for benzene or toluene diffusing through biofilm ($D_{b,e}$ or $D_{t,e}$) did not vary markedly with matric water potential (Table IV). However, the values for $D_{t,e}$ are lower by nearly two orders of magnitude than the published diffusion coefficient for toluene in pure water, e.g., 9×10^{-6} cm²/s (Schwarzenbach et al., 1993).

Kinetic Parameters q_m , K_s , k_{bio} , and p_c

Resting cell kinetic parameters (q_m and K_s), growing cell kinetic parameters (k_{bio}) and cellular protein contents (p_c) for *P. putida* mt-2 as a function of matric water potential are provided in Table V. Also provided in Table V is the culturable population densities of resting cultures. The half saturation constants, K_s , were relatively constant as a function of matric potential and averaged 19 mg/L. This average compares reasonably well with a previously reported half saturation constant of 17.4 mg/L for microbial populations depleting toluene in sandy aquifer material (Alvarez et al., 1991). The maximum intrinsic rate of toluene depletion for resting cells (normalized to cellular protein), q_m , varied irregularly across the matric potentials studied

but was highest at -1.5 MPa. As previously reported (Holden et al., to appear), the value of k_{bio} was significantly higher at 0.0 MPa ($P = 0.05$) as compared to the other water potential conditions; however values of k_{bio} were not significantly different from one another for matric potentials across the range -0.25 to -1.5 MPa. The average of our k_{bio} values, 2.8×10^{-2} , is reasonably similar to a maximum specific toluene utilization constant of 0.03 mg toluene/mg protein-minute previously reported for microbial populations in sandy aquifer material (Alvarez et al., 1991). The protein content per cell was highest at -0.25 MPa; otherwise protein content per cell did not vary significantly with matric water potential.

Predicted and Measured Rates of Toluene Depletion vs. Matric Water Potential

Thiele moduli, ϕ , and internal effectiveness factors, η_i , were calculated using the tabulated parameters (Tables II–V) and Equations (6), (7), (9), and (10). Results are provided in Table VI. As indicated by the low values for η_i , regardless of which kinetic parameters are used (q_m or k_{bio}), the diffusion of toluene through biofilms severely limited the rate of toluene depletion.

Data used to calculate initial rates for the intact biofilm toluene depletion experiments, along with associated regression parameters, are provided in Table VII. The data in Table VII are a subset of the entire time-course of toluene concentrations. At least 10 samples were taken for toluene analysis over time for each incubation. Over the time interval (typically four to five points) for which Table VII rates were calculated, toluene depletion rates were constant. The data on either side of the time intervals indicated in Table VII were used to clearly establish the temporal basis for those intervals. Outside of the time interval over which toluene depletion was linear, toluene depletion rates decreased due to biodegradation and reduced toluene concentrations. The values of R^2 in Table VII are generally high, indicating that the rate was constant over the time interval. The low values of R^2 for the -1.5 MPa data are indicative of the variability of the data at this extreme low matric potential condition.

The net rates of toluene depletion per unit of cellular protein for an initial aqueous phase toluene concentration of 25 mg/L are plotted in Figure 8 for three cases: two predicted and one measured. The two curves based on predictions (“model” in figure legend) were generated by inserting experimentally-determined parameter values into the biofilm model [Eq. (3)], using either the Michaelis-Menten maximum rate, q_m , or the maximum rate by growing cells, k_{bio} . The rate was converted to a per unit protein basis using cellular protein data of Table V. The Figure 8 curve labeled as “composite” was experimentally measured using whole biofilms; plotted rates are the initial rates of toluene depletion (Table VII). As shown in Figure 8, over the range of 0.0 to -1.0 MPa, the pattern of rate vs. matric potential is similar for all curves. The rates predicted from inserting measured parameters (using q_m) into the biofilm mathematical model were lower than rates measured for whole biofilm with the exception that at -1.5 MPa, the predicted overall rate exceeds that measured (Fig. 8). The two patterns of predicted rates (based on q_m or k_{bio}) were reasonably parallel over the entire range of matric water potentials. For the whole biofilm measurements (“composite”), toluene depletion rates did not differ significantly [$P = 0.05$, Tukey's HSD (Daniel, 1991)] across the range of matric potentials from 0.0 to -1.0 MPa. However, the mean rate for the -1.5 MPa matric potential condition was significantly lower than the rates for the 0.0 and -1.0 MPa conditions ($P = 0.05$).

Discussion

We have applied a diffusion-reaction model to describe toluene biodegradation by unsaturated *P. putida* biofilms and have found that the model describes, within an order of magnitude, the rate of toluene biodegradation by whole biofilms. We have also determined that toluene diffusion within active biofilms severely limits the rate of toluene depletion for all matric potentials over the range of 0.0 to -1.5 MPa. The reduced amount of water in unsaturated

biofilms as compared to saturated biofilms is a factor that distinguishes vadose zone biofilms from those in aquatic systems. Unsaturation apparently confers a greater resistance to mass transfer (as compared to saturation) in biofilms: the diffusion coefficients for toluene in biofilm that we have measured are approximately two orders of magnitude lower than a toluene diffusion coefficient in water and one order of magnitude lower than diffusion coefficients for similar compounds in saturated biofilms (Fan et al., 1990).

As reviewed by Fan et al. (1990) diffusion coefficients for solutes in saturated biofilms have been measured using various techniques that result in widely ranging values. Unlike saturated biofilms, there is no existing literature that addresses the diffusional characteristics of unsaturated biofilms. However, we have previously estimated (Holden, 1995) the diffusion coefficient for toluene in unsaturated biofilm as a function of water potential by modifying a published diffusion coefficient for toluene in water using the model of Mackie and Meares for diffusion coefficients in gels (Mackie and Meares, 1955), modified by Westrin and Axelsson (1991) to account for bacterial cells, and water content–water potential relationships (characteristic curves) for microbial exopolymer (Chenu, 1989; Roberson and Firestone, 1992). This approach estimates the diffusion coefficient at between 0.2 to 0.6 of the aqueous values, with the lower value corresponding to driest conditions. Chenu and Roberson (1996) recently reported a decrease in glucose diffusion coefficients with decreasing water potential (hence water content) of pure xanthan. The diffusion coefficients they reported ranged from 0.1 to 0.6 of the value for glucose in water, with the lower value corresponding to the lowest (−1.0 MPa) water potential. These values are comparable to our predictions (Holden, 1995) for unsaturated biofilms. However, our predictions and the measured diffusion coefficients of Chenu and Roberson (1996) are underestimates of the mass transfer resistance in unsaturated biofilms as found in our experimental determinations. We believe that bacterial cells and the presence of an air-biofilm interface, neither of which was present in the studies of Chenu and Roberson (1996), contributed to the high mass transfer resistance that we observed with our biofilms.

Our model and measured parameters approximately describe the rate of toluene biodegradation by whole biofilm. The fact that the rates calculated using the model are different than the directly measured rates may be due to our having measured bacterial kinetic parameters in liquid culture. Exclusive of diffusion, microbial reaction kinetics are often considered to be the same for attached and planktonic organisms (e.g., Mehmetoglu, 1990). Although the composite experimental literature is inconclusive (van Loosdrecht et al., 1990), biofilm intrinsic microbial reaction rates may be higher due to biofilm retention of growth or reaction-promoting compounds (Costerton, 1995; Mignot and Junter, 1990). Thus, our use of liquid culture to determine bacterial reaction rate parameters (q_m or k_{bio} with toluene as the carbon source) may have resulted in inaccurate predictions.

Another characteristic of our unsaturated biofilms that may distinguish them from saturated biofilms is the shape of the biofilm-air interface. In our biofilms, the interface is flat (Fig. 6), reflecting the high interfacial tension of an air-water interface. In saturated biofilms, this regular interface does not exist. Rather, recent observations support that saturated biofilms are a complex network of channels and microcolonies of varying shapes and heights (Costerton et al., 1995). Water flows over the rough biofilm surface and through the biofilm channels (Costerton et al., 1995), perhaps contributing to a complex topography. Although convective flow may occur periodically in unsaturated environments, we grew our biofilms in the absence of turbulence, and thus, our images may reflect the typical biofilm topography of a generally unsaturated system.

The experimental and modeling results in Figure 8 can be compared with the conceptual model presented in Figure 2. Across the range of 0.0 to −1.0 MPa, our measured (Fig. 8) rates were

constant. This differs from our generalized conceptual progression (Fig. 2) in that we expected slightly negative matric water potentials to result in increased rates above those at 0.0 MPa, then for rates to decrease as matric water potentials became more negative. Similar to the proposed progression of Figure 2, we found that a matric water potential of -1.5 MPa resulted in a significantly decreased toluene depletion rate by whole biofilms (Fig. 8). One potentially overriding factor that distinguishes Figures 2 and 8 is that the generalized conceptual progression (Fig. 2) considers macropore mass transfer limitations that are absent from our modeled and measured systems. However, both Figures 2 and 8 would reflect physiological impairment and local mass transfer resistance. Previous studies of bacterial reaction rate in soils as a function of soil water potential have not separated diffusion from reaction at the scale of the soil biofilm (Potts et al., 1984; Rattray et al., 1992). However, where reaction or growth rate is studied independently by liquid culture, the effect of more negative water potential is generally to decrease free enzyme catalytic rate (Andersson and Hahn-Hägerdal, 1987; Laurent, 1971) and bacterial growth rate (Busse and Bottomley, 1989; McAneney et al., 1982) at more negative water potentials. Thus, there is basis within the published literature to expect that negative water potentials would significantly decrease hydrocarbon biodegradation rates due to intrinsic physiological impairment—we have predicted this conceptually (Fig. 2) and found this experimentally for intact biofilms (Fig. 8).

Our mathematical model based on Michaelis-Menten kinetics predicted an increased rate at -1.5 MPa (Fig. 8) because of the high value of q_m at -1.5 MPa. To sort out the differences between predicted and whole biofilm rates at -1.5 MPa, further replication of q_m measurements would be necessary. However, k_{bio} is another measure for the maximum intrinsic rate of toluene depletion and this measure represents the upper extreme of bacterial activity in soil. When comparing the modeled rates and the composite rates, we find that the two modeled rates generally bracket the composite rates over the range of water potentials studied (Fig. 8). We might expect this, given that metabolic activity in the intact biofilms probably lies somewhere in between that of resting and rapidly growing cells. Still, without knowing the total error associated with each predicted rate, it is difficult to quantify the significant differences between “model” and “composite” curves in Figure 8. For the purposes of this study, we have shown that a diffusion-reaction model can be used to predict and better understand VOC depletion by unsaturated biofilms at the scale of the biofilm and that predicted rates are within the same order of magnitude as actual rates.

Oxygen resupply is an important consideration in aerobic metabolism, and it was a factor that we considered in conducting our experiments. There was a potential for oxygen resupply to determine toluene depletion rates in our measurements using whole biofilms. Based on the stoichiometry of mineralization (9 moles O_2 per mole toluene), values of K_s for O_2 in the literature (Button, 1985), an initial biofilm toluene concentration at the biofilm-air interface of 25 mg/L, and our experimental measurements of q_m and $D_{b,e}$ (correlated through Wilke-Chang for oxygen), we estimated that the diffusion of oxygen in our whole biofilms would support a toluene depletion rate of approximately 0.6×10^{-3} mg/mg protein-minute. This estimated rate is comparable to our measured and predicted rates (Fig. 8) that were based on the assumption that toluene was the limiting substrate. Thus, at a toluene concentration of 25 mg/L, oxygen resupply could be as important as toluene diffusion rates in limiting the rate of toluene depletion by unsaturated biofilms.

To our knowledge, there are no previously published relationships for the effects of water potential on hydrocarbon biodegradation rates by unsaturated biofilms, nor evaluations of the diffusion and reaction characteristics of such biofilms. The results of this study provide an indication of how VOC biodegradation rates in unsaturated biological systems will vary as a function of matric water potential. Furthermore, these results reveal the importance of mass transfer resistance within unsaturated biofilms in determining the overall rate of VOC

biodegradation. Finally, our results suggest that saturated biofilm processes may quantitatively differ from unsaturated biofilm processes and that our understanding would benefit from further investigation into these differences.

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Nomenclature

$C_{b,z}$	mass concentration of VOC in the hydrated biofilm at position z into the biofilm (mg/L)
C_g	gas-phase VOC concentration in soil pore (mg/L)
C_0	gas-phase concentration of benzene just above liquid benzene (mg/L)
$C(t)$	average headspace concentration of benzene in upper compartment at time, t (mg/L)
$D_{b,e}$	effective diffusivity of benzene through biofilm (cm^2/s)
$D_{t,e}$	effective diffusivity of toluene through biofilm (cm^2/s)
D_e	effective diffusivity of VOC through biofilm (cm^2/s)
H_c	Henry's Law constant $[(\text{mg/L})_{\text{gas}}]/[(\text{mg/L})_{\text{liquid}}]$
K	overall mass transfer coefficient (cm/s)
k_b	mass transfer coefficient through bacterial biofilm (cm/s)
k_{bio}	intrinsic toluene removal rate per growing cell protein (mg/mg min)
k_m	mass transfer coefficient across membrane (cm/s)
K_s	intrinsic aqueous phase half saturation constant (mg/L)
L_a	length of headspace in upper compartment (cm)
L_b	average biofilm thickness (μm)
L_w	length of liquid column in upper compartment (cm)
p_c	biofilm cellular protein content (mg protein/cell)
q_m	intrinsic toluene removal rate per resting cell protein (mg/mg min)
r	rate of VOC depletion per unit of cell protein (mg/mg min)
V_b	molal volume of benzene (L/g mol)
V_t	molal volume of toluene (L/g mol)
η_i	internal effectiveness factor (dimensionless)

ϕ	Thiele modulus (dimensionless)
ρ_m	number density of bacteria per mass of biofilm (cell/ μm^3)
Ψ_m	matric water potential (–MPa)

Figure 1.
Unsaturated biofilm.

Figure 2.

Conceptual relationship between VOC depletion rate in unsaturated soil and matric water potential. Region 1: thick water films impede mass transfer and intrinsic microbial physiology is unimpaired; Region 2: optimum range of matric water potential over which microbial physiology and mass transfer are relatively unimpaired; Region 3: bacterial physiology slightly impaired by lower water potential and thin water films limit diffusion of ionic nutrients; Region 4: bacterial physiology and diffusion of ionic nutrients through thin films severely impaired (solid line) although greatly improved mass transfer may occur as low resistance flow paths may open through biofilm (broken line).

Figure 3. Conceptual progression of unsaturated biofilm morphology during drying: (a) saturated biofilm, (b) semi-hydrated biofilm, (c) collapsed semi-hydrated biofilm, (d) EPS-embellished semi-hydrated biofilm.

Figure 4.
Biofilm membrane reactor.

Figure 5.
Idealized biofilm reactor with mass transfer regions indicated.

Figure 6.

Transmission electron micrographs of *P. putida* mt-2 biofilms grown at controlled matric water potential with glucose or toluene as the carbon source. BAI = biofilm-air interface. (A) glucose, 0.0 MPa at 16,000 $\times$ magnification; (B) glucose, -0.25 MPa at 16,000 $\times$ magnification; (C) glucose, -0.5 MPa at 16,000 $\times$ magnification; (D) toluene, 0.0 MPa at 5,000 $\times$ magnification. Scale: (A), (B) and (C) bar = 0.5 μ m; (D) bar = 5 μ m.

Figure 7. Benzene diffusion in inoculated (closed symbols, “Biofilm”) and uninoculated (open symbols, “Control”) reactors when matric water potential is 0.0 MPa to -1.5 MPa. *Pseudomonas putida* biofilm impeded diffusion in the inoculated reactor.

Figure 8.
Predicted and measured rates of toluene depletion by unsaturated *P. putida* mt-2 biofilms as a function of matric water potential.

Table I

Selected physicochemical and structural characteristics of toluene and benzene.

Characteristic	Toluene	Benzene
Chemical formula ^a	C ₇ H ₈	C ₆ H ₆
Molecular weight (g/g-mole) ^a	92.1	78.1
Molecular volume (cm ³ /g-mole) ^b	118	96
Vapor pressure (mm Hg, 25°C) ^a	29	96
Aqueous solubility (mg/L) ^a	518	1789
Henry's Law constant (gas/water; unitless) ^a	0.29	0.22
Log K _{ow} ^a	2.69	2.13

^aSchwarzenbach, 1993.^bCalculated (Welty et al., 1984).

Table II
Henry's Law constants for toluene in PEG-8000 amended media (dimensionless; gas/liquid) (Holden et al., to appear).

Matric water potential of PEG-amended media (MPa)				
H _c	0.0	-0.25	-0.5	-1.0
	0.27	0.27	0.26	0.20
				-1.5
				0.17

Table III

Morphological characteristics of unsaturated *P. putida* biofilms as a function of carbon source and matric water potential.^a

Substrate, parameter	Matric water potential, ψ_m				
	0.0 MPa	-0.25 MPa	-0.5 MPa	-1.0 MPa	-1.5 MPa
Glucose					
tortuosity	1.31 (0.02)	1.15 (0.07)	1.29 (0.05)	(not avail.)	1.27 (0.02)
porosity	0.41	0.45	0.17	(not avail.)	0.0
Toluene					
tortuosity	1.14 (0.04)	1.19 (0.03)	1.09 (0.02)	1.08 (0.02)	1.28 (0.02)
porosity	0.35	0.18	0.51	0.52	0.35
ρ_m (cell/ μm^3)	4.2	5.1	5.0	5.8	4.3

^aNumbers in parentheses are standard errors of the means.

Table IV
Mass transfer parameters for unsaturated, toluene-grown *P. putida* biofilms as a function of matric water potential.

Parameter	Matric water potential, ψ_m				
	0.0 MPa	-0.25 MPa	-0.5 MPa	-1.0 MPa	-1.5 MPa
L_b (μm)	25	13	36	37	25
$D_{b,e}$ (cm^2/sec) $\times 10^7$	1.4	0.9	1.3	2.4	1.2
$D_{i,e}$ (cm^2/sec) $\times 10^7$	1.2	0.8	1.2	2.1	1.0

Table V
Microbial kinetic parameters for unsaturated *P. putida* biofilms as a function of matric water potential.^a

Parameter	Matric water potential, ψ_m				
	0.0 MPa	-0.25 MPa	-0.5 MPa	-1.0 MPa	-1.5 MPa
$p_c \times 10^9$ (mg pro./CFU) ^b	0.19	0.35	0.11	0.07	0.06
Resting cells $\times 10^{-8}$ (CFU/mL)	6	7	3	142	2
K_s (mg/L)	21	19	21	15	18
$q_m \times 10^2$ (mg tol/mg pro.-min)	0.45	0.17	0.87	0.95	3.4
$k_{bio} \times 10^2$ (mg tol/mg pro.-min) ^b	4.5 (0.2)	3.4 (0.3)	2.3 (0.5)	1.8 (0.4)	2.0 (0.5)

^aNumbers in parentheses are standard errors of the means.
^bHolden et al., to appear.

Table VI
Internal effectiveness factors and Thiele moduli for *P. putida* biofilms as a function of matric water potential.

Parameter	Matric water potential, ψ_m				
	0.0 MPa	-0.25 MPa	-0.5 MPa	-1.0 MPa	-1.5 MPa
Calculated from q_m					
ϕ	12	8	20	17	23
η_i	0.14	0.22	0.08	0.11	0.08
Calculated from k_{bio}					
ϕ	38	34	33	23	17
η_i	0.04	0.05	0.05	0.08	0.10

Table VII

Data utilized for calculating toluene depletion rate by whole biofilms as a function of matric water potential.

ψ_m (MPa)	Replicate	Elapsed time (min)	Toluene depleted (mg)	Total protein (mg)	Number regressed data	Rate $\times 10^3$ (mg tol/mg pro.-min)	R^2
0	1	60.0	0.108	2.05	6	0.88	0.98
	2	22.7	0.028	1.67	3	0.73	0.99
	3	66.6	0.044	0.95	5	0.69	0.81
-0.25	1	60.5	0.047	1.39	6	0.56	0.96
	2	22.8	0.029	1.90	3	0.69	0.96
	3	24.9	0.014	1.01	3	0.55	0.99
-0.5	1	32.3	0.032	1.28	3	0.77	0.90
	2	49.0	0.058	1.79	4	0.66	0.73
	3	85.4	0.078	1.15	4	0.80	0.83
-1.0	1	48.7	0.035	0.80	4	0.90	0.95
	2	49.2	0.046	1.01	3	0.92	0.99
	3	81.1	0.074	1.04	4	0.88	0.99
-1.5	1	146	0.053	0.54	10	0.67	0.46
	2	130	0.042	0.72	9	0.44	0.64
	3	107	0.020	0.63	6	0.30	0.65