Bacterial Growth on Distant Naphthalene Diffusing through Water, Air, and Water-Saturated and Nonsaturated Porous Media

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The influence of substrate diffusion on bacterial growth was investigated. Crystalline naphthalene was supplied as the substrate at various distances in the range of centimeters from naphthalene-degrading organisms separated from the substrate by agar-solidified mineral medium. Within 2 weeks, the cells grew to final numbers which were negatively correlated with the distance from the substrate. A mathematical model that combined (i) Monod growth kinetics extended by a term for culture maintenance and (ii) substrate diffusion could explain the observed growth curves. The model could also predict growth on naphthalene that was separated from the bacteria by air. In addition, the bacteria were grown on distant naphthalene that had to diffuse to the cells through water-saturated and unsaturated porous media. The growth of the bacteria could be used to calculate the effective diffusivity of naphthalene in the three-phase system. Diffusion of naphthalene in the pore space containing 80% air was roughly 1 order of magnitude faster than in medium containing only 20% air because of the high Henry's law coefficient of naphthalene. It is proposed that the effective diffusivities of the substrates and the spatial distribution of substrates and bacteria are the main determinants of final cell numbers and, consequently, final degradation rates.

The success of efforts to bioremediate polluted environments depends on the availability of the pollutants to microbes. Since in many environments microorganisms and their substrates are unequally distributed, transport processes may control the degradation rates (6). Pollutants exist separated from active microorganisms for several reasons: they may have been accumulated in particles into which degraders cannot penetrate (29), or they may exist as point contaminations in solid state or as separate-phase liquids (1, 23). In some cases, the pollutants may be present in an environment deficient in the appropriate electron acceptor for degradation (8). In subsurface environments, most organisms are immobile because of their association with solid particles (11, 12, 25). Consequently, degradation relies mainly on diffusive or convective transport of the pollutant.

Diffusion of chemicals through soil or in groundwater is impeded by physical barriers like soil particles, by reactive surfaces, and by soil constituents with high resistance to diffusion like, for instance, organic matter (14, 29, 35). Another soil constituent which influences diffusion is the air that is present in the vadose zone. While the biodegradation of pollutants in water-saturated porous environments has been studied intensively, only scarce information exists on bacterial activity in the unsaturated zone of subsurface systems (7, 13, 19, 22) and on the influence of entrapped air on diffusive pollutant transport (2, 18).

Here, the hypothesis was tested that the spatial distribution of a substrate and its transport govern bacterial growth in a heterogeneous system. If this is the case, bacteria with known growth parameters can be used as a tool to determine substrate transport in such systems. The experimental procedure was (i) to determine the bacterial growth parameters in aqueous systems; (ii) to prove that these parameters, together with infor-

mation on substrate transport, allow prediction of growth on a substrate that is separated from the bacteria by air; and (iii) to use bacterial growth on a substrate separated by glass beads, agar, and various volume fractions of air to calculate substrate transport through such a complex matrix.

MATERIALS AND METHODS

Bacteria and culture conditions. Naphthalene-degrading bacterial strain HH4 was used in all experiments. Strain HH4 has been isolated from garden soil from the Zurich (Switzerland) area with naphthalene as the sole source of carbon and energy. It is a gram-positive, aerobic, rod-shaped bacterium. The 16S rRNA sequence was partly elucidated by the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). It showed the highest sequence similarity to *Rhodococcus* species but could not be assigned to a known genus. Strain HH4 was grown in a mineral medium that has been described previously (15). Naphthalene (1 g liter⁻¹) was added aseptically to the autoclaved medium as a carbon source after the medium was cooled to 60°C. Because of its maximum aqueous solubility of 242 µM (at 25°C) (24), naphthalene was present mainly as crystals. The bacteria were grown in 100 ml of medium in 1-liter Erlenmeyer flasks at 25°C on a rotary shaker at 175 rpm. The flasks were equipped with glass stoppers to avoid air stripping of naphthalene. During growth on naphthalene, excretion of degradation products was monitored by high-pressure liquid chromatography. The cells were harvested at the end of the exponential growth phase. Residual naphthalene crystals were removed by filtration through a sterile paper tissue. Cells were centrifuged, washed twice in 10 mM phosphate-buffered saline (PBS) (15), and resuspended in PBS. Solid media were prepared from mineral medium by adding agar (15 g liter⁻¹).

Analytical methods. Protein was determined by the method of Spector (31) and calibrated to an optical density at 578 nm. The high-pressure liquid chromatography system consisted of a model 360 autosampler, a model 325 pump, a model 440 DAD detector, and integration software from Kontron Instruments, Zurich, Switzerland. A 5-µm PRP-1 column (4 by 150 mm) (Hamilton AG, Bonaduz, Switzerland) was used for separation. The mobile phase consisted of 10 mM H₃PO₄ in water and 60% (vol/vol) methanol. The flow rate was set to 1 ml min⁻¹. UV absorption peaks were detected at wavelengths of 210 and 280 nm.

Bacterial parameters. Table 1 explains the abbreviations used in this report. The $\mu_{\max}$ of strain HH4 was calculated from growth curves determined in liquid batch cultures. To increase the dissolution rate of naphthalene (32), substrate crystals were ground thoroughly in a mortar and added to the cultures in amounts ranging from 0.5 to 10 g liter⁻¹. Initial growth rates were independent of the amount of substrate, indicating that the dissolution kinetics did not limit the growth rate. $q_{\max}$ and K_m were determined in batch experiments as described previously (15).

Growth experiments with distant naphthalene. Strain HH4 was grown in test tubes on naphthalene that was spatially separated from the inoculum (Fig. 1A to

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TABLE 1. Abbreviations used in this report

Abbrevia- tion(s)	Definition	Unit
A	Surface area over which diffusion takes place	m^2
b, b'	Microbial maintenance rate	h^{-1}
C_c	Substrate concentration at cell surface	mol m^{-3}
C_d	Distant substrate concentration	mol m^{-3}
C_g	Maximum solute concentration in gas phase	mol m^{-3}
C_w	Maximum solute concentration in water phase	mol m^{-3}
D_{eff}	Effective diffusivity	$\text{m}^2 \text{s}^{-1}$
D_g	Diffusivity in gas phase	$\text{m}^2 \text{s}^{-1}$
D_w	Diffusivity in water phase	$\text{m}^2 \text{s}^{-1}$
H	Henry's law coefficient ^a	$\text{m}^3 \text{m}^{-3}$
k	Mass transfer coefficient	$\text{m}^3 \text{s}^{-1}$
K_m	Half-saturation constant of substrate uptake	mol m^{-3}
$n(t)$	Cell number at time t	
r	Radius of test tube	m
R	Resistance to diffusion ^b	s m^{-1}
q_c	Rate of substrate uptake by one cell	mol s^{-1}
q_d	Rate of substrate flux to one cell	mol s^{-1}
q_{max}	Maximum rate of substrate uptake by one cell	mol s^{-1}
V_w	Volume of agar	m^3
x	Diffusion path length	m
x_g	Linear diffusion path length in glass matrix	m
Y	Yield coefficient	$\text{no. of cells mol}^{-1}$
ϵ	Porosity	$\text{m}^3 \text{m}^{-3}$
μ	Specific growth rate	h^{-1}
μ_{max}	Maximum specific growth rate	h^{-1}
θ	Volume fraction of aqueous medium in pore space	$\text{m}^3 \text{m}^{-3}$

^a $H = C_g/C_w$ ^b Subscripts: s , serial; p , parallel; t , total; w , water; g , gas.

C). The naphthalene had to diffuse over various distances (x) through aqueous medium (experiment 1, Fig. 1A) or air (experiment 2, Fig. 1B) or over a constant distance through porous media varying in θ (experiment 3, Fig. 1C).

Growth experiment 1 was designed to study the influence of the distance between the substrate and the cells and to estimate b (17). It was assumed that the bacteria would grow to numbers at which the substrate flux only fulfills the maintenance requirements of the culture but is insufficient to promote further growth. Final cell numbers were assumed to depend on the distance from the substrate and would allow estimation of the b of strain HH4 (see below). Test tubes (Fig. 1A) were prepared in the following way. One hundred milligrams of crystalline naphthalene was placed into sterile test tubes (length, 160 mm; inner diameter, 12 mm). Tapping of the tubes resulted in flat surfaces of crystals. Various volumes of mineral medium agar were poured on top of the crystals and given time to solidify. The distances between the crystal surfaces and the agar surfaces in individual tubes were ranged from 1.72 to 6.96 cm. The tubes were closed with gas-tight glass stoppers and incubated at 25°C. The naphthalene was given 24 h to diffuse into the agar before further use. Agar has been proven not to hinder the diffusion of low-molecular-weight chemicals (10, 30). The aqueous diffusivity (D_w) of naphthalene ($6.74 \times 10^{-10} \text{ m}^2 \text{s}^{-1}$ at 25°C) was calculated with the Wilke-Chang equation (28).

Growth experiment 2 served to test whether the growth parameters determined in liquid culture and experiment 1 could be used to predict the growth of strain HH4 on naphthalene diffusing through air (Fig. 1B). Various volumes of mineral medium agar were poured into empty test tubes, leaving headspaces of various heights. Naphthalene was added to the tubes after inoculation (see below). An approximate D_g of $10^{-5} \text{ m}^2 \text{s}^{-1}$ for naphthalene was converted from D_w with a diagram included in the *Handbook of Chemistry and Physics* (34). An H of 0.019 for naphthalene has been reported (24).

Growth experiment 3 was designed to determine the D_{eff} of naphthalene in porous media varying in θ from the growth of strain HH4 (Fig. 1C). One-hundred-milligram samples of crystalline naphthalene were placed into sterile test tubes. After tapping, 0.5 ml of mineral medium agar was poured on top of the crystals and given time to solidify. This served to fix the substrate crystals at the bottom of each test tube. The test tubes were incubated at 70°C in a heating

block. Sterile glass beads (average diameter, 450 μm) were used to fill the tubes to a height of 3.5 cm. Subsequently, various amounts of hot mineral medium agar were added to the glass beads with a syringe. The tubes were taken out of the heating block, and the injection needle was used to stir the mixture of agar and glass thoroughly while the agar became solid. Subsequently, the partially saturated glass was compressed to its original height of 3.5 cm with a sterile metal rod. The glass of some test tubes was left without agar to obtain dry porous medium. To obtain agar-saturated glass, the liquid agar was poured on top of the fixed crystals and then glass beads were carefully submerged to avoid entrapment of air. Excess agar was removed from the top layer of the glass beads with a syringe. The tubes were closed with gas-tight glass stoppers and incubated at 25°C, and the naphthalene was given at least 5 days to diffuse into the matrices. The degree of saturation of the glass matrix with agar was calculated by the following equation:

$$\theta = (V_w/x_g \epsilon \pi r^2) \quad (1)$$

The porosity of the packings (ϵ , 0.35) was determined gravimetrically after saturating 100 ml of glass beads with water. Tubes used in growth experiments had θ values of 0, 0.2, 0.4, 0.6, 0.8, and 1. Glass did not sorb naphthalene, as confirmed with a previously described method (15).

Inoculation of the test tubes was done in the following way. Naphthalene-pregrown HH4 cells were plated on mineral medium agar with a 2-mm thickness. The initial cell densities were $1.7 \times 10^5 \text{ cm}^{-2}$ (experiments 1 and 2) and $3.7 \times 10^3 \text{ cm}^{-2}$ (experiment 3). Agar disks (diameter, 1 cm) were cut out with a sterilized cork borer. Growth experiments were started by placing one inoculated agar disk on top of either the agar surface or the porous glass matrix in each test tube with the inoculated side directed to the headspace (Fig. 1A to C). The disks were carefully pressed against the surfaces to remove residual air between the two phases. The tubes for growth experiments 1 and 3 were immediately closed with glass stoppers. Tubes for growth experiment 2 were turned upside down, and glass dishes 1 cm in diameter filled with 100 mg of naphthalene were inserted together with the glass stoppers. The distances between the surfaces of the naphthalene crystals and the inocula ranged from 1.3 to 8.2 cm. Experiments 1, 2, and 3 were performed in duplicate, triplicate, and quadruplicate, respectively. All tubes were incubated at 25°C. Test tubes without a substrate were run as controls to check for growth of strain HH4 on agar. Once every 2 days, all tubes were opened for 1 min to allow oxygen to diffuse in. This procedure of aeration was chosen to minimize loss of the volatile substrate from the headspaces of the tubes. Individual tubes were harvested by removing the agar disks, submerging them in 1 ml of sterile PBS in an Eppendorf tube, and suspending the cells by intensive vortexing for 2 min. Successful separation of cells by this procedure was confirmed microscopically. Cell numbers were determined either by doing plate counts after appropriate dilution (experiments 1 and 3) or by measuring the optical density at 578 nm of the cell suspensions after having calibrated it with respect to plate counts (experiment 2).

Model for calculation of bacterial growth on distant substrates. The microbial μ is a function of the C_c available to the cells (6, 26).

$$\mu = \mu_{\text{max}} \frac{C_c}{K_m + C_c} - b \quad (2)$$

Growth continues only when substrate use by the cells is balanced by substrate diffusion to the cells (6, 21).

FIG. 1. (A to C) Test tubes used in growth experiments. (D) Simplifying assumption about the distribution of agar and water in the unsaturated porous media used in growth experiment 3. (E) Scheme of the resistor model used to calculate effective diffusivities in unsaturated porous media.

$$q_d = k(C_d - C_c) \quad (3)$$

Therefore, C_c and, consequently, μ are governed by both bacterial substrate consumption, since $\mu = q_c Y$, and substrate flux to the cells. Assuming a steady state of substrate diffusion and uptake ($q_d = q_c$) at the cell surface, equations 2 and 3 can be combined as follows (6):

$$\mu = \mu_{\max} \frac{C_d + K_m + q_{\max} k^{-1}}{2q_{\max} k^{-1}} \quad (4)$$

$$\left\{ 1 - \left[1 - \frac{4C_d q_{\max} k^{-1}}{(C_d + K_m + q_{\max} k^{-1})^2} \right]^{1/2} \right\} - b$$

For the derivation of equation 4, see the reports of Best (4), Bosma et al. (6), and Koch (20). Equation 4 allows the calculation of μ as a function of C_d . It was assumed that the amount of naphthalene crystals added to the tubes in growth experiments 1 and 3 was sufficient to maintain saturated aqueous substrate concentrations at the interfaces between crystals and agar throughout the experiments. C_d was therefore set to the C_w of naphthalene. In growth experiment 2, the amounts of naphthalene crystals added to the tubes were assumed to be sufficient to maintain naphthalene-saturated air at the crystal surfaces. C_d was therefore set to $C_g = C_w H$. It was furthermore assumed (i) that the substrate had to partition from the air into the water film surrounding the cells before it could be consumed, (ii) that this water film was infinitely thin, and (iii) that the distribution of naphthalene at the air-water interface was in equilibrium instantaneously. The k for the setup used in all three experiments, i.e., one-dimensional diffusion, was calculated as follows (6):

$$k = D_{eff} A / x \quad (5)$$

with $A = \pi r^2$ (experiments 1 and 2) or $A = \epsilon \pi r^2$ (experiment 3). D_{eff} equaled D_w and $D_g H$ in experiments 1 and 2, respectively.

Model for calculation of D_{eff} in unsaturated porous media. It was assumed that when $1 > \theta > 0$, every transported molecule (i) passes air-liquid interfaces and (ii) bypasses spots of water while diffusing in air bubbles and vice versa. The entirety of spots of water and air bubbles present in the porous medium was therefore regarded as a three-dimensional array of resistors connected in series and in parallel. Water and air represent materials with different conductivities (33). It was furthermore assumed that only diffusion within the phases, and not partitioning at the interfaces, contributes to the overall transport rate. Hence, all aqueous and gaseous sections, respectively, were summed up and a minimum of one partition step was assumed. This partition step was included since continuous channels of the more permeable phase would create an efficient bypass, leading to a drastic increase of the overall transport rate. The x of the molecules is, on average, $x\theta$ in water and $x(1 - \theta)$ in the gas phase. The preparation of the test tubes probably led to an isotropic distribution of water and gas; i.e., serial and parallel arrangements of resistors were of equal probability. To account for the simultaneous presence of both types of resistors, half of the system length was regarded as being composed of a serial resistor block. Figure 1D and E shows how this block was connected in series with a block of parallel resistors in the remaining half. The tortuosity of the pore space lengthening the diffusion distance was neglected in the calculations. The serial resistances in one half of the system length therefore become

$$R_{w,s} = 0.5x\theta/D_w \quad (6)$$

and

$$R_{g,s} = 0.5x(1 - \theta)/D_g H \quad (7)$$

in the aqueous and gas phases, respectively. The total resistance along the alternating phases is

$$R_{t,s} = R_{w,s} + R_{g,s} \quad (8)$$

The mean cross-sectional area fractions available for the passage are θ and $1 - \theta$ of water and gas, respectively. Hence, the parallel resistances in one half of the system become

$$R_{w,p} = 0.5x/\theta D_w \quad (9)$$

and

$$R_{g,p} = 0.5x/(1 - \theta) D_g H \quad (10)$$

in the aqueous and gas phases, respectively. The total parallel resistance is

$$1/R_{t,p} = 1/R_{w,p} + 1/R_{g,p} \quad (11)$$

The total resistance is then

$$R_t = (R_{t,s} + R_{t,p}) \quad (12)$$

and the effective diffusivity in the system is

$$D_{eff} = x/R_t \quad (13)$$

Calculation and fitting of growth curves. Cell numbers $n(t)$ were calculated with standard spreadsheet software. Equation 4 was used to calculate the Δn for 1-h intervals. The cell number at the end of each interval was used as the starting cell number for the next interval. Calculated growth curves were fitted to experimental cell counts. In growth experiment 1, the input value of b as the only fitting parameter was varied iteratively to minimize the sum of squares of differences between calculated cell numbers and experimental cell counts. Calculation of growth curves in experiment 2 involved no fitting procedure. To calculate growth in experiment 3, b values from experiment 1 were used. The input value of D_{eff} as the only fitting parameter was varied iteratively to give the best fit to experimental growth curves.

RESULTS

Kinetic parameters of strain HH4. With naphthalene as the substrate, the $q_{\max}$ of strain HH4 at 25°C was $1.40 \times 10^{-18} \pm 2.04 \times 10^{-19}$ mol s⁻¹, the K_m was 0.5 mol m⁻³, and the $\mu_{\max}$ was 0.32 ± 0.01 h⁻¹. No excretion of metabolites was observed during growth on naphthalene.

Growth experiment 1. Figure 2 shows the growth of strain HH4 on naphthalene that had to diffuse over various distances through agar. Cell numbers in all of the test tubes decreased during the first few days after inoculation. The die-off that was positively correlated with x was due to the fact that the time between preparation of the test tubes and inoculation was insufficient for naphthalene to reach the inoculation sites. The mean distance of naphthalene diffusion at 25°C in water after 24 h is 7.6 mm. Growth was observed in all tubes between days 5 and 9 of incubation. About 14 days after inoculation, the growth stopped and cell counts remained more or less constant for another 22 days. Table 2 summarizes the plateau cell numbers, i.e., the arithmetic means of cell counts after 14, 19, and 36 days. High standard deviations of the plateau cell numbers at x values of 5.68 and 3.18 cm are due to single outlying cell counts. No growth was observed in test tubes without naphthalene. Growth curves were modeled with cell counts determined after 5 days as the starting cell numbers. The b value of 0.0102 h⁻¹ gave the best average fit to all six growth curves (the solid lines in Fig. 2). The modeled growth slightly overestimated the observed growth at large x values and underestimated the observed growth at small x values. Therefore, modeling was also done with b values giving the best fits to individual growth curves, b' (dashed lines). Calculated cell numbers after 36 days and b' values are listed in Table 2. Most b' values differ only slightly from b . The large deviation at $x = 1.92$ cm is caused mainly by the high cell count after 19 days.

Growth experiment 2. Strain HH4 grew efficiently on naphthalene that had to diffuse over various distances through air. Figure 3 shows a comparison between cell numbers counted after 5 and 9 days and cell numbers predicted with equation 4 by using the mean b value (0.0102 h⁻¹) from experiment 1. Cell counts were inversely correlated with x and only slightly below the predictions. It should be noted that most cell counts differed from the predicted culture sizes by less than one doubling. The absolute differences from predicted cell counts increased with higher cell densities. Five times 10⁸ cells of strain HH4 on an agar disk with an area of 0.78 cm² formed about 30 layers. For most of the cells, this may have resulted in an unfavorable balance between the supply of naphthalene and oxygen from the headspace and the nutrient supply from the agar, a fact that may have slightly reduced their growth rate. A general oxygen deficiency is unlikely for all cultures except those at $x = 1.3$ cm, with the highest cell numbers and the smallest headspaces.

Growth experiment 3. Growth of strain HH4 on distant naphthalene that had to diffuse through matrices of glass, air, and various volume fractions of agar started immediately. This

FIG. 2. Growth of strain HH4 on distant naphthalene crystals separated from the inocula by agar. The distances between the substrate crystals and the inocula were 7.16 (A), 5.68 (B), 3.78 (C), 3.18 (D), 2.6 (E), and 1.92 (F) cm. Filled circles represent experimentally observed cell counts. Lines represent modeled growth curves with one b value giving the best fit to all six growth curves (solid lines) or b values giving the best fits to individual growth curves (dashed lines). Standard deviations are indicated by error bars which, in some cases, are obscured by the symbols. The dotted lines indicate the starting points of the modeling.

indicates that 5 days of preincubation of the test tubes was sufficient for diffusion of naphthalene through the matrices. As an example, Fig. 4 shows the growth with $\theta = 0.2$ and 0.8 . Final cell counts were negatively correlated to the water content of the matrix connecting the substrate and the inoculum. Observed growth curves were used to calculate D_{eff} for each value of θ . Each growth curve was modeled with a b value of 0.0028 or 0.0140 h^{-1} , i.e., the highest or lowest b' value from experiment 1. D_{eff} served as the only fitting parameter for each curve. D_{eff} values giving the best fits to the observed growth curves were plotted against the water saturation and compared with D_{eff} values predicted with the resistor model (Fig. 5). Experimental values were slightly lower than the prediction at $\theta = 0$ but were higher at $\theta > 0$. Even at complete water saturation, the observations were higher than predicted by factors of 4 to 10.

TABLE 2. Observed and calculated plateau numbers of strain HH4 cells growing on distant naphthalene and calculated maintenance rate coefficients

Distance (cm)	Observed mean plateau cell no. $\pm$ SD	Calculated cell no. after 36 days for $b = 0.0102 \text{ h}^{-1a}$	b' (h^{-1})	Calculated cell no. after 36 days for $b = b'^a$
7.16	$3.7 \times 10^6 \pm 4.8 \times 10^5$	5.5×10^6	0.0140	3.9×10^6
5.68	$5.2 \times 10^6 \pm 1.9 \times 10^6$	6.9×10^6	0.0135	5.1×10^6
3.78	$7.8 \times 10^6 \pm 5.4 \times 10^6$	1.0×10^7	0.0135	7.7×10^6
3.18	$1.1 \times 10^7 \pm 9.6 \times 10^6$	1.2×10^7	0.0102	1.2×10^7
2.6	$1.9 \times 10^7 \pm 3.8 \times 10^6$	1.5×10^7	0.0070	2.2×10^7
1.92	$5.8 \times 10^7 \pm 5.0 \times 10^7$	2.0×10^7	0.0028	7.0×10^7

^a Equation 4 was used to calculate Δn for 1-h intervals. The cell number at the end of each interval was used as the starting cell number for the next of a total of 864 intervals.

FIG. 3. Comparison of experimental cell counts obtained with naphthalene diffusing over various distances through air after 5 (circles) and 9 (squares) days and predicted cell numbers calculated from bacterial growth parameters and gaseous naphthalene diffusion. The dotted line indicates conformity of counts and predictions. Symbols without error bars indicate that variations were smaller than the symbols used.

DISCUSSION

Growth on distant naphthalene. Immobilized inocula of strain HH4 grew on naphthalene, from which they were separated by aqueous medium, to distance-dependent final cell numbers (Fig. 2). The growth curves could be explained with a mathematical model which used the $\mu_{\max}$, $q_{\max}$, and K_m of the cells and the substrate diffusion to the cells as input parameters. b was used as the fitting parameter. The quasi-immobilized cultures grew to steady-state cell numbers. The available substrate flux could only fulfill their maintenance requirements and compensate for decaying cells but was insufficient to promote further growth. Values of b' giving best fits of 0.0028 to 0.0140 h^{-1} (between 0.9 and 4.4% of $\mu_{\max}$) were in the range of reported values of several organisms growing on glucose, glycerol, and toluene which ranged from 0.002 to 0.25 h^{-1} (9). Except for $x = 1.92$ cm, the deviation of b' from b was small. Accordingly, the mean b value was appropriate for rough prediction of the growth of strain HH4 on naphthalene diffusing through air (Fig. 3). In this second growth experiment, the combination of faster diffusion in air and the high H of naphthalene caused much higher final cell numbers than in experiment 1. In a third growth experiment, it was shown that the presence of air also favored the diffusive transport of naphthalene in porous media (Fig. 4). Here, D_{eff} was used to fit calculated growth curves to those experimentally obtained with various degrees of water saturation in porous media. These experimental estimates of D_{eff} exceeded those predicted with a resistor model at all water saturations, with the exception of $\theta = 0$, but followed the predicted trend at $0.2 < \theta < 1$ (Fig. 5). It is unlikely that this systematic deviation was caused by a lower b of the cells than in experiment 1, since the experimental D_{eff} at $\theta = 0$ was close to the value reported in the literature for diffusion in air and the same inoculum was used for all values of θ . Since the systematic deviation included $\theta = 1$, where D_{eff} calculated with the resistor model is equal to D_w , the

FIG. 4. Growth of strain HH4 on distant naphthalene crystals separated from the inocula by a matrix of glass, air, and volume fractions of 1/5 (circles) and 4/5 (squares) agar. Lines represent modeled growth curves with an input b value of 0.0028 h^{-1} (solid and short-dashed lines) or 0.0140 h^{-1} (long-dashed and dotted lines). Symbols without error bars indicate that variations were smaller than the symbols used.

deviation may reflect increased substrate transport in the presence of glass beads. This would be possible if (i) air films had condensed on the glass surfaces during the preparation of the test tubes (ii) or the agar matrix, during cooling, had exuded water which condensed at the glass surface and allowed convective transport. The drastic upshift of D_{eff} at $0.4 > \theta > 0.2$ may be due to the presence of a continuous gas phase in the porous medium with low agar content. The resistor model assumed that air and water phases alternate along the diffusion path. However, a continuous gas phase creates a parallel connection with high conductivity, which is not accounted for in the model. In a recent study, Batterman et al. (2) measured the effective diffusivity of trichloroethylene in sand (grain diameters, 300 to 600 μm) with varying water content. D_{eff} of trichloroethylene decreased by 1 order of magnitude when θ changed from 0 to 0.8. D_{eff} values from growth experiment 3 decreased by roughly the same factor, whereas the resistor model pre-

FIG. 5. D_{eff} of naphthalene in matrices of glass, air, and agar as a function of the volume fraction of agar in the pore space. Bars represent experimental values obtained with assumption of b values ranging from 0.0028 h^{-1} (lower boundaries) to 0.0140 h^{-1} (upper boundaries). The solid line represents predicted diffusivities. The dashed and the dotted lines indicate the effective diffusivities of naphthalene in gas and in water, respectively.

FIG. 6. Calculated curves of bacterial growth on distant naphthalene. In each plot, either D_{eff} (A), x (B), b (C), μ_{max} (D), or K_m (E) was varied while all other parameters were kept unchanged.

dicted a drop of 2 orders of magnitude. This indicates that mechanisms other than those accounted for in the model, like, e.g., convective transport, may play a role in substrate transport in porous media.

Dependency of the final biomass on system characteristics and cell properties. In the experimental system described here,

the rate of substrate diffusion determines the cell number that can be maintained. Final cell numbers, in turn, determine the long-term degradative capacity of the system. It is therefore worthwhile to identify the relative influence of system parameters and cell characteristics on final cell numbers. Proceeding from the calculated growth curve at $x = 3.78 \text{ cm}$ (the dashed curve in Fig. 2C), the parameters D_{eff} , b , x , μ_{max} , and K_m , were varied individually while the other four were kept constant. The results are summarized in Fig. 6. Final cell numbers were strongly determined by the value of D_{eff} (Fig. 6A). D_{eff} was varied over a wide range, since in soil the presence of solid particles and air substantially affects chemical diffusion. For clarity, Fig. 6A does not comprise the whole range of D_{eff} values observed in soils. Diffusion of halogenated pollutants in soil particles was found to be between 2 and 12 orders of magnitude slower than diffusion in water (5, 29). In contrast, the presence of air in the vadose zone of soils may increase the D_{eff} of volatile chemicals. Favorable transport through the gas phase can be expected for compounds with $H \gg 10^{-4}$. The relative enrichment of such compounds in water compared with the gas phase is overcompensated by their faster diffusion in air. Final cell numbers were also governed by x (Fig. 6B). It

should be noted that in Fig. 6B, x values in the centimeter range were used. However, the mean distances between microcolonies in soil and, as a consequence, the mean distances of microcolonies from point sources of pollutants were estimated to be in the range of only hundreds of micrometers (6). Figure 6B shows that distance effects become more important as x becomes smaller. In nature, distant effects on final biomass may therefore be even more important than in the experimental system used here. The influence of the location of active bacteria and their substrates for the biodegradation rate has been addressed earlier (15, 16). It is well accepted that mixing of soils, i.e., reducing the mean distances, promotes the degradation of sparsely mobile pollutants (6, 29). Although b also determined the final cell number, it does not have an effect on the final degradation rate. With respect to degradation, higher steady-state cell numbers are compensated by lower maintenance requirements per cell. Both $\mu_{\max}$ (Fig. 6D) and K_m (Fig. 6E) influenced mainly the growth kinetics rather than final cell numbers.

Dependency of best-fit b values on x . Varying b values, as obtained in experiment 1, reflect different relative substrate fluxes not resulting in growth. Real variations between the maintenance needs of cells in individual incubations were unlikely, since (i) all inocula were from the same preculture, (ii) the environment of the cells was the same at all x values, and (iii) differences in μ , which have been reported to result in different maintenance needs (27), were small between individual incubations.

Growth experiment 1 was modeled on the assumption that the cells were already growing 5 days after inoculation. However, because of the lack of further datum points around day 5, the possibility cannot be excluded that the modeling was started with a cell number which was still declining and started to increase at some time between days 5 and 9. A later shift from decay to growth is indeed more likely for longer diffusion paths and may therefore contribute to apparently upshifted b' values. However, calculations show that underestimation of the real growth rate would have been balanced in the fitting procedure by an only slightly higher b' value. This is due to the fact that b as the fitting parameter is sensitive to final cell numbers but insensitive to the length of the growth phase.

Additional substrate sinks could account for apparently increased b values. Conceivable sinks for naphthalene in the experimental system were (i) metabolites that were excreted by the organisms; (ii) the agar matrix, that also could have accumulated naphthalene; (iii) the air phase, that was released once every 2 days; and (iv) the biomass formed that could have absorbed naphthalene (3). When growing in liquid culture at $\mu \leq \mu_{\max}$, strain HH4 did not excrete metabolites, but the possibility that cells growing on agar at $\mu \ll \mu_{\max}$ behaved differently cannot be excluded. To account for an apparent upshift of b' by $> 0.01 \text{ h}^{-1}$ at $x = 7.16 \text{ cm}$ compared with $x = 1.92 \text{ cm}$, the final naphthalene concentration in the agar must have been as high as 31 mM. The accumulation of naphthalene to such a high concentration in agar, exceeding the aqueous solubility of naphthalene by a factor of 128, is unlikely. A modest accumulation, however, may have contributed to apparent upshifts of b' . Loss of naphthalene to the air can be excluded as the reason for different b values, since more naphthalene was probably lost at smaller x values because more voluminous headspaces were released. In addition, biosorption of naphthalene also cannot explain higher naphthalene losses at higher x values, at which less biomass was formed.

Conclusion. Measures to promote biodegradation in soils should aim either at enhancing the D_{eff} of the pollutant or at reducing the average distance between the cells and the pol-

lutant. A way to increase D_{eff} could be introduction of air into the soil. However, D_{eff} in soil is governed mainly by intraparticle diffusion (35). Therefore, reduction of transport distances by homogenization of the soil and mechanical breaking of particles appears to be a more promising approach.

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