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Enumeration and Cell Cycle Analysis of Natural Populations of Marine Picoplankton by Flow Cytometry Using the Nucleic Acid Stain SYBR Green I

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The novel dye SYBR Green I binds specifically to nucleic acids and can be excited by blue light (488-nm wavelength). Cell concentrations of prokaryotes measured in marine samples with this dye on a low-cost compact flow cytometer are comparable to those obtained with the UV-excited stain Hoechst 33342 (bis-benzimide) on an expensive flow cytometer with a water-cooled laser. In contrast to TOTO-1 and TO-PRO-1, SYBR Green I has the advantage of clearly discriminating both heterotrophic bacteria and autotrophic *Prochlorococcus* cells, even in oligotrophic waters. As with TOTO-1 and TO-PRO-1, two groups of heterotrophic bacteria (B-I and B-II-like types) can be distinguished. Moreover, the resolution of DNA distribution obtained with SYBR Green I is similar to that obtained with Hoechst 33342 and permits the analysis of the cell cycle of photosynthetic prokaryotes over the whole water column.

The prokaryotic fraction of natural marine communities is composed of both heterotrophic and autotrophic organisms. The two major groups of photosynthetic prokaryotes, *Synechococcus* (31) and *Prochlorococcus* (4, 5) spp., are easily discriminated by flow cytometry due to their different pigment compositions. When cells are excited by blue (and to a lesser extent UV) light, the phycobilins contained in *Synechococcus* emit a strong orange fluorescence which can be recorded separately from the red fluorescence of their chlorophyll. Since the *Prochlorococcus* harvest light mainly using divinyl-chlorophylls *a* and *b* (8), they emit only red fluorescence. *Synechococcus* cells are significantly larger than *Prochlorococcus* (ca. 1- and 0.6-μm diameters, respectively). These two types of cells can be discriminated by their flow cytometry scatter signals that are related to their size (24). These distinctive characteristics have allowed advances in the knowledge of the oceanic distributions of these two prokaryotes (2, 18–20, 30). The other major prokaryotic group in marine environments is constituted of diverse heterotrophic bacteria which generally dominate the 0.2- to 2-μm size fraction in terms of cell number and biomass (1, 6, 13) but which are very poorly characterized at the taxonomic level (7). Absence of pigments in these organisms make them less readily countable by fluorescence methods than the autotrophs. Classical enumeration methods consist of staining bacterial DNA with a fluorescent dye, such as acridine orange or 4′,6-diamidino-2-phenylindole (DAPI), and counting cells by epifluorescence microscopy (11, 22). However, epifluorescence microscopy is not sensitive enough to discriminate *Prochlorococcus* from heterotrophic bacteria without the help of a sophisticated cooled charge-coupled device camera (24). In the case of oligotrophic waters, where *Prochlorococcus* can reach concentrations as high as 2.0×10^5 cells ml^{-1}, i.e., 20 to 40% of total prokaryotes, this bias can cause a significant overestimation of the number of heterotrophic bacteria (1, 24).

In contrast, the sensitivity of flow cytometry allows the discrimination of both groups (17). Moreover, its speed greatly facilitates the accurate enumeration of prokaryotes. It also allows statistically significant cell cycle analyses of autotrophic prokaryotes that have been proved very useful both to compute the in situ growth rate of phytoplankton (3, 28) and to assess the physiological effects of limiting factors (27, 29). Until recently, only UV-excited dyes such as DAPI and Hoechst 33342 (bis-benzimide) were available for flow cytometric analysis of prokaryotic cells (21, 23). However, UV excitation is only available either on low-cost but low-sensitivity flow cytometers equipped with mercury lamps or on very expensive flow cytometers equipped with high-power water-cooled lasers. Since high sensitivity is required to discriminate prokaryotes from background noise satisfactorily, only the latter can really be used for counting heterotrophic bacteria or analyzing the cell cycle of prokaryotes, which explains the limited number of studies using these techniques. A new generation of blue-light-excited nucleic acid dyes has recently been marketed for molecular biology purposes and may bring an alternative to the currently used UV-excited dyes. Li et al. (14) used TOTO-1 and TO-PRO-1 from Molecular Probes Inc., Eugene, Oreg., for staining prokaryotes in marine assemblages. However, they could not discriminate between heterotrophic bacteria and *Prochlorococcus* because in order to make these stains penetrate the cells, they had to use a detergent such as Triton X-100 that removed chlorophyll fluorescence. Recently, we also tested other dyes from the same company, including YOYO-1, YO-PRO-1, and PicoGreen (16). These dyes provide very satisfactory results for both enumeration and cell cycle analysis of prokaryotes in cultures after dilution in an appropriate buffer. However, they are not suitable for use with natural seawater samples because they are very sensitive to ionic strength (16).

In this report, we have tested another blue-light-excited nucleic acid dye SYBR Green I (referred to hereafter as SYBR-I). The manufacturer, Molecular Probes, reports this dye as the most sensitive available for detecting nucleic acids in agarose and polyacrylamide gels. It has a strong binding affinity for double-stranded DNA, but it also binds with single-stranded DNA and RNA with lower affinities (10). It can be excited by

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UV but is optimally excited by 495-nm-wavelength light. On natural samples, it allows both separation of autotrophic from heterotrophic prokaryotes and high-resolution cell cycle analyses. Therefore, it provides a perfect alternative to UV-excited dyes and makes this type of analysis possible on the current generation of low-cost high-sensitivity flow cytometers.

MATERIALS AND METHODS

Cultures. *Prochlorococcus marinus* CCMP 1375 obtained from the Center for the Culture of Marine Phytoplankton, Bigelow, Mass., was grown at 20°C on a modified K/10 medium as described previously (15). *Synechococcus* strains WH 8103 and WH 7803 were grown in f/2 medium (9). The pelagophyte *Pelagomonas* sp. strain EUM 8, a picoeukaryote isolated from the tropical Atlantic Ocean (25), as well as the prasinophytes *Micromonas pusilla* CCMP 490 and *Pycnococcus provasolii* CCMP 1203, were grown in K medium (12). All these strains were used for preliminary experiments which allowed testing of the effectiveness of SYBR-I staining on different types of autotrophs, as well as defining the optimal conditions for staining. A mixture of nonaxenic cultures of *P. marinus*, *Synechococcus* strain WH 7803, and *Pelagomonas* sp. at concentrations of 10^5, 2.0×10^4, and 2.8×10^4 cells ml^{-1}, respectively, was also used to assess the suitability of SYBR-I for staining the DNAs of a picoplankton assemblage including (contaminating) heterotrophic bacteria. All samples were fixed for 15 min with 0.1% glutaraldehyde (final concentration), quickly frozen in liquid nitrogen, and stored at −80°C until analysis (26).

Natural samples. The efficiency of staining was tested on natural samples from different marine regions. Surface seawater was collected in coastal waters of the English Channel (Station Estacade, Roscoff, France) in March 1996 and of the Mediterranean Sea (point B, Villefranche-sur-Mer, France) in February 1995. All samples were preserved as described above.

Seawater samples were collected using Niskin bottles during the OLIPAC cruise in the equatorial Pacific Ocean along 150°W aboard the N.O. *l'Atalante*. At 5°S, 150°W, 13 depths were sampled every 2 h from 6:00 to 22:00 on 19 and 21 November 1994. Picophytoplankton cell concentrations were determined by flow cytometry on fresh samples on board ship. An aliquot (1.5 ml) of each sample was put in a cryovial, fixed, then frozen as described above, and kept at −80°C for delayed analysis ashore.

Dyes and staining conditions. Hoechst 33342 and SYBR Green I nucleic acid gel stain were purchased from Molecular Probes Inc. The SYBR-I commercial stock solution corresponds to a 10,000-fold concentrate of the recommended concentration used for staining electrophoretic gels. The optical density at 495 nm of the SYBR-I batch used in the present study (lot no. 0561-3) was 0.682 for a 10^{-3} concentration of the commercial stock solution in distilled water. Prior to staining, samples were quickly thawed and incubated for 30 min at 37°C in the presence of 0.1 g of a mixture of RNase A and B (Sigma R-4875 and R-5750) (1:1 [wt/wt]) $liter^{-1}$. The optimal incubation duration was estimated on preliminary tests with a culture of *P. marinus* diluted at a final concentration of 1.2×10^5 cells ml^{-1}. SYBR-I was added at a final concentration of 10^{-4} of the commercial solution, just before sample was run, and the acquisition was monitored continuously for 30 min. Concentrations from 10^{-5} to 10^{-3} of the commercial solution of SYBR-I were tested on *P. marinus*, *Synechococcus* strain WH 8103, *Pelagomonas* sp., *M. pusilla*, and *P. provasolii*. Cultures were diluted in filtered seawater (passed through 0.22-μm-pore-size filter) to reach final concentrations between roughly 2.0×10^4 and 2.0×10^5 cells ml^{-1}. Different additives including potassium citrate, magnesium chloride, calcium chloride, ammonium sulfate, dimethyl sulfoxide, urea, EGTA, and EDTA were tested to try to improve cell staining either of *P. marinus* only or of the mixture of the three different picophytoplankters, as described before (data not shown). All additives were passed through 0.22-μm-pore-size filters prior to use. Addition of 30 mM (final concentration) of potassium citrate improved both the staining quality and the stability of the SYBR-I fluorescence.

Natural samples were thawed, incubated with RNases A and B as described above, and then separated into three aliquots of 500 μl each. In the first two aliquots, 5μl of a 1% solution of SYBR-I (final concentration, 10^{-4}) and 45 μl of 300 mM potassium citrate (final concentration, 25 mM) were added. Triton X-100 at a final concentration of 0.1% was added to only one of these two aliquots, as suggested by Li et al. (14) for use with TOTO and TO-PRO. The last aliquot was supplemented with 50 μl of a stock solution of Hoechst 33342, consisting of 300 mM potassium citrate, 10 mM EDTA, and 5 μg of Hoechst 33342 ml^{-1} (final concentration, 0.45 $\mu g \cdot ml^{-1}$). Samples were stored in the dark at room temperature before flow cytometric analysis.

Flow cytometry. All experiments with SYBR-I were performed with a FACSort flow cytometer (Becton Dickinson) equipped with an air-cooled laser providing 15 mW at 488 nm and with the standard filter setup. All parameters were collected as logarithmic signals. For the experiments with the mixture of picoplankters, *Synechococcus* orange fluorescence was collected through a 585/42-nm band-pass filter after reflection on a 560/22.5-nm dichroic filter. However, this setup does not allow the collection of linear DNA fluorescence needed for cell cycle analysis. Therefore, for all other experiments including analyses of natural samples, we collected both linear and logarithmic green fluorescence. To do this, a T-connector was set on the output of the photomultiplier collecting green fluorescence. Then we disconnected the output of the photomultiplier collecting orange fluorescence and used the corresponding analog-to-digital converter to collect the linear signal for green fluorescence. The use of a flow cytometer equipped with five analog-to-digital converters (available as an option for the FACSort) could avoid this technical modification.

All natural samples from the equatorial Pacific Ocean were immediately analyzed live on board the ship with the FACSort in order to enumerate the autotrophic populations. Two subsets of samples were fixed and subjected to further flow cytometric analyses after being stained in the laboratory ashore. The first analysis, aimed at enumerating cells, was gated on green DNA fluorescence. The second run, aimed at analyzing the *Prochlorococcus* cell cycle, was gated on red chlorophyll fluorescence. When the fluorescences of autotrophs and heterotrophs overlapped (in near surface waters), electronic composition was applied by subtracting 1 to 10% of the red fluorescence from the green fluorescence signal. For the enumeration experiments, each sample was put into a 4-ml plastic tube and was weighed before and after analysis in order to determine the analyzed volume. Yellow-green fluorescent microspheres (0.95-μm-diameter beads; Polysciences Inc., Warrington, Pa.) were added in each sample as an internal reference.

To compare SYBR-I and Hoechst 33342 staining, aliquots of natural samples from three vertical profiles from the equatorial Pacific Ocean were also stained with the latter dye and analyzed with an EPICS 541 flow cytometer (Coulter Co., Hialeah, Fla.) equipped with a Biosense flow cell and a tunable laser (Coherent Innova) delivering 0.5 W of UV light (353- to 357-nm wavelength). Samples were run such that the event rate was below 800 cells s^{-1} to avoid coincidence. Each sample was run for a couple of minutes to equilibrate and fill the sample line with the dye and then stopped. At this point, a known volume of sample (200 μl) was run. Sample injection and acquisition were started simultaneously and stopped when about 40,000 events were recorded. The precise volume delivered was calculated by measuring the remaining volume and subtracting it from the initial volume.

Right-angle light scatter (RALS) was collected after reflection on a 400-nm long-pass dichroic filter, chlorophyll fluorescence through a 670-nm long-pass filter after reflection on a 590-nm short-pass dichroic filter and Hoechst fluorescence through a 530-nm short-pass filter. Data acquisition and recording were achieved with the Cicero system (Cytomation Inc., Fort Collins, Colo.).

Data from both flow cytometers were recorded as listmode files (except for enumerating bacteria with Hoechst 33342, for which we simply stored the cytograms of RALS versus DNA fluorescence) and processed with a custom-designed software CYTOWIN (29a) that discriminates cell populations by using a combination of all the parameters recorded. Graphs were drawn with the WinMDI freeware (Joseph Trotter). Cell cycle analyses were performed using MultiCYCLE (P. Rabinovitch).

RESULTS

The staining kinetics of SYBR-I was determined by flow cytometry on *P. marinus* cells. The equilibrium was reached within 5 min (data not shown). Different concentrations of SYBR-I, ranging from 10^{-5} to 10^{-3}, were tested separately on the prokaryotes *P. marinus* and *Synechococcus* strain WH 8103 and the eukaryotes *Pelagomonas* sp., *M. pusilla*, and *P. provasolii* (Fig. 1). For concentrations from 1.0×10^{-5} to 8.0×10^{-4}, an increase of the intensity of green DNA fluorescence and a concomitant decrease of the coefficient of variation of the G_1-like peak (G_1 CV) were observed. Stabilization of the fluorescence intensity and CV were obtained for concentrations above 7.0×10^{-4} to 6.0×10^{-4} of the commercial solution (Fig. 1). For all organisms tested but *P. provasolii*, for which the G_1 CV increased, concentrations of SYBR-I above 2.0×10^{-4} induced no changes in both green DNA fluorescence intensity and G_1 CV (Fig. 1). The ratios between G_2-like and G_1-like peaks of the DNA distribution were about 1.9 for both *P. marinus* and *Synechococcus* strain WH 8103. The fluorescence emission spectrum of SYBR-I (excitation at 488 nm) extends up to 650 nm (data not shown) and is therefore partially collected by the red photomultiplier which detects the fluorescence of the chlorophyll. A significant increase of the red fluorescence intensity (used to estimate chlorophyll on unstained samples) was therefore observed in the presence of SYBR-I for *P. marinus* or *M. pusilla*. In contrast, for *Synechococcus* strain WH 8103, there was no significant alteration of the red signal, while for *Pelagomonas* sp., the red fluorescence slightly decreased (data not shown). However, the ratio of the

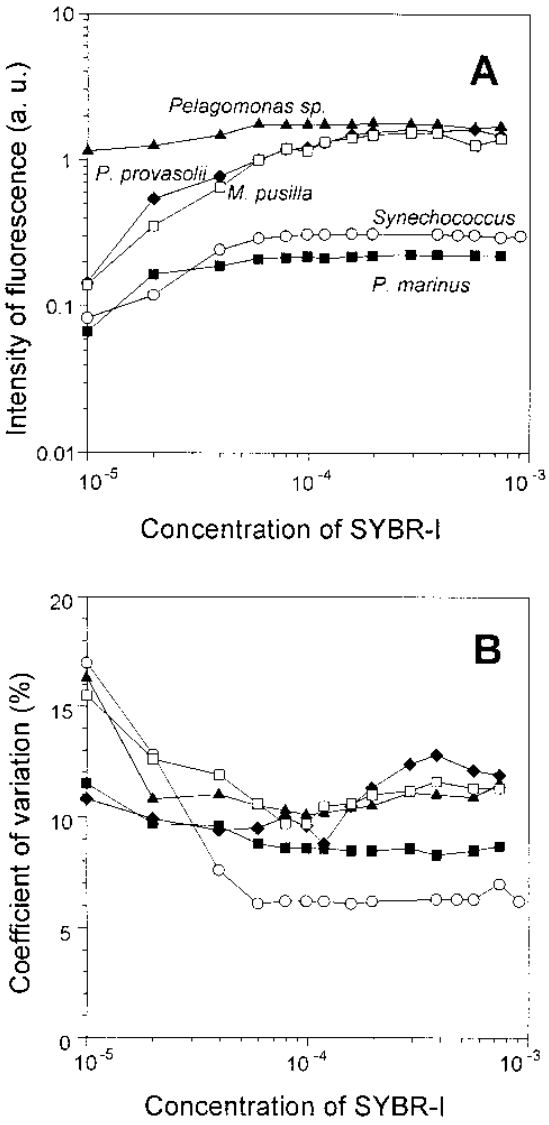

FIG. 1. Fluorescence intensity (A) and CV (B) of the G_1-like peak of the DNA distribution for *P. marinus*, *Synechococcus* strain WH 8103, *M. pusilla*, *P. provasolii*, and *Pelagomonas* sp. as a function of SYBR-I concentration (relative to the commercial solution) in seawater. a.u., arbitrary units.

intensity of red fluorescence of the photosynthetic cells relative to that of the heterotrophic population did not change as a function of SYBR-I concentration above 2.0×10^{-4}. For all other experiments, including the staining of natural seawater samples, the protocol consisted of RNase incubation for 30 min at 37°C, staining with a 10^{-4} concentration of the SYBR-I stock solution in the presence of 30 mM potassium citrate, and an incubation for 15 min or more at room temperature in the dark.

The different populations of a mixture containing *P. marinus*, *Synechococcus* strain WH 7803, *Pelagomonas* sp., and their contaminating bacteria were well separated by flow cytometry after staining with SYBR-I (Fig. 2), especially on the red-versus-green fluorescence cytogram (Fig. 2C). Leaking of SYBR-I green fluorescence on red (chlorophyll) and orange (phycoerythrin) channels is visualized by the significant SYBR-I-conferred red and orange fluorescences of heterotrophic bacteria and the orange fluorescence of *Prochlorococcus* and *Pelagomonas* sp. Thus, the cytograms representing orange versus red fluorescence (Fig. 2B) looked similar to that of green versus red fluorescence (Fig. 2C) except for cyanobacteria, for which the phycoerythrin and SYBR-I fluorescences added up in the orange fluorescence channel. Individual DNA distributions (in logarithmic scales) for the four types of organisms of the mixture (Fig. 2E to H) show clear separation between G_1 and G_2 peaks for *Prochlorococcus* and *Synechococcus*.

Coastal seawater samples from the English Channel and the Mediterranean Sea, exhibited clearly defined prokaryotic populations (Fig. 3). In the English Channel, heterotrophic bacteria ($\sim 3.5 \times 10^5$ cells ml^{-1}) largely outnumbered the photosynthetic cells ($<5.0 \times 10^3$ cells ml^{-1}). Three populations of heterotrophs could be distinguished on the RALS-versus-green fluorescence cytogram (Fig. 3A). Two of them (so-called B-I-like and B-II-like bacteria) probably correspond to the B-I and B-II populations identified by Li et al. (14) in the Atlantic Ocean and in the Labrador Sea, using TO-PRO staining. A third (B-III-like) population, which is the most numerous, can also be distinguished (Fig. 3A). In the sample collected from the Mediterranean Sea, relatively abundant populations of *Prochlorococcus* and *Synechococcus* were present (about 2.0×10^4 cells ml^{-1} for each one and 5.2×10^5 for heterotrophic bacteria). The red chlorophyll fluorescence was sufficiently high to distinguish them from heterotrophic bacteria (Fig. 3D). Two distinct groups could be discriminated for the bacterial population similar to those described by Li et al. (14) (Fig. 3C).

SYBR-I was also used on vertical profiles from the equatorial Pacific (5°S, 150°W), sampled every 2 h from 6:00 to 22:00 (local time) on two separate days (19 and 21 November 1994). In a previous study, the cell cycle of *Prochlorococcus* populations was shown to be highly synchronized by the light-dark cycle (28). The selected profile presented here (Fig. 4) corresponds to the middle of the period of active DNA replication of *Prochlorococcus* cells. Good separation of autotrophs and heterotrophs was possible on red-versus-green DNA fluorescence with both SYBR-I and Hoechst 33342 at 75 m and below (Fig. 4). Because *Prochlorococcus* sp. largely dominated the other picoplanktonic cells, it was the sole autotroph to constitute a well-defined population. SYBR-I also allowed us to resolve two populations of heterotrophs (B-I-like and B-II-like bacteria) on scatter-versus-green fluorescence cytograms (Fig. 4, left panels), while Hoechst 33342 (not shown) did not. Above 110 m, the natural red fluorescence of chlorophyll of *Prochlorococcus* cells was too low due to photoacclimation to completely discriminate *Prochlorococcus* from bacteria. These two prokaryotic populations also overlapped in scatter and DNA fluorescence (Fig. 4). With Hoechst staining, separation of *Prochlorococcus* and bacteria could still be obtained at 65 and 55 m but not above (see Table 2). With SYBR-I staining, electronic compensation between the red and green channels was used for samples above 55 m to increase the discrimination between the autotrophic and heterotrophic populations on the red fluorescence signal.

The *Prochlorococcus* concentrations in SYBR-I-stained samples were highly correlated with those obtained on fresh samples for the data from 55 m and below (i.e., without electronic compensation $r^2 = 0.993$, $n = 113$ [Fig. 5A, closed symbols]). The slope (0.822) was slightly lower than 1, as observed in previous studies, due to the loss of a small percentage of cells during fixation and (or) freezing-thawing processes (17, 20, 26). Correlation was not as good when all data (compensated and noncompensated) were considered ($r^2 = 0.893$, $n = 226$), because the discrimination of *Prochlorococcus* from heterotrophic bacteria was less satisfactory in the upper layer on SYBR-

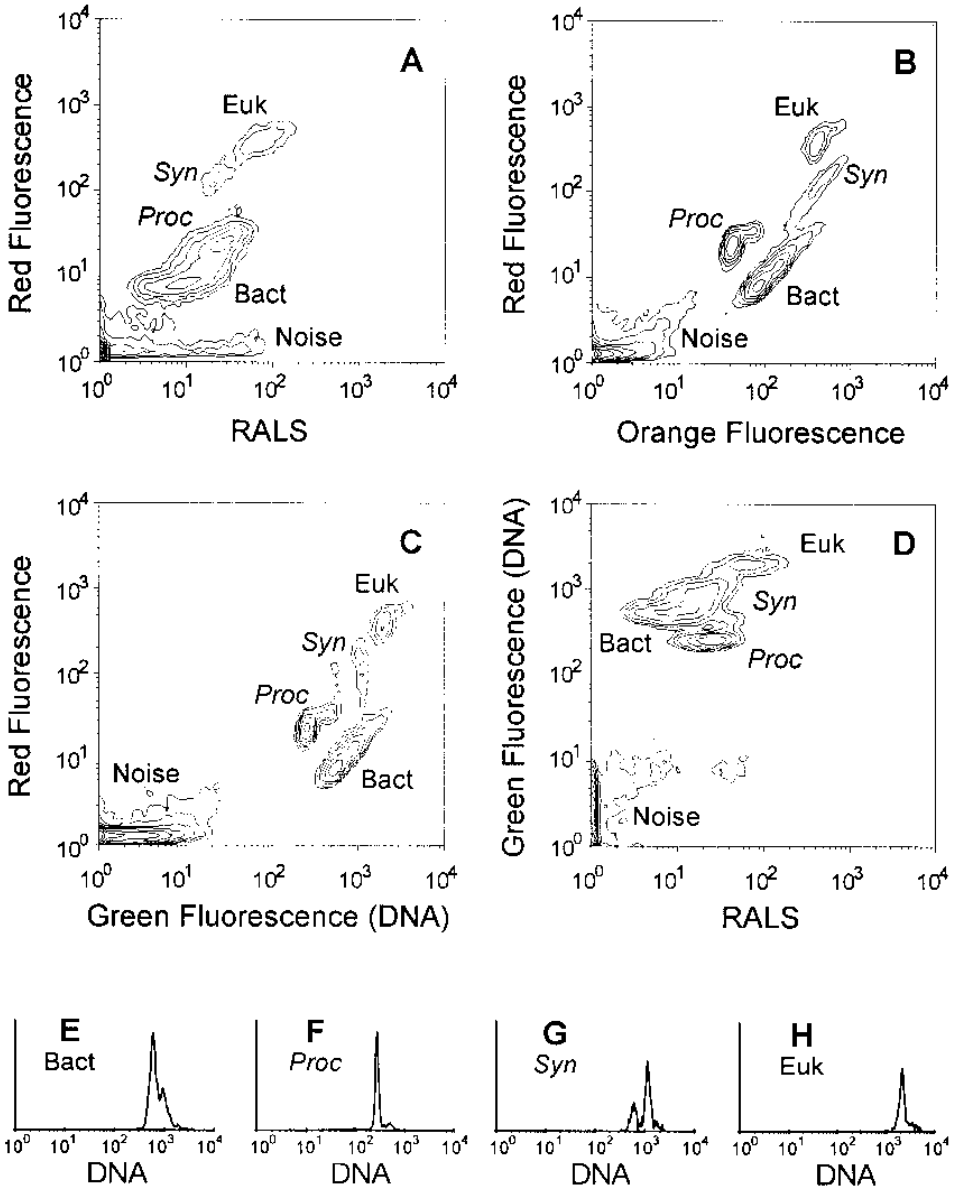

FIG. 2. Flow cytometric analysis of a mixture of cultures containing *P. marinus*, *Synechococcus* strain WH 7803, and the eukaryotic strain *Pelagomonas* sp. stained with SYBR-I. Samples were diluted in filtered seawater and stained with SYBR-I at a final concentration of 10^{-4} of the commercial solution, supplemented by 30 mM potassium citrate. Populations of heterotrophic bacteria (Bact), *P. marinus* (*Proc*), *Synechococcus* (*Syn*), and the picoeukaryote *Pelagomonas* sp. (Euk) were discriminated from each other on different combinations of RALS and green, red, and orange fluorescence signals (A to D) and the corresponding DNA distributions (E to H) were then computed with CYTOWIN ((29a). Contours correspond to increasing cell densities.

I-stained samples (Fig. 5A). The concentrations of prokaryotes (*Prochlorococcus* and heterotrophic bacteria) determined with SYBR-I were slightly lower than with Hoechst 33342 (slope, 0.78) but correlated very well ($r^2 = 0.87$, $n = 39$ [Fig. 5B]).

With SYBR-I, addition of Triton X-100 reported to be beneficial in the case of TOTO and TO-PRO staining of bacteria (14) did not modify estimates of prokaryote concentration (Table 1) or improve the resolution of DNA distributions (not shown). In fact, Triton X-100 induced a decrease of the relative green fluorescence intensity of SYBR-I-stained cells. It also reduced the discrimination between *Prochlorococcus* and bacteria due to the detrimental effects of this detergent on chlorophyll (not shown).

SYBR-I also proved very useful for the cell cycle analysis of natural *Prochlorococcus* populations. DNA histograms and computed percentages of cells within the different phases of the cell cycle were similar between SYBR-I- and Hoechst-stained samples (Fig. 4, inserts, and Table 2). The CVs on the G_1 peak ranged between 8 and 10% for SYBR-I and were systematically 1 to 2% higher than those obtained with Hoechst 33342 on the same samples (Table 2).

DISCUSSION

SYBR-I, a novel dye developed by Molecular Probes, appears extremely useful in analyzing marine picoplankton in natural samples by flow cytometry. Its major advantage is to be

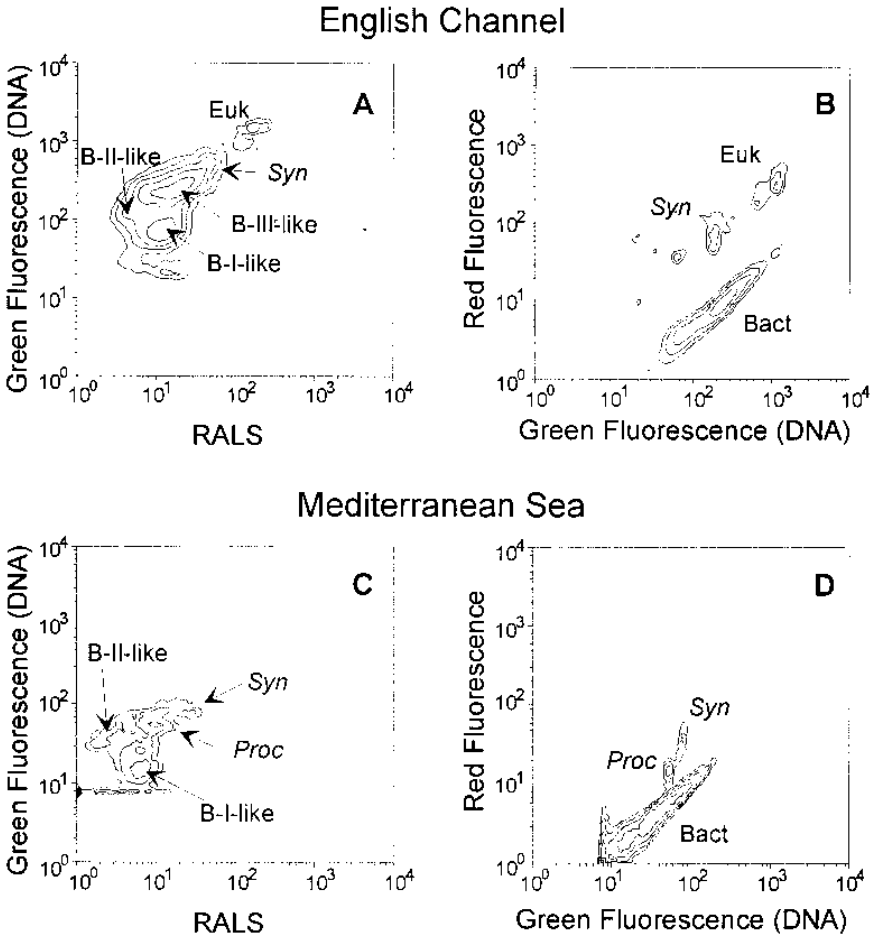

FIG. 3. Flow cytometric analysis of natural marine samples collected in the English Channel off Roscoff in March 1996 (A and B) and in the Mediterranean Sea off Villefranche-sur-Mer in February 1995 (C and D). Samples were stained with SYBR-I at a concentration of 10^{-4} (relative to the commercial solution) in the presence of 30 mM potassium citrate and analyzed with a FACSort flow cytometer. Abbreviations: Euk, picoeukaryote; *Syn*, *Synechococcus*; Bact, heterotrophic bacteria; *Proc*, *P. marinus*.

usable on compact low-cost flow cytometers (such as the FACSort), that can be used on ships. The fluorescence excitation spectra of the SYBR-I (emission wavelength, 525 nm) has two maxima, one in the visible region around 495 nm and one in the UV region near 360 nm. Therefore, this dye is usable on all existing flow cytometers, equipped either with lasers or mercury arc lamps. Although no information is available on its structure and chemical properties and in particular on its binding mode to DNA, we developed a simple staining protocol that permits discrimination between and counting of autotrophic and heterotrophic prokaryotes in a variety of marine samples, separation of several subpopulations within heterotrophic bacteria, and resolution of cell cycle DNA distributions for autotrophic prokaryotes and eukaryotes.

Compared with Hoechst 33342, the dye that was previously used for DNA analysis of marine prokaryotes by flow cytometry (17), SYBR-I offers very similar performances for the estimation of prokaryote concentrations (Fig. 5) and for *Prochlorococcus* cell cycle analysis (Fig. 4 and Table 2). Moreover, blue light excites the chlorophyll of autotrophs better than UV light does. Consequently, SYBR-I allowed a slightly better discrimination than did Hoechst 33342 between *Prochlorococcus* and heterotrophic bacteria on a typical depth profile sampled in the equatorial Pacific Ocean (Fig. 4). Near the surface, we had to use electronic compensation for SYBR-I-stained cells run on the FACSort instrument because some SYBR-I fluorescence leaked into the red channel. In practice, compensation just allows better visualization of the boundary between *Prochlorococcus* and bacteria. When the two populations overlap, as for example at 5 and 25 m (Fig. 4), *Prochlorococcus* cells concentrations could be slightly overestimated, although the comparison of *Prochlorococcus* counts on fresh and SYBR-I-stained samples suggests that in fact, there are few heterotrophic bacteria with the same scatter and DNA fluorescence characteristics as those of *Prochlorococcus*. In more oligotrophic waters (e.g., down to 15 to 20°S, or near Hawaii), the much weaker *Prochlorococcus* red fluorescence in surface water would make them indistinguishable from heterotrophic bacteria. Altogether, the sole advantage of Hoechst over SYBR-I is that it allows to obtain slightly lower CVs for G_1 peaks of DNA distributions, theoretically allowing a more precise computation of the percentage of cells in the different phases of the cell cycle. However, the percentages obtained with these two stains were in fact quite similar (Table 2).

Like with the other blue-light-excitable dyes TOTO and TO-PRO, which have been recently proposed to analyze natural marine prokaryotic populations (14), SYBR-I allowed us to differentiate two subpopulations of heterotrophic bacteria with different DNA and scatter signatures (such as TOTO and TO-PRO) (14). The first group (B-I-like bacteria) has a larger

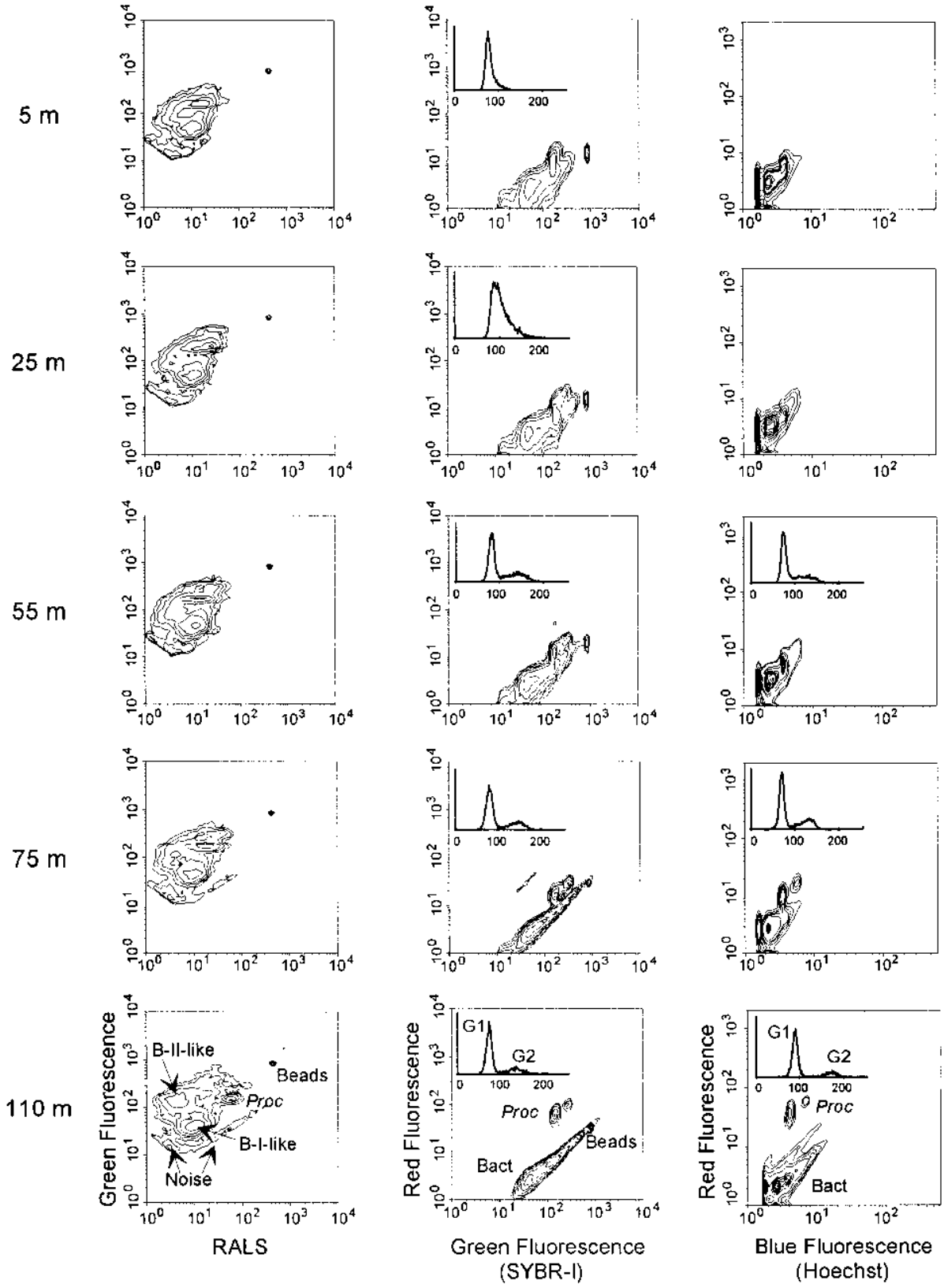

FIG. 4. Flow cytometric analyses of seawater samples collected at different depths on a vertical profile in the equatorial Pacific Ocean (5°S, 150°W) on 19 November 1994 at 18:00 (OLIPAC cruise, cast number 73), after staining by SYBR-I (left and center columns) or Hoechst 33342 (right column). Five representative depths among 13 sampled are shown. Samples were stained with SYBR-I at a final concentration of 10^{-4} and analyzed with the FACSort flow cytometer or stained with Hoechst 33342 at a concentration of 0.5 μg ml^{-1} and analyzed with the EPICS flow cytometer. For the SYBR-I samples, electronic compensation (3 to 5%) was used for the samples from 5 to 55 m in order to increase the discrimination between *Prochlorococcus* and heterotrophic bacteria. Fluorescent microspheres (0.95-μm diameter) (Polysciences) were used as an internal standard. Inserts show DNA distributions of the *Prochlorococcus* population on a linear scale. Note that *Synechococcus* and picoeukaryote populations were present at low concentrations but are not visible on these graphs due to the contour scale selected.

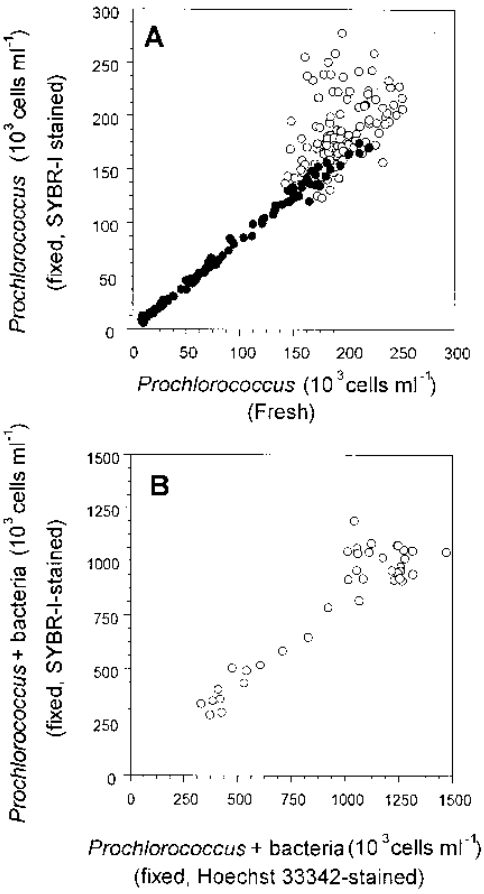

FIG. 5. Samples from the Equatorial Pacific (5°S, 150°W; 19 and 21 November 1994; OLIPAC cruise). (A) Comparison of *Prochlorococcus* analyzed fresh and unstained on board the ship versus *Prochlorococcus* fixed and stained with SYBR-I (pooled data from 18 vertical profiles, $y = 0.97x + 15.5$, $n = 226$, $r^2 = 0.893$). Closed symbols represent the data acquired without compensation ($y = 0.82x + 1.3$, $n = 113$, $r^2 = 0.993$). The dashed line corresponds to a 1:1 relationship. (B) Concentration of prokaryotes (*Prochlorococcus* and bacteria) stained with Hoechst 33342 versus SYBR-I on fixed samples (pooled data from three vertical profiles, $y = 0.78x + 58$, $n = 39$, $r^2 = 0.869$). The dashed line corresponds to a 1:1 relationship.

scatter signal and a lower DNA fluorescence, while the other (B-II-like bacteria) has a smaller scatter signal but a higher DNA fluorescence. Our findings clearly establish that the two groups are not staining artifacts. The increase of the relative abundance of the B-II-like group with depth (Fig. 4), as nutrient levels increased, is consistent with the observation by Li and coworkers (14) that the B-II-like group is associated with less-oligotrophic conditions than the B-I-like group. In the coastal waters of the English Channel, a third heterotrophic population (B-III-like bacteria) can also be distinguished. It seems to match the signature of the B-II-b population observed by Li et al. (14) in Bedford Basin samples that grew rapidly when diluted in seawater. The necessity of using a detergent (Triton X-100) with TOTO and TO-PRO results in chlorophyll destruction and therefore prohibits their use in studying the cell cycle of phototrophs, especially *Prochlorococcus* sp.

SYBR-I theoretically allows the determination of the cell concentrations of four populations within the picoplankton (picoeukaryotes, *Synechococcus*, *Prochlorococcus*, and heterotrophic bacteria) during a single analysis. In practice, however, it is recommended that one should first determine autotrophic picoplankton on fresh, nonstained samples and then analyze fixed, SYBR-I-stained samples for heterotrophic bacterial concentrations and cell cycle analysis. This approach allows the collection of unbiased information on scatter and pigments (red and orange) fluorescence from the autotrophs, since fixation induces changes in these signals (25, 26) and SYBR-I fluorescence leaks into red and orange channels. Moreover, the generally large proportion of bacteria in the picoplankton would make the acquisition of a large number of events necessary in order to obtain statistically significant numbers for autotrophs. This is not compatible with routine flow cytometric analysis.

Clearly, SYBR-I shows promise as a stain of cellular DNA in natural samples, either for cell enumeration or cell cycle analysis, and will help understanding the structure and dynamics of picoplankton populations in oceanic environments.

TABLE 1. *Prochlorococcus* and heterotrophic bacterial concentrations on a vertical profile[a]

Depth (m)	Bacterial concn (10^3 cells ml^{-1})								
	Fresh Proc	SYBR-I			SYBR-I + Triton			Hoechst 33342		
		Proc	Bact	Total	Proc	Bact	Total	Proc	Bact	Total
5	159	147	806	953			994			1,011
15	164	143	766	909			923			981
25	185	171	872	1,043			1,155			1,208
35	172	174	798	972			996			1,023
45	191	172	839	1,011			977			1,047
55	194	186	775	961			991	241	835	1,076
65	208	177	743	920			1,004	191	886	1,077
75	163	142	639	781			762	144	636	780
85	73	61	456	517			518	64	477	541
95	50	46	458	504			471	48	377	425
110	26	25	334	359			358	25	394	420
130	10	11	325	336			329	9	320	329
150	8	9	276	285			269	8	364	371

[a] *Prochlorococcus* (Proc) and heterotrophic bacteria (Bact) concentrations on a vertical profile collected in the equatorial Pacific Ocean (cast number 73; 5°S, 150°W) during the OLIPAC cruise on 19 November 1994 at 18:00. Concentrations were determined either after SYBR-I staining (with or without treatment by Triton X-100 [Triton]) and acquisition with a FACSort flow cytometer or after Hoechst 33342 staining and acquisition with an EPICS flow cytometer.

TABLE 2. Percentages of cells in the different phases of the cell cycle and CVs of the G_1 peak for natural *Prochlorococcus* populations[a]

Depth (m)	SYBR-I Green				Hoechst 33342			
	CV G_1 (%)	%G_1	%S	%G_2	CV G_1 (%)	%G_1	%S	%G_2
5	9.0	88.3	11.7	0.0				
15	9.0	80.7	19.3	0.0				
25	12.0	69.0	31.0	0.0				
35	7.6	21.4	78.6	0.0				
45	7.1	38.4	56.6	4.9				
55	8.6	58.8	29.1	12.1	7.2	59.5	33.0	7.6
65	8.2	77.6	21.2	1.2	7.5	74.5	24.1	1.4
75	8.8	65.5	12.8	21.6	7.8	63.2	9.0	21.5
85	9.5	56.6	10.7	32.6	7.4	55.3	9.9	31.4
95	9.8	71.5	4.5	24.1	7.2	69.5	8.4	22.0
110	8.8	75.8	7.8	16.4	7.2	78.8	7.5	14.8

[a] Data shown are for natural *Prochlorococcus* populations from the equatorial Pacific Ocean (OLIPAC cruise, cast number 73) stained with either Hoechst 33342 or SYBR-I. The data were processed with the Multicycle software.

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