Resuscitation of Starved Ultramicrobacteria to Improve *in Situ* Bioremediation[a]

JAMES D. BRYERS[b,d] AND SELIM SANIN[c]
[b]*The Center for Biochemical Engineering*
[c]*The Department of Civil and Environmental Engineering*
Duke University
Durham, North Carolina 27708

INTRODUCTION

Understanding the mechanisms controlling the migration of bacterial cells, under oligotrophic conditions, within porous media will impact hazardous waste detoxification remedies by either the enhancement of *in situ* biological activity or the suspended cell augmentation of resident soil populations, assessment of the fate of accidentally or intentionally released genetically manipulated microorganisms, and determination of potential hazards associated with deep subsurface hazardous chemical or radiological waste containment. Under oligotrophic or extended starvation conditions, bacteria can exhibit a number of responses that can affect their movement through porus media, including a reduction in cell size, creating ultramicrobacteria (UMB); a reduction in cell endogenous decay or maintenance energy requirements; an increase in the bacteria's ability to attach to surfaces; and a change in the bacteria's production of insoluble extracellular polysaccharides. Thus the ability of a bacterial cell to migrate through complex porous media without catastrophically reducing media permeability runs contrary to its ability to adsorb, attach permanently at a solid surface, and form a biofilm.

BACKGROUND

Interest in the transport of microorganisms in complex porous media arises from concerns over (a) microbes as pollutants that may contaminate drinking water supplies;[1] (b) bacteria as disseminators of pollutants as in the enhanced solubility of certain adsorbed hazardous organics, the metabolic generation of by-products more toxic than original contaminants, and the increased mobility of radionucleotides,[2,3] (c) the fate of genetically manipulated microorganisms released, intentionally or

---

[a] The authors would like to extend their appreciation to the Office of Exploratory Research of the U.S. Environmental Protection Agency for their support of this research (Grant R-816701-0101 to J. D. Bryers) while at Duke University.

[d] Current affiliation and address for all correspondence: Co-Director, The Center for Biofilm Engineering, An NSF Engineering Research Center, and Professor, Department of Chemical Engineering, Montana State University, Bozeman, MT 59717.

inadvertently, into a soil ecosystem; and (d) the efficacy of bioaugmentation protocols as a biological means to reclaim hazardous waste contaminated soil.

Contamination of aqueous industrial effluents and surface and subsurface water resources by refractory, toxic organic substances (*i.e.*, xenobiotics) is a problem of increasing domestic and international focus. Development of novel, efficient, and economical means for mitigating these hazards is an important goal of the chemical industry and environmental regulatory agencies alike *(Bioremediation, Chemical &* Engineering News, 26 August, 1991). Among the most attractive and promising approaches to the problem of soil or groundwater reclamation is the use of selectively enriched microorganisms to either biologically detoxify the xenobiotics directly to benign by-products or to bioconvert the contaminants to less toxic materials, capable of further biotransformation. Resident microorganisms and their extracellular products attach to porous media forming biofilms that extract carbon and energy substrates and other essential nutrients from the surrounding liquid phase, thus "purifying" the water. Biological degradation of xenobiotics does occur, at very slow rates, in groundwater aquifers, clay liners of hazardous waste containment sites, near subsurface fuel and chemical storage tanks, waste pond injection wells, and in oil-bearing substrata. *In situ* biological decontamination can involve the stimulation of existing resident microbial communities through the addition of a nutrient or nutrients that are not naturally present in sufficient concentrations.

In situ bioaugmentation refers to the external supply of selectively enriched microorganisms to intensify resident biological activity in order to increase the rates of detoxification of specific xenobiotic(s) with minimal physical disruption to the site. Bioaugmentation comprises identifying resident microbes or genetically engineering an organism with the heterologous genes capable of degrading the target xenobiotic, quantifying any nutritional limitation preventing cell growth and rapid biodegradation of the xenobiotic, and then increasing the microbial concentration and activity by directly injecting suspensions of the selectively enriched population(s) into the contaminated site. Unfortunately, the efficacy of *in situ* bioremediation is oftentimes limited by the ability to uniformly distribute the selectively enriched cell suspension within the porous media, maintain the cell population within the contaminated site, and supply the population with necessary nutrients to degrade the xenobiotic(s).

Understanding of interactions among specific microbial systems, biofilm formation processes, and porous media mass transport characteristics (FIG. 1) is critical to affecting efficient treatment protocols and an explanation for the existence of diverse microbial communities in remote, unlikely ecosystems.

Biofilm Formation

Microbial cells (predominantly bacteria) and their extracellular polymers associated with a substratum are called *biofilms.*[4,5] Bacterial attachment, biofilm formation, and persistence is governed by a complex set of physical, chemical, and biological processes acting simultaneously. As illustrated in FIGURE 1, development of a bacterial biofilm comprises the following fundamental processes: (1) substratum preconditioning by adsorption of fluid phase organic molecules; (2) bacterial cell

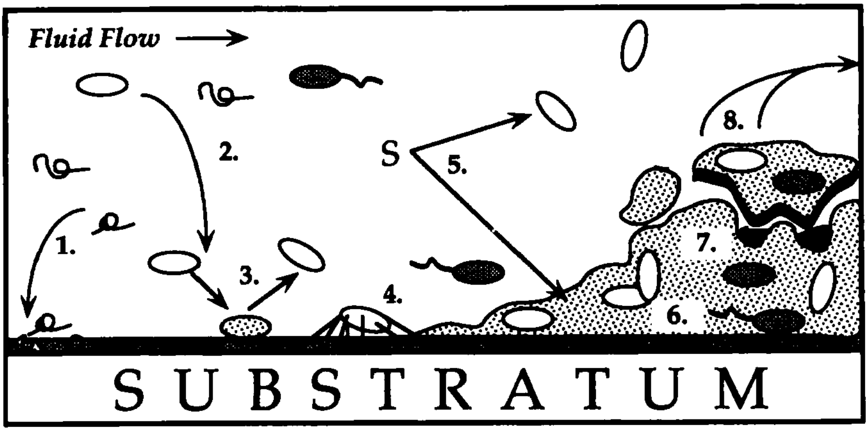

FIGURE 1. Processes governing biofilm formation in flowing fluid systems. 1. Organic pretreatment of substratum. 2. Particle transport to the substratum. 3. Reversible adhesion to substratum. 4. Irreversible adhesion to substratum. 5. Substrate metabolism and cell growth. 6. Cell replication. 7. Extracellular polymer production. 8. Biofilm detachment processes.

deposition to the conditioned substratum, (a) bacterial cell transport to substratum (molecular and convective diffusion, sedimentation), (b) bacterial adhesion to substratum (physicochemical nonspecific adhesion, ligand receptor–specific adhesion), (c) bacterial desorption from substratum (shear stress, turbulent eddy bursts); (3) bacterial metabolism, (a) substrate transport (molecular and convective diffusion), (b) cell substrate conversion, cell growth, and replication, (c) extracellular polymer production (biofilm glycocalyx, secreted pigments, and enzymes), (d) cell starvation, death, lysis); and (4) biofilm removal, (a) cell and biofilm shear stress-related erosion, (b) biofilm sloughing.

BACTERIAL RESPONSES TO STARVATION

Deprived of their primary carbon and energy substrate, microorganisms exhibit a number of physiological responses, including cell dormancy or sporulation (gram positive bacteria); endogenous decay and eventual cell membrane lysis; and a two-step process of cell fragmentation and continuous size reduction.[6] Natural environments are often oligotrophic, that is, deficient in growth-promoting nutrients, such that microorganisms have evolved mechanisms to survive rather than decay and lyse. There are extensive discussions concerning the physiology of vegetal, nonsporulating bacteria during progressive stages of nutrient starvation.[7-12] Such bacteria, mainly gram negative, often respond to nutrient deprivation by forming ultramicrobacteria or so-called viable but nonculturable cells. Of particular importance here is the tendency of numerous species of bacteria to fragment (divide without growth) upon starvation, leading to populations of increased numbers of smaller cells. Microorganisms, typically 1–2 μm, in nutrient-sufficient environments reduce to "micro"-

bacterial sizes of ~ 0.08–0.1 μm or less. Development of small UMB upon carbon/energy substrate starvation has been documented for sometime,[13] and it is suggested as one means for bacteria to survive in oligotrophic environments. Kjelleberg, Marshall, and co-workers[14–18] in a series of works document the sequence of events exhibited by bacteria upon carbon substrate deprivation. Cells experience 45–70% volume reductions, 50% decreases in endogenous decay rate,[19] increases in cell number of 80%, and approximately 55% increases in surface area:volume ratio. The process of "dwarfing" is a sequence of two processes: fragmentation (rapid cell division without growth), followed by a continuous size reduction while still maintaining near 100% viability.[16] Some marine *Pseudomonas spp.* also exhibit an increase in the production of extracellular polysaccharide upon carbon and energy substrate deprivation.[18]

Upon the transition from exponential growth to stationary or death phase, bacteria are known to undergo numerous changes in cell morphology, and physical and biochemical properties, including RNA degradation; a decrease in ATP content; expression of some novel "starvation" genes; and elevated resistance to different kinds of stress.[7–9,11,12,20–23]

Kjelleberg, Marshall, and co-workers[8–12] and Lappin-Scott[24] also report that upon responding to starvation by reducing cell size, UMB in some species also appear to attach to surfaces at higher rates than their freely suspended counterparts. Upon first reflection, the ability to attach better, while attaining a reduced cell size sufficient to navigate porous media interstices, appears to be contradictory for bacteria. One explanation for the survival and abundance of active bacteria in the deep subsurface may be related to these two conflicting traits. Cells with an increased ability to attach to surfaces would be able to avail themselves of adsorbed nutrients as well as a continual supply of any dissolved substrate that "passes" by. Once a colony has established its limits based on nutrient availability, progeny would be eroded from the biofilm and entrained in the liquid. Suspended UMB cells are able to migrate further than full-size bacteria, although, being the density of water, mixing (and thus resupply of nutrients) in the fluid directly adjacent to the cell is minimal. Potentially, one could envision bacteria "leapfrogging" from one surface colony to another, requiring both the ability to attach, to survive on low or no food, and to negotiate the tortuous confines of porous media.

POTENTIAL APPLICATIONS OF UMB

In porous media, microbial cells can be either suspended or attached to media surfaces. The effects of cell particle suspensions and biofilm formation on porous media permeability are only now receiving research attention. Microbial suspensions are percolated over spent mine tailing to enhance the microbial leaching and recovery of valuable metals from ore. Injection of surface waters into petroleum reservoirs constitutes the most common form of secondary oil recovery (*i.e.*, water flooding), but it also introduces active anaerobic sulfate-reducing bacteria into the oil-bearing substrata and aboveground recovery facilities. Sulfate-reducing bacteria form hydrogen sulfide, which at certain concentrations is highly corrosive, and, in higher concentrations, the gaseous form is deadly. Microbial growth within a porous formation can lead to plugging and loss of water injectability. Cunningham *et al.*[25] documents permeability in laboratory porous media columns decreased in 7 days to between 1–5% of its original clean bed values due to the intentional cultivation of bacterial biofilms.

Conventional oil recovery methods are not able to extract more than 40% of oil in place in a reservoir because most oil is trapped in low permeability porous media or is too viscous to flow. Various secondary oil recovery techniques exist to extract these remaining oil reserves, the most prominent being water flooding. Unfortunately, without any control, water floods would be directed through only high permeability zones, yielding poor recovery. Oil companies have devised several techniques to artificially block specific zones of high permeability so that water floods are directed to oil-bearing strata. Such techniques include injection of finely dispersed solids or fibers (< 1 μm) or *in situ* chemical reactions that form insoluble precipitates. Intentional plugging of high permeability, oil-spent formations with bacterial suspensions is now employed in oil-recovery operations to insure that water flooding is directed to oil-bearing but low-permeability regions. This application is termed *selective plugging,* as the bacteria physically prevent the water flood from entering areas of the reservoir already devoid of oil. The major performance criteria in bacterial selective plugging is the successful migration of plugging particles uniformly throughout formation. Microorganisms have been employed with mixed

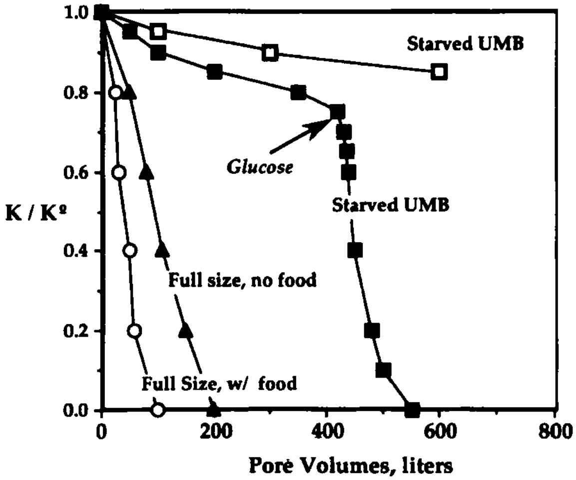

FIGURE 2A. Relative permeability in response to loading sandstone core columns with full-size bacterial cells with (○) and without (▲) nutrients present; UMB (☐), and UMB followed by resuscitation (■). K^{o} is the initial permeability of the core under "clean" conditions; K is the permeability observed during the experiment. K^{o} is the permeability or Darcy's constant as defined by Darcy's law. $Q = K \cdot \Delta H$, where Q = volumetric flow rate and ΔH is the head loss across a known length of porous media.

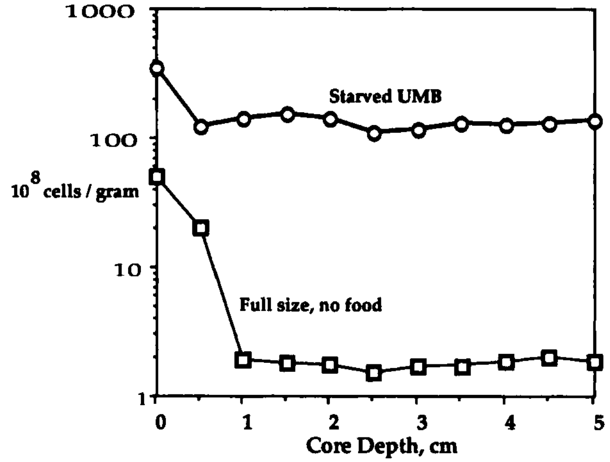

FIGURE 2B. Concentration of cells per gram of support as a function of position in porous media core axial position. ○, UMB; □, full-size cells without food.

results, in that their normal size (1–3 μm), their tendency to adhere to each other and inert substrata, and their propensity to produce exopolymers, the injection of cell suspensions usually creates unacceptable plugging within centimeters of the injection well casing.[26] Lappin-Scott *et al.*[24] report (FIG. 2A) the successful orchestration of controlled porous media plugging by first starving the bacterial culture *(Klebsiella pneumoniae)* over periods from one week to 3 months, which results in UMB (< 0.5 μm) that then migrate easily throughout the porous structure (FIG. 2B) until they are resuscitated by low concentrations of organic nutrient (citrate). Upon addition of citrate nutrients, core permeabilities dropped to zero within 48 hours.

Microbial processes can be harnessed to degrade or transform pollutants contaminating subterranean environments and groundwater aquifers, producing harmless by-products *in situ.*[27,28] Such approaches are tantalizing because they involve the use of indigenous microorganisms, or selectively enriched cultures can be introduced to the polluted site. Crawford[27] and Madsen[29] both summarize recent developments in bioremediation technologies. Unfortunately, most methods relying on the introduction of cell suspensions of enriched consortia are plagued with a serious problem: the very situation promoted in selective plugging, that is, the uncontrolled plugging of the porous media. Uncontrolled plugging by bacteria introduced to a contaminated site creates nonuniform distribution of the cells and poor hydrodynamics, preventing necessary nutrients from reaching the seeded cultures.

Hypothetically, the use of UMB would provide for the uniform and wide distribution of a specific species or culture throughout a contaminated subsurface zone, but two issues remain unresolved. First, can a species or culture selectively enriched (or genetically manipulated) to metabolize a specific xenobiotic compound be starved of that compound in order to solicit size reduction? Second, once distributed throughout a contaminated site,'can the starved UMB be resuscitated? Will they resume to their selective metabolic activity? Can this activity be controlled so as not to adversely affect permeability?

Results presented here address these questions for the study case of a single bacterial species, *Klebsiella pneumoniae,* that can metabolize the xenobiotic *s*-triazine compound, cyanuric acid (CN), contaminating a model porous media system.

EXPERIMENTAL PROTOCOL

Microbial System

Klebsiella pneumonia 99 (KB) (nonmotile) was the bacterial species used in this project. It was isolated and characterized previously by Cook[30] from a *s*-triazine wastewater-receiving outfall. Batch cultures were initiated by inoculating basal nutrient solutions of 2.5 mM CN and 150 mg/L glucose. According to the metabolic pathway for *s*-triazine mixture degradation (Fig. 3), CN is the final triazine ring compound remaining prior to microbial conversion to biuret, urea, then ammonia. This species can degrade CN as carbon and nitrogen source but requires additional carbon, supplied here as glucose, for growth. After batch operation under aerobic conditions for 24 hours, continuous pure cultures were initiated by delivering to a constant volume reactor a CN:glucose nutrient solution. The nutrient solution consists of basal salts medium[30] supplemented with 2.5 mM and 50 mg/L glucose. Agar

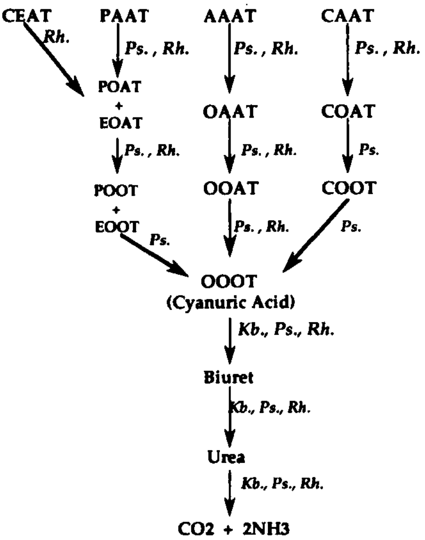

FIGURE 3. Biodegradation pathway of *s*-triazine herbicides. Kb = *Klebsiella spp.*, Ps. = *Pseudomonas spp.*, and Rh. = *Rhodococcus spp.* C = chloro-; E = ethylamino-; A = amino-; P = cyclopropylamino-; O = hydroxy-; T = triazine ring.

slants were maintained at 3°C and reslanted every six weeks. Additional slants were maintained at -20°C.

Reactor Systems

Suspended Cell Reactors

All suspended cell continuous cultures were carried out at 30 ± 1°C within 2-litre (1 litre working volume) fully instrumented bioreactor systems (B. Braun Biotech, Inc.). Each system maintained and monitored bulk liquid phase conditions of dissolved oxygen, pH, temperature, and influent/effluent flow rates. In addition, these systems allowed aseptic addition of CN and removal of culture fluid phase samples.

Porous Media Reactors

Porous media study reactors were constructed using quartz sand of known particle diameter (80–100 μm diameter), packed within circular glass tubes of different inner diameter. Glass tubes were filled with water and placed in a gentle vortex mixer while sand particles were delivered slowly, randomly packing within the tubes. Then the water is drained and contents air dried. Both ends of the tube are sealed temporarily with plugs, and the entire study reactor is heated to 700°C for 2.5 hours to fuse the particles to each other, thus fixing the porosity of the media.

To assess whether *Klebsiella pneumoniae* could be resuscitated in place, in case I experiments, porous beads were packed clean and sterilized in place (FIG. 4A). UMB of the species *Klebsiella pneumoniae* were delivered in a basal nutrient

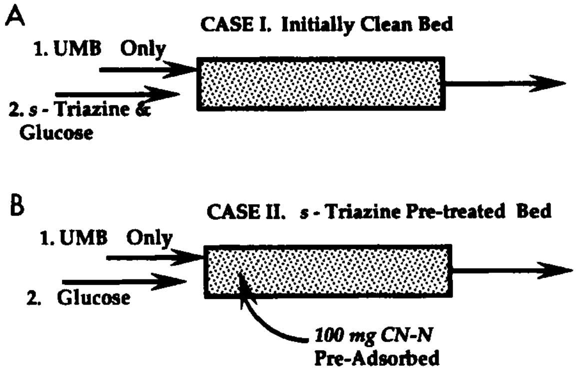

FIGURE 4. A. Case I. Core reactor seeded with UMB, then exposed to a constant loading of cyanuric acid and glucose. Flow rate is 1.73 L/day. **B.** Case II. Core reactor pretreated with adsorbed cyanuric acid only to affect a total adsorbed concentration of 150 mg CN-N. UMB seeded in nutrient media minus CN and glucose until cells appear in effluent. Core then supplied only a glucose medium minus any nitrogen; only available nitrogen present in adsorbed CN.

solution (minus carbon and nitrogen) at a suspended cell concentration of 108 cells/mL until cells were detected in the effluent. Delivery of the cell suspension was then terminated and a CN:glucose–containing nutrient solution was initiated at a flow rate of 1.73 L/day. Effluent CN and ammonia-nitrogen concentrations, effluent suspended cell concentrations, and pressure drop were determined periodically through the experiment. Biofilm mass and thickness at different points within the porous media column were determined after the completion of an experiment.

To simulate an existing CN contaminated porous media, in case II, columns containing fused glass beads were filled with a CN-only solution for three days. The columns were drained and air dried (FIG. 4B). Based on prior adsorption isotherm studies, sufficient CN was employed to affect a total adsorbed amount for the entire column of 150 mg CN nitrogen.

Porous media tube sections were situated within a fluid delivery system. Either cell suspension or dissolved nutrients and xenobiotic solutions were delivered by pump to the porous media at a constant volumetric flow rate (1.73 L/day). Each porous media reactor contained a series of electronic pressure transducers for on-line continuous pressure readings.

After each experiment, to assess the location and activity of the adherent bacteria, porous media cores will be removed from their containment tubes and sectioned, with a diamond saw blade, laterally down the axial length of the core, creating a number of small porous media circular discs.

Analytical Methods

Liquid phase glucose concentrations were determined using a modified enzymatic assay provided commercially by Sigma Chemical Co., St. Louis, MO [Kit No. 510]. CN concentrations were determined by spectrophotometric absorbance readings at 220 nm[31] and confirmed by HPLC separation and UV detection.[32]

Total suspended cell number concentration was determined from both orange and green counts of acridine orange–stained cell samples as per Zimmerman *et al.*[33] Viable cell number concentration, based on the cell's dehydrogenase activity, was determined on the same cell sample using INT staining as per Zimmerman *et al.*[33]

Cell size distributions were determined from direct observation of scanning electron micrographs of cell suspensions filtered through 0.1 μm Nuclepore™ membrane filter. An acridine orange solution was applied to the membrane for 1 min and then removed by vacuum filtration. Epifluorescence total cell counts[33] were tabulated as before but were reported per mass of original glass core sample.

Endogenous respiration measurements were made on cells during progressive durations of starvation by measurement of the oxygen uptake rates of known concentrations of starved cells in the absence on any electron donor. A 3 mL suspension of cells, starved for a known period of time, was placed within a microrespirometer (Rank Brothers, England) and aerated to oxygen saturate the suspension. The vessel was sealed, and the dissolved oxygen concentration was monitored continuously. This particular respirometer was a simple plexiglass cylinder with a 5 mL cylindrical cavity that was situated over a microoxygen electrode. Contents were well mixed by a micromagnetic stirring bar. The entire vessel was jacketed and temperature controlled at 30°C by a circulating water bath. As cells endogenously respired, dissolved oxygen concentration decreased with time. The slope of the oxygen concentration, decreased with time, is defined as the endogenous respiration rate.

RESULTS AND DISCUSSION

Starvation and Batch Suspension Resuscitation Results

Klebsiella pneumoniae was grown aerobically in free suspension continuous culture chemostats at low growth rate (dilution rate = 0.10 h^{-1}; residence time = 10 h) on a basal nutrient medium containing 2.5 mM CN and 50 mg/L glucose. Cells were continuously harvested, centrifuged, and resuspended in buffered (pH = 7.0) nutrient media devoid of any carbon source. Samples were then sealed, placed in a rotary shaker at 200 rpm and 22°C for time periods ranging from hours to two months. Periodically, sample vials were sacrificed to determine cell number and cell size. Subsamples of starved cell suspensions from each vial were used as inoculum for batch suspended growth tests. An inoculum of starved cells at 10^3 to 10^4 cells/mL were injected into sterile buffered nutrient solution containing 2.5 mM CN and 50 mg/L glucose, which was then plugged and placed on a rotary shaker operated at 200 rpm and 22°C.

FIGURES 5, A–C are SEM photographs that illustrate the average size of *Klebsiella pneumoniae* during continuous growth at 0.1 h^{-1} dilution rate (FIG. 5A), *Klebsiella pneumoniae* cells sampled after two weeks of starvation (FIG. 5B), and *Klebsiella pneumoniae* cells at exponential growth stage of a batch suspension regrowth experiment (FIG. 5C). Slowly growing cells averaged ~1.0–1.3 μm rod-shaped nonmotile cells. During the first hours of starvation, *Klebsiella pneumoniae* cells exhibited a classic response to starvation, termed *fragmentation*—cell division without cell growth followed by continuous size reduction of the fragmented cells. By the end of this initial period, cell volume was reduced 40–70%, cell numbers increased by 80%, and endogenous respiration rates decreased by 50%. Cell viability remained near 100%. Cells did not grow, but rather divided, creating larger numbers of smaller cells. This initial fragmentation period led to cells with an average size of ~ 0.5–0.75 μm. Fragmentation was followed by a period of very slow but continual size reduction. This period can last days to months, leading to cells with an average size of 0.1–0.2 μm.

Cells were starved for periods of 1 day, 2 days, 2 weeks, and 4 weeks in this study, with the mean cell size for each time period being 0.75 μm, 0.55 μm, 0.2 μm, and 0.18 μm, respectively. Although fragmentation was observed to occur rapidly in the first few hours of starvation, resulting in cell sizes of 0.6–0.7 μm, the process of size reduction obviously continued for this species for four weeks. All cell samples starved from one day to four weeks retained their ability to metabolize CN, although with varying degrees of efficiency (FIG. 6). Cells starved between one day and two weeks exhibited similar, minor lag phases (15–18 h), followed by exponential growth phases at approximately the same rate (0.6 h^{-1}) for the three different inocula. Only the inoculum starved for four weeks exhibited an extensive lag period (20 h) followed by an exponential growth period at a rate (0.4 h^{-1}) slower than the other

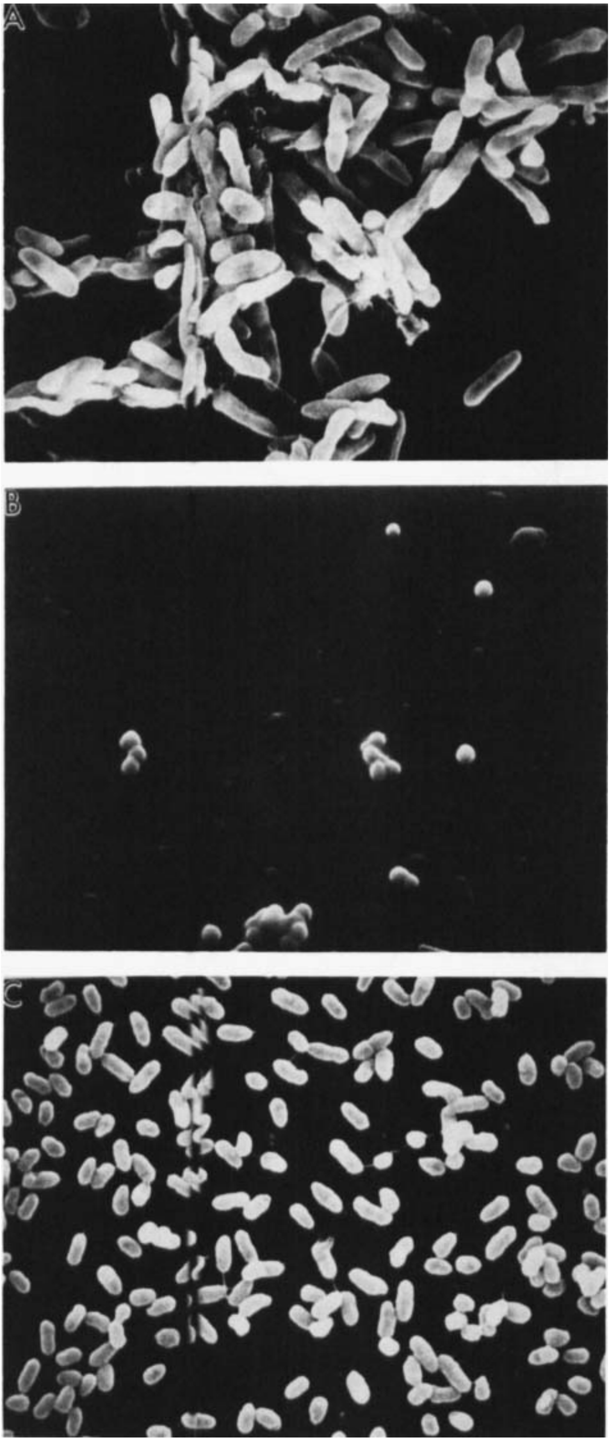

FIGURE 5. Scanning electron micrographs of *Klebsiella pneumoniae* cells samples from **(A)** chemostat culture operated at a dilution rate of 0.1 h^{-1}, **(B)** UMB of *Klebsiella pneumoniae* samples after 2 weeks starvation, and **(C)** *Klebsiella pneumoniae* cells 30 hours after batch-suspended phase resuscitation.

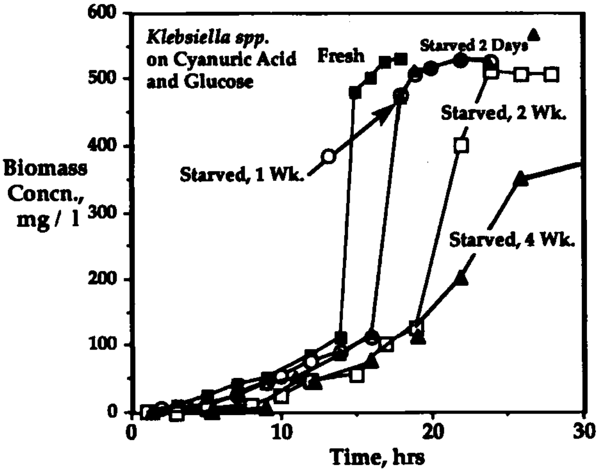

FIGURE 6. Batch-suspended phase regrowth trials for *Klebsiella pneumoniae* cells growing on a cyanuric acid (2.5 mM) and glucose (50 mg/L) nutrient solution. Different trials represent the difference in inoculum starvation period: ■, fresh-growing cells sampled from chemostat; ▲, cells starved two days; ○, cells starved 1 week; ☐, cells starved 2 weeks; ▲, cells starved 4 weeks.

three conditions. From these types of experiments, it appeared that starvation for approximately two weeks minimized cell size without detrimentally affecting the species' ability to metabolize CN. Only *Klebsiella pneumoniae* cells starved two weeks were used for the remainder of the study.

UMB and Porous Media Resuscitation: Case I Results

Porous glass core columns were constructed as described above. Sterile distilled water was delivered to the core reactors at a flow rate of 1.73 L/day in order to determine "clean" core initial pressure drops per length and calculated core permeabilities prior to introduction of the UMB. UMB starved for two weeks were suspended in nutrient solution (minus CN and glucose) to a concentration of 10^6 cells/mL and delivered to the cores at a volumetric flow rate of 1.73 L/day. Once UMB were detected in the core effluent, delivery of the cell suspension was terminated and replaced with sterile nutrient media containing 1.0 mM CN and 50 mg/L glucose. The nutrient solution was preaerated prior to injection into the core, and effluent dissolved oxygen was monitored continuously by way of an in-line microoxygen probe. With the stoichiometry reported by Cook,[30] 1 mole of CN would be biologically converted to 3 moles of ammonia. FIGURE 7 illustrates the time course of dissolved ammonium concentration in the effluent, leaving a porous core reactor and the subsequent decrease in core relative permeability as a response to a constant input feed of 1.0 mM CN. Evolution of ammonium ion indicates that conversion of

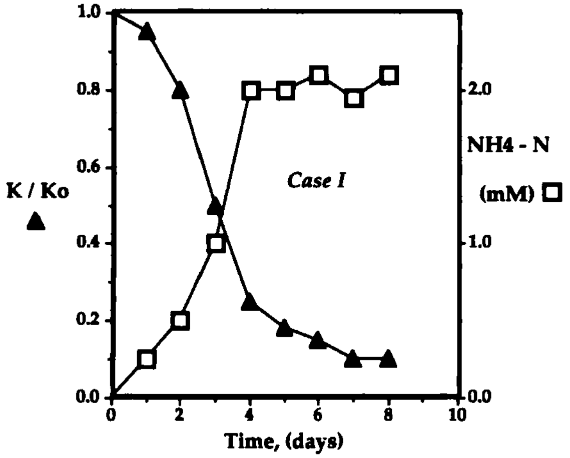

FIGURE 7. Response of relative permeability (▲) and effluent ammonia concentration (□) for a UMB-seeded core column to constant loading of cyanuric acid/glucose nutrient solution. Ko is the initial permeability of the core under clean conditions; K is the permeability observed during the experiment.

CN reaches a steady state after about five days of constant feeding. Almost total plugging of the core column by accumulating bacteria is observed by a 90% drop in core permeability by day 6. FIGURE 8 illustrates the evolution of biofilm thickness values for the first eight days at different locations in the core reactor above. As anticipated for once through column reactors, an axial profile in biofilm amount develops over time, with the most extensive biofilm growth (and the most dramatic drop in permeability) occurring at the entrance of the core where the CN concentration is highest. Not shown here are SEM photographs of the core sections, which

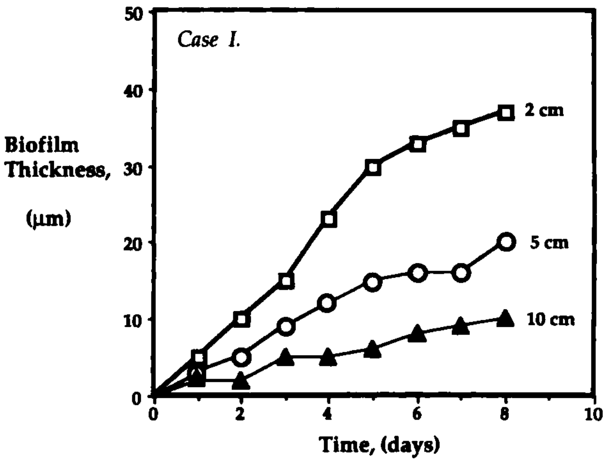

FIGURE 8. Evolution of biofilm thickness in porous media core as a function of axial distance down the core.

indicate under these conditions that considerable amounts of extracellular polysaccharides were produced throughout the core; these polymers also contribute to the dramatic increases seen in fluid-frictional resistance.

UMB and Porous Media Decontamination: Case II Results

In case II, porous core reactors were exposed, under quiescent conditions, to a CN solution of sufficient concentration to preadsorb onto the glass beads a total amount of CN equal to ~ 150 mg of CN-N (CN-N refers to the amount of CN as nitrogen atom). Nutrient solution, minus any glucose (but containing the same dissolved amount of CN as used in the adsorption step), containing the UMB was then delivered to the reactor at 1.73 L/day. Once UMB were detected in the core reactor effluent, the cell suspension was terminated and replaced with sterile, aerated nutrient solution containing 50 mg/L glucose but devoid of any nitrogen source. The only source of nitrogen for cell growth was the CN-N preadsorbed to the porous media. Thus this experiment mimics the situation of delivering a UMB to a waste-contaminated site.

FIGURE 9 illustrates the evolution of the mass effluent rate of ammonium-nitrogen (mg NH_4^+-N/day) leaving the column reactors in case II. This parameter is equal to the product of the constant effluent volumetric flow rate (F in L/day) and the

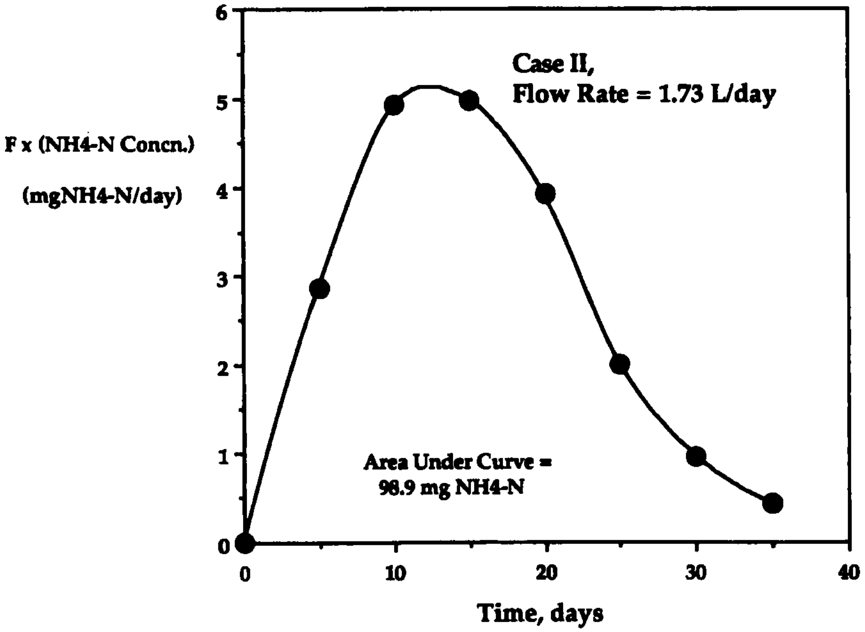

FIGURE 9. Mass rate of dissolved ammonia evolved due to biological degradation of preadsorbed cyanuric acid in core reactor.

instantaneous effluent concentration of ammonium-nitrogen (NH_4^+-N in mg/L). These data indicate that as the cells start to metabolize the fixed amount of adsorbed CN, effluent concentration of product ammonia increases to a maximum and then drops to almost zero. The decline in ammonia concentration is brought on by a decrease in the amount of adsorbed CN and a concomitant drop in CN degradation rate. The integral under the curve in FIGURE 9 represents the total amount of NH_4^+-N produced, which, in this case, should be equal to the mass preadsorbed. Calculations indicate that about 98.9 mg NH_4^+-N was produced from the ~ 150 mg/L CN-N preadsorbed. In replicate experiments, the recovery of preadsorbed CN-N ranged between 64–66%. Note that as the CN concentration dropped to very low levels, the cells were free to metabolize NH_4^+-N as a nitrogen source, which explains the lack of a complete nitrogen inventory in either case I or II.

REFERENCES

1. GERBA, C. P. 1985. *In* Groundwater Quality. C. H. Ward, W. Giger & P. M. McCarty, Eds. J. Wiley, Inc., New York.
2. CHAMP, D. R. 1985. *In* Groundwater Quality. C. H. Ward, W. Giger & P. M. McCarty, Eds. J. Wiley, Inc., New York.
3. WEST, J. M., H. A. GROGAN, I. G. MCKINLEY & E. J. ROWE. 1985. *In* Groundwater Quality. C. H. Ward, W. Giger & P. M. McCarty, Eds. J. Wiley, Inc., New York.
4. CHARACKLIS, W. G. & K. C. MARSHALL, Eds. 1990. Biofilms. J. Wiley & Sons. New York.
5. CHARACKLIS, W. G. & P. A. WILDERER, Eds. 1989. Structure and Function of Biofilms. Dahlem Conference Life Science Research Report Nr. 46. J. Wiley & Sons. Berlin.
6. MASON, C. A., G. HAMER & J. D. BRYERS. 1986. FEMS Microbiol. Rev. **39:** 373–401.
7. MATIN, A. 1990. FEMS Microbiol. Ecol. **74:** 185.
8. MATIN, A. 1991. Mol. Microbiol. **5:** 3.
9. MATIN, A. 1992. J. Appl. Bacteriol. Symp. Suppl. **73:** 49S.
10. ROSNAK, D. B. & R. R. COLWELL. 1987. J. Bacteriol. **174:** 345.
11. SIEGLE, D. A. & R. KOLTER. 1992. J. Bacteriol. **174:** 345.
12. TUNNER, J. R., C. R. ROBERTSON, S. SCHIPPA & A. MATIN. 1992. Biotechnol. Bioeng. **40:** 271.
13. NOVITSKY, S. A. & R. Y. MORITA. 1977. Appl. Environ. Microbiol. **33:** 635.
14. KJELLEBERG, S., B. A. HUMPHREY & K. C. MARSHALL. 1982. Appl. Environ. Microbiol. **43:** 1166.
15. HUMPHREY, B. A., S. KJELLEBERG & K. C. MARSHALL. 1983. Appl. Environ. Microbiol. **45:** 43.
16. KJELLEBERG, S., B. A. HUMPHREY & K. C. MARSHALL. 1983. Appl. Environ. Microbiol. **46:** 978.
17. KJELLEBERG, S. & M. HERMANSSON. 1984. Appl. Environ. Microbiol. **48:** 497.
18. WRANGSTADH, M., P. L. CONWAY & S. KJELLEBERG. 1986. Arch. Microbiol. **145:** 220.
19. PIRT, S. J. 1987. J. Ferment. Technol. **65:** 173.
20. INGRAHAM, J. L., O. MALLOØE & F. C. NEIDHARDT. 1983. Sinauer Associates. Sunderland, MA.
21. KEILIN, D. 1959. The Leeuwenhoek Lecture. Proc. R. Soc. Lond. Biol. Sci. B **150:** 149.
22. KOLTER, R. 1992. ASM News **58:** 75.
23. LANG, R. & R. HENGGE-ARONIS. 1991. J. Bacteriol. **173:** 4474.
24. LAPPIN-SCOTT, H. M., F. CUSACK & J. W. COSTERTON. 1988. Appl. Environ. Microbiol. **54:** 1373.
25. CUNNINGHAM, A. B., W. G. CHARACKLIS, F. ABEDEEN & D. CRAWFORD. 1991. Environ. Sci. & Technol. **25:** 1305.
26. SHAW, J. C., B. BRANHILL, N. C. WARDLAW & J. W. COSTERTON. 1985. Appl. Environ. Microbiol. **49:** 693.

27. Crawford, R. L. 1991. Bioremediation of groundwater pollution. Curr. Opinion Biotechnol. **2:** 436–439.
28. Fredrickson, J., D. Balkwill, J. Zachara, S-M. Li, F. Brockman & M. Simmons. 1991. Appl. Environ. Microbiol. **57:** 402–411.
29. Madsen, E. L., J. L. Sinclair & W. C. Ghiorse. 1991. Science **252:** 830–833.
30. Cook, A. M. 1987. FEMS Microbiol. Rev. **46:** 93–116.
31. Hogrefe, W., H. Grössenbacher, A. M. Cook & R. Hütter. 1985. Biotechnol. Bioeng. **28:** 1291–1296.
32. Beilstein, P., A.M. Cook & R. Hütter. 1981. J. Agric. Food Chem. **29:** 1132–1135.
33. Zimmermann, R., R. Iturriaga & J. Becker-Birk. 1978. Appl. Environ. Microbiol. **36:** 926.