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Phylogenetic Composition, Spatial Structure, and Dynamics of Lotic Bacterial Biofilms Investigated by Fluorescent *in Situ* Hybridization and Confocal Laser Scanning Microscopy

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Abstract

The phylogenetic composition, three-dimensional structure and dynamics of bacterial communities in river biofilms generated in a rotating annular reactor system were studied by fluorescent *in situ* hybridization (FISH) and confocal laser scanning microscopy (CLSM). Biofilms grew on independently removable polycarbonate slides exposed in the reactor system with natural river water as inoculum and sole nutrient and carbon source. The microbial biofilm community developed from attached single cells and distinct microcolonies via a more confluent structure characterized by various filamentous bacteria to a mature biofilm rich in polymeric material with fewer cells on a per-area basis after 56 days. During the different stages of biofilm development, characteristic microcolonies and cell morphotypes could be identified as typical features of the investigated lotic biofilms. *In situ* analysis using a comprehensive suite of rRNA-targeted probes visualized individual cells within the alpha-, beta-, and gamma-*Proteobacteria* as well as the *Cytophaga–Flavobacterium* group as major parts of the attached community. The relative abundance of these major groups was determined by using digital image analysis to measure specific cell numbers as well as specific cell area after *in situ* probing. Within the lotic biofilm community, 87% of the whole bacterial cell area and 79% of the total cell counts hybridized with a *Bacteria* specific probe. During initial biofilm development, beta-*Proteobacteria* dominated the bacterial population. This was followed by a rapid increase of alpha-*Proteobacteria* and bacteria affiliated to the *Cytophaga–Flavobacterium* group. In mature biofilms, alpha-*Proteobacteria* and *Cytophaga–Flavobacteria* continued to be the prevalent bacterial groups. Beta-*Proteobacteria* constituted the morphologically most diverse group within the biofilm communities, and more narrow phylogenetic staining revealed the importance of distinct phylotypes within the beta1-*Proteobacteria* for the composition of the microbial community. The

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presence of sulfate-reducing bacteria affiliated to the *Desulfovibrionaceae* and *Desulfobacteriaceae* confirmed the range of metabolic potential within the lotic biofilms.

Introduction

In contrast to marine and freshwater lake microbiology, the microbial ecology of river and stream ecosystems is a less intensively investigated field [26] and the ecological impact of planktonic and attached bacteria within these ecosystems is poorly understood. Bacteria that can attach and multiply under the high shear forces normally present in rivers are obviously adapted to live in this environment. This ability together with their contribution to overall metabolic activity or production makes them interesting subjects for studying the microbial ecology of these ecosystems. It is also generally accepted that the activity of attached microbes dominates in small streams, whereas planktonic bacteria are more important in large rivers because of the different ratios of the surface (of sediments or suspended particulate matter) to the volume of the floating water [25]. However, it is not known to date whether there are specific bacteria in rivers that can be distinguished from those organisms that are passively transported downstream. Microbiologists are aware that conventional cultural methods do not provide a representative image of the true composition of microbial communities. Therefore, in microbial ecology, methods for the characterization of microorganisms *in situ* without prior cultivation and for the assessment of their metabolic potential within the natural habitats are preferred. Important steps to address these questions are lipid biomarker analysis [14, 38], *in situ* measurements of metabolic rates [54], microsensor measurements [41], and *in situ* hybridization. Among these, *in situ* hybridization with fluroescent oligonucleotide probes in combination with epifluorescence microscopy has become a widely applied method to analyze microbial communities [5]. First studies dealing with bacterial assemblages of low phylogenetic diversity [4] were followed by an increasing interest in using oligonucleotide probes derived from 16S or 23S rRNA sequences as phylogenetic stains for more complex microbial communities such as those in soil [15], drinking water [17, 18, 29], oligotrophic lakes [1, 53], marine systems [42], and activated sludge [30, 32, 33, 51].

The application of *in situ* probing combined with conventional fluorescence microscopy for the analysis of complex microbial biofilms can be impaired by biofilm thickness, background fluorescence caused by humic substances or detritus, and the inherent autofluorescence of phototrophs. Hybridization assays with fluorescently labeled probes in conjunction with confocal laser scanning microscopy offers the possibility of circumventing these problems [52]. The advantage of CLSM for the study of complex environments is that undisturbed samples can be analyzed without removal or homogenization of biofilm material [22]. Biofilm thickness is not limiting since light from out-of-focus planes is excluded. In addition, digital image analysis can be performed after scanning of the biofilm communities, which to some extent alleviates problems with bleaching and fading of fluorescent dyes.

The aim of this study was to analyze the phylogenetic composition, spatial organization, and population dynamics during the development of river biofilm microbial communities. Rotating annular reactors [8, 36] were used as model systems for growing river biofilms on defined surfaces under controlled shear forces and water exchange rates. The nondestructive sampling of biofilm material was achieved by removable polycarbonate slides inserted into the inner wall of the reactor. Subsequently, biofilm development over time, the relative abundance of defined bacterial groups, and the occurrence of characteristic bacterial morphotypes and microcolonies were analyzed by a combination of *in situ* probing, CLSM, and digital image analysis.

Materials and Methods

Experimental Setup

The rotating annular biofilm reactors (Sinis, Dettingen, Germany) used in this study are described in detail by Neu and Lawrence [36]. They were made of polycarbonate with a stationary outer and a rotating solid inner cylinder with a working volume of 650 ml. For biofilm sampling, 12 removable polycarbonate slides (surface area 3285 mm^2) were fitted into the inner side of the outer cylinder. The reactors were fed with natural river water from the South Saskatchewan River, Saskatchewan, Canada, as inoculum and sole nutrient and carbon source. The inner cylinder rotated at 100 rpm, and the flow rate was set at 12.6 liter h^{-1}. The reservoir volume (40 liter) was replaced 3 times per week (semibatch operational mode) and kept at river temperature of 18°C during operation. Carbon, nitrogen, and phosphorus compounds, pH, oxygen saturation, and turbidity were analyzed weekly [36]. Additionally, water samples from the reservoirs were taken once a week immediately prior to the

Table 1. Oligonucleotide probes, target organisms, and stringencies (FA = percent formamide in hybridization buffer) used for biofilm community analysis

Probe	Target organisms	FA (%)	[NaCI] (mM)	Reference
EUB338	Domain *Bacteria*	20	250	2
non-EUB338	Serves as negative control	20	250	
ALF1b	α-subclass of *Proteobacteria*	20	250	28
BET42a	β-subclass of *Proteobacteria*	35	88	28
beta1	β-*Proteobacteria*	35	88	18
beta2–6, 8a	β-*Proteobacteria*	40	62.4	18
beta7, 8b	β-*Proteobacteria*	50	31.2	18
GAM42a	γ-subclass of *Proteobacteria*	35	88	28
LEG705	*Legionellaceae*	35	88	31
CF319a/b	*Cytophaga–Flavobacteria* cluster	20	250	32
HGC69a	Gram-positive bacteria with high GC content of DNA	35	88	44
SRB385Db	Most members of delta subclass of *Proteobacteria* including *Desulfobacteriaceae*	35	88	40
660	*Desulfobulbus propionicus*	60	15.6	10
DSMA488	*Desulfomonile tiedjei*	60	15.6	33
DSR651	*Desulforhopalus vacuolatus*	35	31.5	33
DSS658	*Desulfosarcina variabilis*	60	15.6	33
DSV698	*Desulfovibrio salexigens*	35	31.5	33
DSV1292	*Desulfovibrio desulfuricans*	35	88	33
ARCH915	Domain *Archaea*	20	250	49
CREN499	Most crenarchaeota	35	88	7
EURY498	Most euryarchaeota	35	88	7

water change. Biofilms grown on polycarbonate slides for hybridizations with group-specific probes and digital image analysis were sampled from May to July 1995, on days 7, 15, 21, 29, 40, 49, and 56 after start of the experiment. Additional biofilms grown under the same experimental conditions subjected to hybridization with species-specific probes were obtained from May to July 1997.

Biofilm fixation was done essentially following the protocol of Manz et al. [29]. Some minor modifications were introduced as colonized slides were used instead of cell suspensions. Parts of the polycarbonate slides covered with biofilm were soaked with formaldehyde solution (3.7% vol/vol) and fixed for at least 1 h at 7°C. Slides were washed once with 1 × PBS (130 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2) for 3 min, dehydrated in an ethanol series (50, 80, and 96%, 3 min each) and dried at room temperature. For hybridization with probe HGC69a, biofilms were ethanol-fixed as described by [44]. Finally, small pieces (6 × 15.3 mm) were cut off from the slides with ethanol-sterilized scissors and fixed onto standard microscope slides with acid-free silicone glue (Dow Corning, Midland, MI).

In Situ *Hybridization*

Oligonucleotide probes, references, and target organisms used in this study are summarized in Table 1. Custom synthesized, oligonucleotides were 5′-labeled with fluorescein isothiocyanate (Boehringer Mannheim, Germany), tetramethylrhodamine (TIB MOLBIOL, Berlin, Germany), or the indocarbocyanine dye Cy3 (Biometra, Göttingen, FRG), respectively. All oligonucleotides were stored in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) at −20°C. Working solutions were adjusted to 50 ng DNA/μl. Prewarmed hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl [pH 7.2], 0.01% SDS, formamide concentration as given in Table 1) was mixed with fluorescently labeled oligonucleotide (1ng μl^{-1} hybridization buffer) and applied to a 6 × 15.3 mm biofilm sample. For hybridizations with probes BET42a, GAM42a, and beta8a, unlabeled competitor oligonucleotides (GAM42a, BET42a, and beta6, respectively) were incorporated into the hybridization mixture to ensure hybridization specificity [28]. The slides were placed in humid chambers and incubated for 90 min at 46°C. After this, hybridization buffer was drawn off with tissue placed at the edges of the slides. Subsequently, slides were transferred to 50 ml prewarmed washing buffer (20 mM Tris/HCl, 0.01% SDS, NaCl concentration as given in Table 1) and incubated at 48°C for 20 min. For microscopic analysis, slides were carefully rinsed with distilled water, air dried, and mounted in antifading glycerol medium (Citifluor AF2, Citifluor Ltd., London, UK). All hybridization and washing steps were performed in the dark. To ensure the overall comparability of the microbial community composition within biofilms obtained in 1995 and 1997, control hybridizations with all group specific oligonucleotides given in Table 1 were performed. Fluorescein isothiocyanate- and Cy3-labeled derivatives of the oligonucleotide non-EUB338, which has a sequence complementary to EUB338, served as a negative control for nonspecific binding.

In order to obtain total cell area values and total cell counts,

fixed biofilm slide pieces were also stained with the fluorescent DNA stain SYTO-15 (Molecular Probes Inc., Eugene, OR) according to the manufacturer's instructions.

Image Collection

An MRC 1000 CLSM (BioRad, Hemel Hempstead, UK), equipped with a krypton–argon laser and mounted on a Microphot SA microscope (Nikon, Tokyo, Japan) was used to obtain images of hybridized biofilms. The biofilms were observed using excitation/emission lines of the krypton argon laser as follows: green (excitation (ex) 488, emission (em) 522/32) for fluorescein isothiocyanate (FITC) conjugated probes, and red (ex 568, em 605/32) for CY3 or tetramethyl rhodamine (TRITC) conjugated probes. Samples were also examined using standard epifluorescence microscopy techniques. Observations were made with 60× and 100×, 1.4 numerical aperture oil immersion lenses (Nikon).

Image collection was carried out by scanning 20 microscope fields each equal in area of 109 μm × 73 μm = 7957 μm^2. Thus, the total area analyzed was equal to 159,140 μm^2 (1.6×10^5 μm^2). Each image was collected using Kalman filtration, 8 running average scans per image, and stored in TIFF format for subsequent analysis. The sampling grid was arranged over the strip to cover both the leading and trailing edge of the strip. However, sampling occurred at random with regard to the *xz* location within the biofilm. Additional images of brightly fluorescent bacteria were produced for documentary purposes during a thorough inspection of the slide piece. For observations on the 3D distribution of hybridized cells, fields containing a maximum of information (cells) were selected.

Image Analyses

Digital image analysis of the CSLM optical thin sections were carried out using the analysis software NIH Image 1.61 (http://rsb.info.nih.gov/nih-image/download.html). Image analysis of hybridized cells in the CSLM images were made with reference to the results of epifluorescence observations of the same material. These observations allowed the identification of positive hybridized cells and autofluorescent regions within the image files after CSLM scanning. This information assisted in establishing the threshold for measurement of hybridized cell area and cell counting. Nonspecific fluorescence of polymeric substances was manually eliminated.

Images were thresholded to define the boundaries of cells creating binary images for measurement. The signal-to-noise ratio was improved through application of a single cycle of dilate and erode functions to eliminate one- and two-pixel noise and nonspecific haze. In addition, upper and lower size cutoffs were applied to eliminate objects too small or large to be bacteria [21]. Image analysis resulted in information on such parameters as biofilm depth, cell area (biomass), and cell number at various depths and proportion of cells hybridizing with any of the nucleotide probes. The bacterial biomass was expressed as the percentage of the total microscopic field area. A detailed description of these procedures is given in Lawrence et al. [23, 24].

Results

Image Analyses

The digital image analysis approach used in this study could be effectively applied to identify specifically hybridized microbial cells and colonies within the intact biofilm matrix material. Figure 1A, B shows the original CLSM images, and the corresponding thresholded images are shown in Figure 1C, D, after discrimination, but prior to application of dilation, erosion, object size selection, and measurement of cell area and cell number. These images also illustrate the problems of balancing the loss of faint objects and blooming resulting in the clumping of what are separate hybridized cells or microcolonies.

Dynamics of Biofilm Development

During the study, microbial biofilms developed from a monolayer formed by single attached bacteria, cell chains, and small microcolonies to a more confluent and ridged structure. Mature biofilms contained massive extracellular polymeric substances (EPS) and detritus and showed a distinct orientation to flow.

Figure 2 shows the total and specific cell numbers (left column) and cell areas (right column) covered by different taxonomic groups of lotic biofilm bacteria versus time after hybridization of biofilm samples with oligonucleotide probes specific for *Bacteria* (A, D), alpha- and beta- (B, E) as well as gamma-*Proteobacteria,* and the *Cytophaga–Flavobacteria* group (C, F). Total cell counts and areas were determined after staining with SYTO-15. Because of the heterogeneity of the intact biofilm architecture, cell area and number of hybridized cells were highly variable between and within biofilm samples and were not statistically equally distributed. In mature biofilms consisting of several cell layers, the metabolic potential of the bacteria, as reflected by the fluorescent signal intensity after hybridization, was mostly localized within the outer shell of the biofilm. On an average, 87% of the whole bacterial cell area and 79% of the total cell counts determined by SYTO-15 staining could be hybridized with the *Bacteria* probe EUB338 and affiliated to the major phylogenetic lineages by use of group- and species-specific probes. The only exceptions were samples obtained from 15-day-old biofilms, where just 16% of the total cell area and 23% of the total cell counts could be hybridized. In general, there was a tight correlation between specific cell counts determined with the domain-specific probe EUB338 and phylogenetically more narrow group-specific probes.

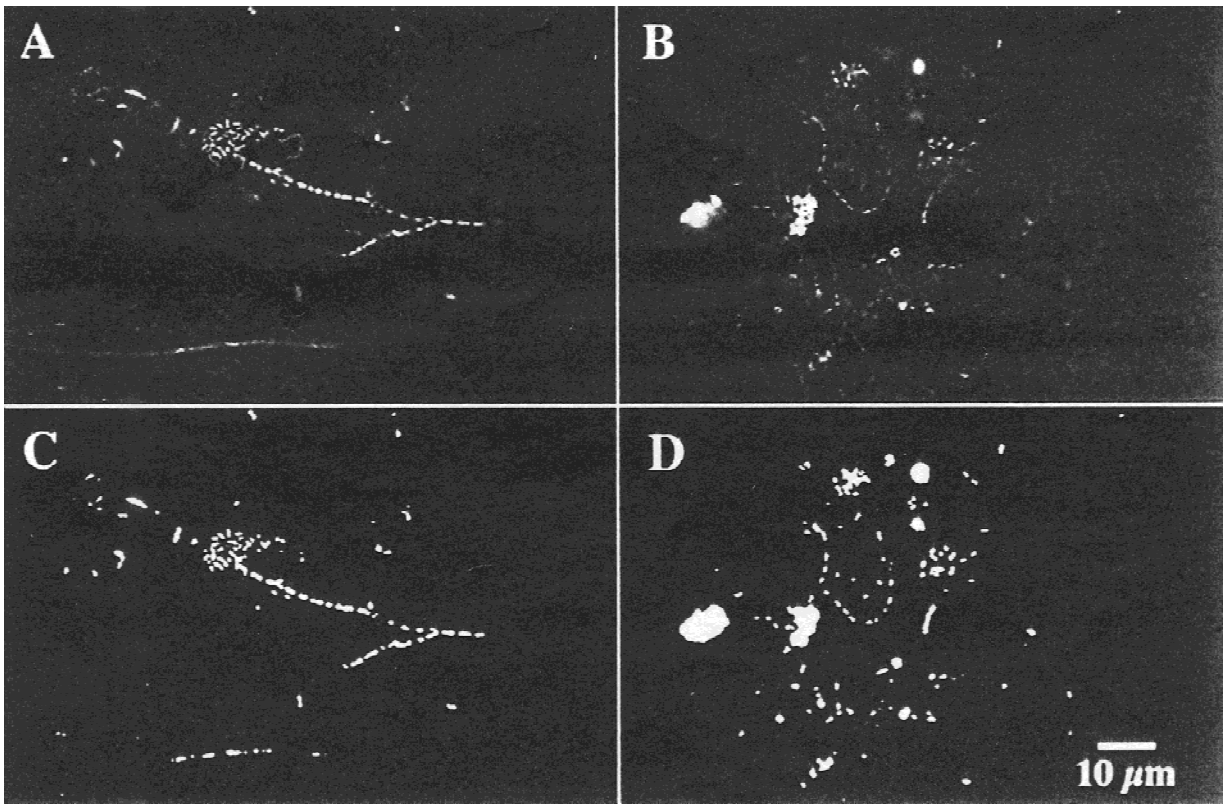

Fig. 1. CSLM optical thin sections illustrating the raw unprocessed image (A, B) of river biofilms hybridized with the probe EUB338, and the corresponding images after the application of thresholding to establish objects for determination of cell number and cell area (C, D). The scale bar given in Fig. 1D corresponds to all images.

In terms of biofilm coverage determined by *in situ* probing, there were different dominant bacterial groups over time. During initial biofilm development, image analysis indicated that beta-*Proteobacteria* accounted for the greatest part of the hybridized bacterial area on day 7 (23%), followed by a rapid increase in the specific area of alpha-*Proteobacteria* on day 15 (135%). On day 21, beta-*Proteobacteria* covered the greatest specific microbial cell area again (65%), whereas on day 29, beta-Proteobacteria abundance declined, and bacteria affiliated to the *Cytophaga–Flavobacterium* group and alpha-*Proteobacteria* showed the highest rates of coverage (55 and 50%, respectively). On days 49 and 56, alpha-*Proteobacteria* strongly increased and continued to be the prevalent bacterial group, corresponding to 111 and 405% of the EUB338-hybridized cell area.

Specific cell counting revealed similar results: In 7- and 21-day-old biofilms, beta-*Proteobacteria* showed the highest specific cell counts with 51 and 59% of the EUB338-hybridized bacteria. For day 15, the predominance of alpha-*Proteobacteria* in the biofilm coverage could be confirmed by cell counting as well (119%). On day 29, alpha-*Proteobacteria* became the relatively most abundant group (61%). On day 49, alpha-*Proteobacteria* (68%) and members of the *Cytophaga–Flavobacterium* group (55%) accounted for similarly high specific cell counts. Alpha-*Proteobacteria* formed the overwhelming bacterial group in 56-day-old biofilms (223%), whereas *Cytophaga–Flavobacteria* remained stable at 52%. Gamma-*Proteobacteria* were never the most abundant bacterial phylum as determined by specific cell area or cell counting.

Phylogenetic and Morphotypical Diversity of Lotic Biofilms

The panels of images are given in Figure 3 show typical morphotypes of lotic biofilm bacteria affiliated to the alpha-, beta-, and gamma-*Proteobacteria* and the *Cytophaga–Flavobacteria* at different stages of biofilm development after *in situ* probing. Probe EUB338 detected a wide variety of bacterial morphotyes, most of which could be characterized by the application of phylogenetically more narrow probes. In terms of morphometry, at an age of 7 days the biofilm community was dominated by microcolonies and cell

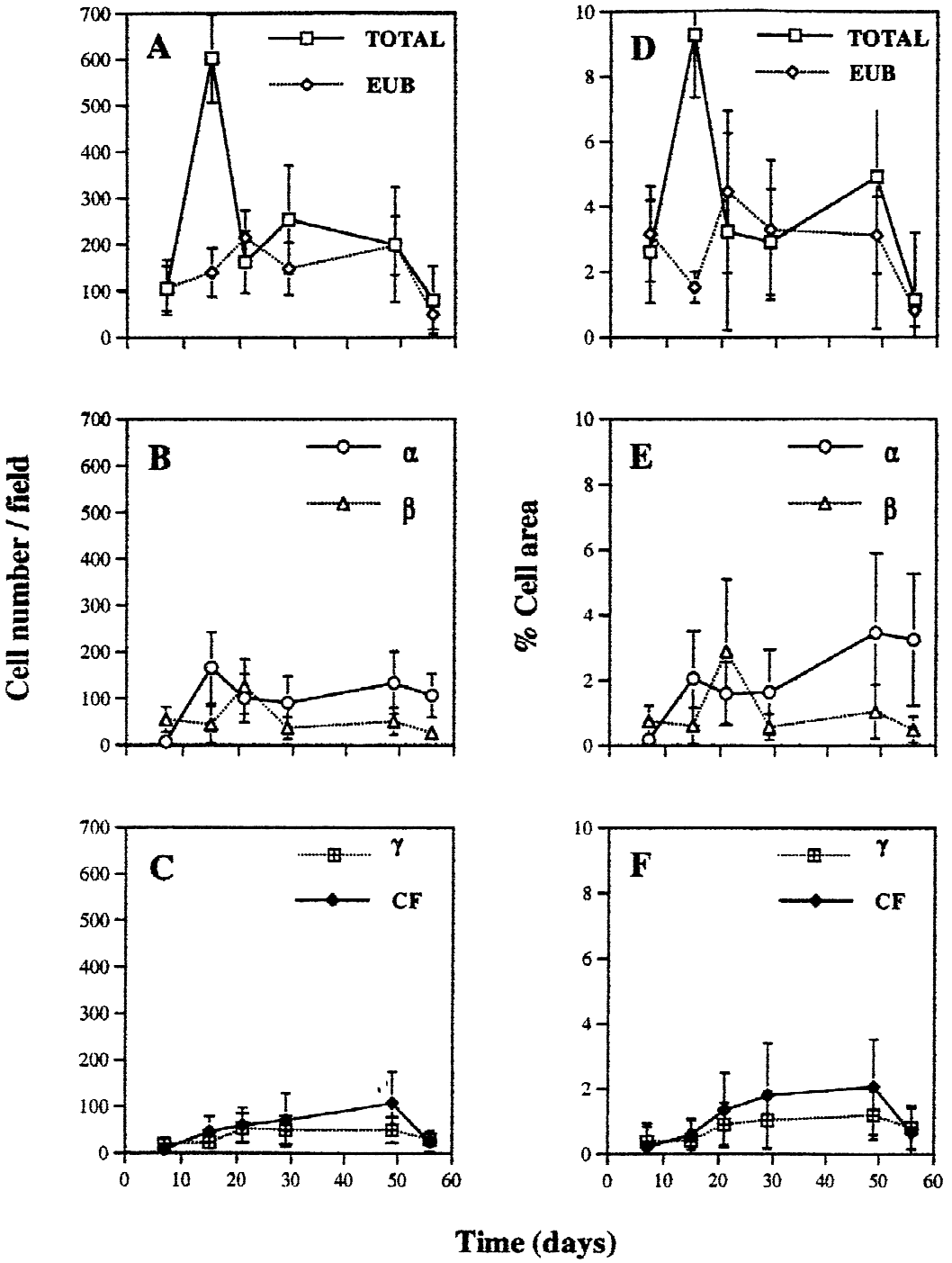

Fig. 2. Total and specific cell counts (left column) and cell areas (right column) covered by different taxonomic groups of lotic biofilm bacteria vs time, resulting from image analysis of CLSM data sets of biofilm samples hybridized with oligonucleotide probes specific for *Bacteria* (A, D), alpha- and beta-(B, E) as well as gamma-*Proteobacteria,* and the *Cytophaga–Flavobacteria* group (C, F). Total cell counts and areas were determined after staining with the fluorescent nucleic acid stain SYTO-15. In each case, $n = 20$ and the error bars are equal to the standard deviation from the mean.

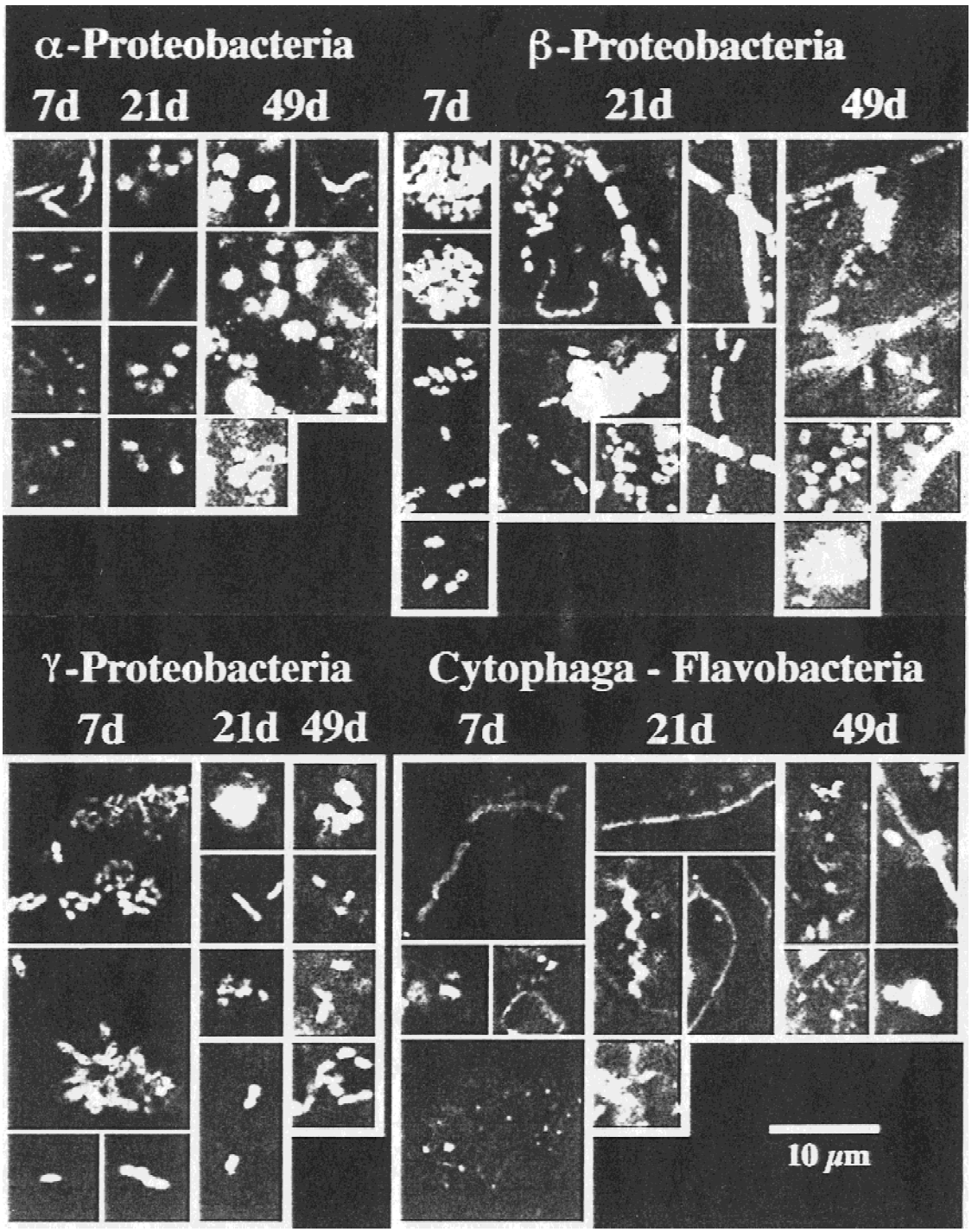

Fig. 3. Sample optical thin sections of lotic biofilms taken at days 7, 21, and 49 showing typical bacterial morphotypes characterized using CSLM in conjunction with fluorescent oligonucleotide probes specific for the alpha-, beta-, and gamma-*Proteobacteria* and the *Cytophaga–Flavobacteria.*

chains. On day 21, 30–50 μm long filaments composed of large (5–8 μm in length) single cells were dominant. Additionally, spirillum-like organisms occurred and large coccoid morphotypes became more frequent. Thin curved rods not hybridizing with any of the group-specific probes could also be microscopically visualized. Biofilm samples obtained on days 29 and 40 showed mixtures of morphologically different bacteria without the appearance of striking new mor-

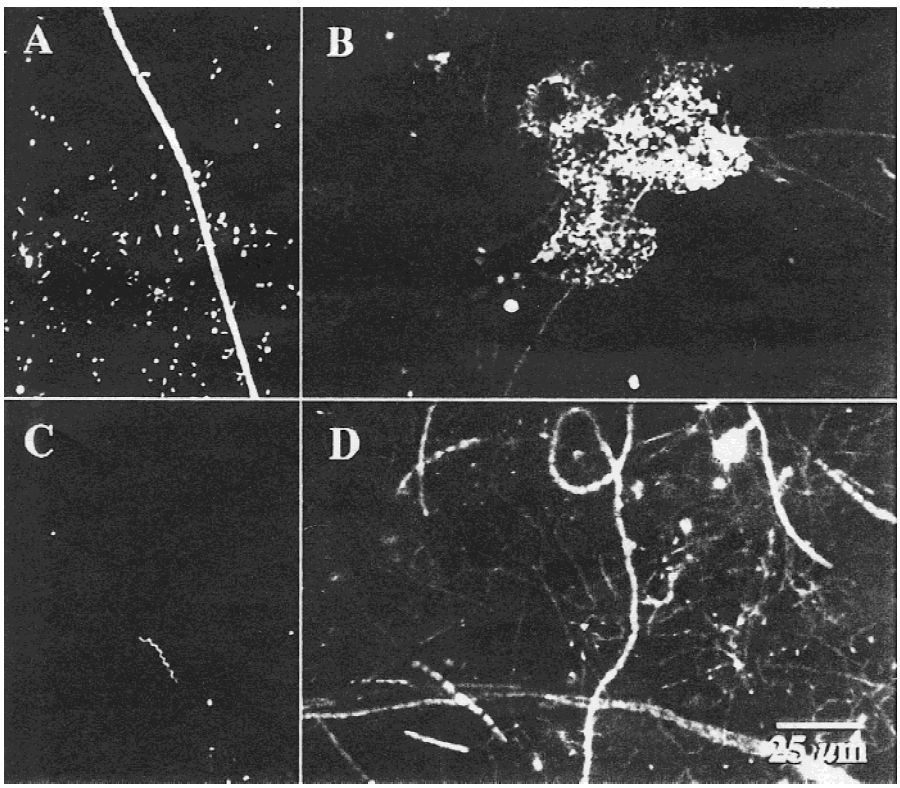

Fig. 4. Selected microscope fields illustrating typical members of the lotic biofilm community after hybridization with probe ALF1b (A), beta8b (B), and CF319a/b (C, D). The scale bar given in Fig. 4D corresponds to all images.

photypes. In 49-day-old biofilm samples, loosely arranged groups of small cocci (0.6 µm maximum size) could also be detected.

Further hybridizations using the group- and species-specific probes revealed some characteristic bacterial morphotyes among the alpha-, beta-, and gamma-*Proteobacteria* and the *Cytophaga–Flavobacterium* group, which are described below.

The predominant members of the alpha-*Proteobacteria* (Fig. 4A) were large (diameter 1.4–1.7 µm) coccoid bacteria growing in groups or, to a minor extent, in cell chains. They were detectable on day 7 and in even higher amounts on all other sampling days. Large rods (0.6 × 2.9–3.4 µm) were also regularly detected with more frequent occurrence in younger biofilms (days 7–21).

Cell chains and filaments were the characteristic appearance of beta-*Proteobacteria,* and most filamentous bacteria observed in the river biofilm belonged to this group. Beta-proteobacterial filaments were detected from day 7 to day 55, but they were less abundant in mature biofilms. Another characteristic morphotype affiliated to the beta-*Proteobacteria* consisted of short oval rods of 1 µm length that possessed polyhydroxybutyrate inclusion bodies. They formed microcolonies of 50 and more cells and could be visualized on days 7, 15, 21, 40, and 49 (Fig. 4B). Specific *in situ* hybridizations using probe beta8b revealed them as members of the beta1-subclass of *Proteobacteria,* putatively related to previously described drinking water bacteria [18]. Another rod shaped beta-*Proteobacteria* could be specifically hybridized using probe beta5.

An additional morphological type observed to hybridize with the BET42a probe formed large, up to 250 µm diameter, colonies with a very specific erect, branching growth habit (Lawrence and Neu, unpublished data).

Among gamma-*Proteobacteria,* long rod-shaped bacteria (0.5 µm in width and up to 3–4 µm in length) occurring singly or in loose small clusters were the characteristic morphotypes for this phylum. Single rod-shaped cells could be identified as members of the family *Legionellaceae* by hybridization with probe LEG705. Coccoid bacteria of similar size (0.9–1.5 µm) as those detected by probe ALF1b were also found. In contrast to these, the cocci detected by GAM42a occurred mostly as single cells or in pairs and to a minor extent in clusters. Smaller coccoid as well as very large (2.3 µm) bacterial cells were seen occasionally. Typical microcolonies of more than 20 closely arranged cells were only found in biofilm samples taken on day 7.

Three bacterial morphotypes were regularly detected within the *Cytophaga–Flavobacteria* group: large Spirillum-like organisms (day 15), shown in Fig. 4C, as well as loose

colonies of rather small cells (days 7, 15, and 56) and thin filaments without visible cell separations (days 7 to 40 and day 56) (Fig. 4D).

In situ hybridization with the group-specific probe SRB385Db and the SRB-specific probes 660, DSV1292, DSV698, DSMA488, DSS658, and DSR651 showed single rod-shaped and vibrioid bacteria affiliated to the families *Desulfobacteriaceae* and *Desulfovibrionaceae* within the lotic biofilm community. The SRB were not restricted to distinct locations within the aerobic biofilms, but they were equally dispersed throughout the whole 200–500 μm thick biofilm structures.

Gram-positive bacteria with high G+C content DNA hybridizing with probe HGC69a formed less than 1% of the attached microbial population.

Members of the domain *Archaea* could not be detected within the river biofilm communities using domain-, group-, or species-specific probes.

Discussion

To our knowledge, the study presented here is the first attempt to monitor bacterial population dynamics in stream biofilms by a combination of FISH, CLSM, and digital image analysis. In addition to an improvement in the descriptive *in situ* investigation of bacteria, CSLM in combination with digital image analysis can also form the basis for quantitative measurements of various parameters. Bloem and co-workers [6] combined CSLM and image analyses and developed a fully automated approach for counting cells in soil smears prepared from homogenized samples. They found that using a sampling frequency with $n = 10$ microscope fields, they could achieve a coefficient of variation of about 20%. Similar results were reported by Viles and Sieracki [50], who enumerated picoplankton in water samples. However, the determination of cell numbers or biomass within undisturbed biofilm matrices presents special concerns. As reported by Korber et al. [19], using a geostatistical approach of representative element analysis, areas exceeding 10^5 μm^2 would be required for statistically valid determinations of cell numbers within intact biofilm materials. In the present study, we used 20 microscope fields covering a total area of 1.6×10^5 μm^2 distributed systematically over the surface of the subsample. This approach was based on the previously observed structural variance and distribution of populations in river biofilms described by Neu and Lawrence [36]. The major problem in quantitative image analyses is the definition of the object pixels of interest before measurements are made. Within intact biofilm materials, the difficulty of distinguishing between specifically stained microbial cells and other fluorescent objects, and the determination of cell numbers despite the presence of microcolonies, clumps, and different cell morphologies are specific problems. The user must determine the accurate boundary of the objects to be measured or counted. In general, this involves the elimination of background through thresholding or defining the gray value of the objects in question [23]. A number of approaches have been used to determine object boundaries in fluorescent or CSLM images [6, 35, 42, 48, 52]. Wilkinson [55] also provides an excellent overview of the various methods used for determination of object boundaries in fluorescent images, including the Marr–Hildreth algorithm and edge strength methods, which may also have advantages for biofilm studies. However, these studies concentrated on the provision of fully automated analyses. We found that our samples required the use of an operator-defined boundary, in combination with erode and dilate treatments to eliminate noise, followed by application of upper and lower size cutoffs to eliminate objects too small or large to be bacteria. This relatively simple approach provided an effective compromise for analysis in the complex biofilm matrix (Fig. 1) and was used to determine both the cell area (biomass) and the cell number in each image. In general, digital image analyses provide more objective quantitative results than visual estimates. However, the variation in automated digital analyses is much higher than that found for visual determinations even in homogenized samples [6]. We also found a high standard deviation in the determination of both cell area and cell number, which is typical for an extremely patchy cell distribution as would be expected in an undisturbed biofilm sample.

As discussed below, comparisons of cell area vs cell number give a somewhat different impression of the relative abundance of the major taxonomic groups during biofilm development. This difference is a function of a comparison of "biomass" or cell area vs abundance as measures of ecological importance. When the factors of numbers, biomass, and activity are effectively brought together, as may be done using rRNA probe techniques in combination with CSLM and digital image analyses, it may be possible to evaluate the ecological significance of specific organisms or groups under *in situ* conditions.

In most studies, cell numbers have been used to compare the relative abundance of different taxonomic groups. This approach is limited by the number of microscopic fields that

can be counted manually, the time needed for this procedure, and the thickness of the sample, because cells have to be clearly visible by epifluorescence microscopy. The aim of the study presented here was to investigate thick complex biofilms applying CLSM and digital image analysis. Area estimates generated by confocal techniques are of superior accuracy compared to other image analysis methods, and fewer than 20 images are often used to calculate cell numbers or biovolumes [42]. Nevertheless, the number of cells included in a calculation is also critical, and sometimes few cells were present on a single image. However, quantitative cell counting and measurement of biofilm coverage and biovolume of specific populations within the biofilm community is difficult for several reasons. First, the strength of fluorescence intensities emitted after *in situ* hybridization is dependent on the rRNA region targeted by the oligonucleotides. Because of this, the threshold setting, which is a necessary prerequisite for digital image analysis, has to be a compromise between the individual signal strength intensity of all probes used in the experiment. Second, the normalization of specific cell counts over a total staining method such as DAPI, SYTO, or hybridization with domain specific oligonucleotides (e.g., EUB338) often leads to an underestimation of specific cell counts, independent of the actual counting method. In this study, the sum of specific cell counts increased to 150% of total cell counts determined by SYTO staining, and to an average of 200% of cells hybridizing with the bacterial probe EUB338. In terms of cell area, the biofilm coverage as determined by hybridization with the probe EUB338 and by SYTO staining compared to specific cell area measurements was underestimated by a factor 2.5 and 1.8, respectively. Furthermore, the shrinkage of the natural biofilm architecture due to the ethanol treatment during the hybridization procedure might cause an overlay of different cell layers, which could be difficult to differentiate by applying general stains or domain-specific probes. In the case of hybridizations with group- or species-specific probes, however, single bacterial cells can be visualized even if they are surrounded by large quantities of other bacteria [30]. Methods for the stabilization of the natural, three-dimensional structure of biofilms, e.g., embedding techniques using plastic matrices and subsequent cutting prior to hybridization, are currently under development. Nevertheless, the ratio of specific populations within the lotic biofilms could be successfully worked out in this study by a combination of *in situ* probing and digital image analysis.

The early colonization of the polycarbonate slides used as substratum by the river bacteria was characterized by an remarkable increase of cell numbers and cell area, mostly caused by the prevailing alpha-*Proteobacteria* with a distinct peak around day 15. A similar increase in cell numbers during the initial phase of biofilm development was shown for drinking water biofilms by Kalmbach et al. [17]. Beta-*Proteobacteria* have been reported to be dominant in freshwater systems such as oligotrophic lakes, lake snow, and drinking water biofilms [1, 17, 29, 53]. In our study, beta-*Proteobacteria* were important parts of the attached community during days 7 and 21. During the course of the experiment, members of the *Cytophaga–Flavobacterium* group increased in terms of cell area and cell counts; on day 29, they formed the greatest specific cell area of the four major groups investigated. On days 49 and 56, alpha-*Proteobacteria* again clearly dominated the attached microbial community. With regard to relative abundance and biomass, gamma-*Proteobacteria* were of secondary importance for the river biofilm community. Indeed, on day 56 of the experiment, the cell area covered by large, rod-shaped gamma-*Proteobacteria* was the second largest among the four major bacterial subpopulations investigated.

Recently, sulfate-reducing bacteria (SRB) were shown to be important members of the microbial communities in habitats not restricted to anaerobic microniches, but often located near oxic–anoxic interfaces [16, 45]. The river biofilms investigated in this study provide by their spatial heterogeneity, presenting valleys and ridges [36], an enlarged area of contact zones between aerobic and more reduced conditions. Thereby, individual SRB affiliated to the *Desulfovibrionaceae* and *Desulfobacteriaceae* were visualized to be equally distributed in the biofilms, reflecting the broad range of metabolic potential within the lotic biofilms. Further studies, including microelectrode measurements of local O_2 and H_2S concentrations [41], could give additional evidence for the ecological impact of SRB in river biofilms.

Several studies showed that freshwater ecosystems are often dominated by gram-negative bacteria [1, 17, 29, 53], whereas gram-positives could play an important role in technical systems, e.g., sewage treatment plants [47]. Applying probe HGC69a, targeted against gram-positive bacteria with high G+C content of DNA, less than 1% of the total biofilm bacteria community showed clear hybridization signals. However, not all gram-positive bacterial cell walls are permeabilized by ethanol treatment; some might require an additional treatment with cell-wall degrading enzymes, e.g., lysozyme. These treatments, however, have to be adjusted

for different types of bacteria, which is difficult to perform for the *in situ* analysis of natural microbial communities [46].

In contrast to marine systems, in which *Eury-* and *Crenarchaeota* have been shown to be present in significant amounts [9, 13], for lotic freshwater systems the contribution of *Archaea* could not be shown *in situ* at present. For aggregated biofilm communities, an enrichment culture under methanogenic conditions prior to hybridization recently elucidated the presence of viable archaebacterial cells affiliated to the genus *Methanosarcina* in the river Elbe, which were putatively in an arrested metabolic state (Böckelmann and Manz, unpublished data).

As previously reported for wastewater biofilms [37], the lotic biofilms examined in this study showed a vertical stratification, where the metabolically active cells were mostly concentrated in the upper biofilm layers distal to the substratum. Neu and Lawrence showed [36] that about 75% of total cell counts were putatively living bacteria, which corresponds well with the 66 to 70% of cells emitting a strong fluorescent signal after hybridization in our study. An average detection ratio of 70% of total cell counts is generally considered satisfactory for community analysis with probes of narrow specificity.

A succession of bacterial morphotypes during biofilm development in rotating annular reactors could be demonstrated. Corresponding structural and biochemical dynamics of biofilm development have been analyzed in detail by a combination of nucleic acid stain and a set of fluorescently labeled lectins [36]. In the present study, the taxonomic diversity of the bacterial community could be shown by FISH using rRNA targeted probes of different phylogenetic levels in a top-to-bottom approach. Additionally, cell morphologies, taxonomic relationships of attached autochthonous river water bacteria, and characteristic growth patterns (chains, filaments, loose or compact colonies) that developed under close to natural conditions with respect to flow regime and water chemistry could be visualized. Distinct growth patterns on surfaces are also reported for well-known culturable bacteria as a species- or strain-specific feature [11, 20] and may thus be helpful for distinguishing different organisms without culture on laboratory media. A variety of distinct river biofilm bacteria could be observed, including large coccoid bacteria (alpha-*Proteobacteria*), typically structured colony-forming rods affiliated to the beta1-*Proteobacteria,* thin filamentous bacteria, and large Spirillum-like organisms affiliated to the *Cytophaga–Flavobacterium* group. Most of them inhabited the biofilm not only transiently, but throughout all phases of the experiment. Further CSLM studies with other lotic biofilms will show if they are ubiquitous, typical surface colonizers in lotic freshwater environments. For example, the Spirillum-like organisms described in this study were previously detected in suspended particulate matter of the river Elbe, Germany, within Elbe river biofilms grown on polycarbonate slides as well as in activated sludge flocs of different sewage treatment plants. Some of the globular microcolony forming beta-*Proteobacteria,* which could be hybridized with the species-specific probes beta8a and beta8b [18], seem to be ubiquitous freshwater bacteria as well.

In conclusion, the rotating annular biofilm reactor in combination with FISH, CLSM, and digital image analysis provides a tool that is well suited for the investigation of model lotic biofilm communities. In the future, questions about the ecological interactions within these important ecosystems, such as interactions between eukaryotes and prokaryotes and their effects on the behavior and growth of attached microbial communities, need to be addressed.

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