APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 2002, p. 661–667 Vol. 68, No. 2
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.2.661–667.2002

Comparison of Fluorescently Labeled Oligonucleotide and Polynucleotide Probes for the Detection of Pelagic Marine Bacteria and Archaea

Annelie Pernthaler,[1] Christina M. Preston,[2] Jakob Pernthaler,[1]* Edward F. DeLong,[2] and Rudolf Amann[1]

Max-Planck-Institute for Marine Microbiology, Bremen, Germany,[1] and Monterey Bay Aquarium Research Institute, Moss Landing, California[2]

Received 8 October 2001/Accepted 15 November 2001

We compared the detection of bacteria and archaea in the coastal North Sea and at Monterey Bay, Calif., after fluorescence in situ hybridization (FISH) either with rRNA-targeted oligonucleotide probes monolabeled with the cyanin dye Cy3 (oligoFISH) or with fluorescein-labeled polyribonucleotide probes (polyFISH). During an annual cycle in German Bight surface waters, the percentages of bacteria visualized by polyFISH (annual mean, 77% of total counts) were significantly higher than those detected by oligoFISH (53%). The fraction of total bacteria visualized by oligoFISH declined during winter, whereas cell numbers determined by polyFISH remained constant throughout the year. Depth profiles from Monterey Bay showed large differences in the fraction of bacterial cells visualized by polyFISH and oligoFISH in the deeper water layers irrespective of the season. Image-analyzed microscopy indicated that the superior detection of cells by polyFISH with fluorescein-labeled probes in bacterioplankton samples was less a consequence of higher absolute fluorescence intensities but was rather related to quasi-linear bleaching dynamics and to a higher signal-to-background ratio. The relative abundances of archaea in North Sea and Monterey Bay spring samples as determined by oligoFISH were on average higher than those determined by polyFISH. However, simultaneous hybridizations with oligonucleotide probes for bacteria and archaea suggested that the oligoFISH probe ARCH915 unspecifically stained a population of bacteria. Using either FISH technique, blooms of archaea were observed in North Sea surface waters during the spring and summer months. Marine group II archaea (*Euryarchaeota*) reached >30% of total picoplankton abundances, as determined by polyFISH. We suggest that studies of pelagic microbial community structure using oligoFISH with monolabeled probes should focus on environments that yield detections ≥70% of total cell counts, e.g., coastal surface waters during spring and summer.

Different staining techniques are available for the microscopic identification of bacteria and archaea in environmental samples (1, 8, 13, 20). Although in principle all of these methods aim at a similar goal, the elucidation of microbial community structure, each approach is currently limited to addressing a particular set of topics. For example, bacterial chromosomal painting and immunofluorescence staining require the prior cultivation of the target organism yet provide high specificity for the detection of individual bacterial strains in environmental samples (20, 43). In contrast, general questions about the abundances of different bacterial groups and of archaea in marine pelagic and sediment microbial communities can be answered more readily by fluorescence in situ hybridization (FISH) with rRNA-targeted oligo- and polynucleotide probes (8, 17, 22). Since it is still poorly understood which microbial lineages substantially contribute to the picoplankton, FISH techniques are an increasingly popular tool for basic ecological research in marine microbiology (5, 7, 11, 12, 18, 21, 29, 31, 38, 39).

Fluorescently labeled rRNA-targeted oligonucleotides are used to specifically stain different members of microbial communities. The specificity of the probes ranges from the phylotype to the kingdom, depending on the targeted region on the rRNA (4, 9). Such probes can be readily developed and tested to detect lineages of uncultured microbes in environmental samples (35, 37). The signal intensity of cells hybridized with oligonucleotide probes is directly related to the cellular rRNA content. This allows a quantification of rRNA concentrations both in single cells and in the environment (26, 32, 36), but it may hamper the microscopic or flow cytometric counting of nongrowing or starving bacteria by FISH with monolabeled oligonucleotides (2, 27). Presently, only the cyanide dye Cy3 provides sufficient signal intensity to serve as fluorescent label for the direct microscopic visualization of microbial assemblages in the marine environment by FISH with monolabeled oligonucleotide probes (11, 17, 19, 31, 39).

Recently, poly(ribo)nucleotide probes to discriminate marine bacteria and uncultured marine archaea have been successfully applied in the open ocean (18). Such probes are RNA transcripts from PCR amplicons of 16S and 23S rRNA genes from either environmental DNA or fosmid clones (8). Numerous fluorescently labeled uridine molecules are incorporated into the polynucleotides during transcription, and the probes are subsequently hydrolyzed under controlled conditions to obtain approximately 100-mer fragments. The fluorescently labeled rRNA-targeted polynucleotide probes have been reported to yield higher signal intensities than oligonucleotide probes (23, 42) and may thus represent a better means of detecting microbes with a low ribosome content in oligotrophic habitats.

We compared the performance of the two FISH approaches

* Corresponding author. Mailing address: Max-Planck-Institut für Marine Mikrobiologie, Celsiusstrasse 1, D-28359 Bremen, Germany. Phone: 49 421 2028 940. Fax: 49 421 2028 580. E-mail: jperntha@mpi-bremen.de.

in different water bodies and during different seasons, using oligonucleotide and polynucleotide probes targeting bacteria and marine group 1 (G1; crenarchaeota) and group 2 (G2; euryarcheota) archaea (8). In addition, image analysis was applied for evaluation of brightness parameters that were considered relevant for microscopic quantification irrespective of the fluorescent label, i.e., the signal-to-background ratios and the bleaching dynamics of FISH-stained cells in environmental samples.

MATERIALS AND METHODS

Study sites and sample preparation. Between January and December 1998, seawater was collected biweekly from a 1-m depth at the sampling site Helgoland Roads (54°9′N, 7°52′E) near the island of Helgoland, which is situated 23 miles offshore in the German Bay of the North Sea (12). Water from Monterey Bay was collected on 12 April and 16 November 2000 with a rosette sampler at a station 10.8 miles offshore near Moss Landing, Calif., with a total depth of 1,097 m. Samples were fixed with 3.7% (vol/vol) formaldehyde solution overnight at 4°C and filtered onto white polycarbonate membrane filters (diameter, 25 or 47 mm; Millipore, Bedford, Mass.). Monterey Bay samples were rinsed with a 2% NaCl (wt/vol)–ethanol (50% [vol/vol]) solution, and North Sea samples were rinsed with particle-free distilled water. Filters were air dried and stored at −20°C until further processing.

Synthesis of polynucleotide probes. Cloning of rRNA operons, preparation of DNA templates, and generation of fluorescently labeled polyribonucleotide probes were carried out as described previously (8). Picoplankton DNA extracts of samples from both the Monterey Bay and the German Bight of the North Sea were used separately as templates to generate bacterial polynucleotide probes for each region.

FISH with oligonucleotide probes (oligoFISH). Sections of filters were hybridized as described previously (16) with the Cy3-monolabeled probes EUB338 and ARCH915 (Interactiva, Ulm, Germany), which are targeted to bacteria and to archaea, respectively (3, 41). The air-dried samples were mounted in a mixture that contained 5.5 parts Citifluor AF1 (Citifluor Ltd., London, United Kingdom), 1 part Vecta Shield (Vector Laboratories, Burlingame, Calif.), and 0.5 parts phosphate-buffered saline; the mixture was amended with 4′,6′-diamidino-2-phenylindole (DAPI) (final concentration, 1 μM) for counterstaining. Filters were then evaluated on a Zeiss Axioplan microscope (Carl Zeiss, Jena, Germany) equipped with an HBO 100 W Hg vapor lamp, appropriate filter sets for Cy3 and DAPI fluorescence (34), and a 100× Plan Apochromat objective. Between 700 and 1,000 DAPI-stained objects were counted per sample. Counts of bacteria from the German Bight after FISH with probe EUB338 were taken from Eilers et al. (12). Bleaching dynamics were determined with both fluorescein isothiocyanate (fluorescein)- and CY3-labeled probes.

FISH with polynucleotide probes (polyFISH). Fluorescein multiply labeled probes targeted to marine pelagic crenarcheota (probe G1), euryarcheota (probe G2), or bacteria (probe EUBAC) were used for FISH counts of environmental samples, and both fluorescein- and CY3-labeled polynucleotide probes targeted to bacteria were synthesized for the determination of bleaching dynamics. For FISH, pieces of filter were placed face up onto glass slides. Only 19 of 22 samples of the time series from the German Bight were available for counts with EUBAC. Depending on their size, the filter sections were covered with 10 to 20 μl of hybridization solution containing 5 ng of hydrolyzed polynucleotide probe/μl. The hybridization solution was prepared as described previously (8). The filter was then covered by a coverslip and placed in a 50-ml plastic tube. A piece of filter paper was placed inside the tube to maintain humidity, and 5 ml of 5× SET (1× SET is 150 mM NaCl, 1 mM Na_2EDTA, 20 mM Tris-HCl [pH 7.8]) was added. Hybridizations were incubated for 4 h at 55°C (50% formamide; probe EUBAC) or 65°C (70% formamide; probes G1 and G2). After hybridizations, the samples were washed for 1 h at 55°C in washing buffer (50% formamide, 0.2× SET). For microscopic evaluation the filter sections were embedded in the above-described mounting mix and the percentage of FISH-stained objects was quantified at blue (fluorescein) and UV (DAPI) excitations (8).

Image acquisition and analysis. Hybridized filter sections were inspected on an Axioplan II Imaging microscope (Carl Zeiss) (100× Plan Apochromat lens). Images (resolution, 1,280 by 1,024 pixels, 12 bits $pixel^{-1}$) were captured with a black and white slow-scan digital camera with approximately constant quantum efficiency between 440 and 580 nm wavelength (ORCA; Hamamatsu, Hersching, Germany) linked to a personal computer. Pictures were recorded and processed with KS400 image analysis software (Carl Zeiss). Exposure times ranged between 100 and 500 ms, and identical exposure times were used for the analysis of parallel samples with Cy3- or fluorescein-labeled oligonucleotide or polynucleotide probes.

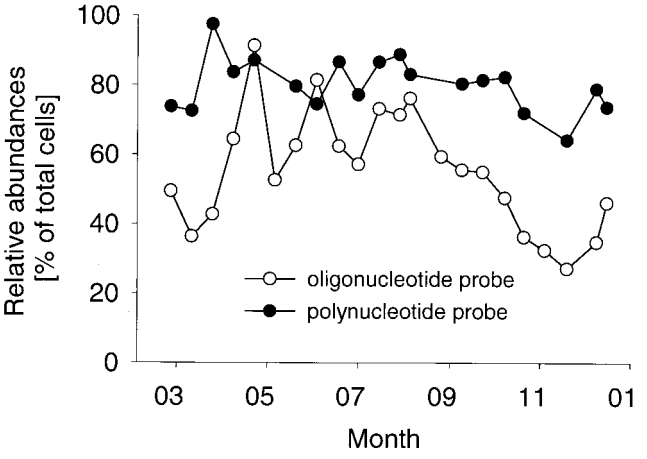

FIG. 1. Percentages of DAPI-stained cells in the German Bight of the North Sea (surface samples, 1998) detected by oligonucleotide (EUB338) and polynucleotide (EUBAC) probes targeted to bacteria. FISH data with the probe EUB338 are from reference 12.

Samples for the measurements of bleaching dynamics were prefiltered (1.2-μm pore size) and incubated for 24 h at in situ temperature in the dark prior to fixation, which typically results in a community shift towards larger gamma proteobacteria with a higher FISH signal intensity (10). For the bleaching time series, 50 images from one microscopic field were captured at intervals of 500 ms at blue (fluorescein) or green (Cy3) excitation and continuous illumination. To extend the observable bleaching period, the intensity of the 100 W mercury arc bulb was reduced by a diaphragm in the beam path; therefore, the acquired curves represent a minimum bleaching estimate. A binary mask image for cell detection was produced for the first image of a series (t_0), as described previously (28). The mask image was subsequently edited, and 10 randomly distributed background areas were added interactively. Changes in the mean grey levels of all positive objects in the t_0 image (between 50 and 200 cells per image) and of the background areas were then measured for the whole image series. Ten image series were averaged per sample, and the signal-to-background ratios were calculated as the quotient of mean object to mean background grey levels.

For a direct comparison of cells stained by either FISH technique, double images were acquired from the same microscopic fields at probe and DAPI excitation wavelengths. Ten randomly selected background areas were added to measurement masks interactively. Triplicate filter sections were analyzed per sample, and 700 cells were randomly chosen from 10 image pairs captured on each filter section. Image pairs were processed as described previously (33), but the evaluation strategy was modified as follows: probe-positive objects without DAPI signal were excluded from measurements. Measurement masks for objects that showed both probe and DAPI fluorescence ("positives") were produced from images of probe staining, whereas masks for all other DAPI-stained objects ("negatives") were deduced from DAPI staining. Such DAPI-positive objects without apparent probe signal were not used for the calculations of mean per cell fluorescence intensities. They are, however, included in Fig. 4, below, in order to depict the difference in signal-to-background ratios between probe-positive and probe-negative cells.

Statistical analysis. The nonparametric Wilcoxon matched pair test was used to analyze the percentages of total DAPI counts with either probe in North Sea samples. All other differences between two groups of data were tested for significance by using the Mann-Whitney rank sign test.

RESULTS AND DISCUSSION

Relative abundances of bacteria by oligoFISH and polyFISH. The percentages of DAPI counts of North Sea bacteria that could be visualized by polyFISH were significantly higher than those detected by oligoFISH ($n = 19$; $P < 0.01$) (Fig. 1). During the spring and summer months (April to September),

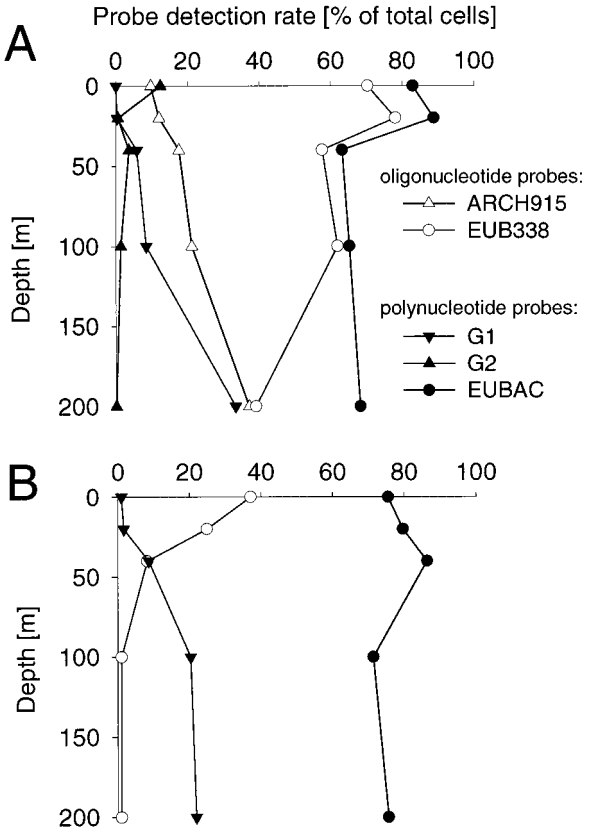

FIG. 2. Percentages of DAPI-stained cells in the upper 200 m of Monterey Bay detected with oligonucleotide and polynucleotide probes for bacteria and archaea, from 12 April 2000 (A) and 16 November 2000 (B). Oligonucleotide probes: ARCH915 (Archaea), EUB338 (bacteria). Polynucleotide probes: G1 (crenarchaeota), G2 (euryarchaeota), EUBAC (bacteria). Probes detecting <1% of DAPI-stained cells throughout the profiles are omitted from the graphs.

abundances determined by polyFISH were on average 10% higher and were less variable (mean, 78%; range, 64 to 87%) than by oligoFISH (66%; range, 52 to 91%) (12), but this difference was not statistically significant ($n = 9$; $P > 0.1$). Detection with probe EUB338 significantly decreased in autumn and winter (before 8 April and after 26 August) (mean, 43%; range, 27 to 55%) ($n_{summer} = 9$, $n_{winter} = 13$; $P < 0.01$), whereas detection with EUBAC remained high during this period (76%; range, 64 to 82%) and was statistically indistinguishable from the percentages of DAPI counts in spring and summer samples ($P > 0.05$). In the spring samples from Monterey Bay (Fig. 2A), the mean relative abundances of EUBAC and EUB338 in depths between 0 and 100 m was 75% (63 to 89%) and 67% (62 to 78%) of total cells, respectively. Less than 60% of EUBAC counts were visualized by EUB338 at 200 m depth. In Monterey Bay autumn samples, we could not detect any cells with oligoFISH below 50 m, whereas the polyFISH counts remained high in all depths (78%; range, 71 to 86%) (Fig. 2B). Test counts revealed no significant differences in the percentages of probe-stained cells between our laboratories with either technique. PolyFISH with probes prepared from DNA templates either from the North Sea or Monterey Bay resulted in equally high abundances in German Bight samples (data not shown).

Image analysis. FISH with Cy3-labeled oligonucleotide or polynucleotide probes clearly yielded higher initial signal-to-background ratios than that with probes labeled with fluorescein (Fig. 3). Although the magnitude of signal-to-background ratio and fluorescence bleaching is certainly related to sample preparation, optical properties of the equipment such as the age and power of the Hg vapor lamp, and to antibleaching agents in the mounting medium, the different dynamics of signal decrease of Cy3- and fluorescein-labeled probes were, nevertheless, striking. In spite of a microscopic embedding solution that had been developed and tested for its superior antifading properties, we observed a decrease in the signal-to-background ratio of bacteria stained with Cy3-labeled oligo- or polynucleotide probes of over 50% within the first 30 s of illumination (Fig. 3B). Initially, the signal-to-background ratios for bacteria stained with fluorescein-multilabeled polynucleotide probes was only marginally higher than that with Cy3-labeled oligonucleotide probes. After 30 s of illumination, the signal-to-background ratios for cells stained by polyFISH with fluorescein-labeled probes were still twice as high as that of cells after oligoFISH with Cy3-labeled probes. Since the counting of a microscopic field may require time periods of several seconds up to minutes, bleaching potentially represents an important feature of a staining procedure. It is very probable that the comparatively low detection with Cy3-monolabeled

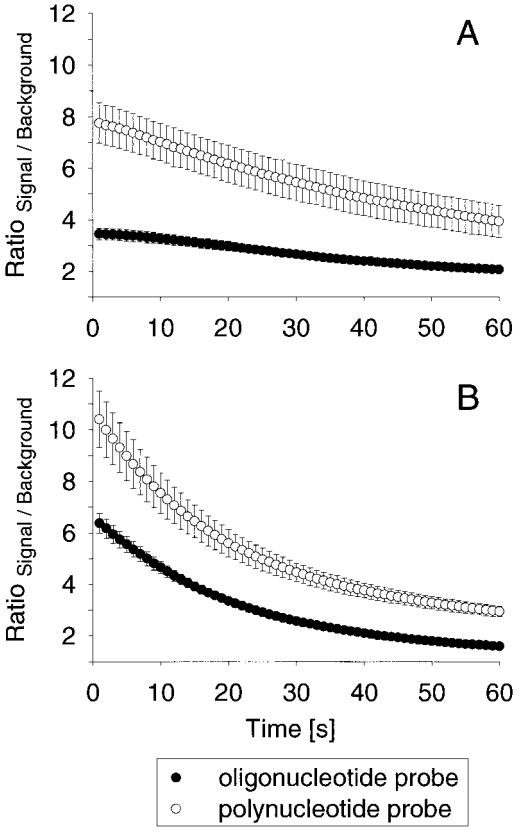

FIG. 3. Bleaching dynamics of North Sea picoplankton enrichments stained with the monolabeled oligonucleotide probe EUB338 or the multilabeled polynucleotide probe EUBAC and labeled with fluorescein (A) or Cy3 (B). A signal-to-background ratio of 1 indicates object brightness equivalent to the background fluorescence.

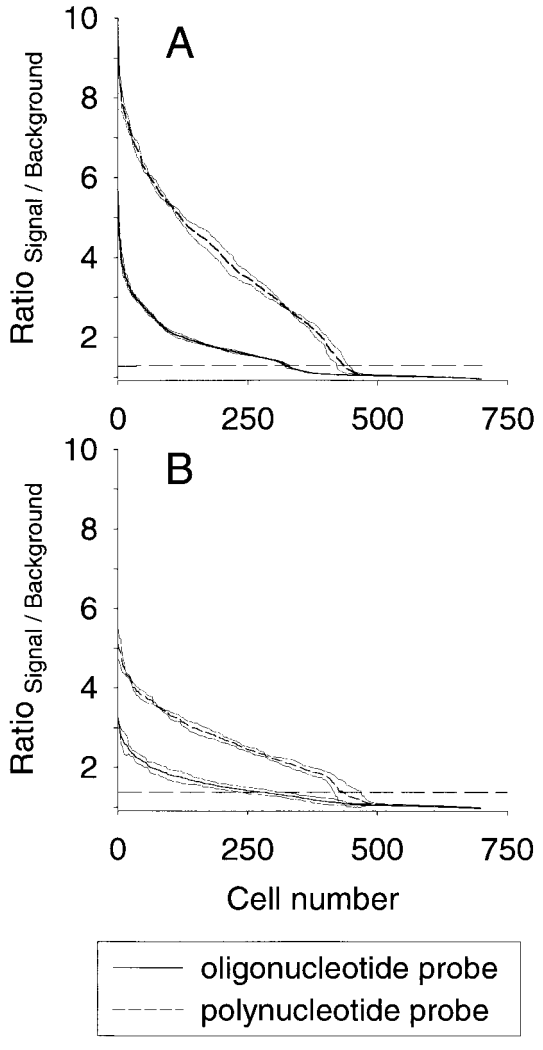

FIG. 4. Signal-to-background ratio spectra of cells hybridized with oligonucleotide probe EUB338 (labeled with Cy3) and polynucleotide probe EUBAC (labeled with fluorescein). Samples were from Monterey Bay from 12 April 2000 (A) or 16 November 2000 (B). A signal-to-background ratio of 1 indicates object brightness equivalent to the background fluorescence, i.e., cells that were not hybridized. The horizontal broken line indicates the lower limit for positive microscopic counts with probe EUB338. Thin lines are ranges of one standard deviation of triplicate samples.

oligonucleotide probes in German Bight winter samples is in part due to bleaching of the dimmest cell fraction.

At identical camera gain settings and image capture times, the mean fluorescence intensities (mean object grey values) of positively hybridized bacteria in surface samples from 12 April in Monterey Bay (Fig. 4A), stained by oligoFISH or polyFISH, were 72 ± 0.7 and 100 ± 1 (mean ± 1 standard error; n_{EUB} = 1,061, n_{EUBAC} = 1,456). In samples from 16 November (Fig. 4B), the mean fluorescence intensities of probe-positive objects after either staining procedure were statistically indistinguishable (97 ± 0.7 and 100 ± 0.8) (n_{EUB} = 1,314, n_{EUBAC} = 1,248; $P > 0.1$), but oligoFISH with Cy3-labeled oligonucleotides resulted in almost twice the background fluorescence as that after polyFISH. Thus, the mean signal-to-background ratio of EUB338-stained cells both in spring and autumn (2.1 ± 0.03 and 1.7 ± 0.02) was significantly lower than those of EUBAC-stained cells (4.2 ± 0.04 and 2.8 ± 0.02) ($P < 0.01$). The signal-to-background ratio spectrum of oligoFISH-stained cells in general declined more gradually, with little or even no clear difference between the dimmest fraction of probe-positive and probe-negative cells (Fig. 4). This presents a major obstacle for the microscopic evaluation of samples with numerous dimly stained cells and may result in more subjective counting at low EUB338 detection. In contrast, such a threshold could be more readily distinguished after polyFISH staining. As mentioned above, accurate counting of dim oligoFISH-stained cells will also be hampered by a more rapid bleaching within the typical period of time for the microscopic inspection of a single field. Altogether, this implies that the fraction of DAPI-stained cells visualized by oligoFISH in such samples might be strongly influenced both by the quality of the microscopic equipment and by the counting strategy.

Detection of archaea. In North Sea samples, the specific counts with the oligonucleotide probe ARCH915 (archaea) during spring and summer were on average 8% higher than the sum of polynucleotide probes G1 (crenarchaeota) and G2 (euryarchaeota) (Fig. 5). A high percentage of cells hybridizing with ARCH915 in April (26% of DAPI counts) was not detected by polyFISH with G1 (<1%) or G2 (5%). Simultaneous hybridizations of this sample with Cy3-labeled ARCH915 and Cy5-labeled EUB338 and inspection by confocal microscopy revealed a double binding of both probes to some cells (Fig. 6). Considering the systematically lower relative abundances determined with the polynucleotide probes, we suggest that the ARCH915 probe unspecifically binds to some pelagic marine bacteria even under stringent hybridization conditions (41). Therefore, other available (14, 31) or newly designed probes might be more adequate for a future quantification of archaea in marine samples by oligoFISH. Nevertheless, blooms of archaea in North Sea picoplankton were detected by both FISH methods between May and September, reaching a maximum of 31% of DAPI counts as determined with the G2 probe and 41% as determined with ARCH915.

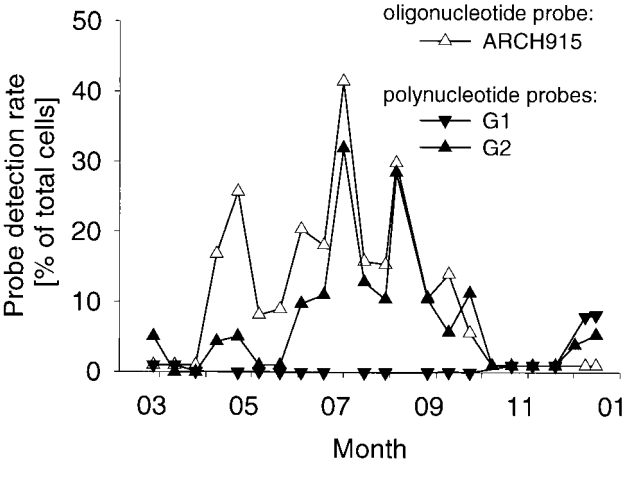

FIG. 5. Percentages of DAPI-stained cells in the German Bight of the North Sea (surface samples, 1998) detected by FISH with oligonucleotide and polynucleotide probes (ARCH915, all archaea; G1, crenarcheota; G2, euryarcheota).

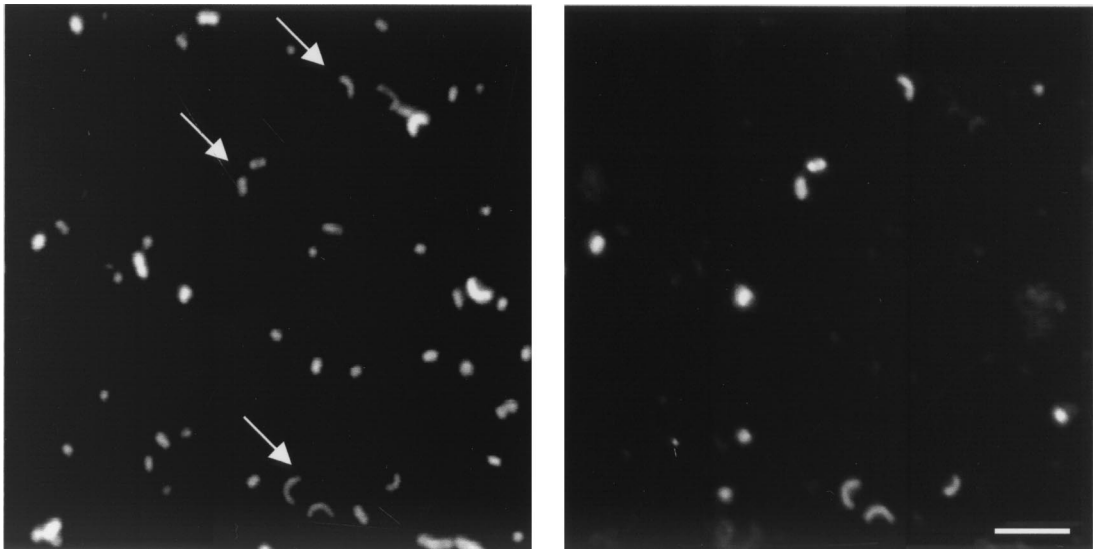

FIG. 6. Confocal laser scanning micrographs of a double hybridization of North Sea picoplankton (July 1998) with oligonucleotide probes EUB338-Cy3 (bacteria, left panel) and ARCH915-Cy5 (archaea, right panel). Arrows indicate cells hybridized by both probes. Bars, 10 μm.

In Monterey Bay samples, the percentage of total counts detected by oligonucleotide probe ARCH915 were high in spring, even exceeding the summed counts of both archaeal polynucleotide probes (Fig. 2). In winter, oligoFISH counts for archaea were below detection limits, whereas archaeal cells stained by polynucleotide probe G1 could still be visualized. Pelagic crenarcheota (cells detected by probe G1) were most abundant in the water column below 100 m depth and represented up to 21 and 33% of DAPI counts in winter and summer, respectively. The G2 probe (euryarcheota) detected 12% of DAPI-stained cells at the surface in summer and less than 1% in winter throughout the sampled water column.

The observed zonation of the two archaeal groups in the depth profiles (Fig. 2A) corresponds well with previous reports about the vertical distribution of pelagic crenarcheota and euryarcheota in the coastal Pacific Ocean and other marine provinces (24–26). In general, G1 archaea (crenarcheota) are regarded as a more prominent component of the marine picoplankton in the deeper water layers (8, 14, 15, 18, 26). In North Sea surface waters, G1 archaea only occurred sporadically, at maximal densities of 3.7×10^3 cells ml^{-1}. In contrast, G2 archaea (euryarchaeota) were detected in high abundances in the shallow and eutrophied coastal North Sea (Fig. 5), reaching total cell numbers between 1×10^5 and 2×10^5 cells ml^{-1}. To our knowledge, this is the first report that a substantial fraction of coastal North Sea picoplankton may be seasonally formed by euryarchaea. PolyFISH results from both the seasonal study and the depth profiles furthermore indicated that the pelagic euryarchaeota were mainly present in the spring or summer plankton but only appeared sporadically during autumn and winter. This may indicate that pelagic archaea are either disproportionately eliminated from the plankton at the end of the productive season or that their productivity decreases significantly below that of the bacteria.

Potential and limitations of either FISH approach. We chose our standard oligoFISH protocol for the comparison of the two approaches (34), using 5′-Cy3-monolabeled probes and direct microscopic evaluation. One advantage of this approach is that the probes can be obtained commercially in high quality at relatively low cost. A modified protocol for oligoFISH in the open ocean reports the use of Cy3 doubly labeled probes in combination with preincubation with chloramphenicol (30) and specialized intensifier equipment for image capture, to visualize "cells with fluorescence considerably below direct detection by eye" (14). However, detection of bacteria and archaea by this modified approach in the San Pedro Channel (approximately 40 to 60% and 15 to 30%, respectively) (14) was not higher than our oligoFISH results in a comparable depth profile from Monterey Bay (Fig. 2A). This indicates that even an oligoFISH staining protocol with greatly enhanced sensitivity would not fundamentally increase the signal-to-noise problem associated with probe sensitivity. High detection of DAPI-stained cells by EUB338 (>70%) using oligoFISH with the original protocol (16) were also reported from surface samples off the California coast (6).

Our study illustrates limitations of oligoFISH with presently available fluorescent dyes and direct microscopic evaluation for the staining of marine picoplankton in deeper water layers and during winter (Fig. 1 and 2). The majority of the microbial communities in such samples, presumably cells with low rRNA content (27), could not be detected. Any analysis of marine picoplankton samples by any of the current oligoFISH approaches should thus be limited to locations and seasons that allow for a detection comparable to that with polyFISH staining. It is, however, difficult to predict the percentages of total counts detectable by oligoFISH in different marine habitats, and there is evidence that high oligoFISH detection of bacterioplankton are not limited to the coastal temperate regions. Simon et al. (39) reported counts with EUB338 in the Antarctic circumpolar vent that exceeded 80% of total picoplankton abundances and increased to >90% at the marginal ice zone.

Our data furthermore indicated that polyFISH is sometimes a superior means to detect and discriminate bacteria and archaea that are low in ribosome content (Fig. 1 and 2). Despite

this obvious advantage, there are also drawbacks to the polyFISH approach. First, the application of oligoFISH probes is not dependent on any other molecular biological technique but mainly relies on the availability of a sequence database and an epifluorescence microscope. In contrast, each batch of polyFISH probes has to be synthesized using PCR and in vitro transcription techniques (8). In addition, RNA polynucleotides are more readily degraded during handling than the DNA oligonucleotide probes, e.g., by repeated freezing and thawing. So far, fluorescently labeled polynucleotides for the detection of marine picoplankton have been designed to distinguish groups that are evolutionarily very distant from each other (8, 18). The probes EUBAC, G1, and G2 are mixes of polynucleotides targeting the entire 16 and 23S rRNAs and, consequently, numerous probe molecules can simultaneously bind to each ribosome. To design more specific polynucleotide probes that discriminate different phylogenetic lineages, highly variable regions of 300 to 600 nucleotides within the 16S or 23S rRNA should be targeted (M. Leclerc, personal communication) (23, 40, 42). Nevertheless, oligoFISH may still offer higher phylogenetic resolution than polyFISH, and conditions for specific hybridization must be rigorously tested. In some cases it has been shown that the hybridization conditions required for a clear discrimination between closely related groups may substantially decrease polyFISH probe fluorescence intensity (42). In summary, any future improvement of the FISH approach for marine microbiology should attempt to preserve the superior signal intensity of multilabeled polynucleotides and at the same time reach the specificity of the oligonucleotide probes.

ACKNOWLEDGMENTS

We thank K.W. Klings (Biologische Anstalt Helgoland) for help during sampling and Marion Leclerc for help with probe synthesis. The officers and crew of the RV *Point Lobos* are acknowledged for sampling opportunities at sea.

This study was supported by the German Ministry of Education and Science (BMBF 01 LC0021/TP4) and the Max Planck Society.

REFERENCES

1. **Amann, R., F. O. Glöckner, and A. Neef.** 1997. Modern methods in subsurface microbiology—in situ identification of microorganisms with nucleic acid probes. FEMS Microbiol. Rev. **20:**191–200.
2. **Amann, R. I.** 1995. In situ identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes. Mol. Microb. Ecol. Manual **3.3.6:**1–15.
3. **Amann, R. I., L. Krumholz, and D. A. Stahl.** 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. **172:**762–770.
4. **Amann, R. I., W. Ludwig, and K. H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.
5. **Boetius, A., K. Ravenschlag, C. J. Schubert, D. Rickert, F. Widdel, A. Gieseke, R. Amann, B. B. Jorgensen, U. Witte, and O. Pfannkuche.** 2000. A marine microbial consortium apparently mediating anaerobic oxidation of methane. Nature **407:**623–626.
6. **Cottrell, M. T., and D. L. Kirchman.** 2000. Community composition of marine bacterioplankton determined by 16S rRNA gene clone libraries and fluorescence in situ hybridization. Appl. Environ. Microbiol. **66:**5116–5122.
7. **Cottrell, M. T., and D. L. Kirchman.** 2000. Natural assemblages of marine proteobacteria and members of the Cytophaga-Flavobacter cluster consuming low- and high-molecular-weight dissolved organic matter. Appl. Environ. Microbiol. **66:**1692–1697.
8. **DeLong, E. F., L. T. Taylor, T. L. Marsh, and C. M. Preston.** 1999. Visualization and enumeration of marine planktonic archaea and bacteria by using polyribonucleotide probes and fluorescent in situ hybridization. Appl. Environ. Microbiol. **65:**5554–5563.
9. **DeLong, E. F., G. S. Wickham, and N. R. Pace.** 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science **243:**1360–1363.
10. **Eilers, H., J. Pernthaler, and R. Amann.** 2000. Succession of pelagic marine bacteria during enrichment: a close look on cultivation-induced shifts. Appl. Environ. Microbiol. **66:**4634–4640.
11. **Eilers, H., J. Pernthaler, F. O. Glöckner, and R. Amann.** 2000. Culturability and in situ abundance of pelagic bacteria from the North Sea. Appl. Environ. Microbiol. **66:**3044–3051.
12. **Eilers, H., J. Pernthaler, J. Peplies, F. O. Glöckner, G. Gerdts, and R. Amann.** 2001. Isolation of novel pelagic bacteria from the German Bight and their seasonal contribution to surface picoplankton. Appl. Environ. Microbiol. **67:**5134–5142.
13. **Faude, U., and M. G. Höfle.** 1997. Development and application of monclonal antibodies for in situ detection of indigenous bacterial strains in aquatic ecosystems. Appl. Environ. Microbiol. **63:**4534–4542.
14. **Fuhrman, J. A., and C. C. Ouverney.** 1998. Marine microbial diversity studied via 16S rRNA sequences: cloning results from coastal waters and counting of native Archaea with fluorescent single cell probes. Aquat. Ecol. **32:**3–15.
15. **Fuhrman, J. A., and A. A. Davis.** 1997. Widespread Archaea and novel bacteria from the deep sea as shown by 16S rRNA gene sequences. Mar. Ecol. Prog. Ser. **150:**275–285.
16. **Glöckner, F. O., R. Amann, A. Alfreider, J. Pernthaler, R. Psenner, K. Trebesius, and K.-H. Schleifer.** 1996. An in situ hybridization protocol for detection and identification of planktonic bacteria. Syst. Appl. Microbiol. **19:**403–406.
17. **Glöckner, F. O., B. M. Fuchs, and R. Amann.** 1999. Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence in situ hybridization. Appl. Environ. Microbiol. **65:**3721–3726.
18. **Karner, M., E. F. DeLong, and D. M. Karl.** 2001. Archaeal dominance in the mesopelagic zone of the Pacific Ocean. Nature **409:**507–509.
19. **Karner, M., and J. A. Fuhrman.** 1997. Determination of active marine bacterioplankton: a comparison of universal 16S rRNA probes, autoradiography, and nucleoid staining. Appl. Environ. Microbiol. **63:**1208–1213.
20. **Lanoil, B. D., and S. J. Giovannoni.** 1997. Identification of bacterial cells by chromosomal painting. Appl. Environ. Microbiol. **63:**1118–1123.
21. **Lee, N., P. Nielsen, K. Andreasen, S. Juretschko, J. Nielsen, K. Schleifer, and M. Wagner.** 1999. Combination of fluorescent in situ hybridization and microautoradiography—a new tool for structure-function analyses in microbial ecology. Appl. Environ. Microbiol. **65:**1289–1297.
22. **Llobet-Brossa, E., R. Rossellò-Mora, and R. Amann.** 1998. Microbial community composition of Wadden Sea sediments as revealed by fluorescence in situ hybridization. Appl. Environ. Microbiol. **64:**2691–2696.
23. **Ludwig, W., S. Dorn, N. Springer, G. Kirchhof, and K. H. Schleifer.** 1994. PCR-based preparation of 23S rRNA-targeted group-specific polynucleotide probes. Appl. Environ. Microbiol. **60:**3234–3244.
24. **Massana, R., E. F. DeLong, and C. Pedros-Alio.** 2001. A few cosmopolitan phylotypes dominate planktonic archaeal assemblages in widely different oceanic provinces. Appl. Environ. Microbiol. **66:**1777–1787.
25. **Massana, R., A. E. Murray, C. M. Preston, and E. F. DeLong.** 1997. Vertical distribution and phylogenetic characterization of marine planktonic *Archaea* in the Santa Barbara channel. Appl. Environ. Microbiol. **63:**50–56.
26. **Murray, A. E., A. Blakis, R. Massana, S. Strawzewski, U. Passow, A. Alldredge, and E. F. DeLong.** 1999. A time series assessment of planktonic archaeal variability in the Santa Barbara Channel. Aquat. Microb. Ecol. **20:**129–145.
27. **Oda, Y., S. J. Slagman, W. G. Meijer, L. J. Forney, and J. C. Gottschal.** 2000. Influence of growth rate and starvation on fluorescent in situ hybridization of Rhodopseudomonas palustris. FEMS Microbiol. Ecol. **32:**205–213.
28. **Oerther, D. B., J. Pernthaler, A. Schramm, R. Amann, and L. Raskin.** 2000. Monitoring precursor 16S rRNAs of *Acinetobacter* spp. in activated sludge wastewater treatment systems. Appl. Environ. Microbiol. **66:**2154–2165.
29. **Ouverney, C. C., and J. A. Fuhrman.** 1999. Combined microautoradiography-16S rRNA probe technique for determination of radioisotope uptake by specific microbial cell types in situ. Appl. Environ. Microbiol. **65:**1746–1752.
30. **Ouverney, C. C., and J. A. Fuhrman.** 1997. Increase in fluorescence intensity of 16S rRNA in situ hybridization in natural samples treated with chloramphenicol. Appl. Environ. Microbiol. **63:**2735–2740.
31. **Ouverney, C. C., and J. A. Fuhrman.** 2000. Marine planktonic *Archaea* take up amino acids. Appl. Environ. Microbiol. **66:**4829–4833.
32. **Pernthaler, A., J. Pernthaler, H. Eilers, and R. Amann.** 2001. Growth patterns of two marine isolates: adaptations to substrate patchiness? Appl. Environ. Microbiol. **67:**4077–4083.
33. **Pernthaler, J., A. Alfreider, T. Posch, S. Andreatta, and R. Psenner.** 1997. In situ classification and image cytometry of pelagic bacteria from a high mountain lake (Gossenköllesee, Austria). Appl. Environ. Microbiol. **63:**4778–4783.
34. **Pernthaler, J., F. O. Glöckner, W. Schönhuber, and R. Amann.** 2001. Fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes, p. 207–226. *In* J. H. Paul (ed.), Methods in microbiology, vol. 30. Academic Press, San Diego, Calif.
35. **Pernthaler, J., F. O. Glöckner, S. Unterholzner, A. Alfreider, R. Psenner,**

and R. Amann. 1998. Seasonal community and population dynamics of pelagic *Bacteria* and *Archaea* in a high mountain lake. Appl. Environ. Microbiol. **64:**4299–4306.
36. **Poulsen, L. K., G. Ballard, and D. A. Stahl.** 1993. Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young and established biofilms. Appl. Environ. Microbiol. **59:**1354–1360.
37. **Ravenschlag, K., K. Sahm, and R. Amann.** 2001. Quantitative molecular analysis of the microbial community in marine Arctic sediments (Svalbard). Appl. Environ. Microbiol. **67:**387–395.
38. **Rosselló-Mora, R., B. Thamdrup, H. Schäfer, R. Weller, and R. Amann.** 1999. The response of the microbial community of marine sediments to organic input under anaerobic conditions. Syst. Appl. Microbiol. **22:**237–248.
39. **Simon, M., F. O. Glöckner, and R. Amann.** 1999. Different community structure and temperature optima of heterotrophic picoplankton in various regions of the Southern Ocean. Aquat. Microb. Ecol. **18:**275–284.
40. **Spring, S., U. Lins, R. Amann, K. H. Schleifer, L. C. S. Ferreira, D. M. S. Esquivel, and M. Farina.** 1998. Phylogenetic affiliation and ultrastructure of uncultured magnetic bacteria with unusually large magnetosomes. Arch. Microbiol. **169:**136–147.
41. **Stahl, D. A., and R. Amann.** 1991. Development and application of nucleic acid probes in bacterial systematics, p. 205–248. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. Wiley & Sons Ltd., Chichester, England.
42. **Trebesius, K., R. Amann, W. Ludwig, K. Mühlegger, and K.-H. Schleifer.** 1994. Identification of whole fixed bacterial cells with nonradioactive 23S rRNA-targeted polynucleotide probes. Appl. Environ. Microbiol. **60:**3228–3235.
43. **Weinbauer, M. G., and M. G. Höfle.** 1998. Distribution and life strategies of two bacterial populations in a eutrophic lake. Appl. Environ. Microbiol. **64:**3776–3783.