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Enumeration of marine microbial organisms by flow cytometry using near-UV excitation of Hoechst 34580-stained DNA

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Abstract

A flow cytometry method for enumerating marine heterotrophic bacteria and phytoplankton in a living or preserved sample using a low power solid state near-ultraviolet laser is described. The method uses Hoechst 34580 to stain DNA in microbial cells in seawater samples. This stain is optimally excited at 375 nm unlike the similar Hoechst 33342, which requires ~ 350 nm excitation only available on more expensive lasers. Phytoplankton abundances from the Hoechst 34580 method are comparable to those of unstained samples and when analyzed by the Hoechst 33342 staining method. With this new method, nonpigmented marine bacteria and phytoplankton abundances are obtained simultaneously in a single sample as the Hoechst emission wavelength (~ 450 nm) is well separated from the emission wavelengths of chlorophyll and phycoerythrin fluorescence. Bacteria abundances are similar between this new method and those obtained with established Hoechst 33342 and SybrGreen I methods. Precision estimates (coefficient of variation) on populations with abundances near ~ 10^5 cells mL^{-1} are 1–3%, increasing to 3–9% at lower cell concentrations of 10^3 cells mL^{-1}. The Hoechst 34580 method is simple, requiring no heating or pretreatment with RNAse, can be used on unpreserved and formaldehyde-preserved cells, and is amenable to at-sea use with portable, compact, low power-requiring flow cytometers.

Marine microbial population abundances have been estimated using flow cytometry (FCM) for the last ~ 40 yr (Olson et al. 1983; Yentsch et al. 1984). This technique uses the intrinsic properties of cells and fluorescent probes to detect particles in a fluid stream that are subsequently separated by their properties into microbial groups. Phytoplankton and nonpigmented marine bacteria have been enumerated using FCM in habitats ranging from the open ocean to coastal areas, as well as in laboratory cultures (Veldhuis and Kraay 2000; Marie et al. 2005).

Flow cytometers used in marine research have the capability to detect phytoplankton chlorophyll fluorescence, usually using lasers with 488 nm excitation and detectors configured to gather emitted light at ~ 680 nm (Olson et al. 1983). One notable oceanographic discovery made with FCM was the detection of *Prochlorococcus*, which are cyanobacteria found in the euphotic zone of marine waters and of great importance to overall primary production (Chisholm et al. 1988; Partensky et al. 1999). Phycoerythrin, and phycobiliproteins in general, are naturally occurring pigments that are easily detected in FCM samples, and allow the enumeration of *Synechococcus*, cyanobacteria commonly found in coastal and open-ocean waters, as well as its separation from *Prochlorococcus*, as phycoerythrin expression is usually absent or minimal from the latter (Hess et al. 1996). Nonpigmented bacteria are usually detected with DNA dyes; this staining also facilitates the separation of living from dead cells or inorganic particles (Gasol and Del Giorgio 2000).

Most DNA stains used in oceanography are excited with a 488 nm laser, and often emit at green wavelengths (~ 525 nm, e.g., SybrGreen I; Marie et al. 1997), although stains emitting at longer wavelengths may be used, such as propidium iodide (Taylor and Milthorpe 1980). For samples containing phytoplankton, however, staining of DNA which results in fluorescence emission overlapping that of phycoerythrin or chlorophyll is of limited use. Probes optimally excited by UV light (~ 300 to < 400 nm) with emission (< 500 nm) are better separated from natural pigments than those emitting in green wavelengths. For instance, Hoechst 33342 binds to the minor groove of ds-DNA preferring AT-rich sequences (Portugal and Waring 1988) and is best excited by wavelengths near 350 nm, emitting at ~ 450 nm (Arndt-Jovin and Jovin 1977; Shapiro 1981; Monger and Landry 1993; Bucevičius et al. 2018). This probe has been used extensively to characterize phytoplankton and marine bacteria in many open ocean

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habitats (Campbell et al. 1994; Binder et al. 1996; Landry et al., 2008, 2011; Taylor and Landry 2018). However, this approach has been limited by the expense of flow cytometers with ultraviolet (UV) lasers emitting ~ 350 nm required to optimally excite this probe.

Described here is the use of Hoechst 34580, which is excitable by a near-UV (375 nm) or violet (405 nm) laser, with a stain excitation maximum of 368 nm, and emission maximum of 437 nm. These laser excitation wavelengths are achieved with relatively inexpensive solid-state lasers (Shapiro and Pellmutter 2001) found on commercially available instruments (Shapiro and Telford 2018). I will describe the use of this probe in detecting marine bacteria and phytoplankton in a single sample, saving time and sample volume. Presented here are data comparing microbial population abundances in preserved and unpreserved samples, as well comparing samples stained with Hoechst 34580, Hoechst 33342, and SybrGreen I.

Table 1. Sampling locations. Station ALOHA and station Ka'ena samples were from HOT 312 in the subtropical Pacific. NF17 samples were from the BLOOFINZ project in the Gulf of Mexico. Waimanalo Beach, Oahu, Hawaii samples were taken from the shore break (surface). CCE-LTER cruise P1908 samples were from a range of stations locations and euphotic zone depths.

Site	Latitude (°N)	Longitude (°W)	Date (M/D/Y)
Station ALOHA	22.7500	158.0000	6/11/19–6/13/19
Station Ka'ena	21.8467	158.3632	6/13/19
NF17 CTD 53	25.9934	89.2511	5/16/17
NF17 CTD 94	26.6339	90.1794	5/27/17
NF17 CTD 121	26.8138	89.9787	5/30/17
Waimānalo Beach	21.3356	157.6955	12/18/20
CCE-P190	34.3534–36.3319	120.8993–122.4957	8/05/19–9/06/19

Materials and procedures

Samples were collected from a variety of locations for method validation and comparisons (Table 1). Open-ocean samples were collected from six depths in the euphotic zone (5–125 m) at the Hawaii Ocean Time-series Stations ALOHA and Ka'ena during HOT 312 (KM19-09, June 2019). Depth profile samples were also obtained from the Gulf of Mexico, aboard the NOAA *Ship Nancy Foster* during May 2017, designated as NF17 samples (5–115 m, further details found in Selph et al. 2021). In addition, surface samples were obtained on 18 December 2020 from Waimānalo Beach (Oahu, Hawaii).

Samples (2 mL) were preserved within 1 h of collection with paraformaldehyde (0.5% final concentration), then flash frozen in LN_2 and stored at −80°C until batch analysis. On the run date, samples were thawed, and 275 μL aliquots were stained with 7 μL of 40 μL mL^{-1} Hoechst 34580 (1 μg mL^{-1}, final concentration) for 1 h in the dark at room temperature. Hoechst is potentially mutagenic and carcinogenic, so gloves should be worn as protection; also it may bind to plastic so staining should be in glass tubes. For stain stability tests, samples were thawed, stained (as above), then analyzed starting at 1 min poststaining and at frequent intervals till ~ 6 h, stored in the dark in the refrigerator (4°C), and then analyzed again at ~ 24 h.

Separate sample aliquots were stained with 1X SybrGreen I using the method of Marie et al. (1997) without RNAse-treatment or heating, for comparison of nonpigmented bacteria abundances relative to the Hoechst-stained samples. Briefly, 5 μL of 1% SybrGreen I solution (2 μL of 10,000X SybrGreen I diluted with 200 μL of distilled water) was added to 45 μL of 300 mM potassium citrate and 500 μL of sample. Another aliquot of sample was analyzed without staining, using intrinsic pigment fluorescence (i.e., chlorophyll and phycoerythrin) to detect phytoplankton cells. Finally, since the Hoechst molecule is small enough to enter unpreserved cells, freshly collected surface samples from Waimanalo Beach were stained with Hoechst 34580 (as above) or Hoechst 34580 plus trifluoperazine dihydrochloride (TFD; 15 μM final concentration).

A Beckman-Coulter CytoFLEX S flow cytometer was used to analyze all samples. This instrument is equipped with four lasers emitting light in spatially separated pathways at 375 nm (60 mW), 405 nm (80 mW), 488 nm (50 mW), and 561 nm (30 mW). Acquisition software was CytExpert (v. 2.3.1.22), generating listmode (FCS 3.0) files. Samples (115 μL) were delivered to the instrument from a 96-well plate and analyzed at 30 μL min^{-1}, with the exception of stain stability test samples which were run in single tube mode at the same acquisition rate. Daily flow rate calibrations (by weight) were done to ensure accurate delivery volume. For Hoechst 34580-stained samples, signal discrimination (with logic as "OR" rather than "AND") was on chlorophyll (EX 488 nm, EM 690 ± 50 nm, gain 500, discrimination 310 area) or DNA (EX 375 nm, EM 450 ± 45 nm, gain 500, discrimination 1950 area). For SybrGreen I-stained samples, the DNA discrimination was on green fluorescence (EX 488 nm, EM 525 ± 40 nm, gain 300, threshold 2000 area). *Synechococcus* was excited for both sample types using the yellow laser (EX 561 nm, EM 585 ± 42 nm, gain 500). Forward scatter gain was 100, while right-angle (90°) light scatter was collected from both the 405 and 488 nm lasers (gains 220 and 100, respectively). For unstained samples, signal discrimination was on chlorophyll fluorescence (EX 488 nm, EM 690 ± 50 nm, gain 500, discrimination 310 area). 0.5 μm-UV and YG beads (Polysciences) were used to normalize the fluorescence and scatter signals (e.g., mean fluorescence/bead fluorescence = normalized signal).

I also present data comparing nonpigmented bacteria in samples collected from off the coast of California (CCE-LTER, cruise P1908, August 2019; Table 1), preserved (as above), LN_2 frozen, stored at −80°C, thawed in batches, and then divided into two aliquots. One aliquot was stained with Hoechst 34580 (as above), whereas the other aliquot was stained with Hoechst 33342 (1 μg mL^{-1} final concentration). The Hoechst 34580 aliquot was analyzed on the CytoFLEX S (as above). The Hoechst 33342 aliquot was analyzed on a Beckman Coulter EPICS Altra flow cytometer, equipped with dual beam colinear excitation by two Coherent I-90 argon gas, water-cooled lasers, emitting at ~ 350 nm (200 mW) and 488 nm (1 W), with quantitative sample delivery controlled by a syringe pump (50 μL min^{-1}). Chlorophyll (680 nm), phycoerythrin (575 nm), and DNA (450 nm) fluorescence, as well as forward and 90° light scatter signals, were collected (more details in Selph et al. 2011).

Linear regressions for data comparisons were reduced major axis regressions, calculated using RMA for Java v.1.21 (Bohonak and van der Linde 2004). This program also calculates 95% confidence intervals using Jackknife estimates. Difference plots (values from one method subtracted from the other method, plotted as a function of the mean of the two values) were used to determine if systematic bias was inherent in the data, and to examine if data fell within the 95% confidence limits of agreement (Hollis 1996).

Assessments

Population definitions

Hoechst 34580-stained sample listmode files were analyzed using a series of two-parameter fluorescence and scatter plots to separate each microbial population using their signatures (Fig. 1). Shown here to illustrate this procedure is a 5 m station ALOHA-preserved sample. First, Hoechst 34580-bound DNA (discrimination parameter, thus including all events detected) was plotted as a function of acquisition time, which allows assessment of sample delivery rate, a key parameter for quantitative sample analysis (Fig. 1a). The CytoFLEX S flow cytometer has an uneven sample flow rate during the first

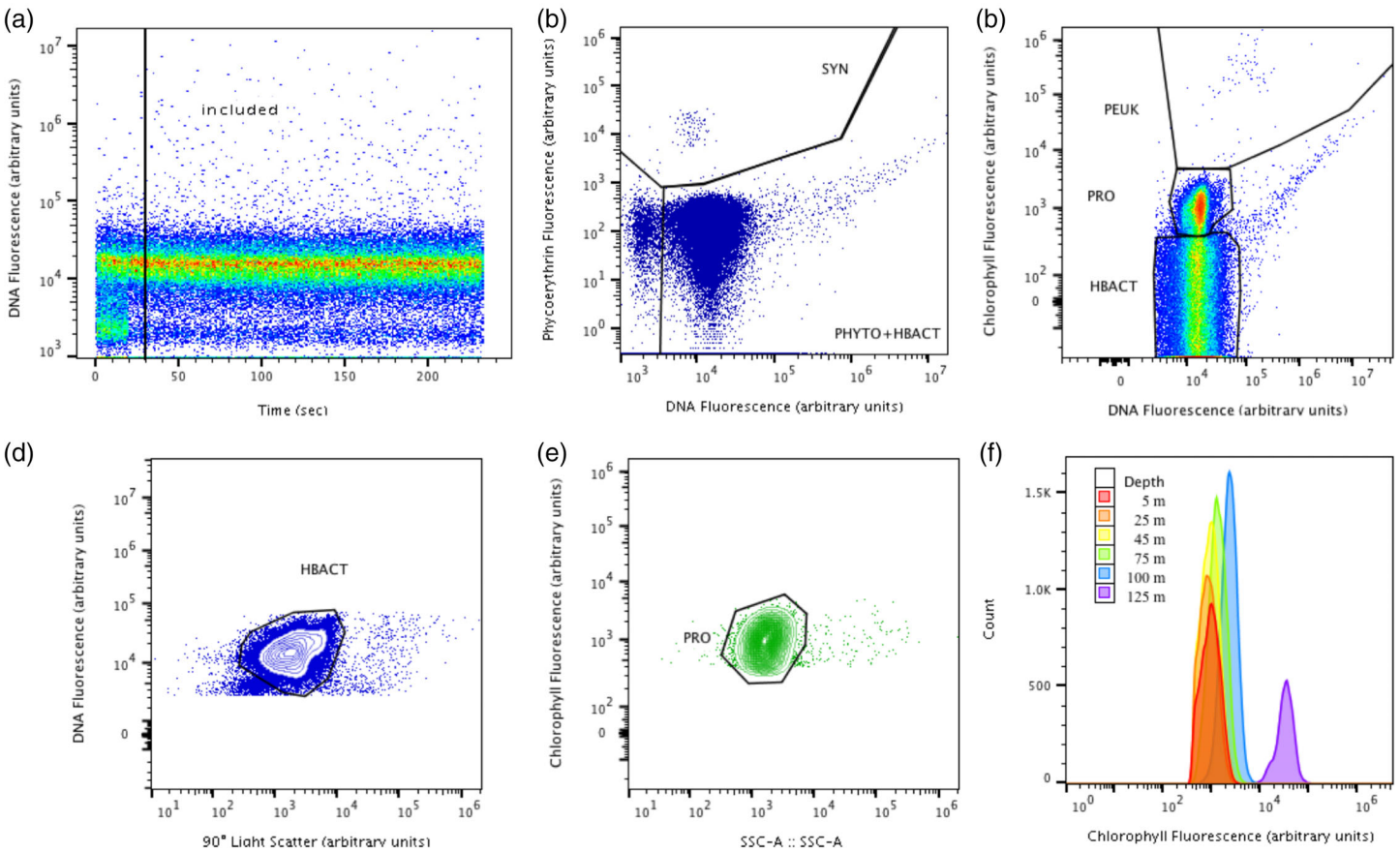

Fig 1. Hoechst 34580-stained samples from station ALOHA during HOT 312. Panels (**a**–**e**) are from a 5 m sample, showing the procedure of designating microbial populations after listmode file acquisition using the software program FlowJo. SYN, *Synechococcus*; PRO, *Prochlorococcus*; PEUK, photosynthetic eukaryotes, PHYTO, PRO+PEUK; HBACT, heterotrophic bacteria (= nonpigmented bacteria). Panel (**f**) shows the increase in chlorophyll (red) fluorescence with depth in the *Prochlorococcus* population.

~ 30 s of sample delivery, so this was excluded in subsequent population analyses, resulting in a sample file representing 100 μL of sample (i.e., "included" particles).

Phycoerythrin fluorescence as a function of DNA fluorescence was plotted for the included particle population, and subdivided into those particles with (e.g., *Synechococcus*) or without phycoerythrin fluorescence (Fig. 1b). It is critical for quantitative purposes to include all ≤ 0 events on the phycoerythrin axis for downstream analyses or non-phycoerythrin-containing particles will be excluded completely from further consideration. Next, chlorophyll and DNA fluorescence are used to separate heterotrophic bacteria, *Prochlorococcus* and photosynthetic eukaryotes from each other (Fig. 1c). The *Prochlorococcus* population DNA fluorescence magnitude is within the range found for heterotrophic bacteria, as is expected since all are prokaryotes. However, *Prochlorococcus* has chlorophyll fluorescence, so it is separable from heterotrophic bacteria by that parameter. Photosynthetic eukaryotes have more chlorophyll and DNA fluorescence than *Prochlorococcus*.

Further refinement of the *Synechococcus* population was achieved by plotting chlorophyll fluorescence as a function of phycoerythrin fluorescence (data not shown). Typically, *Synechococcus* shows less DNA fluorescence with Hoechst than *Prochlorococcus* or heterotrophic bacteria despite having a somewhat larger genome size. This may be due to *Synechococcus*' lower A-T content (Zhaxybayeva et al. 2009), which is the target of the Hoechst molecule. Photosynthetic eukaryote populations were refined to omit machine or other particle noise by plotting chlorophyll fluorescence as a function of 90° light scatter, since they will have both higher light scatter (i.e., they are larger) and chlorophyll fluorescence (data not shown).

The heterotrophic (nonpigmented) bacteria population was refined (from the subset indicated in Fig. 1c) by plotting DNA fluorescence as a function of 90° light scatter (Fig. 1d). Particles with very high light scatter (e.g., > 10^4 arbitrary scatter units in Fig. 1d), but no higher DNA fluorescence than the bulk of the cells, were omitted, as were particles with very low light scatter and low DNA fluorescence. *Prochlorococcus* was further defined using chlorophyll fluorescence as a function of 90° light scatter, excluding large and small particles that do not have appropriate chlorophyll fluorescence (Fig. 1e). Samples collected deeper in the water column typically have more chlorophyll per cell; this is particularly apparent for *Prochlorococcus* (Fig. 1f).

Notably, DNA signals from Hoechst 34580-stained cells showed little difference whether excited with a violet (405 nm) laser or a 375 nm laser (data not shown) using the CytoFLEX S cytometer, despite the expectation that more DNA signal should be obtained from 375 nm excitation based on the inherent Hoechst 34580 excitation-emission properties (Shapiro and Permutter 2001). However, violet-emitting lasers may not always emit at the expected wavelength (Shapiro and Telford 2018), so Hoechst 34580 signals may vary depending upon the instrument laser specifications and their response should be verified.

A second unstained aliquot of the preserved 5 m station ALOHA sample was analyzed within 1 h of the Hoechst-stained aliquot (Fig. 2). Similar to the stained sample, a plot of chlorophyll fluorescence (discrimination parameter) as a function of time was used to omit the first 30 s of uneven sample flow rate (Fig. 2a). Then, chlorophyll fluorescence as a function of phycoerythrin was used to separate *Synechococcus* from *Prochlorococcus* and photosynthetic eukaryotes (Fig.2b). The *Synechococcus* population was further refined by plotting phycoerythrin fluorescence as a function of 90° light scatter (data not shown), while photosynthetic eukaryotes and *Prochlorococcus* were delineated separately with chlorophyll fluorescence as a function of 90° light scatter (data only shown for *Prochlorococcus*, Fig. 2c).

Precision and stability tests

To estimate the precision of the Hoechst 34580 method, replicate (10) samples of a single preserved aliquot from HOT

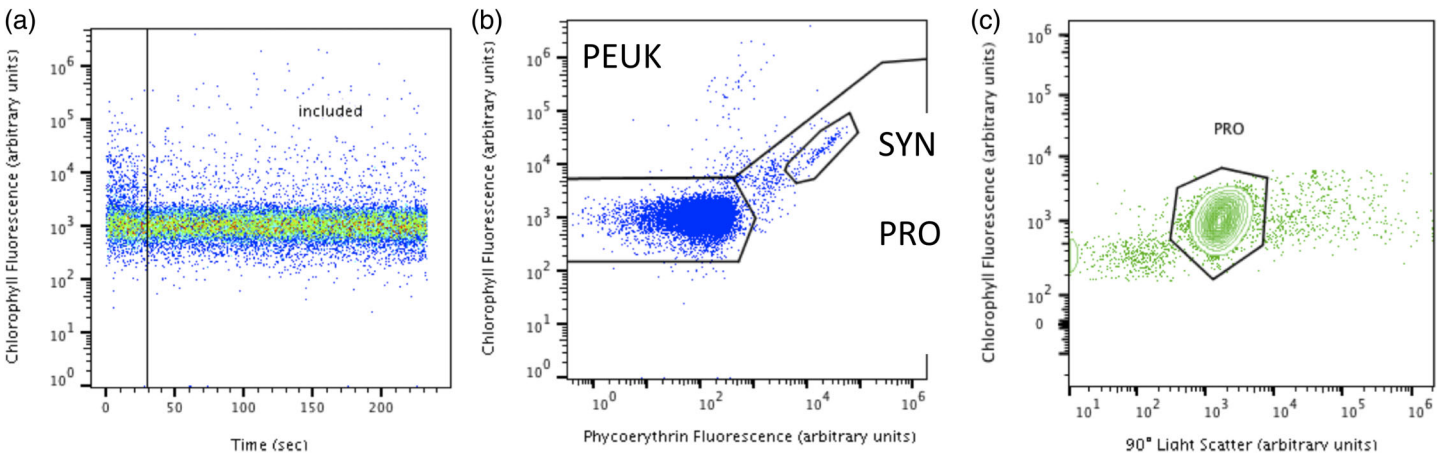

Fig 2. Microbial population designations for a second aliquot of the same 5 m sample from station ALOHA of HOT 312 as the sample shown in Fig. 1a–e, however this aliquot was not stained. SYN, *Synechococcus*; PRO, *Prochlorococcus*; PEUK, photosynthetic eukaryotes.

Table 2. Precision estimate of Hoechst 34580-staining method demonstrated with HOT 312 station ALOHA samples from 5, 75, and 125 m depth. Separate aliquots of each preserved sample ($n = 10$) were analyzed after staining with Hoechst 34580. Populations enumerated are *Prochlorococcus* (PRO), *Synechococcus* (SYN), photosynthetic eukaryotes (PEUK), and nonpigmented (heterotrophic) bacteria (HBACT). Shown are means ± 1 standard deviation ($n = 10$) for cell abundances ($\times 10^3$ cells mL^{-1} for SYN and PEUK and $\times 10^5$ cells mL^{-1} for PRO and HBACT), as well as the coefficient of variation (CV, %) for each population.

Depth (m)	PRO (10^5 mL^{-1})	CV (%)	SYN (10^3 mL^{-1})	CV (%)	PEUK (10^3 mL^{-1})	CV (%)	HBACT (10^5 mL^{-1})	CV (%)
5	1.99±0.05	2	1.10±0.08	7	0.70±0.06	9	3.88±0.11	3
75	3.03±0.03	1	2.02±0.11	5	1.24±0.04	3	5.20±0.04	1
125	0.095±0.003	3	0.09±0.04	45	0.31±0.07	22	2.46±0.04	1

312 station ALOHA were analyzed after staining (Table 2). For *Prochlorococcus*, coefficients of variation (CVs) on the average cell abundance were 1%–3% at the three depths compared (5, 75, and 125 m). Similarly, nonpigmented, or heterotrophic bacteria had very low CVs (1–3%). In the 100 μL aliquot analyzed for both of these populations, at least 1000 and usually more than 10,000 cells were enumerated. In contrast, for the photosynthetic eukaryotes and *Synechococcus*, the 5 and 75 m samples had CVs between 3% and 9%, whereas the deepest sample (125 m) had very high CVs (22% for photosynthetic eukaryotes and 45% for *Synechococcus*). These very high CVs are likely due to these populations' low abundances, i.e., 10s or 100s of cells in a 100 μL aliquot. For lower variances, a higher volume of sample can be analyzed at the same rate (30 μL min^{-1}) for a longer time. However, increasing the sample flow rate to analyze more volume must be done with extreme caution in any system with hydrodynamic focusing, as this broadens the sample stream and may compromise single-cell detection.

To determine how stable the DNA fluorescence was from Hoechst 34580, preserved station ALOHA samples (three casts, with samples from 45 to 100 m) were stained and analyzed in a time course, starting from the initial staining time (~ 1 min), then re-analyzed at intervals over a 24-h period. *Prochlorococcus* abundance time points averaged from 15 min to 6 h were within the precision of repeated measurements (2–3% CV), except for two of the 45 m samples where repeated measurements had CVs of 4–5% (Fig. 3a). In contrast, the average nonpigmented bacteria abundances were within precision estimates (1–2% CV) for all samples from 15 min to 6 h (Fig. 3b). At 24 h after staining, abundance measurements were lower for all samples, but still 83–94% of the mean value for *Prochlorococcus* and 94–98% of the mean value for nonpigmented bacteria (Fig. 3a,b, respectively).

DNA fluorescence in *Prochlorococcus* and nonpigmented bacteria increased after staining from 1 to 15 min, and was relatively stable after 30 min of staining, varying little over 24 h (Fig. 4a,b, respectively). Chlorophyll fluorescence in *Prochlorococcus* was highest initially, but decreased slightly over the first 2 h, stabilizing after that, with the 24-h value still 73–85% of the initial value. One exception was a 45 m sample (1a, Fig. 4c), which increased in chlorophyll

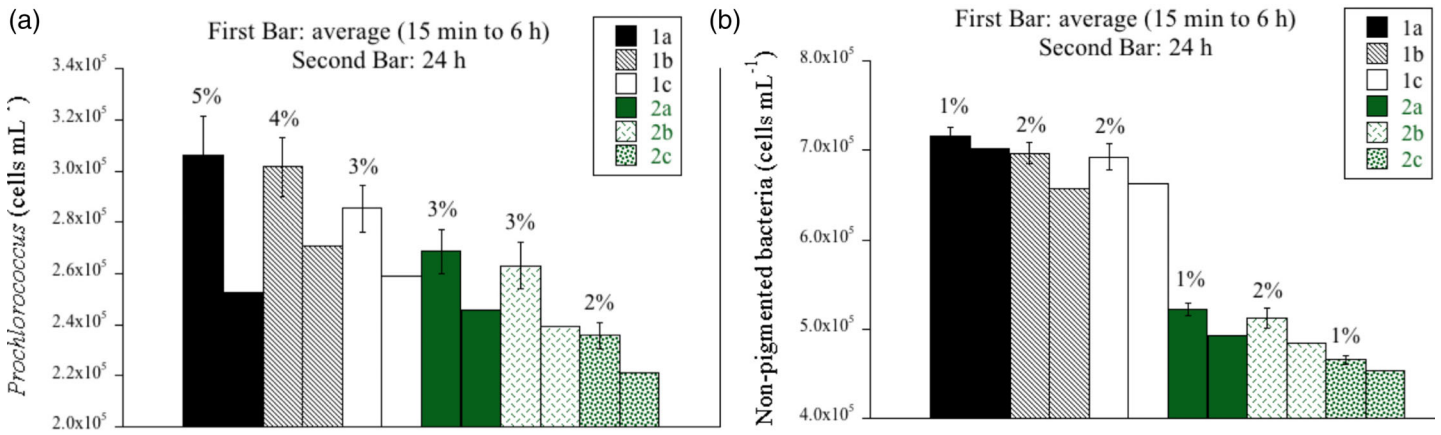

Fig 3. Hoechst 34580 stain stability tests. Samples were stained and repeatedly analyzed to determine the time course of Hoechst staining and the stability of the signal. Shown are data arranged as pairs of bars for 45 m (1a, 1b, 1c) and 100 m (2a, 2b, 2c) *Prochlorococcus* **(a)** and nonpigmented bacteria abundance **(B)** (cells mL^{-1}) from separate casts at station ALOHA during HOT 312. Data in the first bar are the average of the 15 min-6 h time points, followed by the 24 h time point in the second bar. Error bars are 1 standard deviation of the mean ($n = 7$ for 45 m; $n = 6$ for 100 m). Percentages above each mean value are the coefficient of variation for that determination over the 6 h time course.

fluorescence in the first hour before decreasing. However, that sample was an outlier in several respects, showing the most abundance variance (5%) and greatest decrease in abundance and chlorophyll fluorescence after 24-h (1a, Fig. 3a).

Method comparisons

The Hoechst 34580 method was compared the SybrGreen I method using a difference plot (Hollis 1996), with the difference in nonpigmented bacteria abundance measured by each method plotted as function of the average of the two values (Fig. 5a). The data show a mean ± standard deviation of the difference of $-0.01 \pm 0.44 \times 10^5$ cells mL^{-1}, with a standard error of the mean ($n = 72$) of 0.05×10^5 cells mL^{-1}. The 95% confidence interval of these data is $-0.01 \pm 0.89 \times 10^5$ cells mL^{-1}, which includes zero, showing no systematic bias. The percent of values falling between the confidence

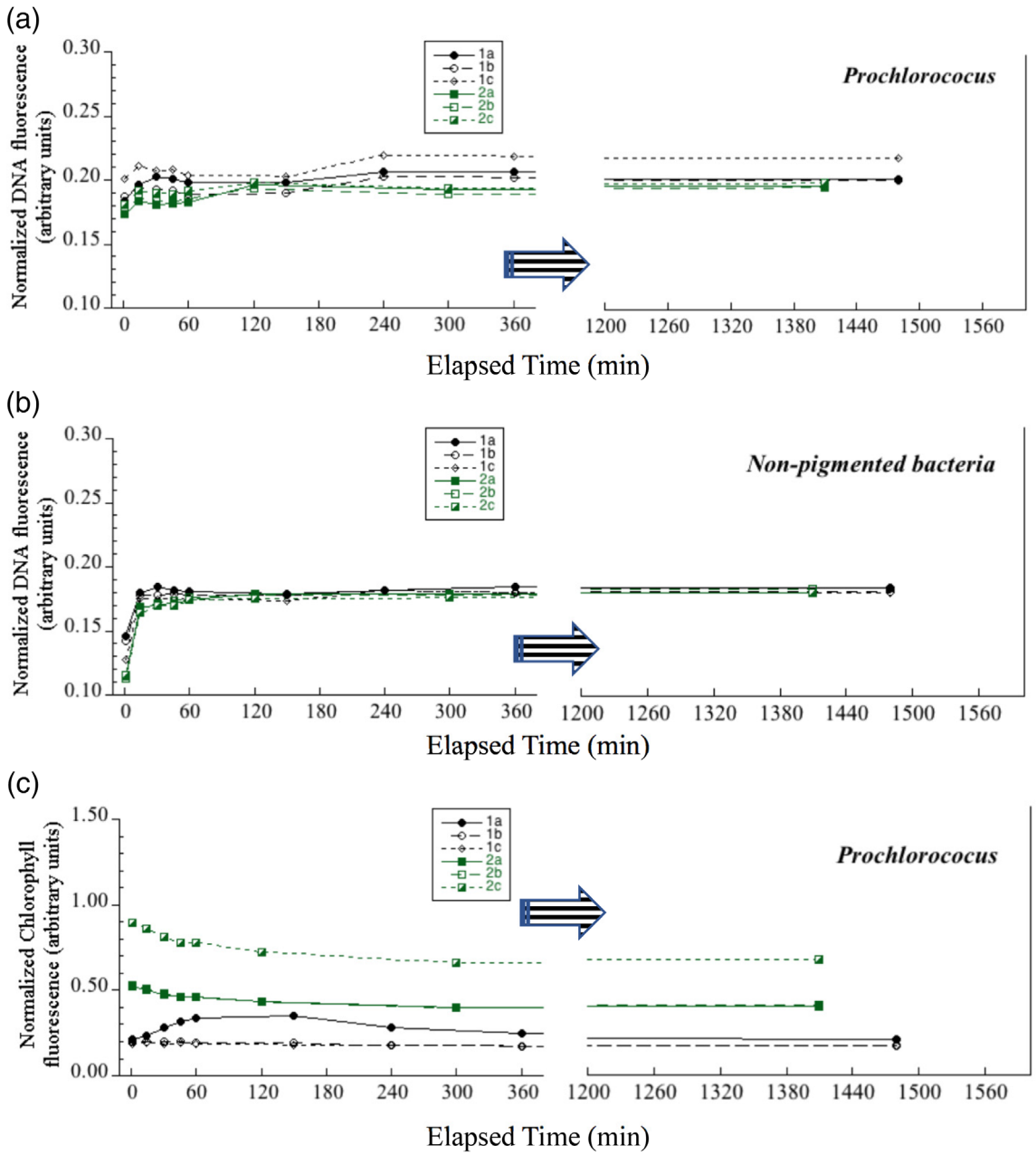

Fig 4. DNA (from Hoechst 34580) and chlorophyll normalized fluorescence signals as a function of staining time. Samples were stained, then repeatedly analyzed to determine the time course of Hoechst staining and the stability of the signal. Shown are fluorescence data (arbitrary units) for 45 m (1a, 1b, 1c) and 100 m (2a, 2b, 2c) *Prochlorococcus* (panel **a** – normalized DNA fluorescence, panel **c** – normalized chlorophyll fluorescence) and nonpigmented bacteria (panel **b** – normalized DNA fluorescence) from separate casts at station ALOHA during HOT 312. Note *x*-axis scale discontinuity (indicated by arrow).

intervals is 94.4%, suggesting good agreement between the methods. Further, reduced major axis regression of these data have a slope overlapping 1.0 (range, 0.9881–1.198) and an intercept encompassing zero (range, −1.127 to 0.06624), within 95% confidence limits (Fig. 5b).

The Hoechst 34580 method was also compared to the Hoechst 33342 method using a difference plot (Fig. 5c). These data show a mean ± standard deviation of the difference of $0.177 \pm 0.591 \times 10^5$ cells mL^{-1}, with a standard error of the mean ($n = 42$) of 0.091×10^5 cells mL^{-1}. All data fall within the 95% confidence interval which includes zero, indicating no systematic bias. Reduced major axis regression of these data have a slope of 1.124, with 95% confidence intervals of 1.046–1.127, indicating that the Hoechst 34580 method gives a slightly higher abundance than the Hoechst 33342 method (Fig. 5d). The intercept is -0.6199×10^5 cells mL^{-1}, and its

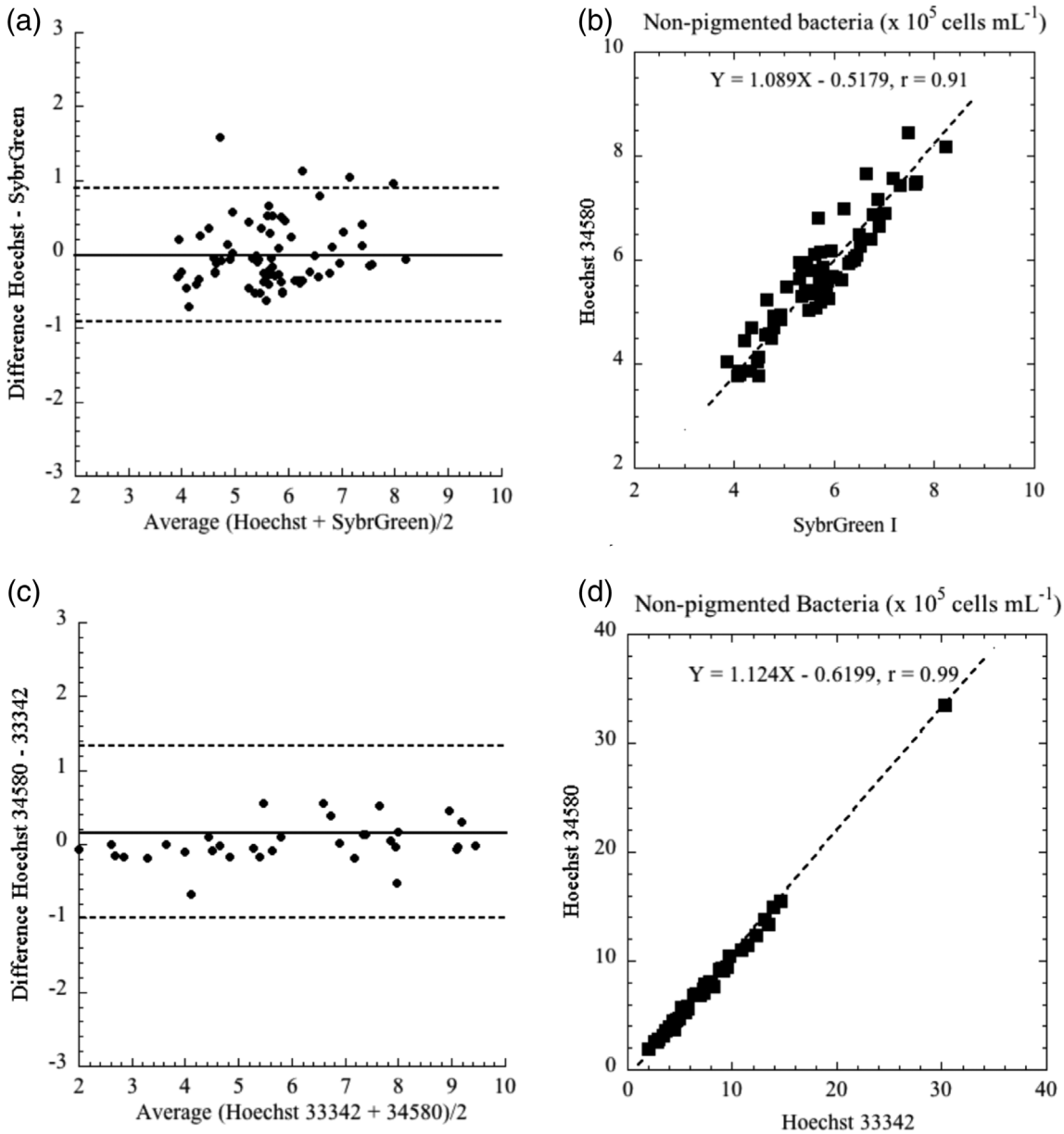

Fig 5. Comparison of nonpigmented bacteria abundance (× 10 5 cells mL^{-1}) from samples stained with Hoechst 34580 and SybrGreen I (**a**, **b**), and Hoechst 34580 and Hoechst 33342 (**c**, **d**). Panels (**a**) and (**b**) show data from euphotic zone samples from the Hawaii Ocean Time-series stations ALOHA and Ka'ena (HOT 312, June 2019) and oligotrophic, deep-water stations in the Gulf of Mexico (NF17, May 2017). Panels (**c**) and (**d**) show data from open-ocean euphotic zone samples ($n = 42$) collected from California coastal waters during the CalCOFI CCE project (P1908, August 2019). Panels (**a**) and (**c**) are difference plots (× 10 5 cells mL^{-1}), with the mean difference (solid line) and limits of agreement (dashed lines). Panels (**b**) and (**d**) are the reduced major axis regressions of these data.

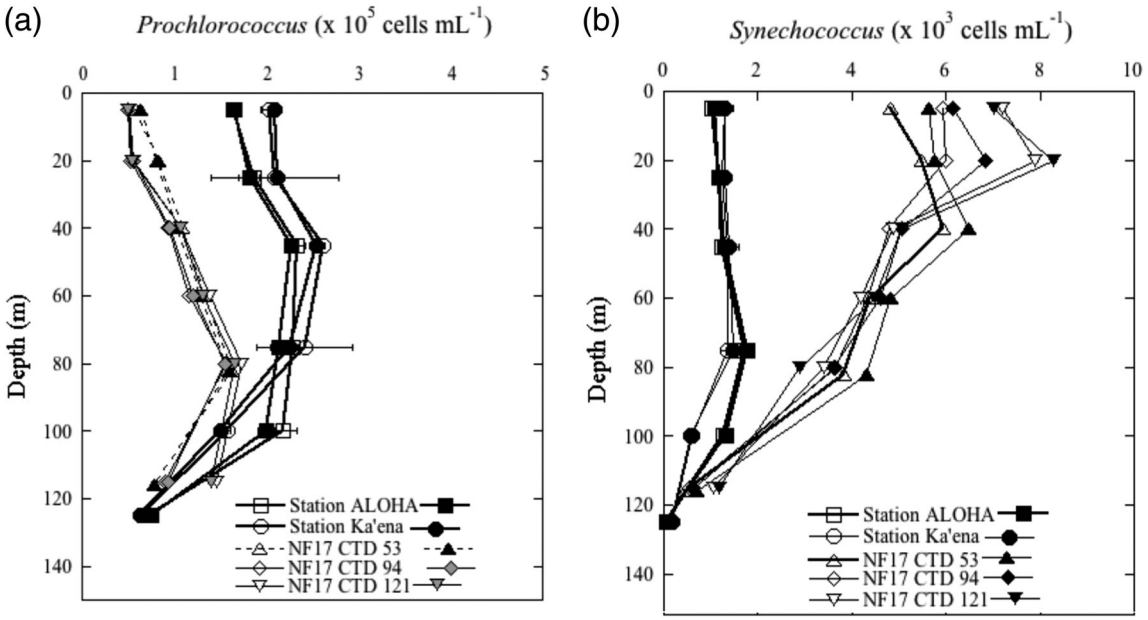

Fig 6. *Prochlorococcus* (**a**) and *Synechococcus* (**b**) abundance (cells mL^{-1}) as a function of depth. Comparison of unstained and Hoechst 34580-stained samples from the Hawaii Ocean Time Series stations Aloha and Ka'ena point (HOT 312, June 2019) and from oligotrophic, deep-water stations in the Gulf of Mexico (NF1704, May 2017, one profile per station). Open symbols are unstained samples, while closed symbols are samples stained with Hoechst 34580. Stations ALOHA and Ka'ena data ($n = 4$ per depth) are means ± 1 standard deviation, whereas NF17 profiles are from single casts.

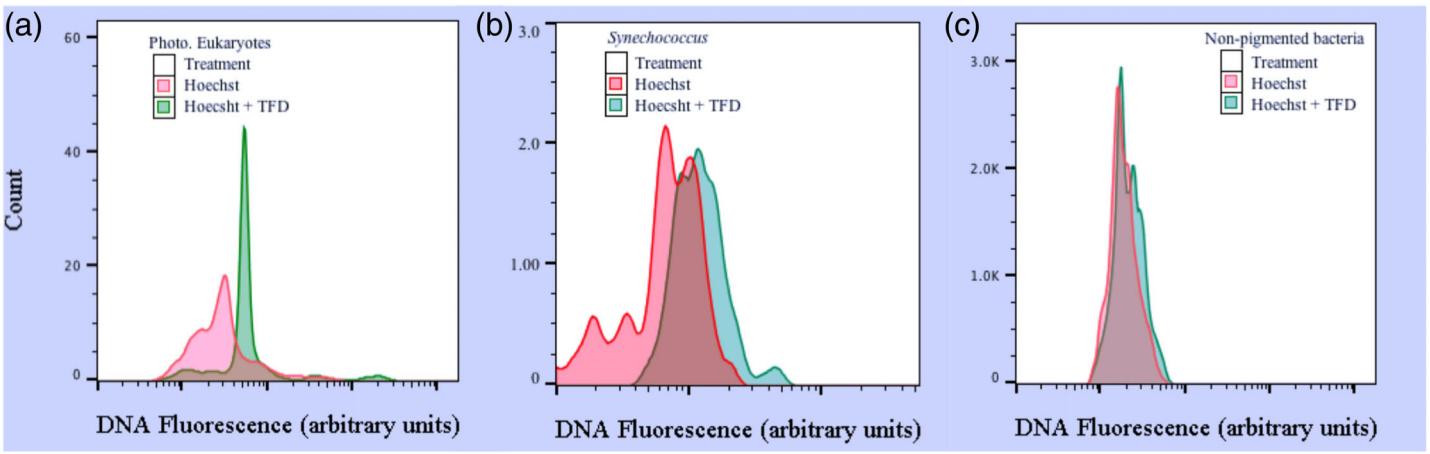

Fig 7. Phytoplankton (photosynthetic eukaryotes (**a**), *Synechococcus* (**b**) and nonpigmented bacteria abundances (**c**) for live (unpreserved) Waimānalo Beach surface samples. Abundances are the number of cells in 100 μL of sample. Samples were stained with (1) Hoechst only (red fill) or (2) Hoechst + TFD (green fill).

95% confidence interval is below zero (range, −0.7025 to −0.2198 × 10^5 cells mL^{-1}). While the single high outlier in this regression (~ 30 × 10^5 cells mL^{-1}) does increase the slope slightly (1.124 relative to 1.061 × 10^5 cells mL^{-1}), its omission still results in a slope with 95% confidence limits higher than 1.0 at the hundredth decimal place (range, 1.026–1.088 × 10^5 cells mL^{-1}).

Depth profiles from HOT 312 and NF17 show that phytoplankton enumerated after staining with Hoechst 34580-stained samples were similar to unstained samples (Fig. 6). *Prochlorococcus* abundances were not significantly different from each other (Fig. 6a). At Stations ALOHA and Ka'ena, where *Synechococcus* concentrations are very low (≤ 2 × 10^3 cells mL^{-1}), means of stained and unstained samples were also similar (Fig. 6b). However, in the Gulf of Mexico, where *Synechococcus* were present at higher concentrations in the euphotic zone and only single casts are compared between methods, Hoechst-stained samples were somewhat higher than unstained samples at a few depths, reflecting the lower precision of the method when applied to low population abundances.

Unpreserved Waimānalo Beach surface samples were stained with Hoechst 34580 (Fig. 7). While Hoechst alone stained the live samples adequately, the addition of TFD greatly increased the cellular fluorescence of the eukaryotic phytoplankton (Fig. 7a). TFD functions to block efflux of Hoechst from living cells, for a brighter fluorescence signal (Krishan 1987). In contrast, *Synechococcus* had only slightly higher DNA fluorescence with TFD (Fig. 7b), while nonpigmented bacteria showed no overall change in fluorescence (Fig. 7c).

Discussion

The Hoechst 34580 method, which can be applied to preserved or living cells, has a precision of ≤ 3% for cell populations at ≥ 10^4 cells mL^{-1}, and < 10% for lower cell concentrations of ~ 10^3 cells mL^{-1} when 100 μL of sample is analyzed. Staining is also relatively stable, with the same DNA signal obtained over ~ 24 h period. However, preserved phytoplankton samples gradually lose pigment fluorescence on shorter time scales so it is advisable to analyze thawed samples as soon as possible for maximum signal. Abundance estimates in preserved samples also decrease somewhat after 6 h at room temperature. The method compares well to other methods, in particular Hoechst 33342 and SybrGreen I staining.

The SybrGreen I staining method is quite effective at separating *Prochlorococcus* from nonpigmented bacteria when the cellular red fluorescence of *Prochlorococcus* is high enough (e.g., deeper natural samples or cultures). However, for natural samples from shallow depths, SybrGreen I fluorescence interference into the chlorophyll fluorescence detector prevents clear separation of these populations, as previously noted by Marie et al. (1997). Marie et al. (1997) also recommends analyzing each sample twice: first, live and unstained for phytoplankton, and second preserved and stained for nonpigmented bacteria. With Hoechst 34580, only a single sample run is required regardless of *Prochlorococcus*' intrinsic chlorophyll fluorescence; and if a suitable instrument is available at the time of collection, this dye also may be used to stain living cells, so again a single sample can be analyzed for obtaining information on all populations.

The instrument used here, the Beckman Coulter CytoFLEX S, is a compact, easily transportable, sensitive flow cytometer, suitable for at-sea use. In addition to the availability of the 375 nm laser on this instrument, it has very sensitive avalanche photodiode detectors that allow the detection of natural populations of surface *Prochlorococcus*, which is not possible for many instruments (Gérikas Ribeiro et al. 2016). Avalanche photodiodes have a higher quantum efficiency than photomultiplier detectors at wavelengths greater than 650 nm (Lawrence et al. 2008). Previously, surface *Prochlorococcus* has usually only been detectable using very expensive, power-intensive flow cytometers which are comparatively difficult to take to sea and require highly trained operators to maintain and use.

The Hoechst 34580 DNA staining method allows enumeration of phytoplankton and nonpigmented bacteria in a single sample, using relatively low power 375 nm laser excitation. This is a distinct improvement over methods requiring expensive, power-intensive UV lasers for excitation, or the use of stains emitting in the green with emitted fluorescence overlapping natural pigment fluorescence. This methodology is effective for staining living or preserved cells, making it versatile in application for shore- and ship-based analyses. The simplicity of the method is also attractive, with no preincubation of sample in RNAse or heating. This new methodology, using a simple to operate and relatively inexpensive instrument, will allow more researchers to have the capability to study microbial populations, and particularly picoplankton, on shore or at sea.

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