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DNA distributions in planktonic bacteria stained with TOTO or TO-PRO

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Abstract

The blue-excitable nucleic acid-binding fluorochromes, TOTO and TO-PRO, were used to stain bacteria in marine plankton communities. Flow cytometric counts of cells stained with these dyes agreed well with counts made by epifluorescence microscopy of DAPI-stained cells. We have used these new dyes in a study of bacteria from various waters in the North Atlantic and the eastern Mediterranean. The results indicate a distinction among bacterial groups of different fluorescence intensities (apparent DNA content). At large spatial and temporal scales, about half of the variation in the percentage of bacteria with high apparent DNA content (termed group II bacteria) could be explained by the variation in chlorophyll. Further, the apparent mean DNA content of group II bacteria was also correlated with chlorophyll.

Bacteria are important in marine food webs, and a quantification of their abundance is de rigueur in modern biological oceanography. Epifluorescence microscopy of fluorochrome-stained cells is the standard method by which bacteria are enumerated. However, precision, speed, and ease of analysis are greatly improved when the enumeration is performed electronically by flow cytometry or image analysis. Furthermore, electronic enumeration affords a characterization of heterogeneity in mixed population assemblages based on a large number (up to tens of thousands) of cells.

Although nucleic acids can be stained by many dyes, recent flow cytometric analyses of marine bacteria have mainly relied on only two: DAPI (4′,6-diamino-2-phenylindole) and HO342 (Hoechst 33342, a bisbenzamide derivative) (Robertson and Button 1989; Monfort and Baleux 1992; Monger and Landry 1993). Both of these dyes require excitation by ultraviolet light and are thus not suitable for use in many flow cytometers restricted to visible-light excitation, which is commonly at 488 nm.

Here, we show that two blue-excitable dyes derived from thiazole orange can be used to stain the DNA of bacteria in marine plankton communities. These stained bacteria are not only counted by flow cytometry, they are also characterized by relative size and relative apparent DNA content. In this paper, we report our application of these dyes to bacteria from various waters in the Atlantic Ocean and Mediterranean Sea. Our results indicate an effective new method for bacterial enumeration and also emphasize a distinction among bacterial groups of different apparent DNA content. This distinction was correlated with chlorophyll.

Acknowledgments

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Methods

Sample collection and storage—Seawater was collected by Niskin bottles from many locations on separate cruises (*see Fig. 6A*): the Labrador Sea and the Grand Banks during May–June 1994; from an east–west transect of the North Atlantic during October 1992; from the eastern Mediterranean Sea by Zohary and Yacobi during October–November 1991 and March 1992; on a transect from Delaware to the Sargasso Sea by Geider in April 1995; and regularly from Bedford Basin, which is a eutrophic coastal site (44°41.3′N, 63°38.3′W). Standard hydrographic information (SeaBird CTD) and bulk phytoplankton chlorophyll measurements (fluorometric assay of acetone extracts) accompany each sample.

For flow cytometry, the plankton samples were placed in 2-ml capacity polypropylene cryogenic vials, fixed in 1% glutaraldehyde (1991–1992 samples) or 1% paraformaldehyde (post-1992 samples) for 10 min at room temperature, then frozen and stored at −70°C (Vaulot et al. 1989; Monger and Landry 1993). For epifluorescence microscopy, plankton samples were placed in 20-ml capacity borosilicate scintillation vials, fixed in 2% Formalin, then stored at +4°C.

Epifluorescence microscopy—The preserved sample was held over a polycarbonate membrane (0.2-μm black Nuclepore) underlain by a prewetted backing filter (Sartorius No. 11306) which aids in distributing cells in an even manner on the Nuclepore surface. Cells were stained for 10 min with DAPI (Porter and Feig 1980) at a final concentration of 2 μg ml^{-1} and then drawn through the filters at a vacuum pressure of <150 mm of Hg. The Nuclepore filter was examined by epifluorescence microscopy (Leitz Orthoplan) under ultraviolet excitation from a mercury arc lamp (HBO 100W).

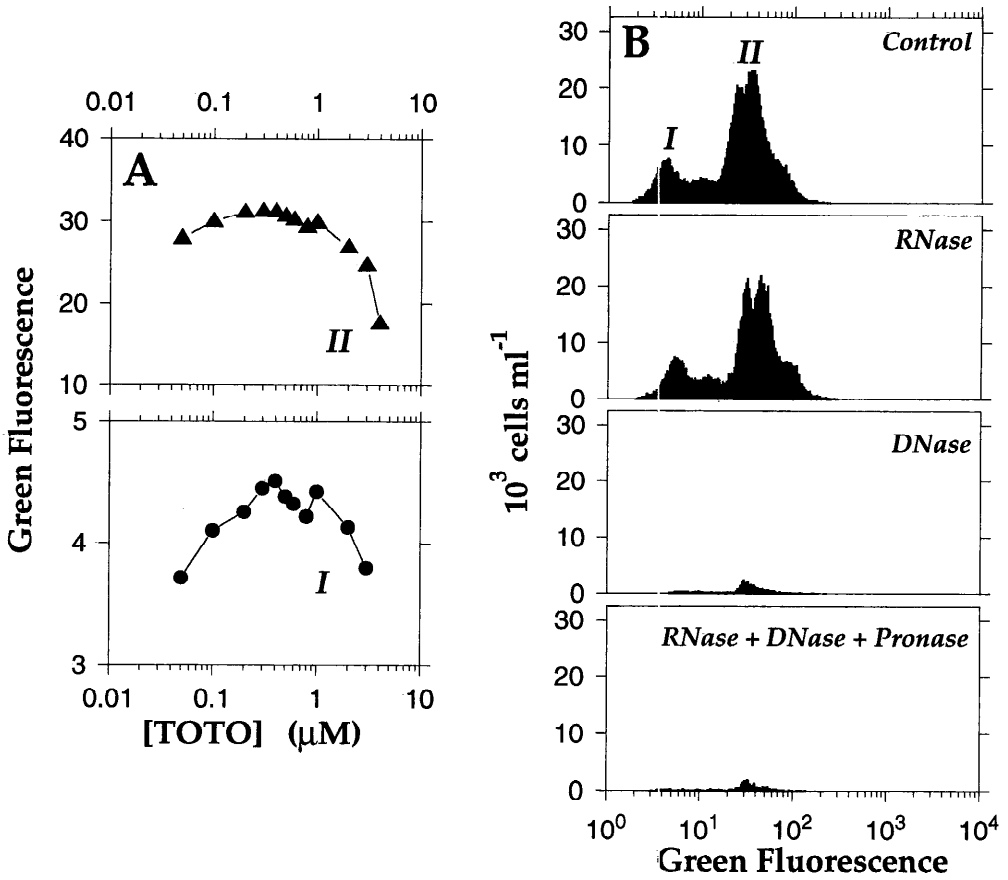

Fig. 1. A. Mean fluorescence intensity in two groups of bacteria (I and II) vs. concentration of TOTO. B. Enzyme digestion of Triton-treated sample from Bedford Basin. The control received filtered seawater; RNase was a mixture of RNase A and RNase T_2. Cells were stained with TOTO after enzyme treatments.

Staining with cyanine dyes—The membrane-impermeant fluorochromes TOTO-1 iodide and TO-PRO-1 iodide were from Molecular Probes, Inc. TOTO is the homodimer of thiazole orange; TO-PRO is a monomeric thiazole orange terminated with a propylammonium side chain. The fluorochromes yield negligible fluorescence in solution but have strong binding affinities to double-stranded DNA (Rye et al. 1992, 1993; Hirons et al. 1994).

Frozen preserved plankton sample was quickly thawed to room temperature. An aliquot was added to a polystyrene tube containing the staining mixture—Triton-X100 (0.1% final) together with either TOTO (0.3 or 0.5 μM final) or TO-PRO (3 or 5 μM final). The sample was vigorously vortex mixed, then incubated in the dark at room temperature for at least 10 min before flow cytometric analysis. The stain concentrations used were those that optimized fluorescence in tests of Bedford Basin bacteria (Fig. 1A). The intensity of fluorescence was stable for at least 8 h at room temperature.

We tested TOTO staining of Triton- (0.1%) permeabilized cells after they had been digested (37°C, 60 min) by the following enzymes, either singly or in combination: RNase A (type 1A, Sigma R-4875) at 400 Kunitz units ml^{-1}, RNase T_2 (Sigma R-3751) at 32 units ml^{-1}, DNase I (type 4, Sigma D-5025) at 2,000 Kunitz units ml^{-1}, and Pronase E (Sigma P6911) at 50 μg ml^{-1} for the possibility of dye-restricting nucleoproteins. Ribonuclease A was rendered free of DNase by incubation at 95°C for 10 min before use on samples.

We find TOTO and TO-PRO to be equivalent in their staining characteristics of marine bacteria. TOTO is more cost-effective to use than TO-PRO because detectable green fluorescence is obtained at a tenth of the concentration.

Flow cytometry—Samples were analyzed in a FACSort (Becton Dickinson) with excitation at 488 nm which is close to the excitation maximum for TOTO-1 and TO-PRO-1 (514 nm). Sample was delivered at a calibrated rate, generally 12 μl min^{-1}, typically for 200 s, but for less time when bacterial concentrations were high. Green fluorescence from bound stain was collected in the FL1 channel (530 ± 15 nm). The electronic circuitry that corrects for the overlap of fluorescence across instrument channels, so-called compensation (Phinney and Cucci 1989), was not invoked, leading to measurable orange

and red fluorescence originating from the dyes whose peak emission is 531–533 nm in the green waveband. In this study, fluorescence intensity was not calibrated against absolute standards. Values are reported in relative units that depend on voltages applied to the photomultiplier tubes. The voltage setting for TO-PRO analysis was different from that for TOTO analysis because of signal-to-noise considerations. For calibration in a relative sense, calf thymocyte nuclei (Riese Enterprises) were stained with TO-PRO. The ratio of green fluorescence intensity between populations of 2n and 1n genome size was 1.98. Although we do not know whether the staining of these nuclei is representative of the staining of marine bacteria, we assume that the ratio of fluorescence was essentially equivalent to the ratio of DNA content.

Separate analyses were also made on unstained plankton samples (Olson et al. 1993) to characterize and enumerate the phytoplankton, especially *Prochlorococcus,* whose red autofluorescence from chlorophyll *a* was collected in the FL3 channel (>650 nm). As results will show, *Prochlorococcus* confounds the green fluorescence of stained heterotrophic bacteria. For all analyses, peak height measurements of fluorescence and light scatter were collected by logarithmic amplification and recorded in relative units in a four-decade range spanned by 256 channels. Initial inspection of data was performed with LYSYS II software (Becton Dickinson) or WinMDI (Joseph Trotter). Bivariate curve-fitting to a sum of lognormal distributions (Li 1990) was used to extract population statistics from overlapping clusters.

Growth experiment—A plankton sample was taken from 1 to 5 m in Bedford Basin on 8 February 1995. Plankton were diluted (0.1 v/v) with freshly filtered seawater (<0.22-μm Millipore membrane) to initiate growth under reduced grazing pressure. The diluted sample was incubated in a sterile polypropylene 50-ml tube (Falcon 2098) at room temperature (~19°C above ambient) and in the dark for 14 h. At hourly intervals, a 2-ml subsample was preserved and stored for subsequent analysis.

Results

Staining characteristics—Bedford Basin bacteria sampled on different days were tested for staining characteristics after enzymatic digestion. Results of a representative experiment (Fig. 1B) indicate that the green fluorescence of TOTO-stained bacterial assemblages was almost completely eliminated by prestaining digestion with DNase. Conversely, the fluorescence was almost completely unaffected by prestaining digestion with RNase, except perhaps for a marginally higher degree of resolution in the major staining peaks. A small percentage (3%) of particles appeared unaffected by any of the tested enzymes.

Cytometric signatures—Bacteria coexist with phytoplankton, many of which are only slightly larger in size but all of which are also stained by the dyes. Phytoplankton therefore confound the cytometric analysis of bacteria. In principle, the red autofluorescence of chlorophyll could be used to exclude phytoplankton. In practice (results not shown), treatment of cells with Triton (necessary for permeation of the dye) reduced the red autofluorescence of phytoplankton so that for *Prochlorococcus* (which is weakly fluorescent), red autofluorescence became an ineffective criterion by which to make a distinction against heterotrophic bacteria. It was therefore prudent to know whether *Prochlorococcus* was present in each sample. This was a relatively simple requirement, which we met by separate autofluorescence analysis of Triton-free, unstained samples.

When *Prochlorococcus* was not present, the recognition of stained heterotrophic bacteria was straightforward because other small phytoplankton (*Synechococcus* and eucaryotic picoalgae) could be recognized by red fluorescence even when they had been treated with Triton (Fig. 2A). In analyzing dye fluorescence vs. light scatter plots from which phytoplankton had been excluded (Fig. 2B), distinct but overlapping clusters of cells were always evident, with the possible implication (discussed later) that these were groups of bacteria differing in DNA content and size. For now, we have labeled the groups I (low DNA) and II (high DNA). Independent of the nature of these groups, parameters of interest could be extracted by bivariate curve-fitting on the assumption that each group was distributed in a log-normal fashion with respect to both fluorescence and light scatter (Fig. 2C,D). In this paper, we focus on two of these parameters: the number of cells and the mean intensity of green fluorescence.

When *Prochlorococcus* was present, its cytometric signature after TO-PRO staining (Fig. 3A,B) or TOTO staining (Fig. 4A,B) was not unambiguously distinct from that of the bacteria. However, a notable difference was the weak degree of correlation between green and red fluorescence for *Prochlorococcus* vs. the strong degree of correlation for bacteria. In the case of *Prochlorococcus,* the red fluorescence was due mainly to chlorophyll (although reduced by Triton treatment); in the case of bacteria, red fluorescence was simply the "uncompensated" overlap from green fluorescence. We have relied on bivariate curve-fitting to yield the distribution parameters for group I and II bacteria as well as *Prochlorococcus* (Figs. 3C,D, 4C,D). By this means, it was possible to exclude *Prochlorococcus* from subsequent data analyses related solely to bacteria. The use of curve-fitting to separate overlapping groups was limited to cases in which *Prochlorococcus,* when present, was abundant enough to form a recognizable cluster. Results presented here pertain to these cases. In any case where *Prochlorococcus* occurs, whether in high or low abundance, its contribution to the overestimate of heterotrophic bacteria is easily removed by subtraction of its count made on a Triton-free, unstained replicate.

The distinction of bacteria into two groups is an obvious simplification because there was evidence of more heterogeneity. In other words, the frequency distribution of stained bacteria often displayed more than two modes (e.g. *see Figs. 1B, 8*). Further, Figs. 3C and 4C show the inadequacy of a three-component model (i.e. groups I, II

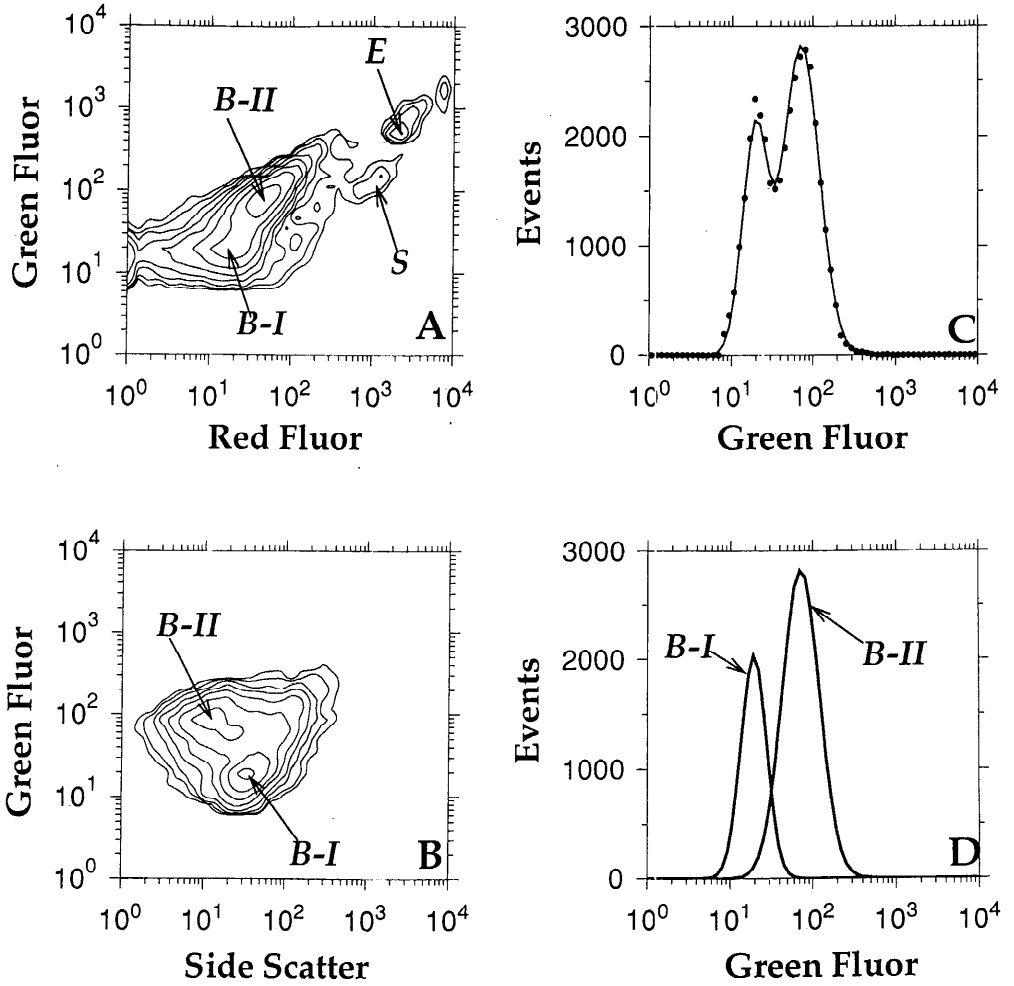

Fig. 2. TO-PRO staining of a plankton sample not containing *Prochlorococcus* from 20 m at 31°13.97′N, 10°51.17′W on 5 October 1992. A. Flow cytometric analysis of green vs. red fluorescence showing heterotrophic bacteria group I (B-I), heterotrophic bacteria group II (B-II), *Synechococcus* (*S*), and eucaryotic picoalgae (E). Contours indicate cell abundance at levels of 2^n, where n is an integer incrementing by 1 starting at 1. B. List-mode analysis showing green fluorescence vs. side scatter for cells excluding *S* and E. Contour levels as in panel A. C. Curve-fit of data in panel B to a sum of two bivariate log-normal distributions by the method of Li (1990). Plot shows the univariate projections of measured data (●) and least-squares fit onto the green fluorescence axis: $U(y) = \sum_x [F_I(x, y) + F_{II}(x, y)]$ where x is side scatter, y is green fluorescence, F_I is the bivariate log-normal distribution for group I, F_{II} is the bivariate log-normal distribution for group II, and U is the projected histogram. D. $\sum_x F_I(x, y)$ and $\sum_x F_{II}(x, y)$.

bacteria and *Prochlorococcus*) to account for a slight right shoulder occurring at midrange of the fluorescence distribution.

Comparison of counts—Counts made by epifluorescence microscopy of DAPI-stained cells were highly correlated with those made by flow cytometry of cells stained with TOTO ($r^2 = 0.83$, $n = 62$) or with TO-PRO ($r^2 = 0.93$, $n = 32$) (Fig. 5). Model 2 regression of cytometric vs. microscopic counts yielded slopes (±95% C.L.) of 0.89±0.09 and 0.96±0.10 and Y intercepts (±95% C.L.) of 0.04±0.3 and 0.03±0.2 × 10^6 cells ml^{-1} for TOTO and TO-PRO respectively. In other words, slopes were ~1 and intercepts were ~0. In these comparisons, *Synechococcus* and eucaryotic algae were excluded from the cyanine dye counts. However, *Prochlorococcus*, when present, was included on the presumption that DAPI microscopy of "bacteria" included both the heterotrophs and *Prochlorococcus* (Campbell et al. 1994).

Survey of various waters—In the Labrador Sea, on the Grand Banks, and on the continental shelf and slope off

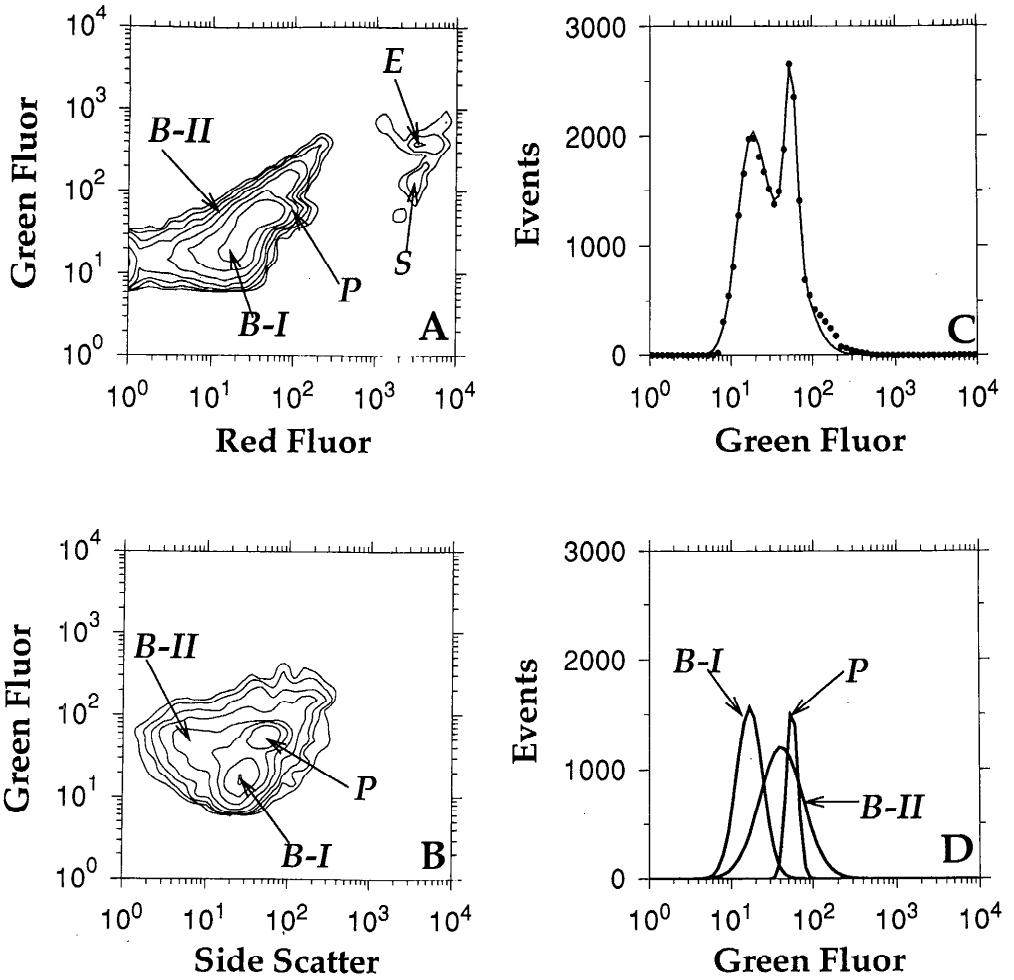

Fig. 3. TO-PRO staining of a plankton sample containing *Prochlorococcus* (*P*) from 80 m at 31°29.99′N, 11°0.00′W on 3 October 1992. A,B. As in Fig. 2A and B. C. Curve-fit of data in panel B to a sum of three bivariate log-normal distributions by the method of Li (1990). As in Fig. 2, except $U(y) = \sum_x [F_I(x, y) + F_{II}(x, y) + F_P(x, y)]$ where F_P = bivariate log-normal distribution for *Prochlorococcus*. D. $\sum_x F_I(x, y)$, $\sum_x F_{II}(x, y)$, and $\sum_x F_P(x, y)$.

Delaware, the waters were demonstrably free of *Prochlorococcus* (data not shown). The group II bacteria averaged 75% of all heterotrophic bacteria in the aforementioned subarctic regions and 65% off Delaware.

In the central North Atlantic gyre, where surface concentrations of *Prochlorococcus* were on the order of 10^4–10^5 cells ml^{-1} (Li 1995), these phytoplankters represented ~11% of the total number of TOTO-stained cells. We subtracted the contribution of *Prochlorococcus* to TOTO-stained cells and found that the group II bacteria averaged 42% of heterotrophic bacteria across the 1992 trans-Atlantic section. The eastern end of our section was at the nutrient-rich upwelling site off Morocco. Exclusion of the Moroccan data lowered the average for group II bacteria to 36% of heterotrophic bacteria in the oligotrophic central North Atlantic.

In the eastern Mediterranean Sea, which is extremely oligotrophic, *Prochlorococcus* cells at the surface were on the order of 10^3–10^4 cells ml^{-1} (Li et al. 1993) where, on average, they comprised only 2% of the TOTO-stained bacterial-sized cells. Group II bacteria averaged 35% of total bacteria here.

A significant correlation ($r^2 = 0.52$, $n = 54$, $P < 0.01$) existed between the relative composition of heterotrophic bacteria and the standing stock of chlorophyll (Fig. 6B). In other words, in phytoplankton-rich waters, the majority of bacteria were group II; conversely, in phytoplankton-poor waters, the majority of bacteria were group I. However, as indicated by the value of r^2, only slightly more than half of the bacterial variation could be explained on the basis of chlorophyll variation. The mean apparent DNA content of group I varied weakly in the waters surveyed ($r^2 = 0.10$, $n = 54$, $P > 0.01$), but that of group II increased strongly with chlorophyll biomass ($r^2 = 0.45$, $n = 54$, $P < 0.01$) (Fig. 6C). The resultant ratio of mean apparent DNA content between groups II

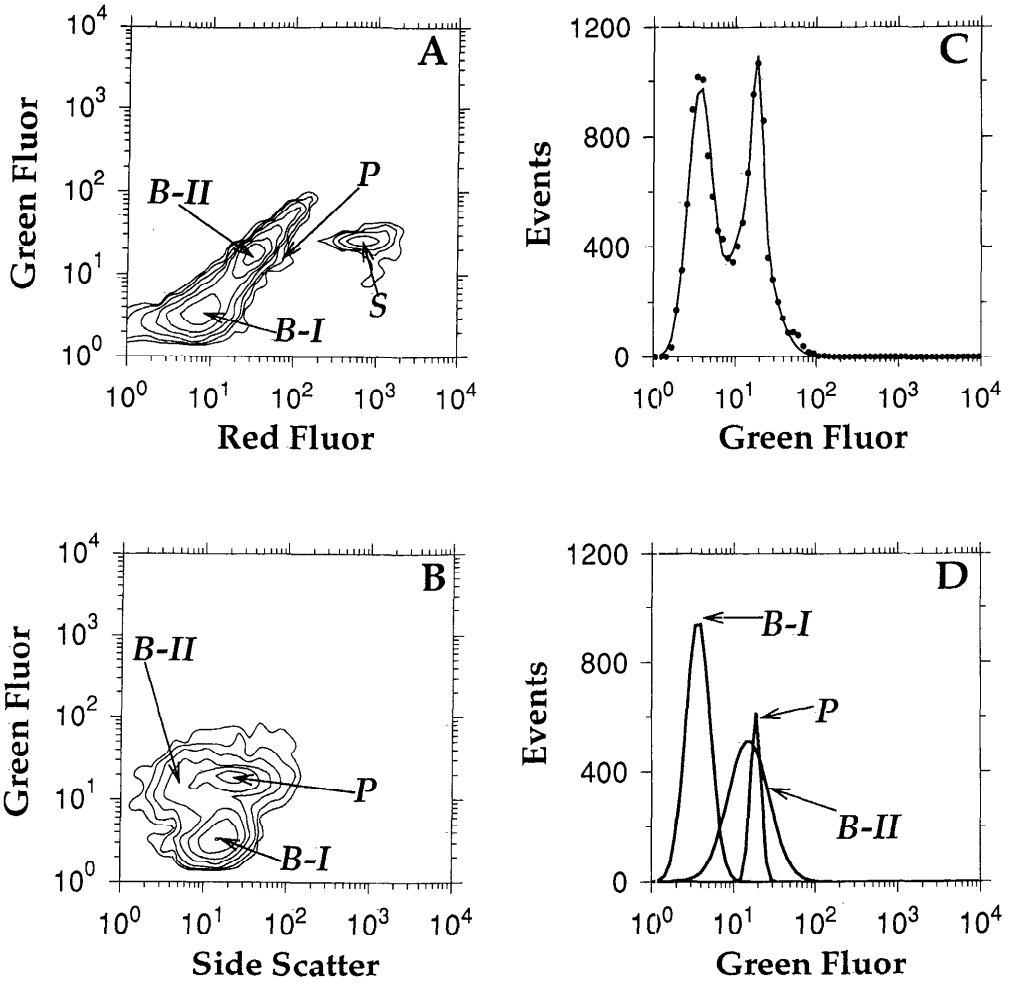

Fig. 4. TOTO staining of a plankton sample containing *Prochlorococcus* (*P*) from 1 m at 36°15.24'N, 72°17.76'W on 3 April 1995. A,B. As in Fig. 2A and B. C,D. As in Fig. 3C and D.

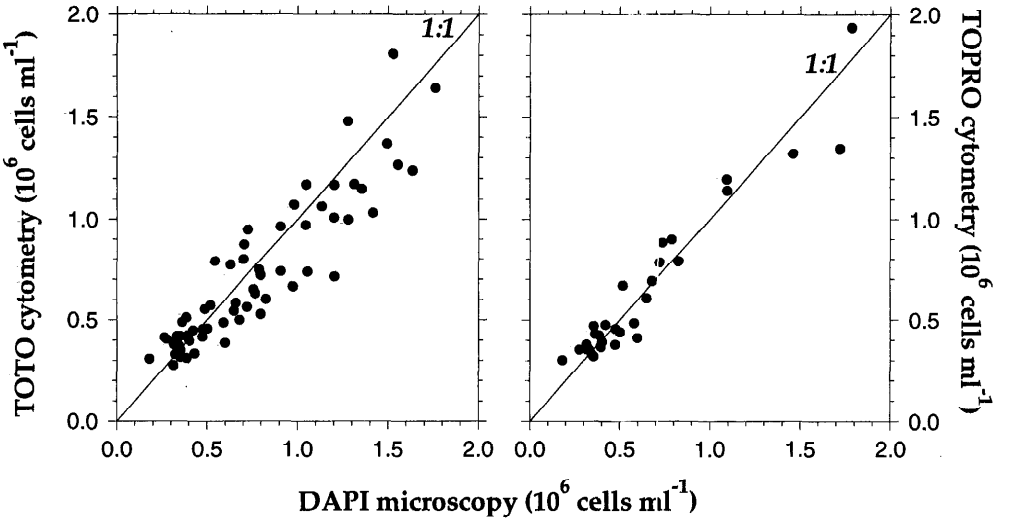

Fig. 5. Counts made by epifluorescence microscopy of DAPI-stained cells vs. those made by flow cytometry of cells stained with TOTO (left) and TO-PRO (right).

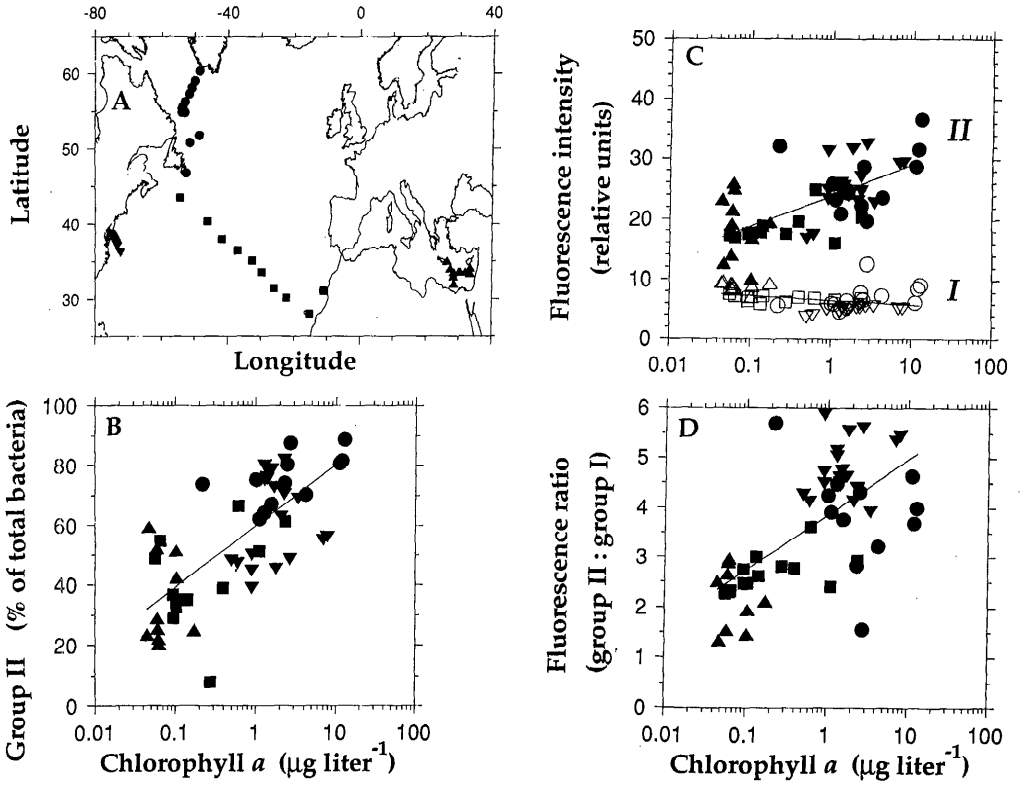

Fig. 6. Correlations with chlorophyll concentration. A. Sampling locations. B. Percentage of TOTO-stained heterotrophic bacteria that belong to the heterogeneous high apparent DNA group II. C. Mean intensity of green fluorescence from group II (closed symbols) and group I (open symbols) bacteria. D. Ratio of fluorescence between groups II and I. Data from Labrador Sea-Grand Banks (● ○), from the Central North Atlantic including the Moroccan upwelling (■ □), from the eastern Mediterranean Sea (▲ △), and on a transect from Delaware to the Sargasso Sea (▼ ▽). All lines through data are based on linear regression.

and I increased from 2 in "blue waters" of the Mediterranean to >5 in "green waters" of the Labrador Sea and the U.S. continental shelf ($r^2 = 0.40$, $n = 54$, $P < 0.01$) (Fig. 6D).

Seasonal variations—Through two annual cycles at a eutrophic coastal site containing no *Prochlorococcus*, group II cells were the dominant heterotrophic bacteria. A seasonal variation in the relative composition of bacteria was discernible: in each year, the contribution from group II cells was lowest in summer months (Fig. 7). On average, the ratio of mean apparent DNA content between groups II and I was ~5; this ratio ranged from 2 to 7 through the period of observation.

Growth experiment—From Bedford Basin, group II bacteria grew more than 3 times as fast as group I bacteria (2.9 vs. 0.9 doublings d^{-1}) (Fig. 8). The heterogeneity within group II was clearly apparent in that of two subpopulations differing in light scatter (labeled a and b); the one with higher mean light scatter (IIb) grew 3 times as fast as the other (4.0 vs. 1.3 doublings d^{-1}).

Discussion

The analysis of biological communities on an individual cell basis allows for a partitioning of the bulk properties of seawater on the basis of component organisms. The opportunities afforded by modern cytometric technologies make such partitioning a realistic goal. For phytoplankton, first-order analysis by flow cytometry is straightforward because of autofluorescence from photosynthetic pigments. For heterotrophic bacteria, first-order analysis by flow cytometry proceeds from staining a universal cell constituent; most often, DNA is chosen. The selection of a suitable fluorochrome depends on several considerations (Boucher et al. 1991), and others have often chosen DAPI or Hoechst and less often acridine orange or mithramycin with ethidium bromide. None of these were suitable for our work. We required a fluorochrome that could be excited by a low-power (15 mW) 488-nm argon laser. At the same time, the fluorochrome needed to have binding properties such that the emitted fluorescence would be detectable in oligotrophic marine bacteria that have DNA content in the femtogram range

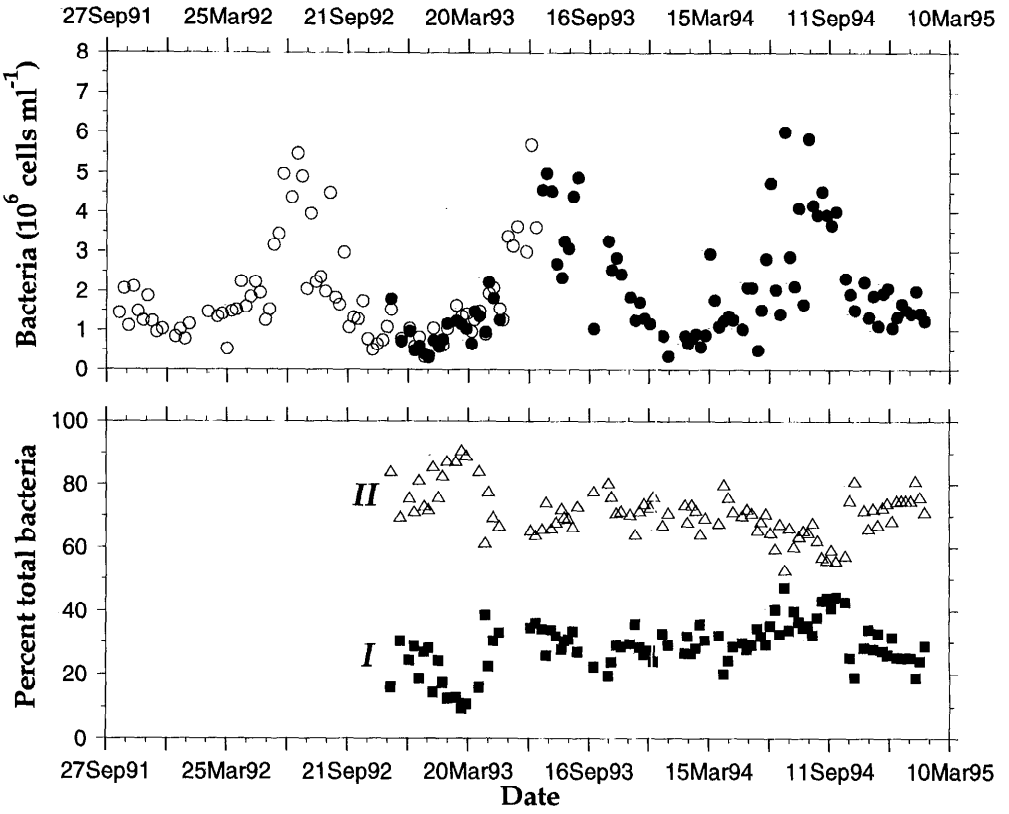

Fig. 7. Bacteria in Bedford Basin. Upper panel shows cell concentration estimated by DAPI microscopy (○) and TOTO cytometry (●). Lower panel shows contribution to total from low DNA group I (■) and high DNA group II (△).

(Robarts and Zohary 1993). Hennes and Suttle (1995) recently used YO-PRO, a cyanine dye based on oxazole yellow, to enumerate aquatic viruses. We chose TOTO and TO-PRO because the fluorescence emission of these stains was better matched to the optical configuration of our instrument. With TOTO and TO-PRO, we have a simple and effective new method for bacterial enumeration by benchtop flow cytometric analysis of suitably preserved samples (Fig. 5).

TOTO and TO-PRO bind to nucleic acids by intercalation (Rye et al. 1992), and spectrofluorometric data on dye-polynucleotide complexes indicate that there is no significant preferential base specificity (Hirons et al. 1994). In contrast, both DAPI and Hoechst complex by nonintercalative binding, specifically to AT base pairs (*see* Monger and Landry 1993). The nonspecificity of cyanine dye binding is advantageous because it allows detection of all bacteria regardless of genomic base composition. On the other hand, TOTO and TO-PRO also bind to RNA, which is undesirable in a DNA-staining procedure. The protocol for sample fixation, storage, staining, and analysis and also the instrument configuration for fluorescence excitation and emission are all important factors that influence the detection of bound-stain fluorescence. Under our experimental conditions, we found no significant effect of RNase digestion on the detection and quantification of marine bacteria (Fig. 1B). This result and the complementary one from the DNase digestion (Fig. 1B) suggest that our method provides information on DNA distribution. The following differences have been demonstrated between TOTO and TO-PRO bound to dissolved DNA vs. bound to dissolved RNA: a red shift of 10 nm in the fluorescence emission for dye RNA (Hirons et al. 1994) and lower fluorescence enhancement for dye RNA (Rye et al. 1993). The extent to which these factors contribute to the seeming RNA insensitivity of our method is not clear.

Detection of bacteria in flow cytometric analyses of TOTO- or TO-PRO-stained plankton is relatively straightforward in waters without *Prochlorococcus*. Eucaryotic algae and *Synechococcus* are usually recognizable in cytometric clusters distinct from the large clusters of stained bacteria or can be excluded on the basis of red fluorescence even when red fluorescence is reduced by Triton treatment (Fig. 2). When *Prochlorococcus* is present, its green fluorescence and light scatter overlap with those of the bacteria. We presume that other species of extremely abundant but very small phytoplankton, such as *Ostreococcus tauri* (Courties et al. 1994), which we have not observed, might interfere in the same manner. In future work, we intend to invoke electronic compensation to reduce the overlap of green stain fluorescence into the red fluorescence channel. This might leave much of the red fluorescence to the phytoplankton, including *Pro-*

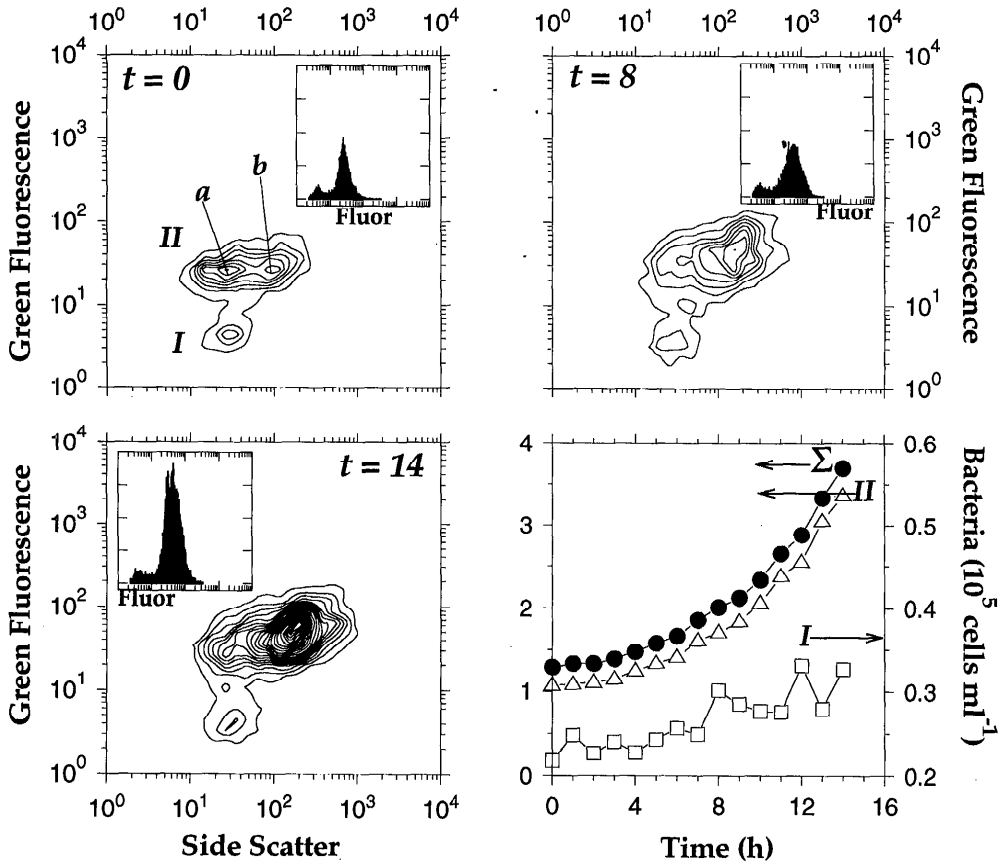

Fig. 8. Growth of Bedford Basin bacteria diluted 1 : 10. Green fluorescence (TOTO) vs. side-scatter contour plots, and projected fluorescence histograms are shown for bacteria sampled at 0, 8, and 14 h. At least three bacterial groups are apparent: a low DNA group I; a high DNA-low scatter group IIa; a high DNA-high scatter group IIb. Contours indicate cell abundance at levels of $5n$ where n is an integer incrementing by 1 starting at 1. In the time-course plot, the growth curve identified with group II is the sum of IIa and IIb. The ordinate axis for group II and Σ = group II + group I is on the left; the ordinate axis for group I is on the right.

chlorococcus. If so, red fluorescence would serve as an effective criterion for the exclusion of all phytoplankton from bacterial analysis.

In the present study, we have shown how bivariate curve-fitting can be used to account for *Prochlorococcus* when its numbers are sufficient to form a recognizable overlapping cluster (Figs. 3, 4). Alternatively, a count of *Prochlorococcus* in a separate, unstained replicate can be used for subtraction from the total number of TOTO-stained bacterial-sized cells to give the number of heterotrophic bacteria. Our results from the central North Atlantic (11%), those of Campbell et al. (1994) from the central North Pacific (31%), and those of Binder et al. (1995) from the equatorial Pacific (17%) indicate that a significant percentage of bacterial-sized DNA-staining cells are in fact *Prochlorococcus*. In summary, cytometric and microscopic counts agree at a high level of confidence (Fig. 5). In comparing methods, the uncertainty is constrained by the variance of replicate microscopic counts (C.V. ≈ 15%), which is much higher than that of cytometric counts (C.V. < 5%).

It is clear that marine bacteria in plankton communities differ in the degree to which they emit green fluorescence when they are stained by TOTO and TO-PRO. The question remains whether this heterogeneity in fluorescence indicates a heterogeneity in DNA content or a difference in the effectiveness of staining DNA of different cells. Our distinction of stained bacteria into two groups is made without knowing whether the differences are based on physiology or taxonomy. For a given clonal entity, various circumstances give rise to differences in DNA content, conformation, topology, or stain accessibility—all of which would influence the degree of emitted fluorescence. These circumstances depend, among others, on the cycle of DNA replication and cell division (Steen and Boye 1980; Kell et al. 1991), on the availability of essential nutrients (Thorsen et al. 1992; LeBaron and Joux 1994), on the susceptibility to programmed cell death (Yarmolinsky 1995), and on the nucleoid state of the DNA (Zweifel and Hagström 1995). It is also clear that heterogeneity in DNA content can be expected because of taxonomic diversity in marine bacterioplankton

(Giovannoni and Cary 1993; Fuhrman et al. 1994). In other marine cytometric studies using DAPI or Hoechst, subpopulations of bacterial-sized particles have also been noted or may be evident in the results (Robertson and Button 1989; Campbell et al. 1994; Heldal et al. 1994; Binder et al. 1995). Most particularly, Sieracki and Viles (1992) presented a scattergram of size vs. fluorescence of image-analyzed particles stained with acridine orange, from which we find it possible to subjectively discern two major clusters in the bacterial-sized fraction (i.e. $\geq \sim$ 0.4-μm diam) centered at $\sim$4.1 and 4.4 log fluorescence units.

Without knowing the nature of the differently fluorescing stained bacteria, we have made the distinction between a general group with low apparent DNA content (group I) and another with high apparent DNA content (group II). Our evidence points to a difference of usually more than 2-fold in mean apparent DNA content between groups II and I (Fig. 6D) and suggests that in Bedford Basin, group II had a much greater growth potential than group I (Fig. 8). Given these differences, a major conclusion of our work is that group II bacteria dominate in phytoplankton-rich waters, while group I bacteria do so in phytoplankton-poor waters (Fig. 6B). The cytometric distinction between groups increases in rich waters as the apparent DNA content of group II increases (Fig. 6C). We suggest that these patterns are underlain by controlling mechanisms operative at spatial and temporal scales where large-area and long-term averaging occur. At a gross level in oceanic environments, bacterial biomass is controlled by phytoplankton production (Ducklow 1992). At the same level, it is plausible to suggest from our results that the characteristics of bacterial assemblages are also under the influence of phytoplankton activities. More particularly, we might speculate whether under conditions where bacteria are resource limited (Ducklow 1992), there exists a general relationship between the intensity of phytoplankton activities and the proportion of bacteria that grow more rapidly. When the spatial and temporal scales are finer, this broad correlation may not necessarily hold. Certainly, the amount of variation in the grouping of bacteria explainable by chlorophyll variation (52%) leaves much to account for. Examples in which this broad correlation may not necessarily hold would be measurements taken in vertical profile at particular hydrographic stations or measurements taken at the same station over time.

The ease with which "cytometric diversity" can be documented provides an impetus for wide application of flow cytometric analysis of DNA-stained bacterioplankton. The biological and ecological nature of cytometrically diverse groups remains to be determined.

Note from author (11 October 1995)

Marie, Vaulot, and Partensky (in prep.) and Hennes and Suttle (1995) indicate that divalent cations, which are at high concentrations in seawater, interfere with DNA binding of the related stains YOYO-1 and YO-PRO-1. We have directly compared the TOTO-staining protocol described in our methods section (modified only by diluting the plankton sample 1 : 10 with filtered seawater) against the following protocol modified from Marie et al.: the paraformaldehyde-fixed plankton sample was diluted 1 : 10 in a buffer of 10 mM Tris and 1 mM EDTA, incubated with 0.1 mg ml^{-1} of a mixture of RNase A and RNase B (Sigma R-4875 and R-5750, 1 : 1, wt/wt) at 37°C for 30 min, cooled to room temperature, and then stained with a mixture of Triton-X100 (0.1%) and TOTO-1 (300 nM) for at least 10 min. We found the count of total heterotrophic bacteria (i.e. the sum of group I and group II) to be the same for the two protocols. In seawater, RNase had no significant effect on green fluorescence (same result as Fig. 1), but in buffer, RNase significantly reduced the fluorescence intensity of stained particles. In light of these new results, we remain confident in the accuracy of flow cytometric counting of TOTO-stained bacteria. However, more work is needed to confirm the significance of the differentially staining subpopulations and also the correlations shown in Fig. 6. We thank D. Vaulot and C. Suttle for preprints of their work.

References

BINDER, B. J., S. W. CHISHOLM, R. J. OLSON, S. L. FRANKEL, AND A. Z. WORDEN. 1995. Dynamics of pico-phytoplankton, ultra-phytoplankton, and bacteria in the central equatorial Pacific. Deep-Sea Res. Part 2 **42:** in press.

BOUCHER, N., D. VAULOT, AND F. PARTENSKY. 1991. Flow cytometric determination of phytoplankton DNA in cultures and oceanic populations. Mar. Ecol. Prog. Ser. **71:** 75–84.

CAMPBELL, L., H. A. NOLLA, AND D. VAULOT. 1994. The importance of *Prochlorococcus* to community structure in the central North Pacific Ocean. Limnol. Oceanogr. **39:** 954–961.

COURTIES, C., AND OTHERS. 1994. Smallest eukaryotic organism. Nature **370:** 255.

DUCKLOW, H. W. 1992. Factors regulating bottom-up control of bacteria biomass in open ocean plankton communities. Ergeb. Limnol. **37:** 207–217.

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.

GIOVANNONI, S., AND S. C. CARY. 1993. Probing marine systems with ribosomal RNAs. Oceanography **6:** 95–104.

HELDAL, M., S. NORLAND, G. BRATBAK, AND B. RIEMANN. 1994. Determination of bacterial cell number and cell volume by means of flow cytometry, transmission electron microscopy, and epifluorescence microscopy. J. Microbiol. Meth. **20:** 255–263.

HENNES, K. P., AND C. A. SUTTLE. 1995. Direct counts of viruses in natural waters and laboratory cultures by epifluorescence microscopy. Limnol. Oceanogr. **40:** 1050–1055.

HIRONS, G. T., J. J. FAWCETT, AND H. A. CRISSMAN. 1994. TOTO and YOYO: New very bright fluorochromes for DNA content analyses by flow cytometry. Cytometry **15:** 129–140.

KELL, D. B., H. M. RYDER, A. S. KAPRELYANTS, AND H. V. WESTERHOFF. 1991. Quantifying heterogeneity: Flow cy-

tometry of bacterial cultures. Antonie van Leeuwenhoek J. Microbiol. Serol. **60:** 145–158.

LEBARON, P., AND F. JOUX. 1994. Flow cytometric analysis of the cellular DNA content of *Salmonella typhimurium* and *Alteromonas haloplanktis* during starvation and recovery in seawater. Appl. Environ. Microbiol. **60:** 4345–4350.

LI, W. K. W. 1990. Bivariate and trivariate analysis in flow cytometry: Phytoplankton size and fluorescence. Limnol. Oceanogr. **35:** 1356–1368.

———. 1995. Composition of ultraphytoplankton in the central North Atlantic. Mar. Ecol. Prog. Ser. **122:** 1–8.

———, T. ZOHARY, Y. Z. YACOBI, AND A. M. WOOD. 1993. Ultraphytoplankton in the eastern Mediterranean Sea: Towards deriving phytoplankton biomass from flow cytometric measurements of abundance, fluorescence and light scatter. Mar. Ecol. Prog. Ser. **102:** 79–87.

MONFORT, P., AND B. BALEUX. 1992. Comparison of flow cytometry and epifluorescence microscopy for counting bacteria in aquatic ecosystems. Cytometry **13:** 188–192.

MONGER, B. C., AND M. R. LANDRY. 1993. Flow cytometric analysis of marine bacteria with Hoechst 33342. Appl. Environ. Microbiol. **59:** 905–911.

OLSON, R. J., E. R. ZETTLER, AND M. D. DURAND. 1993. Phytoplankton analysis using flow cytometry, p. 175–186. *In* P. F. Kemp et al. [eds.], Handbook of methods in aquatic microbial ecology. Lewis.

PHINNEY, D. A., AND T. L. CUCCI. 1989. Flow cytometry and phytoplankton. Cytometry **10:** 511–521.

PORTER, K. G., AND Y. S. FEIG. 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. **25:** 943–948.

ROBARTS, R. D., AND T. ZOHARY. 1993. Fact or fiction—bacterial growth rates and production as determined by [*methyl*-^{3}H]-thymidine? Adv. Microb. Ecol. **13:** 371–425.

ROBERTSON, B. R., AND D. K. BUTTON. 1989. Characterizing aquatic bacteria according to population cell size, and apparent DNA content by flow cytometry. Cytometry **10:** 70–76.

RYE, H. S., J. M. DABORA, M. A. QUESADA, R. A. MATHIES, AND A. N. GLAZER. 1993. Fluorometric assay using dimeric dyes for double- and single-stranded DNA and RNA with picogram sensitivity. Anal. Biochem. **208:** 144–150.

———, AND OTHERS. 1992. Stable fluorescent complexes of double-stranded DNA with bis-intercalating asymmetric cyanine dyes: Properties and applications. Nucleic Acids Res. **20:** 2803–2812.

SIERACKI, M. E., AND C. L. VILES. 1992. Distributions and fluorochrome-staining properties of sub-micrometer particles and bacteria in the North Atlantic. *Deep-Sea Res.* **39:** 1919–1929.

STEEN, H. B., AND E. BOYE. 1980. Bacterial growth studied by flow cytometry. Cytometry **1:** 32–36.

THORSEN, B. K., Ø. ENGER, S. NORLAND, AND K. A. HOFF. 1992. Long-term starvation survival of *Yersinia ruckeri* at different salinities studied by microscopical and flow cytometric methods. Appl. Environ. Microbiol. **58:** 1624–1628.

VAULOT, D., C. COURTIES, AND F. PARTENSKY. 1989. A simple method to preserve oceanic phytoplankton for flow cytometric analyses. Cytometry **10:** 629–635.

YARMOLINSKY, M. B. 1995. Programmed cell death in bacterial populations. Science **267:** 836–837.

ZWEIFEL, U. L., AND A. HAGSTRÖM. 1995. Total counts of marine bacteria include a large fraction of non-nucleoid containing "ghosts." Appl. Environ. Microbiol. **61:** 2180–2185.

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