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The Study of Microbial Biofilms by Classical Fluorescence Microscopy

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15.1 INTRODUCTION

In open environmental systems, the majority of microbial activity is associated with an interface within thin biological layers known as biofilms. Biofilms cause problems ranging from reducing transfer efficiency and deteriorating materials to increasing health risks. Conversely, biofilms are a set of immobilized cells that can facilitate cell-product separation, increase cell concentration and enhance overall productivity. Since these communities are highly heterogeneous in structure and in physiological activity, biofilm research relies on the understanding of the spatial information of composition and metabolic activity within biofilms. Traditional methods of studying these communities, which involve removing biofilm samples from the substratum followed by homogenization, and chemical and biological analyses, fail to provide spatial information about the structure and function of biofilms. To reveal spatial information within biofilms, fluorescent staining coupled with cryoembedding, cryosectioning and image analysis is proving useful in biofilm research. Using classical fluorescent microscopy, spatial patterns of biofilm thickness and species distribution, as well as physiological activity such as respiratory activity and growth rate can be visualized. These spatial patterns indicate that biofilms are highly heterogeneous in both structure and function.

15.1.1 Biofilms: general description

When solid surfaces are submerged in an aquatic environment, suspended microbial cells attach to the surface, the immobilized cells grow, replicate and secrete extracellular polymers that surround the cells within a gelatinous matrix. This constitutes the collective surface community referred to as a biofilm (Characklis and Marshall, 1990). Although the term is usually applied to bacterial cells and their extracellular polymers, any biologically active layer of cells (microbial, plant or mammalian) can be considered a biofilm (Bryers, 1987). There

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are several features unique to microbial biofilms (Hamilton, 1988):

(a) Biofilms are the principal sites of biological activity in many natural and man-made environments.
(b) Biofilms are heterogeneous, with discontinuities in both vertical and horizontal dimensions, involving organisms and biotic and abiotic components, creating physicochemical microenvironments.
(c) Biofilms are dynamic structures: their heterogeneities vary with time.

Biofilms consist of cells and their secreted insoluble extracellular polymers, which are primarily polysaccharides. Biofilm formation, illustrated in Figure 15.1, is the net result of suspended cell deposition, attached cell metabolism and biofilm removal processes (Bryers, 1987). Deposition of cells onto a substratum involves several individual processes: the macromolecular organic preconditioning of the target surface, cellular transport from the bulk phase to the solid substratum, and reversible and irreversible cell adhesion to the surface. Cellular growth, substrate conversion, endogenous decay and extracellular polymer production collectively constitute biofilm metabolic processes. Biofilms can be removed from the surface through chemical challenges, abrasion, shear-related detachment and sloughing. Deposition and biofilm detachment have been studied extensively and mathematical models have been established for each process during the past decade (Characklis, 1990).

15.1.1a Importance of biofilms

Biofilms have been implicated in microbiological problems associated with industrial and drinking water systems. Detrimental effects of biofilm formation

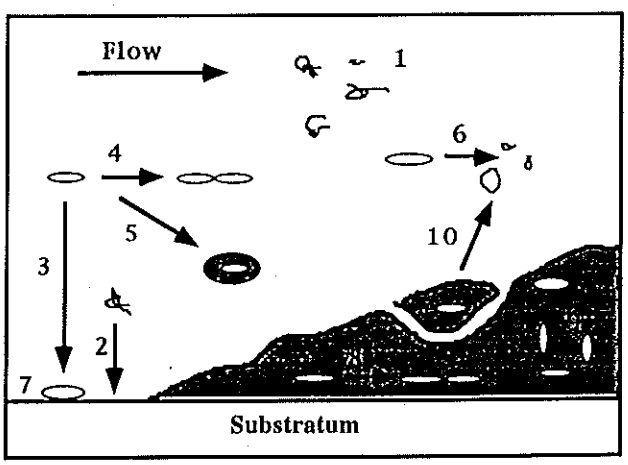

Figure 15.1 The mechanism of biofilm formation, in liquid phase: (1) dissolution of organic macromolecules, (2) pre-conditioning adhesion, (3) cell transport, (4) replication, (5) production of extracellular polymer, (6) death, (7) attachment to the substratum; within biofilm: (8) replication, (9) production of extracellular polymer and (10) biofilm detachment.

on system performance range from operational problems to economic losses. In industrial systems, biofilm formation and persistence create problems in engineered systems by increasing the resistance to mass, momentum and energy transfer, as well as by mediating chemical or biological reactions at the substratum. In drinking water systems, the results of a survey conducted in the US indicate that a relatively high proportion of potable water distribution systems occasionally experience excessive bacterial populations, in the absence of obvious defects within the distribution network (Smith *et al.*, 1990). Other studies have suggested that these unexplained occurrences of excessive heterotrophic and indicator bacteria result from microbial growth on pipe surfaces at the expense of nutrients in the bulk water (LeChevallier, 1990; Camper, 1994; LeChevallier *et al.*, 1996). Although limited direct experimental evidence is available that supports this hypothesis, results obtained from operating systems demonstrated increasing levels of coliforms with distance as water travelled through the distribution system, under conditions that would not allow proliferation of the bacteria within the bulk water (LeChevallier *et al.*, 1987). These reports are typical of a literature providing strong suggestive evidence associating the growth of coliforms and other heterotrophic bacteria within biofilms, in response to occasional physical and chemical conditions that are suitable for the multiplication of sessile bacteria on pipe surfaces. The subsequent release of these micro-organisms into the water column results in recalcitrant problems for the water utility in terms of meeting water quality standards and the erosion of consumer confidence.

Other microbiological problems have also been associated with biofilms in aquatic systems including drinking water networks. For example, biofilms containing sulphate-reducing bacteria (SRB) have been linked to biologically-enhanced corrosion of substrata containing iron (Little and Wagner, 1994). These biofilms require somewhat greater time to develop the necessary microzones and gradients that are found within thicker biofilms. Biocorrosion of other materials used in engineered aquatic systems is also promoted by attached bacteria. Nitrification is another biofilm-associated problem in drinking water systems (Wolfe *et al.*, 1990).

Apart from industrial and drinking water systems, biofilms also cause problems in dental plaque and device-related infections. Many microbiologists work in the area of oral health, and the clinical problems on which they focus increasingly involve microbial biofilms. As the problem of simple caries is partially solved by fluoride treatment and improved prophylaxis, microbiological attention is shifting to periodontal disease and to infections secondary to implantation and surgery. Periodontal disease is one of the most interesting and vexing biofilm diseases. It is clear that this disease is caused by a mixed species biofilm, some of whose microbial components are mobile cells that are only intermittently present in the biofilm itself (e.g. *Treponema*). The biofilm mode of growth in peridontitis promotes the survival of fastidious anaerobes, and the pathological cooperation of different microbial species, while modifying the interactions between the pathogens and the host tissues. Medical device-related bacterial infections have also been known to be caused by bacteria growing in biofilms (Khoury *et al.*, 1992). The surfaces of these devices are colonized by bacteria that are introduced at the time of surgery, or subsequently by haematogeneous spread, and these bacteria proliferate and

produce exopolysaccharide slimes until mature biofilms are formed (Costerton and Anwar, 1994). These infections often develop slowly, as their causative biofilms withstand the sequential attacks of phagocytic cells (Jessen *et al.*, 1990) and antibodies (Costerton *et al.*, 1995), but most of them eventually serve as foci of acute disseminated infections that spread from the colonized surface and produce discernible symptoms.

15.1.1b Control of biofilms

The control of biofilms represents one of the most persistent challenges within engineered systems where these microbial communities are problematic. Mechanical cleaning and antimicrobial chemicals are the most commonly used methods of biofilm control. Mechanical cleaning is often impracticable and can be costly because it usually involves equipment downtime. Therefore, the main strategy of biofilm control relies on chemical biocides to kill the attached micro-organisms and/or remove them from the surface. However, biofilms are found to be hundreds of times more difficult to eradicate than their counterparts in suspended cultures. Bacteria within biofilms, such as those within drinking water distribution pipes, are notoriously difficult to control through the use of disinfectants. Concentrations of chlorine usually employed to disinfect potable water (i.e. 1–2 mg/l) are ineffective in controlling bacterial occurrences thought to be associated with biofilms (LeChevallier *et al.*, 1988). These and other studies have reported that surface-associated bacteria are as much as orders of magnitude less susceptible to the effects of antimicrobial agents (LeChevallier *et al.*, 1984; Wolfe *et al.*, 1990). Difficulties in formulating efficient control strategies are related to our incomplete understanding of biofilm processes and structure.

Mechanisms proposed to explain this enhanced resistance in bacteria within biofilms can be divided into two categories: transport limitation and physiological adaptation. Transport limitation is attributed to the neutralization of the antimicrobial agent in the biofilm faster than it can diffuse in to the community (Chen and Stewart, 1996; Stewart 1996; Xu *et al.*, 1996). Another explanation of biofilm resistance to chemical challenge is sought in physiological differences between biofilm and planktonic cells (Brown and Gilbert, 1993). It is quite reasonable to postulate that micro-organisms deep within a biofilm, where the chemical and physical microenvironment might be quite different from that in the bulk fluid, will be physiologically distinct and therefore less susceptible to disinfection. In particular, slow growing or starving micro-organisms in the interior of the biofilm are candidates for reduced susceptibility (Brown *et al.*, 1988; Tresse *et al.*, 1995).

Since biofilms are highly heterogeneous, traditional research methods that rely on the removal of biofilms from the substratum followed by disaggregation and enumeration provide little spatial information regarding the structure and physiological activity of individual bacteria within biofilms. To improve our understanding about the mechanisms of biofilm recalcitrance, and to develop more effective strategies of biofilm control, it is necessary to describe spatial patterns within biofilms. Using classical fluorescence microscopy in conjunction with

cryoembedding, cryosectioning and image analysis, we are able to study the spatial patterns of biofilm structure (thickness variation and species distribution) and physiological activity (growth rate and respiratory activity).

15.2 APPROACHES TO REVEAL SPATIAL INFORMATION WITHIN BACTERIAL BIOFILMS

15.2.1 Introduction

Conventional microbiological approaches are inadequate for resolving spatial patterns of bacterial activity and species distribution as well as overall structure within biofilm communities. Traditional methods have relied on the quantitative removal of the biomass from the substratum using some type of shear force, such as scraping, followed by disaggregation to form a monodisperse bacterial suspension. This has usually been followed by identification and enumeration using colony formation or some other growth-dependent assay. This analytical approach is deficient for the analysis of biofilms because these communities are known to have established three-dimensional structural and chemical heterogeneities (Costerton *et al.*, 1995; Yang and Lewandowski, 1995) plus spatial and sometimes temporal gradients in physiological activities within individual cells (Huang *et al.*, 1995; McFeters *et al.*, 1995a; Wentland *et al.*, 1996). Hence, biofilm disaggregation obliterates the native community structure and any hope of gaining meaningful spatial information.

Cultural methods have also been criticized for their failure to detect bacteria under a number of circumstances. For example, natural bacterial communities are often composed of various autochthonous organisms, including some that have not been successfully cultivated (Ward *et al.*, 1992). Likewise, the detection and quantification of allochthonous bacteria in natural and some engineered systems can be problematic, because of reduced culturability under environmental conditions. Specifically, a variety of bacteria enter a viable but unculturable physiological state following aquatic exposure (Rozak and Colwell, 1987; see also Section 11.1.1). Some allochthonous bacteria can also become sublethally injured when exposed to sublethal conditions of antimicrobial treatment (McFeters, 1990; Singh and McFeters, 1990), and natural conditions can lead to bacteria that are both injured and viable but unculturable (Smith *et al.*, 1994) using established criteria. This is illustrated by results from controlled laboratory experiments using bacterial biofilm composed of both *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* exposed to monochloramine for 2 hours (4 mg/l), where culturability decreased by orders of magnitude more than cellular respiration as well as net oxygen and glucose utilization (Stewart *et al.*, 1994). Under these circumstances, the bacteria become reversibly incapable of colony formation on certain media, while retaining metabolism and the ability to recover, grow and even initiate infections, in the case of pathogens, following a process of resuscitation (Singh and McFeters, 1990). This is significant since the detection and quantification of both autochthonous and allochthonous bacteria from environmental circumstances can be significantly underestimated when growth-

dependent methodologies are used. These problems are further aggravated by the realization that the removal of biomass from the substratum as well as cellular disaggregation is seldom complete or quantitative.

15.2.2 Visualizing biofilm structure

A longstanding challenge in the study of bacterial biofilms has been the development of a method to visualize the structure of attached communities at the cellular level in their natural configuration without introduced artefacts. The commercial availability of the confocal laser scanning microscope (CLSM) provides this capability and allows the display of vertical sections through biofilms and the potential for two- and three-dimensional reconstruction of such communities (see Chapter 16; Lawrence *et al.*, 1991; Caldwell *et al.*, 1992a, b, 1993; Surman *et al.*, 1996). The cellular exclusion of low molecular weight fluorescent compounds has also been used to follow biofilm formation and structure, when coupled with CLSM and optical sectioning (Caldwell *et al.*, 1992b). However, problems have been encountered in the use of CSLM when studying thick or relatively opaque biofilms, and the instrumentation is still somewhat limited in terms of routine availability. A separate chapter in this volume (Chapter 16) is devoted to the use of CLSM to yield three-dimensional images of biofilms.

15.2.2a Cryosectioning and the study of bacterial biofilms

The examination of very thin biofilms can be accomplished by conventional microscopy coupled with conventional methodology to assess structure, viability and physiological activity (Yu *et al.*, 1993). Computer-enhanced darkfield microscopy has also been used in the quantitative analysis of bacterial growth and behaviour on surfaces (Lawrence *et al.*, 1989). However, biofilm communities are usually thicker than a monolayer and often exceed 100 μm in depth. Cryoembedding and cryosectioning coupled with fluorogenic stains and fluorescence microscopy are ideally suited to the examination of many biofilm types, irrespective of thickness, optical properties or substratum material. This approach has been extensively used in other biological as well as medical applications with great success, and was found practical for the detailed examination of biofilm structure, when used with fluorogenic dyes (Yu and McFeters, 1994a; Yu *et al.*, 1994). The method, illustrated in Figure 15.2, involves embedding the biofilm with a commercial medium (Tissue-Tech OCT, Miles Inc., Elkhart, IN, USA), and rapid freezing by placing the substratum on dry ice. The embedded biofilm is then easily removed from the substratum surface, turned over on the dry ice, and more OCT is added to surround the specimen fully. The resulting frozen block containing the embedded biofilm can then be sectioned with a cryostat to yield 5 μm slices of the community. This method requires minimal sample processing without prolonged fixation, and can be completed in only a few hours with minimal preparative artefacts. As an additional advantage, most of the equipment required for this procedure is readily available and the resulting images can be stored for later electronic processing.

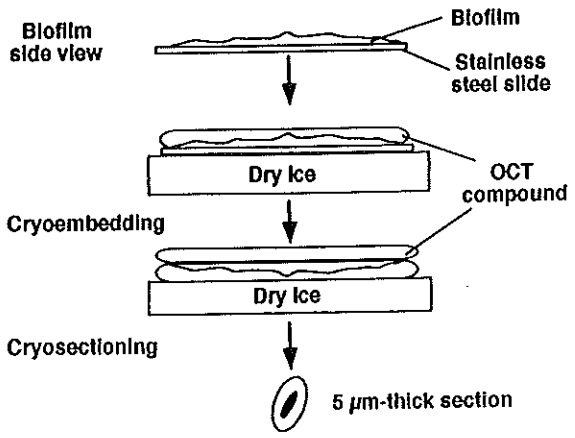

Figure 15.2 Cryoembedding and cryosectioning of biofilms.

Protocol 15.1 Cryoembedding and cryosectioning.

1 Gently dispense a layer of OCT to flood over biofilms accumulated on the substratum.
2 Place on a block of dry ice.
3 Wait until OCT turns white and is completely frozen.
4 Slightly bend the substratum and pop off the semi-embedded biofilms.
5 Turn the semi-embedded biofilms over and place on dry ice.
6 Dispense another layer of OCT.
7 After OCT is frozen, label the substratum side with marker.
8 Chop the embedded sample with Teflon-coated razor blade and mount on a cryostat stand.
9 Section the sample at thickness of 5 μm in −20 °C cryostat chamber.
10 Pick up the 5 μm thick section with a glass slide.

15.2.2b Physiological assessment of biofilm bacteria using specific fluorochromes

The challenge of accurately assessing the physiological status of individual bacteria is especially critical in the arena of biofilm research. Chemical and physical data have established the existence of structural complexity and chemical gradients within biofilms, suggesting the possibility of physiological heterogeneities within thicker biofilms (Costerton *et al.*, 1995; Stewart *et al.*, 1995). However, only limited experimental evidence has been available to test that hypothesis (Van Loosdrecht *et al.*, 1990; Marshall and Goodman, 1994). Such a task seems almost trivial from the perspective of pure culture studies of planktonic bacteria in the laboratory, but it becomes a significant methodological challenge when examining fixed three-dimensional communities like biofilms.

Fluorescent stains are used in a very wide range of biological applications where the response of individual cells can be observed microscopically (see Chapters 4 and 12; Mason, 1993; Haugland, 1996). Although fluorescent techniques are commonly used by microbiologists, including the acridine orange direct count (AODC) and

fluorescent antibody detection techniques, there have been only a few microbiological applications of the numerous potentially useful physiological fluorochromes currently available (McFeters *et al.*, 1995a). However, recent studies have demonstrated the feasibility of describing various physiological parameters within biofilms, including growth rate, respiratory activity, membrane potential and adenylate energy charge with these stains. The combined application of fluorescent stains and cryosectioning is ideally suited for the task of examining spatial and temporal heterogeneities of these and other physiological activities in biofilms and other fixed bacterial communities.

15.2.3 Image analysis of fluorescent images

In order to distinguish specific components of biofilms (cells or extracellular polymers) or physiological activity, most fluorescent stains require counterstaining by a nucleic acid or protein dye. Specific fluorescent stains generally stain specific parts of biofilms, while counterstains provide overall staining of the sample. After counterstaining, positive cells alone and both positive and negative cells can be selected using different optical microscope filters. Once the images are grabbed, they can be digitized using a digital camera, saved in a grey scale TIFF file format, and converted into two-dimensional array numerical data for further analysis.

The image analysis system used at the Center for Biofilm Engineering at Montana State University includes an Olympus BH-2 Epi-Illumination ultraviolet (UV) upright microscope with 100 Watt mercury lamp and 50 Watt halogen lamp, Optronics OPDE-47OT cooled colour charge-coupled device (CCD) camera, with a $\frac{1}{2}$ in Sony ICX038AK chip with pixel resolution of 768(H) × 494(V), a Sony high resolution SVPVM1353 13 in RGB colour monitor, and a Sony UP-1200 thermal dye-sublimation video image printer. The image is processed by a Targa 64+ ADC (Truevision, Inc.) card, and handled by Image-Pro Plus 2.0 software (Media Cybernetics). The image is captured using different microscope filter cubic units and can be saved as either 8-bit grey scale or 24-bit colour image. Table 15.1 lists the specifications of microscope filters used at the Center for Biofilm Engineering. For quantitative analysis, the image is saved as a grey scale. Each pixel is assigned a number between 0 to 255 according to its intensity. An in-house software package, MARK, is used to analyse these digital image data. To avoid the error resulting from different image enhancement set-up, MARK is not used to enhance or sharpen images. The alignment of images is checked by placing a transparent sheet with grid on the monitor.

Table 15.1 Parameters of microscope filters used at the Center for Biofilm Engineering.

Filter type	Excitation filter	Dichroic mirror	Barrier filter
Olympus U	U (UG-1)	DM-400	L-420
Olympus B	B (BP-490)	DM-500	AFC + O-515
Olympus G	G (BP-545)	DM-570	O-590
Omega Optical 5716	485	505	535 ± 17.5
Omega Optical 5721	515	565	>590

15.3 SPATIAL INFORMATION WITHIN BACTERIAL BIOFILMS

15.3.1 Thickness variability in biofilms

Microscopic examination of frozen biofilm sections affords high-resolution information about biofilm structure. Some of the physical properties that have or could be measured through image analysis of biofilm cross-sections are local biofilm thickness, nucleic acid density and extracellular polysaccharide (EPS) density (Kristensen and Christensen, 1982; Ganczarczyk and Zahid, 1994; Yu *et al.*, 1994; Zahid and Ganczarczyk, 1994; Zhang and Bishop, 1994; Stewart *et al.*, 1995).

Murga *et al.* (1995) used MARK image analysis software at the Center for Biofilm Engineering to measure biofilm thickness profiles along transects as long as 1 cm. This was done by catenating information from sequential, slightly overlapping, digitized images. Each image was a few hundred micrometres wide. Digitized images were imported into the custom software. This software allowed the user to draw a line at the location of the substratum and to trace, using the mouse, the outline of the biofilm surface. In their study, Murga *et al.* defined the biofilm surface as the outermost material stained by Gill II haematoxylin. The software then automatically calculated a thickness profile for the image by constructing perpendicular lines from substratum to the biofilm surface trace at defined lateral intervals of a few micrometres. An example of a thickness profile from a single image is reproduced in Figure 15.3. Thickness profiles from sequential images were subsequently catenated by eye in a spreadsheet.

The digitized images and thickness profiles obtained as described above reveal structural heterogeneity consistent with that observed by other techniques, such as CLSM and the Electroscan wet scanning electron microscopy (SEM). Features such

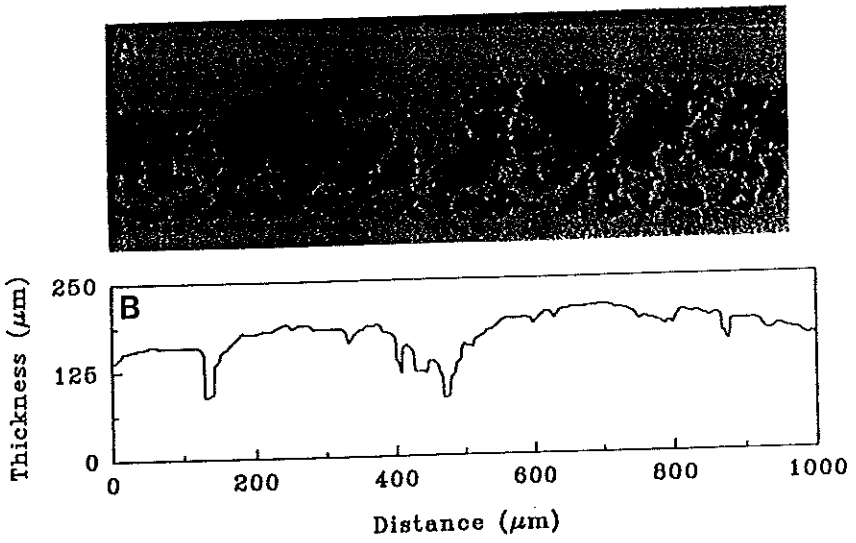

Figure 15.3 (A) Microscopic image of a frozen cross-section of a two-species (*K. pneumoniae*, *P. aeruginosa*) biofilm and (B) the thickness profile obtained from that image.

as thickness variation, void areas and putative water channels are visible. Although staining biofilm internal structure has not been quantified by image analysis, it should be straightforward to convert differences in relative staining intensity, for example with a DNA stain such as DAPI (4′,6-diamidino-2-phenylindole), into maps of relative cell density. An example of the potential for this type of analysis is presented in Plate 3, which compares the distribution of nucleic acids (as stained by ethidium bromide) with the distribution of EPS (as stained by Calcofluor White) in the same section. An overlay of the digitized patterns for the two stains indicates the presence of cell-free regions that nevertheless contain EPS.

15.3.2 Species distribution in biofilms

Spatial heterogeneity is a hallmark of biofilms. Spatial patterns of micro-organism distribution within biofilms have only recently begun to be investigated. To study species distribution within biofilms, a method is required to identify specific species while preserving biofilm spatial structure. The preservation of biofilm structure can be achieved by using cryoembedding techniques or CLSM. Recent advances in immunology and molecular biology have greatly expanded the array of tools that can be applied for speciation. Among these, immunofluorescent staining and oligonucleotide probing are the most frequently used methods in the investigation of species distribution within biofilms.

Various immunological techniques have been used successfully to study the distribution of methanogens in methanogenic granular sludge (Macario *et al.*, 1991; Visser *et al.*, 1991). Recently, Stewart *et al.* (1997) utilized cryoembedding and cryosectioning coupled with immunofluorescent staining to reveal the spatial distribution and coexistence of *K. pneumoniae* and *P. aeruginosa* in biofilms. They cultivated binary population biofilms using annular reactors by inoculating *K. pneumoniae* and *P. aeruginosa* at similar concentrations. Biofilm sections were stained with anti-*Klebsiella* fluorescein isothiocyanate (FITC) labelled monoclonal antibody followed by propidium iodide counterstaining. The spatial heterogeneity of *K. pneumoniae* and *P. aeruginosa* biofilm is shown in Plate 4A–C. They found that most of the biofilms fluoresce red only after staining (Plate 4A), indicating that *P. aeruginosa* dominated the biofilm. In some regions, both *K. pneumoniae and P. aeruginosa* were partially intermixed from the surface to the substratum (Plate 4B), while *K. pneumoniae* appeared as a distinct colony above a *P. aeruginosa* base film in other regions (Plate 4C). Although fluorescent antibodies have been used extensively in the study of microbial ecology, due to their high specificity, this approach has limitations. To prepare specific antibodies, it is necessary to isolate the target micro-organism and to cultivate it in pure culture prior to immunization of the animal. However, not all natural populations are amenable to pure-culture isolation.

In situ hybridization with fluorescently labelled oligonucleotide probes is another approach used in the detection and identification of micro-organisms in biofilms. Although DNA-based hybridization is also used (Holben *et al.*, 1988; Ogram and Saylor, 1988), rRNA-based oligonucleotide probes are most frequently used. Oligonucleotide probes targeting rRNA are attractive because rRNA is an abundant constituent of all living cells. The rRNA oligonucleotide probe has been

successfully applied in the identification of ruminal fibrolytic bacteria (Odenyo *et al.*, 1994), sulphate-reducing bacteria (Amann *et al.*, 1992; Poulsen *et al.*, 1993; Ramsing *et al.*, 1993; Raskin *et al.*, 1996), methylotrophic bacteria (Tsien *et al.*, 1990), methanogenic bacteria (Raskin *et al.*, 1996) and *Pseudomonas fluorescens* (Boye *et al.*, 1995). There are some obstacles to general application of the rRNA approach. Difficulties in the application of the rRNA approach can occur in both sequence retrieval and probing of highly diverse samples (Amann *et al.*, 1995).

Although each method has its limitations, the combination of cryoembedding, cryosectioning, microscopy, immunofluorescent staining, molecular probing and image analysis should provide a better approach in the study of species distribution within biofilms.

Protocol 15.2 Immunostaining of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* biofilms.

1 Fix frozen sections with 1% formaldehyde at room temperature for 1 h.
2 Wash three times with TBS solution for 5 min. To prepare TBS solution, mix 4.5 g NaCl and 0.605 g Tris in 500 ml H_2O and adjust pH to 7.4.
3 Wash with blocking solution for 1 h at room temperature. To prepare blocking solution, add 0.3% Tween-80 and 2% skimmed milk in TBS solution.
4 Wash three times with TBST solution for 5 min. To prepare TBST solution, add 0.03% Tween-80 in TBS.
5 Add primary antibody solution and incubate at room temperature for 2 h. To prepare primary antibody solution, rat *K. pneumoniae* monoclonal antibody was diluted 500-fold by TBST.
6 Wash three times with TBST solution for 5 min.
7 Add secondary antibody solution and incubate at room temperature in darkness for 2 h. To prepare secondary antibody solution, goat anti-rat monoclonal antibody labelled with FITC was diluted 1000-fold by TBST.
8 Wash three times with TBST for 5 min in darkness.
9 Counterstain with 50 mg/ml propidium iodide for 2 in in darkness.
10 Wash once with TBS, shake in TBS and store in the freezer.

15.3.3 Physiological heterogeneities within biofilms

15.3.3a Growth rate

Only a few of the numerous fluorogenic stains with potential to indicate specific physiological and biochemical processes at the cellular level have been applied to microbiological studies (McFeters *et al.*, 1995a; see also Chapter 11). Among these, acridine orange (AO) has been widely used in the AODC technique for determining 'total' bacterial concentrations and has been interpreted with putative physiological implications. Some workers have used the staining reaction with AO to distinguish between active and inactive cells. While that practice has been openly questioned by some, it is based on the well-established differential spectrofluorescence of AO intercalated within single-stranded versus double-stranded nucleic acids and the relationship between the concentration of single-stranded (RNA) versus double-

stranded (DNA) nucleic acids and growth rate in bacteria. Specifically, more rapidly growing enteric bacteria, which contain a higher proportion of single-stranded nucleic acids, emit red to orange fluorescence while inactive bacteria appear green due to the different modes of AO–nucleic acid intercalation. Despite these factors, and considerable disagreement, the physiological validity of the AO stain remained largely untested until a crucial study was done (McFeters *et al.*, 1991). This study examined the physiological interpretation of the AO stain in enteric bacteria, by using *Escherichia coli* grown under a range of controlled laboratory conditions. Both these bacteria and purified nucleic acids and a single-stranded bacteriophage were examined using a number of methodological variables of the AODC staining technique. The findings indicate that the fluorescent outcome of the AO stain is a potentially useful index of physiological activity under carefully controlled conditions with some bacteria. Hence, this concept has been employed successfully to examine growth rate heterogeneities within bacterial biofilms and colonies that were cryosectioned followed by microscopic examination and electronic quantitation by image analysis.

Wentland *et al.* (1996) stained colony biofilm frozen sections, pretreated with acidic fixative (5% acetic acid, 4% formaldehyde, 85% ethanol), using 4 mg/l acridine orange, and examined them using epifluorescence microscopy. Plate 4D shows the growth rate pattern of a real *K. pneumoniae* biofilm section stained with acridine orange. The biofilm was green in the interior with an orange band running along the biofilm–bulk fluid interface. In places where the biofilm was locally thin, the orange band extended to the substratum. On colony biofilms, they found that growth rates correlated with the orange:green intensity ratios. The orange and green images were captured using Omega Optical filter cubic units 5721 and 5716, respectively. The images were saved as grey-scale TIFF files and their intensities were calculated by MARK software. Figure 15.4 shows the comparison of average

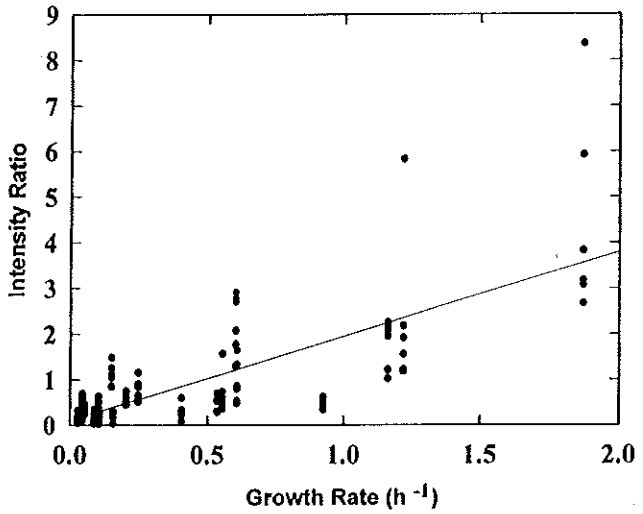

Figure 15.4 Comparison of average orange:green intensity ratio and colony average specific growth rate. The regressed line shown is given by $y = 1.84x + 0.107$ ($r^2 = 0.57$). Reproduced from Wentland *et al.* (1996), by permission of the American Institute of Chemical Engineers.

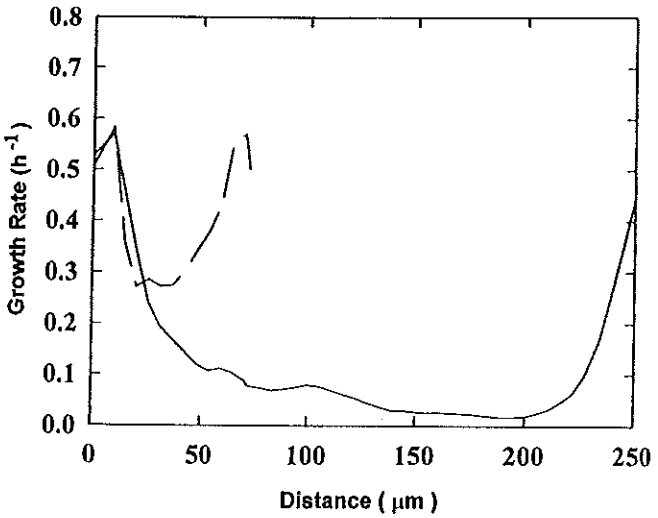

Figure 15.5 Estimated growth rate profiles in aerobically grown colonies. The colony ages and average specific growth rates were (solid line) 30.42 hours old, 0.086 h^{-1} and (broken line) 8.17 hours old, 0.56 h^{-1}. The membrane interface (agar side) was at approximately zero on the x axis. Reproduced from Wentland *et al.* (1996), by permission of the American Institute of Chemical Engineers.

orange:green fluorescent intensity ratios and average specific growth rates determined from cell count data. Using the linear regression of data in Figure 15.4 and profiles for the orange:green intensity ratio, spatial variations in the growth rate across a colony cross-section can be estimated (Figure 15.5). These profiles indicated that bacteria were growing rapidly near the air and agar interfaces, and more slowly in the centre of colonies. The correlation presented in Figure 15.5 is consistent with the differential staining of RNA and DNA by acridine orange and the known dependence of bacterial RNA:DNA ratio on specific growth rate (Moyer *et al.*, 1990; Berdalet and Dortch, 1991; Kerkhof and Ward, 1993).

These results indicate that orange colour corresponds to regions of rapid growth and green to regions of slow growth. The underlying mechanism for this approach for assessing actively growing bacteria has also been demonstrated through the use of fluorescent rRNA probes on planktonic cells (DeLong *et al.*, 1989) and bacteria within activated sludge flocs (Manz *et al.*, 1994).

Protocol 15.3 Acridine orange staining to distinguish growth rate.

1 Fix frozen sections mounted on glass slides in an acidic fixative at 4 °C for 10 min.
2 To prepare the acidic fixative, mix 5 ml 37% formaldehyde, 2.5 ml glacial acetic acid and 42.5 ml 95% ethanol.
3 Rinse the sections twice with 4 °C 85% ethanol.
4 Allow to air dry.
5 Add 5 μl of 4 μg/ml acridine orange on the section and leave in darkness for 1 min.
6 Blot excess acridine orange with tissue paper.

15.3.3b Respiratory activity and membrane potential

The microbiological adaptation of CTC (5-cyano-2,3-ditolyltetrazolium chloride) by Rodriguez *et al.* (1992) has enhanced the evaluation of respiratory activity of individual bacteria because of its fluorogenic properties. Although the mechanism of cellular CTC reduction is known (Smith and McFeters, 1997) and some limitations have been encountered in its use (Pyle *et al.*, 1995; Smith and McFeters, 1996), this compound remains useful in physiological studies of biofilms (McFeters *et al.*, 1995a; McFeters *et al.*, 1995b). When coupled with cryosectioning, this compound has allowed the visualization of spatial and temporal cellular respiratory activity in bacterial biofilms with disinfectant treatment (Huang *et al.*, 1995). CTC has also been used to determine that 5–35% of the bacteria within drinking water biofilms demonstrated active respiration and that starvation resulted in a rapid reduction in electron transport activity (Schaule *et al.*, 1993).

Fluorescent probes that reveal cellular bioenergetic properties have also been useful in the assessment of physiological activities within bacterial biofilms. Rhodamine 123 (Rh123), which signals membrane potential, is such a stain that has been applied to the determination of physiologically active bacterial populations in starved planktonic cultures of *Micrococcus luteus* (Kaprelyants *et al.*, 1996) and within biofilms (Yu and McFeters, 1994b). Enumeration results obtained after staining with Rh123 were comparable with data from CTC activity and an *in situ* direct viable count (DVC) method for enumeration of viable cells (Kogure *et al.*, 1979), but all three yielded significantly higher values than plate counts. Cellular energetic status was also evaluated within biofilm communities by determining spatial heterogeneities in adenylate energy charge, by cryosectioning followed by classical adenylate extraction and measurement (Kinniment and Wimpenny, 1992). Other membrane potential fluorogens will almost certainly be found to be useful in the description of cellular physiological heterogeneities within biofilms.

15.3.4 Physiological response of biofilms during disinfection

Accurate data describing the response of key cellular physiological processes within bacterial biofilms exposed to disinfectants is critical in the study of biofilm control. Such information is essential to (i) describe the mode of action of different disinfectants, (ii) help explain why bacteria within biofilms are less susceptible to antimicrobials and (iii) evaluate the efficacy of new and existing antimicrobial formulations. Therefore, the analytical approach utilizing fluorogenic stains for key physiological activities in combination with cryosectioning, microscopic examination and digital image analysis is ideal for gaining a greater understanding of biofilm control strategies.

Although more traditional methodologies such as DVC and fluorescence microscopy have been useful for describing the effects of biocides on very thin biofilms *in situ* (Yu *et al.*, 1993), alternative approaches are clearly needed when studying the disinfection of thicker biofilms, which are more typical of sessile communities in natural systems. Further, the method for assessing the cellular response to biocide exposure is optimal if it specifically addresses a key

physiological process that is known to be compromised by the disinfectant under investigation. Chlorine-based biocides continue to be widely used as disinfectants in aquatic systems, and their coupling with CTC to assess the physiological response of the treated bacteria satisfies that experimental design criterion. Specifically, chlorine is known to damage bacterial respiratory activity, when used in a way that mimics the disinfection of drinking water (Camper and McFeters, 1979), and CTC provides a compatible microscopic method to evaluate the respiratory activity of the treated biofilm at the cellular level.

Studies were carried out to describe the spatial and temporal physiological response of bacterial biofilms to disinfection with chloramine (Huang *et al.*, 1995). Biofilms containing both *P. aeruginosa* and *K. pneumoniae* were grown on stainless steel coupons in a continuous-flow reactor, which were then treated with monochloramine (2 mg/l) for two hours. This resulted in very little removal of biofilm, but there was a linear one-log reduction of culturable bacteria belonging to both species. Coupons with control and treated biofilms were also examined at various times of disinfection exposure, to discriminate respiring and nonrespiring cells using CTC and DAPI, as a counterstain, followed by cryosectioning, microscopic examination and data acquisition by image analysis. Examination of these biofilms using epi-illuminated fluorescence microscopy clearly revealed uniform activity throughout the community in control samples that were not treated with biocide, but increasing gradients of respiratory activity, seen as striking images of active (orange) and inactive (green) cells, with time of exposure to disinfectant. The gradients in specific cellular respiratory activity were then quantified by calculating the ratio of reduced CTC to DAPI intensities as shown in Figure 15.6. The CTC and DAPI stained images were captured using Olympus G and U filters, respectively, and the intensities were measured by image analysis. These data, which resulted in a family of curves demonstrating respiratory activity

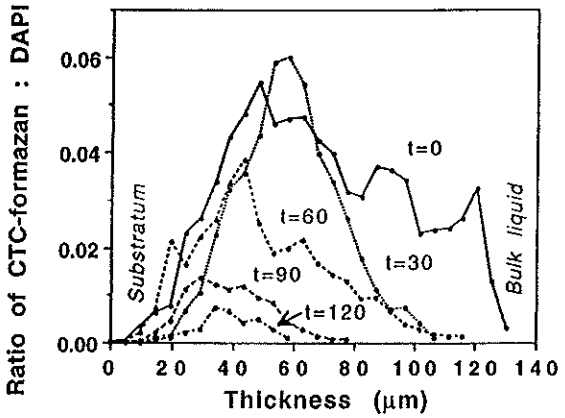

Figure 15.6 Ratio of intensities of CTC–formazan and DAPI obtained from 5 μm frozen sections following exposure to 2 mg monochloramine per litre. The biofilm thicknesses determined by the dimensions of the DAPI-stained region were 121 μm before treatment and 116, 106, 125 and 92 μm at 30, 60, 90 and 120 min, respectively. *t*, Time (min). Reproduced from Huang *et al.* (1995), by permission of the American Society for Microbiology.

versus position within the biofilm for each of the biocide exposure times, graphically revealed the spatial and temporal effects of monochloramine on bacterial cells within this biofilm community. Before application of chloramine, high levels of reduced CTC were seen throughout the entire 130 μm depth of the biofilm, indicating relatively uniform respiratory activity in the community. However, with disinfection, individual cells near the biofilm–bulk fluid interface lost respiratory activity first and after 120 minutes of exposure, only an approximately 60 μm thick region of the biofilm exhibited low levels of respiration. It is also of interest that the remaining active cells following disinfection were near the substratum and centrally located within structured cell clusters.

This study demonstrates the utility of using a specific fluorogenic stain to reveal the non-uniform physiological response of individual bacteria within a biofilm exposed to a disinfectant. The resulting data have provided a greater understanding of biofilm control, and allow the formulation of new hypotheses to stimulate further advances. This development in the area of biofilm research is mirrored by similar advances using flow cytometry technologies to evaluate the physiology of heterogeneous planktonic bacterial populations using some of the same fluorochromes (Davey and Kell, 1996).

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