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[29] Evaluating Biofilm Activity in Response to Mas Transfer-Limited Bioavailability of Sorbed Nutrient

By RYAN N. JORDAN

Introduction

The rate of substrate metabolism (r, mole/liter-time), as describe the classical Michaelis–Menten model, depends on the concentratio the limiting nutrient (S_{aq}, mole/liter) that governs the rate of enzymati action:

$$r = \frac{r_{max} S_{aq}}{K_s + S_{aq}}$$

In a well-mixed aqueous culture, S_{aq} is simply the nutrient concentra in the bulk liquid (aqueous) phase. It is commonly assumed that bac can only metabolize aqueous phase species. Thus, the aqueous phase be referred to as the *directly bioavailable* phase.

However, if the limiting nutrient exhibits a high degree of surface a ity, such that it tends to associate with a solid–water interface, distribu of the nutrient between solid and aqueous phases might be describe a simple linear partitioning process:

$$K_p = \frac{S_s}{S_{aq}}$$

where S_s represents the sorbed nutrient concentration (moles of nutr sorbed per unit area of solid–aqueous interface). Thus, sorption of drophobic compounds (i.e., those having a high partitioning coefficient tends to reduce their concentration in the directly bioavailable phase (S_{aq}). More sorption may reduce S_{aq} to the extent that Michaelis–Me kinetics [Eq. (1)] are reduced to simple, first-order kinetics (by assun that $K_s \gg S_{aq}$):

$$r = k_b S_{aq}$$

where k_b is a first-order biotransformation rate coefficient (1/time).

If the partitioning process [Eq. (2)] is "rapid" relative to the biotrans mation process [Eq. (3)], then S_{aq} will govern the rate of biotransformat This special case is referred to as *concentration-limited bioavailability.* H ever, if partitioning is "slower" than biotransformation, then metabol will occur at a rate that is faster than the rate at which the directly bioav

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able pool can be replenished. Consequently, biotransformation proceeds at progressively slower rates until S_{aq} falls below the minimum substrate concentration required for metabolism (i.e., "S_{min}"). Slow mass transfer from sorbed to aqueous phases can often be approximated by a first-order mass transfer process:

$$\frac{dS_{aq}}{dt} = k_m(S_s - K_p S_{aq}) \tag{4}$$

where k_m is defined as the *first-order mass transfer rate coefficient* (1/time).

For the simple series desorption–biotransformation model described by Eqs. (2)–(4), the characteristic times for mass transfer and biotransformation are defined by Bouwer *et al.*[1]:

$$T_m = \frac{1}{k_m K_p R_{s/w}} \tag{5}$$

$$T_b = \frac{1}{k_b} \tag{6}$$

where $R_{s/w}$ is defined as the surface area of solid sorbent per unit volume of water in the system (i.e., the "solid-to-water" ratio, having units of area/volume).

The characteristic times defined by Eqs. (5) and (6) describe the *process time scales* for the mass transfer and biotransformation processes, respectively. Thus, concentration-limited bioavailability as described earlier (i.e., mass transfer is "rapid" relative to biotransformation) occurs when $T_m \ll T_b$. For $T_m \ll T_b$, bioavailability is *mass transfer limited* (i.e., mass transfer is "slow" relative to biotransformation), indicating that the rate of biotransformation is governed by the rate of replenishment (i.e., solid-to-aqueous phase mass transfer) of the directly bioavailable nutrient pool (i.e., the aqueous phase).

Previous studies have addressed both concentration- and mass-transfer limited bioavailability in solid–aqueous systems (e.g., soil slurries) where the substrate concentration was monitored,[1–4] but few studies[5] have addressed quantification of the growth of the microorganisms responsible for

[1] E. J. Bouwer, W. Zhang, L. P. Wilson, and N. D. Durant, *in* "Soil and Aquifer Remediation: Non-Aqueous Phase Liquids—Contamination and Reclamation" (H. Rubin, N. Narkis, and J. Carberry, eds.). Springer-Verlag, Heidelberg, 1997.
[2] T. N. P. Bosma, P. J. M. Middeldorp, G. Schraa, and A. J. B. Zehnder, *Environ. Sci. Technol.* **31**, 248 (1997).
[3] L. M. Carmichael, R. F. Christman, and F. K. Pfaender, *Environ. Sci. Technol.* **31**, 126 (1997).
[4] C. Fu, S. Pfanstiel, C. Gao, X. Yan, and R. Govind, *Environ. Sci. Technol.* **30**, 743 (1996).
[5] H. Harms, *Appl. Environ. Microbiol.* **62**, 2286 (1996).

substrate biotransformation. Further, most of the methods used ir studies were performed in batch systems, making it difficult to in the separate contributions of concentration and mass transfer proce bioavailability limitations without extensive model calibration.

Thus, the objective of the methodology described herein is to e biofilm activity in response to mass transfer limited bioavailabili flowing system. Of key importance to the method are design para that govern mass transfer rates and reactor flows. System design based on an engineering process approach to modeling the mass tr biotransformation process kinetics as described earlier.

Method Description

While previous methods applied to bioavailability-limited system addressed the fate of the substrate, the goal of this method is to es a biofilm on a surface where the only available substrate is present sorbed phase at the solid–water interface. Thus, this method is de to investigate the fate of the biofilm in a bioavailability-limited sys

In general, an appropriate solid medium (i.e., a coupon or some suitable substratum for biofilm growth, not to be confused with *microl ical* media used to provide nutrition for cell metabolism) is selec manufactured such that sorption of the desired limiting nutrient solid–water interface is encouraged. The coupon is placed in a r inoculated with a selected microbial culture, and exposed to a f nutrient stream for a predetermined period of time. Depending reactor configuration, the coupon is observed nondestructively (e microscopic techniques) or sampled destructively. (Some of thes niques are addressed later.)

As one example of applying this methodology, a system in whi desorption-limited bioavailability of soil-sorbed hydrophobic organi pounds (HOCs) governs biofilm activity will be described. Howev methodology is certainly applicable to monitoring biofilm growtl variety of surfaces harboring low-solubility chemicals. Some appli might include assessing the efficacy of engineered surfaces (e.g., bi rial-coated medical implants) with embedded antimicrobial agents or toring biofilm activity in response to growth on insoluble substrate chitin).

Reactor Systems

A variety of flowing reactor systems exist for growing biofilms o media. Foremost among these systems are porous media (e.g., p

column) reactors, parallel plate (e.g., flow cell) reactors, and other coupon-based reactors (e.g., the Robbins device or the "rotating disk reactor" described by Zelver *et al.*[6]). Any of these systems could be incorporated into this method. Selection of the reactor system will depend primarily on limitations in the physical configuration of the coupon and the types of data to be collected from the system.

For the purpose of illustrating this method, a parallel plate (flow cell) coupon reactor will be described (Fig. 1). The flow cell is approximately 8 (length) by 6 (width) by 1 (depth) cm. The flow cell housing consists of two machined parallel plates (Fig. 1, A and B). A 1-mm (depth) flow channel [Fig. 1, C, 4 (length) by 2 (width) cm] is machined into a polycarbonate bottom plate (Fig. 1, A) having dimensions of 8 (length) by 6 (width) by 0.6 (thickness) cm. In turn, a 1.25 (diameter) by 0.5 (depth)-cm hole is drilled into the center of the flow channel to accommodate the biofilm coupon (Fig. 1, D). Pipe channels (Fig. 1, E and F, 3 mm diameter) are drilled in appropriate locations to introduce laminar flow and to allow effluent to exit the reactor. Inserted into the pipe channels on each end of the plate are sections of 3-mm (outside diameter) tubing (e.g., stainless steel). The top plate (Fig. 1, B) of the housing consists of an 8 (length) by 6 (width) by 0.3 (thickness)-cm sheet of aluminum or stainless steel. An observation window (Fig. 1, G) having dimensions large enough to accommodate a microscope objective [e.g., 4 (length) by 3 (width) cm] is cut from the center of the top plate. After the coupon is inserted into the flow cell, a gasket (Fig. 1, H, i.e., cut from a sheet of Viton rubber having the same dimensions as the top plate) and a glass coverslip (170 μm thickness), used as the observation window pane (Fig. 1, I), are sandwiched between the plates. The plates are held secure by a series of bolts with thumbscrews through holes (Fig. 1, J) drilled through the top plate, gasket, and bottom plate.

The biofilm coupon consists of a 1.25 (diameter) by 0.5 (thickness)-cm machined glass cylinder that is used as a substratum for depositing a reactive surface as described in the next section. (It should be emphasized that the choice of material used for the coupon is entirely up to the researcher—consideration must be given to the type of reactive surface being manufactured.) The flow cell is placed in-line with a nutrient delivery system consisting of a nutrient feed vessel, peristaltic pump, and waste collection container. Alternatively, the flow cell can be operated in a continuously recirculating batch mode, in which the waste stream simply feeds into the

[6] N. Zelver, M. Hamilton, B. Pitts, D. Goeres, D. Walker, P. Sturman, and J. Heersink, *Methods Enzymol.* **310** [45] 1999 (this volume).

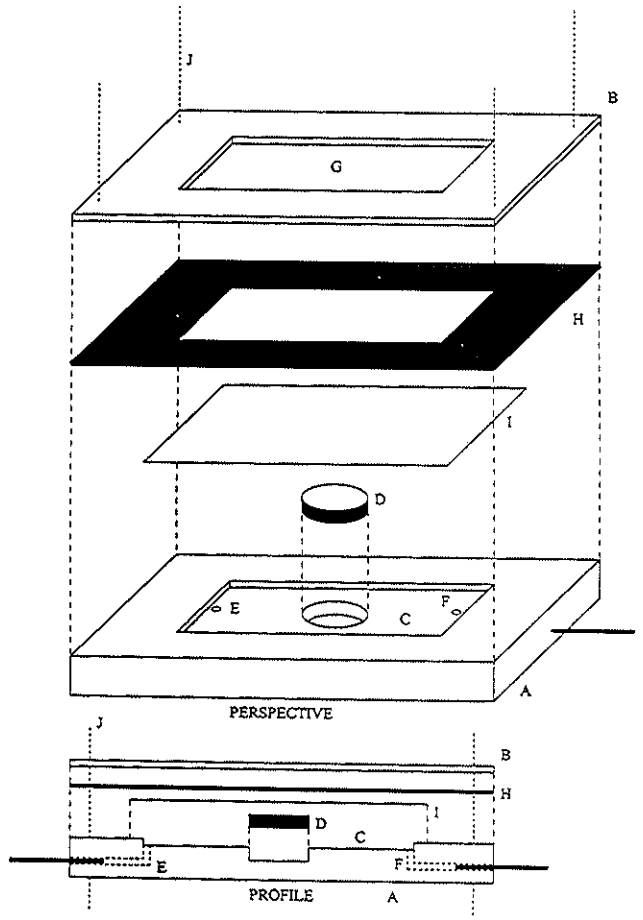


FIG. 1. Flow cell reactor configuration. See text for part descriptions.

inlet, with a pump placed in-line. This recirculating mode is quite helpf for initial inoculation.

Biofilm Coupon Manufacture

Although an engineered surface that mimics the chemical and physic complexity of a natural soil particle does not exist, a variety of analogs c be imagined. The most simple of these analogs is SiO_2 (glass). While Si(is often the most dominant mineral in soils, it is an unrealistic model f recreating the chemical reactions that dominate the sorption of hydrophob

organic chemicals in the subsurface. Key among these sorption processes is the partitioning of HOCs with natural organic matter (NOM)[7,8] Further, NOM is often associated with the highly reactive mineral surfaces of iron oxides such as ferrihydrite, goethite, and hematite. Thus, manufacture of a coupon having a composite iron oxide (Fe_2O_3, hematite) and NOM (humic acid) sorbent phase is described.

A number of methods for depositing iron oxide on a silicon dioxide surface exist.[9,10] These methods are based on the heat-induced growth of α-FeOOH (goethite) from Fe nuclei present at the SiO_2–water interface. Although we have used these methods successfully in our own laboratory, a much simpler approach will be described that takes advantage of the strong electrostatic and hydrophobic attraction between the SiO_2 surface and Fe_2O_3 colloids during thermal dehydration. Following deposition of the iron oxide, the coupon is soaked in a humic acid solution and rinsed, leaving behind only humic acid molecules that are bound tightly to the iron oxide surface functionalities.[11] Finally, the oxide/NOM coupon is aged in an aqueous solution containing a selected HOC and rinsed again, leaving only strongly bound HOC. The result of this procedure is a coupon having an HOC-contaminated oxide/NOM surface that is chemically analogous to those found in HOC-contaminated soils. The procedure is outlined as follows.

Coupon Preparation. Select a glass coupon having dimensions appropriate for incorporation into the flow cell. Clean the coupon by soaking in chloroform (1 hr), 6 *M* H_2SO_4 (1 hr), rinsing with deionized water, and baking in a foil-covered glass beaker at 550° for 6 hr to oxidize residual organic contaminants.

Iron Oxide Deposition. Using a pipetter, place a 100-μl drop of a slurry containing 10 g/liter of Fe_2O_3 (Fisher Chemicals, Pittsburgh, PA; 99%) in deionized water in the center of the coupon. Using the pipetter tip, gently spread the drop so that it covers the entire coupon surface. Bake the coupon in a foil-covered glass beaker at 550° for 6 hr to dehydrate the oxide layer and remove organic contaminants in the oxide matrix.

NOM Deposition. Soak the coupon in a gently stirred solution containing 100 mg/liter of humic acid for 24 hr. Well-characterized and purified humic materials isolated from surface waters, soils, and peat can be obtained

[7] J. J. Pignatello and B. Xing, *Environ. Sci. Technol.* **30,** 1 (1996).
[8] J. J. Piatt and M. L. Brusseau, *Environ. Sci. Technol.* **32,** 1604 (1998).
[9] P. C. Rieke, B. D. Marsh, L. L. Wood, B. J. Tarasevich, J. Liu, L. Song, and G. E. Fryxell, *Langmuir* **11,** 318 (1995).
[10] Y. Chang and M. M. Benjamin, *J. Am. Water Works Assoc.* **89,** 100 (1997).
[11] E. M. Murphy, J. M. Zachara, S. C. Smith, J. L. Phillips, and T. W. Wietsma, *Environ. Sci. Technol.* **28,** 1291 (1994).

from the International Humic Substances Society.[12] Rinse the coupon in three successive solutions (1 hr per rinse) of gently stirred sterile buffer solution (SBS, 0.05*M* HCO_3^- buffer adjusted to pH 7 with HCl).

HOC Aging. HOC bioavailability is largely governed by its aging time with NOM[3,13] Thus, the researcher should select an aging technique with some caution. One simple technique involves soaking the coupon in a gently stirred solution of SBS (as described in the previous step) that contains a selected concentration of HOC (e.g., 1 mg/liter phenanthrene). Aging times ranging from 15 min to several months are suggested. After the aging procedure, the coupon should be rinsed in SBS for 1 hr.

Coupon Storage. Coupons should be used as soon as possible following the surface deposition process to minimize biological and/or chemical degradation of the organic constituents. Careful consideration must be given to coupon storage conditions, including atmospheric moisture, redox conditions, and preservation techniques. Selection of coupon storage criteria will be based largely on the chemical nature of the sorbent surface. At a bare minimum, coupons should be stored at 4°. For the oxide/NOM coupons described herein, it is recommended that they be placed into the reactor and used immediately following manufacture. Under no circumstances should the finished coupons be dried and exposed to the atmosphere so as to avoid chemical degradation of the organic compounds on the surface.

Reactor Start-Up and Inoculation

The objective of the inoculation step is to promote the initial adhesion of cells to the coupon surface. These cells will constitute the population base for further biofilm growth. To maintain repeatability of adhesion during inoculation, the cells should initially be grown in a stable chemostat under exponential-growth dilution rates as opposed to batch culture. (Batch cultures contain cells of different "ages" or growth rates, and consequently they may possess different membrane characteristics that could contribute to differential adhesion behavior.) The cells should be centrifuged and washed (in a sterile salt buffer) to remove traces of growth media constituents that could interact with the coupon and provide nutrient sources preventing the coupon-sorbed nutrient from limiting biofilm activity. The washed cells are then suspended in SBS at a density appropriate for inoculation (e.g., 10^6 cells/ml).

[12] http://www.gatech.edu/ihss/
[13] R. G. Luthy, G. R. Aiken, M. L. Brusseau, S. D. Cunningham, P. M. Gschwend, J. Pignatello, M. Reinhard, S. J. Traina, W. J. Weber, Jr., and J. C. Westall, *Environ. Sci. Technol.* **31,** 3341 (1997).

The simplest method for inoculating the reactor is to recirculate the resulting cell suspension in the flow cell for a predetermined period of time. For example, using the coupons described herein and a culture of *Pseudomonas aeruginosa* (grown in a chemostat with a 5-hr dilution rate on 300 mg/liter of LB media), an inoculation period of 5–15 min is usually sufficient to achieve a 5–10% surface coverage of cells on the coupon.

Reactor Operation

After the inoculation period, the flow configuration should be switched from batch recirculating mode to a once-through flow mode. Selection of the flow rate through the reactor (and the resulting fluid residence time) is the most important operating parameter when evaluating biofilm growth on desorbing nutrients. Thus, particular attention will be paid to this issue.

In addition to the characteristic times for desorption (mass transfer) and biotransformation defined by Eqs. (5) and (6), one must also consider the characteristic time for transport in the reactor (i.e., the fluid residence time). If the flow rate (Q, volume/time) and the reactor volume (V) are known, then the fluid residence time is defined as

$$T_Q = \frac{V}{Q} \tag{7}$$

Process dynamics (hydraulics, biotransformation, and mass transfer) in the reactor must satisfy the following criteria: (1) $T_Q \ll T_b$ and (2) $T_Q \ll T_m$.

Satisfaction of condition (1) is required to prevent the accumulation of active planktonic organisms in the bulk liquid. Condition (2) must be satisfied so any substrate that is desorbed (and not retained by the biofilm) will be washed rapidly out of the reactor. Satisfaction of condition (2) is the key operating principle of this design: it ensures that the biofilm has access *only* to that fraction of the limiting nutrient that is either (1) sorbed at the solid–water interface or (2) retained in the biofilm matrix. Consequently, this design is ideal for examining the effect of unique strategies that bacteria employ to facilitate desorption (e.g., by biosurfactant production) or otherwise increase the bioavailability of sorbed nutrients.

It should be noted that these conditions apply only for the case of mass transfer-limited bioavailability (i.e., $T_b \ll T_m$). If the reactor environment satisfies criteria (1) and (2) listed earlier, but bioavailability is concentration limited (i.e., $T_b \gg T_m$), then the substrate will simply desorb and be washed out of the reactor before the biofilm has the opportunity to metabolize it.

Order-of-magnitude estimates of T_b, T_m, and T_Q can be used to invoke the just-described analysis. Estimation of T_b can be performed in a traditional biokinetics experiment (e.g., planktonic batch culture or chemostat)

by fitting experimental substrate degradation kinetics data to Eq. (3). can be estimated in an abiotic desorption experiment in which the sur harboring the sorbed nutrient is placed in a batch vessel containing nutri free buffer solution. The concentration of the nutrient in the aqueous ph is then monitored over time and data are fitted to Eq. (4) to deterr k_m. At equilibrium, the resulting values of S_{aq} and S_s can be used to comp K_p from Eq. (2). Finally, estimation of T_Q is determined by monitoring flow rate through the reactor by measuring the amount of fluid colle from the effluent in a given period of time. These approaches are somew general; thus, it is advised to review the literature to identify more spe protocols for assessing process kinetics that are appropriate to the exp mental system under investigation.

Nutrient Feed

The composition of the nutrient feed stream must be considered c fully. If it is assumed that the limiting nutrient will be initially presen the coupon-sorbed state (as is the HOC in this example), then this nutri concentration must be zero in the feed. Further, to promote bacterial me olism, the feed must be supplemented with other nutrients required growth (nitrogen, phosphorus, trace metals, etc.). In the example presen herein, the sole source of carbon for energy and growth is the HOC sor to the coupon surface. A supplemental mineral salts medium with no car is then used as the nutrient feed.

Coupon Analysis

The methodology culminates, of course, with analysis of the biofilm variety of approaches can be employed and are limited only by the imag tion of the researcher and the analytical tools available. Two approac compatible with the flow cell methodology described herein are sum rized briefly.

Real-Time Microscopy. The flow cell can be designed in such a way it can be mounted onto a microscope stage with an observation win large enough to accommodate the desired objective. Consequently, user brings the stage up to the objective and can view the biofilm du the experiment in real time. Combined with organisms that exhibit biolu nescence or fluorescence (e.g., green fluorescent protein, GFP), both l and epifluorescent techniques can be used to identify the biofilm w minimizing emission interference from the coupon surface. A wor caution is in order for examining biofilms attached to the coupon hou in the lower plate of the flow cell: the objective working distance mus at least as long as the observation window thickness (e.g., 170 μm)

the vertical height of the flow channel (e.g., 1000 μm) so that the focal plane of the biofilm is within the working range of the objective. While objectives having working distances up to 2 mm are not uncommon, they are not usually standard issue in the 60–100× magnifications required for the useful observation of intact biofilms. Thus, it may be necessary to specify a microscope objective with an "ultralong" working distance from the microscope's manufacturer.

Biofilm Quantitation. It is not the objective of this article to address the art of quantifying (counting) biofilm bacteria. However, a variety of techniques may be employed by removing the coupon from the reactor at the end of the experiment and performing a desired quantitative analysis. These techniques span the range of sophistication from traditional "scrape-and-plate" methods to more elaborate molecular biology techniques.

Conclusion

Methods for growing and monitoring biofilms are not new. However, few methods address the growth and analysis of biofilms under flowing conditions where biofilm activity is controlled by the bioavailability of nutrients localized at a solid–water interface. The objective of the method described herein is to provide a standard protocol based on sound engineering principles that can be used to further investigate biofilm microbiology in these systems. Thus, a reactor-based methodology is introduced that affords the researcher a maximum amount of flexibility in reactor selection, coupon manufacture, and coupon analysis. It is expected that implementation of this methodology will provide particular insight into the physiology of microorganisms possessing unique nutrient scavenging strategies (e.g., biosurfactant production) that facilitate the bioavailability of sorbed nutrients. We are also investigating the applicability of the methodology and process analysis approach described herein to determine the bioavailability of surface-embedded antimicrobial agents in an effort to evaluate the efficacy of engineered antimicrobial surfaces.

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