Flow Cytometric Analysis of Marine Bacteria with Hoechst 33342†

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We investigated the accuracy and precision of flow cytometric (FCM) estimates of bacterial abundances using 4',6-diamidino-2-phenylindole (DAPI) and Hoechst 33342 (HO342, a bisbenzamide derivative) on paraform-aldehyde-fixed seawater samples collected from two stations near Oahu, Hawaii. The accuracy of FCM estimates was assessed against direct counts by using epifluorescence microscopy. DAPI and HO342 differ in two aspects of their chemistry that make HO342 better suited for staining marine heterotrophic bacteria for FCM analysis. These differences are most important in studies of open-ocean ecosystems that require dual-beam FCM analysis to clearly separate heterotrophic bacterial populations from populations of photosynthetic *Prochlorococcus* spp. Bacterial populations were easier to distinguish from background fluorescence when stained with HO342 than when stained with DAPI, because HO342 has a higher relative fluorescence quantum yield. A substantially higher coefficient of variation of blue fluorescence, which was probably due to fluorescent complexes formed by DAPI with double-stranded RNA, was observed for DAPI-stained populations. FCM estimates averaged 2.0 and 12% higher than corresponding epifluorescence microscopy direct counts for HO342 and DAPI-stained samples, respectively. A paired-sample t test between FCM estimates and direct counts found no significant difference for HO342-stained samples but a significant difference for DAPI-stained samples. Coefficients of variation of replicate FCM abundance estimates ranged from 0.63 to 2.9% (average, 1.5%) for natural bacterial concentrations of 6×10^5 to 15×10^5 cells ml^{-1}.

Much of the progress that has been made in elucidating the structure of marine microbial food webs over the past decade can be linked directly to the application of epifluorescence microscopy (EFM) techniques. Specific milestones include the unambiguous demonstration of large populations of heterotrophic marine bacteria (15, 51), the discovery of ubiquitous populations of chroococcoid cyanobacteria (47), and the resolution of autotrophic and heterotrophic modes of existence among small eukaryotic protists (18). More recently, however, the powerful new technology of analytical flow cytometry (FCM) has yielded the discovery of additional photosynthetic bacteria previously overlooked by EFM (11) and has shown promise in addressing the fundamental limitations of EFM, tedium and poor precision.

Applications of FCM have focused largely on autotrophic organisms whose photosynthetic pigments make ideal fluorescent markers (7, 36, 37, 38, 49, 50). Much less attention has been devoted to the development of staining protocols for FCM analysis of heterotrophic organisms. Robertson and Button (43) presented a detailed discussion of instrumentation and staining requirements for detection of heterotrophic marine bacteria by FCM. Cells were stained, as in EFM, with the DNA-specific fluorochrome 4',6-diamidino-2-phenylindole (DAPI) (16, 27), and bacteria were identified from the blue fluorescence of the DAPI-DNA complex and forward light scatter (an index of cell size) as they passed a tightly focused beam of UV light from an argon ion laser. While this technique gives estimates of bacterial abundance comparable to those based on EFM direct counts, neither this method nor the EFM direct-count method accurately measures heterotrophic bacterial abundance in open-ocean ecosystems, where high abundances of minute photosynthetic bacteria (11, 35), *Prochlorococcus* spp. (10), confound their measurement. *Prochlorococcus* and heterotrophic bacteria broadly overlap in size and blue DAPI fluorescence. UV excitation of red autofluorescence from *Prochlorococcus* photosynthetic pigments would distinguish these organisms from heterotrophic bacteria, but the red fluorescence is too dim and short-lived to be detected by EFM (11), and in the well-lighted surface mixed layers it is too faint to be detected by FCM using only the UV line of an argon ion laser (35). Separation of the two populations by FCM can be accomplished in theory by using a second argon ion laser, tuned to 488 nm and aligned colinearly with the UV beam, to better excite the red autofluorescence of the *Prochlorococcus* populations (46).

FCM analysis for the simultaneous determination of red autofluorescence and the blue fluorescence of the DAPI-DNA complex proved to be nontrivial and led us to test alternative dyes. The bisbenzamide dye Hoechst 33342 (HO342) (1, 29) appears to have several advantages over DAPI. We present a qualitative comparison of the performances of HO342 and DAPI stains in terms of background fluorescence, dye specificity, and the coefficient of variation (CV) for blue fluorescence of natural populations. We also provide a quantitative comparison of FCM estimates of bacterial abundance with the two dyes relative to EFM direct counts.

MATERIALS AND METHODS

Sample collection and preparation. Seawater samples were collected in acid-washed polycarbonate bottles (Nalgene) or sterile polypropylene centrifuge tubes (Corning) from just below the sea surface in Kaneohe Bay, Hawaii (central station), and 2.5 km outside the bay (outer station) during March and April 1992. The 4-m deep central station is

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protected from free exchange with oceanic water by a shallow reef (flushing time, ca. 10 days) and has a large population of the chroococcoid cyanobacterium, *Synechococcus* sp. The 200-m deep outer station is characteristic of oceanic waters with a high abundance of *Prochlorococcus* bacteria. Heterotrophic bacteria are a major component of the biomass at both stations (1, 2, 25).

Seawater samples were kept cool and in the dark (<3 h) until they could be fixed with paraformaldehyde (final concentration, 1.0%, wt/vol). For each experiment, working stocks (10 times the desired final concentration) of HO342 (Molecular Probes, Eugene, Oreg.) and DAPI (Sigma Chemical Co., St. Louis, Mo.) were prepared fresh daily by dissolving the dyes in distilled-deionized water and filtering through 0.22-μm-pore-size syringe filters (Gelman, Ann Arbor, Mich.) prior to use. Except for time course experiments, samples were allowed to stain in the dark at room temperature in polypropylene cryogenic tubes (Bio-Rad, Richmond, Calif.). Counts obtained from filtered blanks were subtracted from experimental counts to remove any contribution from unbound dye. Filter blanks consisted of filtered (0.22-μm pore size) and fixed seawater at dye concentrations corresponding to experimental conditions. The contribution from unbound dye was greatest for heterotrophic bacteria, ranging from 1 to 3% of total counts.

Optimal staining. The basic experimental design was to determine optimal dye concentration and minimum staining time for FCM estimation of bacterial abundance and to compare EFM direct counts with FCM estimates for which these optimal conditions were used. To determine the optimal dye concentration, 0.1-, 0.3-, 0.5-, and 1.0-μg-ml^{-1} (final concentrations) HO342 or DAPI were added to separate 1.0-ml aliquots of fixed seawater and incubated for 2 to 5 h prior to FCM analysis. We refer to this as the dye concentration series. Time course experiments were initiated with the addition of HO342 or DAPI to fixed seawater (final concentration, 0.5 μg ml^{-1}) followed by periodic sampling for 12 h. Yellow-green fluorescent microspheres (diameter, 1.1 μm) (Polysciences Inc., Warrington, Pa.) were added as an internal fluorescence standard for dye concentration and time series experiments.

Precision and accuracy. To test the precision of FCM abundance estimates, six separate samples were optimally stained with DAPI or HO342, as determined in the previous set of experiments, and repetitively subsampled four or five times. To test the accuracy of the bacterial abundance estimates obtained by FCM analysis, eight separate seawater samples were similarly stained with DAPI or HO342 and analyzed by FCM and EFM. FCM abundance estimates for *Prochlorococcus* and heterotrophic bacteria were combined for comparison with direct counts, since *Prochlorococcus* bacteria cannot be distinguished from heterotrophic bacteria by EFM (11).

FCM analysis. We analyzed all samples using an EPICS 753 flow cytometer (Coulter Co., Hialeah, Fla.) equipped with two 5-W argon ion lasers (model 90-5; Coherent Inc., Palo Alto, Calif.), a Biosense flow cell (restricted flow), and a syringe-based multiple-sample delivery system controlled with MDADS II software. One laser was tuned to the UV range at a power level of 200 mW to excite DAPI and HO342. The second laser was aligned colinearly with the UV beam and tuned to 488 nm at a power level of 1.3 W to excite the chlorophyll molecules of photosynthetic picoplankton. The laser beams were focused with a confocal lens assembly (Coulter) to produce a beam about 16 μm high and 40 μm wide at the intersection with the sample stream. Fluorescence and right-angle light scatter (RALS) were collected

with a high-numerical-aperture aspheric pickup lens assembly (Coulter) positioned orthogonally to the lasers. Collected light was subsequently split into four color components by three dichroic filters and directed to separate photomultiplier tubes (PMTs). A 488-nm dichroic rejection band filter (Coulter) reflected 488-nm scattered laser light to the RALS PMT while transmitting longer and shorter wavelengths. A 630-nm dichroic short-pass filter (Omega Optical, Brattleboro, Vt.) separated red fluorescence from shorter wavelengths. Blue and orange fluorescence wavelengths were separated with a 560-nm dichroic short-pass filter (Coulter). Interference band pass filters, 680 DF40, 450 DF40 (both from Omega), and 575 DF25 (Coulter), were placed in front of the red, blue, and orange PMTs, respectively. PMT signals were collected in log-integral mode (three decades; 256 channels) without the gated amplifier or fluorescence compensation. The analog-to-digital converter discriminator for the RALS signals was set to 100%. All other analog-to-digital converter discriminators were set just high enough to eliminate PMT or laser noise. Mean fluorescence was calculated on the basis of linearized distributions obtained from log-transformed signals by using calibration factors determined separately for each amplifier.

Between 15 and 30 μl was sampled at a rate of 25 to 40 μl min^{-1}. Count rates ranged between 600 and 1,300 s^{-1}. More than 1,000 counts were obtained for heterotrophic, *Prochlorococcus* (outer-station samples), and *Synechococcus* (central-station samples) bacteria. The volume delivered by the Coulter multiple-sample delivery system was calibrated at the end of each experiment from replicate FCM counts ($n = 4$) of a suspension of 1.98-μm-diameter yellow-green fluorescent microspheres (Polysciences Inc.), the concentration of which was accurately determined (CV, 0.59% [mean of six replicate estimates]) by using an Elzone/Celloscope 80XY particle counter (Particle Data Inc., Elmhurst, Ill.) with a 48-μm-diameter orifice tube.

List-mode files were analyzed on an IBM PC-compatible microcomputer using a custom-designed program (CytoPC, version 3.0) created by D. Vaulot (Station Biologique, Roscoff, France). Identification of *Synechococcus* organisms was based on blue, orange, or red fluorescence and RALS (see Fig. 4). Heterotrophic bacteria were identified on the basis of their RALS, blue fluorescence, and a lack of red or orange fluorescence (see Fig. 3 and 4). *Prochlorococcus* organisms were distinguished by the presence of red fluorescence, the lack of orange fluorescence, and their proximity, in terms of blue fluorescence and RALS, to heterotrophic bacteria (see Fig. 3). Each group was discriminated with analysis gates in the list-mode replay.

EFM. Direct counts were obtained microscopically with a Zeiss epifluorescence microscope with a 100-W mercury vapor lamp, ×100 Neofluor objective, a ×16 ocular with a calibrated grid (50 by 50 μm, with 5-μm divisions), and a standard UV filter set (Zeiss model 48772). Fixed seawater samples (5.0 ml) were stained with DAPI or HO342 for 3 to 4 h (final concentration, 0.5 μg ml^{-1}), filtered onto 0.22-μm-pore-size black membrane filters (Poretics Inc., Livermore, Calif.), and sealed between immersion oil, slide, and coverslip (42). At least 42 ocular grids and 900 bacteria were counted for each slide.

RESULTS

Dye concentration. The blue fluorescence of HO342-stained bacteria increased slightly with dye concentrations from 0.1 to 1.0 μg ml^{-1}, while the blue fluorescence of
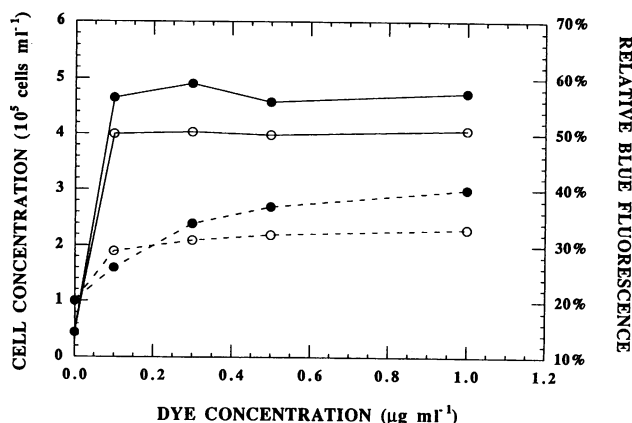
FIG. 1. Effects of dye concentration on abundance estimates (solid lines) and blue fluorescence relative to a 1.1-μm-diameter microsphere standard (dashed lines) for DAPI (●) and HO342 (○).

DAPI-stained cells increased rapidly at concentrations up to 0.5 μg ml^{-1} and at a lower rate beyond this point (Fig. 1). Estimated cell numbers were generally independent of dye concentration over the range tested but were higher for DAPI-stained samples and more constant for HO342-stained samples. A dye concentration of 0.5 μg ml^{-1} was chosen for both DAPI and HO342, because it gave a stable blue fluorescence for heterotrophic bacteria while keeping relative background fluorescence from unbound dye to a minimum.

Dye uptake. Abundance estimates increased dramatically in the first few minutes of HO342 staining and more gradually for DAPI staining (Fig. 2). Estimates were essentially constant after 2 h for both dyes. Blue fluorescence plateaued within 1 h for HO342-stained cells but increased steadily over 12 h for DAPI-stained cells (Fig. 2). *Prochlorococcus* and *Synechococcus* spp. showed dye uptake patterns similar to that of heterotrophic bacteria.

Background fluorescence and variation of blue fluorescence. The relative levels of background fluorescence of both dyes are presented graphically in single-parameter histograms of blue fluorescence in Fig. 3a and d and 4a and d. The sharp cutoff at the left end of each histogram represents the
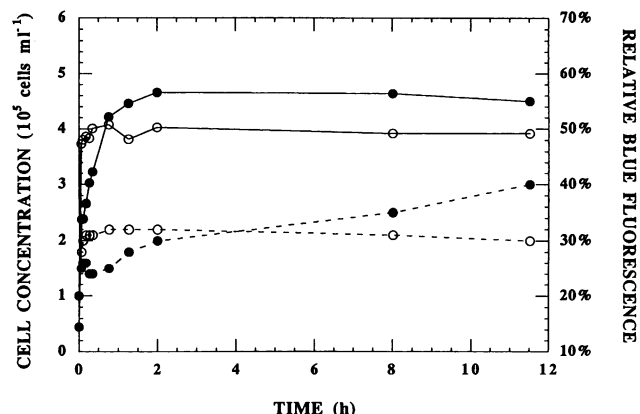

FIG. 2. Time course of abundance estimates (solid lines) and blue fluorescence relative to a 1.1-μm-diameter microsphere standard (dashed lines) for DAPI (●) and HO342 (○).

analog-to-digital converter discriminator position below which low-level noise was not recorded. For both central and outer stations, the HO342-stained populations were better separated from the noise than were the DAPI-stained populations. Moreover, the CV of blue fluorescence was significantly lower for the HO342-stained populations than for the DAPI-stained populations. The CVs for heterotrophic bacteria ranged from 45 to 50% and 75 to 80% for HO342- and DAPI-stained populations, respectively. CVs for *Prochlorococcus* bacteria were lower, typically 10 and 20% for HO342- and DAPI-stained populations, respectively. HO342- and DAPI-stained *Synechococcus* bacteria had CVs of roughly 45 and 60%, respectively. Strong correlations between RALS and blue fluorescence of DAPI-stained samples were apparent, but these correlations were absent for HO342-stained samples (Fig. 3b and e).

Precision and accuracy of FCM estimates. We found general agreement between the results of FCM and direct-counting methods (Table 1). Overall, FCM estimates ranged from 83 to 130% of direct counts and averaged 7% higher. FCM estimates averaged 2 and 12% higher than corresponding direct counts for HO342 staining and DAPI staining, respectively. A paired-sample t test between FCM estimates and direct counts showed no significant difference for HO342-stained samples ($P = 0.84$) but a significant difference for DAPI-stained samples ($P = 0.038$). There was no significant difference between direct microscope counts of samples stained with DAPI and those of samples stained with HO342 ($P = 0.72$). No obvious differences between the CVs of replicate abundance estimates obtained by using DAPI and those obtained by using HO342 were found (Table 1). CVs ranged from 0.63 to 2.9% (average, 1.5%) for bacterial abundances of 6 × 10^5 to 15 × 10^5 cells ml^{-1}.

DISCUSSION

Direct counting of DAPI-stained samples is one of the most widely used methods for estimating bacterial abundance in seawater and, therefore, serves as a standard against which the accuracy of any new method must be assessed. Our results indicate that FCM estimates of bacterial abundance using HO342 are essentially equivalent to direct counts of DAPI-stained samples. Moreover, HO342 appears to be superior to DAPI in terms of lower background fluorescence, smaller CVs of blue fluorescence, and better accuracy of abundance estimates. With the precision offered by FCM analysis of bacterial abundance (average CV, 1.5%), it is possible to distinguish a 6% difference between estimated means of single samples with 95% confidence. In contrast, direct-counting methods cannot resolve a difference smaller than 20% at the same confidence level (9).

HO342 and DAPI have similar excitation and emission maximums (23) and are remarkably similar in terms of binding with nucleic acids. Both dyes have two distinct binding modes, but at high ionic strength (>0.4 M) they form only highly specific complexes by nonintercalative binding with AT base pairs of DNA (24, 27, 28) and with AU base pairs of double-stranded RNA (dsRNA) (27, 34, 48). There are, however, two important factors that distinguish these two dyes, making HO342 better suited for FCM analysis of marine bacteria. First, while the absolute fluorescence quantum yield of the DAPI-AT complex is 17% higher than that of the HO342-AT complex (40), the relative fluorescence quantum yield (fluorescence quantum yield relative to the unbound dye in solution) is 30% higher for HO342 than for DAPI (23). The higher relative fluorescence quantum yield of
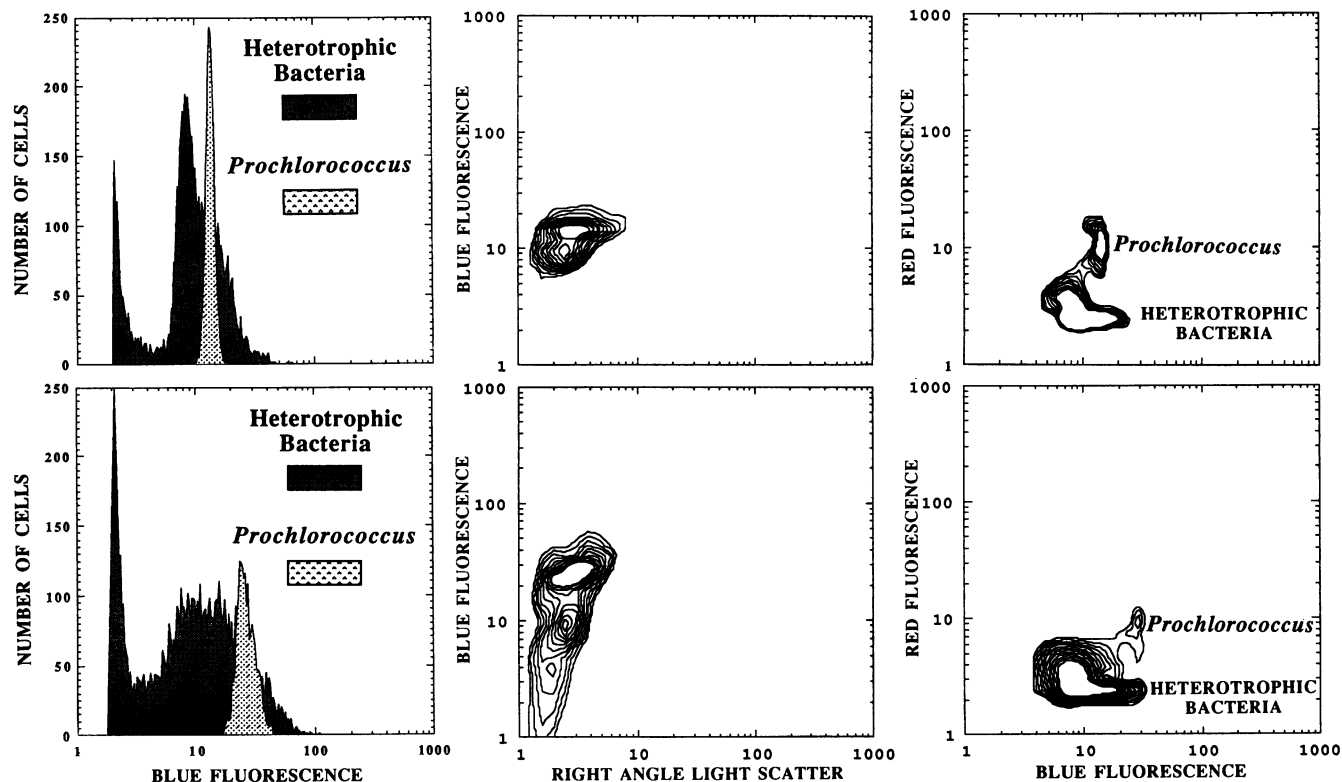
FIG. 3. FCM histograms of samples stained with HO342 (a to c) and DAPI (d to f). Samples were collected from the outer region of Kaneohe Bay and analyzed with identical FCM alignments. Blue fluorescence, dye-DNA complex fluorescence; red fluorescence, chlorophyll autofluorescence. Low-level blue noise signals have been partially gated out of single-parameter histograms and completely gated out of two-parameter histograms.

HO342 explains why cells stained with this dye are better separated from the background fluorescence of the dissolved dye (Fig. 3 and 4). Second, DAPI-AU complexes of dsRNA are fluorescent while HO342-AU complexes are nonfluorescent. Complexes formed between HO342 (also Hoechst 33258) and adenosine-bromodeoxyuridine base pairs are well known to be nonfluorescent (1). However, Müller and Gautier (34) and, more recently, Loontiens et al. (30) have shown that complexes formed between Hoechst 33258 (and presumably HO342) and AU clusters are also nonfluorescent. In contrast, DAPI-AU complexes exhibit a fluorescence quantum yield comparable to that of DAPI-AT complexes (31). Fluorescent DAPI-AU complexes are expected to make a measurable contribution to the CV of blue fluorescence because 40 to 60% of cellular RNA is in the double-stranded conformation (14, 17, 19, 44) and cellular RNA content of marine bacteria is variable (depending on growth conditions), ranging from less than half of to more than double the DNA content (32, 33). Coleman et al. (13) noted that DAPI can bind to cell wall and extracellular material, particularly polyphosphates, and to RNA and that much of this fluorescence is lost after RNase treatment.

The formation of fluorescent DAPI-dsRNA complexes would explain the high CVs of blue fluorescence that arise, in large part, from the size-dependent contribution to blue fluorescence as inferred from the covariation of RALS (an index of size, given that refractive index and cell shape are constant) and blue DAPI fluorescence (Fig. 3 and 4). The fact that no correlation for HO342-stained samples was

found effectively rules out the possibility of analytical artifacts such as spillover of RALS into the blue PMT.

The large CVs of blue fluorescence with DAPI-stained cells can be reduced with the addition of a detergent such as Triton X (final concentration, 0.1%) (8, 39), which permeabilizes the cells to allow better access by the dye. Our attempts to use Triton X (molecular grade) (U.S. Biochemical) with the current FCM configuration produced a large background fluorescence that made analysis impossible. We feel that the probability that detergent addition may cause lysis of some fragile cells or perhaps loss of important water-soluble phytoplankton pigments is great enough to warrant avoiding its use for the analysis of natural seawater samples.

Reliable fixation methods are important in oceanographic studies, in which it is not always possible to analyze samples collected at sea in real time; paraformaldehyde and glutaraldehyde are two commonly used fixatives. We found that blue DAPI fluorescence is strongly affected by fixation method but blue fluorescence of HO342 is not. Boucher et al. (4) analyzed DAPI-stained samples fixed with 1% (final concentration) glutaraldehyde and obtained CVs (10%) of blue fluorescence for *Prochlorococcus* bacteria that were similar to our results for HO342-stained cells. We observed CVs for blue fluorescence of heterotrophic bacteria fixed with 1% glutaraldehyde that were generally equivalent to HO342-stained samples fixed with either glutaraldehyde or paraformaldehyde (unpublished data). Glutaraldehyde and paraformaldehyde differ appreciably in their abilities to fix
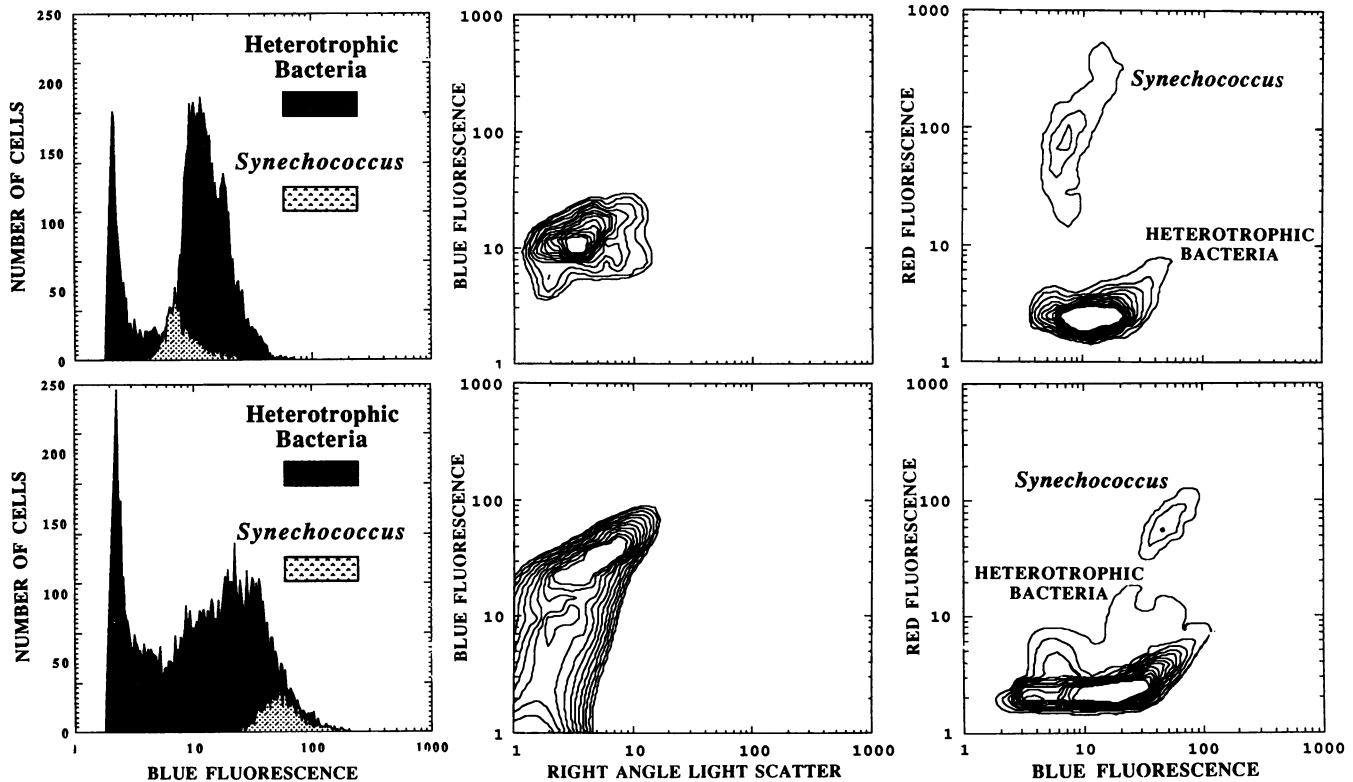
FIG. 4. FCM histograms of samples stained with HO342 (a to c) and DAPI (d to f). Samples were collected from the central region of Kaneohe Bay and analyzed with identical FCM alignments. Blue fluorescence, dye-DNA complex fluorescence; red fluorescence, chlorophyll autofluorescence. *Synechococcus* cells were gated on orange fluorescence (data not shown). Low-level blue noise signals were partially gated out of single-parameter histograms and completely gated out of two-parameter histograms.

nucleic acids in vivo. Paraformaldehyde quickly penetrates into cells and is by far the most effective agent for fixing nucleic acids and associated protein complexes (6). Glutaraldehyde penetrates slowly into cells and probably does not fully permeate gram-negative bacteria (21) or react appreciably with nucleic acids (6, 26). We speculate that glutaraldehyde failed to penetrate the cell, allowing naturally occurring RNases to degrade the dsRNA and leaving only DNA to react with DAPI to produce low CVs of blue fluorescence.

Glutaraldehyde fixation (final concentration, 1%) has been shown to protect most marine microbes from lysis and loss of natural fluorescence upon rapid freezing in liquid nitrogen and long-term cryogenic storage (45). Paraformaldehyde (final concentration, 1%) offers similar protection from cell lysis upon liquid nitrogen freezing (unpublished data) and performs better than glutaraldehyde for long-term cryogenic preservation of natural fluorescent pigments (22). We selected paraformaldehyde because of its value in long-term cryogenic storage and to avoid the potential interference introduced by the autofluorescence exhibited by glutaraldehyde (3; also, unpublished data).

It is worth pointing out that with paraformaldehyde fixation and in the absence of detergent additions the blue fluorescence of DAPI is significantly time dependent (Fig. 2), which will not affect the abundance estimates but will make estimates of DNA content based on blue fluorescence problematic. The blue fluorescence of HO342, however, is extremely time independent after 2 h (Fig. 2).

We found no statistically significant difference in abun-dance estimates among eight pooled DAPI- and HO342-stained samples using direct-counting methods; however, our FCM analysis gave DAPI estimates that were 12% higher than HO342 estimates. Paul (41) was also able to detect a 6%-higher abundance estimate by direct counting for DAPI staining compared with Hoechst staining when he pooled his data from 32 samples. Paul (41) proposed, as one possibility, that HO342 underestimates actual abundances because of poor cell penetration due to its relatively high molecular weight (615 versus 350 for DAPI). This explanation now seems unlikely in light of our time course results, which show that HO342 is taken up into the cells much faster than is DAPI (Fig. 2). It is probable that DAPI overestimates abundances because of noise introduced by the combination of the large CV of blue fluorescence and low relative fluorescence quantum yield. However, the presence of cells or particles similar in size to marine heterotrophic bacteria but low in DNA content and high in dsRNA content cannot be ruled out at this point. Observations by Bratbak et al. (5) of large (0.4-μm-diameter) virus-like particles in temperate coastal waters in the range of 10^4 ml^{-1} suggest an intriguing possible source for such particles if they turn out to be RNA viruses.

We have attempted to optimize the staining conditions for HO342 and DAPI so that meaningful comparisons could be made. Concentrations of 0.5 μg of HO342 ml^{-1} and 0.5 μg of DAPI ml^{-1} were optimal for incubations of >2 h and are roughly comparable in magnitude to those used by other researchers (2, 4, 13, 37). Lower levels of DAPI (e.g., 0.01

TABLE 1. Abundance estimates and CVs for EFM direct counts and FCM analysis

Date (mo/day)	Station	Dye	Abundance (10^5 cells ml^{-1})			
			FCM[a]			EFM[b]
			HBACT	PRO	HBACT + PRO	
3/11	Outer	HO342	4.32 (1)	1.11 (1)	5.46 (1)	5.55 (28, 29)
		DAPI	4.10 (1)	1.09 (1)	5.19 (1)	5.15 (28, 28)
3/18	Central	HO342	15.2 (1)			13.55 (21, 19)
		DAPI	15.6 (1)			11.98 (21, 28)
3/25	Outer	HO342	4.74 (5, 2.5)	1.42 (5, 2.5)	6.23 (5, 2.3)	6.12 (77, 30)
		DAPI	5.45 (5, 3.5)	1.61 (5, 3.3)	7.06 (5, 2.8)	6.29 (77, 30)
	Central	HO342	10.01 (5, 0.93)			8.99 (77, 39)
		DAPI	10.72 (5, 1.1)			8.92 (77, 27)
4/8	Outer	HO342	5.05 (5, 2.9)	1.86 (5, 3.5)	6.91 (5, 2.9)	8.29 (42, 25)
		DAPI	6.45 (5, 1.4)	1.96 (5, 0.92)	8.41 (5, 1.1)	8.29 (42, 24)
	Central	HO342	8.42 (5, 0.65)			8.49 (42, 33)
		DAPI	9.96 (5, 1.9)			9.40 (42, 25)
4/15	Outer	HO342	4.71 (4, 1.4)	1.86 (4, 1.9)	6.57 (4, 1.3)	7.55 (112, 40)
		DAPI	5.38 (4, 0.48)	2.27 (4, 1.9)	7.76 (4, 0.63)	7.03 (56, 31)
	Central	HO342	9.87 (4, 0.92)			10.81 (56, 32)
		DAPI	12.26 (4, 1.8)			11.48 (56, 25)

[a] Outer-station estimates include *Prochlorococcus* bacteria (PRO) in addition to heterotrophic bacteria (HBACT) for comparison with EFM counts. The first number in parentheses is the number of replicate sample deliveries by the multiple-sample delivery system; the second number (if given) is the percent coefficient of variation.

[b] The first number in parentheses is the number of fields counted; the second number is the percent coefficient of variation.

μg ml^{-1} [42]) produced very low fluorescence that could not be reliably analyzed. Robertson and Button (43) observed a continued increase in the blue fluorescence of heterotrophic bacteria at concentrations above 1.0 μg of DAPI ml^{-1} and used 2.5 μg ml^{-1} for best results. However, 0.5 μg of DAPI ml^{-1} has been used in subsequent work by these authors (8). While it is not strictly valid to extrapolate beyond the dye concentrations used in this study, the flatness of the abundance curve beyond 0.5 μg ml^{-1} (Fig. 1) suggests that further increases in the concentration of either dye will not significantly alter the current abundance estimates or the conclusions drawn in this report.

Data presented in this paper demonstrate that HO342-stained heterotrophic marine bacteria can be clearly distinguished from background fluorescence and separated from *Prochlorococcus* populations. With the analytical FCM approach described in this paper, it is now possible to reliably track changes in the numbers of heterotrophic bacteria independently of *Prochlorococcus* bacteria and to do so with greater speed and precision than by EFM counting. Given the ecological importance of the heterotrophic bacteria and *Prochlorococcus* populations in the oceans (12, 20, 46), this analytical method has much to offer in refining our understanding of the structure and dynamics of marine microbial food webs.

ACKNOWLEDGMENTS

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