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Application of the Novel Nucleic Acid Dyes YOYO-1, YO-PRO-1, and PicoGreen for Flow Cytometric Analysis of Marine Prokaryotes

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Novel blue light-excited fluorescent dyes for nucleic acids (YOYO-1, YO-PRO-1, and PicoGreen) were tested on cultures of *Escherichia coli* and of a variety of marine prokaryotes. Results of flow cytometric DNA analyses were compared with those obtained with the UV-excited dyes bis-benzimide Hoechst 33342 or 4′,6-diamidino-2-phenylindole (DAPI). YOYO-1, YO-PRO-1, and PicoGreen can be used only on aldehyde-fixed cells and need to be supplemented with cofactors such as potassium, citrate, or EDTA. They are highly sensitive to ionic strength. Consequently, seawater culture samples cannot be stained directly with these dyes and require at least a 10-fold dilution with distilled water to obtain reliable fluorescence signals. After treatment with RNase, coefficients of variation for the G_1 peak of the DNA distributions of the different strains tested with YOYO-1 or PicoGreen indicated in general an improvement over Hoechst 33342 staining. These novel dyes can be used to enumerate prokaryotic cells by flow cytometry, as demonstrated with *E. coli*. However, their sensitivity to ionic strength makes them unsuitable for cell cycle analysis in natural samples.

In recent years, flow cytometry has been increasingly used to analyze natural communities of marine microorganisms because of its sensitivity, quantification capacity, speed, and lack of interference from dissolved substances. Many studies (reviewed in reference 22) have dealt with quantifying autotrophic picoplankton (i.e., cells smaller than 2 μm in diameter), including the prokaryotic genera *Prochlorococcus* (8, 33) and *Synechococcus* (34), on the basis of their distinctive pigment fluorescences (red for chlorophyll and orange for the cyanobacterium-specific phycoerythrin). Staining of cell DNA has been used for discriminating and enumerating heterotrophic bacteria initially by epifluorescence microscopy (see the review in reference 16) and more recently by flow cytometry (4, 21). The combination of DNA and chlorophyll fluorescence allows us to discriminate between the autotrophic and heterotrophic components of picoplankton, leading to the recognition that the latter had been previously overestimated in very oligotrophic waters such as those of the subtropical Pacific (5). Another area in which DNA stains are useful is cell cycle analysis (27). This application requires good staining conditions to clearly separate the different cell cycle phases. Initially restricted to the study of marine prokaryotes in culture (1, 2, 17), cell cycle analysis is now applied to natural populations of marine phytoplankton, enabling assessment of their physiological status (32) and their growth rate (6, 31).

Combined measurements of DNA and chlorophyll fluorescences are not necessarily straightforward. The necessity to record chlorophyll emission precludes the use of propidium iodide and ethidium bromide, which emit in the red region of the visible spectrum and interfere with chlorophyll. Chromomycin A3, a GC-specific dye excitable at 457 nm and emitting at 570 nm, works well with eukaryotes (29) but poorly with prokaryotes, in particular *Prochlorococcus marinus* (20a), probably because of its low G+C content (14). Thus, only the UV-excitable AT-specific dyes such as 4′,6-diamidino-2-phenylindole (DAPI) or bis-benzimide Hoechst 33342 have provided reliable results to date (4, 21). However, the use of UV excitation has two major drawbacks. First, UV does not excite chlorophyll very well, and therefore discrimination of very small photosynthetic prokaryotes, such as *Prochlorococcus marinus*, is not optimal. Second, small low-cost flow cytometers such as the Coulter XL or the Becton-Dickinson FACScan, which represent a very large fraction of available instruments and are increasingly used for marine applications, are equipped with a low-power laser tuned at a single wavelength of 488 nm.

A variety of new DNA-staining dyes, in particular YOYO-1, YO-PRO-1 and PicoGreen, which are excitable at 488 nm and emit in the green with a high fluorescence yield, have been introduced recently. They potentially could make DNA-staining techniques available to flow cytometers equipped with small 488-nm lasers. YO-PRO-1 and YOYO-1 are cyanine dyes which have a strong binding affinity to nucleic acids. They have been used for the detection of small amounts of DNA in electrophoretic gels (11). Like ethidium bromide and propidium iodide, their fluorescence yield is proportional to DNA concentration and does not depend on the G+C content (26). YOYO-1 has recently been used for flow cytometric analysis of mammalian cells and suspensions of chromosomes (15), while some of its close relatives (TOTO-1, TO-PRO-1, and YO-PRO-1) have been proposed for the enumeration of marine bacteria (18) and viruses (13). PicoGreen is another DNA-specific dye which has a strong affinity for double-stranded DNA and is designed to quantify these molecules in solution. As with Hoechst 33342, the presence of RNA does not interfere with the fluorescence emission of the PicoGreen–double-stranded DNA complex (12).

We have tested these new dyes by flow cytometry on different cultures of marine prokaryotes as well as on the widely used bacterium *Escherichia coli*. After optimization of staining conditions, we have compared these results with those obtained by using the UV-excited dyes Hoechst 33342 and DAPI.

MATERIALS AND METHODS

Strains and culture conditions. The photosynthetic prokaryote *Prochlorococcus marinus* CCMP 1375 was obtained from the Center for the Culture of Marine Phytoplankton, Bigelow, Maine. *P. marinus* was grown in 50-ml polyethylene

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TABLE 1. Molecular weights, absorption, and fluorescence maxima for the dyes and optical configuration of the EPICS 541 flow cytometer for each dye

Dye	Mol wt[a]	λ_A[a] (nm)	λ_F[a] (nm)	Optical configuration of flow cytometer[b]			
				Dichroic 1	Dichroic 2	Fluo 1	Fluo 2
YOYO-1	1,271	491/450[c]	509/550[c]	488LP	590SP	530SP	670LP
YO-PRO-1	629	491/450[c]	509/550[c]	488LP	590SP	530SP	670LP
PicoGreen	NC[d]	480[e]	520[e]	488LP	590SP	530SP	670LP
Hoechst 33342	652	346[e]	460[e]	400LP	590SP	480BP	670LP
DAPI	350	359[e]	461[e]	400LP	590SP	480BP	670LP

[a] λ_A, Absorption maximum; λ_F, fluorescence maximum. Data for dyes are from the manufacturer (Molecular Probes).
[b] Dichroic filter 1 is used to separate RALS signal from fluorescence, and dichroic filter 2 is used to split the fluorescence of the DNA stain from that of the chlorophyll. Fluo 1 and 2 are the filters used to further discriminate the DNA and chlorophyll emission signals. LP, long pass; SP, short pass; BP, band pass.
[c] Value obtained from manufacturer/value obtained in reference 15.
[d] NC, not communicated by manufacturer.
[e] Obtained from reference 12.

culture flasks (Nunc) at 20°C and 8 μmol quanta of continuous blue light m^{-2} s^{-1}. Culture medium was K/10 medium (7) modified as described previously (19). The cyanobacterium *Synechococcus* strain WH8103 (23) was grown at 20°C in an artificial seawater medium (35). We also tested a variety of heterotrophic prokaryotes: *E. coli* DH5α (RecA$^-$, F$^-$ *endA1 gyrA96 thi-1 hsdR17* [r_K^- m_K^+] *supE44 relA1*) was grown on Luria-Bertani medium at 37°C (20); the marine bacteria *Cytophaga drobachiensis* Dsij (25), *Alteromonas carrageenovora* ATCC 43555, and *Pseudomonas alginovora* CIP 102941 were grown on Zobell slant medium (37) at 22°C; *Salmonella typhimurium* CIP 60.62T was grown on sterile Trypcase soja (BioMerieux) at 37°C; *Deleya aquamarina* CIP 103199T was grown on a marine broth (Difco) at 21°C; and the archaon *Pyrococcus abyssi* was grown on YPS medium (10).

DNA staining. YOYO-1, YO-PRO-1, PicoGreen, and Hoechst 33342 were purchased from Molecular Probes, Inc., (Eugene, Oreg.). Stock solutions were 1 μM in dimethyl sulfoxide-H_2O (1:4) for YOYO-1 and YO-PRO-1, 1/10 in dimethyl sulfoxide-H_2O (1:4) for PicoGreen (the exact concentration and molarity of this dye were not communicated by the supplier), and 10 μg/ml in distilled water for Hoechst 33342. DAPI was purchased from Sigma, St. Louis, Mo. The stock solution of DAPI was 1 mg/ml in distilled water. All samples were fixed for 20 min with a mixture of 1% paraformaldehyde and 0.05% glutaraldehyde (final concentrations) at room temperature in the dark, frozen in liquid nitrogen, and then stored at −80°C until analysis, a fixation method modified from that of Vaulot et al. (30). All samples were incubated with the DNA-specific stain at 37°C from 30 min to 2 h with 0.1 g of a mixture of RNase A and B (Sigma R-4875 and R-5750; 1:1 [wt/wt]) per liter. Tests were performed in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 7.2]), distilled water, seawater, or a special buffer used for isolation of nuclei (NI; 30 mM $MgCl_2$, 20 mM sodium citrate, 125 mM sorbitol, 55 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES], 1 mM EDTA) (29). For experiments with *P. marinus*, a culture of this photosynthetic organism was sampled in the exponential phase and fixed as described above. The sample was then aliquoted and quick-frozen. For optimization of stain concentrations, aliquots of fixed *P. marinus* were diluted in TE buffer at a final concentration of 1.25×10^5 cells ml^{-1} before RNase treatment and staining. For optimization of the staining buffer, aliquots of fixed *P. marinus* were diluted in the analysis buffer (final concentration, 1.25×10^5 ml^{-1}) and stained with the following dye concentrations: 40 nM Hoechst 33342, 30 nM YOYO-1 or YO-PRO-1, or 1/1,000 of the stock solution for PicoGreen. These tests allowed us to define the best buffers (see Results), which were TE buffer for *P. marinus*, the *Synechococcus* strain, and *C. drobachiensis* and modified TE buffer with 50 mM potassium citrate and 0.1% (wt/vol) Triton X-100 for the other prokaryotic strains tested.

Flow cytometry. DNA analyses were performed on an EPICS 541 flow cytom-

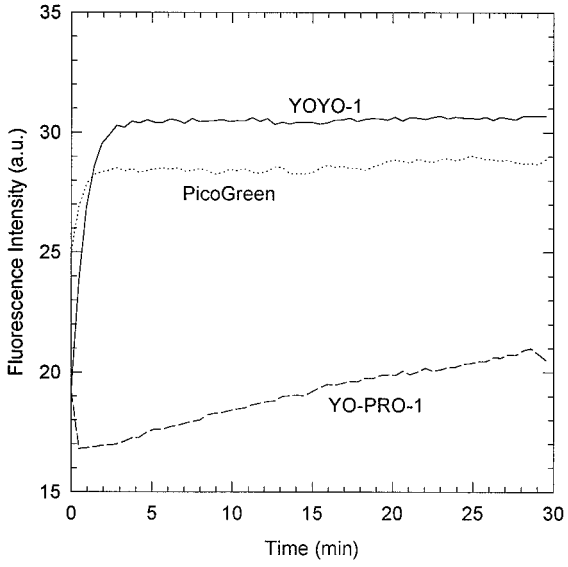

FIG. 1. Staining kinetics of a culture of *P. marinus* fixed and pretreated with RNase before addition of 50 nM YOYO-1, 50 nM YO-PRO-1, and 1/1,000 of the commercial solution of PicoGreen in TE buffer. The fluorochrome was added just before the sample was run, and the intensity of fluorescence was monitored continuously for 30 min.

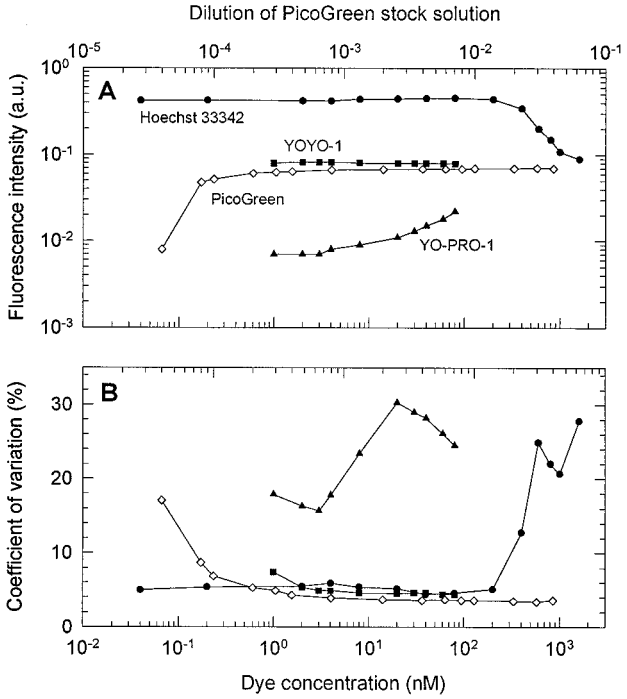

FIG. 2. (A) Variation of the fluorescence intensity of the G_1 peak of the cell cycle of *P. marinus* for YOYO-1, YO-PRO-1, PicoGreen, and Hoechst 33342 in TE buffer as a function of dye concentration. (B) CV of the G_1 peak of the cell cycle of *P. marinus* cells as a function of dye concentration. Note that Hoechst 33342 fluorescence is not directly comparable to that of the three other dyes, since their excitation and emission wavelengths are different.

TABLE 2. Fluorescence intensity and CV of G_1 peaks for *P. marinus* cells resuspended in different buffers or after addition of salts to TE buffer or seawater[a]

Compound	Hoechst 33342		YOYO-1		PicoGreen	
	Intensity[b]	CV	Intensity[b]	CV	Intensity[b]	CV
Buffers						
TE	0.306	4.8	0.083	4.5	0.083	3.3
Seawater	0.240	5.8	0.019	17.7	0.020	9.7
Distilled water	0.094	27.9	0.039	22.7	0.033	14.4
NI	0.424	5.0	0.073	3.9	0.066	4.1
Salts added to TE buffer						
Sodium chloride (50 mM)	0.425	4.9	0.066	4.7	0.060	4.7
Potassium chloride (50 mM)	0.414	5.1	0.073	6.3	0.064	4.5
Magnesium chloride (50 mM)	0.347	6.1	0.034	12.0	0.019	11.8
Calcium chloride (50 mM)	0.365	5.6	0.033	12.5	0.019	9.5
Sodium citrate (50 mM)	0.436	4.9	0.073	6.8	0.054	7.8
Potassium citrate (50 mM)	0.270	5.0	0.081	5.2	0.071	4.9
Ammonium sulfate (50 mM)	0.314	6.4	0.073	7.6	0.058	6.6
Urea (50 mM)	0.404	4.8	0.075	4.4	0.064	4.0
EDTA (5 mM)	0.093	37.3	0.064	7.1	0.054	5.9
Salts added to seawater						
Potassium chloride (50 mM)	0.241	5.8	0.015	18.1	0.024	8.7
Potassium iodide (50 mM)	0.243	5.6	0.010	28.8	0.025	10.3
Potassium acetate (50 mM)	0.245	5.8	0.017	16.1	0.033	8.6
Potassium nitrate (50 mM)	0.248	5.9	0.016	16.0	0.029	9.7
Potassium bromide (50 mM)	0.199	7.1	0.014	16.6	0.029	9.1
Potassium citrate (50 mM)	0.244	5.4	0.016	22.0	0.023	6.7
Sodium citrate (50 mM)	0.257	5.4	0.025	11.9	0.039	6.9
Urea (50 mM)	0.229	5.8	0.018	13.8	0.031	8.1
EDTA (1 mM)	0.051	25.2	0.014	16.7	0.021	7.7
EDTA (5 mM)	ND[c]	ND	0.029	7.9	0.021	10.9
EDTA (1 mM) + potassium citrate (50 mM)	0.244	5.5	0.016	20.1	0.030	8.3

[a] Concentrations of dyes were 40 nM Hoechst 33342, 30 nM YOYO-1 and 1/1,000 of the stock solution for PicoGreen.
[b] Estimated by the modal position of the G_1 peak normalized to 0.95-μm-diameter fluorescent beads.
[c] ND, not determined.

eter (Coulter, Hialeah, Fla.) equipped with a tunable laser (Coherent Innova 90) emitting at 488 nm for YOYO-1, YO-PRO-1, or PicoGreen and 353 to 357 nm for Hoechst 33342 or DAPI. The excitation and emission properties of each dye and optical configurations used are summarized in Table 1. Some experiments, such as enumeration of cells, were performed with a FACSort flow cytometer equipped with a 488-nm laser. Samples were delivered at a calibrated rate of 10, 28, or 88 μl/min for 1 to 4 min. Chlorophyll fluorescence was collected through a 650-mm long-pass filter, and green fluorescence of the dye-DNA complexes was collected through a 530-/30-mm band pass filter. Fluorescent 0.95-μm microspheres (Polysciences no. 17154) were used as an internal standard. For all analyses, forward and right-angle light scatter and fluorescences were collected by using logarithmic amplifications except for the DNA signal, for which both linear and logarithmic data were recorded. All data were processed with CYTOPC (28).

Enumeration of cells. A flask containing Luria-Bertani medium was inoculated with *E. coli* and incubated at 37°C, and aliquots were collected every 0.5 or 1 h for 8 h. To avoid coincidence, samples were diluted by 100- to 1,000-fold in modified TE buffer. Diluted samples were first enumerated after being stained with DAPI, which was preferred over Hoechst 33342 because it better discriminated *E. coli* during the stationary phase. The results were then compared with those obtained for the YOYO-1, YO-PRO-1, and PicoGreen.

RESULTS

Effect of staining time. The kinetics of staining of YOYO-1, YO-PRO-1, and PicoGreen were analyzed on fixed *P. marinus* cells in TE buffer. Working dye concentrations were determined after preliminary experiments (data not shown) and were 50 nM for YOYO-1 and YO-PRO-1 and 1/1,000 of the stock solution for PicoGreen. The fluorochrome was added just before the sample was run, and the intensity of fluorescence was monitored continuously for 30 min (Fig. 1). Equilibrium was obtained within 5 min for YOYO-1 and less than 2 min for PicoGreen. For YO-PRO-1, the intensity of fluorescence decreased during the first 2 min, and then increased; it stabilized only at the end of the experiment (Fig. 1). Thus, for further tests with all three dyes, both stain and RNase were added at the same time and analyses were done following a 30-min to 2-h incubation. Although samples were left at ambient light levels for the duration of the experiment, direct light exposure had no noticeable quenching effect on the fluorescence yield.

Optimization of stain concentration. Once the optimal staining time was determined, dye concentrations were further optimized by testing a range of concentrations. For Hoechst 33342, the fluorescence intensity and coefficient of variation of the G_1-like peak (G_1 CV; see reference 29 and the discussion below) were constant over a large range of concentrations (0.04 to 160 nM [Fig. 2]). Above 160 nM, the fluorescence intensity decreased dramatically (Fig. 2A), whereas both the CV (Fig. 2B) and the noise increased (data not shown). The noise was estimated by dividing the total number of events recorded by the flow cytometer by the number of cells. YOYO-1 also seemed to exhibit very stable performances in TE buffer (Fig. 2). Well-resolved DNA distributions of *P. marinus* were obtained for concentrations above 2 nM, with G_1 CVs comparable to those obtained with Hoechst 33342. Although we did not observe variations in the intensity of fluorescence emission and CV, the noise due to particles taking up the fluorescent dye increased dramatically beyond 40 nM (data not shown). Like YOYO-1, PicoGreen gave satisfactory results for concentrations above 1/10,000, with systematically better

TABLE 3. CV of the major peak and ratio of G_2 fluorescence to G_1 fluorescence for DNA distributions after staining with the dyes

Organism	YOYO-1		YO-PRO-1		PicoGreen		Hoechst 33342	
	CV (%)	G_2/G_1	CV (%)	G_2/G_1	CV (%)	G_2/G_1	CV (%)	G_2/G_1
Escherichia coli	2.9	1.93	6.1	2.06	4.1	1.96	5.7	1.99
Escherichia coli + rifampin	3.4	1.88	7.1	2.14	1.6	1.95	4.1	1.99
Pyrococcus abyssi	5.5	1.94	NA[a]	NA	4.9	1.94	NA	NA
Cytophaga drobachiensis	4.9	1.97	5.6	1.99	4.6	1.98	7.2	2.02
Prochlorococcus marinus	4.5	1.92	11.5	NA	3.3	1.97	4.8	2.04
Synechococcus strain WH 8103	7.2	1.98	NA	NA	7.7	1.99	14.8	1.89
Pseudomonas alginovora	9.1	1.93	NA	NA	8.2	1.94	15.7	NA
Alteromonas carrageenovora	6.7	1.98	NA	NA	4.2	1.95	9.3	1.93
Salmonella typhimurium	10.1	1.88	NA	NA	9.8	1.87	NA	NA
Deleya aquamarina	12.9	2.01	13.7	1.87	7.8	1.93	16.6	1.83

[a] NA, not applicable.

G_1 CVs than with Hoechst 33342 (Fig. 2) and a G_2-to-G_1 ratio of 2. YO-PRO-1 gave more variable and poorer results than the other dyes. For the whole range of concentrations tested (1 to 100 mM), G_1 CV was always higher than 15%. Moreover, the fluorescence intensity increased continuously with the dye concentration.

Optimization of staining buffer. The first step was to optimize the chemical composition of the staining buffer used to resuspend culture samples. The second step was to test whether cells could be stained in seawater either pure or supplemented with salts or detergents. The latter test aimed at assessing the usability of the new stains for work on natural samples of marine prokaryotes, which, in contrast to cultures, require minimum dilution. In both cases, the effects we sought were an increase in the fluorescence intensity of cells and a decrease in the G_1 CV, without a concomitant increase in the noise from fluorescing particles.

We tested a variety of chemicals with known effects on DNA stains (Table 2). Divalent cations, such as Ca^{2+} or Mg^{2+}, are used as cofactors for chromomycin A or mithramycin (9). In contrast, these cations are deleterious to Hoechst 33342 and DAPI stains, and EDTA is often added to chelate them (9). Urea is a denaturing compound, which prevents the formation of hydrophobic bonds. Therefore, it can be used for its detergent-like effect and has the advantage over more commonly used detergents (e.g., Triton X-100, Tween 20, or deoxycholic acid) that it does not affect the chlorophyll fluorescence. Citrate is a chelator and an anti-oxidant and may increase both the condensation of the double-stranded DNA and the fluorescence signal of Hoechst 33342 or DAPI (9).

Hoechst 33342 gave good results in all buffers but distilled water (Table 2), probably because of its extreme hypotonicity. YOYO-1 and PicoGreen yielded good results in NI and TE buffers but not in distilled water or seawater (Table 2). In the last two, however, PicoGreen was better than YOYO-1. Since the results were about similar with NI and TE buffers, we selected TE buffer, which has a simpler basic composition, for testing the effects of other compounds. Divalent cations proved particularly deleterious to the fluorescence properties of both YOYO-1 and PicoGreen. In contrast, addition of urea to TE buffer had no effect on the G_1 CV. EDTA at 5 mM slightly degraded cell staining (Table 2). The supplier of PicoGreen warns against the deleterious effects of chloride used as a sodium, zinc, or magnesium salt on the fluorescence yield of this dye (12). In our tests, however, potassium chloride did not decrease the quality of the signal (Table 2).

This experiment allowed us to define the best staining buffer for *P. marinus* as being TE (Table 2). A variety of cultured marine prokaryotic strains (eubacteria and archaea) were tested with this buffer supplemented with 0.1% Triton X-100 for the heterotrophic bacteria. Triton X-100 facilitates stain penetration and in some cases increases the fluorescence emission of the dye and improves the CV of the G_1 peak of the DNA distribution. We observed that the addition of 50 mM potassium citrate improved staining. The best results were obtained with YOYO-1 and PicoGreen, with CV as low as 1.6% (Table 3). These two dyes allowed us to distinguish DNA peaks corresponding to cells with different DNA contents (one to four DNA complements), whereas a much poorer resolution was obtained with YO-PRO-1, for example, for the archaeon *Pyrococcus abyssi* or the cyanobacterium *Synechococcus* strain WH8103 (Fig. 3). In all cases, the results obtained with PicoGreen were better than with YOYO-1 or Hoechst 33342. For *E. coli* in the exponential phase, cells with two DNA complements were dominant and the number of cells with one, three, or four complements per cell (1C, 3C, and 4C stages) was low. After incubation of an aliquot of culture taken at the beginning of the exponential phase with rifampin, an antibiotic known to block the initiation sites of DNA replication, the sizes of the 3C and 4C peaks but not the 1C peak increased significantly (Fig. 3). More eukaryotic-like DNA distributions were obtained with the other strains, with clearly defined G_1, S, and G_2 phases. *Pseudomonas alginovora* and *Salmonella typhimurium* displayed DNA distributions with a large number of cells in the S and G_2 phases (Fig. 3), which caused an overestimation of the G_1 CV (Table 3).

Results obtained with seawater were not as satisfactory. For both YOYO-1 and PicoGreen, addition of either potassium salts, citrate, or EDTA appeared to lower significantly the G_1 CVs (Table 2). The best results (CV $\sim$ 7%) were obtained for PicoGreen in seawater complemented with 50 mM potassium or sodium citrate (Table 2). Nevertheless, when tested on natural *Prochlorococcus* populations, these additions were deleterious (data not shown). For an unknown reason, the CVs were much larger (>10%). Moreover, the dyes induced a red fluorescence emission in nonphotosynthetic bacteria, such that *P. marinus* cells could not be discriminated any more (data not shown).

Enumeration of prokaryotic cells. Cultures of *E. coli* were used to test whether YOYO-1, YO-PRO-1, and PicoGreen could be used to enumerate heterotrophic bacteria by flow cytometry. Cell concentrations obtained with the novel dyes were similar to those obtained with DAPI (Fig. 4). Only at the highest cell concentrations were counts with DAPI overestimated, because the presence of abundant debris induced poor discrimination of bacteria from the fluorescent noise (Fig. 4).

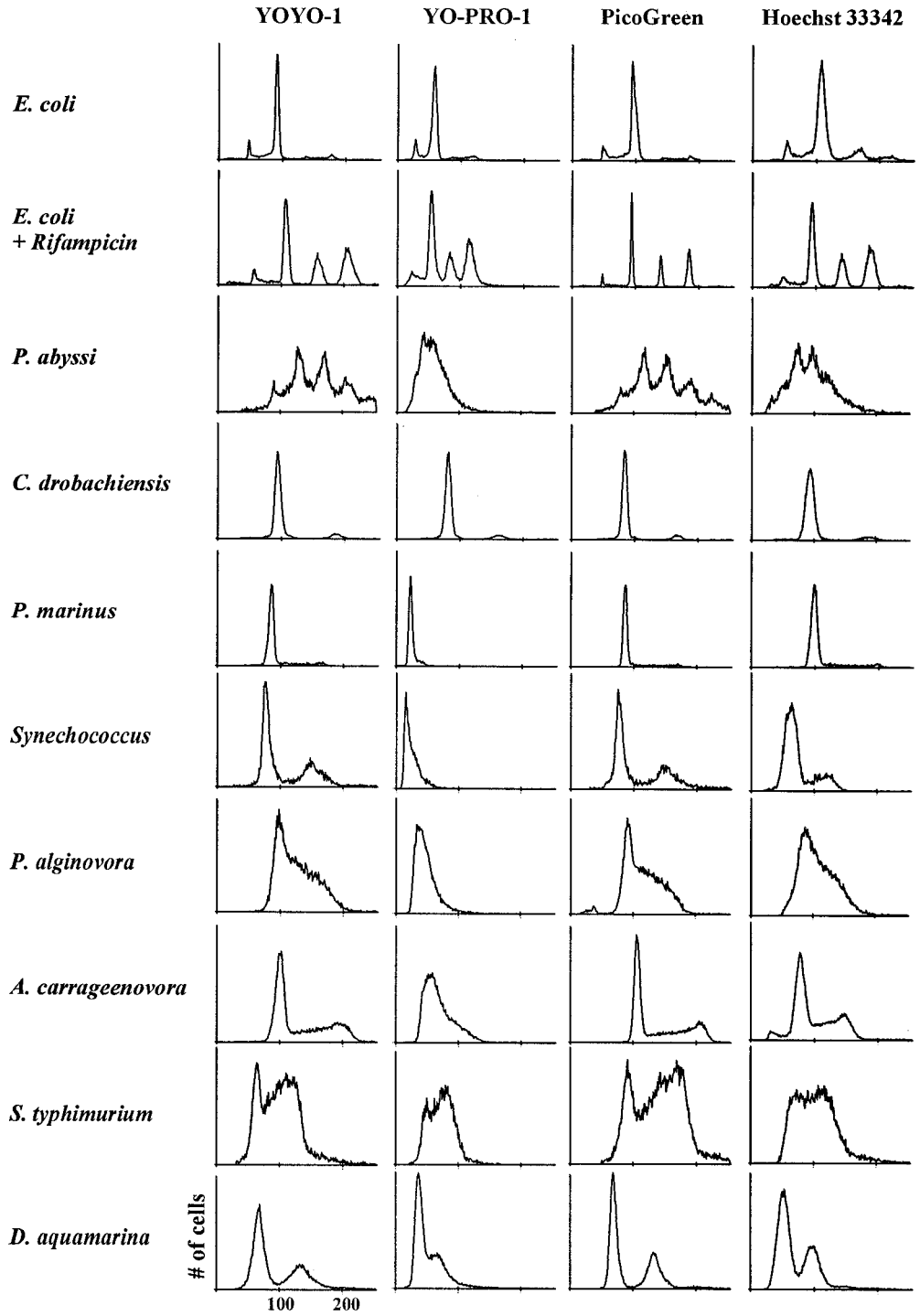

FIG. 3. Histograms of cellular DNA of different prokaryotes after staining with 30 nM YOYO-1, 30 nM YO-PRO-1, 1/1,000 of the commercial solution of PicoGreen, or 40 nM Hoechst 33342. *E. coli* was collected in the early exponential phase. A first aliquot was fixed immediately (first row), and a second was treated with rifampin for 1 h before fixation (second row). *Pyrococcus abyssi* and *C. drobachiensis* were collected in the stationary phase; *P. marinus*, *Pseudomonas alginovora*, and *A. carrageenovora* were collected in the exponential phase. Cells were then fixed and stored at −80°C until analysis. Before staining, *C. drobachiensis*, *P. marinus*, and *Synechococcus* strain WH8103 were resuspended in TE buffer alone, and the other prokaryotic strains were resuspended in a modified TE buffer (see the text).

DISCUSSION

Since the introduction of flow cytometry to marine science at the beginning of the 1980s (36), the applications of this powerful technique have progressively increased because of constant improvements in the sensitivity of optical systems and the progressive reduction in both the size and price of flow cytometers. The most significant advances allowed by flow cytometry in oceanography are certainly the discovery of important marine prokaryotes such as *P. marinus* (8), a better knowledge of

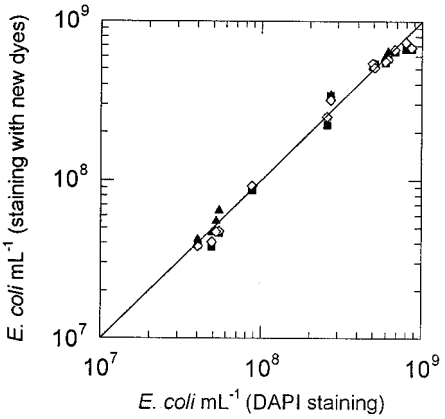

FIG. 4. Comparison between *E. coli* cell concentrations determined by staining with YOYO-1 (■), YO-PRO-1 (▲), or PicoGreen (◇) versus that determined by staining with DAPI. Samples were diluted 100- to 1,000-fold in modified TE buffer before being stained. Plotted numbers are concentrations before dilution. The straight line corresponds to a 1:1 relationship.

the ecological distributions of both heterotrophic and phototrophic microorganisms (see reference 22 for a review), and the possibility to derive in situ growth rates of important species from cell cycle analysis (31). The recent development of a large variety of nucleic acid-specific stains has potential application for the analysis of DNA and cell cycles of marine microorganisms by flow cytometry, even though they were originally designed for the detection of small amounts of nucleic acids on electrophoretic gels.

The main goal of our study was therefore to test the usability of some of these novel fluorochromes for marine prokaryotes. Since small flow cytometers possess a single laser tuned at 488 nm, we selected dyes with optimal excitation close to this wavelength. Another constraint was, in the case of photosynthetic prokaryotes, to choose dyes with emission properties that did not interfere with their pigment (mainly chlorophyll) emission. Finally, we required dyes that could work on fixed cells, since most oceanic samples have to be preserved prior to analysis on shore. In a preliminary screen, we eliminated viable dyes of the SYTO family (Molecular Probes), since they only work on live cells, which prevents the use of RNase, often necessary to obtain the high resolution needed for the cell cycle analysis. The three selected dyes were YOYO-1 (an oxazole yellow dimer), YO-PRO-1 (its analog monomer), and PicoGreen. Observations under an epifluorescence microscope showed that YOYO-1 and YO-PRO-1 were more resistant to photobleaching than PicoGreen (20a). Several other candidates might have been retained, in particular, TOTO-1 and TO-PRO-1 (15).

All three selected stains bind to both RNA and DNA, but, in contrast to SYTO, these dyes can be used on fixed material such that RNA can be selectively eliminated by an RNase treatment. Their kinetics of staining are very rapid. For cell cycle analysis, incubation length therefore depends only on the kinetics of degradation of RNA by the RNase. Like intercalating dyes such as propidium iodide or ethidium bromide, the binding affinity of these new dyes decreases significantly with ionic strength and in extremely hypotonic buffers. Addition of cofactors such as potassium, citrate, EDTA, or urea both improved the resolution of DNA distributions and increased the intensity of stain emission. The best buffer for all three dyes seemed to be either TE (*P. marinus*, *C. drobachiensis*, *Synechococcus* sp.) or modified TE with 50 mM potassium citrate and 0.1% Triton X-100 added (other strains tested). For several reasons, including large CVs on DNA distributions and the absence of saturation of fluorescence at high dye concentrations, YO-PRO-1 gave much poorer results and cannot be recommended for whole cell DNA analyses. PicoGreen gave slightly better CVs than YOYO-1 in all cases except for *E. coli* in the exponential phase. However, the absence of information on the chemical nature and molecular weight of the PicoGreen constitutes a drawback for the understanding of its mode of linking to the DNA molecules.

For the rapidly growing organisms *E. coli* and *P. abyssi*, both YOYO-1 and PicoGreen allowed us to resolve DNA peaks corresponding to one, two, three, or four copies of chromosome more efficiently than Hoechst 33342 did. For the slowly growing microorganisms *C. drobachiensis* and *P. marinus*, these dyes revealed an eukaryotic-like DNA distribution with phases similar to the G_1, S, and G_2 phases, as described previously (32). Noteworthy is the good resolution of the cell cycle distribution of *Synechococcus* strain WH8103, despite the presence of phycoerythrin, which emits at ca. 580 nm. These data suggest that these two dyes can be very useful for the cell cycle analysis of marine prokaryotes (3, 17), providing that the staining buffer is optimized for organisms not yet tested. Both dyes are also satisfactory for the flow cytometric enumeration of prokaryotic cells in culture (24).

Because of their sensitivity to the ionic strength and their possible interaction with some unknown compound(s) of seawater, both YOYO-1 and PicoGreen seem inappropriate for the analysis of natural samples, although Li et al. (18) recently developed a flow cytometric method to enumerate bacterial cells in seawater with TOTO-1 and TO-PRO-1, using Triton X-100 to permeate the cells. Their method, however, suffers from two drawbacks. First, the destruction of chlorophyll by the detergent prevents the discrimination between autotrophic and heterotrophic prokaryotes. Second, the adverse effect of divalent cations does not allow us to distinguish the different cell cycle phases. We are currently exploring the use of other dyes developed by Molecular Probes, which appear more suitable for natural samples (20a).

YOYO-1 and PicoGreen display unique characteristics such as visible-wavelength excitation, high fluorescence yield, low working concentrations, and excellent resolution of cell cycle distributions that should make them very useful for flow cytometric analyses of marine (and also probably nonmarine) prokaryotes in culture by using small, low-cost flow cytometers.

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