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In Situ Identification of Bacteria in Drinking Water and Adjoining Biofilms by Hybridization With 16S and 23S rRNA-Directed Fluorescent Oligonucleotide Probes

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Free-water-phase and surface-associated microorganisms from drinking water were detected and roughly identified by hybridization with fluorescence-labeled oligonucleotide probes complementary to regions of 16S and 23S rRNA characteristic for the domains *Bacteria*, *Archaea*, and *Eucarya* and the beta and gamma subclasses of *Proteobacteria*. Samples of glass-attached biofilms and plankton were taken from a Robbins device installed in a water distribution system. More than 70% of the surface-associated cells and less than 40% of the planktonic cells visualized by 4',6-diamidino-2-phenylindole staining bound detectable amounts of rRNA-targeted probes. These findings are an indication for higher average rRNA content and consequently higher physiological activity of the attached microbial cells compared with the free-living cells. All detectable cells hybridized with the bacterial probe, whereas no *Archaea* and no *Eucarya* cells could be detected. Simultaneous hybridization with probes specific for the beta and gamma subclasses of *Proteobacteria* revealed that microcolonies already consisted of mixed populations in early stages with fewer than 50 cells. These observations provide further evidence that the coexistence and interaction of bacteria in drinking water biofilms may be an integral part of their growth and survival strategies.

The formation and presence of biofilms in drinking water distribution systems have been reported frequently (1, 32, 35). Most reports describe the presence of biofilms and of different morphotypes of microorganisms on the basis of either light microscopic or scanning electron microscopic examinations (10, 24, 25, 27, 29, 32). Conventional culture techniques of scrape samples from surfaces have provided information on the presence of different species of organisms (10, 12, 29). However, only a few percent of the bacteria or other organisms in the biofilm population are culturable on growth media. The application of microscopic staining techniques, e.g., for acid-fast bacilli (36), and genus- or species-directed, labeled antibodies (17, 34) allowed in situ differentiation and enumeration of certain types of bacteria in biofilms. However, the application of these methods is limited by the unavailability of specific staining techniques and antibodies (e.g., for nonculturable bacteria). Additionally, it has been reported that the slime matrices of biofilms may act as penetration barriers for antibodies in thick biofilms (43), and therefore only cells in the upper part of the biofilm are detectable. Other modern techniques applied for the examination of biofilm populations, such as lipid analysis (46) and polymerase chain reaction techniques (20, 31, 41), give mainly qualitative information on the presence of certain groups of bacteria within the biofilm. So far, a clear differentiation between dead, dormant, and active cells in a biofilm is not possible. Staining with acridine orange (26) and formazan formation by actively respiring bacteria (6, 8) only yield incomplete information.

Recently fluorescence-labeled, rRNA-targeted oligonucleotide probes were introduced as a new tool for the in situ identification of bacteria (3, 11). These probes can be designed to be complementary to phylogenetically more or less conserved regions of the rRNA. They are consequently specific on different taxonomic levels ranging from domains to subspecies (15, 39, 40). The detectability of bacteria by such oligonucleotide probes is dependent on the presence of sufficient ribosomes per cell. One advantage of this method is therefore the ability to differentiate bacteria on various phylogenetic levels and, at the same time, to achieve at least qualitative information on the physiological state of the bacteria on the basis of the number of ribosomes per cell (11). Another advantage is that specific rRNA probes can also be obtained for hitherto uncultured microorganisms (4, 38). The applicability of these probes to eutrophic plankton (16) and to biofilm studies has been demonstrated (5). One disadvantage of using fluorescence-monolabeled rRNA-targeted oligonucleotide probes is their limited sensitivity (47). Consequently, with regard to free-living and surface-attached microorganisms in oligotrophic aquatic environments, it was unknown whether the cells would have enough ribosomes to be detected. Yet, because the technique has such potential for the microbiological study of drinking water distribution systems, its applicability was tested.

MATERIALS AND METHODS

Sampling. A flow unit containing a Robbins device (28) was installed in the drinking water distribution system of Norrvatten, which distributes drinking water to the northern part of Stockholm, Sweden, at a distance of 30 km (~60 h distribution time) from the waterworks. The Robbins device consisted of a stainless steel cylinder (200 by 500 mm), which had 40 holes, each threaded and having a diameter of 30 mm (Fig. 1). Stainless steel screws (40 to 60 mm long) with glass

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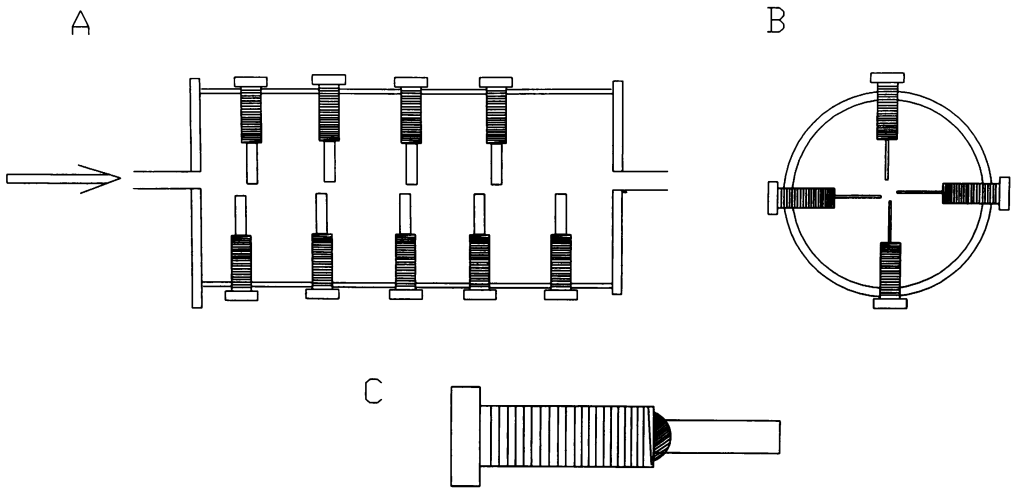

FIG. 1. Schematic diagram of the Robbins device as installed in the water distribution system of Norrvatten, Stockholm. (A) Longitudinal section of the device showing the inflow and outflow of water and the positioning of the screws with mounted glass slides. (B) Cross section demonstrating the arrangement of screws in the Robbins device. (C) Single screw with mounted glass slide.

slides mounted on the front side with silicon rubber were placed in these holes. Thereby, multiple slides could be exposed to flowing water and could be removed independently after various times.

Four-liter samples of drinking water were collected from the outlet of the Robbins device and were centrifuged (5,000 × *g*, 20 min) in 300-ml portions. The cells were resuspended in 10 ml of filter-sterilized (0.2-μm pore diameter) drinking water and were pooled in a glass tube. The pooled suspension was centrifuged (5,000 × *g*, 10 min) again, and the cells were resuspended in 1 ml of filter-sterilized drinking water. This suspension was used for direct microscopic examination of the morphotypes, for staining with the DNA-intercalating stain 4′,6-diamidino-2-phenylindole (DAPI; Sigma, Deisenhofen, Germany) and for the hybridization experiments with the oligonucleotide probes.

TABLE 1. Physical, chemical, and microbiological parameters of the drinking water from the Robbins device

Parameter	Means ± SD for all 1992 measurements
Temp (°C)	9 ± 3.2[a]
Turbidity (FNU)[b]	0.2 ± 0.4
TOC[c] (mg/liter)	4.1 ± 0.2
pH	8.3 ± 0.1
Alkalinity (HCO_3; mg/liter)	71 ± 4.4
Total hardness (°dH)	6.3 ± 0.3
Ca (mg/liter)	36 ± 0.9
Fe (mg/liter)	<0.01
Cl^- (mg/liter)	16 ± 0.6
SO_4^{2-} (mg/liter)	53 ± 2.5
Cl_2 (from monochloramine; mg/liter)	0.04 ± 0.02
Heterotrophic bacteria:	
After 2 days (CFU/ml)	3 ± 7
After 7 days (CFU/ml)	90 ± 140
Total coliform bacteria (CFU/100 ml)	<1

[a] Maximum, 16°C; minimum, 4°C.
[b] FNU, formazine nephelometric unit.
[c] TOC, total organic carbon.

Water chemistry and microbiology. The water from the Robbins device was routinely examined for its chemical and microbiological parameters (Table 1). The values are typical for the drinking water of the distribution system in Stockholm.

In situ hybridization. Mounted glass slides from the Robbins device were removed from the screws after different exposure times, and the biofilms on the surfaces were immediately fixed with 4% paraformaldehyde solution, washed once with PBS (130 mM sodium chloride, 10 mM sodium phosphate [pH 7.2]), and dehydrated in 50, 80, and 96% (vol/vol) ethanol (3 min each). A total of 35 slides were examined, and 232 hybridizations were performed. Planktonic cells were fixed and spotted on gelatin-coated slides (3).

The following oligonucleotides were used: (i) Eub 338, complementary to a region of the 16S rRNA conserved in the domain *Bacteria* (2); (ii) Non 338, complementary to Eub 338, serving as a negative control for nonspecific binding; (iii) Bet 42a and Gam 42a, oligonucleotides complementary to selected regions of the 23S rRNA molecules of the beta (Bet 42a) and gamma (Gam 42a) subclasses of *Proteobacteria* (21); (iv) Arch 915, complementary to a region of the 16S rRNA characteristic for the domain *Archaea* (39); and (v) Euk 516, complementary to a region characteristic for the small subunit rRNA of the domain *Eucarya* (2).

Labeling with tetramethylrhodamine-5-isothiocyanate (TRITC; Molecular Probes, Eugene, Oreg.) or 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS; Boehringer Mannheim, Mannheim, Germany) was performed as described previously (2).

For in situ hybridization, aliquots of 10 μl of hybridization solution (39% formamide, 0.9 M NaCl, 0.01% sodium dodecyl sulfate [SDS], 20 mM Tris-HCl [pH 7.2]) were dotted onto the fixed biofilm and were incubated for 1.5 h at 44°C. The probe was removed gently with washing buffer (20 mM Tris-HCl [pH 7.2], 0.01% SDS, 40 mM NaCl, 5 mM EDTA) and was immersed in 50 ml of washing buffer at 44°C for 20 min. The slides were briefly rinsed with distilled water, air dried, and mounted in Citifluor (Citifluor Ltd., London, United Kingdom).

Probe-conferred fluorescence was detected with a Zeiss Axioskop microscope (Zeiss, Oberkochen, Germany), fitted for epifluorescence microscopy with a 50-W high-pressure bulb and Zeiss filter sets no. 09 and 15. For statistical evaluation, 20 fields per sample were enumerated. Color micrographs were done on Fuji P 1600 color reversal film; exposure times were 2 to 8 s for epifluorescence micrographs. Phase-contrast microphotographs of planktonic cells were taken as described by Pfennig and Wagener (30).

DAPI staining. The protocol of Hicks et al. (16) for dual staining of samples with DAPI and fluorescent rRNA probes was modified. Staining was performed for 5 min at an end concentration of 0.1 μg of DAPI per ml after hybridization and washing. DAPI fluorescence was visualized with Zeiss filter set no. 01.

RESULTS

Morphotypes in the free water and at the surface. The cell suspension of concentrated planktonic cells was dominated by rod-shaped and very thin spirilloid cells as detected by direct phase-contrast microscopy. Appendaged and stalked bacteria similar to *Hyphomicrobium* and *Caulobacter* species, often associated with small particles (Fig. 2A and B), as well as microcolonies of rods were observed. Other appendaged bacteria, probably belonging to the *Planctomyces* group, were regularly observed in low numbers (Fig. 2C). Many of these bacteria are culturable only on special media and cannot be detected by standard plating techniques for heterotrophic bacteria.

At the glass surfaces, only rod-shaped bacteria and a few spirilla were observed. Appendaged or stalked bacteria were never seen on the glass surfaces.

Hybridization of planktonic cells from the Robbins device. About 40% (36.8% ± 7.6%) of the DAPI-stained planktonic cells harvested from the water flowing out of the Robbins device by centrifugation hybridized with the bacterial probe. There was no detectable fluorescence conferred by the archaeal or eukaryotic probe. The same morphotypes observed by direct microscopy were present after hybridization.

Hybridization of surface-associated bacteria. Hybridization experiments were carried out with slides which had been exposed from 3 to 8 weeks in the Robbins device. Under phase-contrast microscopy, some microcolonies and very few single bacteria were visible on the glass surfaces.

After hybridization with the TRITC-labeled bacterial probe, the portion of cells giving a clear fluorescent signal was around 70% (67.8% ± 8.2%) of the cells stained by DAPI. Close to 100% of the cells in colonies were detectable by hybridization (Fig. 3A), whereas for isolated cells this percentage was significantly lower (58.2% ± 15.2%). Hybridization experiments with the archaeal or eukaryotic probe did not result in any staining. We have not determined what percentage of cells was not associated with microcolonies.

Incubation of the glass-attached biofilms prior to hybridization in different nutrient broths containing glucose and yeast extract in different concentrations had no influence on the signal strength or on the percentage of detectable cells. Simultaneous hybridization with probes directed against the beta or gamma subclass of *Proteobacteria* revealed that microcolonies consisted of bacteria from both subclasses (Fig. 2B). This was even true for small colonies with less than 50 cells (Fig. 2C).

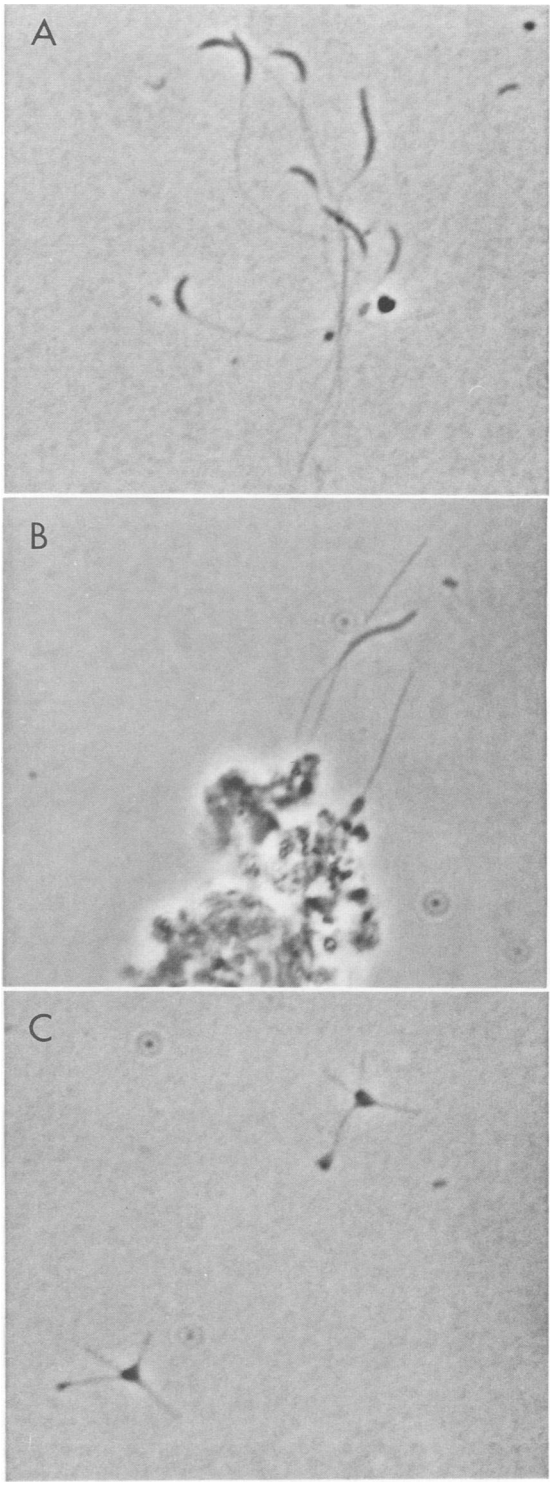

FIG. 2. Phase-contrast micrographs of planktonic bacteria observed in drinking water from the Robbins device. (A) *Caulobacter vibrioides*. (B) *C. vibrioides* and *Hyphomicrobium* sp. associated with a small particle. (C) Bacteria with four appendages and beginning daughter cell formation.

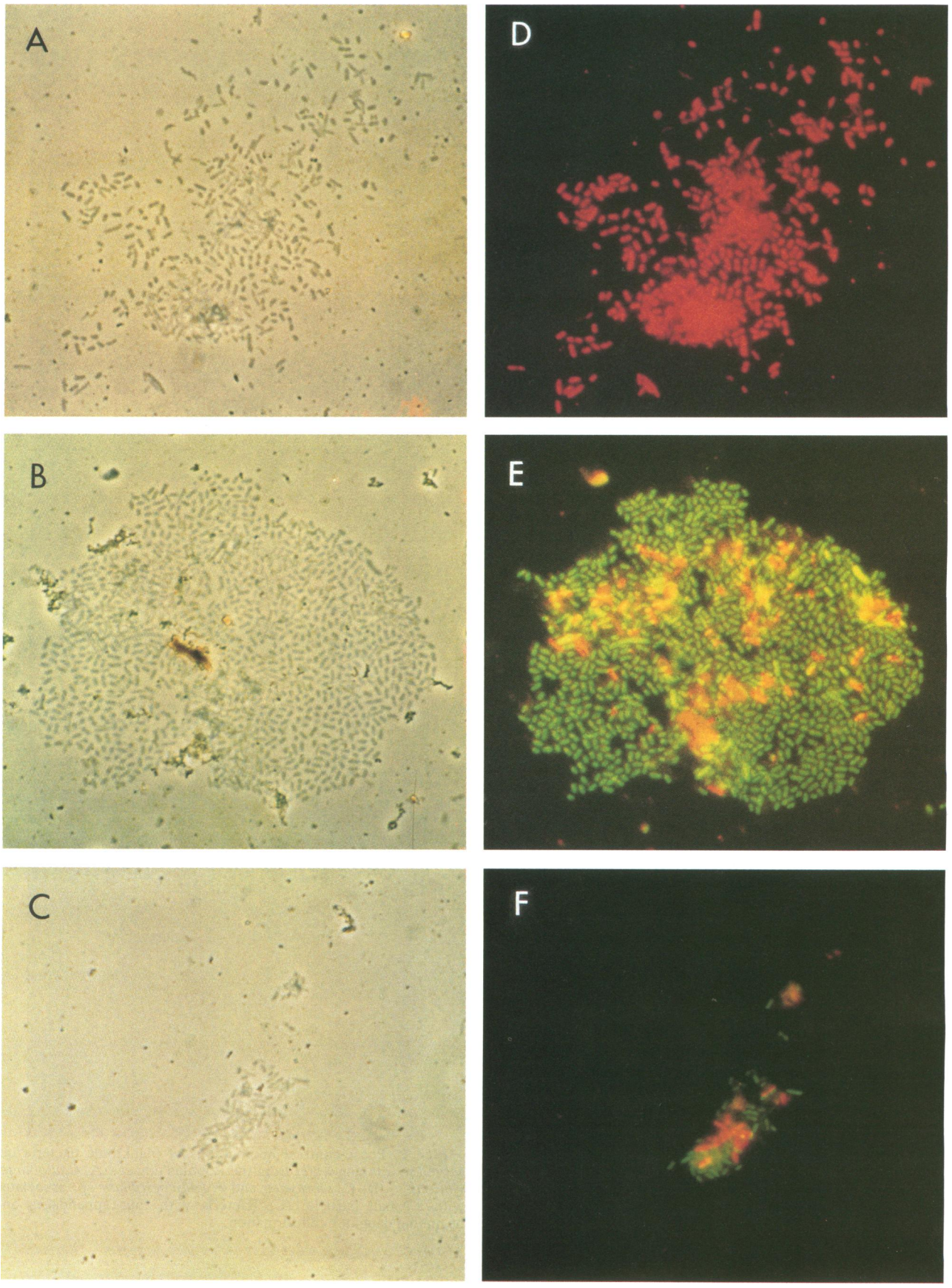
A
D
B
E
C
F

FIG. 3. In situ hybridization of glass-attached biofilms originating from the Robbins device. Phase-contrast (A to C) and epifluorescence (D to F) micrographs are shown for identical microscopic fields. (A) Hybridization of a colony with the TRITC-labeled bacterial probe. (B) Simultaneous hybridization of a colony with the FLUOS-labeled probe specific for the gamma subclass and TRITC-labeled probe specific for the beta subclass of *Proteobacteria*. (C) Simultaneous hybridization of a microcolony with the FLUOS-labeled probe specific for the gamma subclass and TRITC-labeled probe specific for the beta subclass of *Proteobacteria*.

Again, hybridization with the archaeal and eukaryotic probes did not result in the detection of cells.

DISCUSSION

Fluorescence-labeled, rRNA-targeted oligonucleotide probes are not only useful for in situ identification of bacteria but are also useful for a qualitative determination of metabolic activity (11). For oligotrophic environments such as drinking water, it has been postulated repeatedly that bacteria attached to surfaces are more active because of enrichment of nutrients at the surface (13, 18, 45). However, even the opposite, higher activity of planktonic bacteria, has been reported (7, 14). In the present study, we have shown that both planktonic and surface-associated cells could be detected with the probes. Detection is dependent on the rRNA content in the individual cells; with the probes and equipment used in this study, the detection limit is around 10^3 to 10^4 ribosomes per cell.

Interestingly, the percentage of positive cells was significantly higher at surfaces than in the planktonic phase. Within the surface-associated community, nearly all cells in microcolonies and ~60% of the spatially isolated cells were detectable with oligonucleotide probes. Assuming that cells within the two different communities hybridize equally well with the probes and that there is a direct relationship between cellular ribosome content and metabolic activity, our findings indicate that surface-attached cells are more active than free-living cells. However, one should be cautious with general statements on higher activity in biofilms, because it has been demonstrated that the activity of the attached cells is dependent on surface characteristics of the carrier material (13). Glass is a rather selective substratum. This was reflected in this study in the greater variety of morphotypes in the plankton than in the biofilms. The simultaneous hybridization of surface-associated bacteria with probes for the beta and gamma subclasses of *Proteobacteria* clearly demonstrated that microcolonies already consisted of mixed populations in very early stages of development. This observation is of great importance for understanding the mechanisms and dynamics of surface colonization and biofilm development in drinking water.

Currently, information about the complexity and dynamics of mature, steady-state biofilms (19, 33, 44) and the initial steps of adhesion (23) is available. By contrast, much less is known about the processes that occur between first attachment of cells and the fully developed of biofilms (22). Fluorescent rRNA probes have been shown before to be potent tools for the examination of biofilm development (5) and were successfully applied to drinking water in this study. Most studies about biofilm formation were carried out with suspensions of bacteria at relatively high substrate concentrations and therefore consider deposition of cells rather than growth at the surface (9, 37). When the development of microcolonies and subsequent biofilms by an anaerobic bacterium was studied under low-nutrient conditions, a complex life cycle and regulation could be revealed (42). In this study, we could show the formation of multispecies microcolonies in low-nutrient environments, where growth of the cells at the surface is predominant over deposition. Obviously the importance of interspecies interactions, either cooperative, competitive, or both, during surface colonization and biofilm formation has been underestimated.

Multispecies biofilms in oligotrophic environments can be studied with fluorescence-labeled, rRNA-targeted oligonucleotide probes. In the future, they could provide information on the spatial distribution and activity of phylogenetically defined populations and thereby could greatly enhance our understanding of the structure-function correlation in biofilms. More detailed studies are necessary to examine similarities between the surface-associated and planktonic communities. They could help answer the following important questions related to drinking water safety. Is shedding of bacteria from biofilms an important source of drinking water contamination? Do pathogenic bacteria really survive and multiply within biofilms in drinking water systems?

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