APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1997, p. 1208–1213 Vol. 63, No. 4
0099-2240/97/$04.00+0

Determination of Active Marine Bacterioplankton: a Comparison of Universal 16S rRNA Probes, Autoradiography, and Nucleoid Staining

MARKUS KARNER* AND JED A. FUHRMAN

Department of Biological Sciences, Allan Hancock Foundation, University of Southern California, Los Angeles, California 90089-0371

Received 8 July 1996/Accepted 8 January 1997

We compared several currently discussed methods for the assessment of bacterial numbers and activity in marine waters, using samples from a variety of marine environments, from aged offshore seawater to rich harbor water. Samples were simultaneously tested for binding to a fluorescently labeled universal 16S rRNA probe; ^{3}H-labeled amino acid uptake via autoradiography; nucleoid-containing bacterial numbers by modified DAPI (4′,6-diamidino-2-phenylindole) staining; staining with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), a compound supposed to indicate oxidative cell metabolism; and total bacterial counts (classical DAPI staining), taken as a reference. For the universal-probe counts, we used an image intensifying and processing system coupled to the epifluorescence microscope. All of the above-mentioned methods yielded lower cell counts than DAPI total counts. Universal-probe counts averaged about half of the corresponding DAPI count and were highly correlated to autoradiography counts ($r^2 = 0.943$; $n = 7$). Nucleoid-containing cell counts could be lower than DAPI counts by as much as 1 order of magnitude but sometimes matched autoradiography or probe counts. CTC counts were 2 orders of magnitude below DAPI counts. Universal 16S rRNA probe counts correlated well with autoradiography results, indicating a population with at least minimal metabolic activity. The greater variability of the nucleoid-containing cell counts calls for further investigation of the processes involved, and CTC counts were well below the range of the other methods tested.

Enumeration of bacterial cells is the most basic task in aquatic microbial ecology. For over two decades, the standard method used for total bacterial enumeration was the direct counting method, using epifluorescence microscopy after acridine orange or DAPI (4′,6-diamidino-2-phenylindole) staining, on membrane filters. This has been a shift in philosophy compared to the CFU counts used earlier: plate counts were supposed to show the numbers of culturable and, by extrapolation, active bacteria. Total counts, on the other hand, show all bacterium-shaped particles and were usually higher by 2 to 3 orders of magnitude than plate counts (21). This, as well as theoretical considerations (26), led to the view that most marine bacteria in situ must be viable but nonculturable under the conditions used. Total-count methods (13, 27) became standard for bacterial enumeration. One can say that CFU counts likely overlook an unknown but probably large proportion of active marine bacteria (that do not grow on the conventional substrates), while total counts certainly include dead or inactive cells (that look like bacteria). The shift from under- to overestimation in the standard assessment of active bacterial biomass was in fact a far-reaching methodological change. Indeed, for most ecological questions and especially regarding turnover of nutrients, degradation of organic matter, and so forth, one would rather wish to know the numbers of active bacteria than the total numbers of bacterium-like particles (which are included in particulate organic matter measurements anyway). A straightforward standard method that reliably indicates numbers of viable aquatic bacteria would be of great help and most useful in aquatic microbial ecology.

* Corresponding author. Mailing address: University of Southern California, Department of Biological Sciences, AHF 107, Los Angeles, CA 90089-0371. Phone: (213) 740 5759. Fax: (213) 740 8123. E-mail: mkarner@usc.edu.

As a step in this direction, the DAPI staining method (27) has recently been challenged by a modified protocol involving a destaining step (39). The rationale of this new protocol is to avoid unspecific staining of materials which do not contain DNA, thus excluding bacterium-like but DNA-less particles which might have been counted as bacteria under earlier protocols. Only nucleoid-containing cells are supposed to be counted with this new approach. The resulting counts were much lower than conventional DAPI counts: only 2 to 32% of the total DAPI-stained particles were nucleoid positive (39). Thus, it seems appropriate to compare this method with other current methods for the assessment of active or live cells. In this context, autoradiography has been used successfully to assess uptake of organic materials by the actively metabolizing fraction of the microbial community (e.g., see references 8, 14, and 34). To show respiratory activity of single cells, use of the substrate 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) has been proposed (30). CTC is a tetrazolium salt such as 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) (15), which has been used to indicate intracellular redox metabolism. CTC was applied to aquatic ecology (28), for unlike INT, it can be detected by fluorescence and is therefore more suitable to current on-filter techniques.

Molecular biology tools now allow the design of 16S rRNA probes for single-cell detection (11). Used with fluorescent instead of radioisotope markers, this technique can be used with an epifluorescence microscope (2, 6). In natural marine environments however, fluorescence is often weak due to small cell sizes and oligotrophic conditions, making detection difficult. To overcome this difficulty, the use of multiple probes has been suggested (20). Our lab has been using image amplification to enhance the sensitivity of the method, in order to be able to work with natural marine bacterial communities (10). With universal 16S rRNA probes, i.e., those complementary to

the 16S rRNA of nearly all known organisms, one obtains a new method for microbial enumeration that can be easily compared to standard DAPI counts through dual-staining techniques (12). Studies of rRNA content and single-cell hybridization find increasing acceptance, and a number of reports have been published recently (e.g., references 1, 19, and 36; see also the review by Amann [3]).

Up to now, the above-mentioned methods have been used separately. Here, we perform a comparison of simultaneously tested methods in order to assess the consistency of the respective results. Having consistent results with different methods would be highly desirable, as there is still no agreement on the proportions of either live, viable, or active bacteria in marine environments. The methods used here differ in their physiological basis. DAPI staining aims at staining any DNA-containing cell. Nucleoid-containing cell staining is a methodological modification, aimed at eliminating unspecific staining of dead cells that do not contain a significant amount of DNA (39). Universal-probe hybridization will show all cells that contain a threshold amount of rRNA. This is not direct proof of metabolic activity, but it indicates at least potential viability of the cell. The CTC method points at respiratory activity and is therefore a direct indicator of oxidative metabolism. However, dormant or anaerobic cells would not be detected by this method. Autoradiography after incorporation of radiolabeled amino acids points to a minimum of active metabolism, since there has been transport through the cell membrane. Of these methods, the unspecific, classical DAPI staining is the only method likely to include all cells counted by the other methods. It is also the most widely used method for aquatic bacterial enumeration. Therefore, it was taken as a reference for the total number of bacteria.

We compare the above-mentioned methods for a variety of seawater samples. We deliberately include a wide array of water types for a broad range of bacterial activity ratios and total numbers: coastal seawater, seawater cultures, enriched seawater, aged offshore seawater, and harbor water.

MATERIALS AND METHODS

Sampling and fixation. Water samples were taken from Santa Monica Bay at sea surface and transferred into acid-cleaned polycarbonate bottles. One coastal sample (sample 10/16/95 [see Table 1]) was enriched with 50 μM glucose, 16 μM nitrate, and 1 μM phosphate for 6 h prior to measurements as suggested by Kramer and Singleton (17) for enhancement of rRNA content. One sample originated from Marina del Rey harbor waters (sample 4/11/96a). One sample was taken ca. 5 km offshore in the San Pedro Channel and aged under simulated in situ conditions for 3 weeks before measurement (sample 4/11/96b). All others were fresh coastal-seawater samples. For both DAPI counts and 16S rRNA universal-probe hybridization, subsamples were fixed to a final concentration of 2% formalin. For nucleoid-containing cell counts, subsamples were fixed to a final concentration of 0.5 M NaN_3. Fixed samples were usually processed the same day or within 3 days of sampling (storage at 4°C). All counts were done on triplicate subsamples. Incubations for autoradiography and CTC reduction were performed within 2 h of sampling against a formalin-killed control (preincubated for 15 min). Finished slide preparations were either counted immediately or stored frozen until counting (−20°C) to prevent cell loss (35).

DAPI staining (27). We used a final concentration of 2 μg of DAPI per liter and stained for 5 min in the dark before filtering onto black 0.2-μm-pore-size Nuclepore filters.

Nucleoid-containing cell DAPI staining. Sample water was diluted fourfold with filtered (pore size, 0.2-μm) deionized water, amended with Triton X-100 (final concentration, 0.1% vol/vol), and stained with DAPI. After filtration onto a 0.2-μm-pore-size Nuclepore filter, excess DAPI was removed by an on-filter isopropanol washing step, and the filter was mounted in immersion oil. Two modifications of the original method (39) were made: (i) an additional 1-h preincubation of the sample (with DAPI added) before diluting and incubating for 2 h in the dark, as previously suggested (38), and (ii) isopropanol washing extended to 10 min, in order to remove unspecific staining from amorphous particles. We noticed that on shorter washing, some nonbacterial particles could remain visible.

Microautoradiography (34) was done with 1 μCi of amino acid mixture ml^{-1} (15 amino acids, ca. 20 nM final concentration; Amersham): 3-ml subsamples were inoculated with the amino acid mixture and incubated under simulated in situ conditions for 3 h. Uptake was stopped by adding formalin to a final concentration of 2%. After filtering, 0.2-μm-pore-size Nuclepore filters were then placed upside down onto glass slides freshly dipped in 43°C autoradiography emulsion (in a darkroom). These slides were then exposed for 3 days (desiccated and refrigerated in the dark). We used Kodak NTB-2 emulsion, stored refrigerated in the dark, and Kodak Dektol developer.

16S rRNA universal-probe hybridization. Fluorescent oligonucleotide probes were originally described by DeLong et al. (6). We adapted this protocol, including double staining with DAPI (4, 12), as described by Fuhrman et al. (10). Triplicate 5-ml subsamples were stained for 5 min with 2 μg of DAPI $liter^{-1}$ and filtered onto 0.2-μm-pore-size Nuclepore membrane filters over mixed-ester 0.45-μm-pore-size Millipore filters. To optimize cell transfer to the hybridization slides, the Nuclepore filters were kept moist by handling them attached to the moist Millipore underfilter until the transfer. Filters were cut in four pieces; three of the pieces were further used for (i) one 16S rRNA universal-probe hybridization (universal probe) (using universal probe A of Lane et al. [18] with the sequence GWATTACCGCGGCKGCTG, where W = A or T and K = C or G); (ii) one 16S rRNA probe hybridization with a sequence that should not bind to any prokaryote rRNA (nonbinding probe) (sequence CCTAGTGACGCCG TCGAC; no matches in the Ribosomal Database Project [23] allowing for up to three mismatches); and (iii) one autofluorescence control with no probe added. Filter pieces were placed upside down on the wetted surface of the wells of Teflon-coated slides (Cel-line Associates), in which the wells only were previously coated with a thin film of gelatin (0.1% gelatin, 0.01% $CrKSO_4$) and dried. After removal of the filters, the cells were permeabilized for 15 min with a 9:1 (vol/vol) methanol-formalin solution and treated for 30 min with $NaBH_4$-Tris solution (100 mM Tris, pH 8.0, and 50 mM $NaBH_4$) in the dark under rapid agitation. Hybridization buffer containing 50 ng of oligonucleotide probe was added to each well and incubated at 43°C for 4 h or overnight. Hybridization buffer was as follows (final concentrations): 5× SET (1× SET is 150 mM NaCl, 1 mM EDTA, and 20 mM Tris, pH 7.8), 0.2% bovine serum albumin, 10% dextran sulfate, 0.01% polyadenylic acid, 0.1% sodium dodecyl sulfate. The hybridization chamber (50-ml polypropylene tube) was kept humid with a paper towel soaked with 2× SET. After hybridization, the slides were washed three times for 10 min in 0.2× SET at 43°C, air dried, and mounted in a solution of 50% 10× SET, 50% glycerol, and 0.1% *p*-phenylenediamine. Chemicals were purchased from Sigma.

The oligonucleotide sequences were synthesized commercially with 5′ aminolinks and labeled with the fluorochromes tetramethyl rhodamine isothiocyanate (TRITC; Sigma) or Cy3 (Amersham) in our lab, according to the manufacturer's recommendations. Both TRITC and Cy3 fluoresce red under green excitation with our microscopy equipment, but Cy3 yields a brighter image due to higher quantum yield (manufacturer's information). We thus chose Cy3 labeling for samples taken after 27 January 1996. After labeling, unreacted dye was separated from the probe with an STE Select G-25 column (5 Prime → 3 Prime, Inc.). Unreacted oligonucleotide was removed with a nondenaturing polyacrylamide gel (29) and further purified with Nensorb 20 columns (DuPont). Probe stock aliquots were quantitated by the A_{260} of DNA and stored at −80°C.

Probe counts were made with the following equipment attached to our epifluorescence microscope: a Videoscope DS-2000N video camera, a Videoscope KS-1381 image intensifier, and a Dage-MTI DSP 2000 image processor to improve sensitivity (noise reduction by image averaging and background subtraction). The basic epifluorescence microscope was an Olympus Vanox AHBS, fitted with exciter filters BP545 (green) and 365/10 (UV), barrier filters O590 (green) and L420 (UV), and a 100-W mercury lamp as the light source. Cells were counted visually on a monitor under UV (DAPI) and green (probe) epifluorescence excitations for each individual field, by switching filter sets. Electronic superposition of both images allowed for occasional checks that the cells counted under the respective excitation were actually identical. Percentages of probe count versus DAPI count were calculated for each individual field and then averaged. We counted at least 200 bacteria per subsample. Averages given in the figures and in Table 1 refer to triplicate subsamples, not to averages of the fields counted on one individual preparation.

CTC probing. We used the protocol of Rodriguez et al. (28) as an on-filter staining technique. Subsamples (30 ml) were concentrated on polycarbonate filters to near dryness and covered with a 4 mM CTC solution in filtered (0.2-μm-pore-size) sample water. The CTC stock solution in deionized water was kept refrigerated in the dark. The solution was incubated for 3 h, in the dark. Vacuum was applied, and the filters were dried and mounted in immersion oil (Cargille type A). CTC-positive particles were counted under green excitation.

RESULTS

Absolute numbers of nucleoid-containing cells, autoradiography-positive cells, universal-probe-positive cells, and CTC-positive cells are shown in Fig. 1 in comparison to the DAPI total count. DAPI total numbers ranged from 4.9×10^5 to 6.8×10^6 cells ml^{-1}. As indicated by the 45° lines, autoradiography and universal-probe counts were relatively close to the

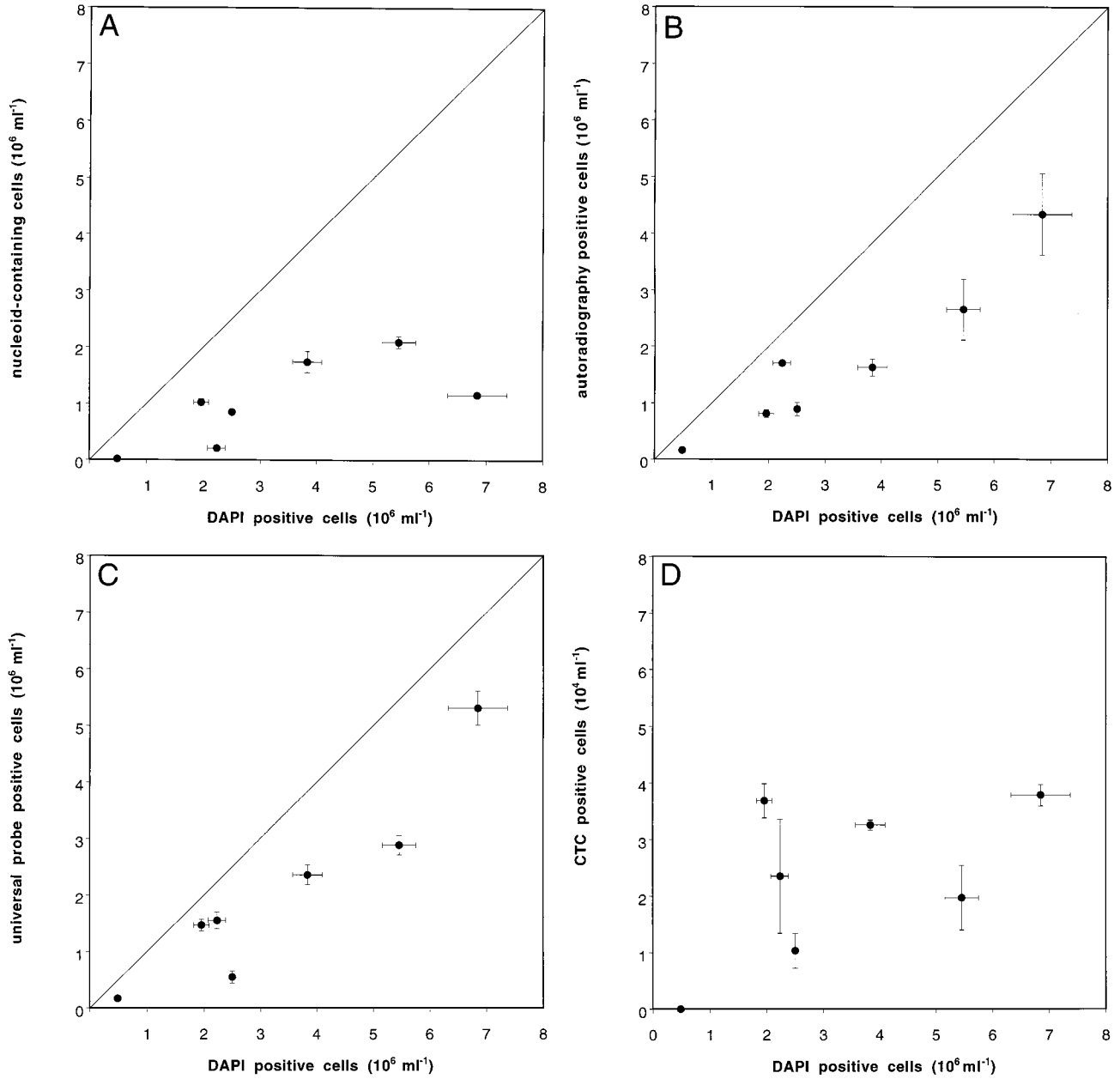

FIG. 1. Comparison of simultaneously tested methods for the assessment of bacterial numbers and activity. Samples are from a variety of marine environments in the Santa Monica Bay area, Calif. Error bars, SE of triplicate subsamples. (A) Nucleoid-containing cells versus DAPI count; (B) autoradiography-positive cells versus DAPI count; (C) 16S rRNA universal-probe-positive cells versus DAPI count; (D) CTC-reducing cells versus DAPI count. Note the different scales of the *y* axes. For further details, consult the text.

respective DAPI count. Nucleoid-containing cells sometimes departed strongly from DAPI numbers. A direct comparison between autoradiography, universal-probe, and nucleoid-containing cell counts (Fig. 2) shows a linear, highly significant relation between autoradiography and universal-probe counts ($r^2 = 0.943$; $P < 0.01$). We found no significant correlation between nucleoid-containing cells and either autoradiography or universal-probe counts. Numbers of CTC-positive cells were 2 orders of magnitude below the respective DAPI counts (Fig. 1D).

Autoradiography and universal-probe counts approached or exceeded 50% of the respective DAPI-count (Table 1). Nucleoid-containing cells sometimes matched these numbers but could drop as low as 4% of the DAPI count (Table 1). CTC counts were usually below 1% of DAPI counts or nil. Formalin-killed controls for CTC and autoradiography were always negligible (<1%). Autoradiography background was very low, and our criteria in this study for positive cells was a clear halo of numerous (>20) silver grains, easily distinguishable from background silver grains and yielding conservative estimates. We also tested for autofluorescence (no probe added) as well as fluorescence with a presumably nonbinding 16S rRNA probe (as indicated by a "no match" with any sequence in the Ribosomal Database Project [23]) along with our universal-probe counts, in six of our seven samples. There was no significant difference between the nonbinding probe and simple auto-

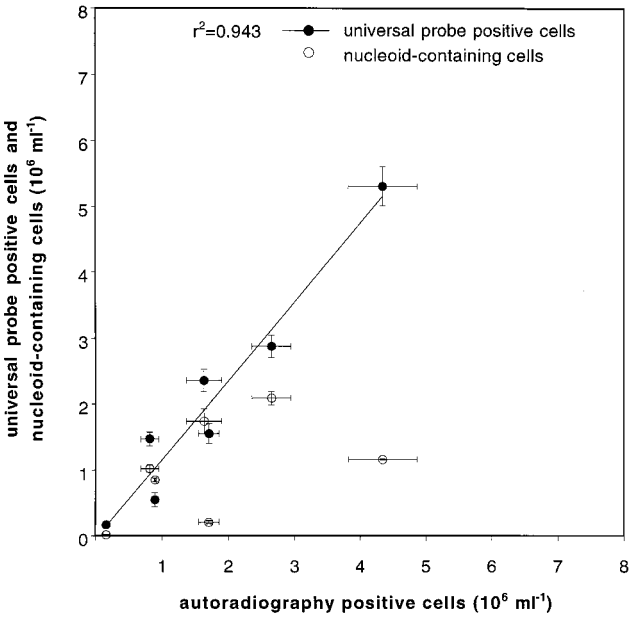

FIG. 2. 16S rRNA universal-probe-positive cells and nucleoid-containing cells versus autoradiography-positive cells in a comparison of simultaneously tested methods for the assessment of bacterial numbers and activity. Samples are from a variety of marine environments in the Santa Monica Bay area, Calif. Error bars, SE of triplicate subsamples. Regression line relates universal-probe-positive cells to autoradiography-positive cells. For further details, consult the text.

fluorescence. Autofluorescence backgrounds averaged 12% ± 4%, and nonbinding probe controls averaged 16% ± 6% of total DAPI counts (averages ± standard errors of the mean [SE] are given in Table 1).

DISCUSSION

This report deals with a comparison of currently discussed, simultaneously tested methods for bacterial enumeration and activity estimation in aquatic environments. The important concerns behind this comparison are expressed by the following questions. Which counts should be taken as total counts? Which counts show viable bacteria and not just bacterium-shaped unidentified particles (or "ghosts" [39])? We hope to contribute to the resolution of these questions. We chose a wide range of natural cell densities and origins of the samples in order to target general relations between the methods tested that should hold true under a broad range of conditions. Coastal and offshore, fresh, aged, and enriched samples were tested. We acknowledge that our results need further corroboration in different regions of the global oceans. As a first result, our deliberate choice of ecophysiologically diverse situations should ensure that the results encountered do not depend on a certain type of sample.

Different methods compared here clearly aim at different physiological characteristics of bacterioplankton. Can it thus be meaningful to compare these methods? We chose DAPI numbers as a reference for total bacterial counts, as this method was initially intended to show DNA-containing particles (27). DAPI total counts are usually lower than the corresponding acridine orange counts (33), which is probably due to unspecific staining of all sorts of organic particles by acridine orange (32). DAPI is therefore the more conservative of the two standard methods, and we choose it as the standard total count reference. However, in combination with formalin fixation of marine samples, DAPI as well has been shown to stain nonliving, organic particles (25). The problem of more accurate direct counting of DNA-containing aquatic bacteria has been studied in detail by Zweifel and Hagström (39). They used DAPI in a slightly different way, yielding numbers of nucleoid-containing cells. This new protocol uses a destaining process with isopropanol and avoids formalin fixation, reportedly to prevent unspecific DAPI retention by non-DNA materials. If this method works as planned, it should yield bacterial numbers higher than our universal-probe counts. Any potentially viable cell must contain at least one chromosome's worth of DNA (readily detected), but it might not have sufficient rRNA to be detected by probe fluorescence.

Despite this expectation of higher counts, the numbers of nucleoid-containing cells are mostly lower than either the universal-probe counts or autoradiography results (Fig. 1A; Fig. 2;

TABLE 1. Cell counts as percentages of DAPI total counts for various methods for the assessment of bacterial numbers and activity

Sample designation[a]	Sample origin (comment[s])	Position	Cell count (% and SE[c])					
			Nucleoid-containing cells	Autoradiography	Universal probe	Nonbinding probe	Autofluorescence (green excitation)	CTC
10/16/95	Playa del Rey (nutrient enriched for 6 h)	33°58′N 118°27′W	9 (1)	76 (1)	70 (7)	ND[b]	ND	1.1 (0.5)
11/20/95	Playa del Rey	33°58′N 118°27′W	52 (3)	41 (3)	75 (5)	20 (3)	14 (2)	1.9 (0.2)
1/18/96	Playa del Rey (2 days after rain)	33°58′N 118°27′W	45 (5)	42 (4)	62 (5)	34 (10)	22 (8)	0.8 (0)
1/27/96	Playa del Rey	33°58′N 118°27′W	34 (1)	36 (5)	22 (4)	11 (4)	8 (1)	0.4 (0.1)
4/9/96	Santa Monica pier	34°00′N 118°30′W	17 (0)	63 (10)	78 (4)	12 (2)	9 (2)	0.6 (0)
4/11/96a	Marina del Rey Harbor	33°59′N 118°26′W	38 (2)	49 (10)	53 (3)	21 (3)	19 (2)	0.4 (0.1)
4/11/96b	San Pedro Channel (offshore, aged 3 weeks)	33°40′N 118°19′W	4 (0)	33 (2)	36 (3)	17 (1)	14 (3)	0.0 (0)
Average (SE)			29 (11)	49 (9)	56 (12)	16 (6)	12 (4)	0.7 (0.4)

[a] Designation is based on date sample was taken (mo/day/yr).
[b] ND, not determined.
[c] SE, standard error of the mean of triplicate subsamples.

Table 1). We were initially concerned about our proper application of this method. However, our numbers of nucleoid-containing cells, as a percentage of DAPI counts, are within the range of published values (39). Variation of staining conditions in preliminary experiments (e.g., incubation times [data not shown]) did not increase these counts. This makes us believe that we applied the method as intended by the original authors. The application of this method is still under development, and new results (5) suggest that for reasons yet to be determined, a certain fraction of cells does not stain well with the original method. Possibly, penetration of DAPI into certain cells is insufficient, or DNA coiling status might make a difference. Choi et al. (5) propose modifications to the protocol of Zweifel and Hagström (39), yielding higher numbers but reverting to aldehyde fixation which was intentionally abandoned by Zweifel and Hagström (39). Further clarification in this area is clearly needed.

Autoradiography aims at showing actively metabolizing cells. We chose amino acid uptake for our autoradiography preparations, because it labels cells more efficiently than other radiolabeled organic molecules (7). Active uptake seems a solid indication for metabolic activity, and formalin-killed controls in our autoradiography assays did not yield any clusters of silver grains. An artificial enhancement of activity by the addition of labeled amino acids seems unlikely, due to the low concentration added (20 nM, clearly below published values for coastal natural amino acid concentrations [e.g., references 9 and 24]) and the short incubation period of 3 h. Even assuming that the addition of amino acids triggered a metabolic response from otherwise inactive bacteria, we would still be counting viable organisms. However, these cells, while taking up amino acids, did not significantly reduce CTC in our series, as shown by the low corresponding CTC numbers. CTC reduction has been linearly related to thymidine incorporation by bacterioplankton ($r^2 = 0.54$) and to the chlorophyll *a* content ($r^2 = 0.78$) of marine waters (22). Still, only 1 to 2% of total cell counts was found to be CTC positive by these authors, corresponding to our results and those of Severin et al. (30). So, despite potential problems that we did not investigate further, possibly including dissolution of CTC crystals by immersion oil (22), our CTC counts are not unusually low. A recent study suggests that CTC is toxic to bacteria (37), so only a fraction of actively respiring cells may reduce CTC at all under certain conditions. We are aware of one study (5) that yielded relatively high numbers of CTC-positive cells in native marine waters, although we cannot specifically determine how this result relates to our own work. Methodology involving CTC clearly needs careful further evaluation.

On the other hand, universal-probe 16S rRNA hybridization did produce results consistent with those obtained from autoradiography. As mentioned above, autoradiography works with tracer amounts of amino acids. From the use of a radiolabeled substrate, one might expect a high sensitivity at even low-level metabolic activity. The threshold of detection via universal probes should depend only on the rRNA content of cells—which similarly might be taken as an indicator for metabolic activity. If this threshold is sufficiently low, then universal-probe 16S rRNA hybridization could detect cells that are barely metabolizing or dormant. Thus, both autoradiography and 16S rRNA hybridization sensitively target metabolic activity indicators, i.e., uptake of tracer amounts of amino acids and presence of minimal rRNA, respectively. The correspondence of both methods is therefore consistent with their physiological bases. Experimental enrichments have been shown to increase rRNA content by a large amount (17). We did conduct an enrichment experiment similar to that of Kramer and Singleton (17), but universal-probe numbers did not increase significantly upon enrichment (data not shown). The rRNA content necessary to make a cell detectable with 16S rRNA hybridization might therefore be low enough to ensure detection with our method, even at low metabolic levels. There is also evidence that total RNA content of cells in situ relates only inconsistently with metabolic activity (16).

The question arises as to how we should deal with autofluorescent cells with respect to the universal-probe counts. We counted two kinds of controls: pure autofluorescence (no probe) and a nonbinding probe control. The nonbinding controls in our samples were statistically indistinguishable from natural autofluorescence (on average, 16% ± 6% versus 12% ± 4% of the DAPI numbers, respectively; averages ± SE are given in Table 1), suggesting that nonspecific-probe binding was low. Autofluorescent cells in the bacterial size range, such as prochlorophytes, are indistinguishable from heterotrophic bacteria in standard DAPI preparations and can reach substantial proportions of bacterial counts (31). An automatic subtraction of autofluorescent cells from universal-probe-positive cells would be wrong, in that it would assume that all autofluorescent cells are inactive, and this is highly unlikely. Similarly, in the DAPI or nucleoid-containing-cell count images, cells will light up, though some of these cells might very well be autofluorescent at the same time. The same holds true for autoradiography: any cell taking up amino acids will develop silver grains, regardless of possible autofluorescence. We cannot distinguish between the autofluorescent cells and the nonautofluorescent cells with either method. A fair and true comparison would not exclude the autofluorescent cells from only one method and not the others. Therefore, we report the total universal-probe numbers in our figures. All our results are to be understood as relating to the prokaryotic community as a whole. To the extent that some autofluorescent cells may be dead, we may somehow overestimate universal-probe numbers. In the most conservative hypothesis (all autofluorescence subtracted), the universal probe/DAPI ratio would be 40% instead of the 56% reported in Table 1. The reported correlation in Fig. 2 would have an r^2 of 0.919 (still highly significant) instead of 0.943 and a slope of 1.031 instead of 1.196.

It seems to us that a cell containing rRNA in quantities detectable by 16S rRNA hybridization probably contains the essential ingredients to be viable, given that rRNA content is regulated in growing cells. So, universal-probe-positive cells may be expected to be live cells with at least a minimum of metabolic activity. This is consistent with the linear correlation between counts obtained with autoradiography and those obtained with 16S rRNA hybridization (Fig. 2), indicating that with these methods we counted the same type of cells.

This result also creates a credible basis for the use of 16S rRNA hybridization with specific probes: if the goal is determining the fraction of the microbial community that belongs to a certain group, one must first define the total number of organisms taken as a reference. Thus, one needs to make sure that universal 16S rRNA probes bind quantitatively to active organisms. The optimal correspondence of autoradiography and universal probe results in our series is an encouraging result in this direction.

ACKNOWLEDGMENTS

We thank Cleber Ouverney for help with 16S rRNA probing and three anonymous reviewers for their valuable comments.

This work was supported by grants to M.K. by the government of Austria (Fonds zur Förderung der wissenschaftlichen Forschung grant no. JO-1122-BIO) and to J.F. by the National Science Foundation (DEB 9401110 and OCE 9218234).

REFERENCES

1. **Alfreider, A., J. Pernthaler, R. Amann, B. Sattler, F. O. Glöckner, A. Wille, and R. Psenner.** 1996. Community analysis of the bacterial assemblages in the winter cover and pelagic layers of a high-mountain lake by in situ hybridization. Appl. Environ. Microbiol. **62:**2138–2144.
2. **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.
3. **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.
4. **Braun-Howland, E. B., S. A. Danielsen, and S. A. Nierzwicki-Bauer.** 1992. Development of a rapid method for detecting bacterial cells in situ using 16S rRNA-targeted probes. BioTechniques **13:**928–932.
5. **Choi, J. W., E. B. Sherr, and B. F. Sherr.** 1996. Relation between presence/absence of a visible nucleoid and metabolic activity in bacterioplankton cells. Limnol. Oceanogr. **41:**1161–1168.
6. **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.
7. **Douglas, D. J., J. A. Novitsky, and R. O. Fournier.** 1987. Microautoradiography-based enumeration of bacteria with estimates of thymidine-specific growth and production rates. Mar. Ecol. Prog. Ser. **36:**91–99.
8. **Fuhrman, J. A., and F. Azam.** 1982. Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: evaluation and field results. Mar. Biol. **66:**109–120.
9. **Fuhrman, J. A., and T. M. Bell.** 1985. Biological considerations in the measurement of dissolved free amino acids in seawater and implications for chemical and microbiological studies. Mar. Ecol. Prog. Ser. **25:**13–21.
10. **Fuhrman, J. A., S. H. Lee, Y. Masuchi, A. A. Davis, and R. M. Wilcox.** 1994. Characterization of marine prokaryotic communities via DNA and RNA. Microb. Ecol. **28:**133–145.
11. **Giovannoni, S. J., E. F. DeLong, G. J. Olsen, and N. R. Pace.** 1988. Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J. Bacteriol. **170:**720–726.
12. **Hicks, R. E., R. I. Amann, and D. A. Stahl.** 1992. Dual staining of natural bacterioplankton with 4′,6-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. Appl. Environ. Microbiol. **58:**2158–2163.
13. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of Nuclepore filters for counting bacteria by epifluorescence microscopy. Appl. Environ. Microbiol. **33:**1225–1228.
14. **Hoppe, H.-G.** 1976. Determination and properties of actively metabolizing heterotrophic bacteria in the sea, investigated by means of micro-autoradiography. Mar. Biol. **36:**291–302.
15. **Iturriaga, R.** 1979. Bacterial activity related to sedimenting particulate matter. Mar. Biol. **55:**157–169.
16. **Jeffrey, W. H., R. Von Haven, M. Hoch, and R. B. Coffin.** 1996. Bacterioplankton RNA, DNA, protein content and relationships to rates of thymidine and leucine incorporation. Aquat. Microb. Ecol. **10:**87–95.
17. **Kramer, J., and F. L. Singleton.** 1993. Measurement of rRNA variations in natural communities of microorganisms on the southeastern U.S. continental shelf. Appl. Environ. Microbiol. **59:**2430–2436.
18. **Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin, and N. R. Pace.** 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci. USA **82:**6955–6959.
19. **Lee, S., and P. F. Kemp.** 1994. Single-cell RNA content of natural marine planktonic bacteria measured by hybridization with multiple 16S rRNA-targeted fluorescent probes. Limnol. Oceanogr. **39:**869–879.
20. **Lee, S., C. Malone, and P. F. Kemp.** 1993. Use of multiple 16S rRNA-targeted fluorescent probes to increase signal strength and measure cellular RNA from natural planktonic bacteria. Mar. Ecol. Prog. Ser. **101:**193–201.
21. **Lee, S. H., and J. A. Fuhrman.** 1991. Species composition shift of confined bacterioplankton studied at the level of community DNA. Mar. Ecol. Prog. Ser. **79:**195–201.
22. **Lovejoy, C., L. Legendre, B. Klein, J.-E. Tremblay, and R. G. Ingram.** 1996. Bacterial activity during early winter mixing (Gulf of St. Lawrence, Canada). Aquat. Microb. Ecol. **10:**1–13.
23. **Maidak, B. L., N. Larsen, M. J. McCaughey, R. Overbeek, G. J. Olsen, K. Fogel, J. Blandy, and C. R. Woese.** 1994. The Ribosomal Database Project. Nucleic Acids Res. **22:**3485–3487.
24. **Mopper, K., and P. Lindroth.** 1982. Diel and depth variations in dissolved free amino acids and ammonium in the Baltic Sea determined by shipboard HPLC analysis. Limnol. Oceanogr. **27:**336–347.
25. **Mostajir, B., J. Dolan, and F. Rassoulzadegan.** 1995. A simple method for the quantification of a class of labile marine pico- and nano-sized detritus: DAPI Yellow Particles (DYP). Aquat. Microb. Ecol. **9:**259–266.
26. **Pomeroy, L. R.** 1974. The ocean's food web, a changing paradigm. BioScience **24:**499–504.
27. **Porter, K., and Y. S. Feig.** 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. **25:**943–948.
28. **Rodriguez, G. G., D. Phipps, K. Ishiguro, and H. F. Ridgway.** 1992. Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. Appl. Environ. Microbiol. **58:**1801–1808.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
30. **Severin, E., J. Stellmach, and H.-M. Nachtigal.** 1985. Fluorimetric assay of redox activity in cells. Anal. Chim. Acta **170:**341–346.
31. **Sieracki, M. E., E. M. Haugen, and T. L. Cucci.** 1995. Overestimation of heterotrophic bacteria in the Sargasso Sea: direct evidence by flow and imaging cytometry. Deep Sea Res. **42:**1399–1409.
32. **Sieracki, M. E., and C. L. Viles.** 1992. Distributions and fluorochrome-staining properties of submicrometer particles and bacteria in the North-Atlantic. Deep Sea Res. **39:**1919–1929.
33. **Suzuki, M. T., E. B. Sherr, and B. F. Sherr.** 1993. DAPI direct counting underestimates bacterial abundances and average cell-size compared to AO direct counting. Limnol. Oceanogr. **38:**1566–1570.
34. **Tabor, P. S., and R. A. Neihof.** 1982. Improved method for determination of respiring individual microorganisms in natural waters. Appl. Environ. Microbiol. **43:**1249–1255.
35. **Turley, C. M., and D. J. Hughes.** 1992. Effects of storage on direct estimates of bacterial numbers of preserved seawater samples. Deep Sea Res. **39:**375–394.
36. **Weiss, P., B. Schweitzer, R. Amann, and M. Simon.** 1996. Identification in situ and dynamics of bacteria on limnetic organic aggregates (lake snow). Appl. Environ. Microbiol. **62:**1998–2005.
37. **Ullrich, S., B. Karrasch, H.-G. Hoppe, K. Jeskulke, and M. Mehrens.** 1996. Toxic effects on bacterial metabolism of the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride. Appl. Environ. Microbiol. **62:**4587–4593.
38. **Zweifel, U.-L.** 1995. Personal communication.
39. **Zweifel, U.-L., and Å. Hagström.** 1995. Total counts of marine bacteria include a large fraction of non-nucleoid-containing bacteria (ghosts). Appl. Environ. Microbiol. **61:**2180–2185.