13 Microbial Biofilms

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Introduction

Biofilms are usually thought of as the slimy layer of microorganisms that covers solid surfaces. However, there are a number of features that distinguish biofilm populations from their planktonic (suspended or free floating) counterparts, namely, the association with a surface, high population densities (on the order of 10^{10} cells per ml of hydrated biofilm), an extracellular polymer (EPS) slime matrix, and a wide range of physical, metabolic, and chemical heterogeneities. However, some biofilms may not have all features. Indeed, a concise universal definition of biofilms has yet to emerge; in part, this is because of the wide diversity of biofilm populations. Although much of contemporary microbiology is based on the study of planktonic "cells," it is now thought that biofilms are the primary habitat for many microorganisms. Microbial mats associated with sediment and suspended microbial flocs or aggregates, although different in appearance from conventional biofilms, have many important features in common and thus are included in the definition of "biofilm." Often biofilm cells are embedded within a highly hydrated EPS matrix, and in the absence of corrosion products or scale, biofilms are estimated to be primarily water. The physical properties of the biofilm are largely determined by the EPS, while the physiological properties are determined by the bacterial cells (*Figs. 13.1* and *13.2*).

Characklis (1990a) identified up to eight processes in the development of biofilms. These can be condensed to three main processes: the attachment of cells to a surface (colonization), growth of the attached cells into a mature biofilm, and the detachment of single cells (erosion) or large pieces (sloughing) (*Figs. 13.3* and *13.4*).

Since free convection is hindered within biofilms, the chemical environment to which the cells are exposed differs from the surrounding water phase. Also, mass transfer to the cells often limits conversion rates. All natural biofilms, mats, aggregates, and flocs can consist of complex microbial communities, and their function is characterized by interactions

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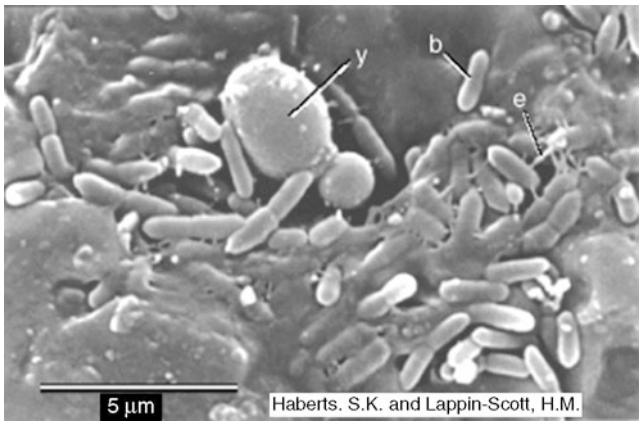

Fig. 13.1
Scanning electron photomicrograph showing biofilm formation after 24-h growth on stainless steel AISI 321. The biofilm was established under laminar pipe flow (*Re* = 2.72) using a modified Robbins device (MRD). The inocula were all environmental isolates and consisted of four Gram-negative rod-shaped bacteria, *Stenotrophomonas maltophilia*, *Pseudomonas alcaligenes*, *Alcaligenes denitrificans*, and *Flavobacterium indologenes*; one nonfermentative yeast, *Rhodotorula glutinis*; and two filamentous fungi, *Fusarium solani* and *Fusarium oxysporum* (Elvers 1998). The image shows a budding yeast cell "y" and bacterial rods "b" attached to the surface. The bacteria are embedded in an EPS matrix. During preparation for SEM the dehydrated EPS has formed characteristic strands "e." Scale bar = 5 µm (Image supplied by Sara K. Roberts, Biological Sciences, Exeter University)

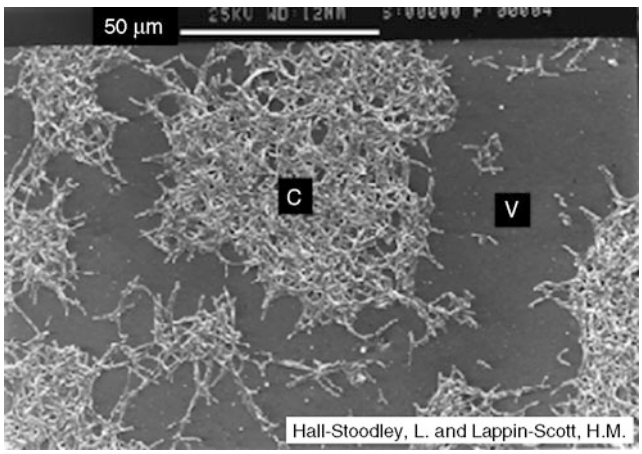

Fig. 13.2
Scanning electron photomicrograph showing biofilm formation of *Mycobacterium fortuitum* after 24-h growth on silicone rubber (Hall-Stoodley 1998). The biofilm was grown under laminar flow in a MRD. The biofilm was composed of cell clusters "c" surrounded by voids "v." Scale bar = 50 µm (Image supplied by Luanne Hall-Stoodley, Biological Sciences, Exeter University)

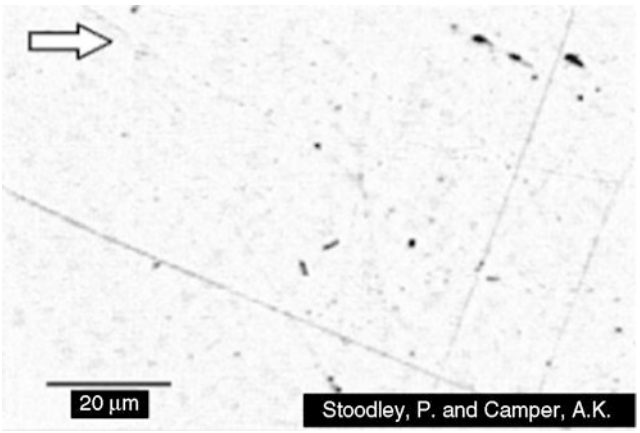

Fig. 13.3
Time-lapse movie showing the initial events of biofilm formation. *Pseudomonas aeruginosa* cells are attaching to a 316L stainless steel coupon over 6.5 h. The *P. aeruginosa* cells were initially grown in a chemostat with a residence time of 5 h. The stainless steel coupon was mounted in a flat-plate flow cell and the inoculum was delivered with an average flow velocity of 2.8 cm·s^{-1} (*Re* = 6). Note how some of the cells divide, detach, and move around on the surface. *Arrow* indicates flow direction. Scale bar = 20 µm (Images were enhanced for clarity using NIH-Image 1.59 (available at http://zippy.nimh.nih.gov). For *Fig. 13.3*, see the online version of *The Prokaryotes*)

between different populations within these communities. Many experimental methods to study aggregated biomass are similar, that is, microscopic and staining techniques. We will refer therefore in this review to all types of aggregated microorganisms. *Table 13.1* gives some examples of biofilm types.

Biofilms, flocs, and microbial mats are responsible for most microbial conversions in natural environments. Natural biofilms can develop on solid surfaces under all conditions facilitating microbial growth; thus, biofilms are ubiquitous in nature, covering rocks and plants in seawater and freshwater, sediment grains, and sediment surfaces. Microbial mats are formed on most sediments, especially under extreme conditions (temperature, salinity) that inhibit the activity of grazers (Karsten and Kühl 1996). Flocs are highly fragile structures suspended in fresh- and seawater (called river and marine snow) and typically occur during bloom periods after an increased input of nutrients. Consequently, biofilms exist almost everywhere, and microbial aggregates are responsible for the majority of the microbial conversions in many aquatic ecosystems. Biofilms have been associated with a wide range of problems both in industry and in medicine (*Table 13.2*) and have been utilized for various processes (*Table 13.3*).

Microbial cells living in biofilms are much more difficult to eradicate or control than suspended cells. Yet the susceptibility of biofilm cells to antibiotics and industrial antimicrobial agents is rarely assessed. In part, this is due to convention and, in part, because standard testing protocols against suspended cultures

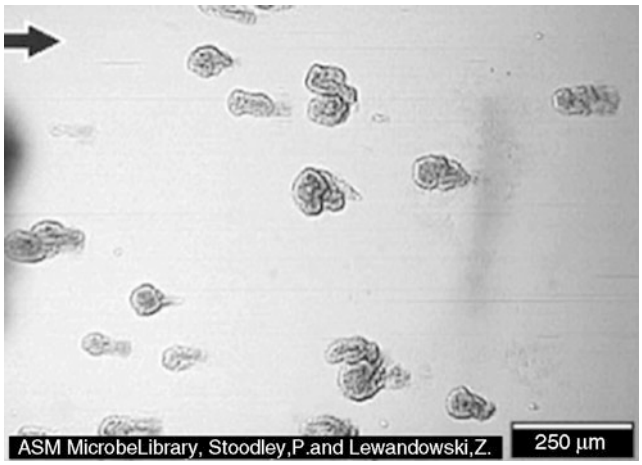

Fig. 13.4
Time-lapse movie showing the accumulation of a bacterial biofilm on a glass surface over 14 days. The biofilm was composed of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Klebsiella pneumoniae* and was grown in a glass flow cell (average flow velocity = 1.8 m·s^{-1}) to simulate conditions in an industrial pipeline. Note the complex structure of biofilm patches (*dark*) and water channels between them. After 12 days, much of the biofilm detached from the surface in a "sloughing event." There was a corresponding decrease in pressure drop across the flow cell, which had been building up as the biofilm accumulated (Lewandowski and Stoodley 1995). The *arrow* shows the direction of fluid flow. Scale bar = 250 µm. This sequence of images has been accepted for use in the ASM Biofilms Collection [{http://www.asmusa.org/edusrc}]. For *Fig. 13.4*, see the online version of *The Prokaryotes*

are much easier to develop. Cell density and growth phase can be easily controlled in both batch and chemostat cultures, whereas biofilms are highly variable.

Currently, the most important practical use of biofilms is for biological wastewater treatment, while many emerging technologies are utilizing biofilms for biodegradation and bioremediation in bioreactors. Municipal wastewater is treated in activated sludge plants that are based on the activity of flocs. Their relevance in natural element cycles as well as their economical and medical impact has been recognized, and the study of immobilized cell systems has gained considerable momentum in the last decade. The knowledge has been advanced because of new techniques to determine the functioning, structure, and microbial populations in biofilms.

Biofilms and microbial mats are thus important microbial communities in most aquatic ecosystems today. Interestingly, the first known fossils of single microbes and microbial communities share almost identical structural characteristics to those found in recent biofilms and microbial mats (Schopf and Klein 1992).

Because of their ubiquity in natural and industrial environments, the study of biofilms lends itself to a multidisciplinary approach involving microbiology and engineering. Originally, an engineering approach was used to study biofilm performance on the macroscale (i.e., for optimization of wastewater treatment plants). Subsequently, engineering concepts were applied to further our understanding of biofilm processes on the microscale. An important task for microbiologists studying biofilms is to determine the types of organisms present and to determine their in situ activities. This chapter will focus on recent findings on biofilm structure, mass-transfer phenomena, microbial activities, and community structure. Brief descriptions of biofilm cultivation methods and new techniques to determine biofilm structure in situ, community structure and population distributions, and in situ microbial activity distributions will be given.

Biofilm Structure

Biofilm structure is the spatial arrangement of bacteria, cell clusters, EPS, and particulates. Since the structure can influence transport resistance, it is a significant determinant in the activity of the biofilm. Various conceptual and mathematical models have been proposed to describe the structure and function of biofilms (Characklis 1990a; Rittmann and Manem 1992; Wanner and Gujer 1986). Mathematical models describing transport, conversion, cell growth, and biofilm development are based on conceptual models. Biofilms were initially considered as planar structures, impermeable and with homogeneous cell distribution. Mass transfer through the mass boundary layer and within the biofilm was assumed to be diffusional and perpendicular to the surface to which it was attached (the substratum).

Biofilms and mats are matrices of cells and extracellular polymers (EPS). The EPS is produced by the cells and consists of polysaccharides, polyuronic acids, proteins, nucleic acids, and lipids (Schmidt and Ahring 1994; Decho 1990; Decho and Lopez 1993). EPS holds the cells together and to the substratum. Owing to the dimensions of microbial mats and biofilms, their structural analysis is strongly dependent on microscopic methods that are briefly discussed and listed in *Table 13.4* (*Fig. 13.5*).

Heterogeneity

Recent microscopic observations indicated that biofilms are not flat and the distribution of microorganisms is not uniform. Instead, multispecies biofilms were observed with complex structures containing "voids," channels, cavities, pores, and filaments and with cells arranged in clusters or layers. Such complex structures were found in a wide variety of biofilms such as methanogenic films from fixed-bed reactors (Robinson et al. 1984), aerobic films from wastewater plants (Eighmy et al. 1983; Mack et al. 1975), nitrifying biofilms (Kugaprasatham et al. 1992), and pure culture biofilms of *Vibrio paraheamolyticus* (Lawrence et al. 1991) and *Pseudomonas aeruginosa* (Stewart et al. 1993).

Depending on growth conditions and age, the thickness of biofilms can range from a few micrometers (a monolayer) up to

Table 13.1
Examples of different types of biofilms

Environment	Biofilm type	Thickness (m)	Community	References
Natural	Photosynthetic microbial mats, hot springs, and hypersaline lakes	10^{-3} to 1	Mixed algal and bacterial community	Stal (1994)
	Stromatolites	1	Bacterial	Stal (1994)
	Benthic/river sediments	10^{-6} to 10^{-3}	Mixed bacterial, algal, and protozoan communities	Baty (1996)
				Costerton (1994)
Medical	Dental plaque	10^{-6} to 10^{-4}	Mixed bacterial community	Kinnement (1996)
	Infectious	10^{-6} to 10^{-3}	Often bacterial or fungal monocultures	Morck (1994)
				Buret (1991)
Industrial	Heat exchangers	10^{-6} to 10^{-3}	Mixed bacterial and fungal communities	Characklis (1990)
	Drinking water pipes	10^{-6} to 10^{-2}	Mixed bacterial and fungal communities	Camper (1994)
				Van Der Kooij (1994)
	Wastewater treatment	10^{-4} to 10^{-3}	Mixed bacterial and fungal communities, biofilms, aggregates, and flocs	Lemmers and Griebe (1995)
	Filtration units	10^{-5} to 10^{-4}	Mixed bacterial and fungal biofilms	Flemming (1996)
	Ship hulls	10^{-4} to 10^{-2}	Mixed bacterial and algal and marine macroorganisms	Cooksey (1995)

Table 13.2
Problems associated with biofilms

Problems	Consequences
Fouling of heat exchangers	Loss of heat exchange efficiency and reduction of flow capacity
Fouling of ships	Energy losses
Oil reservoirs	H_2S souring by sulfate-reducing bacteria
Industrial and drinking water pipelines	Energy losses, pitting and general corrosion, product contamination, pathogen reservoirs
Dental plaque	Dental caries
Medical infections	Colonization of indwelling devices (catheters, artificial joints, contact lenses) – endocarditis

Table 13.3
Processes which utilize biofilms

Processes	Uses
Wastewater treatment	Bioremoval of pollutants
Biobarriers[a]	Immobilization of groundwater contaminants; microbially enhanced oilfield recovery (MEOR)
Metals leaching	Enhanced recovery of metals

[a]See MacLeod 1988

a centimeter. Owing to the microscopic dimensions of microbial mats and biofilms, their structural analysis strongly depends on the microscopic methods used. Most microscopic techniques involve preparation of the sample, such as dehydration and embedding, which causes the soft biofilm structure to collapse and often to be observed as flattened (Stewart et al. 1995). Because this structure most conveniently agreed with the basic assumption for one-dimensional (1-D) modeling, it was accepted as the general structure of biofilms. However, a study by Siebel and Characklis (1990) using interference contrast (Nomarsky) microscopy challenged this assumption. They reported that binary population biofilms of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* could form uneven biofilms consisting of patchy clusters of cells surrounded by a smooth monolayer. After the introduction of confocal scanning laser microscopy (CSLM), however, the perception changed drastically. The findings with new microscopic techniques indicate that the assumptions for 1-D geometry need to be carefully analyzed. CSLM images of undisturbed biofilms show that biofilms can consist of biomass clusters separated by interstitial voids (De Beer et al. 1994). [Voids were made visible with a negative staining by fluorescein that is strongly quenched

Table 13.4
List of microscopic techniques for studying biofilms and mats

Microscopy technique	Spatial resolution	Application	Sample treatment	References
LM	1 μm	EPS and cells	Dehydration, freezing, sectioning, staining	Chayen (1973)
FM	1 μm	EPS and cells	Dehydration, freezing, sectioning, staining	Stewart (1995), Griebe (1995), De Beer (1996)
SEM	1 nm	Cell and EPS surfaces	Dehydration, sputter coating	Beeftink (1986), Paterson (1995)
ESEM	10 nm	Cell and EPS surfaces	None	Little (1991)
TEM	1 nm	Cells and EPS	Dehydration, sectioning, staining	Beeftink (1986), Bakke (1984), Sanford (1995)
CSLM	1 μm	EPS, cells, voids	Staining	Lawrence (1991), De Beer (1994)
AFM	0.1 μm	Cell and EPS surfaces	None	Bremer (1992), Gunning (1996)

Abbreviations: *LM* light microscopy, *EPS* extracellular polymer slime, *FM* fluorescence microscopy, *SEM* scanning electron microscopy, *ESEM* environmental scanning electron microscopy, *TEM* transmission electron microscopy, *CSLM* confocal scanning laser microscopy, *AFM* atomic force microscopy

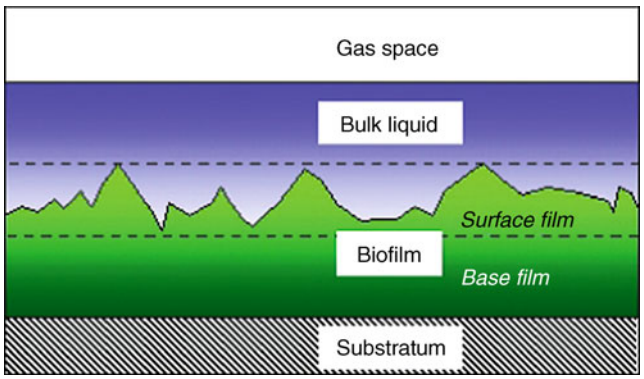

Fig. 13.5
Conceptual model for biofilm structure proposed at the 1988 Dahlem conference (Wilderer 1989). The model divides the biofilm system into specific compartments: the substratum, the biofilm, the bulk liquid, and a possible headspace. The biofilm compartment was further subdivided into a base film and a surface film. Although the model recognized a certain degree of biofilm roughness, it was essentially a planar layered model

by biomass. Cells (stained with a DNA stain) and EPS (stained with calcofluor and Alcian blue) were observed in the clusters, while no cells or EPS could be detected in the voids.] Fluorescent beads (0.3 μm) added to the medium immediately penetrated the voids but not the cell clusters. It was concluded that voids were water channels in open connection with the bulk water phase. Fluid flow in the biofilm was later directly demonstrated and quantified, by using the beads as particle tracers to visualize flow through the water channels (Stoodley et al. 1994). The flow velocity of individual beads at various depths in the biofilm channels were calculated by measuring the bead track length, using confocal microscopy. The resulting flow profiles were consequently used to determine the fluid shear stress acting on the channel wall and the surface of the biofilm cell clusters (deBeer et al. 1994; Stoodley et al. 1994). These observations were made on biofilms grown in the lab, either as undefined culture or as mixed pure culture. Similar observations were reported from both pure culture biofilms and biofilms with undefined microbial communities from various sources (Massol-Deya et al. 1995; Gjaltema et al. 1994; Zhang 1994; Neu and Lawrence 1997; Okabe et al. 1996; Okabe et al. 1997). The presence of voids has considerable consequences for mass transfer inside the biofilms (advection) and exchange of substrates and products with the water phase (effective exchange surface), as will be discussed in the relevant section. These new findings have led to a concept that incorporates two key features: structural heterogeneity and the water flow within the biofilm (*Figs. 13.6–13.11*).

Extracellular Polymeric Substances (EPS)

The proportion of EPS can vary between 50 % and 80 % of the organic matter and is the main structural component of biofilms. The physical properties of the biofilm are largely determined by the EPS, while the physiological properties are determined by the bacterial cells. A common perception was that EPS consists mainly of polysaccharides, and many detection techniques focus on this group of compounds (Christensen and Characklis 1990; Neu and Lawrence 1997; Beeftink and Staugaard 1986; Williams and Wimpenny 1978; De Beer 1996). Also, research relating EPS to biofilm functioning, cell-cell and cell-surface interactions was concentrated on the polysaccharide

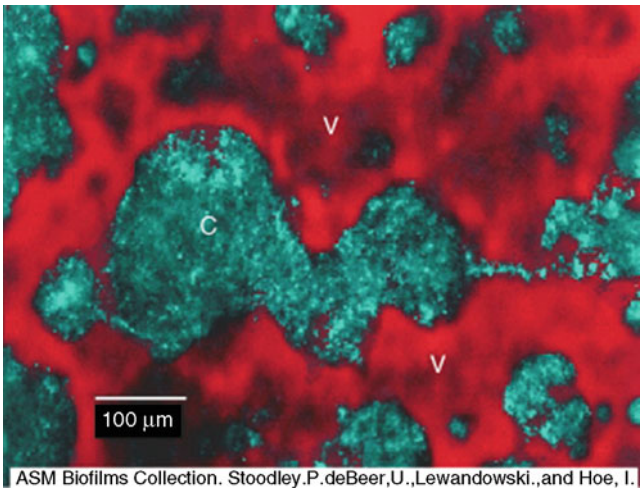

Fig. 13.6
Biofilm composed of *P. aeruginosa*, *P. fluorescens*, and *K. pneumoniae* grown in a glass flow cell for 5 days (de Beer et al. 1994a). The image was taken using CSLM which allows high resolution 3-D imaging of fully hydrated samples. Differential staining with propidium iodide (a nucleic acid stain) and fluorescein (*red*) showed that the biofilm consisted of cell clusters "c" separated by interstitial voids "v" or water channels. Scale bar = 100 μm (Image available from the ASM Biofilms Collection [http://www.asmusa.org/edusrc/edu34.htm])

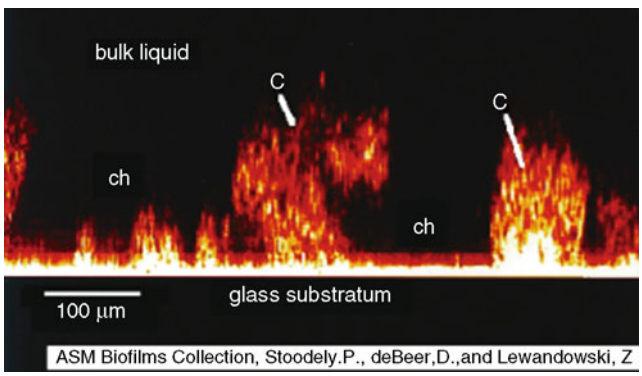

Fig. 13.7
Side view of the same biofilm in *Fig. 13.6*. Cells appear *red* and *orange* and are grouped in distinct cell clusters "c" separated by water channels "ch." Some of the cell clusters formed "mushroom" shapes, which greatly increase the available surface area for nutrient and waste product exchange with the bulk liquid. The horizontal *white line* is the glass surface. Scale bar = 100 μm (Image available from the ASM Biofilms Collection [http://www.asmusa.org/edusrc/edu34.htm])

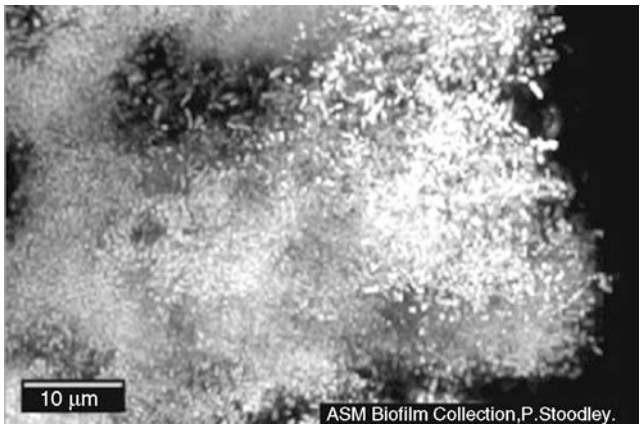

Fig. 13.8
Movie sequence showing 3-D structure of a mixed-species biofilm taken by CSLM. The biofilm was composed of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Klebsiella pneumoniae* and was grown in a flow cell with an average liquid flow velocity of 6.6 cm·s^{-1}. The biofilm was heterogeneous and was made up of microbial cell clusters (individual cells are stained with propidium iodide and appear as *bright dots*) held in an EPS matrix (not stained in this image). The biofilm was approximately 150-μm thick and protrudes out toward the viewer. The image is composed of 27 overlaid optical sections taken at 6-μm depth intervals. The motion is an artifact used to give the 3-D effect. Scale bar = 10 μm. For *Fig. 13.8*, see the online version of *The Prokaryotes*

fraction of EPS. However, recent analyses showed that biofilms contain EPS consisting of a mixture of protein, polysaccharides, lipids, and nucleic acids (Nielsen et al. 1997; Schmidt and Ahring 1994; Jahn 1995; Frolund et al. 1996). Protein appeared the most abundant EPS component (50 % or more) in activated sludge (Frolund et al. 1996), biofilms (Jahn and Nielsen 1995), and anaerobic aggregates (Ahring et al. 1993), while polysaccharides were much less abundant (5–20 %). Detailed knowledge is available on the polysaccharide content of both laboratory grown and natural biofilm EPS (Sutherland 1994, 1996); however, data on the actual composition of nonpurified biofilm EPS as it occurs in situ are lacking. Thus, we face the situation that the actual composition of EPS, including the protein fraction, is largely unknown, as are its chemical and physical properties. Since EPS is the second important fraction of biofilms, beside cells, research on the chemistry and properties of EPS has a high priority.

More research on the composition and function of EPS is needed, since EPS has been linked with many processes and properties integral to biofilm behavior, that is, attachment, detachment, mechanical strength, antibiotic resistance, and exo-enzymatic degradation activity. The mechanical stability of a biofilm is important for stable process maintenance (sloughing of biofilms, floc stability). To remove unwanted biofilms, surfactants are used to weaken the strength of the matrix. Furthermore, there is evidence that biofilms maintain their structural heterogeneity by releasing EPS-degrading enzymes (Davies et al. 1998). This interesting process is thought to involve cell-cell communication, that is, quorum sensing, through the generation of homoserine lactones (Greenberg 1997).

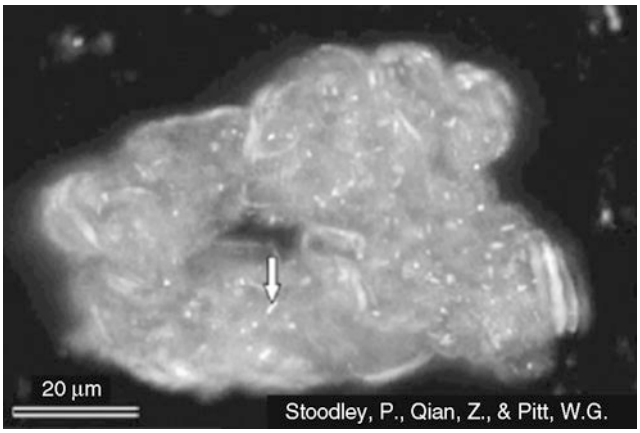

Fig. 13.9
Movie sequence showing 3-D structure of a pure-culture *Pseudomonas aeruginosa* biofilm taken by CSLM (Qian et al. 1996). The biofilm was heterogeneous and consisted of cell clusters and surrounding water channels. This image shows a *donut-shaped cell* cluster that protrudes out toward the viewer. The *bright dots* are stained bacterial cells (representative cell indicated by *arrow*) and the lighter, hazy material is probably EPS slime. The biofilm was grown in a polycarbonate flow cell on a glass slide. Scale bar = 20 µm. For *Fig. 13.9*, see the online version of *The Prokaryotes*

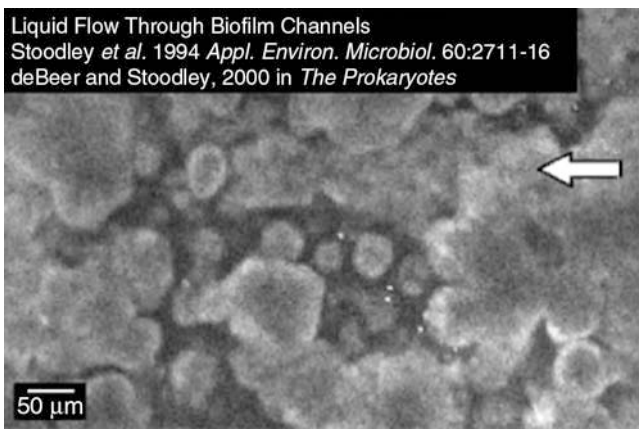

Fig. 13.10
Time-lapse CSLM movie sequence showing fluorescent latex beads moving through biofilm water channels (Stoodley et al. 1994). The bacterial biofilm, composed of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Klebsiella pneumoniae*, was grown in a flow cell on a glass coverslip. The sequence of images was taken over 44 s. The beads were moving with a velocity of approximately 15 µm·s^{-1}; the average liquid flow velocity of the bulk liquid was 6.6 cm·s^{-1}. The *arrow* indicates flow direction of the bulk liquid. Note that in some cases, the flow around the cell clusters is counter to that of the channel current. The biofilm clusters were autofluorescent and appear lighter than the surrounding water channels. The optical section was taken at a depth of 70 µm in the 175-µm thick biofilm. Scale bar = 50 µm. For *Fig. 13.10*, see the online version of *The Prokaryotes*

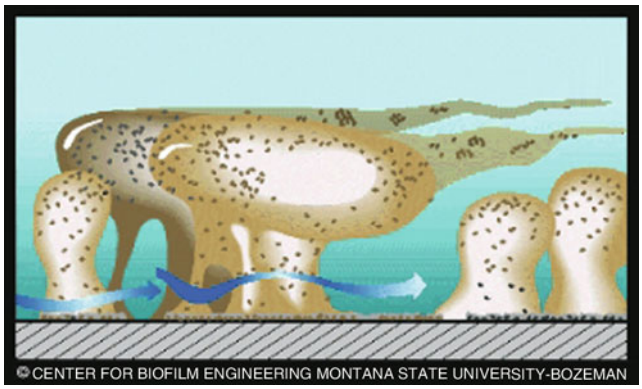

Fig. 13.11
Recent "Center for Biofilm Engineering" conceptual biofilm model incorporating structural complexity and liquid flow through biofilm channels. The model also incorporates biofilm streamers, which form as a function of fluid shear. The schematic was composed by Peg Dirckx of the Center for Biofilm Engineering (With permission from the Center for Biofilm Engineering, Bozeman, Montana, USA)

Binding of water is important for dehydrating activated sludge (Nielsen et al. 1996). Pollutants may bind considerably to the EPS of biofilms; 60 % of biofilm-bound BTX, but less than 20 % of the biofilm-bound heavy metals (Späth et al. 1998), was located in the EPS.

EPS can mask the original surface properties of the cells and render hydrophobic surfaces hydrophilic. The phenomenon of flotation of anaerobic aggregates occurs by attachment of gas bubbles to the hydrophobic aggregate surface. Aggregates with low amounts of EPS showed a strong tendency to float, leading to severe biomass losses from the reactors. The presence of carbohydrates in the feed increases the amount of EPS, especially on the aggregate surface, inhibiting attachment of gas bubbles and preventing flotation (De Beer 1996; Neu and Lawrence 1997).

The diffusion coefficient of solutes in biofilms is influenced by the microstructure of EPS (Neu and Lawrence 1997; De Beer et al. 1997). Biofilms have been considered to be highly porous polymer gels (Christensen and Characklis 1990) and diffusion studies demonstrate their gel-like characteristics (De Beer et al. 1997). Also, recent in situ rheological testing of *P. aeruginosa* biofilms, grown in the absence of divalent cations, showed that the biofilm behaved like a non-cross-linked polymer gel (Stoodley et al. 1999c). In this case, the EPS matrix can be considered to be a two-phase system with a solid network of polymers and free interstitial water as depicted by Stewart (1998). Only then does the structure of the network effect diffusivity (Westrin 1991), particularly when the pore size of the network is of the same order of magnitude as the molecular diameter of the solute. Based on this assumption, it is possible to infer some properties of the microstructure of EPS from the diffusional behavior of large molecules. It was found that the diffusion of small molecules is not strongly inhibited by the

biofilm matrix, whereas diffusion of large molecules is impeded (Bryers and Drummond 1996). Similar effects of the molecular size were found using microinjection of fluorescent dyes (fluorescein, MW 332, Ø > 1 nm, diffusivity not affected, and phycoerithrin, MW 240,000, Ø = 11 nm, 40 % reduction of diffusivity; De Beer et al. 1997). The pore size of the biofilm matrix (ca. 80 nm) was calculated from these data.

However, it has been reported that the forces keeping the polymers together are not strong covalent bonds, but weak hydrophobic and electrostatic interactions and hydrogen bonds (Flemming 1998). These forces are weakened by surface active agents, complexing agents, pH and ionic strength. Flemming concluded that a significant portion of the EPS may not be in bound form; if so, the polymers would increase the viscosity and reduce the diffusion coefficient, which could explain in part contradictory findings on D_{eff}. Boyd and Chakrabarty (1994) hypothesized that altering the length of polymer chains through the activity of alginate lyase may control the viscosity of *P. aeruginosa* biofilms. It is clear that further research is needed on both the physical and chemical properties of EPS, which appears to play a critical role in the structure and function of biofilms.

Morphogenetic Factors

In conclusion, the architectural features of biofilms can be viewed in terms of a hierarchical arrangement, the basic components of the biofilm being the cells and the EPS. These can combine to form secondary structures such as discrete cell clusters (which may take on various forms and dimensions) and a base film. Finally, the arrangement of base film, cell clusters, and the void areas between the clusters gives the overall biofilm architecture. The relative importance of each of these features in determining the biofilm heterogeneity can be highly variable.

Hydrodynamics

Hydrodynamic conditions control two interdependent parameters (mass transfer and shear stress) and will, therefore, significantly influence many of the processes involved in biofilm development. Two types of flows are relevant to most natural and industrial processes: laminar flow and turbulent flow. Generally, when the flow rate of a liquid is low, flow will be laminar, and when the rate is high, it will be turbulent. Transition between these two types of flow will be dependent on channel geometry and fluid properties and, in many cases, can be predicted by the Reynolds number (*Re*), a dimensionless parameter commonly used by engineers (Vogel 1994). In closed pipes, flow is generally turbulent at a *Re* above 1,200. The *Re* is also useful as a comparative indicator of flow conditions in a diverse range of systems. Briefly, in laminar flow shear stresses are low and mixing is poor, whereas in turbulent flow shear stresses are high and mixing is good. Under these conditions, shear and mixing have opposing influences on biofilm accumulation and on the resulting biofilm structure (van Loosdrecht et al. 1995). Increased shear tends to increase the detachment rate by the physical removal of individual cells (erosion) or larger pieces of biofilm (sloughing), while increased mixing tends to increase the growth rate by reducing transport limitations and increasing the nutrient supply. The rate of transport of dissolved and particulate species (nutrients, biocides, etc.) into the biofilm and the removal of waste products from the biofilm also will have a profound influence on the chemistry (pH and eH, etc.) of the local microenvironment. It has been hypothesized that there may be an optimal flow for biofilm formation below which accumulation would be limited by mass transfer and above which accumulation would be limited by detachment (Lewandowski 1991).

To date, most of the detailed investigation on biofilm structure has been conducted on biofilms grown in the laboratory under laminar flows. These biofilms tend to be cell clusters which are roughly circular or amorphous and in which there is no obvious axial alignment. However, in turbulent flows, the influence of drag becomes apparent, and biofilms form filamentous "streamers" which can oscillate rapidly in the flow (Bryers and Characklis 1981; McCoy et al. 1981; Siegrist and Gujer 1985; Stoodley et al. 1998, 1999a, b). The increased energy losses in pipelines have been attributed to the possible formation of streamers (Picologlou et al. 1980). More recently, it has been shown that mixed biofilms growing in turbulent flow can form ripple structures that steadily migrate downstream (Stoodley et al. 1999d). The ripple morphology and migration velocity varied with bulk liquid flow velocity, with a response time on the order of minutes. The ripples had a maximum migration velocity of approximately 1 $mm \cdot h^{-1}$. Dalton et al. (1996) have observed cyclical colonization by marine *Vibrio* and *Pseudomonas* species growing in laminar flow, in which microcolonies repeatedly formed and dispersed over periods between 1 and 2 days. However, in this case, it appears that the structural changes were caused by gliding motility of the individual cells, possibly in response to nutrient conditions in the biofilm. It is generally assumed that microcolonies (also termed cell clusters) are formed mainly through cell division during the early stages of biofilm formation. The observation by Dalton et al. (1996) reveals that clusters also can form by the grouping together of attached cells. Both of these observations (Dalton et al. 1996; Stoodley et al. 1999d) demonstrate that the structural arrangement of biofilms is not only spatially but also temporally complex.

Influence of hydrodynamics on the structure of a *Pseudomonas aeruginosa* PAO1 biofilms grown in parallel glass flow cells under laminar and turbulent pipe flow (Stoodley et al. 1999b). The biofilms were grown on a minimal salts medium with glucose (400 $mg \cdot l^{-1}$) as the carbon source. Images were taken 7 days after inoculation. Black arrow indicates direction of bulk liquid flow. Scale bar = 50 µm (*Figs. 13.12–13.14*).

It may be possible to predict biofilm morphotypes from theoretical consideration of the relative influences of mass transfer and shear (Stoodley et al. 1999a). At high shear flows, where the influence of drag is high but mass-transfer limitations are low, drag-reducing planar structures may be expected. In low

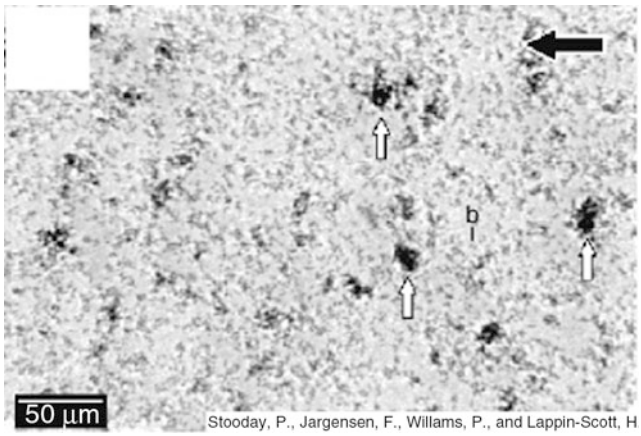

Fig. 13.12
The biofilm grown under laminar flow (flow velocity = 0.033 m·s^{-1}, *Re* = 120) was composed of small cell clusters (*white arrows*) with single cells "b" in the void spaces

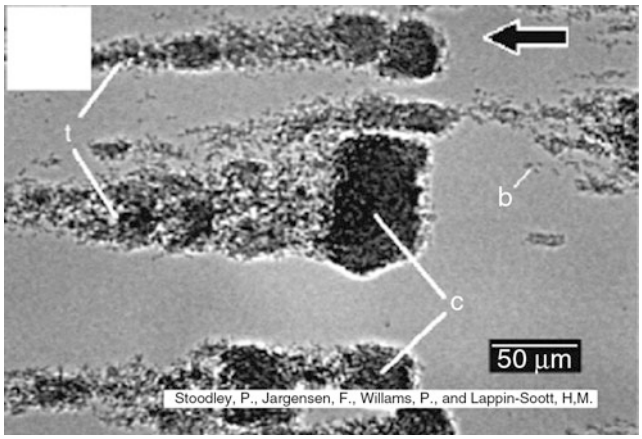

Fig. 13.13
The biofilm grown under turbulent flow (flow velocity = 1.0 m·s^{-1}, *Re* = 3,600) was composed of larger clusters which had become elongated in the downstream direction to form tapered streamers. Each streamer consisted of an upstream "head" cell cluster "c," which was attached to the glass surface, and a downstream tail "t." Some of the tails were free to oscillate in the flow, while others were more firmly attached to the substratum. The void spaces between the streamers were almost devoid of single cells "b"

shear flows, where the mass-transfer limitations are high but drag is low, highly porous structures with high surface exchange areas might be expected. Intermediate forms may exist between these extreme conditions.

Biofilm Viscoelasticity

In addition to the long-term influence of hydrodynamics on the structure of biofilms grown under steady shear, biofilm structure

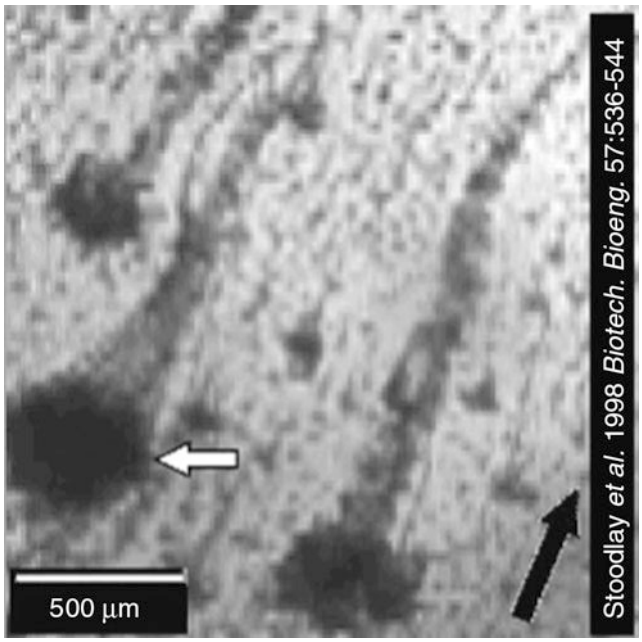

Fig. 13.14
Time lapse movie showing biofilm streamers oscillating in turbulent flow. The bacterial biofilm, composed of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Klebsiella pneumoniae*, was grown on a glass observation window in a polycarbonate flow cell. The oscillation frequency of the streamers was directly proportional to the velocity of bulk liquid, suggesting that the oscillations were caused by vortex shedding of the upstream "head" (*white arrow*) of the streamer (Stoodley et al. 1998). *Black arrow* indicates flow direction; scale bar = 500 µm. For *Fig. 13.14*, see the online version of *The Prokaryotes*

also can be influenced by short-term changes in fluid shear. Structural changes to mixed and pure culture biofilms caused by variations in fluid shear demonstrate that biofilms can be viscoelastic and have a very low elastic modulus (ca. 30 Pa, i.e., biofilms are highly compliant; Stoodley et al. 1999c). The biofilms exhibited liquid flow when the fluid shear stress exceeded the yield point. The yield point occurred between 1.2 and 2.0 times the shear at which the biofilm was grown. It is possible that liquid-like behavior may explain the formation of flowing ripples in similar biofilms (see *Figs. 13.15* and *13.16*).

Also the thickness of cell clusters was reduced by up to 30 % when the flow velocity was increased from 0 to 1.5 m·s^{-1}. It is thought that the flexibility of certain seaweeds, anemones and other benthic macroorganisms may allow the organisms to withstand the large variations in drag to which they are subjected by wave action (Koehl 1984). In these types of organisms, drag reduction can be achieved when the organisms "collapse" into a more streamlined shape. It is possible that the flexibility of some biofilms is an adaptive characteristic, which allows these biofilms to remain attached when exposed to varying shears (as would be expected in turbulent flow and many natural flowing water systems).

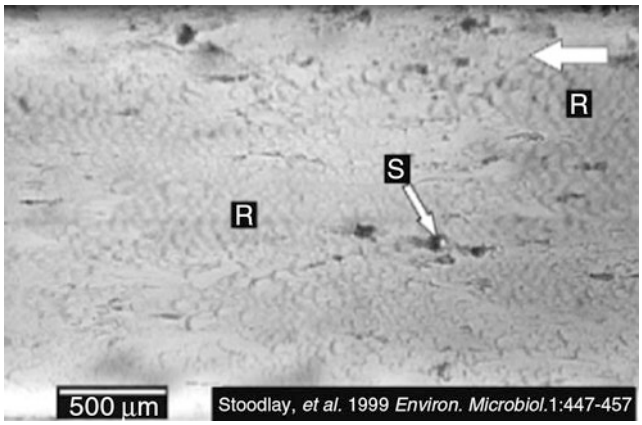

Fig. 13.15
Time-lapse movie showing the migration of biofilm *ripple-like* structures across a glass surface. The mixed species biofilm was composed of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Stenatrophomonas maltophilia*, and *Klebsiella pneumoniae* (Stoodley et al. 1999d). *Ripple patches* are labeled "R." Elongated streamers "S" also formed in the biofilm. The streamers did not migrate across the surface, but some of them were observed to detach during the observation period (see labeled streamer "S"). The biofilm was 15 days old and grown in turbulent pipe flow at a bulk liquid flow velocity of 1 $m \cdot s^{-1}$ (*Re* = 3,600). The flow direction is indicated by the *arrow*. Frames were captured at 1-h intervals over 15 h. Scale bar = 500 µm. For *Fig. 13.15*, see the online version of *The Prokaryotes*

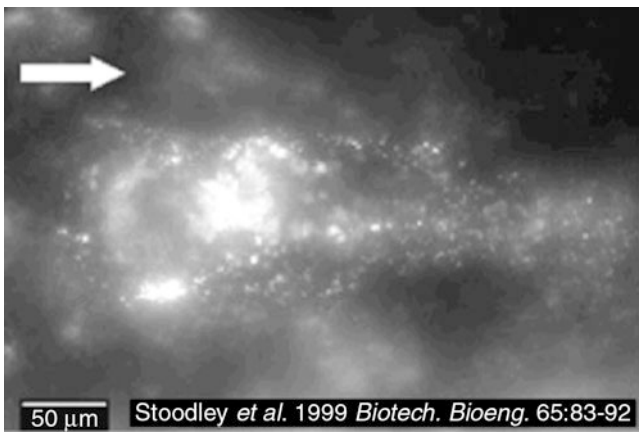

Fig. 13.16
Time-lapse movie showing the influence of fluid shear on biofilm structure (Stoodley et al. 1999c). The biofilm streamer was stained with fluorescent beads, which appear as *bright dots* and were used as fiducial points to monitor structural changes. The sequence of images shows the changes to structure as the fluid shear was increased stepwise from 0 to 10.11 Pa and then reduced stepwise back to 0. When the load was removed, the biofilm "sprang" back, clearly demonstrating an elastic response. Arrow indicates flow direction. Scale bar = 50 µm. For *Fig. 13.16*, see the online version of *The Prokaryotes*

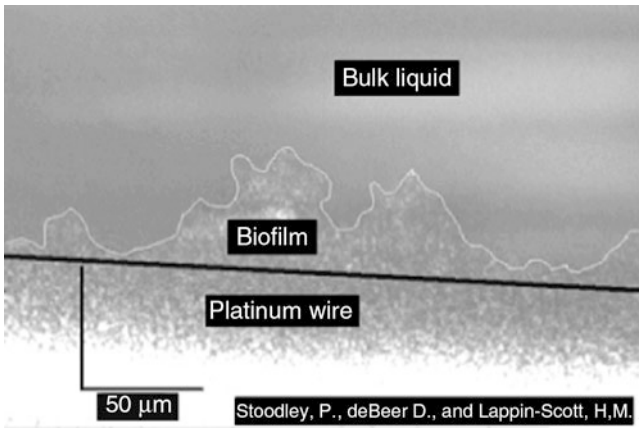

Fig. 13.17
Time-lapse movie sequence showing the expansion and contraction of a mixed-species biofilm growing on a platinum wire (Stoodley et al. 1997). The biofilm expanded when the wire was cathodic but contracted when it was anodic. The biofilm could fully expand and contract at a maximum frequency of approximately 5 Hz. At higher frequencies, the biofilm appeared to fibrillate. Similar contractions and expansions could be induced by pH alone. The edge of the wire is indicated by the *black line*. The edge of the biofilm has been outlined in *white* for clarity. Scale bar = 50 µm. For *Fig. 13.17*, see the online version of *The Prokaryotes*

Biofilm rheology also may help explain the large energy losses that biofilms can cause in water pipelines. High-pressure drops (δP) have been linked to the observed formation of filamentous streamers, and it has been calculated that the measured δP was significantly greater than that expected from an equivalent rigid structure (Picologlou et al. 1980). It is known that rigid structures that are anchored in flowing fluids can dissipate the kinetic energy of the fluid through skin friction and pressure drag (Vogel 1994). Skin friction is dependent on surface area and is more significant in laminar flows. Pressure drag is shape-dependent and is more significant in turbulent flows. Biofilms that are behaving viscoelastically also can dissipate kinetic energy through both elastic and viscous action (Stoodley et al. 1999c). Rapid elastic deformations, which may occur when biofilm streamers oscillate, would result in the generation of heat in the biofilm matrix as bonds repeatedly stretch and contract. It is expected that, because of the thin nature of the biofilm, this heat would be quickly transferred to the bulk liquid. Viscous behavior also can generate heat through friction as individual polymer strands move past each other when biofilm flows. As yet, it is not clear what the relative contribution of each of these mechanisms is to the formation of pressure drops in pipe flow (*Fig. 13.17*).

In addition, biofilm structure may be influenced electrochemically. It has been found that electrical fields can cause biofilms to rapidly contract and expand (Stoodley et al. 1997). It was found that the thickness of a mixed species biofilm cell cluster was reduced to 74 % of the original thickness when the platinum wire which it had been grown on was cathodic. This

change was similar to that caused physically by hydrodynamic shear (see above). This effect is common in gels and may lead to increased exchange between water phase and biofilm and partly explain the bioelectric effect (Wellman 1996).

Growth and Detachment

Detailed studies on growth and detachment have been performed by van Loosdrecht and his group. Tijhuis et al. (1996) suggested that the degree of heterogeneity is determined by the balance between the growth rate and abrasion. Indeed, slow-growing organisms (e.g., nitrifiers and methanogens) form relatively flat biofilms or spherical aggregates; faster (heterotrophic) growth results in formation of more heterogeneous biofilms with cell clusters and streamers. Characklis (1990a) has demonstrated the relative contributions of nutrient loading and flow velocity on biofilm thickness. At low loading rates (0.1 g carbon·m^{-2}·h^{-1}) biofilm thickness (ca. 50 µm) was nutrient-limited and relatively independent of liquid flow velocity. However, at higher loading rates (2.4 g carbon·m^{-2}·h^{-1}), the biofilm thickness was approximately 1,000 µm at a liquid flow velocity of 1.5 m·s^{-1} but was reduced to 200 µm when the biofilm was grown at a liquid flow velocity of 3 m·s^{-1}. In this case, the biofilm thickness was presumably limited by shear-induced detachment as the flow rate was increased. It is well established that generally thinner biofilms form (and less biofilm biomass accumulates) under low nutrient conditions, and it has been proposed that biofilm accumulation may be limited by removing nutrients under controlled conditions in an upstream biofilter (Griebe and Flemming 1998). However, there is less information on the influence nutrients have on the structure of biofilms. Møller et al. (1997) reported that the structure of a mixed microbial community grown under laminar flow changed in response to a switch in substrate (while maintaining a constant labile carbon loading rate) (Møller et al. 1997). When grown on 2,4,6-trichlorobenzoic acid, the biofilm consisted of mounds of cells separated by void areas. However, when the substrate was switched to trypticase soy broth, the biofilm became thicker, and growth in the void areas resulted in a less heterogeneous structure. Also, a change in nutrient concentration can cause a change in the structure of an established biofilm as well as changes in thickness and surface coverage. Stoodley et al. (1999a) reported that the structure of a 21-day mixed-species biofilm growing in turbulent flow initially consisted of ripples and streamers (see *Fig. 13.15*) but changed to large cell clusters (ca. 500 µm in length) when the carbon and nitrogen concentration was increased by a factor of 10. The biofilm also significantly increased in thickness and surface coverage. When C and N concentrations were reduced to their original levels, there was a loss of biomass and the ripples and streamers reappeared. We speculate that cell surface properties, in particular hydrophobicity or hydrophilicity, also can determine the biofilm structure. Cell surface hydrophobicity results in minimization of the contact surface between liquid and biofilm and thus in planar biofilms or spherical aggregates. Hydrophilic cells will more easily form protrusions like streamers and cell clusters. Typically, dividing cells (Allison et al. 1990) and many (facultative) aerobic heterotrophs (Daffonchio et al. 1995) are hydrophilic. Heterotrophic conditions thus result in heterogeneous biofilms. Examples of hydrophobic microorganisms are benthic cyanobacteria (Fattom and Shilo 1984), methanogens, syntrophic bacteria, and, to a lesser extent, sulfate reducers (Daffonchio et al. 1995). Indeed, cyanobacterial mats and methanogenic biofilms are usually relatively flat; however, detailed observations on mat structure and heterogeneity have not been conducted to the same extent as on bacterial biofilms.

Cell-Cell Signaling

Finally, cell-cell communication must be considered as a morphogenetic mechanism. By sensing cell-produced compounds, for example, *N*-acyl-homoserine lactones where the acyl group determines action or strain specificity, cells recognize the local cell density (therefore called "quorum sensing") and react by switching on or off certain sets of functional genes. Quorum sensing regulates the expression of the lux genes in the bioluminescent bacterium *Vibrio fisheri* and the release of virulence genes in pathogens like *Pseudomonas aeruginosa*, and it plays a role in the symbiotic host association of *Rhizobium leguminosarum* in root nodules. Genes for quorum sensing have been found in ca. 25 different bacterial species, and this communication mechanism is believed to be common among Gram-negative bacteria (Greenberg 1997). Quorum sensing also determines the structure of *P. aeruginosa* biofilms (Davies, personal communication): the presence of *N*-3-oxododecanoyl-L-homoserine lactone enhances the production of polyuronic acids, which are important components of bacterial EPS. The lactone concentration is increased, due to restricted out-diffusion, at higher cell densities or after adhesion to a surface, thereby enhancing biofilm formation. At higher concentrations (as can occur in dense and thick biofilms), the same compound induces production of *N*-butyryl-L-homoserine lactone, which then induces the production of alginate lyase that can dissolve EPS and lead to rapid cell mobilization and formation of voids in the biofilm matrix. Mutants of *P. aeruginosa*, defective in quorum sensing, form flat and homogeneous biofilms, while the wild-type organism forms heterogeneous biofilms (Davies et al. 1998). If these mutants are grown with *N*-3-oxododecanoyl-L-homoserine lactone added to the medium, a patchy biofilm resembling the wild-type biofilm is formed. Two counteracting lactones, one stimulating cell aggregation (biofilm formation) and one stimulating biofilm dissolution, can thus regulate biofilm structure. Halogenated furanones, produced by marine algae, interfere with the cell-cell signaling mechanism, resulting in strongly decreased biofilm accumulation (Maximilien et al. 1998). It can be expected that the newly found mechanism of cell-cell signaling will be very important in future biofilm studies. This will lead to an explanation of many biofilm characteristics and to more ways to manipulate biofilms.

Mass Transfer and Microbial Activity

Substrates for biofilm growth usually are supplied by the water phase, and metabolic products are eventually released into the water phase. The rates of exchange between the biofilm and the water phase are determined by the mass-transfer processes of diffusion and advection. Microbial conversions in biofilms are, therefore, dependent and often limited by mass transfer. The process rates should be determined in situ. Activity determinations on biofilm samples cannot give reliable data as the microenvironment cannot be accurately replicated in vitro. Conversions are related mostly to cell growth and division, and the development of reliable methods to determine growth rates of single cells in situ has proved difficult. However, the combination of two photon confocal microscopy with fluorescent gene activity reporters should remove much of the ambiguity associated with current techniques.

Mass Transfer in Biofilms

A common property of microbial mats, biofilms, flocs, and aggregates is the occurrence of mass-transfer resistance. This is due to the limited water flow inside the matrix and the presence of a hydrodynamic boundary layer between the matrix and the surrounding water phase (Jørgensen and Revsbech 1985; Jørgensen 1994; Lewandowski et al. 1993; De Beer et al. 1993, 1994; De Beer and Stoodley 1995; Ploug and Jørgensen 1998). Transport of solutes is thought to be primarily by diffusion inside the biofilm matrix and in the boundary layer adjacent to the solid surface. Consequently, when the internal chemical composition (substrates and products) differs from bulk water conditions, steep gradients develop. This has strong effects on the type and rates of microbial conversions. Mass-transfer resistance often limits conversion rates. However, many processes can occur only inside biofilms because of special prevailing conditions. For example, anaerobic conversions like denitrification, sulfate reduction, and methanogenesis primarily take place in anoxic environments found in the deeper zones of biofilms and mats. However, recent studies showed that anaerobic processes also can occur in the oxic part of sediments and mats, indicating that anaerobic bacteria have special physiological adaptations or that anaerobic microniches may exist in the oxic zone (Canfield and Des Marais 1991; Frund and Cohen 1992; Krekeler et al. 1997). A characterization of the microenvironments and their interaction with mass-transfer processes is needed to understand conversions inside biofilms.

The simplest biofilm concept is a planar geometry, with microbial activity distributed homogeneously and all transport parameters constant regardless of depth. Adjacent to the biofilm is a mass boundary layer (MBL) in which the transport gradually changes from diffusional to advective in the mixed bulk liquid. This is illustrated in *Fig. 13.18*, showing an O_2 microprofile in and above a respiring biofilm. The strength of this concept is its simplicity, which facilitates mathematical modeling of

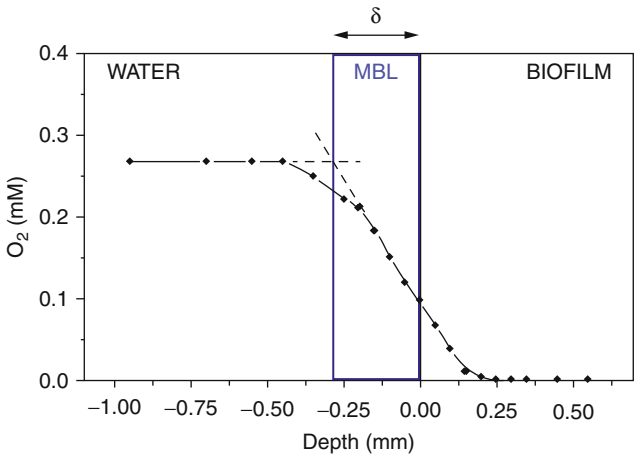

Fig. 13.18
Oxygen profile measured in a moderately active biofilm. The crosspoint of the *dashed lines* indicates the upper boundary of the hypothetical mass boundary layer (MBL), having a thickness δ. The image shows that a significant part of the mass-transfer resistance can be situated outside the biofilm

transport, conversion, and growth (Wanner and Gujer 1986; Rittmann and Manem 1992; Wanner and Reichert 1996) (*Fig. 13.18*).

The mass-transfer resistances can be separated into external, in the MBL, and internal, in the matrix itself. The resistance in the MBL is proportional to its thickness, which depends mainly on the flow velocity of the liquid (Jørgensen and Des Marais 1990). The mass transport coefficient, k_s, and the thickness of the MBL, δ, can be calculated from the liquid flow velocity ($_u\exists$). For example (Shaw and Hanratty 1977):

$$k_s = 0.0889 u_\infty Sc^{-0.704} \qquad (13.1)$$

with Sc as the Schmidt mumber,

$$Sc = \frac{\eta\rho}{D_{eff}} \qquad (13.2)$$

with η as the dynamic viscosity, ρ as the density of the water phase, and

$$\delta = \frac{D_{eff}}{k_s} \qquad (13.3)$$

Ultimately, the effective diffusion coefficient (D_{eff}) and the penetration depth (ρ; diffusion distance) of the limiting substrate determine the mass-transfer resistance (η) in the matrix.

The relative importance of the MBL and intra-matrix resistance for conversion rates has been described for flat geometry with first- and zero-order kinetics (De Beer 1998). Qualitatively expressed, the greater is the microbial activity of the matrix, the smaller is the penetration depth of the limiting substrate. Consequently, the relative contribution of the mass-transfer resistance inside the matrix decreases. Therefore, the higher the microbial activity, the more important the MBL is for the regulation of microbial activity. The penetration depth of O_2, often the limiting substrate, is typically 100 µm in

active biofilms, while the thickness of the MBL is in the range of 50–300 μm. Consequently, external mass transfer, and thus flow velocity (see Eq.), often determines the activity of biofilms.

Internal mass transfer is usually considered to be diffusional and, consequently, frequently described using a single effective diffusion coefficient (D_{eff}). The flux of a compound, therefore, depends both on its diffusion coefficient and the slope of the concentration profile

$$J = D_{eff} = \frac{dc}{dx} \tag{13.4}$$

where J is the flux ($mol \cdot m^{-2} \cdot s^{-1}$), D_{eff} is the effective diffusion coefficient in the biofilm, and dc/dx is the concentration gradient.

Both diffusion and advection of solutes are important in biofilms. The biofilm matrix hinders both phenomena; obviously the matrix is an effective barrier not only for water movement (advection) but also for the random movement of solutes (diffusion). The effective diffusion coefficient (D_{eff}) is proportional to the biofilm porosity (θ) and inversely proportional to the square of the diffusional distance, the average path length (φ):

$$D_{eff} = \frac{D_w \theta}{\varphi^2} \tag{13.5}$$

Diffusion is the only transport mechanism when there is no flow inside the biofilm, while advection usually becomes the dominant mechanism when the matrix is sufficiently permeable to allow liquid flow. Contrary to advectional transport, diffusion becomes rapidly less effective with increasing distance. A simple calculation example demonstrates this; the root-mean-square displacement (x) due to diffusion is described by Berg 1983:

$$\langle x^2 \rangle^{1/2} = \sqrt{2Dt} \tag{13.6}$$

where t = time.

From this equation, it is evident that displacement due to diffusion is time dependent: for a diffusion coefficient with a representative value of 1×10^{-9} $m^2 \cdot s^{-1}$, the average displacement due to diffusion is 100 μm in 5 s, 1 mm in 10 min, 1 cm in 14 h, and 1 dm in 2 months. Diffusion is a very effective transport process for short distances (the size of bacterial cells), but it is much slower over longer distances (the thickness of biofilms). Solute transport due to advection equals the velocity of the liquid, and even if it is in the order of 1 $\mu m \cdot s^{-1}$, advection will be as important as diffusion for biofilms, which typically range from a few hundred μm to several mm thick. Microscopic observations have showed that biofilms can be highly porous, thus the common assumption that diffusion is the sole transport mechanism must be treated with care. Indeed, it was shown that water can flow between the cell clusters as described in the heterogeneity section. Fluorescent beads quickly penetrated the voids, and their movement, followed by confocal microscopy, showed that water can flow through the channel-like structures (De Beer 1994; Stoodley 1994, 1997). With oxygen microsensors, it was further demonstrated that the oxygen concentration inside the voids is much higher than that in the adjacent cell-clusters, and thus diffusion will occur in a horizontal direction or even from voids at the base of the biofilms in an upward direction into the cell clusters (Fig. 13.19; De Beer 1994). It was calculated that

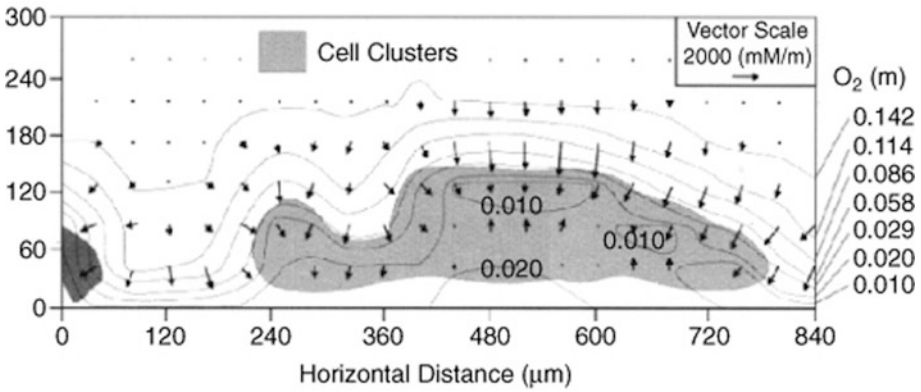

Fig. 13.19

Cross section through a heterogeneous biofilm showing dissolved oxygen (DO) contours associated with the cell clusters (*shaded*) and surrounding water channels. Twenty two adjacent DO profiles were measured by microelectrode to give a 2-D array of DO measurements. The microelectrode was guided by micromanipulator, and confocal microscopy was used to locate the positions of the clusters and channels. In this particular biofilm, the cell clusters were held away from the flow-cell wall (located toward *the bottom of the figure*) by "stalk-like" structures. Since this cross section did not intersect any of these stalks, the biofilm cluster appears to be "floating" above the wall of the flow cell. The *arrows* are the calculated DO fluxes and the length of the *arrow* is proportional to the magnitude of the flux. Note the penetration of DO into the biofilm channel which is centered at 120 μm on the x-axis. Also note that in the center of the large cell cluster the flux arrows are pointing upward, indicating that DO was being supplied from underneath the cell cluster. In this case, the anaerobic region occurred in the *center* of the cell cluster, approximately 100 μm away from the substratum, and not in a layer adjacent to the wall as would be predicted for a flat biofilm. The vertical distance in microns is shown on the *left* Y-axis

in such heterogeneous biofilms, the interfacial oxygen flux is two to three times higher than that for a flat biofilm. At low flow velocity, this effect disappears and a 1-D model can be applied (De Beer 1995). Upon injecting a fluorescent dye in the voids of a biofilm, an elongated plume developed, while injection inside the pores resulted in a spherical plume (De Beer 1995). Therefore, it was concluded that in voids flow occurs and induces advective transport at higher flow velocities, but in cell clusters diffusion is the only transport mechanism (*Fig. 13.19*).

Numerous studies reporting measurements of the D_{eff} of various compounds in biofilms and microbial aggregates have been reviewed (Christensen and Characklis 1990; Libicki et al. 1988; Siegrist and Gujer 1985). Literature values show a wide range of variation, D_{eff} being 1–900 % of the diffusion coefficient in water (D_w), reflecting the variety of biofilms studied as well as the different measurement methods. The D_{eff}/D_w ratios for substrates with small molecular weights, such as oxygen, glucose, ammonium, and nitrate, in spontaneously growing biofilms and microbial aggregates are assumed to be around 0.9 (Christensen and Characklis 1990). Diffusion of macromolecules such as DNA, dextrans, and proteins may be more strongly impeded by biofilm matrices, resulting in decreased D_e/D_w ratios. Diffusion experiments with such molecules have been reported only for gel matrices; however, biofilms have been considered to be highly hydrated gels (Christensen and Characklis 1990). An extensive review on diffusion phenomena in gels is given by Westrin (1991). It has been shown that the D_e/D_w of proteins diffusing through agarose gels is inversely correlated with their molecular weight (Boyer and Hsu 1992; Arnold et al. 1985). This is due to gel-matrix polymers obstructing diffusion (Rodbard and Chranbach 1970) as well as to hydrodynamic drag at the matrix polymer-solvent interface (Brenner and Gaydos 1977). The impeded diffusion of large molecules in gels is strongly influenced by the microstructure of the gel matrices; consequently, information about the microstructure of the biofilm matrix may be derived from diffusion data.

Diffusivities in biofilms have been estimated by measuring transient or steady-state fluxes through biofilms in diffusion chambers or in uptake experiments (Libicki et al. 1988). If the experiments are performed with a nonreacting compound or with killed biofilms, D_{eff} can be calculated by fitting the measured fluxes with a diffusion model. In the case of a reacting compound, a reaction-diffusion model is necessary.

The determination of diffusivities by these methods assumes a homogeneous and flat biofilm. It was, however, shown that biofilms are not always flat but may contain streamers, cell clusters, voids, pores, and channels that may affect strongly the transport properties of the biofilm. Thus, the diffusivity may be overestimated from advective transport through the pores. This may explain why D_e higher than D_w values have been reported (Libicki et al. 1988; Siegrist and Gujer 1985).

To describe transport inside biofilms, transport in the voids (advection and diffusion) and in the base film and cell clusters (diffusion only) must be distinguished. For this, measuring techniques with high spatial resolution are needed. A powerful technique for determining local diffusional properties of biofilms is the fluorescence recovery after photobleaching (FRAP) method (Axelrod et al. 1976). Application of optical techniques is limited to transparent structures. First, a biofilm is soaked with fluorescently labeled compounds. The FRAP method measures the diffusion of fluorescent molecules into a small area in which all molecules are bleached by a high-intensity laser beam. After that volume is depleted of fluorescent molecules by the laser flash, other molecules diffuse in from the surrounding area. By quantitatively monitoring this diffusion using CSLM and by fitting these data using a mathematical model, the diffusion coefficient of the compound can be calculated. The spatial resolution of the method is ca. 30 µm. It was found that the diffusion of small molecules (MW 300) was not significantly impeded by biomass. Large molecules, such as dextrans, proteins, and DNA, were impeded (ca. 30 % of that in water for large proteins, MW 200,000, and 20 % for DNA, MW 3×10^6; Bryers and Drummond 1996).

A conceptually related technique is based on microinjection of a fluorescent tracer and on monitoring the expansion of the fluorescent plume by CSLM (De Beer et al. 1997). Instead of diffusion into a depleted area (FRAP), the out-diffusion is followed into the surrounding area. The distribution pattern of the dye is fitted with an implicit equation to obtain a local value for D, the diffusion coefficient. A refinement of this technique is to detect the fluorescence with an optical fiber connected to the microinjection capillary (De Beer et al. 1997; Kühl and Revsbech 1998). This microsensor can be used in thick nontransparent biofilms. With this technique, similar observations were obtained as with FRAP: the diffusion coefficient of small molecules (MW 300) is close to that in water, while the mobility of large molecules (MW 240,000) is decreased to ca. 30 %.

Both FRAP and microinjection are non-steady-state methods of measuring the diffusivity at a particular location in the biofilm. The value obtained is the molecular diffusivity, corrected for the tortuosity of the matrix (D_w) but not the porosity (Libicki et al. 1988). For determination of D_{eff} (D_w/j), flux measurements during steady-state transport are needed.

Recently, two microsensor approaches were developed to determine local diffusivity (D_{eff}) with high spatial resolution. A microsensor method to determine local diffusivities or local mass-transfer properties (Yang and Lewandowski 1995; Rasmussen and Lewandowski 1998) is based on measuring the limiting current during the reduction of Fe^{3+}. A strong inhibition of Fe^{3+} transport was measured in biofilms, especially in deeper parts of the cell clusters, with values lower than 10 % of that in water. These findings contradict others that concluded small molecules are not impeded in their motility (Bryers and Drummond 1996; De Beer et al. 1997). Possibly, because of the measuring conditions employed (0.5 M KCl and 25 mM $K_3Fe(CN)_6$), the viscosity in the biofilm was increased by cell lysis or weakening of the EPS bonds, thus decreasing the diffusivity. Recently, a diffusion microsensor was developed, based on out-diffusing of a tracer gas from

a needle-type capillary, with a tip size of 140 µm. The concentration of the tracer (H_2 or C_2H_2) in the tip of the capillary is measured using a normal microelectrode incorporated into the capillary. The signal is governed by the diffusional resistance in a sphere around the tip of the sensor and, thus, is proportional to the local diffusion coefficient (Revsbech et al. 1998). With this sensor, the D_{eff} in methanogenic aggregates was estimated at 50 % of that in water (Santegoeds 1999a).

In a recent study using NMR imaging, D_{eff} was determined in microbial mats, which were stratified; their diffusivity values ranged from 30 % to 60 % of that in water (Wieland 1999). These measurements were confirmed with the gas-diffusion sensor (Revsbech et al. 1998).

In conclusion, data on the diffusion coefficient in biofilms are highly variable. This may be partially due not only to differences in the techniques used but also to the possibility of large variations within individual biofilms and between different types of biofilms.

Stratification (E-Donor) and Internal Cycling

In sediments, a stratification into zones with different microbial conversions is well documented (Berner 1981). Sediments are subjected to a continuous or intermittent influx of organic matter, which is mineralized in several steps. Therefore, a wide diversity of conversions takes place, and as a consequence several profiles develop. The deeper regions are more anoxic and reduced than the top, which is usually aerobic. The organics are degraded and oxidized by bacteria using electron acceptors in the characteristic sequence O_2, NO_3^-, MnO_2, Fe^{3+}, SO_4^{2-}, and CO_2 (Reeburgh 1983). This sequence coincides with the standard free-energy changes of the reactions involved, and it is assumed that the larger the energy yield of a conversion, the greater the likelihood it will dominate other competing reactions. The mineralization processes involved are aerobic mineralization, denitrification, iron reduction, sulfate reduction, and methanogenesis. Since electron acceptors are usually supplied from the water phase, the different processes will occur adjacent to each other, going from surface to deeper zones, in the same characteristic sequence mentioned. The stratification is not necessarily strict, and processes do not necessarily exclude each other, for example, there is no thermodynamic argument why methanogenesis does not occur under aerobic conditions. Inhibition and regulation on the cell level are strong determinants for the stratification. Denitrification is inhibited usually by oxygen, although an exception was reported (Robertson 1983). Consequently, denitrification is located directly adjacent to the aerobic zone, with some possible overlap in the microaerobic zone (Lorenzen 1998; De Beer and Sweerts 1989; Robertson 1995). Sulfate reducers were thought to be very sensitive to oxygen, and thus restricted to anoxic zones. However, recently sulfate reducers were found in oxic zones of biofilms, sediments, and microbial mats (Dilling 1990; Krekeler et al. 1997). Furthermore, there is evidence that sulfate reducers can be sulfidogenically active under aerobic conditions (Canfield and Des Marais 1991). Even more confusing was the finding of sulfate reducers that oxidize sulfide aerobically (Fuseler 1996). Sediments are not fundamentally different from biofilms, and both can be considered as matrices with localized (evidence suggests that cells are not immobilized but can swim around inside the cell clusters) microorganisms. All these processes may occur in biofilms as well, although in the relatively thin and heterogeneous biofilms, a stratification of processes and organisms may not be as pronounced and may be more difficult to study.

Owing to the short distances in biofilms, the different conversion processes can be tightly coupled and internal cycling occurs. Organic matter is subjected to hydrolysis and fermentation, producing volatile fatty acids and hydrogen. These products and the original organic matter serve as e-donors for anaerobic and aerobic respiration and methanogenesis. In the oxic zone, products from anaerobic redox processes like sulfide, methane, and Fe^{2+} are oxidized. Thus, internal cycles are possible, which cannot be quantified from interfacial fluxes. Such sequences of processes can only be detected by invasive techniques with high spatial resolution, that is, by microsensors.

An important internal cycle in biofilms is sulfate reduction coupled to sulfide oxidation. It is well known that sulfate reduction contributes considerably to the mineralization process in marine sediments (Jørgensen 1977, 1985; Jørgensen and Des Marais 1990; Thamdrup 1996). Because of reoxidation by aerobic and anaerobic processes, sulfide does not reach the top of the sediment. The importance of sulfate reduction in marine environments is usually explained by the high sulfate concentrations in seawater. However, also in freshwater systems, the sulfur cycle can be important. In aerobic wastewater biofilms, a significant part of the mineralization occurs by sulfate reducers. It was demonstrated with microsensors for S^{2-} that 50 % of the oxygen was used for sulfide oxidation, implying that 50 % of the mineralization of organic matter is degraded by sulfate reduction (Kühl and Jørgensen 1992). In similar biofilms, a combination of molecular techniques and microsensor techniques was used to relate microbial activity with microbial population distributions (Ramsing et al. 1993). A good correlation was found with the distribution of sulfate reducers and sulfide production in these biofilms, which were both confined to the anoxic zones. However, owing to the metabolic versatility of sulfate reducers and their resistance to oxygen, such a good correlation between microbial populations and microbial conversions is not obvious. Recently, the development of sulfate reduction in an aerobic biofilm was studied with microsensor and molecular analyses (Santegoeds et al. 1998). It was found that sulfate reduction coupled to sulfide oxidation began only after 6 weeks, although anoxic zones were present by the first week. Once started, this process became of major importance, resulting in up to 70 % of the mineralization in the biofilm. Thus, sulfate reduction can be an important process even in thin biofilms that are exposed to oxygen.

In the absence of nitrate, sulfide is oxidized by oxygen, and thus, the oxygen and sulfide profiles overlap. Upon addition of

nitrate, a separation between the sulfide and oxygen profile occurs because nitrate penetrates beyond the oxic layer and becomes the e-acceptor for sulfide oxidation (Kühl and Jørgensen 1992). Furthermore, iron and manganese form important shuttles for redox equivalents in marine sediments (Canfield 1991). Their role in biofilms is not known.

When illuminated, photosynthesizing biofilms are found. In such communities, a most complex internal cycling exists as these biofilms are in principle self-supporting. In the photic zone, CO_2 is bound in the biomass and O_2 produced. In the aerobic and anaerobic zones, the biomass is degraded to CO_2. In such systems, a large array of microbial processes can be found. Especially in microbial mats from extreme environments, such as hot springs or hypersaline lakes, undisturbed stratification of the different processes occurs. Microbial mats are actually complete ecosystems, where primary production is balanced by aerobic and anaerobic respiratory processes. Such communities are therefore highly interesting model systems. For further information on these systems, we refer to reviews (Pearl 1996; Stal 1994).

The main processes in the nitrogen cycle are ammonification due to degradation of organic matter, nitrification, and denitrification. Since the product of denitrification is N_2 gas, the nitrogen cycle in biofilms is not closed but depends on input of nitrogen compounds. Since nitrification is an aerobic process and denitrification proceeds primarily in the absence of oxygen, a clear stratification can be expected. Indeed, using microsensors, it could be confirmed that nitrate is formed in the oxic and consumed in the anoxic zone (Schramm and Amann 1999; Schramm et al. 1996; De Beer 1998; De Beer et al. 1997b). Nitrification is usually limited by oxygen penetration and confined to an outer layer of ca. 100-µm thick (De Beer et al. 1993; Schramm 1998a). The intermediate nitrite can be found in a narrow zone near the oxic-anoxic interface, where it is formed by either incomplete nitrification or denitrification (De Beer et al. 1997b). In biofilms, denitrification is regulated by oxygen in several ways: firstly by inhibition, secondly by nitrification in the oxic zone, and finally by transport. Denitrification takes place adjacent to the oxic zone. At higher oxygen levels, the thicker oxic zone forms a transport resistance for nitrate from the water phase.

The competition between sulfate reduction and methanogenesis for e-donors has been investigated intensively. Sulfate reduction has more favorable thermodynamics than methanogenesis (Widdel 1988). Thus, methanogenesis is typically a more important process in low-sulfate environments (freshwater), and sulfate reduction dominates in marine sediments. Anaerobic reactors are usually designed for methane production, while sulfide production is an unwanted process because of odor and corrosion problems. In practice, both processes are active, resulting in biogas that is polluted with sulfide. Based on modeling, it was concluded that, under sulfate-limiting conditions, the outer layers of anaerobic biofilms or aggregates would be sulfidogenic, leaving a microniche for methanogens in the center (Overmeire et al. 1994). Such a division was indeed found: methane and sulfide microprofiles showed that sulfate reduction is confined to the outer 100 µm, while methanogenesis occurs in the center (Santegoeds 1999a). This was also observed in aggregates preincubated for months in excess sulfate and e-donor, that is, where sulfate reducers were expected to be present in the center as well. It was hypothesized that methanogens are needed for initial aggregate formation, while sulfate reduction develops subsequently. However, it is strange that sulfate-reducing bacteria (SRB) do not eventually colonize the aggregate center.

From the previously reviewed literature, it can be concluded that the sequence of e-acceptor use found in sediments is also present in biofilms. This was almost comprehensively demonstrated with microsensors in wastewater biofilms (De Beer 1999). First, O_2 is used by heterotrophic and autotrophic processes (nitrification and sulfide oxidation). Then NO_2^- and NO_3^-, formed by nitrification or originating from the water phase, are consumed in the zone directly adjacent to the oxic layer. Denitrification can be coupled to sulfide oxidation. Sulfate reduction is found below the denitrifying zone. Methanogenesis is also spatially separated from sulfate reduction and occurs in the deepest zones of the biofilms.

Special Physiology of Biofilm Cells?

Since biofilms function differently in many aspects from planktonic cells, it has often been speculated that a special biofilm physiology exists (Cochran et al. 2000). Biofilms usually have a lower specific conversion rate, high resistance toward biocides and antibiotics, and less sensitivity to temperature changes. It has been argued that all these phenomena can be explained by mass-transfer resistance (van Loosdrecht et al. 1990). Mass-transfer resistance reduces the transport of substrates and biocides to the cells, even if they form only a monolayer. Due to mass-transfer resistance, only a partial penetration of substrate occurs, but under conditions of reduced cellular activity (e.g., by cooling), a larger part of the biofilm gets penetrated with substrate, which counteracts the reduced specific conversion rates in each cell. Two other explanations for the reduced efficacy of biocides and antibiotics are the relatively low growth rates of biofilm cells, which make them less susceptible (Brown and Gilbert 1993), and the reduced penetration either by binding of compounds to the biofilm matrix or, in case of reactive biocides, by deactivation in the outer layers of the biofilm (Stewart et al. 1996). It appears that rather than a special biofilm physiology, the growth and activity of the cells within the biofilm may be governed by the physiochemical conditions that prevail in the biofilm microenvironment.

However, more and more evidence indicates that cells recognize and respond to the presence of other cells and surfaces in their environment. It has been shown that after attachment certain genes required for EPS synthesis are activated (Davies and Geesey 1995) and that the production of pheromones (homoserine lactones) induces biofilm formation (Davies et al. 1998). Thus, the development of a mature biofilm involves the same compounds involved in quorum sensing (Greenberg 1997). It was carefully hypothesized that cell-cell signaling might be involved in the resistance of biofilms to biocides (Costerton et al. 1999). See further in the paragraph on section "Morphogenetic Factors."

Microbial Populations

Previously, microbial population analysis was based on enrichment and cultivation techniques. It has become clear that plate counts very often do not represent the true microbial community, as many strains are resistant to cultivation. Microbial analysis has become much more reliable (and easier) due to the development of noncultivation techniques. Owing to the relative ease of molecular techniques, many data are collected from a wide variety of microbial communities; however, the role of the detected populations is often not known. This can be attributed to the difficulty of functional analysis of complex communities: the conversions of a community can be measured, but it is difficult and often impossible to assign the conversion to certain populations. However, there are some exceptions. Some microbial populations can be analyzed with a combination of microsensor and molecular techniques. Then, it is possible to determine the location of certain microbial processes (with microsensors) and to determine the location of certain microbial populations (with fluorescent in situ hybridization, FISH). Comparing these data can lead to estimations of activities and kinetics of populations in situ.

The combination of molecular and microsensor techniques was first used in a biofilm from a trickling filter (Ramsing et al. 1993). Sulfide profiles were measured with AgS microsensors from which the distribution of sulfate reduction and sulfide oxidation was determined. With oligonucleotide probes (SRB385), the distribution of SRB was determined, although it is now known that the probe is not targeting SRB exclusively. Once a reasonable agreement between the distribution of SRB and sulfate-reducing zones was found, the in situ activity could be estimated. However, SRB were also found in the oxic zones. Although 50 % of the mineralization was done by sulfate reduction, only 10^8–10^9 SRB per ml were found, which was probably less than 1 % of the total number of cells.

In a more detailed study, the development of the number and distribution of SRB as well as that of sulfate reduction was followed in a biofilm developing in a wastewater treatment plant. Although anoxic conditions were present from the first week on, no sulfate reduction could be detected until the sixth week. More surprisingly, SRB were present also in the initial biofilm as shown with various molecular techniques (DGGE, denaturing gradient gel electrophoresis; FISH; Santegoeds et al. 1998). A better correlation between molecular and functional analysis was found when comparing the presence of the functional gene for bisulfite reductase (DSR) and sulfate reduction (Santegoeds 1999a), as the start of the sulfate reduction coincided with a strong increase of the signal for DSR. Thus, the presence of a functional gene in a complex microbial community seems more predictive for its behavior than a population analysis. However, in activated sludge DSR could be clearly demonstrated, but sulfate reduction was absent, even upon exposure to anoxia in the presence of a suitable cocktail of e-donor (Schramm and Amann 1999). This points again to the fact that observed populations and biodiversity do not necessarily reflect the behavior of the community at the time of sampling. Many ecosystems and microbial communities are open, and thus, exchange of strains is occurring frequently. For example, wastewater treatment systems, which are the subject of many diversity studies, have an enormous input of microorganisms that are collected from a wide area or are grown in the sewer system. It is likely that the observed sulfate reducers in activated sludge originate from the input and the SRB populations need time to adapt to the conditions in the wastewater treatment plant.

In methanogenic aggregates a good correlation was found between the distribution of SRB and sulfate reduction, as well as between the distribution of methanogenic bacteria (MB) and methanogenesis. SRB were mostly found on the outside, while MB form the core of the aggregates. In between was a layer of syntrophic bacteria, which were found to supply both the SRB and MB with H_2 and acetate. In aggregates from a reactor optimized for sulfate reduction, few MB and little methanogenesis were found. In aggregates from a reactor optimized for methanogenesis, no SRB or sulfate reduction was detected (Santegoeds 1999a). The difference between anaerobic aggregates and the previously studied biofilms and activated sludge is age: while the biofilms and flocs had a life cycle of months or weeks, anaerobic aggregates develop over years. Probably, the SRB populations found in flocs and biofilms originated from outside the biofilms and flocs, and since they did not reduce sulfate, they were not adapted to the environment inside the studied communities. Thus, population analysis based on nucleic acid analysis must be regarded with care. It can be concluded that although certain physiological groups may be present, they may not necessarily be active. The presence of a population will only reflect the functioning of a microbial community if the community is mature and its populations well adapted to its environment.

A good correlation between activity and presence of populations is often found in nitrifying biofilms. In a high-loaded biofilm reactor from a fish hatchery, nitrate microprofiles showed nitrification in the aerobic surface layer and denitrification in the deeper anoxic zone. With FISH, populations of ammonium- and nitrite-oxidizing *Nitrosomonas* and *Nitrobacter* strains were found predominantly in this outer zone (Schramm et al. 1996). Nitrification is generally thought to be mediated mainly by *Nitrosomonas* and *Nitrobacter* strains because these are the main species that can be isolated from environmental samples. However, with molecular techniques these strains are seldom detectable in environmental samples. In aggregates from a rather low-loaded, fluidized bed reactor, intense nitrification was measured, but no nitrifying populations were found with the probes for *Nitrosomonas* and *Nitrobacter*. DNA was extracted, 16S-RNA coding fragments amplified with PCR and sequenced, and after comparison with the databases, new probes were designed. The nitrifiers that were found were new *Nitrosospira* and *Nitrospira* strains, which could not be grown in culture. With FISH and by using these probes, quantification of the different populations was possible, and from the microprofiles, local activities were obtained. Thus, nitrifying activities could be estimated under different

well-chosen conditions, even allowing in situ determination of Monod parameters (Schramm et al. 1998b) of up-to-now uncultured strains. The newly found strains had much lower specific activity and K_s than the known Nitrosomonas and Nitrobacter, implying a different survival strategy. In biofilms, they were found mainly in areas with low oxygen concentrations (Schramm 1998a). *Nitrosospira* and *Nitrospira* strains are adapted to low nutrient and oxygen concentrations (K-strategy), while *Nitrosomonas* and *Nitrobacter* can compete with their much higher conversion (and probably growth) rates at high nutrient and oxygen concentrations (G-strategy).

Previously described in situ studies, activity of populations was determined using a combination of FISH and microautoradiography, but activity determinations of single cells are also possible (Nielsen et al. 1998). Populations of filamentous bacteria from activated sludge were identified with probes for type 021N (strain identification according to Eikelboom key) and for *Thiothrix*. Uptake of six different organic substrates (^{14}C- or ^{3}H-labeled) under aerobic conditions revealed that no filaments took up all substrates and that strains, indistinguishable by morphology and molecular probes, showed differences in uptake patterns. This means again that great care must be taken when interpreting population structure of a complex community from the way its components function (Okabe 1997).

Biofilm Control

Biofouling is the detrimental development of biofilms in engineered systems, such as industrial process equipment, drinking water distribution systems, and ship hulls. Biofilms can decrease heat transfer in heat exchangers, increase the pressure drop in pipelines, enhance corrosion, and may be a source of bacterial contamination of drinking water (McCoy 1987; Camper 1994; Characklis 1990b). Biofilms are a nuisance in these systems and control of their development may be necessary to maintain process efficiency and safety. Biofilm control is often performed with biocides, of which the most commonly used is chlorine, a strong oxidizing agent and disinfectant. Biocides are much less effective against biofilms than suspended cells (Chen et al. 1993; Nichols 1988; LeChevallier et al. 1988). Cells in biofilms are protected from biocide action and are killed only at biocide concentrations orders of magnitude higher than those necessary to kill suspended cells. It has been speculated that the lower sensitivity of biofilm cells to biocides is the result of physiological differences associated with lower growth rate (Brown and Gilbert 1993). Alternatively, biocide may not reach the cells due to diffusional resistance of the biofilm matrix or to neutralization of biocide inside the matrix (Stewart et al. 1996). There is evidence for both theories.

Using a microsensor for chlorine, it was shown that chlorine penetrates very slowly in biofilms (De Beer et al. 1994). The shape of the chlorine profiles, the long equilibration times, and the dependence on the bulk chlorine concentration showed that the penetration was a function of simultaneous reaction and diffusion of chlorine in the biofilm matrix. Frozen cross sections of biofilms, stained with metabolic stain 5-cyano-2,3-ditolyl tetrazolium chloride (CTC; a redox dye), showed that the chlorine penetration overlapped with non-respiring zones near the biofilm-bulk fluid interface. Thus, chlorine was reduced effectively at the surface of the biofilm, which prevented its penetration to the cells in the deeper layers and thereby protected them. See further information in the section on "Cell-Cell Signaling."

Methods for Studying Biofilms

Cultivation

Laboratory-Scale Cultivation

All reactors used for cultivation of biofilms must be designed so that a selective advantage exists for cells to grow in biofilms and not in suspension. It must be kept in mind that cells in biofilms have a transport limitation not shared by suspended cells, which gives suspended cells in a reactor a selective advantage. In general, growth of suspended cells will interfere with biofilm behavior and thus complicate the interpretation of the experiment. Suspended cells can more efficiently compete for the limiting substrates, leaving little available for biofilms attached to the reactor surface. Consequently, in biofilm experiments suspended growth must be suppressed. Alternatively, certain selective pressures may favor biofilm formation. For example, biofilm formation may protect cells within the biofilm from the action of biocides or grazing predators (Costerton et al. 1994; Stewart et al. 1996). When biofilm growth is to be studied under controlled conditions, for example, pure or defined mixed cultures and constant conditions, ideally reactor conditions should be chosen so that the residence time of the liquid is shorter than the generation time of the cells. Then the suspended cells will be washed out, and the substrate is available for biofilm growth. This was nicely demonstrated by experiments that stepwise decreased the residence time of a culture (Beeftink 1987a). When the residence time became shorter than the generation time of the cells, washout occurred accompanied by a temporary increase of the substrate concentration. Subsequently, attached cell mass proliferated and the substrate concentration decreased again.

The particular design of a reactor system depends on the type of experiment, that is, the type of process, conditions desired, and monitoring techniques applied. Besides the residence time of the medium, also the hydrodynamic regime and the microenvironment in the reactor, that is, axial gradients, should be considered. The presence of stagnant zones must be avoided. The hydrodynamics should be predictable, and sudden expansions or contractions or sharp bends should be avoided where possible. For the medium supply, one can choose a once-through system, in which fresh, sterile medium is pumped through the biofilm compartment. This ensures good control over the substrate concentrations. However, to obtain a sufficiently high flow rate to avoid axial gradients and stagnant zones, large amounts of medium are needed. Most reactor

systems are operated with a continuous supply of medium, sufficient to ensure that the residence time of the medium is shorter than the generation time of the cultures, and a recycle allowing reconditioning of the medium (e.g., aeration and pH control) and control over the hydrodynamics of the biofilm. The design is often a compromise dictated by common sense. If experiments are to be done on pure cultures, a simple system that can be sterilized is preferred. Certain accommodations must be made for specific measurements, for example, observation windows for in situ microscopy, removable slides for biofilm sampling, openings for the introduction of microsensors, and so forth.

Flow Cells

One of the most widely used reactors for biofilm study is the flow cell. The system can be very simple, made of two microscopic slides and a spacer (Caldwell and Lawrence 1988; Wolfaardt et al. 1995; Lawrence et al. 1991), which is ideal for microscopic monitoring of biofilms. The volume is small; therefore, a one-way medium supply can be used. It can easily be sterilized and so be used for pure cultures. A more complicated flow cell is needed when the biofilm is monitored with microsensors. Either a closed flow cell with openings for introduction of the sensors (De Beer et al. 1994) or an open flow cell is used (Horn and Hempel 1997). Since these flow cells are larger, a recycle is needed to obtain good mixing of the water phase. A special type of flow cell is the Robbins device (Whiteley et al. 1997; Sly et al. 1990), essentially a pipe with sample holders, with the surface flush with the lining of the pipe. The sample holders are removable, allowing multiple biofilm sampling. Also, flow cells with observation windows for microscopy have been equipped with pressure- and flowmeters to study the interaction of hydrodynamics and biofilm accumulation (Stoodley et al. 1998).

A special flow cell was developed for immobilization of marine snow flocs (Ploug and Jørgensen 1998). The device (called net-jet) consists of a cylinder with a fine stocking separating the top and bottom part. The hydrodynamics are not well understood, but this simple device allows fixation of flocs in an upward water stream. The flocs can be penetrated by microsensors under settling conditions, resembling the normal hydraulic regime. In this flow cell, also activated sludge flocs with a diameter of 0.2–1.5 mm were investigated (De Beer 1998). If flow cells are well designed, the hydrodynamics can be well characterized (in contrast with many other types of bioreactor), allowing the influence of fluid shear and mass transfer on biofilm processes such as adhesion, detachment, and biotransformation rates.

Annular Biofilm Reactor

The annular reactor is essentially a chemostat consisting of a cylinder rotating in an outer cylinder with the reactor content between the outer and inner cylinder (Characklis 1988). The outer cylinder can be equipped with removable sample plates that are flush with the surface. The inner cylinder rotates at variable speed, thus allowing adjustment of the hydraulic regime over a wide range, independent of the residence time of the medium. With this device, many studies have been done on initial biofilm formation (Escher and Characklis 1990), the effect of biofilms on shear stress (Characklis 1990), and the effect of hydraulics on biofilm formation (Gjaltema et al. 1994). It is difficult to maintain a pure culture in these reactors. The hydraulics are not well described and not uniform (Gjaltema et al. 1994). Therefore, it now is recognized that this device cannot easily be used for quantitative studies.

Fowler Cell

Hydrodynamics are important for the attachment of cells and development of biofilm. The Fowler cell (Fowler 1988) is a radial flow cell, consisting of two plates mounted parallel to each other. The inlet is mounted in the center so that flow occurs radially, from the center to the periphery. The flow velocity is the highest in the center and decreases with increasing radial distance. The shear forces can be calculated assuming a flat geometry.

The Modified Robbins Device (MRD)

The MRD consists of a square or rectangular channel in a polycarbonate block in which 25 sampling-port studs are inserted along the length of the MRD (Hall-Stoodley et al. 1999). The studs can be fitted with different materials to investigate biofilm formation on different surfaces. The MRD is usually sterilized with ethylene oxide and the studs can be removed aseptically. The advantage of the system is that several samples can be taken simultaneously at different times to study biofilm development. Quantification, such as viable and total cell counts, total protein, and carbohydrate content, is possible on scraped samples. Although it is not possible to observe the biofilms in situ, microscopic analysis is possible using conventional staining techniques of slide-mounted samples or by electron microscopy of the colonized surfaces. The MRD is also relatively inexpensive. It can be used in both batch recirculating and once-through culture systems or can be connected to a chemostat. The major disadvantages of the MRD are the inability to directly observe the biofilm, the possible formation of significant nutrient gradients along the length of the device, and possible eddy generation around the sampling studs.

Constant-Depth Fermenter

Biofilms actually never reach a steady state, in which cell growth is balanced by decay and abrasion. Instead, biofilm development is characterized by colonization, growth, and sloughing

events and then by regrowth. A special type of biofilm reactor is designed in which the top of the biofilm is constantly (a few times per minute) scraped off and a constant thickness is maintained (Peters and Wimpenny 1988). Biofilms are grown in plugged holes in a plate, over which a plastic scraper rotates. In this model system, flat biofilms were obtained, which have several advantages including ease of microprofile interpretation, facilitated determination of D, multiple sampling (30 biofilm surfaces per plate), mass balances throughout the reactor accurately related to the biofilm surface, successional population changes to a steady-state situation followed, and spatial heterogeneity of populations determined (Wimpenny 1996). The obvious disadvantage of this approach is that the control of the biofilm thickness is very artificial. While in normal conditions an irregular surface develops, perfectly flat biofilms are formed in this model system.

Reactors for Biofilms on Carriers

A step in the direction of applied biofilm reactors is to downscale them to laboratory-size systems. Most applied biofilm reactor systems are based on biofilms on carrier-aggregated biomass, such as gas-lift reactors (GLR), fluid-bed reactors (FBR) and upflow anaerobic sludge blanket (UASB) reactors. Gas-lift reactors are attractive laboratory systems as the behavior is almost scale independent (Beeftink 1987b), facilitating good comparison with full-scale systems. Moreover, they are well mixed and the amount of samples that can be taken without disturbing the reactor is large. The GLR consist of two reactor compartments, a riser and a downcomer. Gas is pumped in the riser, resulting in an upflow of the water-gas-aggregate mixture. At the top of the riser, the gas is separated, and the water-aggregate mixture goes down in the reactor through the downcomer. In aerobic GLR, the gas is used for efficient aeration and mixing (Van Houten et al. 1994; van Loosdrecht et al. 1997; Gjaltema et al. 1997); in an anaerobic GLR oxygen-free gas is recycled for mixing only (Beeftink and Staugaard 1986; Van Houten et al. 1994). The advantage of mixing with gas over mechanical devices (impellers) is the relatively low power input and thus low shear forces. The reactor is completed with an internal or external settler to separate aggregates from the effluent stream.

The FBR is based on suspending aggregates on an upwardly directed liquid flow. This reactor is less well mixed, and axial substrate and product gradients develop. This may be a disadvantage for practical use, but for microbial ecology studies such gradient systems can be very useful (Csikor et al. 1994; Buffiere et al. 1995; Shieh and Hsu 1996; Schramm 1998a; De Beer et al. 1993).

The UASBs are used commonly for anaerobic treatment of concentrated wastewater. In these reactors, the hydrodynamic regime is so quiet that the aggregates are constantly settling in a sludge layer at the bottom (Hulshoff Pol 1989; Lettinga 1995). Laboratory-scale UASB with a volume of 1–3 l have been used to study methanogenic and sulfidogenic consortia (Thaveeshi et al. 1995; Harada et al. 1994; Koster 1989) as well as start-up phenomena of methanogenic biofilm reactors (Hulshoff Pol 1989).

Microscopic and Staining Methods

Microscopy

Scanning electron microscopy (SEM), transmission electron microscopy (TEM), normal light microscopy (LM), fluorescence microscopy (FM), and confocal scanning laser microscopy (CSLM) all have been used to study biofilms (Surman et al. 1996). Most microscopic methods involve some preparation of the sample, including staining, fixation, freezing, dehydration, embedding, and sectioning. For this reason, it is important to realize that biofilms are soft and mostly consist of water (<95 %) (Christensen and Characklis 1990). Preparations for microscopy may significantly change the matrix structure by shrinking and deformation (Stewart et al. 1995), and the resulting artifacts have influenced the concept of biofilm structure for years. Most relevant is the underestimation of the spatial heterogeneity, as several steps in the preparation may level the soft biofilm structures. Then, EPS appears as strands connecting the cells. EPS morphology changes by dehydration: diffuse polymeric matter is condensed to strands, leading to overestimation of the pore size. From SEM images the pore size appears to be in the order of 1 µm. Good TEM preparations show a pore size of ca. 100 nm (Beeftink and Staugaard 1986).

Images acquired by ESEM (Little et al. 1991) and atomic force microscopy (AFM; Bremer et al. 1992) with sub-µm resolution (no dehydration) do not show these strands but rather a smooth smear. A possible artifact from ESEM is the filling of recesses by water, "drowning" the roughness elements of the surface. The sensor needle of the AFM might disturb the surrounding water, causing the polymers to move and resulting in a blurred image (Bremer et al. 1992). Samples examined by LM, FM, AFM, ESEM, and CSLM can be unfixed. The recent application of CSLM has been especially instrumental in changing our concepts of biofilm structure (Lawrence et al. 1991; Massol-Deya et al. 1995; De Beer et al. 1994). With the CSL microscope, living transparent tissues can be sectioned optically, under growth conditions. In as much as out-of-focus fluorescence is effectively removed by the pinhole, the images are much sharper than standard microscopic images. Lawrence et al. (1991) published an excellent description of confocal microscopy techniques for biofilm research. Scanning electron microscopy, ESEM and AFM can be used for surface scanning, while the other techniques to some extent allow observation below the surface.

Staining

Specific staining is an important tool to unravel the spatial distribution of different biofilm components, most importantly

Table 13.5
Dyes for structural analysis of biofilms and microbial mats

Structure	Dye	Microscopy	Staining
Cells	Classical stains (crystal violet, Gram, etc.)	LM	All cells
	Acridine orange DAPI	FM and CSLM	All cells
	Ethidium bromide	FM and CSLM	Dead cells
	Eropidium iodide		
	Hexidium iodide	FM	Living cells
	CTC, formamide	FM and CSLM	Respiratory active cells
Voids and channels	Dextran conjugate	FM	Voids
	Beads	FM and CSLM	Voids
	Fluorescein	CSLM	Voids
EPS	Alcian blue	LM	EPS (carbohydrates)
	Lectins	FM and CSLM	EPS (carbohydrates)
	Calcofluor	FM and CSLM	EPS (carbohydrates)
	FITC	FM and CSLM	EPS (proteins)
	Heavy metals	TEM	Cells, EPS

Abbreviations as stated in *Table 13.4*; *DAPI* 4′,6-diamidino-2-phenylindole, *CTC* cyanoditolyl tetrazolium chloride

in cells, EPS and voids (*Table 13.5*). For viewing cells, stains nonspecific for DNA, such as acridine orange, diamidinophenylindole (DAPI), ethidium bromide, and hexidium iodide are most useful. These dyes can be combined with confocal microscopy, thus giving an image of cell distributions in undisturbed biofilms or mats. Species-specific staining by oligonucleotide probes or antibodies will be treated elsewhere.

Much less attention has been paid to visualization of EPS. Staining of EPS for fluorescent microscopy or CSLM is possible for proteins (fluorescein isothiocyanate), polyuronic acids and polysaccharides (lectin conjugates, calcofluor). Calcofluor stains most polysaccharides (attaching to β-1,4 and β-1,3 polysaccharides; Haigler et al. 1980), while lectins are more specific. Also, EPS dyes will stain cells that become visible as discrete points, whereas EPS appears as a continuous sheet. *The Handbook of Fluorescent Probes and Research Chemicals* (a catalog of molecular probes) is a highly valuable source of information about dyes and staining techniques (Haugland 1996). Also, EPS can be stained by ruthenium red for TEM or observed directly by SEM. Voids can be made visible with negative staining using fluorescein that is quenched by the presence of biomass. Using CSLM, voids appear as bright fluorescent areas, while biomass remains dark (see *Fig. 13.6*, De Beer et al. 1994; Lawrence et al. 1991). Also fluorescent microbeads can be used that penetrate the voids but not the cell clusters (Stoodley 1998) (*Fig. 13.6*).

In conclusion, several new microscopic techniques now make it possible to get a much more detailed view of biofilm structure.

Microbial Activity

As biofilms consist of thin but often dense layers of cells, many trophic interactions between different populations may occur on a small scale. Examples are nitrification and denitrification, sulfate reduction and sulfide oxidation, and photosynthesis and respiration. Few methods are suitable to measure in situ these coupled processes on such a small scale. Activity tests on subsamples give at best an impression of the distribution of potential activities but do not reflect the actual rates (Okabe et al. 1996). Analysis based on in situ detection of mRNA is not developed as yet for complex communities, and its value as a quantitative method is doubtful. With autoradiography, it was possible to detect substrate use on a cell level (Andreasen and Nielsen 1997); however, this method does not give local rates. Microsensor techniques are probably the most suitable for unraveling processes in thin complex communities. The local net consumption or production rates can be calculated from microprofiles with a spatial resolution of 25–50 µm. The main prerequisite is that the different processes must be spatially separated, with a distance of some tens of microns (with the exception of coupled photosynthesis and respiration).

Microsensors

Depending on growth conditions and age, the thickness of biofilms, aggregates and flocs can reach from µm to cm, and the structural heterogeneity can be pronounced. The active zones are typically on the order of a few mm or less. This requires experimental techniques with a high spatial resolution, and here microsensors have proven highly useful tools to study the biofilm and mat microenvironments and microbial activities in immobilized cell systems (including sediments). Microsensors are needle-shaped devices with a tip size of 1–20 µm and can measure the concentration of a specific compound. Owing to the small sensing tip, highly localized measurements

are possible. Although the technique is invasive, the small tips do not influence structures or processes significantly. With microsensors, the spatial distribution of substrates and products can be determined, and from this, the distribution of microbial activity can be inferred.

Niels-Peter Revsbech introduced microsensors in microbial ecology (Revsbech 1983) and also constructed the first reliable O_2 microsensors for profiling sediments and biofilms (Revsbech 1989). Other microsensors relevant for microbial ecology were developed and used, such as for N_2O (Revsbech et al. 1988), pH (Hinke 1969; Lee and De Beer 1995), NH_4^+ (De Beer and van den Heuvel 1988), NO_3^- (De Beer and Sweerts 1989; Larsen et al. 1996), S^{2-} (Revsbech 1983), H_2S (Jeroschewski 1996), NO_2^- (De Beer et al. 1997), CH_4 (Damgaard and Revsbech 1997; Damgaard 1995), CO_2 (De Beer et al. 1997), and HClO (De Beer et al. 1994) determination. Reviews have been published on construction and use of microsensors and on interpretation of results (Thomas 1978; Revsbech 1986; Kühl and Revsbech 1998; De Beer 1998, 1999). Here only a brief summary is given. Microsensors are based on amperometric, potentiometric, or optical principles.

Amperometric sensors are based on current measurements induced by the electrochemical reduction or oxidation of the substrate in the tip, with a rate proportional (usually linearly) to its concentration. Useful O_2, N_2O, H_2S, and HClO sensors are based on this principle. Many research groups have used O_2 microsensors for study of photosynthesis and respiration. Such studies are done in biofilms (De Beer et al. 1994; Kühl and Jørgensen 1992; Lewandowski 1991; Zhang 1994; Harmer and Bishop 1992; Zhang 1996), activated sludge flocs (Schramm 1998a; Lens et al. 1995), and marine snow (Ploug et al. 1997). The N_2O sensor has been used for denitrification studies in biofilms and microbial mats (Revsbech et al. 1988); the development of nitrate sensors has made this sensor obsolete for this purpose. The new H_2S sensor is used to study sulfate reduction and sulfide oxidation in biofilms (Santegoeds et al. 1998) and activated sludge (Schramm et al. 1998b). The HClO microsensor is used in biofilm disinfection studies (De Beer et al. 1994; Xu et al. 1995).

The variety of measurable substrates has been expanded by applying enzymes or bacteria as catalysts for the formation or consumption of redox-active compounds in the sensor. Glucose oxidase has been used for glucose sensors (Cronenberg et al. 1991), cultures of methane oxidizers in methane sensors (Damgaard and Revsbech 1997), and pure cultures of incomplete denitrifiers in nitrate and nitrite sensors (Larsen et al. 1997, 1996).

Potentiometric microsensors measure electrical potential generated at the tip by charge separation. The oldest potentiometric microsensor is the full glass pH sensor (Hinke 1969). It is versatile (Revsbech 1986), has a low spatial resolution owing to its 100-µm long tip, and has a ca. 30-s response time. The AgS-membrane S^{2-} electrode has been very useful in studies of the sulfur cycle in microbial mats and biofilms (Kühl and Jørgensen 1992; Revsbech 1983), but it must be used with care and in absence of oxygen. The H_2S sensor has no such problems and can be used for the same research. The H_2S sensor is most suitable for environments with low pH (>8), whereas the S^{2-} sensor may still be necessary in environments with high pH. The liquid membrane ion-exchanging (LIX) microsensor technique was developed by cell physiologists for intracellular measurements (mostly of CO_3^{2-}, Mg^{2+}, Li^+, Na^+ and K^+). These sensors can be very small (with a tip diameter of less than 1 µm, the size of a bacterial cell) and are used to measure NH_4^+ (De Beer and van den Heuvel 1988), NO_3^- (De Beer and Sweerts 1989; Jensen 1993), NO_2^- (De Beer et al. 1997), H^+ (Schulthess et al. 1981), CO_2 (De Beer et al. 1997), or CO_3^- (Müller et al. 1998). The NH_4^+, NO_3^-, and NO_2^- sensors are used to study the nitrogen cycle in biofilms and in sediments from freshwater environments (De Beer et al. 1991; Sweerts and De Beer 1989; Jensen 1993; De Beer et al. 1997), and the H^+ and CO_2 sensors are used for studies on photosynthesis and respiration in algal mats.

Ion-selective microsensors have some disadvantages. Often their selectivity is not very high. Because of interference by Na^+ or Cl^- ions, measurements cannot be made in marine environments, with the exception of pH, S^{2-}, and Ca^{2+}. However, their value for studies in freshwater environments is high, and no alternative exists for NH_4^+, NO_2^-, and NO_3^- microsensors. They last only ca. 1 day; however, they are easy to construct. Finally, these sensors have behaved unpredictably in some circumstances, readings drifting radically, for example, upon penetration of the biofilm. Most likely this is caused by dissolution of hydrophobic biofilm compounds in the LIX membrane. The microsensor can be protected from this phenomenon with a hydrophilic protein layer (De Beer et al. 1997).

Micro-optodes are based on the change of optical properties (fluorescence intensity or fluorescence lifetime) of a layer covering an optical microfiber. Microsensors are developed for O_2, pH, and temperature. The presence of the substrate induces quenching of the fluorescence intensity or decrease of the fluorescence lifetime. Klimant et al. (1997) gave a description of the theory and practice of this technique. Advantages of optical sensors are their ease of manufacture, insensitivity to noise, stability of calibration, and mechanical strength. Disadvantages include their size (ca. 20 µm), limited types of sensors available, and cost of the opto-electronics.

Typical experiments measure transient concentration changes at a fixed position and concentration profiles. Transient concentration changes are measured during photosynthesis with the fast light-dark shift (Revsbech 1983) or for the in situ determination of diffusion coefficients (Cronenberg 1994a, b). For these types of experiments, we refer to the literature; the interpretation of profiles will be discussed below.

Interpretation of Profiles

Profiles give information on microbial activity as well as insight into the microenvironments in biofilms. Microprofiles depend on mass transfer and microbial conversions. Consequently, if the transport processes are known, information on the distribution of microbial activity can be derived from the measured profiles.

Because of microbial conversion and mass-transfer resistance effects, substrate and product profiles develop inside biofilms. If the biofilm is impermeable, diffusion is the only transport mechanism inside the matrix. The turbulent bulk liquid is usually well mixed by advective transport (transport by liquid flow). Adjacent to the matrix is a viscous boundary layer in which the mixing and flow velocity gradually decrease as the surface is approached. Consequently, the mode of transport changes gradually from advectional in the bulk liquid to diffusional in the laminar boundary layer. Diffusional transport is driven by the concentration differences as expressed in Fick's law:

$$J = D\frac{dc}{dx} \tag{13.7}$$

where J is the flux ($mol \cdot m^{-2} \cdot s^{-1}$), D is the diffusion coefficient ($m^2 \cdot s^{-1}$), dc is the change in concentration (in mols) over the distance dx (in meters), and dc/dx is the concentration gradient. In steady state, local conversion rates are equal to transport rates. Assuming a constant D, the mass balance becomes for a planar geometry

$$D\frac{d^2c}{dx^2} = r \tag{13.8}$$

where r is the conversion rate. With Eq. 13.8, the concentration profiles can be calculated for zero- and first-order kinetics, assuming a homogeneous activity distribution. In reality, the assumptions are often too simple: conversions are of mixed-order kinetics (Monod) and the distribution of activity is varying with depth. Then only with iterative computer modeling, concentration profiles can be calculated. By fitting calculated with measured profiles, a good estimation can be made of the distribution of microbial activity (Revsbech 1986; Berg 1998). The executable code of the procedure from Berg et al. (1998) is available by e-mailing the author (pb8n@virginia.edu). A more direct approach is to derive the local activities from the profiles. Assuming a measured profile consisting of three measured points (a, b, and c), the flux between a and b and b and c can be calculated from Eq. 13.7:

$$J_{ab} = D_{ab}\frac{(c_a - c_b)}{(x_a - x_b)} \qquad \text{and} \qquad J_{bc} = D_{bc}\frac{(c_b - c_c)}{(x_b - x_c)}$$

This gives the best estimate for the fluxes through the intermediate points between the measurements, $0.5(x_a + x_b)$ and $0.5(x_b + x_c)$. If the system is in steady state, then the difference in fluxes through these points is equal to the local conversion in point b, which is approximated by

$$r_b = \frac{J_{ab} - J_{bc}}{0.5(x_a + x_b) - 0.5(x_b + x_c)} \tag{13.9}$$

If D is constant with depth and we measure with constant depth intervals, δx, then

$$r_b = D\frac{c_a - 2c_b + c_c}{\Delta x^2} \tag{13.10}$$

This approach needs high spatial resolution microprofile measurements. Since noise is magnified, a smoothing procedure is recommended. Alternatively, the profiles can be fitted with a polynome. Then the local fluxes are given by the product of the derivative of that polynomial function and D. The local activities are calculated by the product of the second derivative of the function and D.

When D_{eff} is varying with depth, the local fluxes must be calculated with the local D_{eff}, using Eq. 13.7, and local activities calculated with Eq. 13.9. All these calculations can be conveniently done with a spreadsheet.

An example of such an analysis is given in *Fig. 13.19*. In a biofilm, profiles of O_2, NO_2^-, NO_3^-, and H_2S were measured. From these profiles, the aerobic respiration, nitrate consumption (denitrification), and sulfate reduction rates could be calculated. The analysis shows nicely the stratification of processes, that is, the sequence of used e-acceptors (*Fig. 13.20*).

Although activity profiles are valuable, combining these data with bacterial population distribution allows a more complete analysis of the microbial activities in biofilms. By aligning population and activity distributions, it can be determined which microorganisms are responsible for certain conversions (Ramsing et al. 1993; Santegoeds et al. 1998). The use of molecular techniques, techniques for population analysis (e.g., DGGE), and population distribution (using fluorescent in situ hybridization, FISH) has resulted in discovery of new species and their distribution within biofilms. With microsensor analysis and FISH, the ecological niche and kinetic data could be determined from these up-to-now uncultivable species (Schramm 1998a; Schramm et al. 1998b).

Population Analysis

Cultivation Techniques: Microslicing, Most Probable Number (MPN) Technique, and Plate Counting

The classical microbial approach for population analysis is based on cultivation techniques, such as plate counting and MPN. However, it has become clear that these techniques select for certain organisms and thus do not give quantitative data (Staley and Konopka 1985). Subpopulations of environmental samples cannot be cultivated and thus do not appear in MPN or plate counts (Torsvik et al. 1990; Wayne et al. 1987). Even key players of a community may not be identified (Wagner et al. 1993, 1994; Schramm et al. 1998b). Consequently, cultivation techniques give a strongly biased picture of complex microbial communities in biofilms. Moreover, assessment of microscale distribution of microorganisms is very difficult. Okabe et al. analyzed the distribution of heterotrophic and nitrifying organisms by slicing living biofilms horizontally in 100–250-μm-thick sections and enumerating organisms by MPN and plate counting (Okabe et al. 1996a, b). However, since active zones in biofilms are often in the order of 50–100 μm, even this fine-scale technique is too coarse. To avoid these limitations, techniques were developed for identification and counting without cultivation steps.

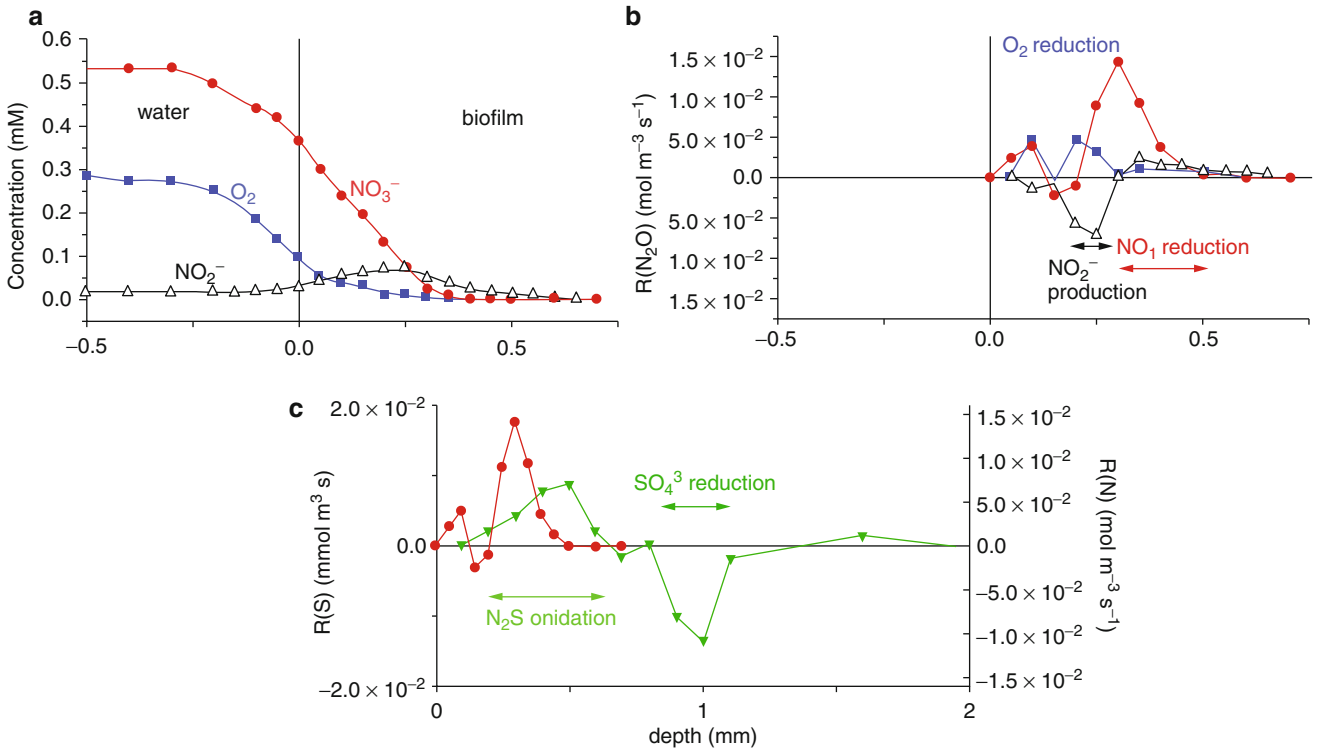

Fig. 13.20

Oxygen, nitrate, and nitrite profiles in a thick biofilm from a wastewater treatment plant (a) and local conversion rates calculated from these profiles (b). In (c) also the total sulfide conversion rates are plotted (sulfide profiles not shown), demonstrating that sulfide production occurs in the deep biofilm (ca. 1-mm depth) and sulfide oxidation overlaps with the denitrifying zone

Molecular Techniques

Species-specific detection of strains is possible with antibodies and analysis of nucleic acids. Both techniques have been used on preparations of dispersed communities (fingerprinting) and on intact biofilms or preparations in which the integrity is maintained (in situ detection). Fingerprinting with antibodies has been described for nitrifiers (Sanden et al. 1994; Both et al. 1992) and methanogens (Kobayashi et al. 1988). More often, in situ analyses (with fluorescently labeled antibodies) are used to detect the spatial distribution of certain microorganisms (Zellner et al. 1995; Kobayashi et al. 1988; Buswell et al. 1998; Coughlin et al. 1997; Sonne Hansen and Ahring 1997; Zellner et al. 1997; Stewart et al. 1997; Morin et al. 1996; Roberts and Keevil 1992; Hunik et al. 1993). Beside technical difficulties, such as nonspecific binding, the preparation of specific antibodies requires pure cultures. Consequently, the antibody technique is not really culture-independent, as only antibodies can be developed for organisms that can be cultivated.

With the nucleic acid approach, population analysis is possible without cultivation. Currently, most efforts are directed to the analysis of ribosomal RNA, recently reviewed by Schramm and Amann (1999). For several reasons, 16S RNA sequence analysis is a powerful tool for the classification of microorganisms (Woese 1987; Maidak et al. 1994). Ribosomes are present in all organisms; thus, this piece of genetic material is universal. Part of the RNA molecules is identical for all microorganisms, while other regions are less well conserved. Thus, sequences can be found that are specific for different taxonomic levels, from species, genera, kingdoms, and domains. Public databases contain 16S RNA sequences from many of the described bacterial species. Microbial cells contain 1,000–30,000 copies of 16S RNA molecules, allowing sensitive assays and identification of single cells by fluorescent oligonucleotide hybridization.

Fingerprinting techniques for populations based on 16S rDNA analysis are ARDRA (amplified ribosomal DNA restriction analysis; Heyndrickx et al. 1996), DGGE (Muyzer and De Waal 1994) or TGGE (temperature gradient gel electrophoresis), and T-RFLP (terminal-restriction fragment length polymorphism; Liu et al. 1997). These techniques all involve isolation of DNA and amplification of the genes or gene fragments encoding for 16S RNA by PCR (polymerase chain reaction).

In the procedure of ARDRA and T-RLFP, first the complete 16S RNA genes are amplified by PCR; this is followed by a digestion with restriction enzymes and size separation of the fragments by gel electrophoresis. ARDRA is used for fast screening of isolates, particularly giving information on similarities. ARDRA is less suitable for community analysis because of the complexity of resulting band patterns. T-RLFP results in community fingerprints which can demonstrate the diversity and dynamics of microbial communities.

However, different species have often similar fragment lengths, leading to an underestimation of the diversity. The more sensitive alternatives are DGGE and TGGE (Schramm and Amann 1999).

For DGGE and TGGE, only fragments are amplified (200–500 bp) and a GC-rich sequence of 40 bp is added at one end. With an increasing gradient of DNA denaturing agents, denaturation of double-stranded to single-stranded DNA fragments will occur at a position in the gel that depends on the composition of the DNA fragment (G + C content, sequence). Upon denaturation, the migration of that gene fragment stops. Consequently, fragments of the same length but with different sequences can be effectively separated, resulting in a band pattern reflecting the microbial diversity of the community. The sensitivity of DGGE is ca. 1 %, meaning that bacterial populations making up 1 % or more of the total community can be detected (Muyzer and Smalla 1998). These methods (DGGE and TGGE) are very useful for detecting population changes of complex microbial communities. Bands in the gel can be further identified by hybridization analysis with specific probes (complementary fluorescently labeled DNA fragments). Also, bands can be retrieved from the gel and sequenced after amplification and cloning. Comparing the sequence with a database allows identification or affiliation of the band within a phylogenetic tree. Although molecular methods may suffer from biases, they are probably less biased than cultivation methods. DNA has to be extracted, and not all cells may lyse. Furthermore, preferential amplification of DNA during the PCR can occur, and consequently, the band intensity in DGGE gels must not be interpreted as a quantitative measure for species abundance (Schramm et al. 1998b). Quantitative analysis is possible with hybridization, either dot-blot hybridization (Stahl et al. 1988) or in situ hybridization (Amann 1995) with labeled oligonucleotide sequences targeting rRNA. After sequencing, it is possible to design a probe (with a fluorescent marker) targeting that sequence and use it for FISH. Then, by a combination of FISH with microscopic analysis and cell counting, quantitative analysis is possible of uncultivated, and even uncultivable, species in environmental samples.

Fluorescent in situ hybridization (FISH) is a recently developed, very powerful tool to quantify populations within a microbial community and to determine the spatial distributions of populations on different taxonomic levels. Instead of extracting nucleic acids, the cells are gently permeabilized so that fluorescently labeled 16S RNA probes, small fragments of up to 20 bp, can enter the cells and hybridize with rRNA. Probes can be labeled with different fluorescent dyes, each with distinct excitation and emission spectra. Thus, two or more probes can be used simultaneously to detect different (up to seven) populations within one sample (Amann et al. 1996). However, the spatial distribution of populations as determined with FISH does not give information on the activity of the cells. The populations detected are there but can be inactive or dead. Conversion rates can be derived from microprofiles determined with microsensors. At the moment, the combination of in situ techniques, that is, molecular and microsensor techniques, gives the most accurate characterization of microbial interactions and activities in complex microbial communities.

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