

Biofilms as Biobarriers

• JOHN LENNOX, JEFFREY ASHE



Tank Car Derails & Spills Toxic Chemical

Altoona, PA. July 2008.

The Pennsylvania Department of Environmental Resources reported today that a team of engineers and scientists is working furiously to contain a 1000 gallon (4000 l) spill of highly toxic vinyl chloride. Vinyl chloride, a carcinogenic compound used in plastics manufacture, was released from a Con Rail tank car that derailed and split open four miles north of the city last week. The dramatic containment effort was spurred by the close proximity of the Little Juniata River that serves as a water supply for Tyrone, Huntingdon, Harrisburg and other towns down stream from the accident site.

Hazardous materials (hazmat) teams from as far away as Philadelphia descended on the site and quickly contained the surface

spill. Cleanup of vinyl chloride-contaminated soil continues. Of more immediate concern to health professionals is the fact that the soil in the vicinity is sandy and quite porous. Test drilling has confirmed that a significant amount of the spilled material has seeped into the soil and contaminated the water table. A plume of toxic material has been detected, carried by the groundwater in the direction of the Little Juniata. Environmental Protection Agency spokesperson Allison Jefferies stated, "Contamination of the river would be a disaster for communities all the way down the Susquehanna River and into the Chesapeake Bay."

EPA specialists are employing a new "biobarrier" technology to stem the flow of vinyl chloride that has entered the groundwater and

is flowing inexorably toward the river. Using heavy drilling equipment, the scientists are directing the boring of a series of holes into which they will inject volumes of "starved" harmless bacteria. The wellfield, drilled in the shape of a funnel, is being placed in front of and at right angles to the developing subsurface plume.

The bacteria are being prepared in the laboratories of the American Type Culture Collection in Manassas, Virginia. Cells of a bacterium (*Klebsiella oxytoca*) have been cultured in large fermentation tanks and are now being "starved;" a process that reduces their size and increases their ability to penetrate pores in the soil. After injection underground, the bacteria will be resuscitated by a cocktail of nitrate and molasses. The molasses is a nutrient

and the nitrate will serve in place of oxygen to enable the bacteria to metabolize the molasses in the oxygen-deficient environment. The scientists expect that, in the soil the bacteria will produce large amounts of slime that will clog pores in the soil and greatly reduce the movement of vinyl chloride-contaminated water. It is now a race against the clock. The plume is moving at an estimated rate of 200 feet (67 m) per day in the sandy soil. The river is only a quarter mile from the wreck site. That gives workers about a week to stem the flow and the tank car derailed three days ago. If successful, the biobarrier is expected to reduce the flow of vinyl chloride by more than 99% to just 2 inches (~5 cm) a day, buying time for the protracted cleanup to follow.

The scenario described here is fictitious. Numerous laboratory scale and field studies have been carried out, but few full-scale tests of this technology have been attempted. One exception, at a gasoline spill in Port Hueneme, California, has demonstrated the potential benefit of this technology (Johnson et al., 2003).

In a climate of increased concern for the environment and its protection, teachers in disciplines as diverse as biology, microbiology, environmental studies, and environmental engineering may be seeking teaching materials and laboratory exercises that will enable them to introduce these new concepts into their classrooms and laboratories. The materials and exercise presented here are intended to enable teachers to illustrate the seamlessness of the intersection between the theory of microbiology and the practice of environmental protection.

○ The Concept

Microbiologists have known for over a decade that the number of bacteria attached to surfaces in substrates

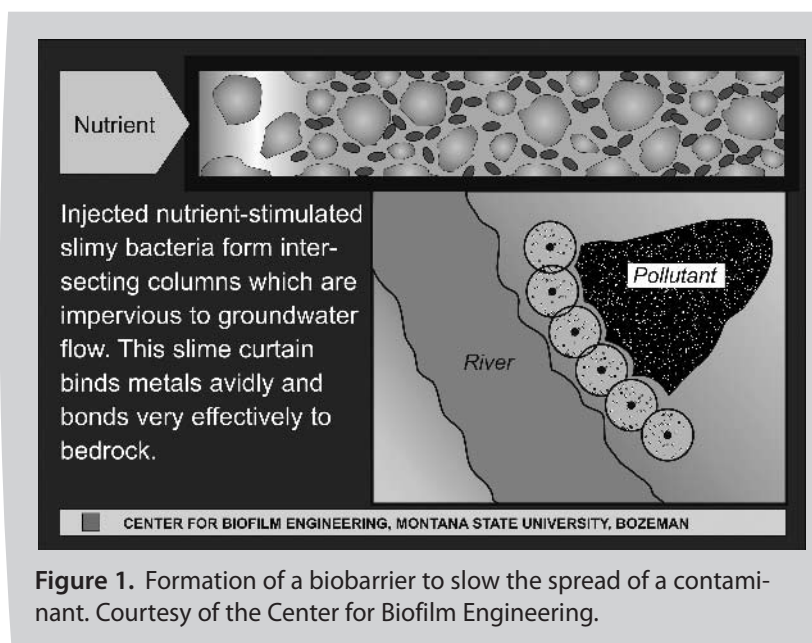


Figure 1. Formation of a biobarrier to slow the spread of a contaminant. Courtesy of the Center for Biofilm Engineering.

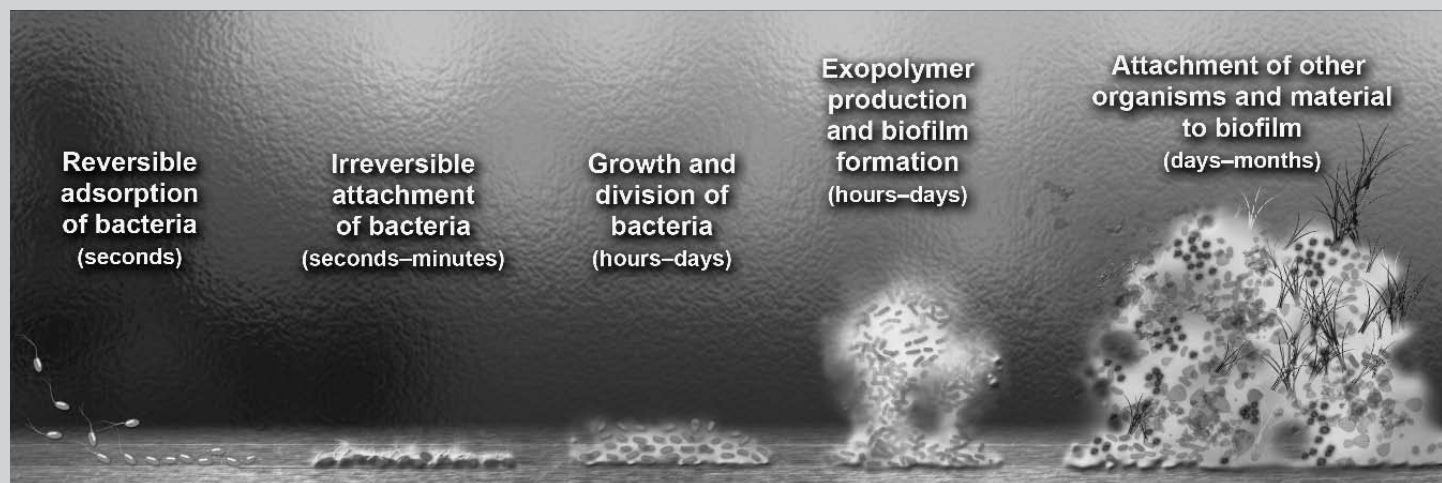


Figure 2. The typical sequence of biofilm development. Courtesy of the Center for Biofilm Engineering.

such as soil may exceed the number of cells suspended in the soil water by several orders of magnitude. These sessile cells account for most of the metabolic activity of bacteria in the soil (Van Loosdrecht et al., 1990).

Scientists are also aware that bacteria may produce many times their own weight in extracellular polysaccharide (EPS) and other polymers. This slimy material can cause plugging of channels in substrates like sand, soil, or porous rock strata, reducing the flow of groundwater and creating biobarriers. In the past, the interest of engineers and microbiologists has usually been in how to get rid of the “slime plug” which may interfere with some desirable process in water or waste treatment plants for example. The plugging of pipes, heat exchangers, and the fouling of ships hulls are other examples of biofouling to be avoided because of the corrosion and loss of efficiency they cause (Characklis, 1990).

The concept of using biobarriers to contain noxious materials is the flip side of the of the biofouling coin and is more recent in origin (Cunningham et al., 2003). In instances where spills of potentially harmful materials have occurred, the ability to use microorganisms injected into the soil that will produce extracellular polymers and reduce the flow of groundwater can be viewed as a potentially valuable technology. It has been discovered that reducing the size of the bacteria used in biobarrier formation increases their ability to penetrate sand, soil, rock and other porous substrates. Starvation of cells either under natural or laboratory conditions has been shown to cause the formation of ultramicrobacteria in many but not all species (Lappin-Scott & Costerton, 1990; Novitski & Morita, 1976). Using such starved bacteria permits increased infiltration of the target area, allowing faster and more effective containment.

Containment of spills is one potential use of this technology, but not the only one. Others include the enhancement of oil recovery by plugging highly permeable rock (Cusack et al., 1990), the control of acid mine drainage by capping acid-producing mines and mine tailings (Blenkinsopp et al., 1992), the bioremediation of toxic materials (Cunningham, 2000), and the enhanced recovery of valuable metals by microbial leaching of low grade ores such as copper (Lennox & Blaha, 1991).

○ Biobarrier Theory

The formation of biofilms is generally considered to be controlled by four processes: 1) bacterial transport into the target area, 2) bacterial adsorption to the substrate matrix surface, 3) growth and develop-

ment of the bacterial biofilm community and 4) detachment and dispersal of cells. In a steady state, biofilm growth and detachment are in equilibrium. Dissolution of the biofilm by scheduled release of cells or sloughing of significant fragments puts many cells back into suspension and represents a source of cells for the establishment of biofilm colonies downstream (Purevdorj-Gage et al., 2005). In soil, the entrapment of bacteria is influenced by the size of the bacterium and the average pore size of the substrate matrix. Small bacterial dimension and high porosity increase the rate at which bacteria migrate through the soil. This migration is also influenced by the rate and volume of groundwater flow and by the “stickiness” of the individual bacterium. This stickiness varies with the nutritional state of the bacteria and with the presence of adhesive proteins and polysaccharides on their surfaces. Some bacteria possess flagellae and pili that may aid bacteria in approaching and adhering to soil particles. Adhesion and entrapment are not independent functions in that it has been found that starvation influences not only the size of bacteria but also the concentration of adhesive materials on their surfaces (Costerton, 2007; Lappin-Scott & Costerton, 1990).

○ Biofilm Development

Initially, attachment of a bacterium to a substrate is tenuous and reversible. Through the formation of extracellular polymeric substances (EPS) the bacterium may become much more firmly attached to the surface. As colonization begins, the bacterium multiplies and the micro-colony thus formed becomes a focus for the adhesion of foreign particles including soil particles, organic matter, and other bacteria.

The nascent biofilm often develops a complex architecture, frequently, but not always, in the form of towers or “mushrooms.” The architecture developed appears to be due to the combined effects of physical forces (flow rate, shear, biofilm viscoelasticity) and biochemical interactions within the biofilm itself (gene expression, cell-to-cell signaling, and chemical gradients). These complex structures are permeated by numerous pores and channels through which water can flow, thus even cells deep within the biofilm may be quite close to the bulk fluid (Costerton et al., 1995).

The typical biofilm consists of a consortium of organisms forming a complex community in which cells of various types interact and compete for resources. These interactions are often mutualistic in that waste products of one colony member may serve as substrate for another. One frequently finds organisms

with related function growing side-by-side or interspersed in the biofilm. Species playing successive roles in nitrate reduction ($\text{NO}_3 > \text{NO}_2 > \text{N}_2\text{O} > \text{N}_2$), aromatic hydrocarbon dissimilation (Naphthalene $> \text{CO}_2$ and H_2O) or iron ($\text{Fe}^{++} > \text{Fe}^{+++}$) oxidation are examples.

Cells detach from a biofilm in a number of ways. Some cells are eroded from the surface of the biofilm as a result of abrasion by particles in the flowing water. Occasionally, large fragments of biofilm may slough off or be torn away from the biofilm due to shear or internal decomposition. Finally, portions of a mature biofilm may experience a genetically controlled dissolution in which biofilm cells are converted into free living planktonic ones which are released into the surrounding aqueous medium (Purevdorj-Gage et al., 2005). "Taken to the extreme, we may view the planktonic or free-swimming microbial phase primarily as a mechanism for translocation from one surface to another" (Watnick & Kolter, 2000).

The site of accumulation of biofilm is dependent largely on the ability of the bacteria to penetrate the soil matrix. Fluid flow and the nutrient concentration of the aqueous phase as it travels through the tortuous flow paths among the soil particles affect this accumulation. It is typical in "engineered" biofilms that more biofilm is found near the site of bacterial injection than further downstream. With time, this difference diminishes as cells and biofilm fragments released from existing biofilms upstream lodge downstream and establish new biofilm sites (Cunningham et al., 1990; Purevdorj-Gage et al., 2005).

The major contribution to the effectiveness of a biofilm in blocking water flow is the formation of extracellular polymeric substances (EPS). These EPS consist largely of water, containing significant amounts of long chain polysaccharide (alginate, for example), proteins, and even DNA, in some species (Whitchurch et al., 2002). In a mature biofilm, living bacterial cells constitute only a few percent of the total biomass (Dunne, 2002).

○ Starvation Effects & Ultra-Microbacteria

Soil and subsurface rock strata are in general low nutrient environments. Many bacteria, but not all, react to these conditions by adopting a starvation response in which cells, though viable, become metabolically dormant, and reduced in size (increase surface-to-volume ratio). In this "starvation state," bacteria can remain viable for extended periods of time until more favorable nutrient conditions return. There is evidence that the starvation state is the "normal" condition for bacteria living in soil, freshwater, and marine environments (Novitsky & Morita, 1976).

This starvation response may affect the penetration of bacteria through soil or rock in at least two ways. The size reduction already mentioned permits easier penetration of the pores in the soil or rock structure. In addition, many starved bacteria alter the array of "sticky" exopolysaccharides displayed on their surface. Although studies differ, many indicate that these adhesins are reduced in starved cells, thus permitting further penetration into the soil or rock matrix (Lappin-Scott & Costerton, 1990; Cunningham et al., 2003).

Some experiments have shown the increased efficiency of penetration of starved cells over vegetative ones. In one experi-

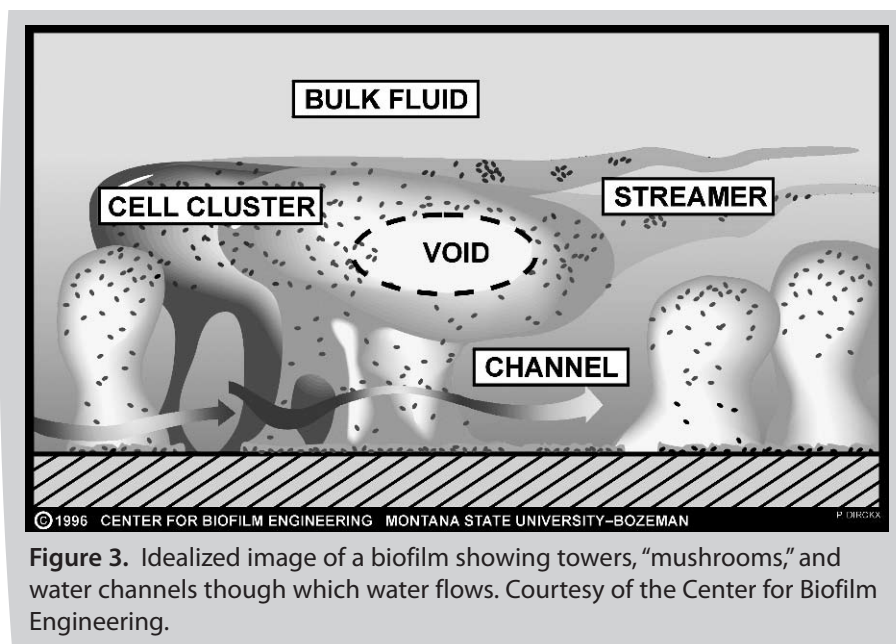


Figure 3. Idealized image of a biofilm showing towers, "mushrooms," and water channels through which water flows. Courtesy of the Center for Biofilm Engineering.

ment, for example, Sharp et al. (1999) compared the penetration of vegetative and starved cells of a slime-producing strain of *Klebsiella oxytoca* through a silica sand column 50 feet high. Most vegetative cells accumulated in the first four to six feet of the column and their numbers decreased by orders of magnitude further down the column. At the outlet, 50 feet from the injection point, 4%-37% of the starved cells were recovered while the vegetative cells were recovered in numbers more than an order of magnitude lower.

○ Laboratory & Field Tests

Studies of the potential for bacteria to be used to construct biobarrriers have been carried out on a variety of materials and in various geometric configurations. The most uniform material commonly used in these laboratory systems is glass spheres, usually of 0.1-1 mm in diameter. Other studies employed sand, consolidated sand, soil, crushed brick, and cores of sandstone or limestone rock. A column packed with the selected material is charged with a bacterial population and fed an appropriate medium. The bacterial population produces EPS that adheres to the matrix particles and reduces the pore volume. This results in a reduction in the flow of water through the matrix, often of more than 99%. The EPS has a strong affinity for organic molecules and particulates in the bulk water. Passage through the biofilm dense column reduces organic and particulate concentrations greatly, a fact that is put to use commercially in water and wastewater treatment facilities (Cunningham et al., 2003).

In a Butte, MT field scale study begun in 1999, a test cell pit 40 m wide, 56 m long and 6.1 m deep was constructed with appropriate input, effluent, and sampling ports to detect groundwater flow through the system. The test cell was filled with a coarse-grained soil and flow rate (hydrologic conductivity) through the system was determined to average 4.1×10^{-2} cm/sec. The test cell was then injected with a highly mucoid strain of *Pseudomonas fluorescens* and fed a nutrient solution containing molasses as the carbon and energy source, and nitrate which served as both the nitrogen source and terminal electron acceptor. The system was fed multiple times over the 22-month course of the experiment. Within 100 days, flow rate had dropped to 9.6×10^{-1} cm/sec— a 98% reduction (Cunningham et al., 2003).

THANK YOU SUSTAINING MEMBERS!

In 1984, it was discovered that fuel lines exiting an underground tank storing gasoline were leaking and that over 10,000 gallons of regular leaded and premium unleaded gasoline had been lost. The tank was located at an NEX service station at the Naval Base Ventura County, Port Hueneme, CA. Although the leaking tanks were removed, by 2004 the gasoline entering the groundwater had produced a plume measuring 150 m wide (~500 ft) and 1500 m (~5000 ft) long. A significant component of this contamination plume was methyl tertiary butyl ether (MTBE), a compound added to gasoline in varying proportions as a fuel oxygenate. Unlike many of the volatile components of gasoline called BTEX (benzene, toluene, ethylbenzene, and xylene) MTBE is more water soluble and highly resistant to natural biodegradation mechanisms in groundwater and soil.

Beginning in 2000, investigators from Equilon Enterprises LLC, Arizona State University, and the Naval Facilities Engineering Service Center constructed a reactive microbial barrier intended to slow the rate of flow of the MTBE containing groundwater and to reduce the concentration of MTBE using a microorganism capable of completely mineralizing this compound to carbon dioxide and water. A grid of injection and sampling wells 500 feet long was drilled completely across the contaminated groundwater plume at varying depths corresponding to the depths of the contaminated aquifer. Using these wells, samples could be obtained from the experimental field.

Several strategies for reducing the MTBE concentration were attempted. These included injection of air into the contaminated ground zone, injection of oxygen, and injection of oxygen and a known MTBE-degrading microorganism, a species of *Rhodococcus*. The injection of air and oxygen only was intended to examine the effectiveness of possible native degraders in reducing the concentration of MBTE. It was expected that these bioaugmented sites would not only reduce the flow of contaminated groundwater by reducing hydrologic conductivity but would also lower the concentration of MTBE by metabolically mineralizing the compound. Significant decreases in the MTBE concentration in the groundwater plume were achieved within 30 to 60 days. Upstream MTBE concentrations ranged from 1000 to 10,000 $\mu\text{g/l}$ while concentrations downstream from the biobarrier were reduced from 50 $\mu\text{g/l}$ to less than the detectable limit of about 1 $\mu\text{g/l}$. Concentrations of MTBE were also reduced in the plots treated with oxygen only, presumably reflecting the activity of native degraders. The concentration of MTBE was reduced to less than 100 $\mu\text{g/l}$ and this reduction was achieved only after 240 days of operation (Johnson et al., 2003).

○ **Reactive Biobarriers**

The ability to create subsurface zones containing bacteria that alter the movement of ground water admits another strategy for remediating toxic spills. It has been demonstrated that one can create biofilm-forming consortia that will reduce the concentration of the toxic compound concerned as well as its spread. This sort of bioremediation strategy is called a reactive biobarrier. Unlike static biobarriers that simply reduce groundwater transport, reactive biobarriers must be “loose,” that is, in order to effectively reduce the concentration of the offending compound, the material must flow past the metabolically active microbial population. The complete limitation of groundwater transport would be counter-productive. The bioremediation of the MTBE spill at Port Hueneme, previously described, is a classic example of the application of this technology.

Laboratory field tests and field trials have demonstrated the effectiveness of this strategy in reducing concentrations of crude

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oil, aromatic hydrocarbons (benzene, toluene), polycyclic aromatic hydrocarbons (naphthylene, phenanthrene), and chlorinated cyclic hydrocarbons (pentachlorophenol). Other experiments have demonstrated the ability of bacteria to immobilize metals (mercury, zinc, manganese, selenium) and to remove other inorganics such as nitrate from groundwater.

○ **A Classroom Exercise Demonstrating Biobarrier Technology**

The exercise described here represents a safe and inexpensive method for introducing biofilms and biobarrier technology into the undergraduate or precollege curriculum either as a classroom exercise or as the focus of an independent student project. It demonstrates the close relationship between microbiology, environmental engineering, and environmental protection and shows how biofilm grown in a porous matrix can reduce underground water movement and the transport of any contaminants the water might be carrying.

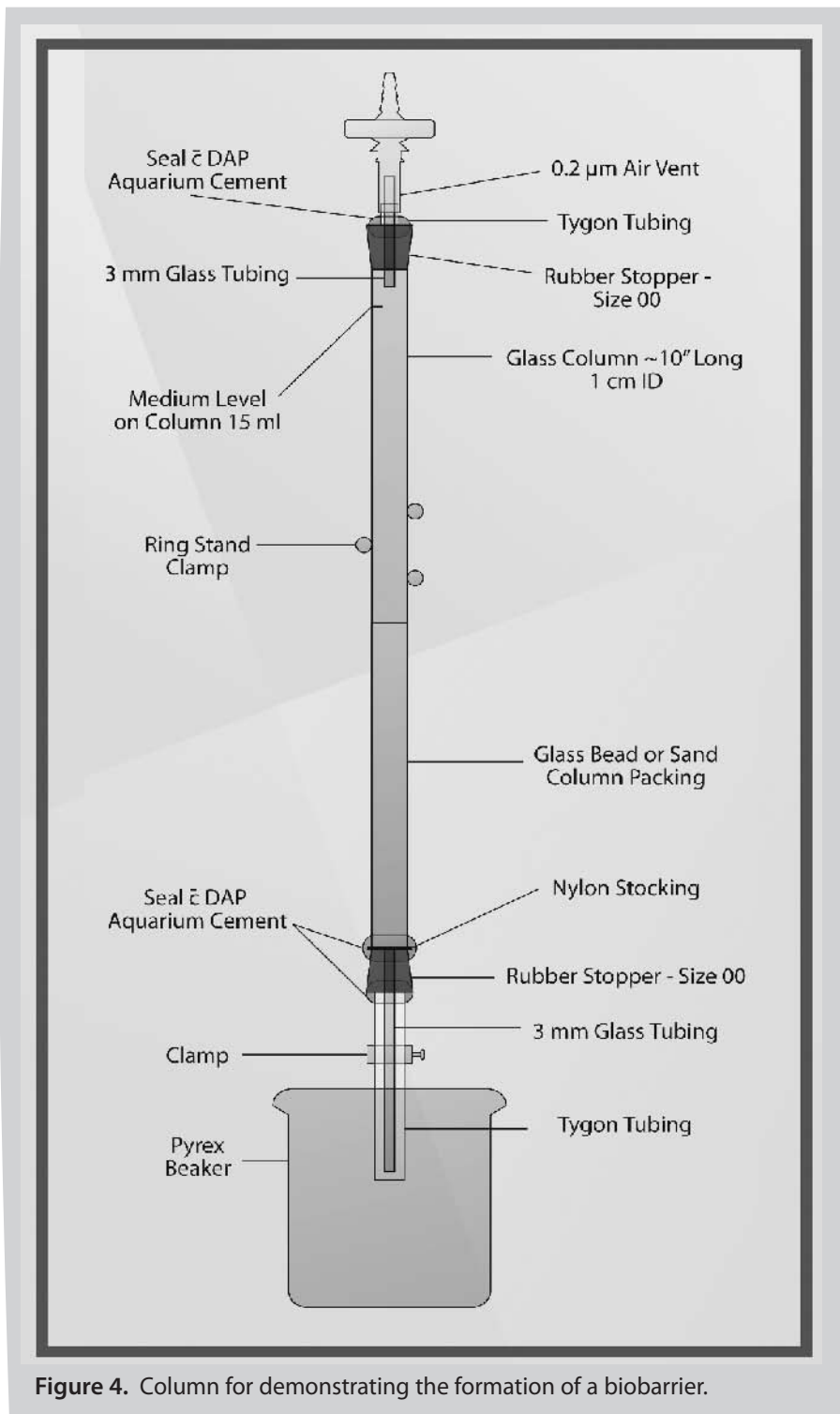


Figure 4. Column for demonstrating the formation of a biobarrier.

One of the defining characteristics of biofilm is the production of a slimy matrix made of polysaccharide, protein, and sometimes DNA. This extracellular polymeric substance (EPS) has often been considered a nuisance, particularly when it accumulates on the hulls of ships, in industrial pipes, or on teeth. More recently, microbiologists and engineers have been looking at biofilms and their EPS production as a means of solving serious environmental pollution problems. When injected into the soil and fed an appropriate diet, organisms will grow and plug the interstices between soil particles with EPS. If drill holes are sited in advance of a contaminated groundwater plume, this “biobarrier” can significantly reduce the rate of flow.

○ Materials & Methods

This demonstration of biobarrier technology employs a column partially packed with tiny glass beads or sand. A significant space (head space) is left above the packing for medium so that the rate of flow through the column can be measured. The column is inoculated with a microorganism and fed a medium formulated to produce large volumes of EPS. At intervals the column is filled with a measured volume of fresh medium, and the time required for the medium to drain from the column is measured. The rate of flow (ml/sec) is a measure of the efficiency with which the flow of medium is being retarded. The effects on flow rate of various media, sand pore size, BSL-1 organism, temperature, etc. can be measured by different student groups. Alternatively, flow rate measured by the entire class in multiple trials can serve as the basis for statistical analysis.

Notes on Safety: Latex or other gloves are highly recommended for all procedures involving the addition of culture or medium to the column and in measuring flow rate. It is strongly suggested to use only BSL-1 organisms for this exercise. As a precaution and to guard against “catastrophic failure” of the column or spillage, it is advisable to place the entire apparatus in a pan or tray capable of containing the entire volume of the column and waste disposal beaker. We have not had such a failure but consider this precaution prudent. Students and technicians should wear hand and eye protection during the cutting of glass tubing and the fitting of this tubing into rubber stoppers.

○ Detailed Instructions

In an advanced class you could have students assemble the columns themselves. In an introductory class it might be best to assemble the columns ahead of time. This is a complex preparation but once the columns are made they are reusable.

1. From 1 cm flint glass tubing stock, cut a section of glass tubing to form a column approximately 10-12 inches (25-30 cm) long. Grind or fire-polish the column ends. Into each end of the column, fit a Number 00 one-hole rubber stopper.

Each stopper should have a glass or autoclavable plastic tube extending beyond the end of the stopper (see Figure 4).

2. Column top: During draining of the tube, sterile air must enter the top of the column to maintain the tube as a monoculture. To allow sterile air to enter the column, fit a bacterial air vent 0.2 μm pore size (e.g., Pall Life Sciences # 4210) to the rubber stopper at the top of the column using a plastic tubing sleeve (Figure 4). As an alternative to using the air vent, extend the length of the glass tube and lightly pack it with roll cotton. After autoclaving, this will act as a bacteriological filter.

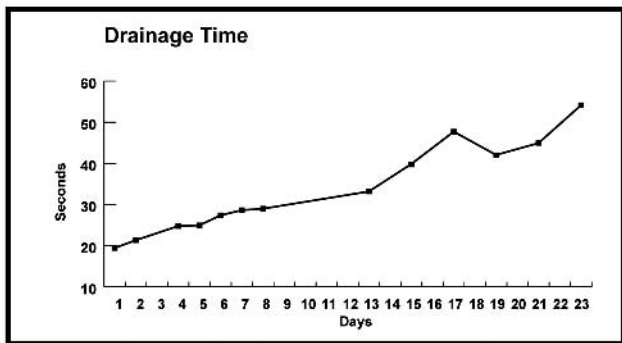


Figure A

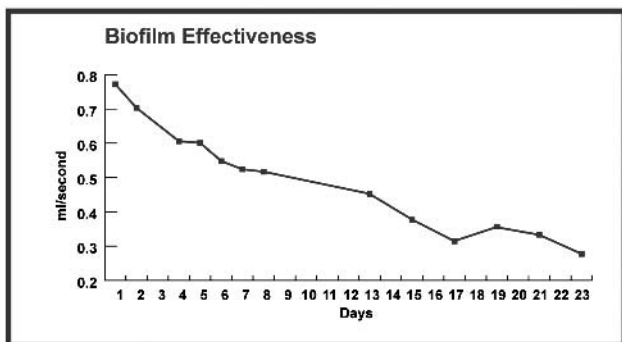


Figure B

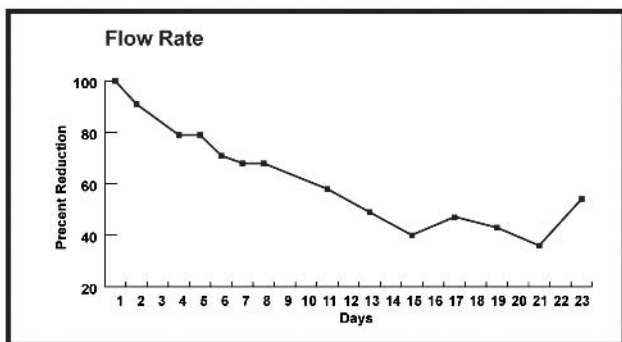


Figure C

Figure 5. Average drainage time, biofilm efficiency, and flow rate of two biobarrier columns as a function of days post inoculation.

- Column base: When inserting the lower rubber stopper, place a small square nylon stocking between the column and the rubber stopper. Trim the nylon as close to the column as possible. This barrier holds the packing material in the column and out of the delivery tube. Attach an 8 cm length of flexible plastic tubing to the glass tubing extending from the bottom stopper, and tighten a screw clamp on this tubing (see Figure 4).
- Using a double thickness of aquarium cement, seal the bottom stopper to the column, and seal the plastic tubing to the glass tubing (see Figure 4).
- Pack the lower 8 cm of the column with glass beads or washed builders sand. The smaller the particles, the more quickly one will see the biobarrier effect. Close the clamp and add just enough water to fill the column to the surface of the packing material.

- Add 15 ml of water to the column and mark the 15 ml level with a permanent marker or piece of autoclavable tape. Then drain the column.
- Cover the lower delivery tube and the top of the column (including the filter) with aluminum foil and autoclave.
- Fix the sterile column securely in a clamp attached to a ring stand.

The Culture

- Any bacterium that produces abundant EPS will serve as a biobarrier agent, but for this application we recommend using only BSL-1 agents. The organism used in this study was *Pseudomonas fluorescens*. Grow the organism up in an overnight culture in molasses medium (see formula on page 26) on a shaker at room temperature. **Note:** Many “domesticated” strains, grown through hundreds of passes in the laboratory, lose much of their ability to form biofilm. If you are unsure if your culture produces sufficient “slime,” grow it up on an agar plate. Touch the top of a colony with a sterile loop and lift. The presence of a slimy stringy mass indicates that the culture is a likely candidate.

Lab Period One

- Add enough of the overnight bacterial culture to the top of the column to bring the level to the 15 ml line previously marked. Remove the clamp while holding the plastic tubing closed. Release the plastic tubing, and with a stopwatch record the time taken for the culture to reach the surface of the packing material. Record this value as the pre-biofilm drainage time. Re-attach the clamp and incubate the column at room temperature until the next laboratory period. Make sure the medium is just at or slightly above the packing material surface. Add more medium if necessary. All waste medium should be collected in appropriate containers (e.g., 150 ml beakers), and disposed of by autoclaving or disinfection (e.g., chlorine bleach).

Lab Period Two

- Add molasses medium to the column up to the 15 ml mark. As before, remove the clamp while holding the plastic tubing closed. Release the tubing and record the time taken for the medium to just reach the surface of the packing material. We find it convenient to provide the molasses medium in individual 20 ml aliquots in capped culture tubes. Dispose of the waste medium as described previously. With growth, one should see the drainage time lengthening. This can be recorded in seconds (Figure 5A), but recording ml/sec may be a more useful measure of biobarrier formation (15 ml/drainage time in sec = ml/sec; see Figure 5B). One can track flow rate as a percentage by dividing the drainage time for any given trial by the original, pre-biofilm drainage time (Figure 5C).

Subsequent Lab Periods

- Continue to take readings on the flow rate of the column as long as any significant change is noted. The data from multiple columns can be used to introduce some basic statistics, or this basic protocol may be modified to introduce variables for independent investigations. Some variables that might be explored are the effect of matrix pore size, the effectiveness of different organisms or combinations of organisms, medium composition, growth temperature, and the effect of treatment with biocides (e.g., 10% Clorox®).

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- 0.04 g/l KH_2PO_4
- 1 g/l NaCl
- 3 g/l $NaNO_3$
- 2 g/l NH_4NO_3
- 0.005 g/l yeast extract
- 1 l distilled or deionized water

The exercise described here is given in greater detail, including a version for student use at Biofilms Online, as part of a growing collection of biofilm related materials and exercises sponsored by the Center for Biofilm Engineering. See: http://www.biofilmsonline.com/cgi-bin/biofilmsonline/ed_biofilms_biobarriers.html.

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JOHN LENNOX is Professor of Microbiology (Emeritus), Penn State Altoona College, Altoona, PA 16601; e-mail: jel5@psu.edu. JEFFREY ASHE is

a former undergraduate student at Montana State University, The Center for Biofilm Engineering, Bozeman, MT 59717.