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HETEROGENEITY OF NATURAL BIOFILM COMMUNITIES

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Abstract

Scanning confocal laser microscopy (SCLM) studies have shown that microbial biofilms consist of a spatially-heterogeneous arrangement of bacterial cells, biogenic extracellular material, and void space. Biofilm architecture is dominated by the presence of cell aggregates interspersed by channels, the arrangement of which varies for different hydrodynamic regimes, ages, species composition, or nutrient status. This organization may regulate the flux of nutrients and oxygen, creating chemical microenvironments (Eh, pH) facilitating the growth of aerobic heterotrophs and anaerobes. The presence of microenvironments has been confirmed using laser microscopy and environmentally sensitive fluorescent probes. In addition, changes in cell number and growth rate with depth and location have also been documented. Elegant methods using fluorescent rRNA probes and reporter gene systems have allowed *in situ* visualization of high genetic diversity and the regulation of genes in microbial biofilms. Through the use of scanning confocal laser microscopy (SCLM) and fluorescent probes, both qualitative and quantitative analyses of many aspects of the ecology of biofilms may be studied.

Key Words: Biofilms, microenvironment, light microscopy, confocal, digital imaging.

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Introduction

Many studies over the last decade have provided a better understanding of the structure and function of biofilms. However, very little has been done to study the spatial organization within these communities. Although it has always been apparent that biofilms do not fit a model of thin homogeneous layers, comparatively little effort has been focused on the description and quantification of biofilm heterogeneity. Recent studies have emphasized the presence of extensive structural complexity and heterogeneity within biofilms (Lewandowski *et al.*, 1989, 1993; Lawrence *et al.*, 1991; Korber *et al.*, 1993; Stewart *et al.*, 1993; Wolfaardt *et al.*, 1994a). Much of this change in our view of microbial biofilms arises from the development and application of scanning confocal laser microscopy (SCLM) and fluorescent molecular probes in biofilm studies.

The development of heterogeneity in microbial biofilms is a function of a series of factors extrinsic to the bacteria and those that may be considered intrinsic factors. Extrinsic factors include: qualities of the substratum, environmental stresses, nutrient availability, flow rates, and effects of grazing. In contrast, factors intrinsic to the colonizing bacteria include: behavior, chemotactic and sensory responses, metabolic pathways, and interactions such as co-aggregation and aggregation. The many factors that have been demonstrated to influence bacterial attachment and biofilm development have been recently reviewed (Lawrence *et al.*, 1995).

Bacteria live in a diffusion-dominated environment and, as individuals, are unable to alter the environment in which they live (Koch, 1991). As a consequence of microbial activity and diffusion limitation, the development of the fundamental biofilm characteristic, the microenvironment, occurs.

This article reviews SCLM based studies of biofilms and the approaches that may be used to apply high resolution digital microscopy to biofilm research. Reviews of digital imaging and SCLM for environmental microbiology applications have been presented previously (Caldwell *et al.*, 1992a; Lawrence *et al.*, 1995, 1996).

Instrumentation and Probes

More basic biological and ecological knowledge is needed to understand the growth of bacteria in multi-species biofilms. For such a purpose, traditional methods are not the appropriate tools. Isolation studies extract the organism from its natural context and restrict studies to a narrow spectrum of naturally occurring microorganisms (Torsvik *et al.*, 1990; Ward *et al.*, 1990). Light microscopy may be used to visualize microorganisms in their natural habitat, however, resolution is limited, particularly for epifluorescence approaches, and information regarding the microenvironment and taxonomic affiliation of microorganisms may not be readily obtained.

There have been several advances in the areas of digital imaging and light microscopy which allow extensive use of non-destructive techniques on a wide range of sample matrices. Digital image processing and analysis methods are now affordable for many laboratories. Highly sensitive charge-coupled device cameras can visualize extremely low levels of emitted light. Ramsing *et al.* (1993) utilized this approach to examine the distribution of sulfate reducing bacteria in biofilms. Confocal scanning laser microscopy allows detailed, non-destructive examination of thick microbial biofilms. Using high numerical aperture 1.4 60X lenses, biofilms 80-100 μm have been optically sectioned; when 0.55 NA extra long working distance lenses or water immersible lenses are used, images may be obtained throughout 500 μm thick biofilm materials. These capacities were demonstrated for biofilms by Lawrence *et al.* (1991) and reviewed by Caldwell *et al.* (1992a). The technique of optical sectioning removes out-of-focus information and leads to sharp, clear digital images. As such, it is an ideal tool for studying spatial distribution of cells in immobilized biofilm communities. When SCLM is coupled with the large number of environmentally sensitive probes that are available (Haugland, 1992), direct data may be acquired on diffusion (Lawrence *et al.*, 1994), redox, as well as pH and ion concentrations. Other probes reveal information on cell viability. SCLM used in conjunction with fluorescent antibodies or oligonucleotide probes provides vital information on the taxonomic affiliation (Assmus *et al.*, 1995) and growth rates (Poulson *et al.*, 1993). Bloem *et al.* (1995) described a fully automatic image analysis system capable of measuring cell numbers, volumes, length and frequency of dividing cells using SCLM images. SCLM, light microscopy, and digital image analysis techniques for application in environmental microbiology have recently been reviewed by Lawrence *et al.* (1996). A comparison of a variety of microscopic techniques for studying biofilms has been presented by Surman *et al.* (1996). In addition, Laurent *et al.* (1994) have provided a more general evaluation of biological applications of SCLM.

Scanning confocal light microscopy may also be combined with physical probes, i.e., microelectrodes to provide a visual record of the environment in which the sensing tip is operating. This approach has been successfully used for examination of oxygen distribution in biofilms (Costerton, *et al.*, 1994; de Beer *et al.*, 1994a). Ramsing *et al.* (1993) correlated the distribution of sulfate reducing bacteria with O_2 and H_2S levels in photosynthetic biofilms using oligonucleotide probes and microelectrodes. Microelectrodes used alone have also provided valuable information on heterogeneity of biofilm communities. Microelectrodes can be used to perform direct concentration measurements on biofilms with a high degree of spatial resolution for a variety of parameters (Revsbeck, 1989; Kuhl and Jorgensen, 1992; de Beer *et al.*, 1993, 1994a,b; Lens *et al.*, 1993). Measurements may be made with relatively little disturbance using sensing tips from 1 to 10 μm for O_2, H_2S, nitrate, ammonium, chloride and glucose.

Biofilm Architecture

When examined using SCLM, living biofilms appear to consist of a variable distribution of cells, cell aggregates, extracellular polymers, and void spaces. This pattern has been defined by Lawrence *et al.* (1991) as the architecture of the biofilm and has been shown to be influenced by a range of factors. These include: the species of bacteria, the nutrient source provided, the flow rate in the liquid phase, grazing by protists, and various environmental stresses.

Lawrence *et al.* (1991) used SCLM with negative staining (Caldwell *et al.*, 1992b) and image analysis techniques to describe the architecture of *Pseudomonas (P.) fluorescens* and *Vibrio parahaemolyticus* biofilms. The *P. fluorescens* biofilms had the highest number of cells at their attachment surfaces and became increasingly diffuse near their outer regions whereas the *Vibrio* biofilms exhibited the opposite trend. In general, the biofilms were highly hydrated, open structures composed of 73 to 98% extracellular materials and space. They went on to indicate that species specific architectures represent the optimal arrangement for influx of nutrients, transfer of wastes, and establishment of microenvironmental conditions. An example of images used for architectural analysis of a biofilm is shown in the series of optical thin sections in Figure 1.

Keevil and Walker (1992) have shown, using SCLM, environmental scanning electron, and episcopic differential interference contrast microscopy, that many native biofilms have long, finger-like stacks that rise from the attachment surface. The basal layer is 5 μm in

depth with stacks or fronds extending 150 μm from the basal layer. The biofilm structure was also described as being penetrated by many channels. The existence of channels within the biofilm has also been noted by Massol-deya *et al.* (1995) for biofilms growing on granular activated carbon. The work of Stoodley *et al.* (1994) has demonstrated the existence of extensive channeling by using SCLM to trace the movement of 0.28 μm beads in biofilms formed by 3 bacterial species growing on glucose. de Beer *et al.* (1994b) also found complex structures in aerobic biofilms, consisting of discrete aggregates and interstitial voids. They further indicated that these structures have considerable impact on mass transfer within the biofilm.

The heterogeneity of biofilms has been shown to be dependent on physical parameters such as laminar flow velocity. High flow rates have been associated with increased substrate loading capacities of biofilms, increased biofilm densities, increased mass transport of compounds across the liquid/biofilm interface, and increased sloughing of biofilm materials (Trulear and Characklis, 1982; Bryers, 1987; Characklis *et al.*, 1990).

Korber *et al.* (1994a) reported effects of laminar flow velocity on the structure of *P. fluorescens* biofilms, measured in terms of biofilm depth and variability of depth measurements. It was found as discussed below, that the occurrence of channels varied with the rate of laminar flow, all other factors remaining constant. These observations may be significant with respect to nutrient limitations which occur within actively growing biofilms and are influenced by flow. For example, biofilms grown at low laminar flow velocities were thinner (0.01 cm s^{-1}, mean depth = 12.3 ± 6.8 μm) than those grown at higher laminar flow velocities (0.21 cm s^{-1}, mean depth = 83.7 ± 10.2 μm). The variability in depth measurements (coefficient of variation) also correlated inversely with rate of laminar flow, indicating that as biofilms became thicker, channels became less prevalent. *P. fluorescens* biofilm bacteria may have adapted to stresses imposed by nutrient limitation by forming large water channels which provide an alternate (lateral) route for the diffusion/advection-limited transport of nutrients or electron acceptors to the base region of biofilms, and similarly, the escape of metabolic wastes. However, nutrient limitation at the lower flow velocities may simply prevent growth, thereby limiting distribution of cells in the biofilm. Similar observations on the existence of pores and channels have been made in studies of biofilm formation on granular activated carbon and other substrata (Lawrence *et al.*, 1991; de Beer *et al.*, 1994a; Stoodley *et al.*, 1994; Massol-deya *et al.*, 1995).

Wolfaardt *et al.* (1994a) showed a dramatic effect of switching from trypticase soy broth to a chlorinated hydrocarbon as carbon source on the architecture of a biofilm community. In this case, the community changed from a relatively thin 5-10 μm homogeneous biofilm structure to one with diversity in morphology of microcolonies and the development of mounds extending up to 40 μm into the overlying medium. The time scale for this switch was also remarkable in that the changes were apparent within 48 hours of the change in nutrients. Changes in biofilm architecture were also observed when this consortium was grown on 2,4-dichlorophenol and 1,3-dichlorobenzene (Wolfaardt *et al.*, 1994a). Although it has been presumed that aerobic degradation of hydrocarbons would not involve extensive interspecies interactions and growth coordination, this study and that of Massol-deya *et al.* (1995) indicate this is not the case.

In most environments, bacteria are subject to periodic stresses which may disrupt the biofilm community, alter growth rates, or select for certain populations. The end result of these effects is the creation of variability in biofilm architecture. Depth-dependent variability in growth rates for biofilm bacteria has been inferred by Korber *et al.* (1994b), where treatment with a fluoroquinolone (fleroxacin, which results in cell elongation) caused significantly more cell elongation at the bulk-liquid interface than at the base of the biofilm, even though determinations of fleroxacin penetration (based on fluorescence) indicated that migration of this compound was not hindered by the bacterial/exopolysaccharide matrix. While cell elongation was evident throughout fleroxacin-treated biofilms, the mean cell length at the 15 μm section depth was 8.1 ± 2.4 μm (max. ~15 μm) following 48 hour fleroxacin exposure, whereas the length of cells located at the base of the same biofilm was 4.2 ± 1.8 μm. In contrast, control biofilm cells at the 15 μm section depth at 72 hours were approximately 1.7 ± 0.6 μm in length, as compared with measured cell lengths at the biofilm base of 1.5 ± 0.5 μm. Generally, cell elongation following the application of fleroxacin was most obvious in regions closest to the bulk phase of the irrigation medium, presumably because these cells were closer to the nutrient source, and thus, were actually growing more rapidly. These observations agree with the premise that biofilms grow mainly at the biofilm-liquid interface and not from the biofilm base, and have implications not only for understanding the ecology of biofilm systems, but also for interpreting the effects of anti-microbial agents on heterogeneous biofilms where not all cells are likely to be growing rapidly or at the same rates (Gilbert *et al.*, 1990; Eng *et al.*, 1991). Gorman *et al.* (1994) also used SCLM to examine the nature of catheter biofilms and noted that they were dominated by microcolonies and clusters of pseudomonads and staphylococcal cells; they attributed this architectural form to the effects of antibiotic therapy on the biofilm development.

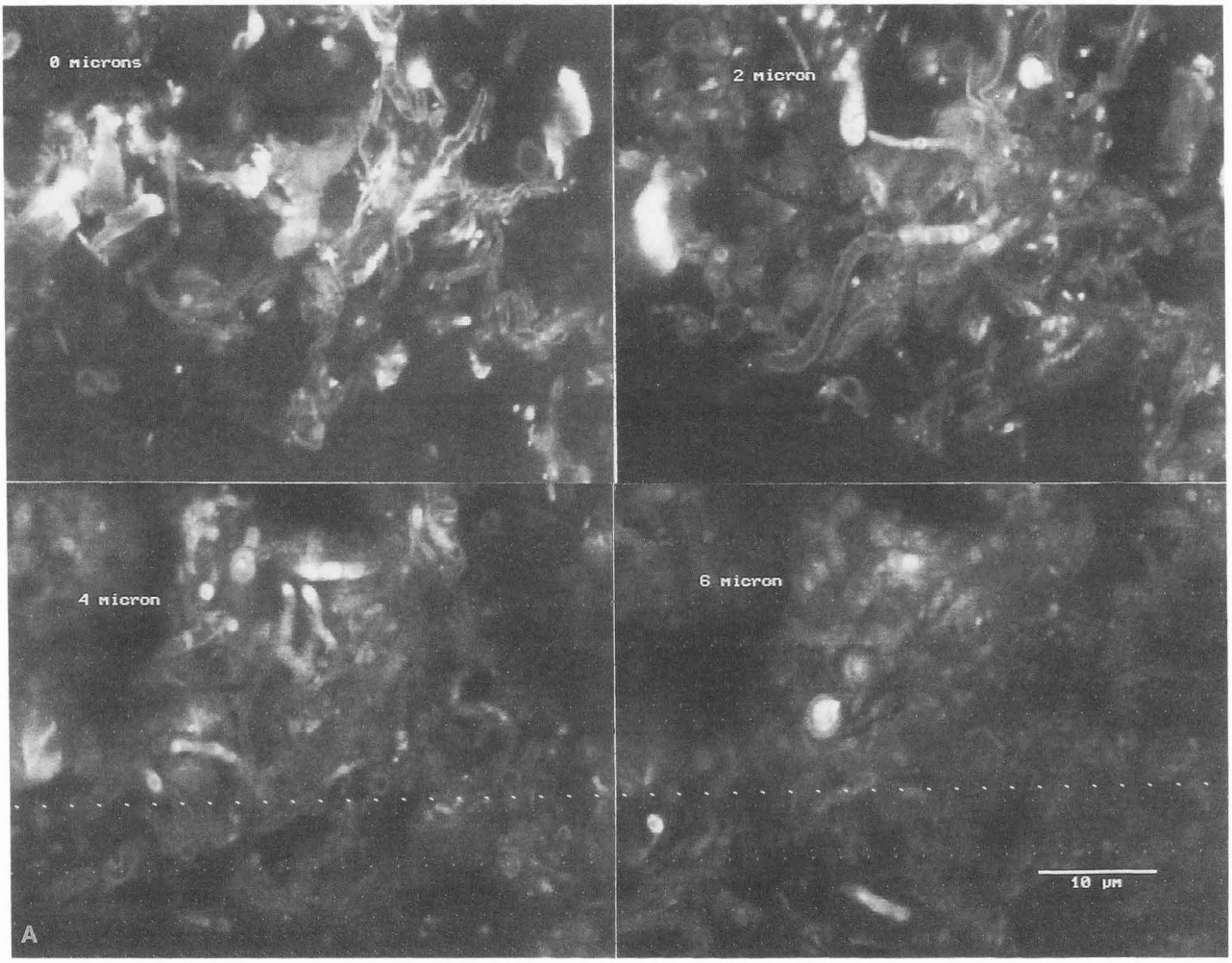
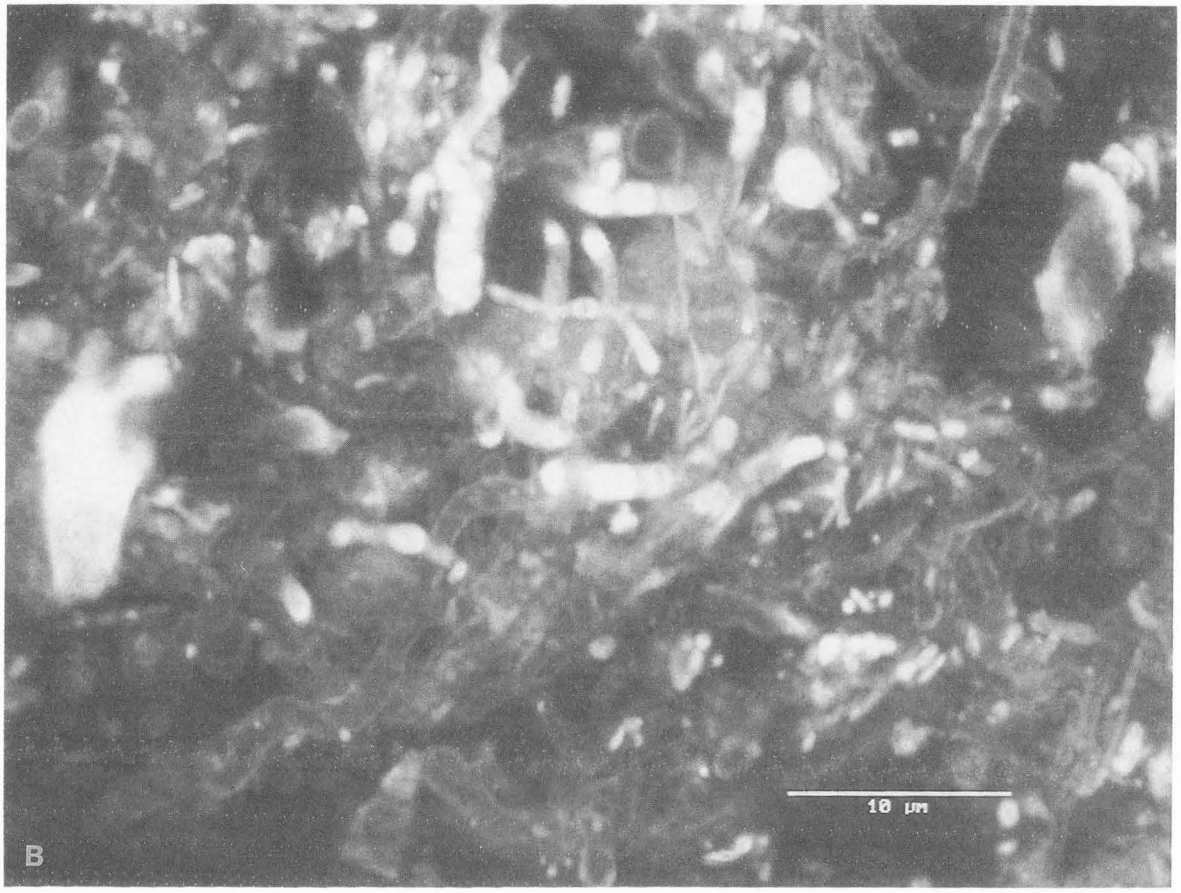


Figure 1. A series of SCLM xy optical thin sections at 0, 2, 4 and 6 μm shown in (**A**) were combined to create a through-view projection of a mixed species biofilm developing on a wood chip support in a bioreactor (**B**). Bar = 10 μm. *Figure 1 (A) above, Figure 1(B) on facing page.*

In one of the few statistically-based studies of biofilm architecture, Korber *et al.* (1993) used a computer controlled stage and SCLM to construct large montages of contiguous regions of *P. fluorescens* biofilms. The large biofilm montages were analyzed to define the extent of analysis required to provide representative biofilm biomass data. It was found that the spatial variability contained within pure culture *P. fluorescens* biofilms was large, requiring analysis areas exceeding 1 x 10^5 μm^2 for statistically-representative results. Thus, biofilms may be extensively heterogeneous and provide a subject for study that can be extremely problematic if representative results are to be obtained.

The Microenvironment

Microbial consumption of nutrients, production of wastes, and synthesis of cellular and extracellular materials all act in concert to physicochemically define what is known as the microbial microenvironment (Hamilton, 1987). These microenvironments persist over time due to diffusion limitations in combination with sustained metabolic activity. The presence of concentration gradients can influence bacterial population diversity and spatial distribution as well as microbial metabolic activity within biofilms, allowing the survival of other organisms with specific, and often stringent, growth requirements. The microenvironment is therefore believed to be the foundation for much of the observed microbial diversity in nature. Many studies have demonstrated the stability



of biofilm communities against disturbances (Brown *et al.*, 1988; Brown and Gauthier, 1993; Korber *et al.*, 1994b; Huang *et al.*, 1995). The basis for this resistance has often been hypothesized to be the existence of physiological, physical and chemical gradients.

Although this is a fundamental concept, its presence has proven difficult to directly verify. The use of SCLM in conjunction with fluorescent probes is one of most attractive approaches to study spatial gradients within biofilms. Evidence has been provided for the existence of physiological variation within biofilms. Korber *et al.* (1994b) has described gradients in nucleic acid staining and cell length in *Pseudomonas* sp. biofilms treated with fleroxacin. Huang *et al.* (1995) reported a spatially non-uniform loss of microbial respiratory activity with biofilms after treatment with monochloramine, with the greatest loss at the biofilm bulk-phase interface. They also described how the highest rates of respiratory activity after treatment were restricted to the base of the biofilm and the centers of cell clusters. The likely explanation for this phenomenon is the development of concentration gradients within the biofilm as a consequence of hindered diffusion. The existence of these gradients for chlorine based disinfectants was confirmed by de Beer *et al.* (1994a).

The presence of hindered diffusion within biofilms was also demonstrated by Lawrence *et al.* (1994) who used SCLM and size-fractionated fluorescent dextrans to demonstrate the existence of regions of low mobility. These authors also showed that extensive binding of both positively and negatively charged molecules occurred within the biofilms which would contribute to incomplete penetration by reactive molecules. Thus, the existence of gradients such as those shown in Figure 2, can be confirmed through direct visualization with SCLM and various staining or probe based techniques.

The application of pH-sensitive fluorescent probes has provided some information on microenvironments, indicating < 1 μm zones of increased fluorescence (higher pH) surrounding cells within biofilms as shown in an SCLM micrograph (Fig. 3). In contrast, Caldwell *et al.* (1992a) showed images of 1-3 μm zones of reduced fluorescence surrounding cells of *Vibrio parahaemolyticus* in biofilms. This change in fluorescence was interpreted as a possible zone of reduced pH. It has been noted that interpretation of these fluorescence patterns may be confounded by differential penetration-concentration effects, or quenching of the fluorescent compound by interaction with proteins in the vicinity of the cell (Caldwell *et al.*, 1992a).

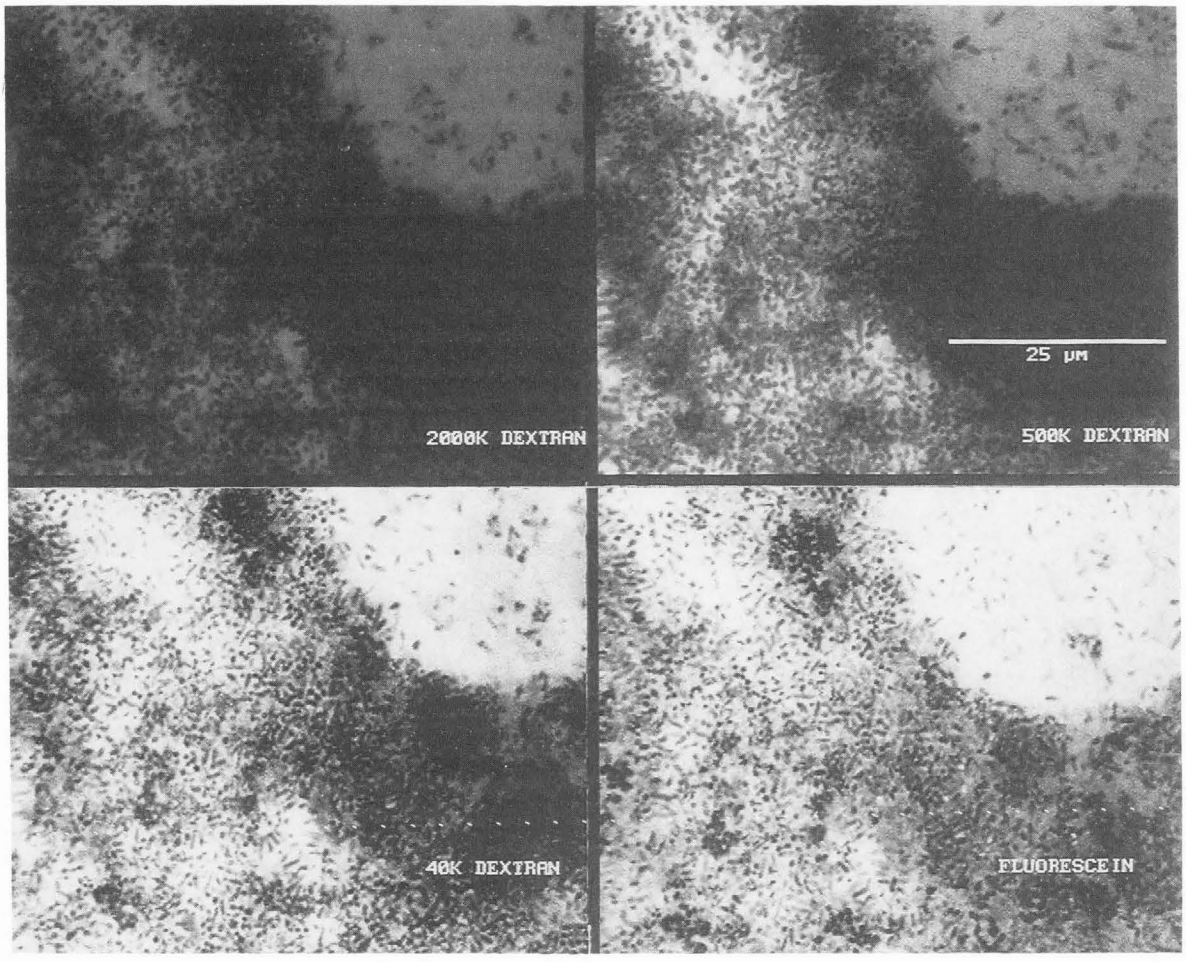


Figure 2. A series of xy images showing the relative penetration of 2,000K, 500K, 4K dextrans and fluorescein at the same location in the biofilm. Dextrans were added from highest to lowest molecular weight and the system was purged with fluor free medium between experiments. Reprinted from Lawrence *et al.* (1994), with permission of Appl. Environ. Microbiol. Bar = 25 μm.

The occurrence of these microzones is also related to the extreme importance of exopolymers in biofilm development (Vandevivere and Kirchman, 1993; Lawrence *et al.*, 1995). Distribution of unique extracellular polymers (EPS) surrounding different members of biofilm consortia may also contribute to the development of chemical microzones (Fig. 4) within the biofilm. Analysis of EPS through traditional means does not allow definition of their spatial distribution within biofilms and their association with specific cells and cell types. Lawrence *et al.* (1995) described the preliminary results of applying a suite of fluor-conjugated lectins to assess the distribution of lectin binding sites within the EPS of a biofilm community grown on the herbicide diclofop methyl as sole carbon source. They noted specific EPS were associated with the binding of the herbicide within the biofilm. In a series of parallel SCLM based studies, Wolfaardt *et al.* (1995) have shown the distribution of the herbicide within biofilms, its local accumulation and subsequent utilization as a carbon source by the bacterial consortium under starvation conditions. They have also shown, that the nature of the EPS and the binding of pesticides has considerable environmental relevance resulting in biotransfer to grazers feeding on the EPS of biofilms (Wolfaardt *et al.*, 1994b).

The dynamic nature of these gradients can be demonstrated through the application of pH buffered solutions to adjust the pH of the bulk phase and assess the impact on microenvironmental pH. The results of pH shifting are shown in Figure 5, where a biofilm consortium growing in continuous flow slide culture was sequentially exposed to macroenvironmental pH's of 4, 7 and 9. The resulting series of images indicated the existence of regions that adopted the pH of the macroenvironment

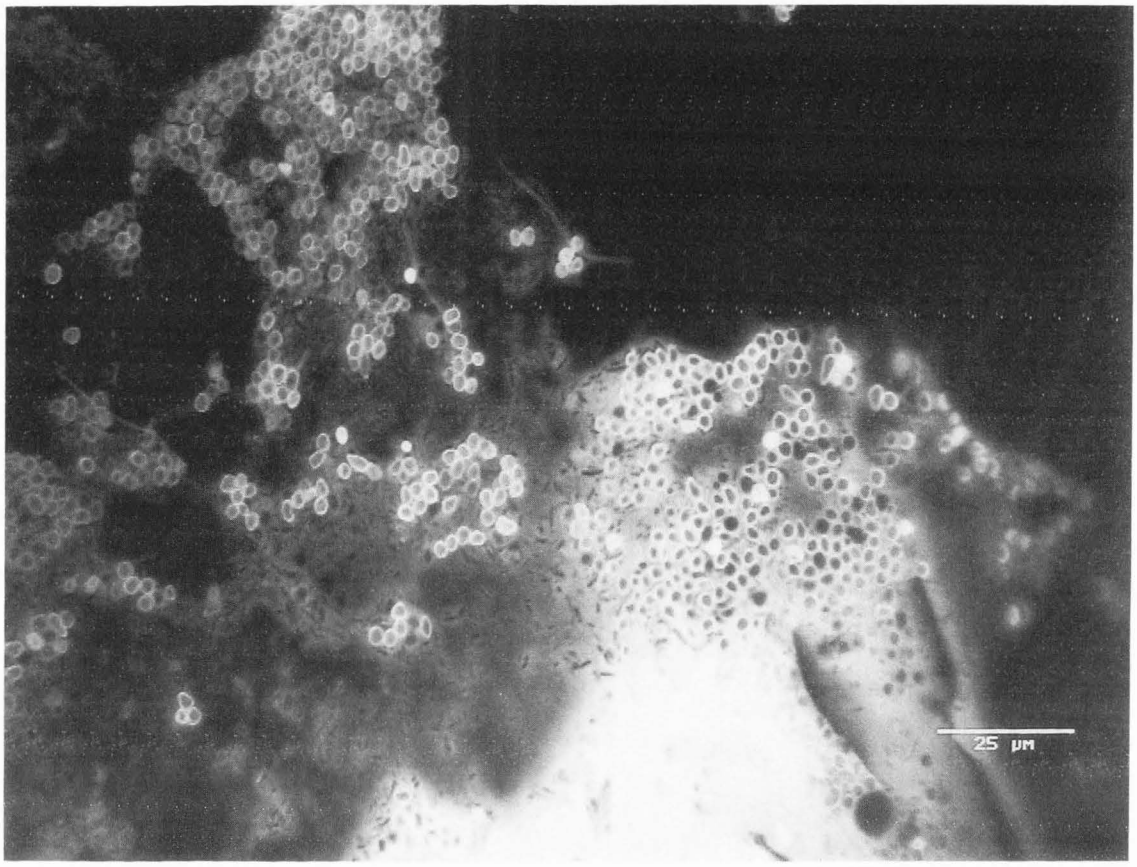


Figure 3. SCLM image providing evidence for microzonal variation in pH within a mixed species biofilm growing on a wood chip support. The fluorescence of fluorescein is quenched at pH 5 or below and increases to pH 9, in this case the sample was flooded with a 0.1% fluorescein solution at pH 7 and covered with a #1 coverslip prior to observation with SCLM. Cells in the top left of the image show a fluorescent halo indicative of a pH > 5 and are surrounded by a dark background indicative of pH < 5. In contrast, in the lower left the cells occur on a fluorescent background, indicating that the pH has shifted to > pH 5. Bar = 25 μm.

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and others that retained pH's lower or higher than that of the macroenvironment.

Another critical component of the biofilm community is the ratio of active or living cells to those that are moribund. Lloyd and Hayes (1995) reviewed viability probes and provided examples of their use in conjunction with SCLM. Mason *et al.* (1995) assessed the usefulness of membrane potential dyes and calcafluor white for their ability to detect living and dead bacterial cells, they indicated that bis 1,3-dibutylbarbituric acid and calcafluor were the most useful probes. The use of LiveDead stains produced by Molecular Probes, Eugene, OR, to determine the distribution of living and dead or moribund cells within biofilm materials is shown in a representative dual channel image (Fig. 6). Caldwell and Lawrence (1989), showed that redox sensitive probes such as resazurin, used in conjunction with SCLM and optical sectioning, could indicate the viability of bacterial cells within cell masses. Rodrigues *et al.* (1992) reported on a promising fluorescent probe for visualization of actively respiring bacteria on surfaces.

Bacterial cells living in a multilayered matrix may be viewed as being diffusion limited and the application of microelectrodes has shown the existence of steep oxygen gradients within aerobic biofilms, confirming the importance of diffusion processes in these systems (Jorgensen and Des Marais, 1990; Dalsgaard and Revsbech, 1992; de Beer *et al.*, 1993, 1994b). De Beer *et al.* (1994b) combined SCLM with microelectrode studies to provide some of the best evidence for the existence of oxygen gradients within aerobic biofilm communities. They showed that oxygen distribution was strongly correlated with the structure of the biofilm, with low oxygen levels in the cell clusters and with depth and higher oxygen values at the biofilm bulk-phase interface as well as the voids and channels.

With the broad range of fluorescent environmentally sensitive probes that are available, the potential of SCLM to visualize and quantify gradients within biofilms is significant, but to date, under utilized.

Analysis of Populations and Communities

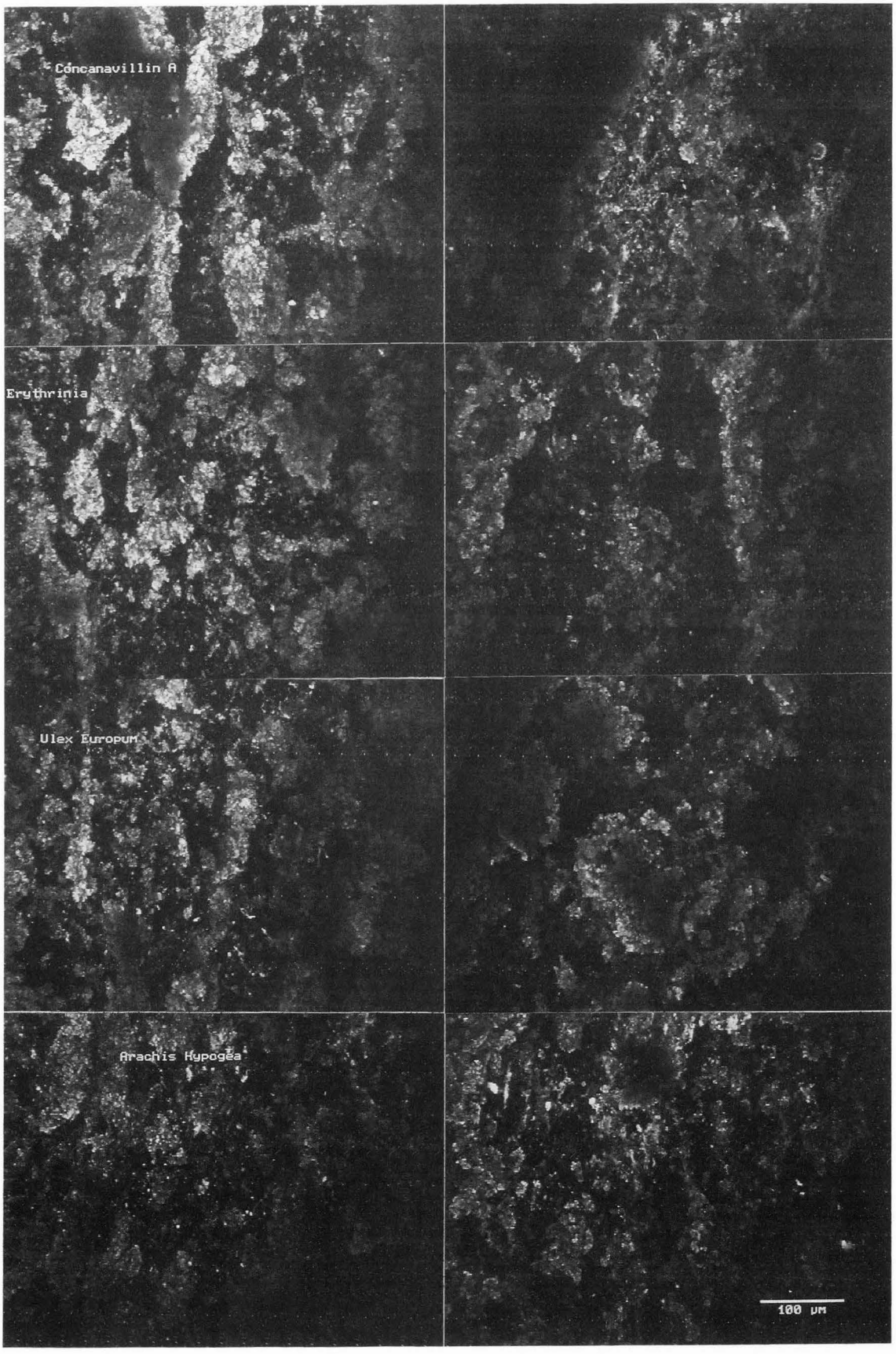
Diversity and dynamics of microbial populations in biofilms and aggregates have mainly been analyzed using culture dependent methods. However, the development of fluorescently conjugated mono- and polyclonal antibodies, and 16S and 23S rRNA oligonucleotide probes provide complementary methods which allow the *in situ* identification of individual cells growing in biofilms. Immunofluorescence techniques, although requiring initial isolation, are rapid and simple and may be used to detect individual cells in complex habitats. Their use in the study of biofilms appears relatively recent, (Zambon *et al.*, 1984). These authors used a battery of 5 antisera to monitor the contribution of 5 species of bacteria to marine biofilms. Although they have been used extensively with conventional epifluorescence microscopy, the application of monoclonal antibodies (Mabs) in conjunction with SCLM is much more limited. Singleton *et al.* (1995) used Mabs to study a mixed species oral biofilm consisting of a consortium of nine oral species. The results simply indicated that it was possible to stain and image *Streptococcus mutans* at the base of a 40 μm thick biofilm using fluorescently labeled Mabs and SCLM. James *et al.* (1993) presented preliminary results regarding the distribution of an *Acinetobacter* sp., *Pseudomonas fluorescens* and *Aeromonas hydrophilia* in biofilms composed of these three species. The authors used SCLM and digital image analysis to determine the contribution to biomass by each member of the biofilm. An excellent example of the immunolabelling approach to biofilm studies is provided by Rogers and Keevil (1992), who tracked the fate of *Legionella pneumophila* in aquatic biofilms. Schloter *et al.* (1993) used Mabs and SCLM to localize *Azospirillum* in the rhizosphere of wheat plants. Hausner, Lawrence and Hartmann (unpublished data) utilized immunolabelling to track the fate of *Agrobacterium* sp. in a degradative biofilm community. In this case, the organisms persistence and spatial distribution when added to the nine-member consortium described by Wolfaardt *et al.* (1994a) was examined. Figure 7 shows a dual channel image with the immunolabelled *Agrobacterium* on the left, while the other biofilm members are shown on the right image. Thus, antibody techniques in conjunction with SCLM are very useful for examination of fully hydrated biofilm material, providing detailed information on identification, location, number and biomass.

SCLM and epifluorescence microscopy, in conjunction with 16S and 23S rRNA probes, have been used to document microbial diversity in a range of environments including sewage sludge and the rhizosphere (Hahn *et al.*, 1992; Wagner *et al.*, 1993, 1994; Amann *et al.*, 1995; Assmus *et al.*, 1995; Wagner *et al.*, 1995). An in depth review dealing with the synthesis and application of oligonucleotide probes for microbiological systems with some specific reference to biofilms has been provided by Amann *et al.* (1995). This review also includes a series of very good SCLM optical thin sections and red-green anaglyph projections of rRNA probed materials.

Figure 4. (*on facing page*) Distribution of unique extracellular polymers surrounding different members of biofilm consortia may also contribute to the development of chemical microzones within the biofilm. A lotic biofilm community was stained with glycoconjugate specific lectins, *Canavalia ensiformis*, *Erythrinia sp.*, *Ulex europaeus*, and *Arachis hypogaea*, the images show the binding sites for these 4 lectins at the several locations within the biofilm. Bar = 100 μm.

One of the first applications of molecular and microscopic identification of defined bacterial populations in multispecies biofilms was carried out using an anaerobic fixed-bed reactor community (Amann *et al.*, 1992). Using *in situ* hybridization, they were able to distinguish two morphologically distinct populations and monitor the colonization of new surfaces. The study of Ramsing *et al.* (1993) examined the distribution of sulfate reducing bacteria (SRB) in photosynthetic biofilms from a trickling filter. They were able to demonstrate the uneven distribution of SRB in the biofilm and the negative correlation of SRB numbers and oxygen profiles. Assmus *et al.* (1995) showed that SCLM and 23S rRNA probes could be used to localize two *Azospirilla* within the rhizosphere of winter wheat plants. Examination of drinking water biofilms using proteobacterial subclass-specific probes indicated that these biofilms are composites of many phylogenetically diverse bacteria (Manz *et al.*, 1993). Manz *et al.* (1995) used SCLM and 16S rRNA probes to visualize *Legionella* sp. in model biofilms and within cells of the protistan *Tetrahymena pyriformis*.

These rRNA probes have also been used in conjunction with image analysis to determine the growth rates of individual cells within biofilms based on quantitative hybridization with ribosomal probes (DeLong *et al.*, 1989; Poulsen *et al.*, 1993; Møller *et al.*, 1995). More recently, the application of SCLM to these enumeration problems has resulted in the development of a fully automatic system for determination of not only cell numbers, but also cell volumes and frequency of dividing cells (Bloem


Concanavillin A
Erythrinia
Ulex Europum
Arachis Hypogea
100 µm

et al., 1995). Møller *et al.* (1995) also used acridine orange staining to obtain simultaneous estimates of cellular RNA and DNA contents of single cells. These determinations may be made very effectively using the combination of SCLM and image analysis of the fluorescence signals.

Genes and Gene Expression

All microorganisms are capable of sensing changes in their environment and can respond to these changes by altering patterns of gene expression as well as genotypic and phenotypic adjustments.

Studies of *V. parahaemolyticus* represent the most comprehensive investigations of surface-induced gene expression (McCarter *et al.*, 1992). Dagostino *et al.* (1991) reported that genes related to specific functions may only be switched on at surfaces and not while the bacteria are growing planktonically. Goodman *et al.* (1993) showed that the marine *Pseudomonas* S9 has a gene that was only switched on at a hydrophobic polystyrene surface. Dalton *et al.* (1994) found that biofilms of a marine bacterium were tightly packed on hydrophobic surfaces, whereas they were sparse and consisted of up to 100 long cell chains on hydrophilic surfaces. Davies *et al.* (1993) utilized a direct visualization approach to demonstrate that algC genes of *P. aeruginosa* were upregulated by adhesion to the surface and growth in the biofilm compared with planktonic cells in the liquid medium. In a subsequent study, Davies and Geesey (1995) demonstrated changes in expression of algC in *P. aeruginosa* following attachment to the substratum. The application of *in situ* PCR (polymerase chain reaction; Hodson *et al.*, 1995) provides a powerful tool for visualization of genes and gene products. Thus, it is possible to see the genetic composition of the biofilm members and gene expression in real time.

Predictability versus Serendipity

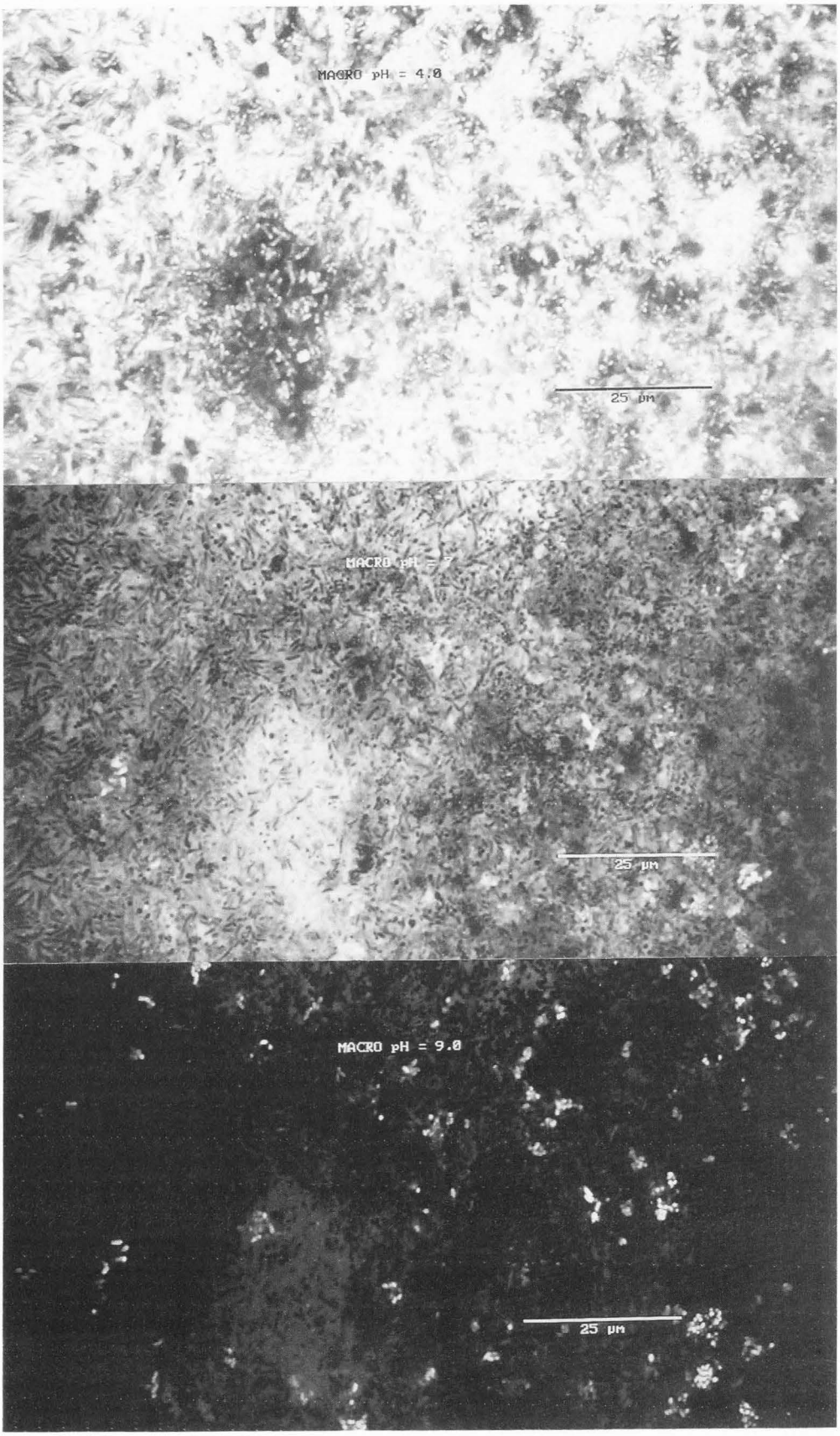
Any microscopic examination of natural biofilms reveals a high degree of structural variation within a biofilm; variation is evident in the distribution of morphologic types of bacteria and that of microcolonies, polymer, and space. These heterogeneities arise from a wide variety of factors including those extrinsic and intrinsic to bacteria, encompassing bacterial/microbial interactions. Various requirements for inter and intra species interactions, as well as growth coordination, all play dominant roles in biofilm development. As suggested by several authors (Lawrence *et al.*, 1991; Wolfaardt *et al.*, 1994a; Massol-Deya *et al.*, 1995) such organization represents an optimal arrangement providing for influx of nutrients and transfer of wastes and metabolites within and out of the biofilm. Using SCLM and O_2 microelectrodes, de Beer *et al.* (1994b) showed that oxygen concentrations were higher in voids than in cell clusters. The results of this study also showed that 50% of the oxygen required for biofilm metabolism was supplied from the void spaces. This provides direct evidence that the specific arrangement of cells and voids was relevant to biofilm growth and activity.

Figure 5. (*on facing page*) The results of pH shifting are shown in a series of optical thin sections where a herbicide degrading bacterial community {community described in Wolfaardt *et al.* (1994a)} was irrigated sequentially with pH 4, pH 7 and pH 9 fluorescein solutions, and the change in fluorescence pattern was monitored using SCLM. The results of this experiment indicate differential resistance at the microscale to changes in the bulk phase pH (fluorescein fluorescence is greatest at pH 9 and quenched at pH 4). For example, when the macroscale pH is 4, all cells (internal pH approx 7) and some regions appear as bright objects (pH 7) on a dark background (pH 4). Bars = 25 μm.

However, physical positioning is only one of the benefits of this architectural organization. This organization also provides for greater degradative capabilities than those exhibited by a single organism (Deming and Baross, 1993). Wolfaardt *et al.* (1994c) showed that a community grown planktonically was much less efficient than its biofilm equivalent during the degradation of the herbicide diclofop methyl. Biofilm organization therefore seems to be critical to the utilization of substrates, and this is true for both anaerobic and aerobic communities (Lawrence *et al.*, 1995; Massol-deya *et al.*, 1995; Wolfaardt *et al.*, 1995). Although all the underlying events leading to the development of these highly structured communities are not yet evident, examination with SCLM and other parallel studies indicate that these structures do not occur by chance.

Conclusions

The studies discussed above suggest that models of biofilms must be adapted to include heterogeneity. However, if researchers only replace homogeneity with a concept of heterogeneity, we will have failed to appreciate the relationships between structure and function and the coordinated nature of these developments. The use of direct visualization techniques provides an opportunity to correlate spatial relationships, functions of populations, and the nature of the microenvironment in which they are functioning. It is also important that these approaches be focused increasingly on natural environments as well as suitable and effective models. This


MACRO pH = 4.0
25 µm
MACRO pH = 7
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MACRO pH = 9.0
25 µm

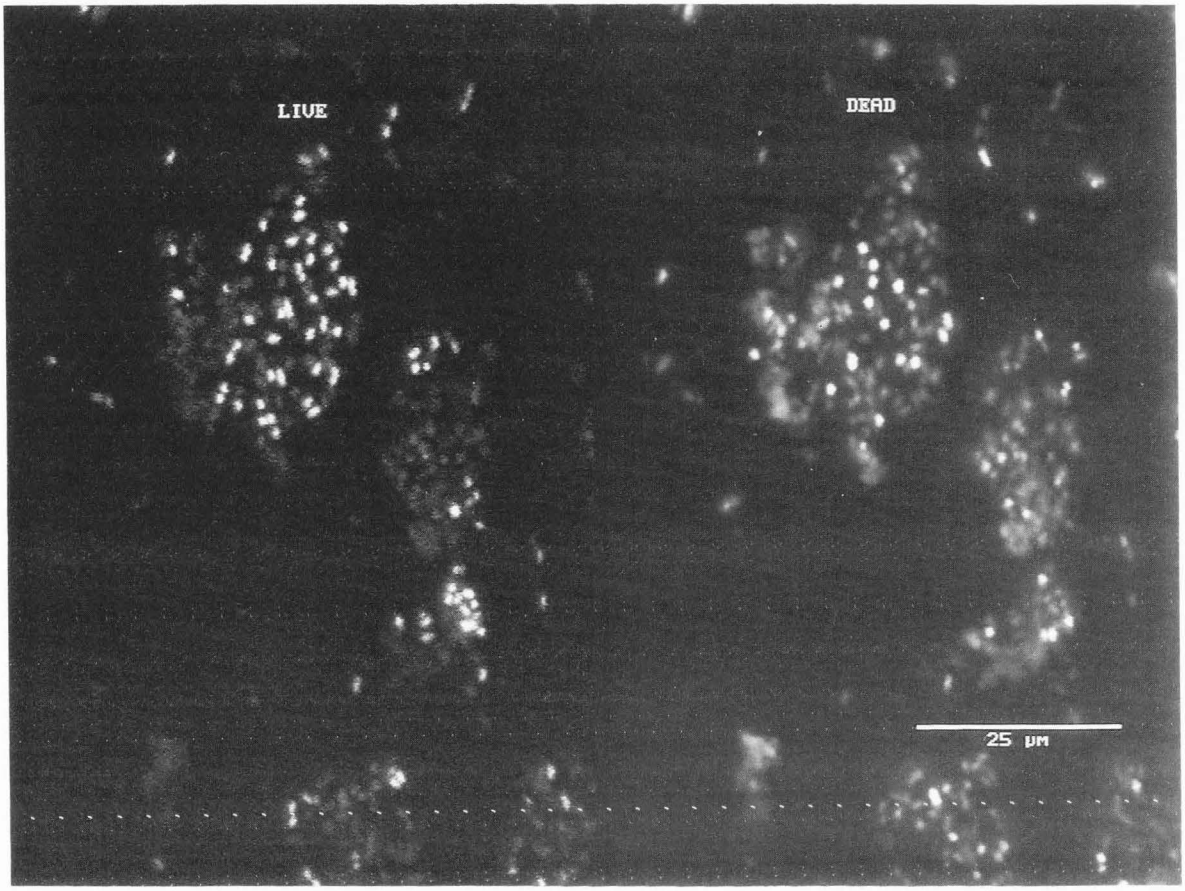

Figure 6. A dual channel (simultaneous imaging of green and red fluorescence in the same sample) SCLM micrograph showing the distribution of both live (left image) and dead (right image) cells within a mixed species biofilm. Bar = 25 μm.

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should allow fundamental studies of the natural history of bacteria, and a basis for greater developments in microbial ecology, particularly regarding biofilms.

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References

Amann RI, Stromley J, Devereux R, Key R, Stahl DA (1992) Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. Appl Environ Microbiol **58**: 614-623.

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol Rev **59**: 143-169.

Assmus B, Hutzler P, Kirchhof G, Amann RI, Lawrence JR, Hartmann A (1995) *In situ* localization of *Azospirillum brasilense* in the rhizosphere of wheat using fluorescently labeled rRNA-targeted oligonucleotide probes and scanning confocal laser microscopy. Appl Environ Microbiol **61**: 1013-1019.

Bloem J, Veninga M, Shepherd J (1995) Fully automatic determination of soil bacterium numbers, cell volumes and frequency of dividing cells by confocal laser scanning microscopy and image analysis. Appl Environ Microbiol **61**: 926-936.

Brown ML, Gauthier JJ (1993) Cell density and growth phase as factors in the resistance of a biofilm of *Pseudomonas aeruginosa* (ATCC 27853) to iodine. Appl Environ Microbiol **59**: 2320-2322.

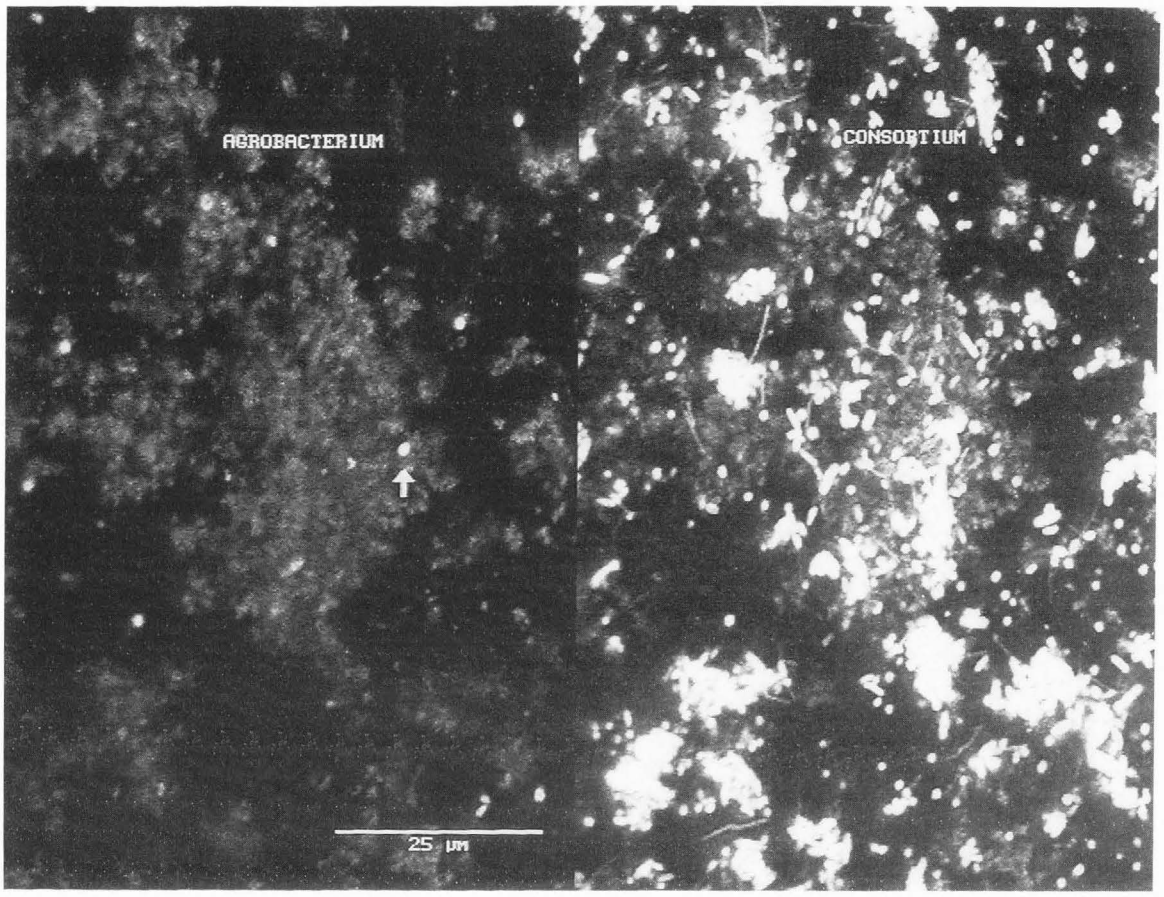

Figure 7. Dual channel optical thin section (simultaneous imaging of green and red fluorescence in the same sample) stained with fluorescein isothiocyanate (FITC) labelled antisera for *Agrobacterium sp.* (arrow) on the left and the other biofilm members shown in the right image counter stained with the red fluorescent stain Syto 17 (Molecular Probes, Inc., Eugene, OR). Bar = 25 μm.

Brown MRW, Allison DG, Gilbert P (1988) Resistance of bacterial biofilms to antibiotics: A growth rate related effect? J Antimicrob Chemother **22**: 777-783.

Bryers JD (1987) Biologically active surfaces: Processes governing the formation and persistence of biofilms. Biotechnol Prog **3**: 57-68.

Caldwell DE, Lawrence JR (1989) Microbial growth and behavior within surface microenvironments. In: Proc. ISME-5 (Internatl. Soc. Microbial Ecology). Hattori T, Ishida Y, Maruyama Y, Morita RY, Uchida A (eds.). JSS press, Tokyo. pp. 140-145.

Caldwell DE, Korber DR, Lawrence JR (1992a) Confocal laser microscopy and digital image analysis in microbial ecology. Adv Microbial Ecol **12**: 1-67.

Caldwell DE, Korber DR, Lawrence JR (1992b) Imaging of bacterial cells by fluorescence exclusion using scanning confocal laser microscopy. J Microbiol Meth **15**: 249-261.

Characklis WG, McFeters GA, Marshall KC (1990) Chapter 10: Physiological ecology in biofilm systems. In: Biofilms. Characklis WG, Marshall KC (eds.). Wiley, New York. pp. 341-393.

Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, de Beer D, James G (1994) Biofilms: The customized microniche. J Bacteriol **176**: 2137-2142.

Dagostino L, Goodman AE, Marshall KC (1991) Physiological responses induced in bacteria adhering to surfaces. Biofouling **4**: 113-119.

Dalsgaard T, Revsbech NP (1992) Regulating factors of denitrification in trickling filter biofilms as measured with the oxygen/nitrous oxide microsensor. FEMS Microbiol Ecol **191**: 151-164.

Dalton HM, Poulsen LK, Halasz P, Angles ML, Goodman AE, Marshall KC (1994) Substratum-induced morphological changes in a marine bacterium and their relevance to biofilm structure. J Bacteriol **176**: 6900-6906.

Davies DG, Geesey GG (1995) Regulation of the

alginate biosynthesis gene algC in *Pseudomonas aeruginosa* during biofilm development in continuous culture. Appl Environ Microbiol **61**: 860-867.

Davies DG, Chakrabarty A, Geesey GG (1993) Exopolysaccharide production in biofilms: Substratum activation of alginate gene expression by *Pseudomonas aeruginosa*. Appl Environ Microbiol **59**: 1181-1186.

de Beer D, van den Heuvel JC, Ottengraf SPP (1993) Microelectrode measurements of the activity distribution in nitrifying bacterial aggregates. Appl Environ Microbiol **59**: 573-579.

de Beer D, Stoodley P, Roe F, Lewandowski Z (1994a) Effects of biofilm structures on oxygen distribution and mass transfer. Biotechnol Bioeng **43**: 1131-1138.

de Beer D, Srinivasan R, Stewart PS (1994b) Direct measurement of chlorine penetration into biofilms during disinfection. Appl Environ Microbiol **60**: 4339-4344.

DeLong EF, Wickham GS, Pace NR (1989) Phylogenetic stains: Ribosomal RNA-based probes for identification of single cells. Science **243**: 1360-1362.

Deming JW, Baross JA (1993) The early diagenesis of organic matter: Bacterial activity. In: Organic Geochemistry. Engel MH, Macko SA (eds.). Plenum, New York. pp. 119-144.

Eng RHK, Padberg FT, Smith SM, Tan EN, Cherubin CE (1991) Bactericidal effects of antibiotics on slowly growing and nongrowing bacteria. Antimicrob Agents Chemother **35**: 1824-1828.

Gilbert P, Collier PJ, Brown MRW (1990) Influence of growth rate on susceptibility to antimicrobial agents: biofilms, cell cycle, dormancy, and stringent response. Antimicrob Agents Chemother **34**: 1856-1868.

Goodman AE, Angles ML, Marshall KC (1993) Genetic responses of bacteria in biofilms. In: Trends in Microbial Ecology. Guerrero R, Pedrós-Alío C (eds.). Spanish Society for Microbiology, Barcelona, Spain. pp. 119-122.

Gorman SP, Adair CG, Mawhinney WM (1994) Incidence and nature of peritoneal catheter biofilm determined by electron and confocal laser scanning microscopy. Epidemiol Infect **112**: 551-559.

Hahn D, Amann RI, Ludwig W, Akkermans ADL, Schleifer K-H (1992) Detection of micro-organisms in soil after *in situ* hybridization with rRNA-targeted, fluorescently labelled oligonucleotides. J Gen Microbiol **138**: 879-887.

Hamilton WA (1987) Biofilms: Microbial interactions and metabolic activities. In: Ecology of Microbial Communities. Fletcher M, Gray TRG, Jones JG (eds.). Cambridge Univ. Press., Cambridge, UK. pp. 361-385.

Haugland RP (1992) Molecular Probes: Handbook of Fluorescent Probes and Research Chemicals. Molecular Probes, Inc., Eugene, OR. pp. 111-274.

Hodson RE, Dustman WA, Garg RP, Moran MA (1995) *In situ* PCR for visualization of microscale distribution of specific genes and gene products in prokaryotic communities. Appl Environ Microbiol **61**: 4074-4082.

Huang C-T, Yu FP, McFeters GA, Stewart PS (1995) Nonuniform spatial patterns of respiratory activity within biofilms during disinfection. Appl Environ Microbiol **61**: 2252-2256.

James GA, Caldwell DE, Costerton JW (1993) Spatial relationships between bacterial species within biofilms. Abstract CSM/SIM Annual Meeting, Toronto, Canada. Canadian Society for Microbiologists, Ottawa, ON, Canada. p. 16.

Jorgensen BB, Des Marais DJ (1990) The diffusive boundary layer of sediments: Oxygen microgradients over a microbial mat. Limnol Oceanog **35**: 1343-1355.

Keevil CW, Walker JT (1992) Nomarski DIC microscopy and image analysis of biofilms. Binary **4**: 93-95.

Koch AL (1991) Diffusion: The crucial process in many aspects of the biology of bacteria. In: Advances in Microbial Ecology. Marshall KC (ed.). Plenum, New York. pp. 37-70.

Korber DR, Lawrence JR, Hendry MJ, Caldwell DE (1993) Analysis of spatial variability within mot^+ and mot^- *Pseudomonas fluorescens* biofilms using representative elements. Biofouling **7**: 339-358.

Korber DR, Caldwell DE, Costerton JW (1994a) Structural analysis of native and pure-culture biofilms using scanning confocal laser microscopy. In: Proceedings of the National Association of Corrosion Engineers (NACE). NACE, Calgary, AB, Canada. pp. 347-353.

Korber DR, James GA, Costerton JW (1994b) Evaluation of fleroxacin activity against established *Pseudomonas fluorescens* biofilms. Appl Environ Microbiol **60**: 1663-1669.

Kuhl M, Jorgensen BB (1992) Microsensor measurements of sulfate reduction and sulfide oxidation in compact microbial communities of aerobic biofilms. Appl Environ Microbiol **58**: 1164-1174.

Laurent M, Johannin G, Gilbert N, Lucas L, Cassio D, Petit PX, Fleury A (1994) Power and limits of laser scanning confocal microscopy. Biol Cell **80** 229-240.

Lawrence JR, Korber DR, Hoyle BD, Costerton JW, Caldwell DE (1991) Optical sectioning of microbial biofilms. J Bacteriol **173**, 6558-67.

Lawrence JR, Wolfaardt GM, Korber DR (1994) Monitoring diffusion in biofilm matrices using confocal laser microscopy. Appl Environ Microbiol **60**: 1166-1173.

Lawrence JR, Korber DR, Wolfaardt GM, Caldwell DE (1995) Bacterial behavioural strategies at interfaces. In: Advances in Microbial Ecology. Vol. 14. Jones JG (ed.). Plenum Press, New York. pp. 1-75.

Lawrence JR, Korber DR, Wolfaardt GM, Caldwell DE (1996) Analytical imaging and microscopy techniques. In: Manual of Environmental Microbiology. McInerney M (ed.). Am Soc Microbiol Press, Washington, DC, in press.

Lens PNL, de Beer D, Cronenberg CCH, Houwen FP, Ottengraf SPP, Verstraete WH (1993) Heterogeneous distribution of microbial activity in methanogenic aggregates: pH and glucose microprofiles. Appl Environ Microbiol **59**: 3803-3815.

Lewandowski Z, Lee WC, Characklis WG, Little B (1989) Dissolved oxygen and pH microelectrode measurements at water immersed metal surfaces. Corrosion **45**: 92-98.

Lewandowski Z, Altobelli SA, Fukushima E (1993) NMR and microelectrode studies of hydrodynamics and kinetics in biofilms. Biotechnol Prog **9**: 40-45.

Lloyd D, Hayes AJ (1995) Vigour, vitality and viability of microorganisms. FEMS Microbiol Lett **33**: 1-7.

Manz W, Szewzky U, Erikson P, Amann R, Schleifer KH, Stenstrum TA (1993) *In situ* identification of bacteria in drinking water and adjoining biofilms by hybridization with 23S rRNA-directed fluorescent oligonucleotide probes. Appl Environ Microbiol **59**: 2293-2298.

Manz W, Amann R, Szewzyk R, Szewzyk U, Stenstrom T-A, Hutzler P, Schleifer K-H (1995) *In situ* identification of Legionellaceae using 16S rRNA-targeted oligonucleotide probes and confocal laser microscopy. Microbiol **141**: 29-39.

Mason DJ, Lopez-Amoros R, Allman R, Stark JM, Lloyd D (1995) The ability of membrane potential dyes and calcafluor white to distinguish between viable and non-viable bacteria. J Appl Bacteriol **78**: 309-315.

Massol-deya AA, Whallon J, Hickey RF, Tiedje JM (1995) Channel structures in aerobic biofilms of fixed-film reactors treating contaminated groundwater. Appl Environ Microbiol **61**: 769-777.

McCarter LL, Showalter RE, Silverman MR (1992) Genetic analysis of surface sensing in *Vibrio parahaemolyticus*. Biofouling **5**: 163-175.

Møller S, Kristensen CS, Poulsen LK, Carstensen JM, Molin S (1995) Bacterial growth on surfaces: Automated image analysis for quantification of growth rate-related parameters. Appl Environ Microbiol **61**: 741-748.

Poulsen, LK, Ballard G, Stahl DA (1993) Use of rRNA fluorescence *in situ* hybridization for measuring the activity of single cells in young and established biofilms. Appl Environ Microbiol **59**: 1354-1360.

Ramsing NB, Kuhl M, Jorgensen BB (1993) Distribution of sulfate reducing bacteria, O_2, and H_2S in photosynthetic biofilms determined with oligonucleotide probes and microelectrodes. Appl Environ Microbiol **59**: 3840-3849.

Revsbech NP (1989) Diffusion characteristics of microbial communities determined by use of oxygen microsensors. J Microbiol Meth **49**: 111-122.

Rodrigues GG, Phipps D, Ishiguro K, Ridgway HF (1992) Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. Appl Environ Microbiol **58**: 1801-1808.

Rogers J, Keevil CW (1992) Immunogold and fluorescein immunolabelling of *Legionella pneumophila* within an aquatic biofilm visualized by episcopic differential interference contrast microscopy. Appl Environ Microbiol **58**: 2326-2330.

Schloter M, Borlinghaus R, Bode W, Hartmann A (1993) Direct identification and localization of Azospirillum in the rhizosphere of wheat using fluorescent-labelled monoclonal antibodies and confocal scanning laser microscopy. J Microsc **171**: 173-177.

Singleton S, Albiston L, Treloar R, Mahers E, Hodgson R, Watson K, Schilling K, Allison C (1995) Optical imaging and characterization of oral biofilm structures using vital stains and specific antibody probes. In: The Life and Death of Biofilm. Wimpenny J, Handley, Gilbert P, Lappin-Scott H (eds.). BIOLINE, School of Applied Biology, University of Wales College Cardiff, Box 915, Cardiff, CF1 3TL, UK. pp. 33-36.

Stewart PS, Peyton BM, Dury WJ, Murga R (1993) Quantitative observations of heterogeneities in *Pseudomonas aeruginosa* biofilms. Appl Environ Microbiol **59**: 327-329.

Stoodley P, de Beer D, Lewandowski Z (1994) Liquid flow in biofilm systems. Appl Environ Microbiol **60**: 2711-2716.

Surman SB, Walker JT, Goddard DT, Morton LHG, Keevil CW, Weaver W, Skinner A, Hanson K, Caldwell D, Kurtz J (1996) Comparison of microscopic techniques for the examination of biofilms. J Microbiol Meth **25**: 57-70.

Torsvik V, Goksoyr J, Daae FL (1990) High diversity of DNA of soil bacteria. Appl Environ Microbiol **56**: 782-787.

Trulear MG, Characklis WG (1982) Dynamics of biofilm processes. J Water Pollut Cont Fed **54**: 1288-1301.

Vandevivere P, Kirchman DL (1993) Attachment stimulates exopolysaccharide synthesis by a bacterium. Appl Environ Microbiol **59**: 3280-3286.

Wagner M, Amann R, Lemmer H, Schleifer K-H (1993) Probing activated sludge with oligonucleotides specific for proteobacteria: Inadequacy of culture-dependent methods for describing microbial community structure. Appl Environ Microbiol **59**: 1520-1525.

Wagner M, Assmus B, Hartmann A, Hutzler P, Amann R (1994) In situ analysis of microbial consortia in activated sludge using fluorescently labelled, rRNA-

targeted oligonucleotide probes and confocal scanning laser microscopy. J Microsc **176**: 181-187.

Wagner M, Amann R, Kampfer P, Assmus B, Hartmann A, Hutzler P, Springer N, Schleifer KH (1995) Identification and *in situ* detection of gram-negative filamentous bacteria in activated sludge. System Appl Microbiol **178**: 405-417.

Ward DM, Weller R, Bateson MM (1990) 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature (London) **345**: 63-65.

Wolfaardt GM, Lawrence JR, Robarts RD, Caldwell DE (1994a) Multicellular organization in a degradative biofilm community. Appl Environ Microbiol **60**: 434-446.

Wolfaardt GM, Lawrence JR, Headley JV, Robarts RD, Caldwell DE (1994b) Microbial exopolymers provide a mechanism for bioaccumulation of contaminants. Microb Ecol **27**: 279-291.

Wolfaardt GM, Lawrence JR, Robarts RD, Caldwell DE (1994c) The role of interactions, sessile growth and nutrient amendment on the degradative efficiency of a bacterial consortium. Can J Microbiol **40**: 331-340.

Wolfaardt GM, Lawrence JR, Robarts RD, Caldwell DE (1995) Bioaccumulation of the herbicide diclofop in extracellular polymers and its utilization by a biofilm community during starvation. Appl Environ Microbiol **61**: 152-158.

Zambon JJ, Huber PS, Meyer AE, Slots J, Fornalik MS, Baier RE (1984) *In situ* identification of bacterial species in marine microfouling films by using immunofluorescence technique. Appl Environ Microbiol **4**: 1214-1220.

Discussion with Reviewers

R.J. Doyle: If the pH is probed in a biofilm with suitable probes, such as fluorescent dyes would one expect the pH to vary with metabolic rates? Biofilms with sufficient carbon may produce more protons than those with limiting carbon. Does the microenvironment of the biofilm lead to trapping of protons?

Authors: The metabolic rate and the nature of the carbon source will influence the nature of the microenvironment; see, for example, Lens *et al.* (1993). Gradients at the level of the cellular microenvironment may exist. The pH is known to vary in the vicinity of bacterial cells, and pH changes are detectable in the surroundings of bacterial cells. In addition, the effective pH of the periplasmic space of a Gram-negative bacterium may differ by more than two units from that in the extracellular environment (Cheng *et al.*, 1970). In this latter instance, the periplasmic space has be shown to be a microniche in which conditions change sharply at the cell boundary (Costerton *et al.*, 1974). However, these results are inferred from indirect means, and the existence of such zones around bacterial cells has been questioned (Van Loosdrecht *et al.*, 1990). Further, the visualization of pH sensitive fluorescence in the vicinity of bacteria may be confounded by interactions between the fluor and the EPS or proteins in the vicinity of the cell.

R.J.C. McLean: Once a biofilm and its structure is established, can it be changed upon alteration of the chemical or physical environment (addition or deletion of nutrients, presence of toxic compounds, changes in temperature, pH or Eh, etc.)?

Authors: Disruption of biofilms is certainly possible given extensive changes in any of these parameters. The study of Wolfaardt *et al.* (1994a) showed a dramatic change in biofilm structure when a degradative consortium growing on tryptic soy broth was switched to the herbicide diclofop methyl as sole carbon source. In this case, a uniform 10 μm thick undifferentiated biofilm changed within 48 hours to one with extensive structure and up to 40 μm thick. McLean *et al.* (1991) showed that significant reduction of the pH of the macroenvironment was required to disrupt a *Proteus mirabilis* biofilm and eliminate struvite crystals within the biofilm.

R.J.C. McLean: There has been considerable literature dealing with bacterial extracellular polymers. Has there been any indication if an organism will produce a different extracellular polymer when growing in a biofilm than when it is growing on conventional media?

Authors: Other authors have found that various factors may influence the composition and yield of EPS produced by a bacterial strain (Uhlinger and White, 1983). Bacteria are able to produce different types of polymer; for example, changes in the composition of EPS occurred during the growth cycle of two *Pseudomonas* species (Uhlinger and White, 1983; Christensen *et al.*, 1985). Omar *et al.* (1983) demonstrated the existence of different polysaccharides in the capsule and slime of *Rhodopseudomonas capsulata* Sp11. A study by Uhlinger and White (1983) concluded that uronic acid-rich EPS were formed during periods of metabolic stress. The work of Wolfaardt *et al.* (1996) indicates a change in the nature of the biofilm polymers, their conformation or relative abundance when the same degradative consortium is grown on labile carbon or chlorinated compounds.

D.J. Stickler: Does this perception of bacterial biofilms as highly hydrated open structures with the aggregates of cell and associated extracellular polymer separated by water channels and penetrated by pores apply to biofilms such as those formed by *Staphylococcus epidermidis* on

implanted prosthetic devices or by *Proteus mirabilis* on encrusting indwelling bladder catheters?

Authors: Without direct observation of actual samples using SCLM, this would be difficult to answer with certainty. Based on SEM and TEM images of these structures, this may not be the case. *Proteus* biofilms that we have grown in continuous flow slide culture using artificial urine media and flow rates of 10 ml/h have been patchy with mounds and open surface areas. Similar reports were made by Gorman *et al.* (1994) using SCLM, although they attributed the nature of the biofilm to treatment with antibiotics. Factors such as flow rates do have a considerable impact on biofilm architecture, as a consequence of their influence on nutrient availability, and considerably more effort is required in the examination of biofilms using the approaches reviewed above.

D.J. Stickler: Could the authors provide more information about the image presented in Figure 3? It would be interesting to know more about the nature of this biofilm and the technical preparation of the sample.

Authors: The biofilm has developed on the surface of wood chips used as support medium in a large scale bioreactor. The biofilm was prepared for study by removal from the reactor, storage at 4°C for 48 hours, rinsing, application of pH 7.0 fluorescein solution, application of a cover slip and immediate observation using SCLM.

Additional References

Cheng K-J, Ingram JM, Costerton JW (1970) Alkaline phosphatase localization and spheroplast formation of *Pseudomonas aeruginosa*. Can J Microbiol **16**: 1319-1324.

Christensen BE, Kjosbakken J, Smidsrød O (1985) Partial chemical and physical characterization of two extracellular polysaccharides produced by marine, periphytic *Pseudomonas* sp. Strain NCMB 2021. Appl Environ Microbiol **50**: 837-845.

Costerton JW, Ingram JM, Cheng K-J (1974) Structure and function of the cell envelope of Gram-negative bacteria. Bacteriol Rev **38**: 87-110.

McLean RJC, Lawrence JR, Korber DR, Caldwell DE (1991) *Proteus mirabilis* biofilm protection against struvite crystal dissolution and its implications in struvite urolithiasis. J Urol **146**: 1138-1142.

Omar AS, Weckesser J, Mayer H (1983) Different polysaccharides in the external layers (capsule and slime) of the cell envelope of *Rhodopseudomonas capsulata* Sp11. Arch Microbiol **136**: 291-296.

Uhlinger DJ, White DC (1983) Relationship between physiological status and formation of extracellular polysaccharide glycocalyx in *Pseudomonas atlantica*. Appl Environ Microbiol **45**: 64-70.

Van Loosdrecht MCW, Lyklema J, Norde W, Zehnder AJB (1990) Influence of interfaces on microbial activity. Microbiol Rev **54**: 75-87.