Experimental biofilms and their applications in the study of environmental processes

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Joanna C. Rayner and Hilary M. Lappin-Scott

1. Introduction

1.1. Why Study Biofilms?

The trend in research in recent years has been to extrapolate results from studies of planktonic bacteria into environmental systems. This method of studying planktonic bacteria under in vitro conditions has undoubtedly yielded important data in a wide range of areas; however, the examination of several environmental habitats, extreme or otherwise, such as a drinking water pipeline has revealed only relatively low numbers of planktonic cells. In aquatic systems the biofilm bacterial count per square centimeter of surface has been estimated to be approx 1000-fold higher than the corresponding planktonic count per cubic centimeter ***(1)***. Surface colonization by microorganisms was first recognized as significant as early as 1943 ***(2)***, and there is now a realization that we need to study microorganisms not only as biofilms but also in the context of the biofilm interactions with their immediate surroundings and the influences they exert on the environment. The environment has a significant effect on the metabolic activities of bacteria, and studies of biofilm bacteria represent the best tool for examining growth in natural and pathogenic ecosystems ***(3)***. The study of biofilms is relevant to a wide range of areas, and a multidisciplinary approach is the most productive route forward in the quest to understand the interactions occurring not only between the cells and the surfaces to which they adhere, but between the microcolonies that coexist within multispecies biofilms ***(4)***.

1.2. What is a Biofilm?

A microbial biofilm is essentially microbial cells immobilized at an interface, covered with a microbially produced exopolysaccharide layer. The initial colonization of surfaces and subsequent growth as a biofilm is the bacterial survival response to environmental stimuli such as low nutrient levels ***(5)***, and may occur as a response to the nutrient accumulation that is thought to occur at air-liquid or liquid-solid interfaces ***(6,7)***. Adhesion to a submerged surface by starved *Vibrio* cells resulted in the cells regaining their normal morphology and growth characteristics ***(5)***. However, attachment has also been observed in systems with an increased dissolved organic carbon content ***(8)***, and higher nutrient and substrate concentrations alone are insufficient to explain the overall effects of surfaces in terms of bacterial activity ***(9)***. Benefits of the attached mode of growth include increased protection against antimicrobial agents ***(10–13)*** and the body's defense mechanisms—phagocytosis, opsonization, and so on. Initially the biofilm was viewed as a homogeneous distribution of cells in a confluent, blanket-like exopolysaccharide matrix ***(3)*** but confocal scanning laser microscopy (CLSM) has been the driving force behind altering our understanding of the processes and the structures within the biofilm. This form of nondestructive visualization has allowed the three-dimensional and real-time visualization of hydrated biofilms. Biofilms are now modeled as microcolonies or clusters of cells enclosed within a hydrated matrix, with pores or channels throughout the nonconfluent biofilm ***(14,15)***. The pores and channels facilitate transport of oxygen and nutrients to the microcolonies and removal of waste and secondary products, but the biofilm matrix or exopolysaccharide has a postulated role in antimicrobial resistance, possibly acting as an ion exchange resin ***(11)*** or ionically hindering the inward diffusion of cationic molecules ***(16)***. Far from being a random structure, the biofilm represents an optimized arrangement of cells to facilitate maximal nutrient diffusion to enable the establishment of microbial consortia allowing metabolic exchange and recycling of essential nutrients ***(17)***, and to facilitate the transfer of plasmids enclosing drug and heavy metal resistance as a result of the close proximity of the cells within the biofilm ***(17,18)***. The biofilm has been likened to a primitive eukaryotic tissue, with homeostatic control mechanisms and a high level of physiological cooperativity ***(19)***.

1.3. The Role of Biofilms in Understanding Environmental Processes

Our understanding of environmental systems and processes has largely been obtained through the extrapolation of data obtained from laboratory systems involving planktonic cells; but this is increasingly inapplicable to real ecosystems ***(3,15)***. Direct observations using some of the techniques described in this

chapter have confirmed the dominance of biofilm bacteria, both numerically and metabolically, in nutrient-sufficient environments ***(1,15)***. Data that can be viewed as unequivocal, owing to its acquisition using nondestructive *in situ* techniques, has demonstrated that biofilm bacteria possess a wide range of phenotypical differences in contrast to their planktonic counterparts ***(15)***. Biofilms have enormous potential to be utilized in the biotechnology industry, because they exhibit a number of capabilities such as the ability to localize a specific biological response (biosensors), production of specific metabolic compounds, and an increased level of performance in a reactor system compared to planktonic bacterial cultures ***(20).*** Wastewater treatment systems commonly utilize trickling filter and porous/nonporous fluidized beds in a number of areas such as denitrification, xenobiotic detoxification, and heavy metal removal from water. Biofilm reactors have been developed and utilized for toluene degradation, removal of uranium, and the degradation of hydrocarbons from wastewater systems ***(20)***. Biofilms play an important role in the biodegradation of organic compounds and the transformation of inorganic compounds, subsequently acting to minimize the buildup of pollutants ***(21)***. Most sections of the human and animal gastrointestinal tract are colonized by bacteria that form tissue-protective biofilms, preventing adhesion by foreign bacteria ***(22)***.

1.4. Detrimental Effects of Biofilms

The uncontrolled and undesirable accumulation of biofilms in biomedical and engineering systems has three primary effects: physical damage, e.g., corrosion and tooth decay; reduction in proper function of the surface, e.g., reduced efficiency of heat exchangers ***(7)*** and turbine power losses in hydroelectric pipelines ***(23)***; and the creation of a reservoir of potential pathogens. Biofouling has been defined as damage to surfaces or the environment as a direct result of surface-associated microbial growth ***(23)***.

1.4.1. Physical Damage and Reduction in Surface Efficiency

Metal corrosion of ships, pipelines, and oil rigs is an expensive problem, with biofilm formation occurring rapidly following immersion of the surface. Marine macrobiofilms on ships act to increase drag and frictional forces, resulting in increased fuel consumption; an 18% difference in power consumption was observed in trials to determine the effect of biofilm removal from ship hulls ***(8)***. The physical thickness of the biofilm reduces the pipe diameter in industrial heat exchanger systems, affecting flow, and the exchange of heat between the liquid and the cooling surface is reduced ***(24)***, with a subsequent estimated cost of £500 million annually ***(23)***. Anaerobic zones are formed within the biofilm as depletion of oxygen occurs by the aerobic microorganisms present in the microbial consortium, favoring the growth of primary corro-

sion organisms such because the sulfate-reducing bacteria (SRB) ***(23)***. Physical damage occurring as a result of biofilm formation can also be observed in the body. Dental caries are an indirect consequence of the formation of a multispecies oral biofilm ("plaque") on the enamel surface of the tooth. Demineralization of the enamel occurs as a result of by-products of the bacterial metabolism, such as organic acids, which become trapped at the tooth surface ***(19)***. The oral microflora utilize biofilm formation not only as a mechanism to avoid the antimicrobial action of salivary components such as lysozyme and mechanical removal, but also to facilitate the optimal utilization of the abundant nutrient supply.

1.4.2. Creation of a Reservoir of Pathogens

The primary concern of the food, water, and medical industries is to determine the potential of the biofilm to act as a pathogen reservoir and to develop effective control strategies ***(6,23,25)***. In most cases, planktonic cell counts do not accurately represent the extent to which biofilm formation is occurring. The contamination of food products may occur following contact with potentially detrimental bacteria sequestered within surface-associated biofilms ***(6)***. The accumulation of coliform bacteria in biofilms in water distribution systems may act to mask the presence of indicator organisms occurring as a result of deficiencies in the treatment processes ***(25,26)***. *Legionella pneumophila* has been demonstrated to be harbored within biofilms that would be present in cooling towers and water systems ***(27)***. The recent increase in the use of indwelling medical devices and advances in intravenous therapy can be correlated with a corresponding increase in nosocomial infection ***(28)***. Extensive bacterial biofilms, formed in the presence of optimal growth conditions provided by the nutrient-rich body fluids, have been observed on sutures, cardiac catheters, central venous lines, pacemakers, heart valves, and prosthetic hip joints ***(7)***, and these biofilms may act as sites for further dissemination of infection. The inherent resistance of the bacteria to phagocytosis and antibiotic chemotherapy may result in the surgical removal of infected devices, in order to dispel chronic device-associated infections ***(24)***.

1.4.3. Strategies for Biofilm Control

Treatment regimes against biofilm-associated infections are normally developed using data that measure measures the efficacy of an antimicrobial agent against planktonic organisms, subsequently resulting in ineffectual eradication of the biofilm ***(29–31)***. Biofilm control can be divided into two areas: the prevention of initial colonization and subsequent biofouling, and the development of removal/control strategies against the established biofilm.

The efficacy of several different antifouling coatings and repellents against marine biofilms has been examined ***(32)***, but there must be a balance between efficiency against biofilms and the level of toxicity to other marine life. The use of impregnated or coated catheters has been examined as a method of reducing the incidence of catheter-associated urinary tract infections; inhibition of primary adhesion to silver-coated latex catheters has been observed in vitro ***(33)***. The incorporation of biocides such as 10,10-oxybisphenoxyarsine (OBPA) has been shown to reduce adhesion to polyvinyl chloride ***(34)***. However, it appears that regardless of the surface roughness, charge, hydrophobicity, or incorporated antimicrobial agents, bacteria will eventually adhere to any surface, and the search for a completely effective antifouling/antiadhesion surface is ongoing.

1.4.3.2. Sanitization/Removal Strategies

In industrial systems, chemical biocides represent the primary strategy for biofilm control. Chlorine, in four different forms—monochloramine, hypochlorous acid, hypochlorite, or chlorine dioxide—is the most commonly used biocide for chemical treatment of water. Monochloramine has been found to be the most effective in the inactivation of biofilm bacteria ***(35)***. Environmental factors, such as nutrient loading, shear stress, and physiologic properties of the bacteria (e.g., growth rate and metabolic status), will affect the overall properties of the biofilm, subsequently affecting biocide efficiency. The nonuniform pattern of microbial respiratory activity that occurs following monochloramine treatment ***(36)*** is suggestive of variations in antibiotic penetration rates ***(36,37)*** and the presence of distinctive biocide gradients within the biofilm ***(38)*** suggests that local differences occur within biofilms in terms of resistance to chlorine and other disinfectants. The food industry uses sanitizers or disinfectants following detergent treatment; commonly used chemical disinfectants include chlorine, iodine, and ammonium-based compounds. Antibiotic treatment of device-associated infections is largely dependent on the organism, or organisms, involved. However, the inherent resistance of the bacterial biofilms ***(29)*** may result in the surgical removal of the infected device in order to dispel chronic device-associated infections ***(24)***. There is currently a trend toward the development of methods that will enable the testing of the susceptibility of the organism of interest as a biofilm.

2. Materials and Equipment for Studying Biofilms

2.1. Model Systems for Establishing Experimental Biofilms

The complexity of biofilms and the need to study them under laboratory conditions has led to the development of model systems for the establishment

Table 1
Experimental Variables and Parameters for the Investigation of Biofilms Using Laboratory Model Systems

Variables	Parameter
Physical	Temperature, surface composition, surface charge, surface roughness
Chemical	pH, substrate concentration, dissolved oxygen concentration
Biological	Organism type, organism concentration

and study of experimental biofilms. Model systems enable the testing of hypotheses and the extrapolation of data under defined, controlled conditions. There are two main types of experimental biofilm models ***(39)***: replicative, which encompass a wide range of complex environmental variables, and investigative which are generally simpler and enable the control of a variety of influencing factors. Most laboratory systems are of the latter type and tend to examine biofilm formation at solid/liquid interfaces utilizing fixed surfaces. **Table 1** lists several variables and parameters that can be examined using laboratory model systems, and **Fig. 1** lists some analytical methods for the measurement of biofilm parameters. This section describes only a number of the most common laboratory model systems in use; for further information on other systems, *see* **refs. *4***, ***7***, ***15***, and ***40***.

2.1.1. The Robbins Device

The Robbins device was developed at the University of Calgary to examine biofouling in industrial pipelines *in situ* ***(41)***. Initially composed of brass or stainless steel, it was later modified for use in examining medical device–associated biofilms. The modified Robbins device (MRD) is a rectangular Perspex block 44 cm long, 2 cm high, and 2.5 cm wide, with a 2 mm high by 1 cm central lumen and a series of removable studs placed along its length to which different surfaces can be fitted. The system is sterilized using ethylene oxide gas since high temperature/pressure results in warping of the Perspex. This system allows the examination of a range of physical, chemical, and biological parameters on biofilm formation and analysis of the response of biofilms to antibiotic and biocide treatment.

2.1.2. Continuous Culture Flow Cell

The continuous flow of media prevents the accumulation of waste and metabolic products and the depletion of oxygen and nutrients ***(42–44)*** and subsequently enables the control of the bacterial growth conditions ***(44)***. Several different designs of continuous culture flow cell are currently in use, utilizing

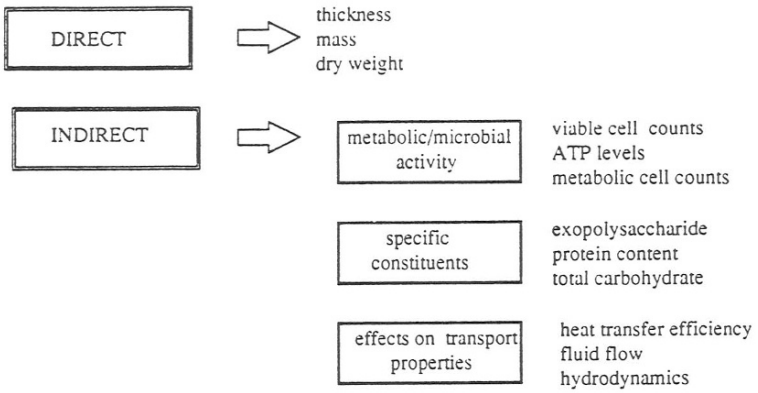

Fig. 1. Experimental variables and parameters for the investigation of experimental biofilms established using laboratory model systems.

materials such as glass and Perspex and ranging from relatively simple, such as two, sealed, glass cover slips with silastic rubber tubing in and outlets, to the more complex. The limitations with the use of flow cells are primarily due to the restriction on the use of transparent surfaces, but when used in conjunction with CLSM, microscopy, image analysis, and metabolic stains, flow cells can provide a great deal of information concerning initial attachment, biofilm structure and functional organization within single and multispecies biofilms.

2.1.3. Perfused Biofilm Fermenter

The perfused biofilm fermenter system was developed to enable distinction between the effects of growth rate and adhesion by selecting synchronous bacterial populations ***(45)***. Midexponential phase bacterial cultures were filtered onto a cellulose acetate membrane; the impregnated membrane was removed and inserted upside down in a continuous fermentation apparatus ***(46)***. Fresh medium was then perfused from below through the filter, with the numbers of eluted cells reaching a steady state after approx 2 h—a situation similar to that of bacterial surface infections of soft tissues. This system has applications for use in examining the effects of antibiotic therapy on soft tissue infections.

2.1.4. Rototorque

Also known as the annular reactor, the rototorque system is composed of two concentric cylinders with a number of removable slides in a continuous culture system. Rotation of the inner cylinder creates a shear field independently of the medium flow ***(47)***. The system is highly sensitive to changes in fluid frictional resistance and is capable of varying fluid shear and stress and

residence times independently. The surface area of the slides are exposed to uniform shear stress values and complete mixing of the liquid in the system enables the analysis of a range of biofilm processes.

2.1.5. Constant Depth Film Fermenter

The constant depth film fermenter is an enclosed fermenter that has been used to examine a river water community ***(48)*** and a *Pseudomonas aeruginosa* metalworking fluid biofilm ***(49)***. It contains a rotatable steel or polytetrafluoroethylene (PTFE) turntable, with a series of removable film pans, each containing six removable plugs. The biofilm is maintained at a constant depth by a scraper blade and, based on protein levels, viable counts, dry weight measurements, and carbohydrate levels, is considered to be "quasi steady-state" ***(40)***. Biofilm formation can be controlled, is reproducible, and is easily sampled under specified nutrient and gas conditions.

3. Methods

3.1. Microscopy

The ability to visualize the biofilm is important in defining the architecture of biofilms and the interactions occurring between the cells and the surfaces. Microscopy has been widely used for the direct visualization of initial attachment and subsequent biofilm formation ***(44,50–53)*** and phenotypic changes following adhesion ***(15,54)***.

3.1.1. Electron Microscopy

The major advantage of electron microscopy is its ability to resolve objects that cannot be seen using light microscopy; the resolution of electron microscopes is approx 0.5 nm compared with the 0.2-µm resolution of differential light absorption microscopes ***(55)***. In electron microscopes, the heating of a tungsten filament generates an electron beam that is focused by a series of magnetic lenses under high vacuum onto the specimen.

3.1.1.1 Scanning Electron Microscopy (SEM)

Unlike conventional bright-field and phase contrast microscopy, SEM does not require a transparent surface. Scanning of the specimen surface by the electron beam causes the emission of secondary electrons that enter the detector and strike a scintillator, generating light flashes, which are converted to an electrical current by the photomultiplier. Subsequent amplification and transmission to a cathode ray tube produce a raster display image ***(55)***. The number of electrons detected is dependent on the surface topography; the presence of depressions causes electrons to be trapped, and consequently, the area appears darker compared to raised and therefore lighter areas. Sample preparation

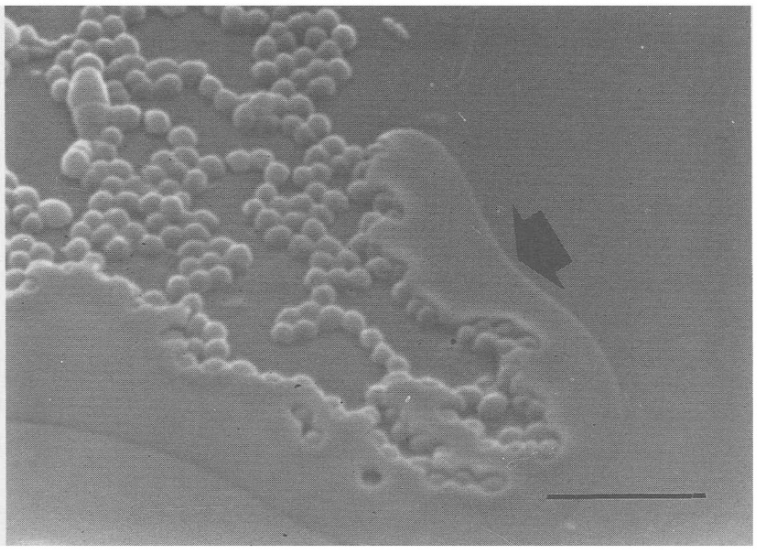

Fig. 2. Laminar flow biofilm (flow rate of 0.8 mL · min^{-1} formed on glass surfaces in a flow-through culture MRD and visualized using SEM. The *Enterococcus faecium* biofilm was 72-h old; microbially produced exopolysaccharide is visible as a blanket-like layer (black arrow), revealing underlying coccoid bacterial cells. Scale bar = 5 μm.

involves the fixation of the surface and attached biofilm using glutaraldehyde or formaldehyde, followed by dehydration and either air or critical-point drying. The specimen is coated with a fine layer of metal particles, placed in the microscope chamber, subjected to a vacuum, and bombarded with electrons ***(56)***. **Figure 2** shows a biofilm on glass visualized by SEM.

3.1.1.2. Electroscan SEM (ESEM)

ESEM, a modified form of SEM enables imaging of hydrated specimens ***(57)*** by placing the specimen in a chamber at pressures exceeding 20 torr—the saturated partial pressure of water at room temperature ***(58)***. This enables the visualization of hydrated specimens under high magnification, with minimized shrinkage and generation of artifacts compared to conventional SEM techniques. However, as is the case with standard SEM, the electron beam damages the specimen in a relatively short period of time.

3.1.1.3. Transmission Electron Microscopy (TEM)

In TEM the electrons are scattered as they pass through the specimen, then focused by magnetic lenses to form an image on a fluorescent screen. TEM has

been used to produce information on biofilm thickness and on the interactions occurring at a cellular level among members of a biofilm. It enables detailed analysis of the spatial arrangements and cellular structure of cells present within the biofilm.

3.1.1.4 Atomic Force Microscope (AFM)

The AFM is a scanning probe microscope, in which variations in voltage occur owing to deflection of the electron cloud at the AFM tip by surface atoms ***(59)***. When a sample is scanned in a raster pattern, variations in the surface topography cause undulations of the cantilever to which a silicon nitride tip is attached. A laser measures this movement and feeds back a signal to the piezoscanner, causing the cantilever deflection to be kept at a constant level. The voltages applied to the piezo scanner can then be converted to an artificially colored image, which consequently mimics the topography of the surface at a constant rate of deflection ***(57)***.

3.1.2 Light and Phase Contrast Microscopy

Studies using bright-field and phase contrast microscopy coupled with image analysis have examined colony development, effects of nutrient concentration on attachment, and so on ***(53,60)***. Phase contrast microscopy has been used to demonstrate reversible and irreversible attachment of marine bacteria to glass surfaces ***(53)***. However, most bright-field and phase contrast microscopy is heavily reliant on the use of transparent surfaces, severely limiting their application to the study of biofilms on opaque materials, except in situations in which stains such as acridine orange and 5-cyano-2, 3-ditolyltetrazolium chloride (CTC) can be used and then visualized using epifluoresence microscopy.

3.1.3. Differential Interference Contrast (DICM) Microscopy

DICM has a marked level of superiority compared to phase contrast microscopy, allowing the observation of biological samples without the generation of artifacts. The DICM microscope is a conventional light microscope with ultraviolet fluorescence, which has undergone reconfiguration of the epifluorescence and episcopic DICM sections to above the microscope stage. These and other adaptations allow the visualization of opaque specimens, and the light intensity can be enhanced by mirrors present in the mercury lamp casing ***(51)***. DICM can provide details of the surface topography of the biofilm and allow visualization of the biofilm exopolysaccharide (EPS) ***(57)***.

3.1.4. Confocal Laser Scanning Microscopy

In CLSM, penetration into thick biofilms is made possible owing to the use of a krypton/argon laser, which excites fluorophore dyes present within the

sample. The resulting fluorescence is detected by photomultiplier tubes and a digital image is obtained. Alteration of the focal (*z* plane) depth and the subsequent collection of the *x*-*y* plane images (parallel to the surface) enables the collection of a series of optical sections that can then be computer processed using image analysis software to create a 3D image ***(61)*** . CLSM is an effective tool for the study of a wide range of biofilm features, including physiologic profiles and structural heterogeneity (*see* also Chapter 17). Because of its ability to allow the *in situ* study of intact, fully hydrated biofilms; the measurement of pH, oxygen, and nutrient profiles and microcolonies using microelectrodes; the analysis of velocity and diffusional processes and a number of other features, CLSM represents a technique of major importance in the study of medical, industrial, and environmental biofilms ***(62)***.

3.1.5 Metabolic/Vital Stains

Laboratory techniques utilized in the enumeration of planktonic bacteria, such as viable cell counts, possess an inherent tendency to underestimate the total number of viable bacteria present owing to the presence of viable but nonculturable cells or a biofilm. Direct microscopic techniques coupled with the use of vital stains represent a more accurate technique for the enumeration and visualization of such bacteria. Metabolic stains such as 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and INT (2-[*p*-iodophenyl]-3-[*p*-nitrophenyl]-5-phenyltetrazolium chloride) have been used to detect metabolically active bacteria present in water samples ***(63)***, on pipelines, and in disinfected biofilms ***(64)***. In the presence of an active electron transport chain, CTC undergoes reduction, resulting in the formation of an insoluble purple CTC-formazan crystal that fluoresces red when excited with a certain wavelength of epifluorescent light. CTC has the advantage over the related compound INT in that it allows the visualization of actively respiring cells on membrane filters and other optically opaque surfaces such as wood, metal, and plastic ***(65)***. Other fluorogenic compounds that have been used to assess biofilm physiological activity include rhodamine, which determines membrane potential ***(66)***, and 4,6-diamidino-2-phenylindole (DAPI), which stains living and dead cells.

3.2. Additional Techniques for Studying Biofilms

3.2.1. Attenuated Total Reflection Fourier Transform Infrared Spectrometry (ATR/FTIR)

In the study of biofilms, ATR-IR radiation is directed through an internal reflectance element (germanium or zinc selenide crystals) to which bacteria are attached. IR radiation is absorbed by a molecule when the energy of the radiation is equal to that required to put the molecule in an excited, vibrational state. This absorption only occurs at discrete frequencies, and the number of

molecules present is proportional to the amount of radiation absorbed. This frequency-dependent absorption produces a unique absorbance pattern of the spectrum that is defined by the structure of the molecule. In biofilms this spectrum is the composite of the spectral signatures of each of the biomolecules present. The frequency at which a molecule absorbs radiation is determined by the presence of specific groups of atoms within the molecule. The group frequency is defined as the individual wave number range at which a specific group of atoms absorbs radiation. Differences in molecular structure can subsequently be identified and quantified using tables of characteristic frequencies to identify specific IR absorbance bands ***(52)***.

3.2.2. Cryoembedding

Cryoembedding is applicable to biofilms of variable thickness on an assortment of opaque or transparent surfaces, and involves the fixation of a biofilm using a cryoembedding compound that contains a number of water-soluble polymers to maintain the intact biofilm structure ***(64)***. The embedding compound is placed onto the biofilm while it is still attached to the surface. This process is carried out on dry ice in order to freeze the sample rapidly, avoiding the formation of ice crystals. The embedded biofilm is removed from the surface and the opposing side embedded, so that the frozen biofilm is sandwiched between the embedding compound ***(66)***. Cross sections of variable thickness can then be cut using a cryostat and imaged using microscopy. Minimal sample disruption ensures that individual cells, microcolonies, and water channels all remain visible; physiologic gradients of metabolic activity, such as those present following antibiotic treatment, can be observed using a combination of metabolic dyes and fluorescence microscopy.

3.3. Model Systems and Experimental Biofilms

Experimental biofilms established using model systems represent a useful tool for the laboratory-based study of sanitization and disinfection strategies, metabolic processes, nutrient utilization, gene transfer, and biodegradation. They allow the examination of a wide range of hypotheses or the determination of those parameters that have a role in influencing biofilm formation, architecture, and functional characteristics. The production of reproducible biofilms under laboratory conditions represents an important factor in the study of biofilms, with particular relevance to environmental processes.

3.4. Applications of Biofilms to Study Industrial Systems

Despite the applicability of experimental biofilms and associated techniques for the study of environmental processes, it should be recognized that biofilms are the site where the majority of environmental processes occur, rather than

simply a tool to facilitate their study. The study of biofilms encompasses a wide range of disciplines and has many important applications in furthering our understanding of key environmental processes. The primary use of biofilms, from an industrial point of view, is in the control of unwanted biofilms, e.g., in the development of antifouling coatings or surfaces that will reduce or prevent microbial adhesion.

3.4.1 Influence of Surface Type on Adhesion and Biofilm Formation

SEM (**Subheading 2.2.1.1.**) has been used to examine the attachment mechanisms utilized by marine-fouling bacteria to glass, plastic, and antifouling painted surfaces ***(67)***. The MRD (**Subheading 2.2.1.**) enables the testing of a wide range of surface types in batch or continuous culture and is a good model for studying flow system biofilms ***(68)***, although it does not allow distinction between factors attributable to growth rate and those owing to adhesion. Mild-steel surfaces exhibited a 10-fold difference in the number of colonized heterotrophic bacteria relative to polycarbonate surfaces when examined using the annular reactor (**Subheading 2.2.4.**) in a study of the persistence of coliforms in mixed-population biofilms ***(69)***.

3.4.2. Physiological Effects of Biocides

To evaluate a particular antimicrobial agent for utilization in treatment regimes, it is necessary to determine the effects on the biofilm in terms of alterations to the physiology or metabolic activity of the bacterial cells. Biofilms that form in heat exchangers, pipelines, and drinking water systems ***(26,38)*** are notably resistant to chlorine which is frequently the main disinfectant of choice. This poses several questions; e.g.: Is this the result of an inability of the biocide to penetrate the biofilm as a result of the presence of an EPS matrix or owing to transport or diffusional processes? Are all of the cells within a biofilm equally affected by the treatment? The use of chlorine microelectrodes and CLSM for the visualization of chlorine penetration into a mixed *P. aeruginosa* and *Klebsiella pneumoniae* biofilm grown in a rototorque revealed the presence of reaction-diffusion interactions, which resulted in limited chlorine penetration into the biofilm ***(38)***. The gradients of physiologic activity within a biofilm following biocide treatment that have been visualized using cryoembedding and image analysis have shown a nonuniform loss of respiratory activity within the biofilm ***(36)***. Cryoembedding has also been used to visualize the physiological responses of bacteria in biofilms to treatment with chlorine ***(37,64)***. To date, there seems to be no single factor that can be identified as being solely responsible for the observed recalcitrance of biofilms. One thousand to 10,000-fold higher concentrations of antimicrobial agents may be required to cause levels of killing equivalent to those observed with planktonic

cells ***(29)***. It is postulated that a combination of altered antibiotic permeability with regard to the cell envelope, binding of antibiotic molecules or modification of molecular targets by EPS ***(29)***, altered physiological status of the cells at different sites within the biofilm ***(36,37)***, and growth rate ***(12)*** all influence the effectiveness of a particular treatment strategy.

3.4.3 Biocorrosion and Pitting

The biofilms existing in the majority of natural ecosystems are present as complex mixed communities, which possess complementary metabolic functions, resulting in the formation of several localized microenvironments. Biofilms are recognized as playing an important role in biocorrosion, and this role can be attributed to a number of features of the biofilm ***(70)***. The heterogeneity inherent of many biofilms ***(47,71–73)*** results in the establishment of localized corrosion cells; anaerobic zones created by the utilization of oxygen by the aerobic and facultatively aerobic organisms favor the growth and activity of SRB. Under optimal conditions, the SRB are important contributors in corrosion. Enhancement of their activity may occur as a result of the EPS, which is capable of acting both as a metal binder and in the retention of corrosion products. AFM (**Subheading 3.1.1.4.**) has been used to examine a bacterial biofilm on a copper surface (previously assumed to be toxic to microorganisms) and has shown that the organism tested was directly associated with the pitting corrosion of copper ***(59)***. AFM does not require sample dehydration and can provide information on the association between the cells, the EPS produced and the surfaces to which they attach. Positioning of a microelectrode tip (<10 μm) in relation to microcolonies and water channels has been used to examine the pH and dissolved oxygen levels in biofilms present at metal/artificial seawater interfaces ***(74)***. In a *P. aeruginosa* biofilm, the levels of dissolved oxygen decreased as the microelectrode was moved away from the biofilm interface and deeper into the less aerobic central zones of the microcolony ***(3)***.

3.4.4 Fluid Flow Systems

Laboratory studies involving experimental biofilms are relevant to industrial and natural systems. Knowledge concerning the effects of factors such as flow rate, hydrodynamics, and shear stress is applicable not only to the undesirable biofilm causing a reduction in the flow rate of a pipeline but also to the aquatic biofilms on rocks in fast-flowing rivers or streams. Biofilm accumulation in pipelines can affect the hydrodynamics of the system, with consequences for heat and mass transfer properties. Even under conditions of turbulent flow, which are common in both natural and engineered systems, a laminar flow sublayer probably exists in the vicinity of the pipe wall ***(68)***.

Variations in the flow rate will influence diffusion rate and nutrient availability and, subsequently, colonization levels. Liquid flow velocity in model systems is an important factor in predicting the effect of a biofilm on system hydrodynamics. **Figure 3** shows a biofilm developed under turbulent conditions and visualized using CLSM. The MRD has been used in an examination of the relationship between biofilm formation and laminar flow conditions ***(68)***. By tracking fluorescently labeled latex beads through the biofilm present on the surface of a flow cell using CLSM, it is possible to link flow velocity with various physical parameters such as biofilm structural heterogeneity ***(71)***. The structural heterogeneity of a biofilm may correspond with heterogeneity in some physiologic parameters such as dissolved oxygen gradients. Transport processes occurring within biofilms will influence the supply of oxygen and nutrients and the overall efficiency of biocides such as chlorine.

3.4.5. Food and Water Treatment Industries **(75)**

Biofilms possess the potential to act as reservoirs for potentially harmful microorganisms capable of affecting the quality of the finished product ***(25)***. This is highly undesirable and there is a need to determine the extent to which existing strategies used in cleaning and sanitization are effective in eradication of these microorganisms ***(6)***, and to develop further strategies for the prevention of bacterial adherence, e.g., by polymer surface modification ***(75)***. The use of a combination of two fluorogenic compounds and cryoembedding to examine gradients in respiratory activity in a mixed culture biofilm following disinfection with monochloramine revealed a nonuniform loss of respiratory activity within the biofilm following monochloramine treatment ***(36)***. The highest loss of activity occurred at the surface of the biofilm near the biofilm and bulk fluid interface, leaving underlying organisms relatively unaffected in terms of their respiratory and metabolic activity. Biofilm bacteria may directly affect water quality by entering the bulk phase liquid or may represent a site for the sequestration of viruses ***(76)***, coliform bacteria ***(69)***, or pathogens such as legionella. Since these organisms will not be detected during standard sampling procedures, this will consequently mask the true quality of the finished water in terms of microbial load. The indirect effects of the presence of biofilm bacteria may include changes in taste or odor, and discoloration of the finished water owing to microbial biodegradation. Fluorescence microscopy of a laboratory-grown drinking water biofilm stained with a β-galactosidase probe showed the presence of *Escherichia coli* (containing a l*ac*Z reporter gene) ***(25)***, demonstrating the ability of coliforms such as *E. coli* to become situated within a biofilm. Coliforms acclimatized to oligotrophic conditions similar to those inherent in water distribution systems were found to be successful in colonization of the mild steel and polycarbonate surfaces of an annular reactor ***(69)***. Differential interference contrast microscopy (DICM) (**Subheading 2.2.3.**) combined with fluorescein

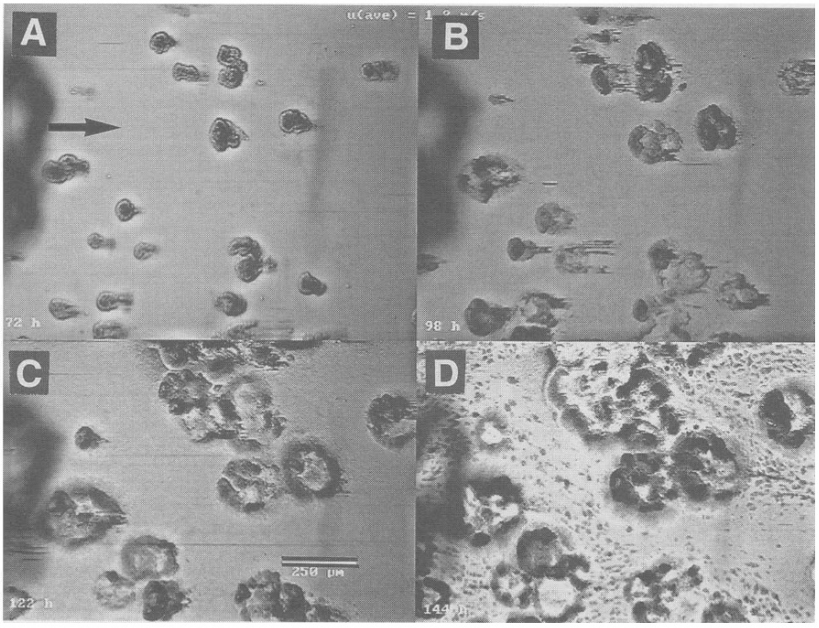

Fig. 3. Biofilm developing under turbulent flow conditions on a glass cover slip in a polycarbonate flow channel at 72 h **(A)**, 98 h **(B)**, 122 h **(C)**, and 144 h **(D)** using CLSM in transmitted light mode. The biofilm was composed of *P. aeruginosa*, *Pseudomonas fluorenscens*, and *K. pneumoniae*. The average flow velocity was 1.8 ms^{-1}, the flow direction is indicated by the arrow in (A). The large black mark on the left edge of each panel is a relocation mark drawn on the outside of the cover slip. Scale (C) = 250 µm. (Image supplied by Paul Stoodley, University of Exeter.)

immunolabeling has demonstrated the presence of *L. pneumophila* within a multispecies tap water biofilm grown on plumbing material surfaces ***(77)***.

3.5. Medical Environments

Any foreign implant introduced into the body, such as a catheter, an artificial joint, or heart valve, represents a potential site for biofilm formation owing to the absence of the normal host defence mechanisms associated with, e.g., the mucous layer ***(78)***. The physical damage/disruption of tissues or organs by invasive techniques may result in a loss of the protective antiadhesion coatings maintained by the body and the establishment of an opportunistic infection ***(79)***.

3.5.1. Development of Antifouling/Adhesion Coatings

The development of antiinfective devices such as catheters is desirable due to the high incidence of associated nosocomial infection and primary septice-

mia *(80)*. The MRD represents a good in vitro model system for testing the efficacy of various incorporated antimicrobial agents *(80)*. A combination of minocycline and rifampin were found to be effective against Gram-positive cocci, Gram-negative bacilli and yeasts such as *Candida albicans*.

3.5.2. Evaluation of Antibiotic Susceptibility

Current tests for the determination of antibiotic sensitivity on which treatment strategies are based, such as the disk diffusion assay and minimum inhibitory concentration tests *(30,81)*, rely heavily on the use of planktonic cells. The role of biofilms in clinical disease is well documented *(7,28)*, and recent increases in the use of indwelling medical devices such as catheters and artificial joints have been closely shadowed by an increased incidence of implanted device-associated infections *(82)*. Coagulase-negative staphylococci account for more occurrences of device-associated infection than any other microorganism *(83)*. The MRD represents a good in vitro model for studying this colonization with a view to determining effective concentrations of antibiotics, either for treatment of the infection or as a coating on the catheter surface to decrease initial attachment of the cells *(80)*. ATR/FTIR has been used to study the effects of biofilms on substrate and in the examination of biofilm composition *(37,52,84)*, and also in a medical context to investigate the penetration of ciprofloxacin into a *P. aeruginosa* biofilm; the penetration of the antibiotic from the bulk fluid to the surface was significantly reduced by the presence of the biofilm *(37)*. The perfused biofilm fermenter closely mimics the situation which occurs with soft-tissue infections, and represents a good model for examining the role of cell growth rate in antibiotic resistance by biofilms *(12)*.

3.6. Natural Systems

Biofilms were first studied with particular relevance to aquatic systems *(79)*, and research into this ubiquitous mode of bacterial growth has subsequently expanded into all natural environments. Growth as a biofilm enables exploitation of the nutrients which may be concentrated at a surface *(5,9)*, protects against desiccation and changes in the pH, temperature, or osmolarity of the environment, and may offer increased protection from grazing predators such as amoebae and protozoa *(85)*.

3.6.1 Gene Transfer/Exchange

The requirement to understand gene transfer, as it occurs in terms of the ability of the natural population to uptake exogenous DNA, has been prompted by concerns about the ability of indigenous populations to uptake genetic sequences from engineered organisms. Transformation occurring in the river epilithon *(86)* has been suggested to represent a possible mechanism by which

resistance genes could be spread through natural populations; the transfer of mercury-resistant plasmids from epilithic communities to *Pseudomonas pudita* recipients ***(17)*** has been demonstrated. Further studies of genetic transfer between microbial communities in aquatic and terrestrial environments should account for the existence of the majority of microorganisms, as biofilm communities and experimental systems should be designed accordingly.

3.6.2. Influence of External Factors on Microbial Biofilm Formation

Environmental forces such as temperature and nutrient concentration exert an effect on microbial behavior in the natural environment. Lawrence and Caldwell ***(43)*** used light microscopy and continuous-flow slide cultures to demonstrate a number of colonization maneuvers shown by bacteria from a natural stream community. Computer-enhanced microscopy has been used to examine colony development on surfaces ***(43)***, examine the behavior of bacterial stream populations within the hydrodynamic surface layers of microenvironments ***(43)*** and to look at the effects of different concentrations of organic nutrients on bacterial colonization ***(5)***.

3.7. How Representative Are Experimental Biofilms?

Experimental biofilms represent a compromise between two extreme types of models; holistic, which involves a study of the complete systems, and reductionistic, which enables wider predictions to be made following the study of individual elements of the system, but which often does not take into account community interactions ***(87)***. Most model systems and experimental biofilm studies fall somewhere in the middle of these two classifications. The use of holistic/replicative models for producing experimental biofilms represents the best system in terms of their relative similarity to environmentally occurring biofilms, but the variety and number of ecologic niches means that these types of models can vary widely ***(39)***. Reductionistic/investigative models simplify the environment, producing reproducible biofilms for the examination of features common to different systems. These enable a wider degree of control over specified experimental factors that have a postulated role in influencing structural and functional processes ***(87)***.

The biofilms present in the environment, on riverbeds, oil rigs, or ship hulls represent a consortia of bacteria, fungi, algae, and protozoa; biofilms in the body are composed of a number of different bacterial species. For example, in the case of dental plaque the primary plaque formers are the oral streptococci, which are then followed by secondary formers such as fusobacterium ***(88)***. Monoculture (single species) biofilms are widely used in laboratory studies but are more usually only present in soft-tissue infections such as endocarditis. Biofilm characteristics are reflective of their growth environment ***(89)*** and are

influenced by nutrition, fluid dynamics, species composition, and physicochemical properties. The laboratory environment differs significantly from the external environment in terms of fluctuations in nutrient supply and demand, and growth conditions should therefore attempt to mimic those observed in vivo ***(29)***. The nature of the growth-limiting nutrient is important in influencing the phenotypic characteristics of the cells ***(90)*** and, consequently, must be considered prior to the extrapolation of data from in vitro models to *in situ* biofilms ***(39)***. In the environment, biofilms represent dynamic systems, with complex interactions such as predator-prey relationships, e.g., the grazing of protozoa on biofilms ***(85)***. These relationships and others, such as specific changes in the physicochemical properties of the surface or microenvironment and the interactions occurring between complex microbial communities, can often prove difficult to model under a laboratory environment. For example, in the perfused biofilm fermenter model, the nutrient concentration will be similar for most of the cells; however, in a naturally occurring biofilm, nutrient gradients and subsequent differences in cell physiology exist owing to the spatial distribution of the cells ***(85)***. The modified Robbins device (MRD) allows no distinction to be made between features occurring as a result of adhesion and those owing to growth rate ***(40,45)***.

3.8. Visualization of the Biofilm

Since CLSM, light, and phase contrast microscopy are limited to transparent surfaces, this restricts the variety of substrates that can be examined. Although electron microscopy enables a variety of surfaces to be examined, dehydration of the sample prior to examination severely condenses the hydrated glycocalyx ***(58)***, destroying the complex architecture of the biofilm (with the exception of environmental SEM ***[58]***). Structural components of the biofilm are often lost during preparation for SEM, sampling is sacrificial, and artifacts are common—a cell may appear present in a pit, suggesting breakdown of the surrounding substratum, but the pit may actually be the condensed residue of the dehydrated glycocalyx ***(7)***. Despite its applicability with the study of biofilms, there are some of disadvantages with CLSM: the use of autofluorescing environmental samples may cause problems when used in conjunction with a fluorescent stain in visualizing objects within a biofilm; shadowing may sometimes occur owing to the presence of objects that are not penetrated by the laser beam, and extremely thin specimens may be difficult to find ***(91)***. ATR/FTIR only examines the base layer (approx 1 μm) and averages the picture to apply to all of the exposed area ***(52)***.

3.9. Sampling Techniques

Primary methods for the enumeration of bacterial viability and activity are largely dependent on the ability of the bacteria to form colonies on laboratory

media. Sublethal injury following exposure to antimicrobial agents, reduced culturability, and the varied microbial composition of biofilm communities may hamper the accurate assessment of surface-associated bacteria ***(92)***. Physical removal of the adherent cells results in changes in the physiologic characteristics of the cells. The spatial distribution of the cells and interspecies interactions may be important in influencing the biodegradative efficiency of a biofilm ***(93)*** or in determining the effects of antimicrobial agents on biofilm processes ***(92)***. There is, therefore, a need to develop techniques that enable the nondestructive analysis of biofilms, as opposed to destructive sacrificial procedures such as colony counts, total cell counts, and SEM. Metabolic stains such as CTC ***(65)*** and rhodamine 123 ***(94,95)*** have been coupled with microscopic visualization and used for the *in situ* study of bacterial metabolic activity ***(96)***.

3.10. Future Applications for Biofilms in the Study of Environmental Processes

It is now clear that biofilms represent the primary tool in the processes of gaining a clearer understanding of a wide range of environmental processes that have previously relied on the extrapolation of data obtained from planktonic microbial cultures ***(15)***. The study and use of biofilms in environmental processes is applicable to a wide range of areas; general areas of future interest may include the following:

1. The further determination of important biofilm structural and metabolic processes that will enable the development of a model of structure/architecture applicable to both high- and oligotrophic nutrient environments.
2. The development of systems to enable the more accurate assessment of the efficacy of biocides and antimicrobial agents; i.e., less reliance on the use of planktonic cell systems in the assessment of antibiotic efficacy prior to the treatment of biofilm-associated infections or in the development of sanitization strategies in the food industry.
3. A more detailed understanding of both cell-cell and cell-interface interactions to enable the development of antifouling surfaces/coatings for use across a wide range of industries, coupled with an understanding of the mechanisms/signaling processes involved in defining biofilm structure/architecture.
4. A study of the underlying genetic processes that influence biofilm formation, such as the expression of genes related to alginate or EPS production; the production of cell signaling factors, and the genetic characteristics that account for the observed physiologic differences between the planktonic and biofilm cells.
5. The determination of the degradation rates of pollutants that occur in the environment by biofilm bacteria rather than owing to planktonic cells. For example, the adsorption of organic pollutants and surfactants onto sediments present in

soils or rivers may act to stimulate attachment, resulting in accelerated biodegradation and depletion of the absorbed surfactant ***(97)***.

6. An understanding of the interactions that occur between the bacterial populations present in a multispecies biofilm in terms of nutrient exchange and recycling, utilization of oxygen, and subsequent effects on metabolic activity, cell distribution, and interspecies cooperation.

3.11. Biofilms and Environmental Monitoring

A model system is essentially a smaller scale reproduction or simplification of a complex system, which allows calculations to be made, along with the testing of hypotheses and predictions. The choice of the system to be used for the production of an experimental biofilm is a process that involves an analysis of the ultimate end-point requirements: Do we require qualitative (SEMs, AFM, light microscopy images) or quantitative (viable counts, metabolic counts, total carbohydrate levels) data? Are we interested in biological, physical, or chemical parameters? How accurate a representation of the natural environment do we require? Once determined, we can then balance the desirable features against the disadvantages of the system of interest. No single model will produce a biofilm capable of examining all areas of interest simultaneously. Whether looking at the chemical factors influencing biofilm formation, the effects of antimicrobial treatment regimes, or the influence of structure on degradative ability, an awareness of the inherent problems connected with the experimental model systems can allow the selection of a system with optimized applications for the area of interest being investigated. A knowledge of the movement of particles and fluids, physiological conditions within the biofilm, the presence of chemical and physical gradients, the spatial arrangement of cells, and diffusional and transport processes occurring within biofilms is important in furthering our understanding of dynamic processes such as nutrient transport and the diffusion of antimicrobial agents. Biofilms are ubiquitous and represent the site for the majority of environmental process. As such, they therefore represent an essential experimental tool in the quest to understand those environmental systems and processes of interest to us.

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