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.

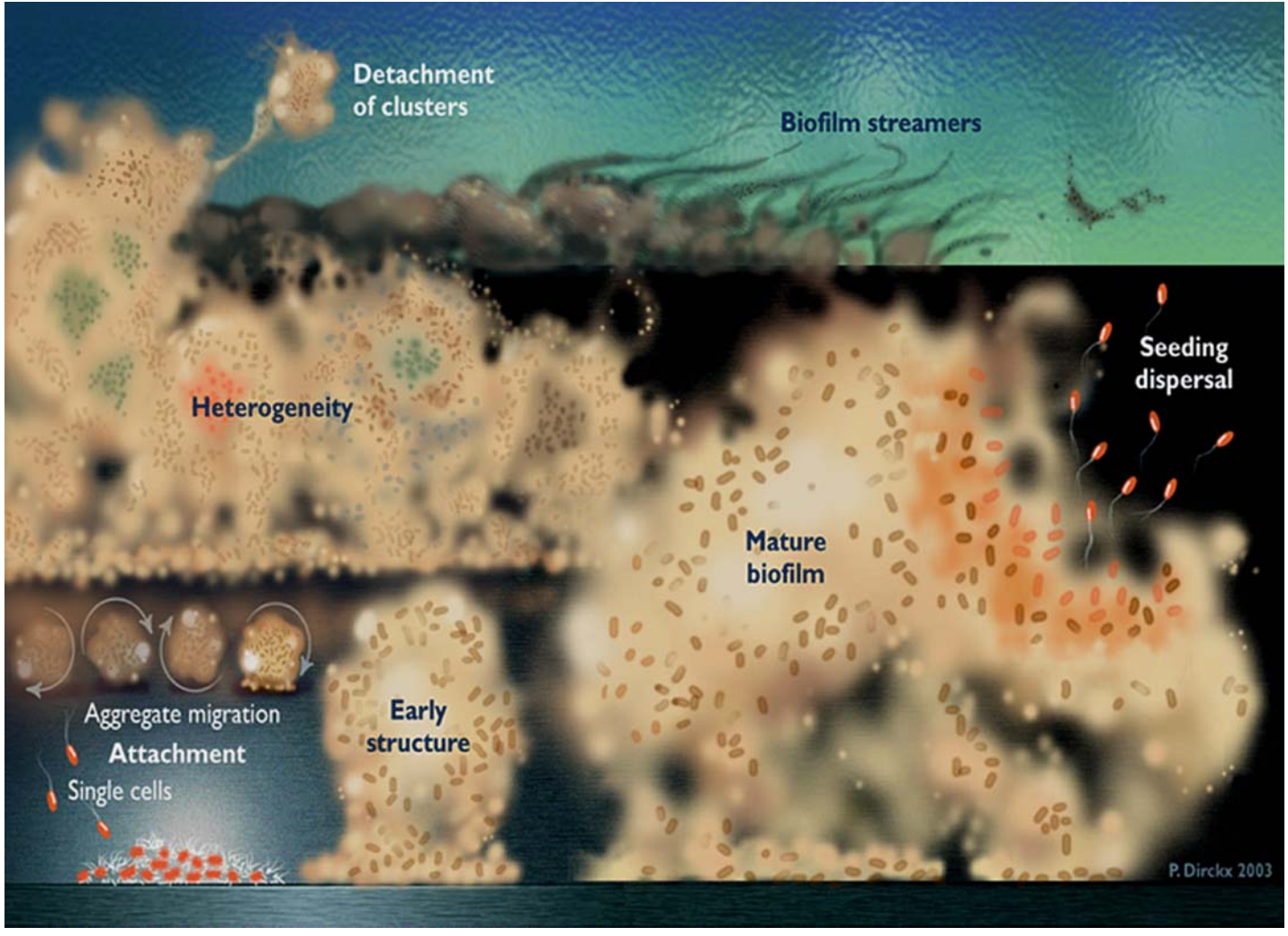
We sometimes discount direct macroscopic examinations of surfaces, when we are accustomed to high-tech microscopy, but the simple observation that cobble surfaces are covered with clear slime actually alerted us to

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