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Series Editor: J. William Costerton

The Biofilm Primer

Volume Author: J. William Costerton

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Series Editor and Volume Author:

Dr. J. William Costerton
Director, Center for Biofilms
School of Dentistry
University of Southern California
925 West 34th Street
Los Angeles, CA 90089
USA

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Preface

Most human activities require a framework that may begin with a kindergarten, extend through sports, and culminate in the medieval institution of a university devoted to teaching, scholarly pursuits, and physical and emotional aggrandizement of its members. There is a certain pleasant symmetry in fitting into this framework being seen as a competent scholar, a journeyman athlete, and a member-in-good-standing of a collegial group that teaches bright youngsters and extends the boundaries of human perception. You play the game by its sensible and evolving rules, the endorphins flow, and you pass contented through the "seven stages of man."

I was blessed to have chosen a warm and wonderful wife who would let me disappear to climb a mountain, or write a grant, and then have our wonderful children all excited to "do something interesting" when Daddy returned. Janet Halliwell customized science funding in Canada, my lab at the new and vigorous University of Calgary grew to more than 40 people and multimillion-dollar funding, and Kan Lam managed the whole group so effectively that we drove the biofilm field forward with 38 refereed papers in a single year (1987). The pace was frantic, the team was winning and the atmosphere heady, and we poured over the goal line like a rugby team on steroids. But the rules of the game limited us to detailed incremental papers and tightly referenced reviews, biofilm perceptions jerked forward unevenly with provocative data in fields as diverse as pipelines and veins, and I woke up one morning and realized I was bored.

At the age of 58, and acutely bored with incremental science in the framework of the single investigator lab, I received an exciting invitation to replace the charismatic leader and founder of the Engineering Research Center (ERC) at idyllic Montana State University. The engineers taught me how to bring a field forward by conducting well-designed experiments that allow generalization and by an ingenious iterative process in which you cycle between concepts and applications until they fit. At Montana State the best all-round scientist I will ever know, Ann Camper, let me "poach" the research of good students and postdocs in her lab, so I didn't have a lab of my own but I got to drink coffee with a succession of young geniuses—you know who you are! I was flying again, I consorted with a mobile cluster of "young turks," I brokered ideas among people of the stature of Pete Greenberg and Buddy Ratner, and

the biofilm concept that lies at the center of this book began to take shape. It is an engineering concept, with a scientific base, and it is meant to solve practical problems and to provide a coherent rationale for research in the field. Lynn Preston runs the ERC program at the NSF, and she rubs the noses of errant ERC directors in wet newspaper, until they embrace this engineering "systems" approach—bless her.

Hal Slavkin hired me, in the School of Dentistry at the University of Southern California, because he endorses the biofilm concept and wants to see it applied in all fields of dentistry and medicine. This will happen, and the team is being assembled, but the serendipity is awesome because Ken Nealson is here and because USC has made a "cluster hire" of the brightest and best microbial ecologists whose modern techniques are used to analyze the microbial populations of the oceans. So I stand on a peak in Darien, on West 34^{th} Street, from which I can see buildings in which modern microbial ecologists will use molecular techniques to analyze bacterial populations and brilliant engineers will invoke combustion theory to model biofilm growth. From my fourth-floor aerie I can also see buildings in which microbiology students will earn PhDs without ever seeing a real bacterial population under a microscope and in which specimens from biofilm infections will be streaked on agar plates on which they will not grow. All concerned are good people who play by the rules of their academic frameworks, but they operate in isolation. Some of them must be wrong, very wrong, and the consequences are far from trivial. Hence this diatribe. Hence this manifesto. Hence this blueprint for a new framework and this primitive map for a way forward for microbiology.

October 2006 J. William Costerton

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Introduction

The origins of the sciences of microbiology and virology are sharply different from those of other biological sciences. While intrepid explorers dissected animals and studied their behaviors in exotic locations, and English vicars described hedgerow plants in loving detail through their gentle seasons, microbiology emerged from the fetid fever hospitals of Europe in the mid-1880s. In these grim times, when millions were dying of plague and children were suffocating with diphtheria, the objective was not to describe bacteria as biological entities but to control their depredations on the human race. The mindset and the methods of the early heroes of microbiology were distillation of data and reduction to a useful conclusion, and they thought of themselves more as detectives (de Kruif 1926) than as cloistered academics contemplating the structure and habits of viruses and bacteria.

The continuing strength of microbiology and virology and mycology has been and still is in the protection of man, and his domestic plants and animals, from diseases caused by specialized pathogens. For more than a century we have trained hundreds of thousands of medical and veterinary microbiologists, and large numbers of plant pathologists, and this small army has virtually eradicated the diseases whose causative agents they have so assiduously detected and controlled. These microbe hunters were schooled in Koch's postulates (Koch 1884), the first of which demands the isolation of the pathogen in pure monospecies culture (Grimes 2006), and arcane art forms emerged in which practitioners vied with each other to grow specific pathogens in various complex media. Transport media were developed for the recovery of such pathogens as *Legionella pneumophila*, egg-based media were developed for the growth of *Mycobacterium tuberculosis*, and microbiological gatherings came to resemble recipe exchanges. This relentless focus on the recovery and growth of specific pathogens was successful in that vaccines and antibiotics have been developed for the control of virtually every bacterial or viral scourge, and the stated objectives of the early microbiologists have been largely achieved.

The recovery and culture methods that served the disease detectives so well have been much less successful in the study of the structure and behav-

ior of viruses, bacteria, and fungi in the communities in which they actually live. Because bacteria are not visible to the unaided eye, and because light microscopy presented us with mind-numbing complexity, we have trolled through complex bacterial populations and have grown what we recovered in the same cultures used in medical microbiology. In its infancy the field of microbial ecology benefited from this reductionist approach, in that the metabolic machinery of nitrogen fixation could be studied in bacteria recovered from ecosystems in which this process had been shown to be both operative and important. We studied cellulose digestion by a bacterial species recovered from the bovine rumen, but we found that we could not extrapolate back to the functional organ in the animal, because this organism was part of a complex community of which we only studied one or two members. The metabolic machinery of cellulose digestion was operative in the cultured organisms, and the active enzymes were the same as those that digest cellulose in the rumen, but the metabolic partnerships that control rates and feedback loops in the real system were missing. Marine microbiologists concluded that less than 1% of the different bacteria they distinguished on the basis of morphology actually grew in any type of culture, and most of the species groups detected by modern DGGE techniques fail to grow in any type of medium. A junior student at the Center for Biofilm Engineering probably said it most succinctly when she said that recovery and culture is like running a rake through soil and bushes and trees along a trail, shaking the rake above some potting soil, and basing your study on the plants that grow up in the greenhouse at 37 °C.

This book, and the whole series of biofilm books that will be published by Springer, is based on our understanding of the structure and behavior of bacterial communities that is drawn from the direct examination of these communities. We have, in essence, used new microscopic and molecular techniques to walk along the path and peer intently at the soil and the plants, and to study the whole complex integrated community, not just the seeds and propagules.

1 Direct Observations

In the traditional microbiological recovery and culture techniques, the assumption is made that each living bacterium in the sample gives rise to a colony, following placement on the surface of agar containing suitable nutrients, and incubation under suitable conditions. This assumption breaks down if the medium or conditions are not permissive for growth, if the cells are aggregated or if several are attached to the same particle, and if any cells are not in a physiological state that permits their rapid growth in the water film on the agar surface. The development of culture systems has usually been driven by our urgent need to grow a particular human pathogen, for purposes of diagnosis and etiological studies, and the system developed by the CDC to grow cells of *Legionella pneumophila* provides an excellent example. When elderly gentlemen sickened and died in that ill-fated hotel in Philadelphia, every effort was made to develop transport media and culture media that would grow this elusive pathogen, and success crowned these labors, but we still cannot grow most of the bacteria in air-conditioning systems. Quite simply, we develop media and culture systems for specific pathogens, as they impinge on our lives, but no one pretends that we can culture all or even most of the bacteria in any given ecosystem. For these reasons, we have developed media and methods to grow most human animal and plant pathogens that cause diseases in which they clearly predominate, but we lack the media and methods to grow more than 1% of the organisms that cause multispecies diseases or simply occupy natural ecosystems. In spite of their narrow focus, these traditional methods have the advantage of yielding continuing cultures of organisms that can be speciated on the basis of their metabolic properties, and whose properties (e.g., antibiotic sensitivity) can be determined in subsequent tests.

Direct observations of microbial biofilms have recently been facilitated by the application of confocal scanning laser microscopy (CSLM), by the development of optically favorable flow cells, and by the proliferation of specific probes to determine species identity and viability. Direct observations of bacterial populations have always constituted the gold standard of bacterial enumeration in natural ecosystems, especially when the cells were stained with

acridine orange, but the CSLM now allows us to count bacteria on opaque surfaces. Our ability to visualize bacterial cells on opaque surfaces such as plastics and tissues provides solid and unequivocal data on bacterial numbers, because the observation is direct, but it also provides information on the mode of growth of the organisms. Bacteria may simply adhere to surfaces as individual cells or they may grow in matrix-enclosed biofilms, in which their Brownian motion is constrained and they are separated by distances ranging from 3 to 10 μm. Phase contrast light microscopy can be equally useful in the determination of the numbers and the mode of growth of bacteria if fluid from a single- or mixed-species system is simply passed into a modern flow cell with an optically correct coverslip as one of its structural components. The usefulness of these numerical and spatial data can now be enhanced by the use of antibodies or 16 S-directed oligonucleotide probes to identify cells of a particular species, and by the use of a live/dead probe that determines the membrane integrity of each individual cell. We can now state unequivocally that direct observation techniques yield accurate data on bacterial cell numbers, mode of growth, species composition, and viability in both planktonic and surface-associated microbial populations.

While modern direct microscopy techniques are clearly well honed and ready to replace culture techniques, in the study of the etiology of disease, the new molecular methods that microbial ecologists use in population analyses of natural ecosystems are equally poised for adoption. These molecular techniques share an advantage with culture techniques in that they examine bacterial populations within large volumes and yield data on the relative prevalence of species in whole ecosystems. While polymerase chain reaction (PCR) is not notably quantitative, the denaturing gradient gel electrophoresis (DGGE) technique is more sensitive and more quantitative, and it yields "bands" that correspond to the species that are present in the whole sample (Amann et al. 1995). The DGGE technique is now being widely applied, in medical and dental fields as well as in ecology, and it is being refined by the production of clone libraries (Burr et al. 2006) and by the replacement of simple gels by high-pressure liquid chromatography (HPLC) (Liu et al. 1998). A useful link can now be made between the molecular techniques and direct microscopy, in that DGGE and related methods can yield information on the 16 S rRNA sequences of the species present, so that 16 S rRNA probes can be constructed for fluorescence in situ hybridization (FISH) analysis using direct microscopy. Now that we can map a bacterial population in situ in infected tissues and gather accurate data on the number, species identity, viability, and mode of growth of all of the organisms present there seems to be little value in extrapolating from cultures of the species that happened to grow when the system was sampled.

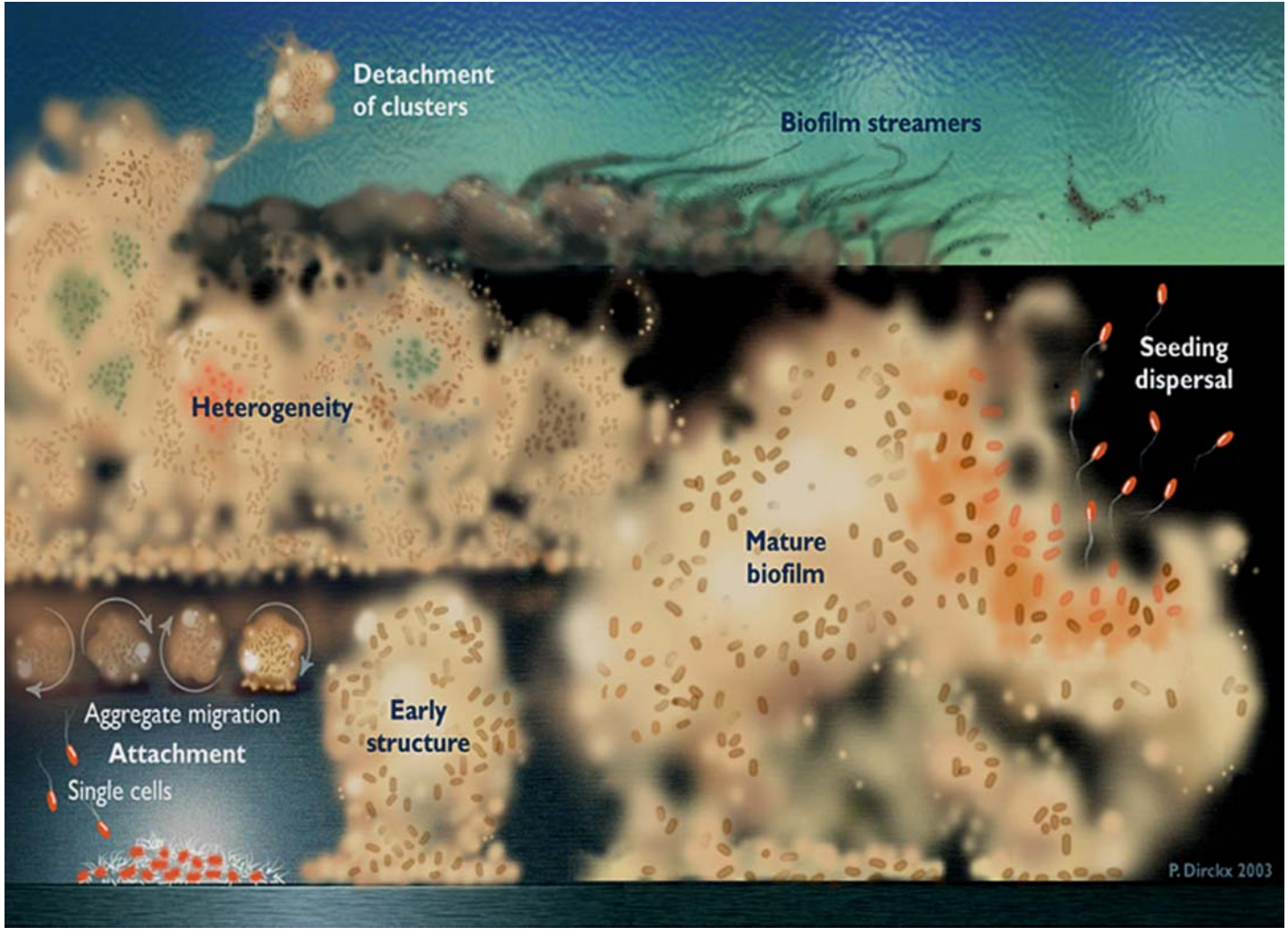
We sometimes discount direct macroscopic examinations of surfaces, when we are accustomed to high-tech microscopy, but the simple observation that cobble surfaces are covered with clear slime actually alerted us to

the preponderance of biofilms in alpine streams. The slime could be recovered by scraping with a penknife, our fingers told us that it was slippery while our noses told us that anaerobes seemed to be absent, and simple observation with a dirt-encrusted field microscope in direct sunlight introduced us to our first natural biofilm! Simple logic encourages us to favor direct observation over extrapolation, but recent studies that document the failure of recovery-and-culture methods tip the balance even more clearly in favor of the new methods of direct observation and molecular analysis. In a recent study of human vaginal microbiology (Veeh et al. 2003) and of "aspectic loosening" of the acetabular cups used in orthopedic surgery (see details in Sect. 4.3), it became apparent that bacteria living in biofilms on healthy or diseased tissues simply fail to grow when they are placed on the surfaces of agar plates. While this failure of biofilm cells to grow on plates is important, our primary contention is that all culture methods are complicated by factors that result in "counts" that are lower than the number of cells actually present, and that direct observation by suitable microscopic methods is the real "gold standard" of quantitative microbiology. My few desultory attempts to explain "most probable numbers" to engineers, who put man on the moon using very real numbers, have met with more confusion than censure, but it is probably high time that we abandon this arcane practice and embrace direct observation.

1.1 The Predominance of Biofilms in Natural and Engineered Ecosystems

Biofilms predominated in the first recorded direct observations of bacteria, when Antonie van Leuvenhoek examined the "scuff" from his teeth, and many pioneers of microbial ecology watched biofilms develop as they placed seawater in glass containers. In fact, ZoBell (1943) noted a "bottle effect" in that colony counts of fresh seawater declined steadily as planktonic (floating) bacteria adhered to glass surfaces and were lost to the bulk fluid. Civil engineers interested in wastewater treatment realized that most of the bacteria that removed organic molecules from sewage lived in sessile populations on surfaces, and they produced elegant models that predicted the efficiency of both biofilms and flocs in nutrient removal. But these isolated observations were not collated and coordinated until we declared the general hypothesis of the predominance of biofilms in natural ecosystems (Fig. 1), using a more rudimentary cartoon, in Scientific American in 1978 (Costerton et al. 1978).

Gordon McFeters and Gill Geesey took advantage of their outstanding physical condition to gallop tens of miles into the alpine zones of the Absorka and Bugaboo mountains, where they plated and cultured water from icy streams crashing down boulder fields (Fig. 2a). These cultures yielded only ± 10 bacterial cells per milliliter, but it soon became obvious that rocks


Detachment
of clusters
Biofilm streamers
Seeding
dispersal
Heterogeneity
Mature
biofilm
Aggregate migration
Attachment
Single cells
Early
structure
P. Dirckx 2003

◀ **Fig. 1** Comprehensive conceptual drawing showing (*front*) attachment of planktonic cells and sequential stages of biofilm formation, including seeding and detachment. The capability of migration is illustrated (*left*), as is the tendency to form mixed and integrated microcolonies (*middle*) for optimum metabolic cooperation and efficiency. The kelp bedlike configuration of biofilms found in natural aquatic ecosystems (*back*) is also illustrated, as is the tendency of these communities to detach large fragments under shear stress

in the streams were covered with slippery biofilms, and direct examination of these clear slime layers showed the presence of millions of bacterial cells (Fig. 2b) encased in transparent matrices (Geesey et al. 1977). As so often happens in biology, a general truth was revealed by the fortuitous examination of a simple system in which nutrients were severely limited and in which a single species (*Pseudomonas aeruginosa*) formed biofilms on all available surfaces and released a few planktonic cells that were rapidly removed by high flow rates. When we examined a wide variety of rivers and streams, from pristine oilsand rivers (Wyndham and Costerton 1981) to abattoir effluents, this preponderance (> 99.99%) of biofilm cells was sustained in all of these ecosystems (Costerton and Lappin-Scott 1995), and these sessile communities were shown to be proportionately active in nutrient cycling. Biofilms have since been found to constitute the predominant mode of growth of bacteria in streams and lakes in virtually all parts of the world and in the nutrient-rich parts of the ocean, and these sessile populations have been found to be both viable and metabolically active (Lappin-Scott and Costerton 1995; Hall-Stoodley et al. 2004).

Once the tendency of bacteria to form biofilms had been reported, and the appearances of biofilm matrices in light and electron microscopy described (Jass et al. 2003), ecologists reported the presence of biofilms in virtually every natural environment, from tropical leaves to desert boulders. We were inspired to search for biofilms in engineered water systems, with the objective of understanding and controlling processes like corrosion and fouling, because of the enormous cost associated with these problems to the oil-recovery and water-distribution industries. The gradual decay in efficiency of heat exchangers was linked to biofilm formation on the water side of shell and tube units, the removal of these adherent slime layers returned the exchangers to full efficiency, and several companies now ply the biofilm removal trade in industrial water systems. Pipeline engineers had noted that the physical scraping (pigging) was more effective than the use of biocides in the control of microbially influenced corrosion (MIC) in seawater pipelines. The mechanism of MIC was examined, and we found that biofilms on metal surfaces contain areas of differential metal binding capacity and different electrical potentials (Nielsen et al. 1993), and that simple corrosion cell theory can explain how cathodes and anodes within these sessile communities (Fig. 3) can drive MIC at high rates (Lee et al. 1995). Because biofilms mature and begin the MIC process in a matter of weeks, pipeline companies now scrape

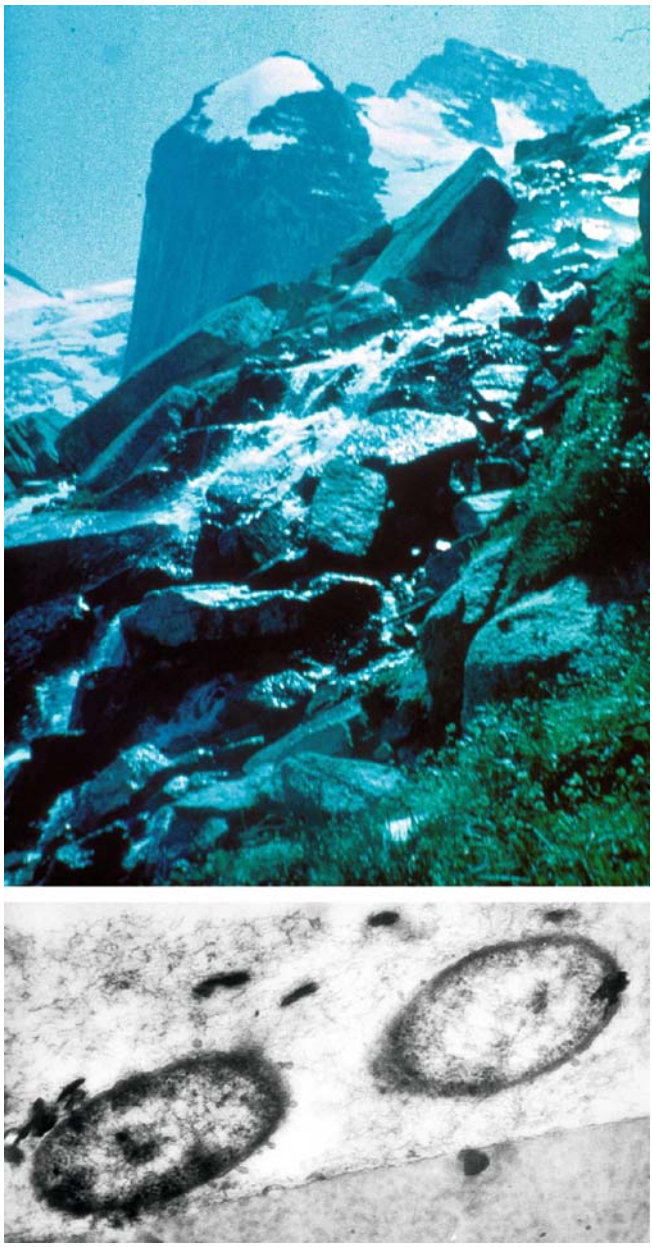


Fig. 2 *Top*: alpine stream under Marmolata Spire in the Bugaboo Mountains of southern British Columbia. *Bottom*: TEM of a section through the microbial biofilm that developed on a methacrylate surface immersed in this stream for 30 min. Note the Gram-negative bacterial cells in an ecosystem that grew only *P. aeruginosa* on culture, the extensive matrix composed of exopolysaccharide (EPS) fibers, and the electron-dense clay platelets trapped by the biofilm

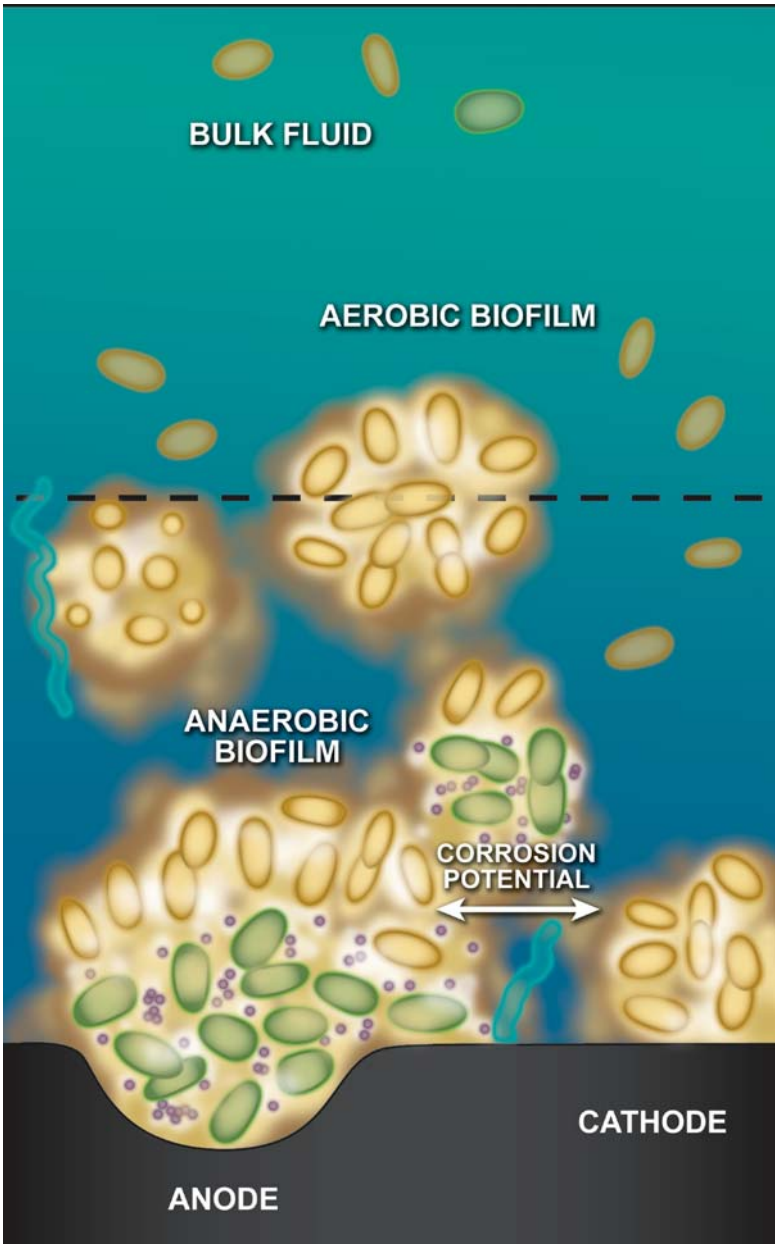


Fig. 3 Conceptual drawing of a multispecies biofilm in whose deeper anaerobic zone a metabolically integrated consortium has developed into an anode, with respect to a neighboring microcolony whose metabolic activities and metal-binding activities have combined to make it relatively cathodic. A corrosion potential has developed between the consortium and the microcolony, in a "classic" corrosion cell, and metal loss occurs at the anode

their lines at regular intervals with pairs of "pigs", with biocide in the intervening fluid, and much less pipe is lost to microbial corrosion. Biofilms also predominate in soils, and the outsides of the same pipes are protected from MIC by the systematic imposition of cathodic protection currents. As we examine more and more ecosystems, from the aerial surfaces of leaves to the ghastly chaos of rumen contents, we always note the predominance of biofilms. We can conclude that the bacteria that live in the biosphere, between the Earth's molten core and outer space, grow almost exclusively in matrix-enclosed communities and that new strategies are urgently needed to study them and to integrate them with the many biological systems currently studied by molecular analysis and direct observation.

Microbial ecologists have embraced the biofilm hypothesis, which states that these sessile communities predominate in the natural and industrial ecosystems of the biosphere, but other bacterial strategies clearly operate in the areas beneath this nutrient-rich crust. Direct observations of the vast nutrient deserts of the deep oceans and the deep subsurface have shown that bacteria adopt a radically different survival strategy in these regions. Dick Morita and his colleagues recovered water from deep oceans and found that it contained very few bacterial cells that could be resolved by ordinary light microscopy, but that the addition of simple nutrients produced direct and culture counts of $\pm 1 \times 10^5$ cells/ml in as few as 20 min (Novitsky and Morita 1976). Further examination produced the fascinating "starvation survival strategy" hypothesis (Fig. 4), which has now been fleshed out and canonized by Staffan Kjelleberg's group (Kjelleberg 1993), in which it is established that starvation triggers the production of very small ($\pm 0.3\ \mu$m) dormant ultramicrobacteria (UMB). These UMB represent a bacterial mode of growth that is antithetical to the biofilm mode of growth in that the cells are naked, nonadherent, and almost completely metabolically dormant (Fig. 4, top and middle) but capable of resuscitation to form normal vegetative cells (Fig. 4, bottom). UMB have now been found, in approximately equal numbers ($\pm 1 \times 10^5$ cells/ml), in groundwater from as deep as 5000 ft (1500 m) below the Earth's surface, and in the abyssal areas of the oceans. Bacteria can thus be seen to have adapted to Earth's biological realities by adopting the starvation survival strategy in the nutrient-deprived regions of the deep oceans and the deep subsurface and by adopting the biofilm strategy in the nutrient sufficient biosphere. The consequence of this remarkable plasticity of the bacteria is that they exist as a vast metabolically dormant genomic reservoir in the nutrient-poor regions immediately underlying the relatively thin layer at the Earth's surface. When dead sailors enter their Spartan ecosystems, they leap into action and, when currents and deep springs carry them to the surface where nutrients are available, they vie with each other and with existing populations for space and reproductive success.

When rare episodes like the injection of carbon tetrachloride into the subsurface, or the sinking of the Titanic, introduce organic nutrients into the

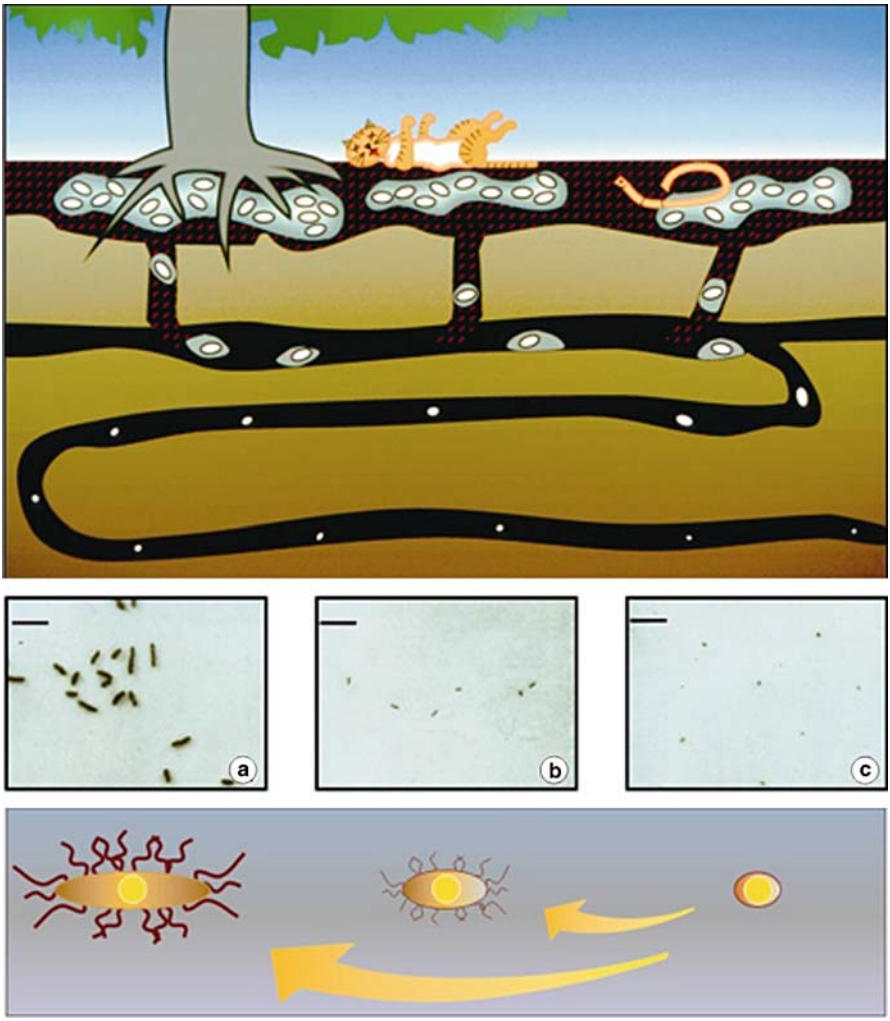


Fig. 4 *Top*: conceptual drawing of biofilm-forming vegetative cells in nutrient-rich upper horizons of soil, which give rise to large numbers of very small starved UMB as planktonic cells are carried down into the nutrient-poor deeper regions. *Middle*: light micrographs of marine vibrio being transformed from vegetative cells (**a**) to much smaller rods (**b**) and to spherical UMB only 0.3 μm in diameter (**c**) by starvation over a 6-week period. From Novitsky and Morita (1976). *Bottom*: cartoon showing resuscitation of UMB to form full-sized biofilm-forming vegetative cells

domain of the UMB, these tiny cells return to their normal vegetative size and resume their tendency to form biofilms (Fig. 4, bottom). We have taken advantage of this starvation-induced shrinkage and nutrient-induced recovery of bacteria to develop a commercial technology for the manipulation of water movement in the subsurface (Fig. 5, top). We select strains of subsurface

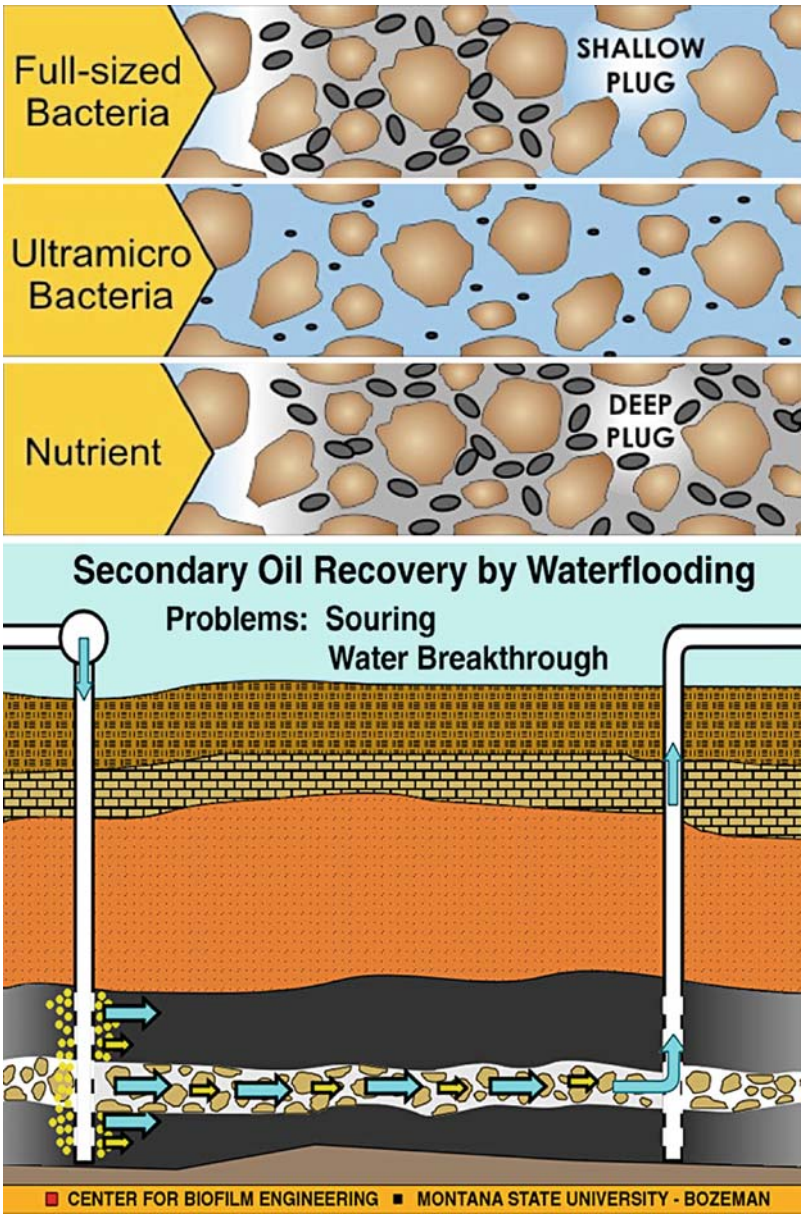


Fig. 5 *Top*: conceptual drawing showing shallow penetration of full-sized vegetative bacterial cells into a porous medium, while UMB can travel (literally) miles through any porous medium > 50 mD in permeability. UMB can be returned to their full size and their full biofilm-forming capability by the addition of nutrients. *Bottom*: this biobarrier technology can be used to plug high-permeability "stringers" that carry injected water past oil deposits, in secondary oil recovery, and the tendency of bacterial biofilms to produce H_2S (*yellow dots*) by the reduction of SO_4 can be controlled by nitrite injection

bacteria, avoiding any tendency to sulfide production or iron deposition, and we grow vegetative cells of the selected strains to very high density in large reactors. The cells are recovered by centrifugation and resuspended in ionically supported distilled water, so that starvation produces very large volumes of suspended UMB that can be transported as stable concentrates. The UMB are injected into the subsurface, where water flow causes problems of pollutant dispersal from point sources, or where the failure of secondary oil recovery is attributed to high permeability "stringers" that carry the injected water past oil reservoirs (Fig. 5, bottom). The UMB are carried as far as 1 km, through any subsurface formation > 50 mD in permeability, and then nutrients are injected by the same route and pumping is suspended to allow the UMB time to return to the full-sized vegetative state (Cusack et al. 1992) and begin biofilm formation. These biofilm "biobarriers" are currently in commercial use for pollutant containment (Dutta et al. 2005), and this technology offers compelling hope that pollutants can be contained and oil can be recovered from established fields that have been abandoned because they were "watered out" (Fig. 5, bottom) (Cusack et al. 1990).

1.2 The Architecture of Biofilms

When microbial biofilms were first visualized, by light microscopy, individual cells could only be resolved in relatively thin sessile communities, and thick biofilms were difficult to visualize with phase contrast optics, especially when they contained crystalline inclusions. Where individual cells could be resolved, it was clear that they were embedded in a translucent matrix that filled the 3- to 6-μm spaces between the cells (Fig. 6) and limited their Brownian movement. Transmission electron microscopy (TEM) of biofilms showed bacterial cells whose structures resembled those of the planktonic cells, but the exopolysaccharide matrices were severely affected by dehydration and could only be resolved if they were stained with electron-dense ruthenium red (Fig. 7). Scanning electron microscopy (SEM) is bedeviled by even more dehydration artifacts than TEM, and attempts to image biofilms were complicated by eutectic bridges that form between cells when their intervening exopolysaccarides are condensed by dehydration (Fig. 8). These bridges appear to connect the cells in biofilms, and they are almost always misinterpreted as intercellular pili. In short, we knew that bacteria lived predominantly in matrix-enclosed biofilms in all nutrient-sufficient ecosystems, but light microscopy was too primitive to reveal the structural details of these ubiquitous and very successful communities, and electron microscopy was fraught with potentially crippling artifacts.

The structural moment of truth came, 15 years after biofilms were seen to predominate in these ecosystems, when we applied confocal scanning