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Flow Cytometric Analysis of Activated Sludge with rRNA-Targeted Probes

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Samples from a wastewater treatment plant were hybridized with fluorescein-labeled oligonucleotide probes specific for members of the domains *Bacteria* and *Eucarya*; the alpha, beta, and gamma subclasses of the class *Proteobacteria*; or the genus *Acinetobacter*. Subsequently, they were counterstained with the DNA-specific dye Hoechst 33342 and analyzed by flow cytometry. By quantifying forward angle light scatter and Hoechst- and probe-conferred fluorescence as measures for cell size, DNA content, and rRNA content, respectively, not only relative abundances but also assessments of general metabolic activity for each of these groups were obtained. Hybridizations with a positive control probe binding to all bacteria showed that in the activated-sludge samples examined, 70 to 80% of the Hoechst-stained cells could unambiguously be identified by this method. The majority of the detected cells (approximately 40%) were beta-subclass *Proteobacteria*. Flow cytometric and microscopic counts were in general agreement. Discrepancies were found in particular for those populations that occurred predominantly in flocs (alpha subclass of the *Proteobacteria*) or chains (*Acinetobacter* spp.). Although the dispersal of aggregates needs to be improved, flow cytometry combined with rRNA-based in situ probing appears to be a powerful tool for the rapid and highly automated analysis of the microbial communities in activated sludge.

The treatment of wastewater with activated sludge is one of the most important biotechnological processes. Nevertheless, knowledge of microbial community structure and function and of their correlation with plant performance is still rather limited. One reason for this is that most studies on microbial composition have been based on culture-dependent methods, but even optimized culture media and conditions can detect only 1 to 15% of the bacteria in activated sludge and are selective for certain species (18, 31). Therefore, these conventional methods miss a high percentage of the microorganisms actually present and cause heavily biased shifts in community composition.

Direct detection and identification of individual cells in activated sludge by immunofluorescence (13, 29) or fluorescent in situ hybridization with oligonucleotide probes directed against rRNA (18, 31, 32) reflect microbial community structure more accurately. Compared with antibodies, which require pure cultures of target organisms for their production and are usually species or strain specific, rRNA-targeted probes offer several advantages. Their specificities can be rather freely and deliberately chosen between the subspecies and the kingdom levels (26), depending on the evolutionary variability of the target region. They can even be constructed for as-yet-unculturable organisms by using PCR for retrieval of rRNA sequences (1, 25). In addition, rRNA contents and, consequently, intensities of the hybridization signals are directly correlated with growth rates of bacterial cultures (8, 21, 33). Thus, the application of rRNA-based probes to environmental samples provides information on both the identity and the activity of individual cells in situ.

Flow cytometry (FCM) is a technique for the rapid analysis (more than 10^3 cells per s) and sorting of single cells. Several physical and chemical properties of individual cells can be measured simultaneously on the basis of fluorescence emitted from specifically and stoichiometrically bound dyes as well as light scattering. FCM has a higher throughput and can more readily be automated than microscopic quantification of specifically stained microbial populations and should therefore facilitate a more rapid and frequent monitoring of the compositions and dynamics of microbial communities. It has previously been combined with immunofluorescence for the detection and identification of bacteria in saliva (23), cooling tower water (28), milk (9), or activated sludge (29). This report demonstrates the potential of the combination of FCM and rRNA-targeted probes, which has until now been applied only to cultured cells (2, 16, 33, 34), for the identification and characterization of microbial cells in activated sludge.

MATERIALS AND METHODS

Organisms and culture conditions. *Pseudomonas fluorescens* (DSM 50090^T [DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany]) and *Comamonas testosteroni* (DSM 50244^T) were grown aerobically in YT broth (containing [in grams per liter] tryptone, 10; yeast extract, 5; glucose, 5; and sodium chloride, 5 [pH 7.2]). The cells were harvested during exponential growth, fixed with 3% paraformaldehyde (2), and stored in 50% ethanol in phosphate-buffered saline (PBS) (130 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2).

Activated-sludge samples. Grab samples of mixed liquor were taken from aeration stage 1 of the municipal wastewater treatment plant München II (Gut Marienhof, Germany; 1 million population equivalents) and immediately fixed with paraformaldehyde as described above. Experiments for dispersal of activated sludge flocs were performed with a mechanical blender (IKA-Ultra-Turrax T25; IKA Labortechnik, Staufen, Germany) and a sonifier (Branson Sonifier B-12; Branson, Danbury, Conn.).

Fluorescent-oligonucleotide probes. The oligodeoxynucleotides were 16- to 18-mers complementary to selected regions of 16S or 23S rRNA. They were labeled with a 6-carboxyfluorescein phosphoramidite (Applied Biosystems, Weiterstadt, Germany) at the 5′ end during chemical synthesis and were purified with an oligonucleotide purification cartridge (Applied Biosystems) according to the manufacturer's instructions. Sequences, specificities, and references for the probes are listed in Table 1.

In situ hybridization and DNA staining. The cells were hybridized in a buffer containing 0.9 M NaCl, 0.1% sodium dodecyl sulfate, 20 mM Tris-HCl (pH 7.2),

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TABLE 1. Oligonucleotide probes

Probe	Specificity	Sequence	%FA[a]	Reference
EUB	*Bacteria*	5′-GCTGCCTCCCGTAGGAGT-3′	0	3
EUK	*Eucarya*	5′-ACCAGACTTGCCCTCC-3′	0	3
ALF	Alpha subclass of *Proteobacteria*	5′-CGTTCG(C/T)TCTGAGCCAG-3′	20	17
BET	Beta subclass of *Proteobacteria*	5′-GCCTTCCCACTTCGTTT-3′	35	17
GAM	Gamma subclass of *Proteobacteria*	5′-GCCTTCCCACATCGTTT-3′	35	17
ACA	*Acinetobacter* spp.	5′-ATCCTCTCCCATACTCTA-3′	35	32
NON	Negative control	5′-ACTCCTACGGGAGGCAGC-3′	0	33

[a] Percent formamide (FA) in hybridization buffer.

and 2 ng of oligonucleotide probe per μl at 46°C (33). For probes that require more stringent hybridization conditions, formamide was added to the buffer (Table 1). An equimolar amount of unlabeled competitor probe was added to probes BET and GAM for enhanced single-mismatch discrimination (see Results). After 3 h, the cells were centrifuged, washed, and resuspended in PBS (pH 8.4) containing 1 μM Hoechst 33342 (Sigma, Deisenhofen, Germany) for FCM analysis. Parts of the samples were also filtered on 0.2-μm-pore-size polycarbonate filters (Reichelt, Heidelberg, Germany), mounted in Citifluor (Citifluor Ltd., London, United Kingdom), and examined microscopically in order to assess the effect of hybridization in suspension on floc dispersal. Standard microscopic counts were determined for samples that had been hybridized after immobilization on microscopic slides and counterstained with DAPI (4′,6-diamidino-2-phenylindole) (Sigma), as described previously (18, 31).

FCM. FCM analyses were performed with a FACStar Plus (Becton Dickinson, Mountain View, Calif.) equipped with two argon ion lasers. The first laser was adjusted to the 488-nm line (500 mW) for the measurement of forward angle scatter (forward scatter; 488-nm band pass filter for detection) and right-angle light scatter (side scatter; 488-nm band pass) as well as fluorescence of the fluorescein-labeled probes (green probe fluorescence; 530-nm band pass). The second laser (UV multilines 351.1 to 363.8 nm, 200 mW) was used to excite Hoechst 33342 (blue Hoechst fluorescence; 424-nm band pass). The four parameters were acquired in list mode as pulse height signals (four decades in the logarithmic scale). The system threshold was set in the side-scatter rather than the forward-scatter channel because the side-scatter channel was less noisy, allowing analysis even of the smallest cells in the samples. In order to exclude further noise signals, events lacking Hoechst fluorescence were rejected from data storage (gated acquisition) (Fig. 1A and B).

Data were analyzed by using the DAS software package (Data Analysis System, DAS V4.2 [4]). Gates for enumeration and graphic presentation were set in two-dimensional histograms. The gate "all cells" was defined on the basis of the parameters forward scatter and Hoechst fluorescence, and "probe-positive cells" were defined by their forward scatter and probe-conferred fluorescence (Fig. 1C and D). In Fig. 1, 3, 4, 5, and 6 each dot represents a cell; its position is given by the respective values for the parameters on the *x* and *y* axes measured for this cell, in arbitrary units. Frequencies can be estimated from the density of the dots and the overlayed contour lines (3, 6, 12, 24, 48, and 90% of maximum frequency, outer to inner lines). Cells within the gate "probe positive" are shown in a darker shade of gray.

For the instrument settings used in our measurements, the mean "probe fluorescence" of polychromatic 0.53-μm-diameter beads (Polysciences Inc.; catalog no. 19507) was 85 (linearized value), and the mean "Hoechst fluorescence" of blue 0.35-μm-diameter beads (Polysciences Inc.; catalog no. 18339) was 125.

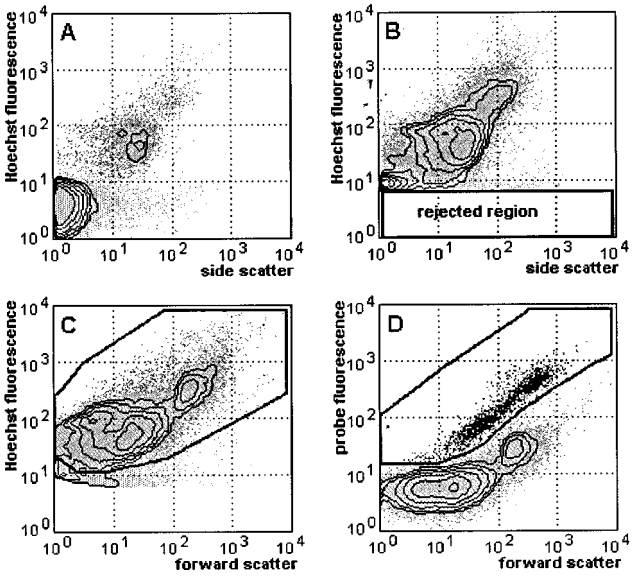

FIG. 1. Ungated (A) and gated (B) data acquisition. Events without Hoechst fluorescence were rejected from storage to a file. Gates "all cells" (C) and "probe-positive cells" (D) were set in two-dimensional histograms.

RESULTS

DNA staining. Fluorescent in situ hybridization was combined with DNA staining in order to discriminate signals from microbial cells from those from noncellular particles and instrument noise. In contrast to findings reported by Monger and Landry (20), cells from activated-sludge samples that were fixed and stored in 0.5 or 3% paraformaldehyde were stained much more efficiently by DAPI than by Hoechst 33342. For samples that were resuspended in 50% ethanol–PBS after paraformaldehyde fixation (which is our routine fixation protocol for in situ hybridization), however, there was essentially no difference in the distributions of DAPI and Hoechst 33342 fluorescence, and they could be used interchangeably as counterstains at concentrations of 1 μM. Both dyes tend to (i) reduce probe-conferred fluorescein fluorescence and (ii) increase nonspecific cell fluorescence in the fluorescein channel at higher concentrations (data not shown).

DAPI and Hoechst 33342 require a second laser capable of UV excitation. Alternative standard nucleic acid dyes might be excited together with fluorescein by the 488-nm line of a single argon ion laser. However, proflavine and acridine orange strongly superimposed, and propidium iodide and ethidium bromide almost completely quenched fluorescein emission by energy transfer and could therefore not be combined with fluorescein-labeled probes (data not shown).

Competitor probes for single-mismatch discrimination. The probes BET and GAM are directed against the same region within the 23S rRNA and differ in only 1 of 17 nucleotides. For dot blot hybridizations or in situ hybridizations of whole cells immobilized on microscopic slides, the addition of 45% formamide to the hybridization buffer creates conditions sufficiently stringent to discriminate this single mismatch (17) because a washing step to remove unbound probe can be performed within a few seconds. During a washing step involving centrifugation after hybridization of whole cells in suspension, however, hybridization conditions could not be kept stringent enough, and strong nonspecific staining occurred.

This problem could be overcome by the application of a competitor probe. Unlabeled (or labeled with a different dye) GAM oligonucleotide was added to fluorescein-labeled probe BET to block binding sites of bacteria belonging to the gamma subclass of the class *Proteobacteria*, and vice versa. The competitor did not have to be added to the cells in advance.

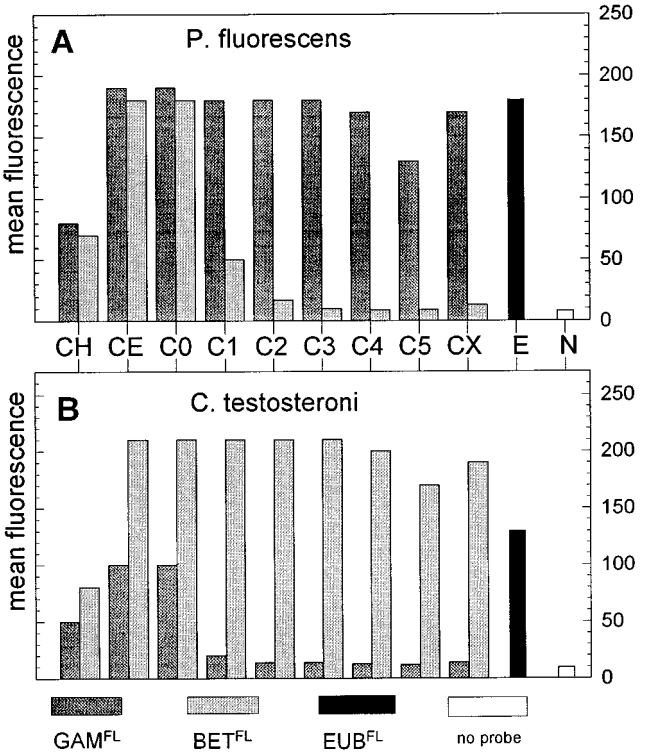

FIG. 2. Effects of competitor probes on specificity and sensitivity, showing the mean fluorescence (arbitrary units; measured by FCM) of *P. fluorescens* (A) (perfect match with GAM) and *C. testosteroni* (B) (perfect match with BET) cells after hybridization with fluorescein-labeled probes, as indicated by the shadings, in combination with the following unlabeled probes (ratio of unlabeled to labeled probe): same probe unlabeled (1:1; homologous competition [CH]); probe EUB unlabeled (25:1; control [CE]); no competitor (C0); specific competitor (unlabeled BET for labeled GAM and vice versa; 1:25 [C1], 1:5 [C2], 1:1 [C3], 5:1 [C4], and 25:1 [C5] with 35% formamide in the hybridization buffer and 1:1 [CX] without formamide). Labeled probe EUB was used as a positive control without competitor (E); no probe was added to determine autofluorescence (N).

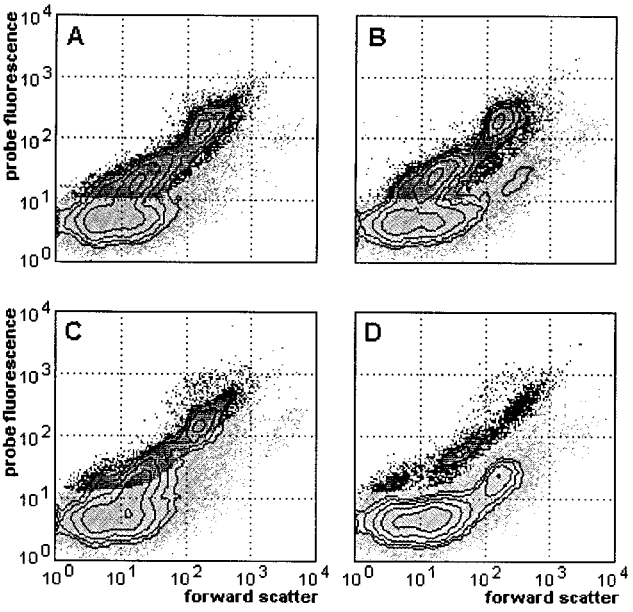

FIG. 3. Use of competitor probes in a complex microbial community. Activated sludge was hybridized with probes BET (A and B) and GAM (C and D) without competitor (A and C) and with unlabeled competitor GAM (B) and BET (D).

Obviously, the probe and competitor bind highly specifically during hybridization and remain at these binding sites as stringency decreases in the washing procedure.

In Fig. 2 the effects of different competitor concentrations on the specificity and sensitivity of the hybridization signal are shown for *P. fluorescens* (a member of the gamma subclass of the *Proteobacteria*) and *C. testosteroni* (a member of the beta subclass of the *Proteobacteria*). Approximately equimolar concentrations of labeled probe and competitor were found to be optimal. A surplus of the competitor decreased the specific signal, and an excess of the probe increased nonspecific staining. With a competitor probe, nonspecific staining was only slightly increased even if the hybridization stringency was largely reduced by using a hybridization buffer without formamide at 46°C. Figure 3 demonstrates the increase of specificity by competition in activated-sludge samples. Without competitor the percentages of cells stained by BET (49%) and GAM (32%) are much higher than they are with a competitor added (BET, 40%; GAM, 11%) because nontarget cells also are stained.

Group-specific relative counts in activated sludge. The relative abundance of a phylogenetic group was calculated as number of cells in the gate "probe-positive cells" divided by the count for the gate "all cells" (Fig. 1). The populations stained by the probes EUB, ALF, and BET were not completely resolved from the rest of the cells by their probe-conferred fluorescence (Fig. 4). Therefore, the respective gates were defined in samples stained with probes EUK (excluding the size range of eukaryotic cells) and NON as negative controls in such a way that at most 0.1% of the cells gave false-positive background counts due to autofluorescence or nonspecific staining. For the other gates background counts were also thus determined as about 0.1% or less and were subtracted from the specific counts. The sensitivity of this method with respect to the relative abundance of a certain phylogenetic group, however, depends on the fluorescence intensity of the cells stained by the respective probe and may be lower than 0.1% for a population with high cellular rRNA content.

The specific relative counts for a sample from aeration stage 1 of München II (obtained on 7 December 1993) as determined by FCM (Fig. 4) and epifluorescence microscopy are given in Table 2. Members of the beta subclass of the *Proteobacteria* (41%) are dominant in this sample. The majority of the members of the gamma subclass (7.7%) belong to the genus *Acinetobacter* (4.7%), and only 1.7% of the bacteria hybridize with the probe specific for the alpha subclass. The cells that are stained by probe EUK (and not by EUB, which acts here as a negative control) are, according to their sizes and DNA contents, presumably yeast cells (0.2%). Most protozoa would be off scale at these instrument settings and are probably too fragile to endure fixation and hybridization. Probe EUB as a positive control for all members of the domain *Bacteria* stains 79% of all cells above the fluorescence background level in this sample and stains about 70 to 80% of the cells in three other samples taken from the same plant at different times.

Reproducibility of FCM counts. In order to assess the reproducibility of the FCM counts, coefficients of variation (CVs) (sigma/mean) for three parallel hybridizations were calculated (Table 2). The variability was low for probes targeting the more-abundant groups. For probes EUB, BET, GAM, and ACA, the CVs were less than 5% for replicate hybridizations and less than 3% for replicate measurements with the same sample. The CV increased for the less-abundant groups

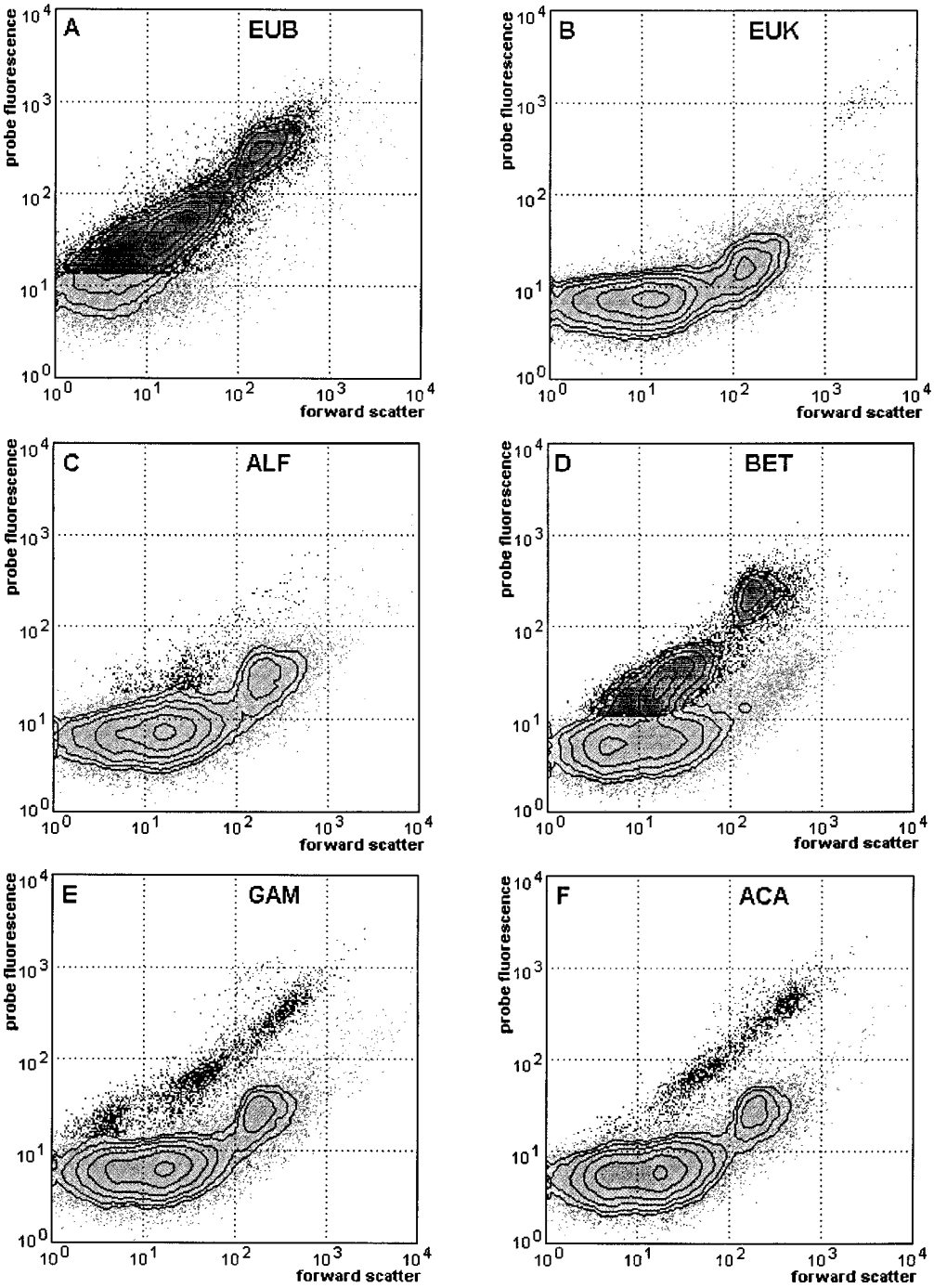

FIG. 4. Distribution of cell size (forward scatter) and probe-conferred fluorescence in activated-sludge samples hybridized with probes specific for members of the *Bacteria* (A); the *Eucarya* (B); alpha (C), beta (D), and gamma subclasses of the *Proteobacteria* (E); or *Acinetobacter* spp. (F).

(probes ALF and EUK) if the same number of total cells (50,000) was measured, but the CV remained below 25% for triplicate hybridizations and below 15% for repeated measurements. In all cases the variation among replicate hybridizations was higher than that among repeated measurements of the same sample, indicating that part of the variability might be due to heterogeneities in the microbial composition of subsamples. The accuracy of enumerations can be increased by replicate hybridizations and, especially for less-abundant groups, by counting higher total numbers of cells. Accuracy is also higher for populations clearly resolved from the rest of the cells, because the definition of count gates is less ambiguous.

Comparison of FCM and microscopic counts. Relative group-specific counts determined by FCM and fluorescence microscopy are in good agreement for probes EUB (79 versus 83%) and BET (41 versus 34%). Part of the differences may be due to statistical errors with fluorescence microscopy counts, which exhibit higher variation than FCM enumerations for

TABLE 2. Group-specific counts for a sample from aeration stage 1 of München II

Probe	Counts (% of total cells) by:			
	FCM[a]		FM[b]	
	Mean	% CV	Mean	% CV
EUK	0.2	24	ND[c]	
EUB	79	4	83	3
ALF	1.7	19	10	18
BET	41	5	34	11
GAM	7.7	4	17	33
ACA	4.7	5	12	47

[a] Results are for three parallel samples; there were 50,000 cells per measurement.
[b] FM, fluorescence microscopy. Results are for six different slides; on each slide at least 3,000 cells in different fields of view were counted.
[c] ND, not determined.

most probes. Nevertheless, discrepancies were highest for the probes that target groups which occurred predominantly in flocs (ALF, 1.7 versus 10%) or chains (ACA, 4.7 versus 12%). Attempts to disperse cell aggregates by blending or sonication were only partially satisfactory. Although microscopic examination showed that flocs became smaller and the fraction of free cells was increased after such treatments, there was essentially no change in the relative counts or in the size distribution according to FCM data. This could be explained by the observation that hybridization in solution by itself results in considerable floc dispersal, which might outweigh the effects of blending and sonication. In samples that were hybridized in suspension and subsequently filtered on membrane filters for microscopic examination, the activated-sludge flocs were significantly smaller than those in samples that had been immobilized on microscopic slides prior to hybridization. Only dense floc structures remained, whereas single cells were released from looser floc structures. When free and attached cells on filters were counted separately, we found that approximately 12% of the cells within the remaining flocs and 2% of the free cells hybridized with probe ALF. The latter value correlates well with the FCM count (1.7%), which considers only the free cell fraction. For the other probes no major differences in the abundances within the free and the attached populations were found. Lower FCM counts for probes ACA, and accordingly also GAM, are probably due to the fact that a larger fraction of the ACA-positive cells were filaments of the Eikelboom type 1863 (11), consisting of about 10 or more individual cells. Each chain was counted as one particle by FCM, whereas all individual cells were enumerated by microscopy. Consequently, the abundance of organisms that occur predominantly within flocs or filaments is underestimated by FCM.

Group-specific cell characterization: size, DNA content, and rRNA content. The multiparametric FCM measurement of individual cells provides information not only for their differentiation but also for their cellular characterization and for assessing their activity. Cell size or biomass (forward scatter), DNA content (Hoechst fluorescence), and ribosome content (probe-conferred fluorescence) are correlated with growth rate and render possible at least an assessment of the general metabolic activities of specific microorganisms. For instance, within the beta subclass of the *Proteobacteria*, two main subpopulations with regard to size, DNA content, and rRNA content are discernible (Fig. 4D, 5D, and 6D). The BET-positive particles with higher DNA and rRNA contents and, accordingly, strong activity correspond to large single or double rods (1.0 by 1.5 to 1.5 by 2.5 μm, as determined by microscopy); the smaller and less active cells are mainly rods of 0.4 by 1.0 to 1.5 μm. The cells which are stained by the probe specific for the genus *Acinetobacter* are both phylogenetically and by their FCM properties (Fig. 4F, 5F, and 6F) a subset of the gamma subclass of the *Proteobacteria* (Fig. 4E, 5E, and 6E). They are short rods of intermediate size (0.5 by 1.2 to 0.8 by 1.5 μm) and chains thereof or large cocci (1 to 3 μm in diameter).

Within the largest and presumably most active cell population (with a forward scatter higher than 100 in Fig. 4 and 5), more than 60% belong to the beta subclass. Probes GAM and ACA stain about 20% each, which means that almost all of the

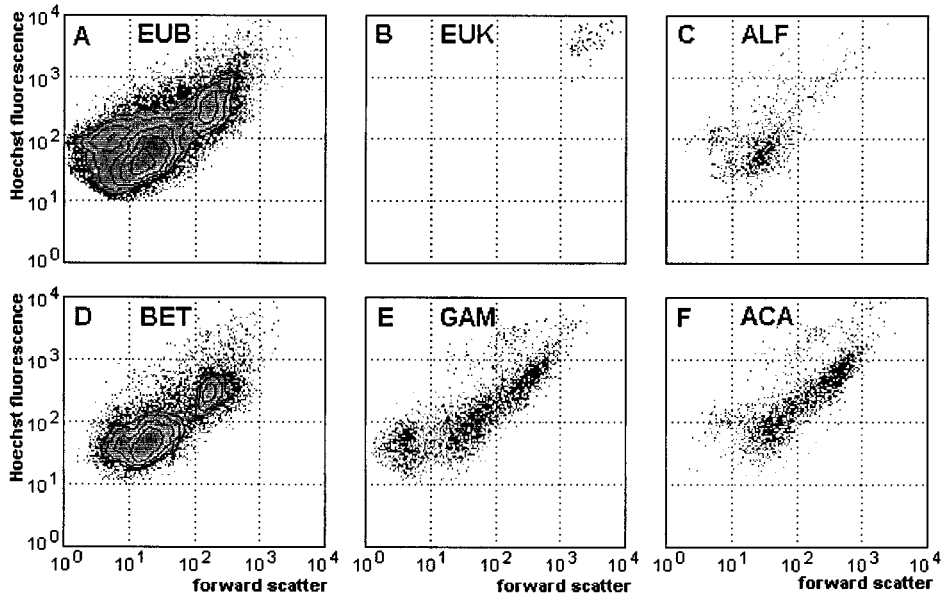

FIG. 5. Distribution of cell size (forward scatter) and DNA content (Hoechst fluorescence) for the same samples as shown in Fig. 4. Only cells within the gate "probe-positive cells" are plotted.

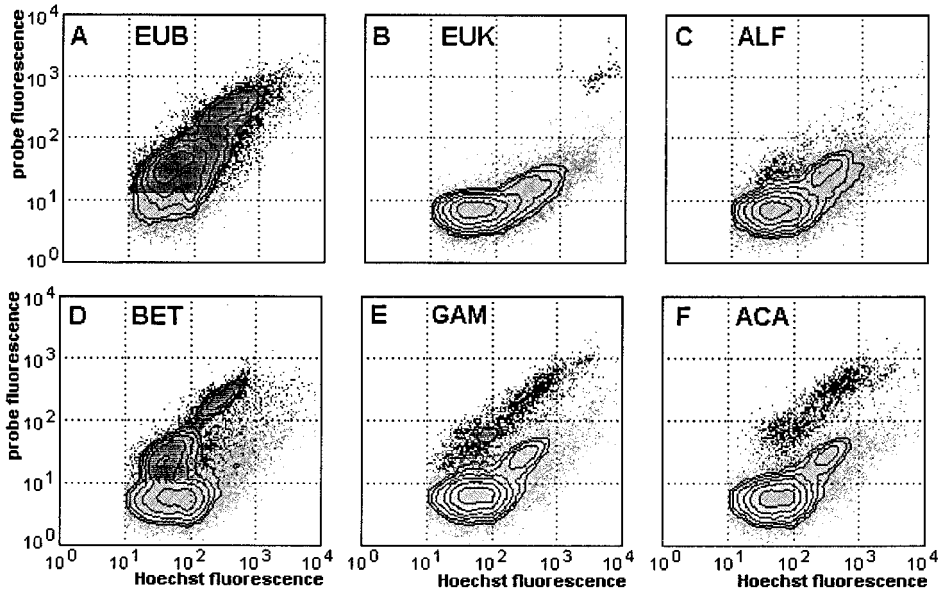

FIG. 6. Distribution of Hoechst and probe fluorescence for the same samples as shown in Fig. 4.

larger cells affiliated with the gamma subclass of the *Proteobacteria* are *Acinetobacter* species.

DISCUSSION

In the present study we could show that the combination of FCM and rRNA-targeted probes, which before had been used only for the analysis of cultured cells (2, 16, 33, 34), can indeed successfully be applied to such complex microbial communities as activated sludge. Because most cells are very active in this rich environment, they have high cellular ribosome contents and give bright hybridization signals. Accordingly, in samples taken at different times from aeration stage 1 of the wastewater treatment plant München II, as many as 70 to 80% of the Hoechst 33342-stained cells showed probe-conferred fluorescence clearly above the background level after hybridization with probe EUB. This means that some 20 to 30% of the cells still cannot be unambiguously identified by this method because their ribosome content is too low or because they are impermeable to probes. However, the percentage of identifiable cells is still much higher than that with culture-dependent methods, which detect only 1 to 15% of the direct total cell counts (18, 31). Additionally, the cells that cannot be identified because of their low ribosome content are presumably not very active and less significant in situ, whereas such cells may be favored by standard culturing conditions and therefore their importance may be overestimated.

By double staining of the samples with the DNA-specific dye Hoechst 33342 and fluorescein-labeled oligonucleotide probes specific for the domains *Bacteria* and *Eucarya*, the alpha, beta, and gamma subclasses of the class *Proteobacteria*, and the genus *Acinetobacter*, we could obtain not only relative counts but also information on the metabolic activity for each of these groups. The dependence of cell size, cellular DNA content, and ribosome content on the growth rate of enterobacteria has long been known (5, 24). In this study, these cellular parameters were estimated by FCM with light scattering at low angles or the fluorescence of DNA-specific dyes and of fluorescent-dye-labeled, rRNA-targeted hybridization probes, respectively. When interpreting the resulting data, the following general aspects have to be taken into consideration.

Determination of size, rRNA and DNA contents, and growth rate. It has previously been demonstrated that the growth rates of bacterial cells in pure culture are well correlated with the intensity of the fluorescence conferred by rRNA-targeted oligonucleotide probes as a measure for the ribosome content of individual cells by microscopy (8, 21) and FCM (33). Linear correlations of light scatter with cell volume as well as of mithramycin-ethidium bromide, Hoechst, or DAPI fluorescence with DNA content in FCM analyses of bacteria from pure cultures have also been shown (6, 22, 27). Such unequivocal relationships, however, should not be taken for granted for complex microbial communities. Some of the cellular properties that modulate these relations by inter- and intraspecies variations are as follows. (i) Probe-conferred fluorescence depends not only on the original rRNA content but also on retention of rRNA during storage and hybridization, accessibility of target sites with respect to cell permeability and ribosome structure, the number and kind of mismatches, and hybridization conditions (26). If different probes are used, their lengths, base compositions, and labeling efficiencies also have to be considered. (ii) DAPI or Hoechst fluorescence is influenced by the genomic percent G+C content (both dyes are AT selective) and, seemingly, other factors (7). (iii) The forward light scatter of cells is determined mainly by their size but also by differences in refractive index, shape, or internal and surface structures (reviewed in reference 12).

Calculation of growth rates from the cellular contents of RNA (which is mainly rRNA) and DNA, or the RNA/DNA ratio (10, 14, 15, 21), may also be less exact for complex communities. Even if single species in the community are analyzed by use of species-specific probes and standard curves for isolates of these species cultured at different growth rates exist, variations in the chemical composition due to differences between strains (19) or to the factors that limit growth (24) are possible.

Although these variations may render exact quantifications of cellular size, DNA content, and rRNA content more diffi-

cult, at least good estimates are possible. The high correlation of forward scatter, Hoechst fluorescence, and probe fluorescence seen in Fig. 4 to 6 is a good indication for the plausibility of these parameters. It can also be generally assumed that the higher the metabolic activity of individual cells and the growth rate of a population are, the larger the cell volume, DNA content, and rRNA content will be. Consequently, high DNA and rRNA contents should at least be regarded as good indicators for viability and considerable activity.

Dispersal of aggregates. One basic problem in the microbiological analysis of activated sludge, by FCM as well as by plating on solid media and (to a lesser extent) by microscopy, is the fact that a major fraction of the microbial cells is attached to aggregates. In FCM individual particles are analyzed; i.e., for free-living bacteria the properties of single cells are measured, and for attached cells the integrated values of the whole aggregate are measured. Therefore, cells within activated-sludge flocs cannot reasonably be identified by FCM, and discrepancies between FCM and microscopic counts were highest for bacteria that were preferentially attached to flocs. Various treatments tested for the dispersal of flocs gave only partially satisfactory results. Hybridization in solution by itself, implying high salt concentrations (35), detergent, and formamide in the buffer at elevated temperature, appears to be quite effective for dispersal, but further attempts for a more efficient release of cells from flocs are necessary. Nevertheless, as complete disaggregation without destruction of a larger fraction of the microbial cells seems unlikely to be achieved, there may remain some bias in specific counts by FCM (but still clearly less bias than with culture-dependent methods) against microorganisms that predominantly occur in flocs or chains.

Competitor probes. The advantage of competitor probes for single-mismatch discrimination with whole-cell hybridizations in suspension was quantitatively shown in this study. They guarantee a high specificity even under low-stringency conditions and facilitate single-mismatch discrimination also in other hybridization formats. This is especially helpful for the simultaneous application of other probes that require less-stringent hybridization conditions.

Conclusions. Classical culture-dependent methods for the microbiological analysis of activated sludge miss a high percentage of the bacteria actually present and cause heavily biased shifts in community composition. The in situ identification of microorganisms with rRNA-targeted probes yields a much more realistic view of the microbial community structure (18, 31, 32). The microscopic evaluation of hybridizations also supplies information on the morphology of cells and, especially by confocal microscopy, on the floc structure and spatial distribution of cells (30). Quantitative microscopy, however, is time and labor intensive and, even in combination with automated image analysis, will not reach the speed of FCM. In contrast, by FCM a highly automated and rapid analysis of the microbial community in activated sludge with rRNA-based probes for routine monitoring becomes possible. Within a few hours (from sampling to data analysis), millions of individual cells can be counted, identified, and characterized by size, rRNA and DNA contents, and, accordingly, general metabolic activity. Supplementary qualitative or semiquantitative microscopic examination can provide further morphological and structural information and allow correction for FCM biases due to cell aggregates.

Comparison of the resulting data with operating conditions and plant performance will increase our knowledge about the correlation of microbial community structure and function in activated sludge. This should yield reliable and predictive indicator populations which could be used to monitor or predict functions such as biological phosphorus removal or nitrification and malfunctions such as sludge bulking by checking their presence and cellular properties.

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