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Dual Staining of Natural Bacterioplankton with 4′,6-Diamidino-2-Phenylindole and Fluorescent Oligonucleotide Probes Targeting Kingdom-Level 16S rRNA Sequences†

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A method for quantifying eubacterial cell densities in dilute communities of small bacterioplankton is presented. Cells in water samples were stained with 4′,6-diamidino-2-phenylindole (DAPI), transferred to gelatin-coated slides, and hybridized with rhodamine-labeled oligonucleotide probes specific for kingdom-level 16S rRNA sequences. Between 48 and 69% of the cells captured on membrane filters were transferred to gelatin-coated slides. The number of DAPI-stained cells that were visualized with eubacterial probes varied from 35 to 67%. Only 2 to 4% of these cells also fluoresced following hybridization with a probe designed to target a eukaryotic 16S rRNA sequence. Between 0.1 and 6% of the bacterioplankton in these samples were autofluorescent and may have been mistaken as cells that hybridized with fluorescent oligonucleotide probes. Dual staining allows precise estimates of the efficiency of transfers of cells to gelatin films and can be used to measure the percentage of the total bacterioplankton that also hybridize with fluorescent oligonucleotide probes, indicating specific phylogenetic groups.

Direct observation of bacterial cells from aquatic environments has become an important technique that has advanced the understanding of microbial populations and communities. However, there are two well-recognized limitations of this approach. Cell concentrations are usually so dilute that they must be concentrated before observation, and noncellular debris can often obscure cells (17). The membrane filter technique can concentrate dilute natural bacterioplankton so that more precise estimates of cell abundance can be made (13). Also, staining nucleic acids in cells by using fluorescent molecules such as acridine orange, 4′,6-diamidino-2-phenylindole (DAPI), and Hoechst 33258 can significantly increase the visibility of bacterioplankton (13, 19, 20). However, some debris in aquatic samples binds these stains. Similarly, some photosynthetic pigments autofluorescesce in the same region as that of acridine orange fluorescence. Nevertheless, the use of the membrane filter technique in combination with these fluorochromes has permitted microbiologists to observe bacterial cells directly and to estimate their density in many habitats.

One drawback of using nucleic acid stains is that both prokaryotic and eukaryotic cells containing intact nucleic acids will fluoresce. Prokaryotic and eukaryotic cells can usually be separated by their cell morphology. Yet, only the abundance of all bacterial cells can usually be determined because most bacteria are morphologically indistinguishable from each other (17). Stains for specific bacterial taxa can be developed by using immunological techniques, but these methods are limited to studies of organisms whose properties are sufficiently characterized to allow their cultivation as pure populations (4, 8, 17, 21). Recent isolates from the habitat being investigated often are required to ensure that the fluorescent antibodies will react with the cells from the natural environment (9). Despite these methodological problems, immunological staining has made it possible to study specific portions of bacterial communities.

In some ecological studies, it is desirable to have a reagent less specific than antibodies that could identify more general assemblages of microorganisms (e.g., class, phylum, kingdom, or domain). Comparing sequences of rRNA molecules has provided a molecular approach that has been widely used to infer phylogenetic relatedness among prokaryotes (11, 18, 28). The 16S-like and 23S-like rRNA molecules have been particularly useful for establishing relationships between microorganisms (27). More recently, these molecules have been used as targets for oligonucleotide probes to assess the environmental abundance and distribution of microorganisms (12, 24). Importantly, the specificity of oligonucleotide probes developed from the comparative sequencing of the rRNAs is not limited to the most specific taxonomic groups (e.g., genus or species); higher phylogenetic groupings (e.g., kingdoms and phyla) can also be determined (12, 24). Fluorescently labeled oligonucleotide probes complementary to 16S rRNA sequences have been used with epifluorescence microscopy to identify single cells of specific phylogenetic groups in cultures and the environment (2, 7, 23, 26). Recently, these probes have also been used to analyze mixed bacterial populations of actively growing cells (1 to 5 μm in diameter) by flow cytometry (1).

The goal of this study was to extend the use of fluorescent oligonucleotide probes to the microscopic characterization of small bacterioplankton cells in dilute aquatic communities. The specific objectives of our work were to develop a dual staining technique using DAPI and fluorescent oligonucleotide probes simultaneously and to evaluate this method for quantification of specific microbial domains in natural bacterioplankton communities. This method could allow microbiologists to directly quantitate the density of bacterial cells at other taxonomic levels within natural aquatic communities.

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MATERIALS AND METHODS

Sources of cultures and bacterioplankton samples. Two species of eubacteria were used to test the dual staining procedure. *Pseudomonas fluorescens* (ATCC 13525) and *Escherichia coli* (Connecticut Valley Biological Supply, Southampton, Mass.) were cultured in tryptic soy broth (Difco) diluted 1:2 with distilled water. Samples were taken from these cultures after 22 and 48 h, respectively, and fixed as described below. An additional sample of the *P. fluorescens* culture was removed after 9 days. Culture samples were immediately fixed as described below. Natural bacterioplankton samples were collected from two sources. Water samples from an experimental aquaculture pond (Illinois Natural History Survey, Champaign, Ill.) were collected in acid-washed medium bottles immediately below the water surface. The second source of natural bacterioplankton was an artificial pond in the University of Minnesota, Duluth, greenhouse. This pond was constructed from a galvanized cattle trough and contained extensive growth of an aquatic macrophyte, *Elodea* sp. Water samples were collected immediately below the surface in sterile 50-ml centrifuge tubes. All bacterioplankton samples were immediately returned to the laboratory and fixed.

Cell fixation and storage. Bacterial samples were fixed with paraformaldehyde (2). A 10-ml portion of culture or water sample was added to 30 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS) (10 mM $NaPO_4$ [pH 7.2], 120 mM NaCl). This fixative was made fresh daily. Samples were fixed overnight in the cold (4°C).

DAPI staining. Three filters were prepared for direct counts of each sample, and another six filters were prepared for the dual staining procedure. Each subsample of fixed cells (5 to 2,000 μl) was diluted to 2 ml with Milli-Q water (0.22-μm-pore-size-filtered water) and stained for 5 min in a filter tower by adding 200 μl of a 38.5-μg/ml solution of DAPI (20). The cells were then concentrated onto black polycarbonate membrane filters (25-mm diameter; Poretics Corp.) with a 0.2-μm pore size by using slight vacuum (<10 cm Hg [ca. 13 kPa]). Each filter was washed twice with 1 ml of 0.1% Nonidet P-40 (nonionic detergent; Sigma Chemical Co.) to completely remove excess stain and the paraformaldehyde fixative. Three filters were mounted on microscope slides in Cargille type A immersion oil, and the cells were counted immediately by epifluorescence microscopy to determine the density of total and autofluorescent cells. The cells on the remaining six filters were transferred to gelatin-coated microscope slides.

Cell transfer to gelatin-coated slides. Glass slides with heavy Teflon coating forming 9-mm-diameter wells were purchased from Cell-Line Associates, Inc. (Newfield, N.J.). These slides were cleaned, coated with a 0.1% gelatin solution, and dried as described by Amann et al. (2). Gelatin from Sigma Chemical Co. (G-2500, 300 bloom) and Difco Bacto-Gelatin (200 bloom) worked equally well. Three microliters of Milli-Q water was added to each well, and the membrane filter containing the DAPI-stained cells was placed cell side down on the gelatin-coated surface of the slide. The filter was air dried in a desiccator and then peeled away from the slide. This usually took less than 5 min. Three slides were prepared for hybridization with the fluorescent eubacterial probes, and the remaining three slides were used with the eukaryotic oligonucleotide probe.

Oligonucleotide probes. The sequences of the three oligonucleotide probes used in this study and their complementary positions in the *E. coli* 16S rRNA sequence are given in Table 1. Two probes, EUB338 (2) and EUB008 (22), target 16S rRNA sequences that are unique to eubacteria. The third probe, EUK1379, targets a sequence unique to eukaryotic cells (22). These probes were designed, synthesized, and purified by the method of Amann et al. (2). Each probe was labeled with tetramethylrhodamine isothiocyanate (Research Organics, Cleveland, Ohio), dissolved in Tris-EDTA buffer (pH 7.2) to a final concentration of 50 ng/μl, and then stored at −20°C until used.

TABLE 1. Sequences of oligonucleotide probes used in this study that are complementary to 16S rRNA sequences

Probe name	Oligonucleotide sequence	Location[a]	Reference
EUB338	5′-GCTGCCTCCCGTAGGAGT-3′	338–355	2
EUB008	5′-TGAGCCAGGATCAAACTCT-3′	008–026	22
EUK1379	5′-TAGAAAGGGCAGGGA-3′	1379–1394	22

[a] Homologous position on *E. coli* 16S rRNA.

Hybridization of DAPI-stained cells. For whole-cell hybridization, 9 μl of hybridization buffer [6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 20 mM Tris HCl (pH 7.0), 0.1% sodium dodecyl sulfate (SDS), 0.01% poly(A)] and 1 μl of a fluorescent oligonucleotide probe solution (50 ng/μl) were added to each hybridization well on Teflon-coated slides. One or both of the eubacterial probes were added to one well on three slides. A second well, which did not contain a probe, was checked for autofluorescent cells. The eukaryotic probe (EUK1379) was substituted for the eubacterial probes in the remaining three slides. Each slide was placed in a hybridization chamber (50-ml disposable centrifuge tube) equilibrated with the wash buffer (0.9 M NaCl, 20 mM Tris HCl [pH 7.2], 0.1% SDS) and incubated at 45°C. Hybridization times varied from 1 to 3 h. The hybridization mixture was then removed by flushing each slide with several milliliters of the wash buffer. The slides were immersed in the wash buffer and incubated at 48°C for 10 min. This was repeated once, and then each slide was gently rinsed with Milli-Q water. The slides were air dried, stored in the dark, and viewed within 3 h because of DAPI fading.

Competition studies. Bacterioplankton from the greenhouse pond were stained with DAPI and transferred to duplicate gelatin-coated slides. The cells were hybridized for 3 to 4 h under hybridization conditions similar to those described above. One well on each slide served as the control and contained the EUB338 fluorescent probe (50 ng) and a 50-fold excess of the unlabeled EUK1379 oligonucleotide (2.5 μg). A second well contained the EUB338 fluorescent probe (50 ng) and a 50-fold excess (2.5 μg) of the EUB338 oligonucleotide that was not conjugated with rhodamine.

Direct counting of bacterial cells. A subset of filters stained with DAPI alone (not hybridized with an oligonucleotide probe) were mounted in immersion oil to quantify bacteria in the sample (20). This was done to estimate the loss of cells resulting from the transfer to gelatin-coated slides and from the subsequent hybridization and washing steps. The gelatin-coated slides were mounted in Milli-Q water and viewed with a 100× Neofluar objective on a Zeiss Standard 16 microscope equipped with epifluorescence optics and a mercury light source. Two excitation and emission filter sets were used. The first filter set (Zeiss G365, FT395, and LP420) excited in the UV region of the spectrum and was used to observe the DAPI stain. The second filter set (Zeiss

TABLE 2. Efficiency of transfer of natural bacterioplankton to membrane filters and the proportion of cells from two freshwater habitats that hybridized with oligonucleotide probes[a]

Sample source[b]	Bacterial density ($10^6 \cdot ml^{-1}$)	% Auto-fluorescent cells	Cell transfer (%)	Probe(s) used	% Bacteria staining as:	
					Eubacteria	Eukaryotes
Illinois pond	2.1 (0.1)	1.8 (2.5)	69	EUB338	67 (14)	
Illinois pond	2.8 (0.3)	6.4 (11.0)	48	EUB338	45 (14)	
			67	EUK1379		2.3 (2.7)
Greenhouse pond	3.2 (0.4)	0.1 (0.1)	65	EUB338 and EUB008	35 (2)	
			59	EUK1379		3.5 (3.0)

[a] Numbers in parentheses are standard deviations.
[b] Three independent slides were prepared and counted for each sample ($n = 3$).

BP546/12, FL580, and LP590) excited the rhodamine-labeled oligonucleotide probes in the green region of the spectrum. At least 10 fields were counted in each well by using both filter sets to determine the number of total cells (DAPI stained) and the number of cells fluorescing in the rhodamine emission region (with or without hybridization). An Olympus BH2 microscope with a UV Fluorite 100× objective and corresponding epifluorescence filter sets worked equally well. Kodak Ektachrome 400 daylight film or Tmax 400 was used to photograph cells. A Zeiss automatic exposure system was used to determine exposure times, which varied from 10 s to more than 1 min, depending on the cells and the fluorochrome being photographed.

RESULTS AND DISCUSSION

Transfer of cells to gelatin films. It is necessary to concentrate natural bacterioplankton prior to examining them by epifluorescence microscopy because of the low cell densities. We chose to concentrate cells on membrane filters to avoid problems encountered when cells are concentrated by centrifugation and then resuspended (e.g., cell disruption or cell clumping). Poretics and Nuclepore membrane filters both provided good transfers to gelatin-coated slides. We examined the use of three different gelatins for transferring cells. Five-percent gelatin films (gelatin powder USP; J. T. Baker Chemical Co.) on microscope slides gave highly fluorescent backgrounds and bound the membrane filter so tightly that it was very difficult to remove the filter without ripping it, even when the slide was dipped in a 1% glycerin solution (25). Slides coated with a 0.1% gelatin film gave better and more consistent transfers. When this technique was used, between 48 and 69% of the bacterioplankton on the membrane filters were transferred and remained on the gelatin-coated slide during the subsequent hybridization and washing steps (Table 2). Our transfer efficiencies were lower than Tabor and Neihof (25) reported for transferring bacterioplankton cells from membrane filters to gelatin films; they reported recovering up to 99% of the bacterial cells. However, Tabor and Neihof (25) stained and washed cells while the filter still adhered to the gelatin film, and later they embedded the cells in a gelatin mist after removing the filter.

Whole-cell hybridization with oligonucleotide probes. Staining young cultured cells (<48 h old) with DAPI and transferring them to gelatin films did not prevent whole-cell hybridization with the fluorescent oligonucleotide probes targeting 16S rRNAs (Fig. 1). All cells that stained with DAPI also hybridized with the eubacterial probes. This technique was used to quantify eubacterial cells in bacterioplankton samples since the transfer efficiency of every slide could be determined.

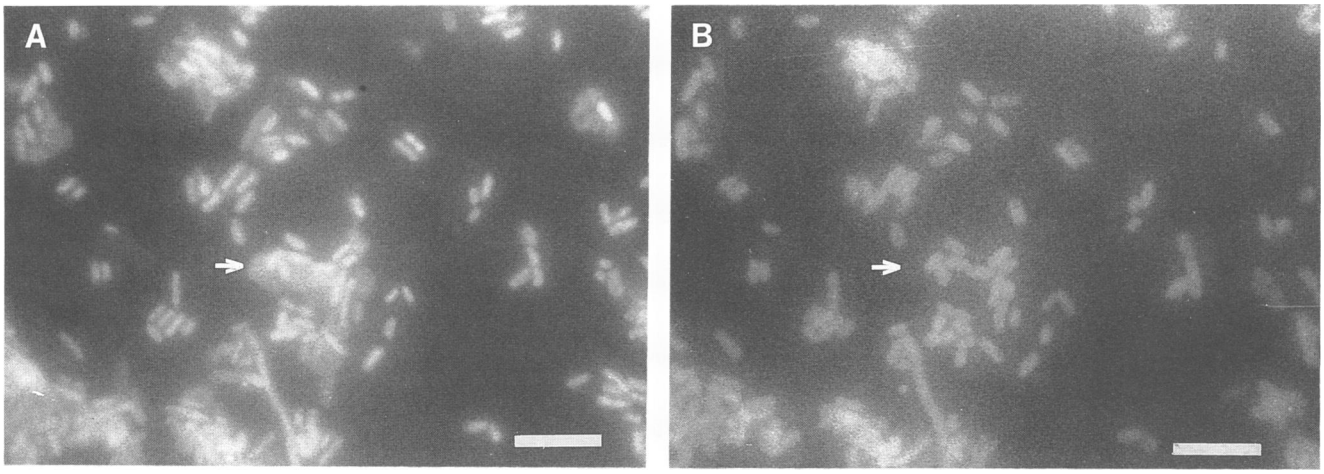

FIG. 1. *E. coli* cells stained with DAPI (A) and then hybridized with two tetramethylrhodamine-labeled oligonucleotide probes (EUB338 and EUB008) that target eubacterial sequences (B). Cells were fixed after 48 h with paraformaldehyde, stained with DAPI, and transferred to gelatin-coated slides. The cells were hybridized at 45°C for 3 h and then subjected to two 10-min washes at 48°C. Milli-Q water was used as the mounting medium. Note that the nonbiomass debris in the culture medium fluoresced with DAPI-stained cells but did not hybridize with the oligonucleotide probes (arrows). Bars = 2 μm.

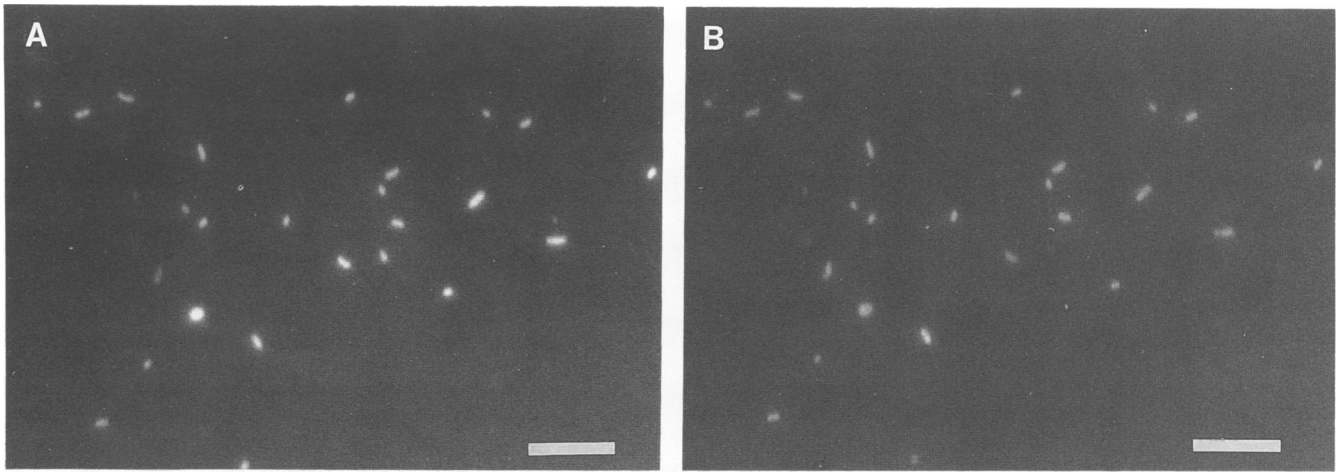

FIG. 2. Photomicrographs of bacterioplankton cells from a greenhouse pond, showing the DAPI fluorescence (A) and rhodamine fluorescence (B) of the two oligonucleotide probes (EUB338 and EUB008). The cells were hybridized at 45°C for 2 h and then subjected to two 10-min washes at 48°C after being stained with DAPI and transferred to gelatin-coated slides. Buffered glycerol (pH 8.0) containing *p*-phenylenediamine was used as the mounting medium. Note that the cell sizes are smaller than those of the cultured cells in Fig. 1. Bars = 2 μm.

We also tried to hybridize unstained cells directly on membrane filters. Few cells were observed on the filters when this was done. This may have occurred for two reasons. First, some cells loosely attached to the membrane filters may have been lost from the filters during the hybridization and washing steps. To determine whether this occurred, we misted some membrane filters with 0.1% gelatin after staining the cells with DAPI but before hybridizing them. This was ineffective because the resolution of the DAPI-stained cells was decreased, and none of the cells appeared to hybridize with the oligonucleotide probes. Second, all of the membrane filters we examined (Poretics, Nuclepore, nylon hybridization membrane, and Anapore) had high levels of background fluorescence that may have obscured cells hybridized with the rhodamine-oligonucleotide probes. The background of the thin gelatin films was much dimmer.

Microscopy. Although the background fluorescence of the gelatin films was adequate for counting, we tried several techniques to reduce it further. Membrane filters were treated with 50 mM sodium borohydride (for 5 min) either before or after bacterial cells were stained in the filtration tower. Other membrane filters were rinsed with 80% ethanol before the cells were transferred to gelatin-coated microscope slides. Some slides containing DAPI-stained bacteria were dehydrated in an aqueous ethanol series (50, 80, and 100%) before hybridization. None of these methods significantly reduced the background fluorescence of the gelatin films.

We routinely used distilled water as the mounting medium. DAPI- and rhodamine-stained cells faded slowly when water was used, but the cells could be counted. Cells on slides mounted in water, examined, dried, and remounted in water 4 days later remained fluorescent enough to be observed. However, water may not be an acceptable mounting medium for other dyes, such as fluorescein. S. Newell has observed that mounting acridine orange-stained cells in an aqueous Irgalan black solution (<1%) can reduce the background fluorescence without masking the cell fluorescence (16a). Mounting hybridized slides in a 2% aqueous Irgalan black solution did not reduce the background fluorescence of fields illuminated with green light, but it did mask most of the DAPI fluorescence. A glycerol mounting medium containing *p*-phenylenediamine reduced the fading of the fluorescent cells but turned brown after a few hours and had to be made fresh daily (15). Mounting slides in immersion oil was not as effective as mounting them in water or the glycerol medium.

Sensitivity and visualization of natural bacterioplankton. Nonspecific binding of the EUB338 probe to other microorganisms was not a problem in the greenhouse pond bacterioplankton assemblage we examined. Nonspecific binding was evaluated by competition studies using hybridization with labeled EUB338 probe in the presence of excess (50×) unlabeled heterologous or homologous oligonucleotides. Seventy-one percent (±14%) of DAPI-stained cells in these samples also hybridized with this probe in the presence of excess unlabeled EUK1379 oligonucleotide, but only 2.1% (±3.0%) of the cells were fluorescent when excess unlabeled EUB338 oligonucleotide was present.

We and others (2, 7, 26) have found that most cultured bacterial cells, yeast spores, and eukaryotic cells examined seem freely permeable to oligonucleotide probes of the size used in this study. However, natural bacterioplankton (Fig. 2) fluoresced more dimly than cultured cells (Fig. 1) when hybridized with the kingdom-specific oligonucleotide probes. This could reflect reduced permeability of fixed bacterioplankton compared with that of fixed cultured cells. Our fixation procedure did not include an additional treatment with alcohol and sodium borohydride, which was used in some studies (1, 26).

The dim fluorescence of the bacterioplankton could also result from low cellular rRNA concentrations. This would limit the number of target sequences per cell for the oligonucleotide probes. DeLong et al. (7) demonstrated that individual *E. coli* cells growing at a rate close to $0.3 \cdot h^{-1}$ had a fluorescence intensity only five times greater than either the background fluorescence or the fluorescence resulting from nonspecific oligonucleotide binding. The growth rates of natural bacterioplankton can be 10 times slower than this (16). If the fluorescence intensity per cell is even lower for bacteria that grow more slowly than $0.3 \cdot h^{-1}$, we would expect the fluorescence intensity of natural bacterioplankton

to be considerably reduced. Consistent with this, we found that the fluorescence of older *P. fluorescens* cells was less intense than that of younger cells hybridized with the same oligonucleotide probes.

Hybridizing natural bacterioplankton from a second greenhouse pond sample with two eubacterial probes simultaneously increased the observed fluorescence intensity of the cells. Previous studies demonstrated that this increase can be additive (e.g., two probes conferred twice the fluorescence intensity [1]). The enhanced fluorescence per cell conferred by two probes was reflected in the reduced variability of our replicate counts compared with that of the other bacterioplankton samples (Table 2).

In addition to their value as determinative tools, fluorescent oligonucleotide probes may become valuable indicators of the metabolic condition of bacterioplankton cells in aquatic ecosystems. Although dual staining with DAPI and fluorescence-labeled antibodies permits total and specific counts of bacteria (14), it does not differentiate active from inactive cells (5, 17). The fluorescent-antibody technique can be combined, however, with autoradiography or the use of redox dyes indicating electron transport activity to count total and viable cells (3, 10). Fluorescent oligonucleotides, like the fluorescent antibodies, cannot differentiate viable from nonviable cells. However, the rRNA content of microorganisms is related to growth rate, and the amount of a fluorescent probe that binds to cells might then serve as an indirect measure of growth rates (7). If the fluorescent signal intensity of natural bacterioplankton cells can be increased further by using multiple probes or indirect labeling with fluorescence-labeled "reporter" groups (6), then it may be possible to estimate the productivity of cells in a specific bacterial taxon with this method.

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