Mass Transfer Effects on Microbial Uptake of Naphthalene from Complex NAPLs

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Abstract: The bioavailability of naphthalene present as a component of a complex nonaqueous phase liquid (NAPL) comprised by nine aromatic compounds was investigated. Specifically, the effects of naphthalene mass transfer from the NAPL to the aqueous phase on rates of its microbial degradation were examined. The investigations were conducted using a pure culture, ATCC 17484, and a mixed culture of naphthalene-degrading bacteria, the former having been implicated previously in the direct uptake of sorbed naphthalene. The studies were conducted in mass-transfer-limited, segregated-phase reactors (SPRs) in which both the NAPL and aqueous phases were internally well-mixed. A 30-day active biodegradation period was preceded and followed by a 5–7-day period devoid of bioactivity, during which time the rates and extents of mass transfer of components from the NAPL to the aqueous phase were quantified. The NAPL-phase naphthalene mass depletion profiles during biodegradation were compared to those predicted by assuming maximum mass depletion under mass-transfer-limited conditions using both pre- and post-biodegradation dissolution rate and equilibrium parameters. The observed mass depletion rates were high during the initial stages of biodegradation but decreased significantly in later stages. Throughout biodegradation, even in the initial rapid stage, mass depletion rates never exceeded maximum predicted rates based on pre-biodegradation mass transfer parameters. Reduced depletion rates in the later stages appear to relate to mass transfer hindrance caused by formation of biofilms at the NAPL–water interface. © 1998 John Wiley & Sons, Inc. *Biotechnol Bioeng* **60**: 750–760, 1998.

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INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs), a class of organic compound characterized by fused multiple benzene rings, are recognized by the USEPA as priority pollutants (Keith and Telliard, 1979). PAH contamination of groundwater and surface water results largely from slow dissolution of complex PAH-containing non aqueous phase liquids (NAPLs) such as coal tars, creosotes, and petroleum oils. Such environmental NAPLs typically vary widely in composition and are often incompletely characterized. Bioremediation is a favored technology for clean-up of PAH contamination. Indigenous microorganisms in contaminated aquifers are known to evolve remarkable pollutant degrading capabilities (Heitkamp and Cerniglia, 1988; Madsen et al., 1992; Cerniglia, 1992). Microorganisms that can use specific PAHs as sole sources of carbon and energy have been isolated from contaminated soil and water samples (Heitkamp and Cerniglia, 1988; Mueller et al., 1989), and the ability of these microorganisms to cometabolize several different PAHs is well documented (Mueller et al., 1989).

The rate and extent of PAH biodegradation in subsurface NAPL-contamination scenarios may be significantly influenced by bioavailability; i.e., by issues relating to the feasibility of specific mechanisms for microbial uptake. It is generally believed that uptake of PAHs occurs only from the aqueous phase, as for most other compounds; i.e., that PAHs in their pure solid state, in multicomponent NAPL states, and in sorbed states are used by microorganisms only after these compounds are transferred to the aqueous phase by processes such as dissolution and desorption (Wodzinski and Bertolini, 1972; Wodzinski and Coyle, 1974; Ogram et al., 1985; Thomas et al., 1986; Stucki and Alexander, 1987; Weissenfels et al., 1992; Volkering et al., 1992, 1993; Harms and Zehnder, 1995; Bouchez et al., 1995; Ghoshal et al., 1996). In such cases, slow mass transfer rates may limit compound availability, and thus limit biodegradation rates. Several researchers conducting studies with separate phase PAHs and low spontaneous dissolution rates have demonstrated a transition in microbial growth rates from exponential to linear, the transition time being related to microbial population size, and the rate of linear growth being dependent on the surface area of the separate phase. In contrast, if the microbial population size throughout a study is sufficiently low that the spontaneous dissolution rate is much greater than the biodegradation rate, exponential growth

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rates independent of solid surface area are noted throughout. In studies with sorbed PAHs, biodegradation rates have been similarly correlated with desorption rates. Robinson et al. (1990) demonstrated that for toluene the characteristic two-stage sorption/desorption on soils (a slow stage following an initial rapid stage; Weber and Huang, 1996), was mirrored by a similar two-stage microbial uptake of toluene from these soils by acclimated bacteria. Volkering et al. (1992) demonstrated that PAH desorption from soils is a slow mass-transfer-limited process that controls the rate of microbial growth. The desorption of PAHs and, hence, their bioavailability have been found to decrease with aging (Sherman, 1989; Landrum et al., 1992; Carmichael et al., 1997; Huang and Weber, 1997), thereby leading to persistence of PAHs in aged contaminated soils.

Mechanisms other than passive uptake from the aqueous phase after dissolution/desorption have been suggested for PAHs and other intermediate and low solubility compounds (Mihelcic et al., 1993). Microorganisms that can secrete biosurfactants and other extracellular surface active agents can enhance mass transfer rates either by micellar solubilization or by emulsification of a NAPL phase (Thomas et al., 1986; Stucki et al., 1987; Rosenberg et al., 1988; Koch et al., 1991). A direct uptake mechanism has also been suggested for PAHs (Wodzinski and Larocca, 1977; Efroymson and Alexander, 1991; Guerin and Boyd, 1992; Ortega-Calvo and Alexander, 1994; Crocker et al., 1995). It is known that certain microorganisms may attach to interfaces using special surface structures such as thin fimbriae and capsules, and directly uptake substrate from a separate/sorbed phase. If direct uptake occurs at a significant rate, biodegradation rates will not be limited by slow mass transfer because the two processes can proceed in parallel. A microorganism may simultaneously consume a substrate from the dissolved state and directly from the NAPL–water interface at different rates. The predominant uptake mechanism may depend on the compound, on the microorganism mediating biodegradation, and on certain environmental factors.

The direct uptake mechanism has been conclusively demonstrated for degradation of low solubility compounds, such as alkanes, from NAPL phases (Nakahara et al., 1977; Rosenberg et al., 1989). For compounds of very low solubility, passive uptake after dissolution is generally negligible because the solution phase concentrations are so low. There is, however, considerable ambiguity concerning direct uptake of compounds of intermediate solubility, for which passive uptake after dissolution/desorption is not insignificant. For example, Wodzinski and Larocca (1977) suggested direct utilization of naphthalene dissolved in a non-biodegradable solvent (heptamethylnonane, HMN), based on observations of bacterial colonization of the solvent–water interface, and an increase in growth rate with an increase in interfacial area and concentration of naphthalene in the NAPL phase, but these observations might apply as well for dissolution-limited uptake from the aqueous phase. Microorganisms may attach passively at interfaces by virtue of hydrophobic interactions, and dissolution-limited uptake is a function of the overall dissolution rate, which in turn depends on the interfacial area and the concentration/mole fraction in the NAPL phase. For compounds dissolved in HMN, Efroysom and Alexander (1991) provided strong evidence for direct utilization of an alkane (hexadecane) but no conclusive evidence for direct utilization of the PAH (naphthalene) tested. The *Arthrobacter* sp. used was observed to passively attach to the NAPL–water interface, but when attachment was hindered by external addition of a surfactant the utilization of hexadecane was prevented, whereas that of naphthalene was enhanced. Some evidence for direct utilization by this same *Arthrobacter* sp. of naphthalene dissolved in a different non-biodegradable solvent (di-2-ethylhexyl phthalate) was, however, provided by Ortega-Calvo and Alexander (1994). A phase of slow dissolution-limited uptake was subsequently followed by a phase of rapid uptake that could be correlated with the formation of an interfacial biofilm. External addition of a surfactant that hindered biofilm formation prevented onset of the rapid uptake phase.

The objective of this work was to investigate the influence of mass transfer on the bioavailability and biodegradation of a representative PAH, naphthalene, present as a component of a complex NAPL. Experiments were specifically designed to determine whether or not direct microbial uptake of naphthalene from the NAPL phase is a significant process relative to uptake from the aqueous phase. Dissolution rates and extents were quantified abiotically before and after biodegradation to determine if they were affected by biological activity. The experiments were conducted in liquid–liquid systems free of solids. A compositionally defined synthetic complex NAPL was used to facilitate a more rigorous quantitative study than would be possible with environmental NAPLs, which would involve compositional uncertainties and variabilities that can affect bulk property estimates. Two naphthalene-degrading cultures, one a pure culture and the other a mixed culture, were used for these studies. The pure culture, ATCC 17484, has been reported previously to uptake sorbed naphthalene directly from soils and modified clays (Guerin and Boyd, 1992; Crocker et al., 1995). The initial mineralization rates in slurries with this culture were significantly higher than those with another culture and concentration-corrected soil-free controls. In contrast, the mixed culture has been reported to uptake naphthalene only from the aqueous phase (Ghoshal et al., 1996). Rates of naphthalene uptake by this culture from heptamethylnonane and coal tar in stirred batch systems were fitted with a dissolution–degradation model having no direct uptake component, and overall biodegradation rates were shown to be significantly lower in systems having low interfacial areas (Ghoshal et al., 1996).

INFINITE-SINK MODEL

A modeling framework referenced here as an infinite sink model (ISM) was developed to facilitate bioavailability in-

terpretations based on NAPL-phase composition analysis and rigorous determination of mass transfer characteristics. Mass transfer of hydrophobic components across a NAPL–water interface can be conceptualized in terms of a linear-driving-force single-impedance model (Weber and DiGiano, 1996). In this model mass transfer through the boundary layer on the aqueous side of the interface constitutes the rate-limiting step, and equilibrium conditions exist at the interface. The flux across the NAPL–water interface is thus proportional to a linear driving force, given by the difference between the aqueous phase concentration at equilibrium and the actual aqueous phase concentration; the constant of proportionality is referred as the film transfer coefficient. On the basis of multicomponent liquid–liquid equilibrium theory, the aqueous concentration of any NAPL component at equilibrium can be expressed as a function of the NAPL composition, yielding Eq. (1) (Mukherji et al., 1997):

$$C^{a}_{e,i} = \frac{\alpha^{n}_{i} X^{n}_{i} C_{S,i}}{(f^{s}_{i}/f^{l}_{i})}, \tag{1}$$

where the subscript i denotes a quantity pertaining to a component i, and the superscripts a and n denote quantities pertaining to the aqueous phase and the NAPL phase, respectively. The term $C^{a}_{e,i}$ in Eq. (1) is the aqueous-phase concentration in equilibrium with the NAPL phase (mg/L), $C_{S,i}$ is the aqueous solubility (mg/L), α^{n}_{i} is the NAPL-phase activity coefficient, X^{n}_{i} is the mole fraction in the NAPL phase, and f^{s}_{i} and f^{l}_{i} are the fugacities in the pure solid and pure liquid state, respectively.

The infinite-sink model assumes that mass loss from the NAPL phase is due only to mass transfer into the aqueous phase; that the aqueous phase represents an infinite sink for all biodegradable components such that their aqueous-phase concentrations are maintained at zero and the driving force for mass transfer is maximum (given by $(C^{a}_{e,i} - 0) = C^{a}_{e,i}$); that there is negligible mass loss of non-biodegradable components from the aqueous phase. For a biodegradable component, the zero aqueous phase concentration assumed by the infinite-sink model can be achieved by the combined action of bacteria in aqueous suspension and bacteria adhering to the NAPL–water interface. Once the substrate available in the aqueous phase is consumed, and the substrate dissolving from the NAPL phase is also consumed immediately upon dissolution, the aqueous concentration is maintained at zero. For a hydrophobic component with low aqueous solubility, present in a system with low NAPL–water interfacial area, the infinite-sink condition can be achieved quite rapidly. For a biodegradable component, mass loss predicted by this model is given in Eq. (2):

$$\frac{dm^{n}_{i}}{dt} = \frac{d}{dt}(V^{n}C^{n}_{i}) = -\frac{A^{\circ}k_{f,i}C^{a}_{e,i}}{1000}, \tag{2}$$

where m^{n}_{i} is the mass of component i in the NAPL phase (mg), V^{n} is the NAPL-phase volume (mL), C^{n}_{i} is the concentration of component i in the NAPL phase (g/L), $k_{f,i}$ is the film transfer coefficient (cm/day), A° is the interfacial area (cm^2), $A^{\circ}k_{f,i}$ is the area-lumped mass transfer coefficient (cm^3/day), and t is time (days).

The mass profile for a biodegradable component in a closed system having a constant interfacial area can be generated by solving Eq. (2) using a finite difference numerical scheme, assuming constant values of the mass transfer parameters (i.e., the area-lumped mass transfer coefficient and the NAPL-phase activity coefficient) but allowing for variations in NAPL composition and NAPL volume. The equilibrium aqueous-phase concentration, $C^{a}_{e,i}$, can be expressed in terms of NAPL-phase activity coefficient and mole fraction in the NAPL phase as given in Eq. (1), and the latter can be expressed in terms of NAPL-phase concentration as shown in Eq. (3):

$$X^{n}_{i} = \frac{(C^{n}_{i}/MW_{i})}{\sum_{i}(C^{n}_{i}/MW_{i})}, \tag{3}$$

where MW_i is the molecular weight of component i (g/mol).

The NAPL volume variation can be quantified on the basis of mass depletion of all the components as shown in Eq. (4):

$$dV^{n} = \Sigma dV^{n}_{i} = \Sigma \frac{dm^{n}_{i}}{1000\rho_{1,i}}, \tag{4}$$

where V^{n}_{i} is the volume of component i in the NAPL phase (mL), and $\rho_{1,i}$ is its liquid density (g/mL).

Thus, although the mass profile of a component based on this model is most directly affected by the product of its mass transfer parameters, it is also indirectly affected by the mass transfer parameters of other components by accounting for variations in NAPL composition and NAPL volume.

NAPL-phase mass profiles of naphthalene generated by the ISM using experimentally determined mass transfer parameters can be compared to NAPL-phase mass profiles of naphthalene obtained experimentally during biodegradation. Comparison of the slopes and positions of predicted and experimental profiles with respect to each other can illustrate the impacts of mass transfer and elucidate bioavailability. This approach to interpretation of bioavailability does not involve explicit determination of biodegradation rates. A predicted profile based on the ISM defines the maximum rate of depletion of a component from the NAPL-phase in the absence of any form of direct uptake; i.e., the maximum rate attainable if mass transfer controls overall rates of solute mass depletion. Hence, experimental profiles having slopes exceeding those of corresponding predicted profiles, or profiles lying below corresponding predicted profiles, are indicative of direct uptake from the NAPL phase at the NAPL–water interface. Conversely, experimental profiles having the same slopes as corresponding predicted profiles, or lying above corresponding predicted profiles are indicative of uptake rates limited by rates of mass transfer and dissolution into the aqueous phase. Such interpretations inherently assume that (i) direct uptake at the interface does not exclude uptake subsequent to dissolution;

(ii) the rate of direct uptake is high enough to be reflected in the profiles; and (iii) other than mass transfer into the aqueous phase, direct uptake is the only mechanism by which mass loss from the NAPL phase can occur. If direct uptake excludes uptake subsequent to dissolution or if the rate of direct uptake is insignificantly low, a false negative conclusion regarding the direct uptake mechanism may result. Conversely, if alternative mass loss mechanisms exist, a false positive conclusion may result.

MATERIALS AND METHODS

Complex NAPL

A complex NAPL was designed to serve as a well-characterized and quantified model for coal tars and creosotes. It contained no single predominant component and was composed as follows (note that the compound abbreviations used subsequently and the component mole fractions are listed in parentheses): toluene (TOL, 0.03); naphthalene (NAP, 0.25); 1-methylnaphthalene (MNAP, 0.22); 2-ethylnaphthalene (ENAP); acenaphthene (ACE, 0.11); fluorene (FLE, 0.05); phenanthrene (PHE, 0.10); fluoranthene (FLA, 0.09); and, pyrene (PYR, 0.04). The PAHs were obtained in purity $\geqslant$98% from Aldrich Chemical Co., and toluene was obtained from Fisher Scientific. The composition was designed such that the mixture existed as a stable organic liquid at ambient temperatures although several of the components exist as solids at the same temperature when in their pure form. The synthesis procedure and design considerations for ensuring liquid-phase stability of this and similar NAPLs containing large amounts of solid-phase pure components have been discussed by Peters et al. (1997). The complex NAPL had an average molecular weight of 152.2 g/mol, a density of 1.055 g/mL (20°C) and a viscosity of 5.05×10^{-2} g/cm s (20°C). The composition of this NAPL was determined and monitored by chromatographic techniques.

Culture and Culture Conditions

A naphthalene-degrading pure culture, ATCC 17484, and a mixed culture of bacteria were used for these studies. ATCC 17484 was obtained directly from American Type Culture Collection (Rockville, MD), and the mixed culture was provided by Dr. R. G. Luthy (Carnegie Mellon University, Pittsburgh, PA). Both cultures could be grown in suspension using naphthalene as the sole source of carbon and energy. The composition of the mineral media used was as reported by Ghoshal et al. (1996); all of its components were obtained from J. T. Baker Co. or from Mallinckrodt Chemical Co. Preliminary studies indicated that the pure culture could use only naphthalene among the NAPL components involved as a growth substrate, whereas the mixed culture could use both naphthalene and toluene. For both cultures, naphthalene-grown resting cells could degrade all components other than fluoranthene and pyrene. Preliminary studies were also conducted to choose an inactivating agent capable of rapidly killing and inactivating both cultures at concentrations of 10^7–10^8 CFU/mL. Electron microscopy grade glutaraldehyde, obtained as a 25% aqueous solution from Sigma Chemicals, was tested over the concentration range from 0.05 to 0.5%. In these studies cells were allowed to grow in batch cultures with excess naphthalene, and glutaraldehyde at the desired concentration was added at the end of the logarithmic growth phase. After glutaraldehyde addition, samples were withdrawn at various time intervals, starting from 10 min up to a period of 7 days. Viability was tested by plating 100 μL of sample on mineral media plates containing excess naphthalene, incubating these plates at 30°C for 4 days, and counting the number of colonies formed. With 0.2% glutaraldehyde no colonies were observed in any of the samples plated. Thus, 0.2% glutaraldehyde was chosen for rapid inactivation of the cultures in the post-biodegradation mass transfer phase of the bioavailability studies.

Segregated Phase Reactors (SPRs)

Each reactor consisted of a lower NAPL reservoir of 7-mL volume connected to an upper reservoir consisting of a 1000-mL three-necked flask containing about 720 mL of the aqueous phase, as depicted in Fig. 1. Each reservoir was provided with a sampling port. The upper reservoir had additional stopcock-fitted ports for venting and drainage and connectors fitted on the side necks for headspace aeration and for introducing the aqueous phase without disturbing the interface. A glass impeller introduced through the central neck and operated at 39 rpm was used for mixing the aqueous phase. A magnetic stirrer with a Teflon-coated stirring bar rotated at 200–250 rpm was used for stirring the NAPL phase. These conditions were determined to provide adequate mixing within each of the two bulk phases while causing minimal disturbance at the interface. Ground glass joints were employed to ensure that the reactor was completely sealed. The cross-sectional area of the reactor at the interface location was 4.9 cm^2.

Experimental Procedure

Each experiment consisted of three stages, a pre-biodegradation mass transfer stage, a biodegradation stage, and a post-biodegradation mass transfer stage. The experiments were set up using aseptic techniques and were conducted at a constant temperature of 25°C. After a reactor was assembled, 720 mL of sterile mineral media was pumped in, 7 mL of NAPL was introduced through the NAPL-phase sampling port, and stirring was initiated. In the first mass transfer stage thus initiated, the aqueous phase was sampled four times a day until equilibrium was achieved between the two phases, over a period of 5–7 days; the NAPL phase was sampled occasionally over the same period. The biodegradation stage was then initiated by spiking freshly harvested

naphthalene-grown cells into the aqueous phase to attain an initial biomass concentration of 10^7–10^8 CFU/mL. In this stage, the concentration profiles in both phases were obtained by daily sampling over a period of about 30 days, and the aqueous-phase biomass concentration was occasionally determined by plate counts. Moreover, every day after sampling, aeration and venting were performed for about 20 min, and about 100 mL of the mineral media was drained and refilled to ensure that biodegradation was not limited by depletion of oxygen or nutrients. After 30 days of active biodegradation the post-biodegradation mass transfer stage was initiated by spiking glutaraldehyde at a concentration of 0.2% to terminate biodegradation. Sampling was performed for 5–7 days as in the pre-biodegradation mass transfer stage. Aqueous-phase samples were occasionally plated to test the effectiveness of microbial inactivation.

In all experimental stages the aqueous and the NAPL samples were diluted by factors of about 1.5–2 and about 1000, respectively, with methanol for purposes of analysis. Additionally, the aqueous samples in the biodegradation stage and the inactivation stage required centrifugation and filtration (0.2-μm PTFE membrane filters) to exclude biosolids prior to analysis. Concentrations of all components in these samples were determined chromatographically using external standards, as reported by Mukherji et al. (1997).

Data Analysis

Mass transfer parameters were determined in both the pre- and post-biodegradation experimental stages. For a closed NAPL–water system with segregated phases, where mass transfer from the NAPL to the aqueous phase constitutes the only source/sink terms, an aqueous-phase solute mass balance yields the following equation:

$$C_i^{\mathrm{a}} = C_{\mathrm{e},i}^{\mathrm{a}} - (C_{\mathrm{e},i}^{\mathrm{a}} - C_{i,\mathrm{o}}^{\mathrm{a}})_{\exp}\left(-\frac{A^{\circ}k_{\mathrm{f},i}}{V^{\mathrm{a}}}(t - t_{\mathrm{o}})\right), \qquad (5)$$

where C_i^{a} is the aqueous-phase concentration (mg/L), $C_{i,\mathrm{o}}^{\mathrm{a}}$ is the initial aqueous-phase concentration at $t = t_{\mathrm{o}}$ (mg/L), t_{o} is the initial time corresponding to the beginning of a mass transfer stage (days), and V^{a} is the aqueous-phase volume (mL).

This equation assumes negligible change in aqueous-phase volume and NAPL-phase composition as a result of dissolution. These assumptions are expected to hold due to the low aqueous solubility of the hydrophobic NAPL constituents. Aqueous-phase concentration–time profiles (C_i^{a} versus t) obtained as the aqueous phase approached and achieved equilibrium in the pre- and post-biodegradation mass transfer stages were fitted with Eq. (5) using a nonlinear parameter estimation routine (SYSTAT Inc., Evanston, IL). The two parameters obtained included the area-lumped mass transfer coefficient, $A^{\circ}k_{\mathrm{f},i}$ (cm^3/day), and the equilibrium aqueous-phase concentration, $C_{\mathrm{e},i}^{\mathrm{a}}$ (mg/L), which yielded the NAPL-phase activity coefficient, α_i^{n}. The parameter estimates were obtained by the method of least squares; i.e., by minimization of the sum of squared residuals achieved by the quasi-Newton method, an iterative numerical search scheme. Use of an alternate search scheme (the Simplex method) and choice of various initial guesses covering a wide range had no impact on the parameter estimates, indicating the robustness of the estimation procedure. The 95% confidence intervals on the estimates were obtained to reflect their precision. The α_i^{n} values obtained by rearranging Eq. (1) were based on average values of X_i^{n} over the duration of a mass transfer stage. The fugacity ratios used were refined values based on compound-specific entropies of fusion. Values of compound-specific inputs and details concerning estimation of mass transfer parameters in nondispersed liquid–liquid systems have been discussed by Mukherji et al. (1997).

The NAPL-phase naphthalene profile over time was measured in the biodegradation stage to establish the rate and extent of solute biodegradation. The ratio of naphthalene to pyrene concentrations served as a surrogate measure of naphthalene mass; i.e., pyrene mass in the NAPL phase is expected to remain constant due to its orders of magnitude lower solubility and its lack of biodegradation by the cultures studied. This surrogate was used because the experimental system had no provision for measuring NAPL-phase volume variations. The NAPL-phase surrogate mass profiles for naphthalene during biodegradation were also predicted using the ISM by substituting the two sets of mass transfer parameters determined in the pre- and post-biodegradation mass transfer stages. These profiles are subsequently referred as the pre- and post-biodegradation ISM predictions. Both sets of model predictions were generated using initial conditions corresponding to the beginning of the biodegradation stage. The infinite-sink condition was assumed for all biodegraded components; i.e., all components other than fluoranthene and pyrene. System-specific inputs to the model were the initial NAPL-phase volume and interfacial area. Liquid density values reported by Davis and Gottleib (1962) and other compound-specific inputs reported by Mukherji et al. (1997) were used in generating the predictions.

RESULTS AND DISCUSSION

Three replicate experiments were conducted with the pure culture and two replicate experiments were conducted with the mixed culture. In all experiments, the aqueous phase developed a greenish-yellow color and the NAPL–water interface was covered by a visible biofilm within 5–10 days of the onset of biodegradation. For the pure culture experiments, biofilm coverage, after it once formed, was observed at the interface throughout the experiment. In contrast, for the mixed culture experiments, the entire biofilm was sloughed from the interface after 22 days in reactor A and after 29 days in reactor B. By the time the post-biodegradation mass transfer stage was initiated, a new interfacial biofilm coverage had developed in reactor A but not in reactor B. Sloughing is widely reported for biofilms formed

on solid substrata and is generally attributed to systematic changes over large portions of the biofilm; e.g., death/lysis and anaerobic fermentation at the base of the biofilm (Wimpenny et al., 1993). In the mixed culture studies, sloughing of the interfacial biofilm probably reflects starvation/loss of viability of the biofilm community, particularly those in the base of the biofilm, resulting from oxygen/nutrient diffusional limitations. Due to differences in the temporal dynamics of the two reactors involved in the mixed culture experiments, the results are presented individually. The pure culture results are presented as pooled results because the three replicate reactors behaved similarly.

Pre- and Post-Biodegradation Mass Transfer Parameters

The pre- and post-biodegradation mass transfer parameters for the pure culture experiments are presented in Table I, and those for the mixed culture experiments in reactors A and B are presented in Tables II and III, respectively. Pooled parameters for the pure culture studies were obtained by first fitting the C_i^a (t) profile for each SPR to obtain parameters, and subsequently pooling parameters from the three SPRs. In the pure culture studies, the post-biodegradation aqueous-phase profiles failed to level off over the duration of this stage (6 days). Hence, the component activity coefficient, α_i^n, during this stage were assumed to be the same as those in the pre-biodegradation stage and the aqueous-phase profile of each component was fitted with a single parameter; i.e., the area-lumped mass transfer coefficient. For both sets of bioavailability studies, post-biodegradation mass transfer parameters could not be determined for toluene because this component was depleted from the NAPL phase during biodegradation to such an extent that its corresponding equilibrium aqueous-phase concentrations were close to the analytical detection limits. Nor could post-biodegradation parameters be obtained for the non-biodegraded components pyrene and fluoranthene because their aqueous concentrations remained close to equilibrium concentrations throughout the biodegradation and post-biodegradation stages.

Post-biodegradation mass transfer parameters were measured to determine whether this process had been altered in any way by the presence and activity of microorganisms during biodegradation. For these parameters to be truly representative, the inactivating agent used to stop biodegradation in the bulk phase and in the interfacial biofilm should cause no alteration in the rate and extent of mass transfer or in the microbial distribution within the system. Glutaraldehyde is reported to act rapidly by crosslinking with proteins, by reacting with nucleic acids, and by binding to the outer layer of cells, thereby hindering cellular uptake and fixing the cells to prevent lysis (Scott and Gorman, 1991; Russell et al., 1994). Preliminary abiotic studies indicated that 0.2% glutaraldehyde had negligible impact on the mass transfer parameters, although it caused development of a yellowish-orange coloration in the aqueous phase and a slow drop in pH. These changes in color and pH were also noted in the post-biodegradation phase of the bioavailability experiments. Glutaraldehyde did not cause biofilm detachment from the NAPL–water interface. Moreover, by virtue of its inactivation mechanism, it prevented cell lysis, thus, essentially maintaining the microbial distribution that prevailed towards the end of the biodegradation stage. Some minor modifications in cell distribution in the aqueous phase were caused by the agglutination tendency of the inactivated cells. No viable cells were detected in any of the aqueous samples plated out over a period ranging from 1 h after glutaraldehyde addition to the end of the post-biodegradation mass transfer phase, thus confirming the speed and effectiveness of inactivation. We assumed that glutaraldehyde effectively penetrated the biofilm to kill the biomass attached at the interface. Although bacteria in biofilms often demonstrate reduced sensitivity to biocides (Nichols, 1989; Wilson, 1996), it has been demonstrated that the bactericidal action of glutaraldehyde is not significantly influenced by the attached/dispersed state of target bacteria (Sharma et al., 1987; Cook et al., 1993; Russell, 1994).

The post-biodegradation activity coefficients for the mixed culture experiments were found to be about 0.6–0.9 times the pre-biodegradation values for both the reactors (Tables II and III). This effective lowering of equilibrium

Table I. Mass transfer parameters for the pure culture studies.

	Pre-biodegradation				Post-biodegradation	
	α_i^n		$A^o k_{f,i}$ (cm^3/day)		$A^o k_{f,i}$ (cm^3/day)	
Compounds	Mean	95% CI	Mean	95% CI	Mean	95% CI
Toluene	1.06	0.017	922.32	62.40		
Naphthalene	0.98	0.013	855.12	45.36	78.00	0.67
1-Methylnaphthalene	1.13	0.017	801.84	45.67	67.20	0.74
2-Ethylnaphthalene	1.17	0.022	796.08	58.58	61.20	1.15
Acenaphthene	0.91	0.015	709.20	43.46	70.08	2.11
Fluorene	0.92	0.014	746.88	43.87	72.00	0.89
Phenanthrene	1.12	0.019	671.04	43.75	61.20	1.97
Fluoranthene	1.12	0.030	510.24	40.03		
Pyrene	1.12	0.044	518.16	49.27		

Table II. Mass transfer parameters for the mixed culture study in reactor A.

	Pre-biodegradation				Post-biodegradation			
	α_i^n		$A^o k_{f,i}$ (cm^3/day)		α_i^n		$A^o k_{f,i}$ (cm^3/day)	
Compounds	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI
Toluene	1.16	0.042	724.32	89.57				
Naphthalene	1.07	0.034	697.68	75.24	0.71	0.026	328.08	27.58
1-Methylnaphthalene	1.25	0.044	637.20	70.92	0.79	0.025	327.12	24.02
2-Ethylnaphthalene	1.29	0.057	588.72	77.06	0.82	0.036	364.08	40.06
Acenaphthene	1.00	0.050	550.32	78.62	0.61	0.025	364.80	38.23
Fluorene	1.01	0.015	970.32	61.66	0.76	0.042	358.56	49.54
Phenanthrene	1.19	0.043	631.44	70.34	1.08	0.114	233.04	49.68
Fluoranthene	0.97	0.043	565.68	71.38				
Pyrene	0.95	0.044	537.36	70.25				

aqueous-phase concentration may be attributed to the products of biotransformation or to the interaction of glutaraldehyde with biotransformation products. The post-biodegradation lumped mass transfer coefficients for the pure culture experiments obtained in the presence of the interfacial biofilm are about 0.1 times the pre-biodegradation values (Table I). For the mixed culture experiments the values obtained in the presence of a newly formed biofilm in reactor A are about half the pre-biodegradation values, and the values obtained in the absence of a biofilm in reactor B are comparable to the pre-biodegradation values (Tables II and III). These results clearly indicate that the presence of the biofilm at the NAPL–water interface hinders mass transfer. The mass transfer hindrance is more severe for the pure culture biofilm having an age of about 20–25 days (when the cultures were inactivated) than the mixed culture biofilm in reactor A, which was formed after the original biofilm dislodged and had an age of only about 4–5 days.

Although interfacial biofilms can lower mass transfer coefficients by reducing turbulence at the NAPL–water interface, this effect is insignificant if the mass transfer coefficients are intrinsic and measured under relatively quiescent conditions. The primary impact of the interfacial biofilm is to lower the overall mass transfer coefficient by posing an additional impedance for mass transfer of components from the NAPL to the aqueous phase. Biofilms consist of a porous and heterogeneous matrix of cells, cellular debris, and extracellular polymeric substances (EPS) that can act as diffusion barriers and molecular sieves (Wimpenny et al., 1993). Reduced solute diffusivities in biofilms formed on solid substrata in various system configurations have been reported; i.e., fluidized aerobic bioreactors, rotating biological reactors, trickling filters, and batch cultures. The diffusivities of various substrates (e.g., glucose, oxygen, phenol) in biofilms have been reported to vary from 2% to 97% of the diffusivities in water depending on the age, origin and porosity of the biofilm (Fan et al., 1990; Beyenal et al., 1996). Zang and Bishop (1994b) have also demonstrated variations in effective diffusivity with depth within heterogeneous biofilms as a result of systematic variations in biofilm properties (i.e., porosity and tortuosity). When a homogeneous biofilm formed in a diffusion-dominated system for which aqueous side resistance controls is assumed, additional resistance due to the interfacial biofilm can be expressed as follows (Zang and Bishop, 1994b; Weber and DiGiano, 1996):

$$R_b = \frac{\tau L_b}{\varepsilon D_i^a} = \frac{1}{k_{f,i}} \left(\frac{1}{r_b} - 1 \right), \tag{6}$$

there τ is the tortuosity, ε is the porosity, L_b is the thickness

Table III. Mass transfer parameters for the mixed culture study in reactor B.

	Pre-biodegradation				Post-biodegradation			
	α_i^n		$A^o k_{f,i}$ (cm^3/day)		α_i^n		$A^o k_{f,i}$ (cm^3/day)	
Compounds	Mean	95% CI	Mean	95% CI	Mean	95% CI	Mean	95% CI
Toluene	1.27	0.059	468.24	61.08				
Naphthalene	1.19	0.058	417.36	52.63	0.82	0.051	415.92	73.63
1-Methylnaphthalene	1.36	0.075	387.60	52.51	0.93	0.062	417.84	78.53
2-Ethylnaphthalene	1.43	0.113	356.88	65.83	0.98	0.076	496.56	126.12
Acenaphthene	1.08	0.104	343.68	75.38	0.72	0.061	510.96	147.86
Fluorene	1.63	0.245	240.48	66.19	0.96	0.063	415.20	93.00
Phenanthrene	1.39	0.127	317.04	62.38	1.22	0.071	366.72	62.16
Fluoranthene	1.23	0.115	277.44	51.74				
Pyrene	1.36	0.216	215.28	58.61				

of the biofilm (cm), D_i^a is the free liquid diffusivity of the component of interest (cm^2/day), r_b is the factor by which the overall mass transfer coefficient is reduced by the interfacial biofilm, and R_b is the additional resistance due to the interfacial biofilm (day/cm).

Free liquid diffusivities for the PAHs of interest lie in the range 0.8–0.5 cm^2/day (Mukherji et al., 1997), and biofilm porosity and tortuosity lie in the range of 0.9–0.5 and 1.1–1.5, respectively (Zang and Bishop, 1994a,b). Based on these typical ranges of values, a 50% reduction in the overall mass transfer coefficient can be caused by a biofilm 20–100 μm thick, and a 90% reduction can be caused by a biofilm 100–700 μm thick. Thus, experimentally observed reductions in overall mass transfer coefficients can be explained on the basis of interfacial biofilms of reasonable thickness (expected range 10–2000 μm; Zang and Bishop, 1994a,b). Since the SPRs used had no provision for accessing the interfacial biofilms, biofilm thickness could not be experimentally measured. Studies in the context of biofilms formed on separate phase liquids serving as substrates have not previously reported additional resistance due to biofilms.

Effects of Mass Transfer on Microbial Uptake and Bioavailability Interpretations

NAPL-phase surrogate mass profiles for naphthalene during biodegradation along with pre- and post-biodegradation ISM predictions are presented in Fig. 2 for the pure culture experiments and in Figs. 3 and 4 for the mixed culture experiments in reactors A and B, respectively. Note that the time scale in these figures extends backward for a period of 10 days prior to initiation of the biodegradation phase of each experiment. Error bars represent standard errors. For the experimental profiles, standard errors could be determined for the pure culture experiments in three replicate reactors but not for the mixed culture experiments in individual reactors. The standard errors for the experimentally determined surrogate mass for naphthalene never exceeded 6%. For the predicted profiles, standard errors were determined by error propagation assuming that only the activity coefficient and mass transfer coefficient of naphthalene contribute significantly to prediction errors, and that these two coefficients are mutually independent. The standard errors in the post-biodegradation surrogate mass predictions were found to be negligible, and are thus not included in the plots. Also, because the post-biodegradation mass transfer parameters could not be determined for some of the components, additional assumptions were needed for generating post-biodegradation ISM predictions. When activity coefficients were unavailable, they were assumed to be same as the pre-biodegradation values. When mass transfer coefficients were unavailable, they were assumed as the average of the mass transfer coefficients of all other compounds for which values could be determined in the post-biodegradation experimental stage. Mukherji et al. (1997) demonstrated that mass transfer coefficients are similar for compounds having similar molecular free-liquid diffusivities.

Biomass concentrations and concentration profiles for the various compounds in the aqueous phase during the biodegradation stage were also determined experimentally. Plate counts indicated that for both the pure culture and the mixed culture studies the aqueous-phase biomass concentrations remained relatively constant. For both sets of studies the aqueous-phase naphthalene concentrations decreased rapidly to values below detection limits in 1–2 days and remained so throughout the duration of the biodegradation stage. The aqueous-phase concentration of all other compounds, other than fluoranthene and pyrene, also decreased to values below their detection limits within 5–10 days. The aqueous-phase concentrations of fluoranthene and pyrene exhibited notable variability although they were expected to remain relatively constant. Initially the concentrations were higher than the expected equilibrium values, but later they decreased significantly; biofilm sloughing was accompanied by significant rises in their concentrations. These observations are indicative of the sorption of these compounds on biomass, initially on suspended biomass and later on the biofilm after it accumulated. The biofilm was probably responsible for lowering the aqueous concentrations in several ways; i.e., by biodegradation within the biofilm, by reducing overall mass transfer rates, and by sorption to the film, depending on the type of compound under consideration.

Mass transfer effects on naphthalene uptake for both the pure culture and mixed culture experiments can be summarized on the basis of the relative positions and slopes of the experimental and ISM predicted NAPL-phase surrogate mass profiles given in Figs. 2–4 and from other observations noted above. The NAPL-phase mass profiles during biodegradation indicate an initial rapid decay followed by an extended period of slow decay. The initial period of rapid decay lasts for about 5–10 days, and an interfacial biofilm develops during this period. During the initial period, the rate of mass decay due to biodegradation is almost equal to the decay rate of the pre-biodegradation ISM prediction, such that the mass data almost coincide with the model profiles. During this period mass decay in the NAPL phase is noticeable. The overall extent of decay depends on the rate of decay and the duration of this initial rapid decay period. During the subsequent period, biodegradation rates are significantly reduced. This reduction cannot be explained by depletion of oxygen or nutrients in the bulk aqueous phase because both were replenished daily, nor can it be explained by the toxicity of biodegradation products accumulating in the bulk aqueous phase, since the aqueous-phase concentrations of all biodegradable compounds were maintained at near zero levels. This reduction can, alternatively, be attributed to changes in mass transfer parameters as a result of biodegradation; i.e., reduction in lumped mass transfer coefficients due to accumulation of interfacial biofilms and potential reduction in activity coefficients due to accumulation of bio-transformation products. The pure culture results clearly demonstrate how reductions in lumped mass transfer coefficients caused by mature biofilms reduce the slope of the post-biodegradation profiles. For the mixed

culture study in reactor A, the post-biodegradation profile reflects both the impact of reduction in lumped mass transfer coefficient caused by the biofilm and reduction in the activity coefficient caused by bio-transformation products. In contrast, for the mixed culture study in reactor B, the post-biodegradation profile obtained with parameters determined in the absence of an interfacial biofilm reflects only the impact of reduction in the activity coefficient. This post-biodegradation profile is not representative of the later stage of biodegradation when a biofilm covers the interface. Correspondingly, the mass depletion rate predicted by this profile is much higher than the observed depletion rate in the later stage of biodegradation. When the post-biodegradation parameters are obtained in the presence of an interfacial biofilm, the mass depletion rates predicted by the post-biodegradation profiles are similar to those of the corresponding experimental profiles in the later stages of biodegradation.

For neither culture studied is there evidence for direct interfacial uptake by attached bacteria. The mass remaining in the NAPL phase is never notably less than the corresponding pre-biodegradation ISM prediction; i.e., the biodegradation rate never exceeds the maximum possible mass transfer rate. It thus appears that naphthalene is utilized only after it is transferred into the aqueous phase. Subsequent lowering of biodegradation rates can be correlated with reduction in mass transfer rates due to formation of interfacial biofilms and potential accumulation of bio-transformation products. The above bioavailability interpretations rely only on the mathematical formulation of the ISM and on the mass transfer coefficients determined before and after biodegradation. The robustness of the method lies in the fact that it determines the mass loss due to biodegradation by directly analyzing the NAPL phase. In most other systems it is not possible to analyze the separate/sorbed phase directly, hence flux out of that phase due to biodegradation is usually determined on the basis of mineralization rate or growth rate data. Such an analysis is less robust due to the possibility of errors in the correction factors involved (Volkering et al., 1993).

Despite the robustness of these bioavailability interpretations, some ambiguity remains due to compromises of some of the assumptions of the ISM. Although the model profiles were generated assuming no mass loss for the non-biodegradable components, pyrene and fluoranthene, from the NAPL phase, some mass loss of these compounds due to sorption on the interfacial biofilm was indicated by the nature of the aqueous-phase profiles. Because the sorption capacity of the biofilm and the mass of the biofilm is unknown, the extent of this loss cannot be determined. However, due to the large volume of the NAPL phase, these sorption losses are expected to be negligible. The ISM further assumes zero aqueous-phase concentrations for all biodegradable components. Although the component of interest, naphthalene, achieved this condition within 1–2 days, it took about 5–10 days for the other biodegradable components to do so. Under these conditions, values of surrogate mass are likely to be higher than those predicted by the ISM, so that the possibility of direct uptake cannot be rule out. However, the shift is expected to be negligible due to the relatively high aqueous-phase solubility and content of naphthalene in the NAPL phase compared to most of the other components. The ISM also assumes that values of the mass transfer parameters remain constant, but significant variations were noted in parameter values measured before and after biodegradation. Due to reduction in mass transfer parameters as a result of biodegradation, the slopes of the post-biodegradation profiles were lower, and their positions were above the corresponding pre-biodegradation profiles. The experimental profiles lie between the pre- and the post-biodegradation model profiles. This can create ambiguity in bioavailability interpretations since the exact functionality of variation in mass transfer parameters over time is unknown. Our interpretations assume that the pre-biodegradation profiles are representative of conditions in the initial stages of biodegradation, and that the post-biodegradation profiles are representative of conditions in the later stages of biodegradation. Because decreases in the lumped mass transfer coefficients correlated with development of interfacial biofilms and increase in biofilm thickness, the initial stage was identified as the period before significant biofilm accumulation occurs, and the later stage as the period after significant biofilm accumulation occurs. Potential reductions in activity coefficients due to accumulation of transformation products are also likely to affect the later stage of biodegradation. In view of the above compromises in some of the assumptions of the ISM, our conclusion of no direct uptake may be a false negative conclusion. It appears that any direct uptake that might have occurred caused significant enhancement in biodegradation rates over and above those expected on the basis of uptake after dissolution.

The mixed culture results (i.e., uptake only after dissolution) are consistent with results reported by Ghoshal et al. (1996) for naphthalene uptake from coal tars and heptamethylnonane. Our results for the pure culture (i.e., no direct uptake) are inconsistent with results reported by Guerin and Boyd (1992) and Croker et al. (1995) for uptake of napththalene sorbed on soils and modified clay. These investigators concluded direct uptake for the pure culture, ATCC 17484, based on higher initial uptake rates in systems with soils compared to soil-free systems at the same initial aqueous-phase concentration. Slow desorption, unlikely to affect initial uptake rates, formed a basis for their conclusion. Abiotic desorption measurements were not performed, but the uptake by another culture, Np-Alk, was also studied and this culture was found to be incapable of direct uptake. The apparent discrepancy between the results of these studies and our results may be explained as follows. First, the basic assumption of the criterion chosen by Guerin and Boyd (1992) and Croker et al. (1995) may have been violated in their studies. Several studies have shown the desorption of labile sorbed compounds to be a rapid process, particularly when contamination occurs over short time pe-

riods as in the laboratory (Robinson et al., 1990; Carmichael et al., 1997). Rapid degradation of dissolved naphthalene could have created a large gradient for desorption, thereby increasing desorption rate. The other culture, Np–Alk, may have been less effective in creating a gradient for rapid desorption, or could have physically hindered desorption by irreversible attachment to soils. Guerin and Boyd (1992) noted that the attachment of ATCC 17484 to soils was a reversible process whereas Np–Alk attached strongly and irreversibly. The other explanations relate to differences in bacterial sorption/attachment at soil–water and NAPL–water interfaces in terms of reversibility of the process and viability of attached cells, which may have affected the direct uptake mechanism. In contrast to reported reversible attachment to soil–water interfaces, ATCC 17484 demonstrated extensive and irreversible attachment to NAPL–water interfaces to form biofilms. Also, attachment at NAPL–water interfaces could have caused a loss of viability or loss of activity of the part of cells in direct contact with the NAPL.

Our results indicate that biodegradation of PAH components of complex NAPLs occurs subsequent to dissolution. This is in accord with the general belief concerning biodegradation of sorbed and separate phase compounds. The direct uptake mechanism observed for lower solubility compounds like alkanes could not be demonstrated for the intermediate solubility compound naphthalene. Even a culture previously implicated in direct uptake from sorbed phases did not demonstrate any ability to utilize PAHs directly from NAPL–water interfaces. Extensive biofilm accumulation was noted at the NAPL–water interface in our studies with both mixed and pure cultures. Thus, microbial attachment at interfaces does not necessarily imply direct uptake. To the contrary, our results demonstrate that interfacial biofilms can hinder both mass transfer and biodegradation rates. To our knowledge, mass transfer hindrance due to biofilms formed on separate phase liquids acting as substrates has not been previously reported.

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