

Rapid estimation of microbial numbers in water using bulk fluorescence

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Summary

Enumeration of microbial cells without culturing is an essential technique for microbial ecology and water quality evaluation. Here we show that bulk fluorescence using the SYBR Gold DNA stain can be used to rapidly quantify microbial cells per millilitre in fresh, marine and estuarine waters. The bulk fluorescence method is comparable to estimating cell concentrations in cultures using optical density; however, this enhanced method enables the user to estimate microbial numbers at lower concentration ($> 10^5$ cells ml⁻¹) found in environmental samples. The technique worked in both single-cell and 96-well plate fluorescent spectrophotometers. Differences of $\sim 10^5$ cells per millilitre were discernible and the precision of the bulk fluorescence was higher than direct counting by epifluorescent microscopy. Treatment with DNase I increased sensitivity by lowering background noise attributed to free DNA. This technique is simple, rapid, inexpensive and adaptable for automatically estimating microbial numbers in water samples.

Introduction

A major breakthrough in microbial ecology was direct counting of microbial cells via DAPI staining and epifluorescence microscopy (Hobbie *et al.*, 1977). Using this method, it was established that there are $\sim 10^6$ prokaryotic cells per millilitre of seawater and $\sim 10^9$ prokaryotic cells per gram of soil or sediment, despite the fact that only 1%

of these cells are readily culturable. Direct count protocols have been modified to incorporate newer nucleic acid stains, such as SYBR Gold (Breitbart *et al.*, 2004) and SYBR Green (Noble and Fuhrman, 1998). The SYBR dyes have a greater fluorescence enhancement (i.e. increase in fluorescence when the dye binds to DNA) than ethidium bromide or DAPI. SYBR nucleic acid stains also have a higher fluorescence quantum yield, making them more sensitive. These characteristics have made the direct counts of prokaryotic cells, and even viruses, relatively routine (Noble and Fuhrman, 1998). Further improvements have included the adaptation of flow cytometry and automated image analyses software.

Despite these improvements, current methods for counting microbes are relatively slow and require expensive/sophisticated equipment (e.g. flow cytometers or epifluorescent microscopes). An inexpensive, rapid technique for estimating microbial concentrations would be valuable to investigators in several fields including water quality assessment, aquaculture and microbial ecology. Here, we present a rapid method for estimating microbial numbers in marine, estuarine and fresh water samples using simple fluorometry and the nucleic acid stain SYBR Gold.

Results and discussion

Correlation of microbial numbers to SYBR Gold bulk fluorescence

To determine whether bulk fluorescence could be used to quantify microbes, a dilution series was created using varying proportions of 0.45 μm (+ microbes) and 0.02 μm filtered seawater (- microbes). The different dilutions were stained with SYBR Gold and the relative fluorescence units (RFUs) measured on a Hitachi f4500 fluorometer. As shown in Fig. 1A, there was a correlation between the dilutions and bulk SYBR Gold fluorescence. There was also a strong correlation ($r^2 = 0.94$) between the relative fluorescence as measured by the fluorometer and the number of microbes in the samples, which were counted directly using epifluorescence microscopy (Fig. 1B).

A similar dilution series was also measured on the Gemini XS 96-well fluorometer. There were significant differences ($P \leq 0.05$) between 0.45 μm samples diluted by 40% with 0.02 μm filtered seawater (e.g. 20% versus 60% and 60% versus 100% in Fig. 1C). The r^2 value for the seawater dilution series measured on the Gemini flu-

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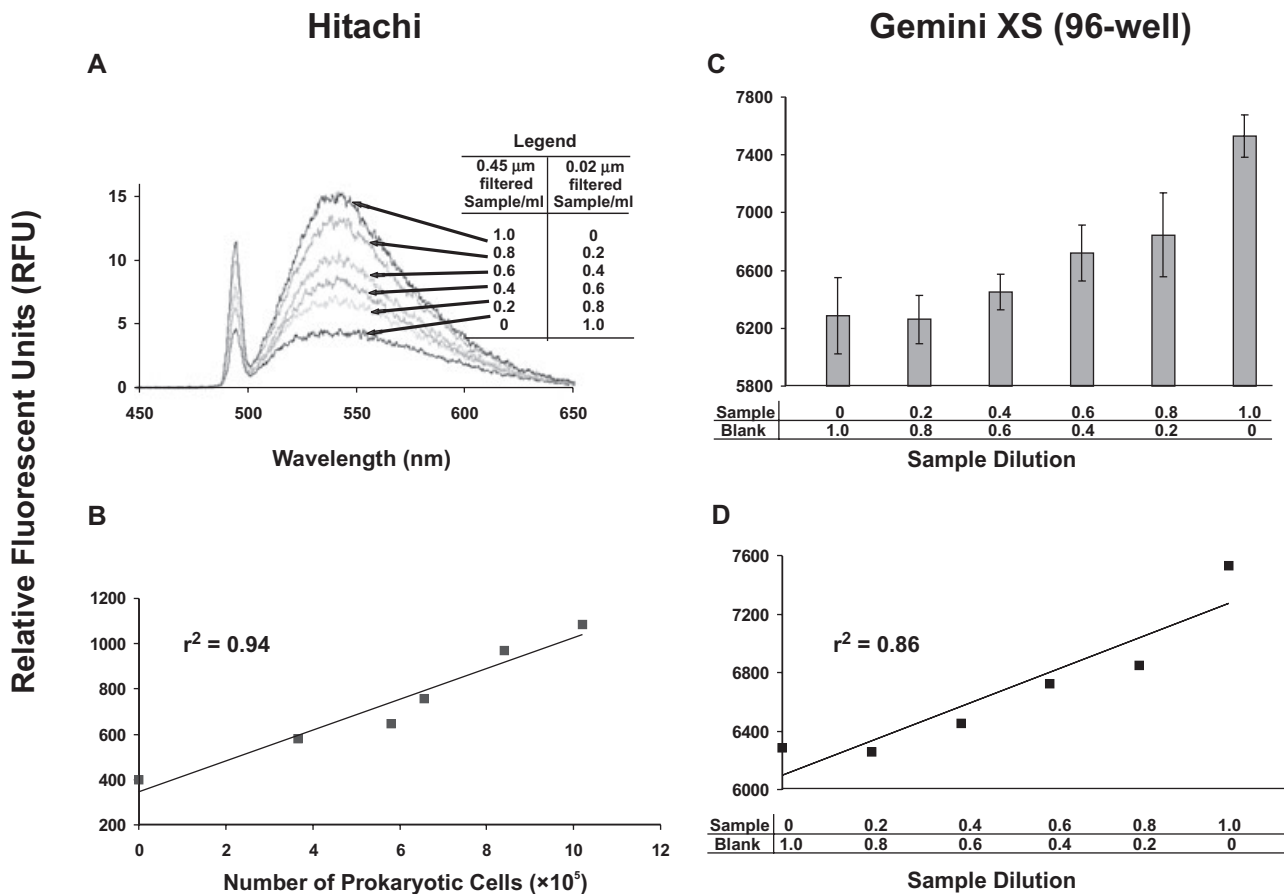


Fig. 1. Measurement of bulk fluorescence in seawater dilution series using the Hitachi and Gemini XS fluorometers. A. The relative fluorescence was measured on the Hitachi fluorescent spectrophotometer using an excitation wavelength of 495 nm and an emission spectra ranging from 450 to 650 nm. The area under the curve was calculated and an XY scatter plot was constructed. B. Correlation between bulk fluorescence and direct cell counts in the seawater dilution series. C. Measurement of relative fluorescence in a seawater dilution series using Gemini fluorometer. The bulk fluorescence was estimated by calculating the area under the emission spectrum and a bar graph was constructed. Four replicates per sample were measured and averages were calculated. D. Correlation between relative fluorescence and percentage of whole seawater in sample. The Gemini fluorometer had the capability of measuring several replicates simultaneously, while the Hitachi could only measure single samples.

rometer was 0.86 (Fig. 1D). These preliminary analyses suggested that bulk fluorescence with SYBR Gold may be used to enumerate microbes on both single-cell and multi-well fluorometers.

Microbial numbers in environmental water samples

Bulk fluorescence and epifluorescent microscope direct counts were performed on fresh, marine and estuarine water samples collected from the San Diego area. As shown in Fig. 2, there were visibly discernible differences between samples that vary by at least 5×10^5 cells ml^{-1} .

Precision

The precision of the bulk fluorescence method versus direct counts was compared in three water samples. The

error between samples was estimated using the standard deviation of the mean from the three replicate samples measured per site. Fluorometer measurements for the three Samples ranged from 427 to 461 RFUs and the standard deviation in RFUs ranged from 12 to 18 (Fig. 3). Estimates of total bacterial numbers using epifluorescent microscopy ranged from 9.1×10^5 to 1.0×10^6 and the standard deviation ranged from 7.2×10^4 to 1.3×10^5 (Fig. 3). In the South San Diego Bay water sample, error with the fluorometer was 2.8% and error with direct counting was 12%; in the Harbor Island sample, error with fluorometer was 2.9% and error with direct counting was 15%; and in the Shelter Island sample, error with fluorometer was 4.2% and error with direct counting was 7.1%. Therefore, the precision was higher with the fluorometer than with directly counting using epifluorescent microscopy.

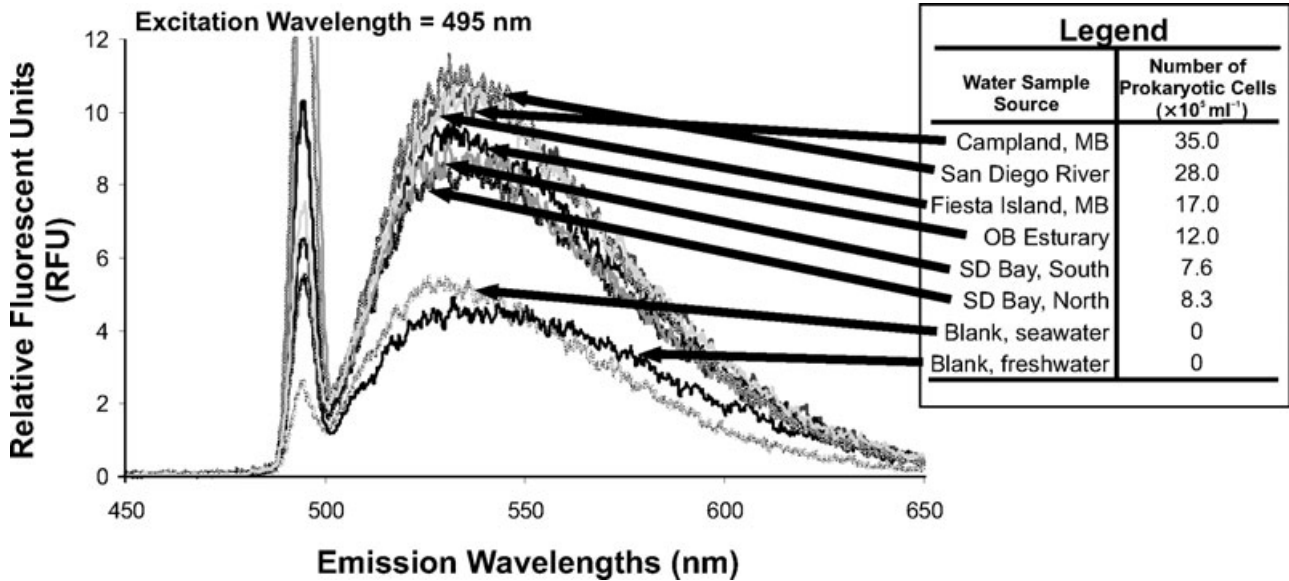


Fig. 2. Measurement of bulk fluorescence in six San Diego water samples. Samples were collected from Mission Bay (MB), Ocean Beach (OB) Estuary, Point Loma, San Diego (SD) Bay and the San Diego River. Each water sample was measured for the total fluorescence using a Hitachi f4500 fluorescent spectrophotometer. The emission spectrum of 450–650 nm was collected (excitation = 495 nm). The area of each wavelength scan was calculated for all water samples. After a water sample was scanned, it was immediately pipetted out of the cuvettes and fixed with 2% paraformaldehyde for the direct counts by microscopy.

Treatment with DNase I lowers background and increases sensitivity

Dissolved DNA has been found at concentrations as high as 5–44 $\mu\text{g l}^{-1}$ in estuaries and 2–15 $\mu\text{g l}^{-1}$ for coastal oceanic environments (DeFlaun *et al.*, 1987; Paul *et al.*, 1987; Boehme *et al.*, 1993; Jiang and Paul, 1995). Therefore, it is likely that dissolved DNA will cause background noise in the bulk fluorescence signal. To test this, five water samples were treated with DNase I and assayed with the bulk fluorescence protocol. The addition of the DNase I lowered background noise associated with the Blank

(Fig. 4). For example, the signal in the Laurel St., South Fiesta Island and Anthony's Pier samples was completely masked by the free DNA noise. Therefore, we recommend that a DNase I step be included when using this method. The SYBR Gold and DNase I can be added simultaneously (data not shown).

The effects of preservatives

To determine whether the bulk fluorescence method was compatible with common preservation techniques, three

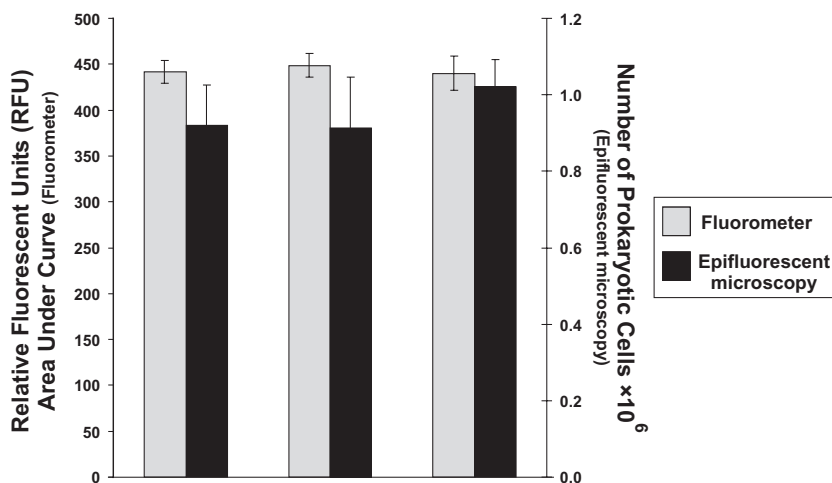


Fig. 3. Precision of the measurements for relative fluorescence in three seawater samples using the Hitachi fluorescent spectrophotometer. The standard error using the fluorometer was 3.3%, while the average error for direct counts was 11%. Direct counts of cells using epifluorescent microscopy were less precise than measuring with the fluorometer.

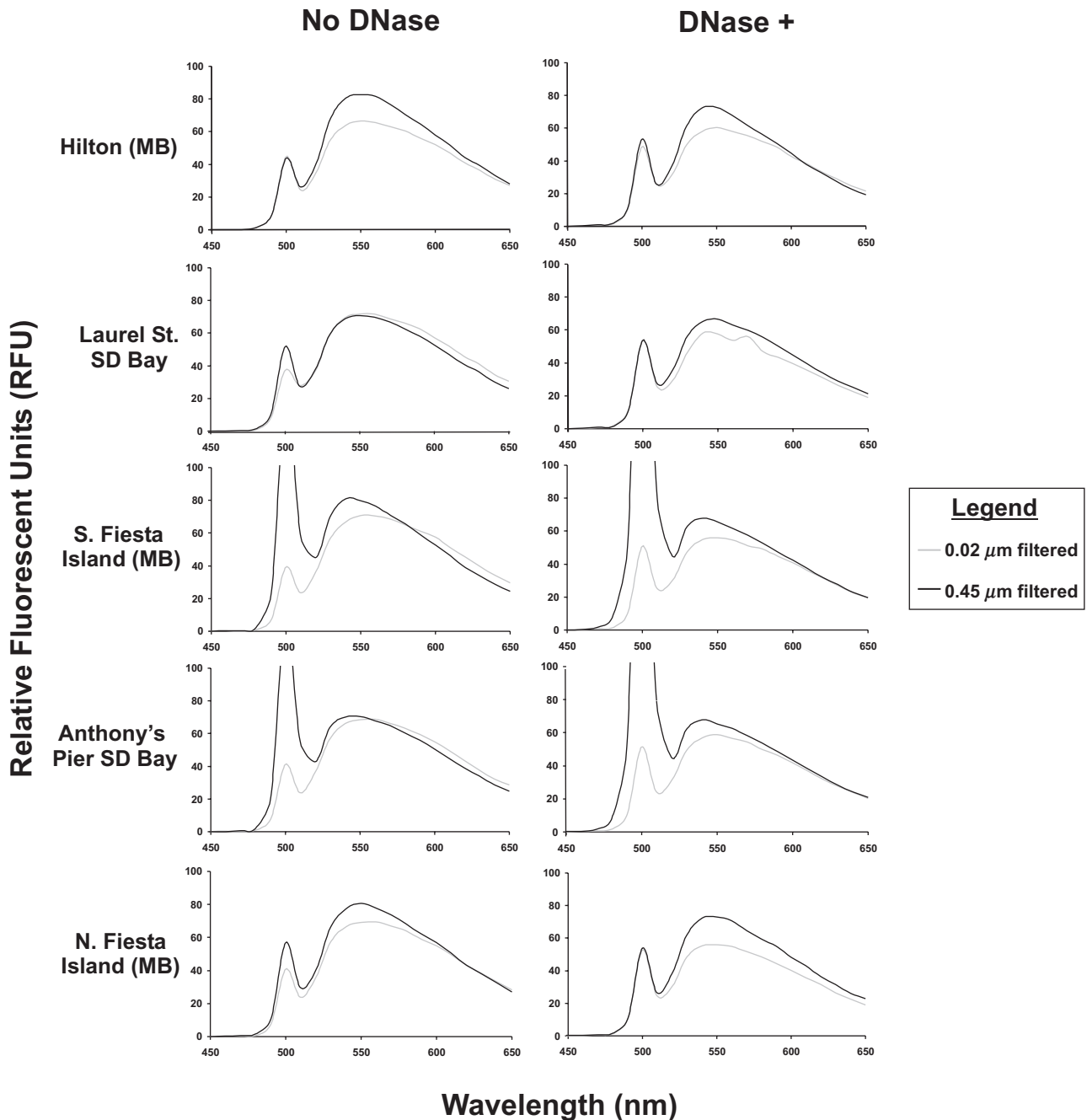


Fig. 4. The effects of DNase I on the relative fluorescence of seawater samples. Five water samples were treated with DNase I and compared with untreated samples with the bulk fluorescence protocol. The 0.02 and 0.45 μm filtered curves represent averages of four replicates.

different samples were harvested and stored at 4°C or -20°C or treated with 2% paraformaldehyde or 0.1% sodium azide. The differences in the RFUs for the Samples and Blanks were not consistent for any of the preservation techniques over time. For example, Samples stored at -20°C showed an increase in fluorescence. This result does not suggest microbial growth at -20°C, but that preservation in this manner is not sufficient for mea-

surement using the bulk fluorescent method. Paraformaldehyde fixing seemed to be the best, but even those samples show variation over time. Other research has shown that microbial numbers, as determined by direct counts, can change over time even after the addition of preservatives (Wen *et al.*, 2004). Therefore, samples should be analysed immediately, without fixation in order to obtain the most accurate reading.

Table 1. Measurement of freshwater and seawater samples using recommended protocol.

Water source	RFU after background subtraction	Direct cell counts ($\times 10^6$)	Correlation between RFU and direct counts
Freshwater			
Goldfish Pond, San Diego State University	567	0.41	$r^2 = 0.96$
Lake Murray	2298	1.72	
Old Mission Dam, SD	2428	1.81	
Lake Lindo	4666	2.80	
Seawater			
Cortez St., IB	1299	1.53	$r^2 = 0.92$
Camp Surf Jetty, IB	1548	2.59	
Seacoast Dr., IB	1598	2.89	
Tijuana Slough	1790	3.58	
Coronado Dog Beach	1834	3.74	
OB Dog Beach	2055	5.54	
Capistrano Wy., MB	2276	5.08	

Freshwater samples were collected from three lakes and one stream all located in the San Diego (SD) area. Seawater samples were collected from four open ocean locations in Imperial Beach (IB) and Coronado Island, as well as three bay locations [Tijuana Slough, Ocean Beach (OB) and Mission Beach (MB)]. Relative fluorescent units (RFUs) after background subtraction calculations were generated by subtracting the Sample measurement from the Blank measurement. Direct cell counts were conducted using epifluorescent microscopy. Correlation between RFU and direct counts represents the r^2 value generated from the four freshwater samples and from the seven seawater samples.

The effects of high versus low DNA/RNA content in cells

Vibrio parahaemolyticus was used to determine whether there was a significant difference in the fluorescence of actively growing cells versus dormant cells. A high concentration sample ($\times 10^7$ cells ml^{-1}) and a low concentration sample ($\times 10^6$ cells ml^{-1}) were both used for the comparison. Stationary cells had RFUs that were ~44% lower than those cells in log phase. Presumably, the increased fluorescence was due to higher RNA content. Actively growing cells also have a higher DNA content than stationary cells which could contribute to increased fluorescence. This implies that bulk fluorescence will be higher for actively growing microbial communities. In natural environments, ~50% of the microbes are categorized as high DNA/RNA content cells (Gasol *et al.*, 1999). Therefore, in most environmental samples, the high and low DNA/RNA content cells would balance.

Recommended protocol

Based on the results presented above, we suggest the following protocol. Syringe filter (0.45 μm for Sample and 0.02 μm for Blank) 1 ml of water sample into an eppendorf tube containing 1 μl of 10 000 \times SYBR Gold and 13 units ml^{-1} of DNase I. Incubate for 1 min. Measure the emission spectrum 450–650 nm with an excitation of 495 nm. Calculate area under the curve, $\Sigma([f(x_n)\Delta x - f(x_{n-1})\Delta x]/2)$. If a plate reading fluorometer is available, replicates should be measured and averages calculated. To calibrate the curve for a particular water type, initial direct counts with epifluorescent microscopy should be performed.

Measurement of environmental samples using recommended protocol

Water samples were collected from freshwater and seawater in order to test the recommended protocol. Both freshwater and seawater fluorescent measurements showed a strong correlation, $r^2 = 0.96$ and 0.92, respectively, when compared with direct counts (Table 1). It can be concluded from these measurements that this bulk fluorescence method may be useful in estimating microbial abundances in both freshwater and seawater.

Approximation of microbial concentrations without direct counts

Table 2 represents a guide for estimating cell concentrations using relative measurements of similar water samples. This is only a guide for rapid approximations.

Table 2. Estimates of a range of cell concentration based on relative fluorescent values from freshwater and seawater samples.

RFU range ($A_{\text{Sample}} - A_{\text{Blank}}$)	Cells ml^{-1} low estimate	Cells ml^{-1} high estimate
Freshwater		
300–1000	1.46E+05	6.64E+05
1000–2000	4.85E+05	1.33E+06
2000–3000	9.69E+05	1.99E+06
3000–4000	1.45E+06	2.65E+06
4000–5000	1.94E+06	3.32E+06
Seawater		
300–1000	4.66E+05	2.59E+06
1000–2000	1.55E+06	5.18E+06
2000–3000	3.11E+06	7.77E+06
3000–4000	4.66E+06	1.04E+07
4000–5000	6.21E+06	1.29E+07

Investigators should construct similar tables for their environment of choice. Consumers of this method should be aware that this technique provides estimations of microbial numbers and will potentially reflect noteworthy changes in microbial communities over time. These estimates should not be misconstrued as accurate cell counts as sources may contribute to over- and underestimates (i.e. DNA/RNA content or background noise).

Potential for automation

An automated version of this protocol would need to include 0.45 and 0.02 µm filtration, an injection unit for mixing the SYBR Gold and DNase I, a fluorometer and a data recorder. Miniature fluorometers are commercially available (e.g. Ocean Optics; Dunedin, FL) and custom-built basic fluorometers should be inexpensive to build. Such systems would be very good at estimating relative microbial concentrations. This would be particularly useful for routine monitoring of aquaculture ponds or storm drains in a city.

Experimental procedures

Water sampling

All water samples were collected from the San Diego area, including Mission Bay, San Diego Bay, La Jolla Shores, Imperial Beach, Ocean Beach, Ocean Beach Estuary, Torrey Pines, Lake Lindo, Lake Murray, the Old Mission Dam and the San Diego River. Portions of each sample were put through a 0.45 µm or 0.02 µm pore syringe filter. The 0.45 µm filter, Millex-HV, Cat. No. SLHV033RS (Millipore, Bedford, MA) removes protists and eukaryotic algae and was used as the Sample. The 0.02 µm, Anotop 25, Cat. No. 6809-2102 (Whatman, Maidstone, UK) filter removes the microbial cells and viral particles and was used as the Blank.

Filter testing

All Samples were filtered through a 0.45 µm, low protein binding Durapore (PVDF) filter to remove small eukaryotes, such as nanoflagellates (Azam and Hodson, 1977; Wilcox and Fuhrman, 1994) This procedure will, inadvertently, remove larger bacteria; however, it was thought that the presence of protists would significantly affect the fluorescent signal. To check whether the pre-filtering step considerably lowered bacterial numbers, seawater was filtered through a series of various pore size filters and microbes were counted using epifluorescent microscopy. All filter membranes used in this experiment were either polycarbonate or PVDF, both of which have been shown to have low protein binding. There was a 20–30% decrease in bacteria in seawater filtered through both the 0.8 µm polycarbonate filter, Poretics, Cat. No. K08BP02500 (GE Osmonics, Fairfield, CT) and the 0.45 µm PVDF filter. There was not a noticeable difference

in bacterial counts between seawater that was filtered through the 0.8 µm filter versus the 0.45 µm filter (data not shown). Therefore, the 0.45 µm filter was chosen for these experiments because it would better filter out the small protists.

Staining with SYBR Gold and bulk fluorescent measurements

Both a Hitachi f4500 (Schaumburg, IL) and a Gemini XS (Molecular Devices Corporation; Sunnyvale, CA) were used in these experiments. Water samples measured using the single-cell Hitachi fluorometer were prepared by mixing 1 ml of the Sample or the Blank with 1 µl 10 000× SYBR Gold nucleic acid stain (Molecular Probes; Eugene, OR). The relative fluorescence was measured using an excitation wavelength of 495λ and an emission spectra ranging from 450 to 650λ. For measurements using the Gemini fluorometer 1 µl of 10 000× SYBR Gold was added to 1 ml of Sample or Blank, then 200 µl aliquots were pipetted into four wells on a 96-well plate. The parameters set for the excitation and emissions wavelengths on the Gemini fluorometer were identical to those set on the Hitachi, except a cut-off filter at 515λ was used. A cut-off filter was not available on the Hitachi; nevertheless, the emission curves generated by both fluorometers were similar.

Epifluorescence microscopy

Cells were counted by filtering samples fixed in 2% paraformaldehyde onto a 0.02 µm Anodisc (Whatman), staining with SYBR Gold (Molecular Probes), and directly counting by epifluorescent microscopy. Cells were visualized at 1000× using a Leica DM RBE microscope equipped for epifluorescence with a mercury bulb (100 W) and filter set XF57-1 (Omega). Images were captured using a CCD camera (Olympus America) and cells were counted (> 200 per sample) in 10–20 fields selected at random.

Seawater dilution series

One litre of seawater was collected from the San Diego Bay. The seawater was 0.45 µm filtered and then diluted by 1/5, 2/5, 3/5 and 4/5 using 0.02 µm filtered seawater collected from the same site. One microlitre of 10 000× SYBR Gold DNA stain was added to 1 ml of each dilution. The whole seawater and the four seawater dilutions were measured for their total fluorescence using both the Hitachi f4500 fluorescence spectrophotometer and the Gemini XS fluorometer. The area of each scan, $\Sigma([f(x_n)\Delta x - f(x_{n-1})\Delta x]/2)$, was calculated for the diluted Samples and the Blank (1 ml of 0.02 µm filtered seawater). For the dilution series measured using the Gemini fluorometer, two-tailed *t*-tests were used to determine whether significant difference existed between the averages obtained from the four replicates measured per Sample and the percentage of bacteria in the Sample. Three millilitres of each dilution was also fixed in 2% paraformaldehyde and the microbes (> 200 cells) were counted using epifluorescent microscopy.

Field tests

Water samples (50 ml) were collected from six different sites in San Diego, California. Samples were collected from Fiesta Island, San Diego Bay, Mission Bay, Point Loma, Ocean Beach Estuary, and the San Diego River. Each water sample was 0.45 µm syringe filtered to remove large particles and eukaryotic organisms. One microlitre of 10 000× SYBR Gold DNA stain was added to 1 ml of each Sample and measured for its total fluorescence using a Hitachi f4500 fluorescent spectrophotometer. The emission spectrum of 450–650 nm was collected (excitation = 495 nm). After a water sample was scanned, it was immediately pipetted out of the cuvette, and fixed with 2% paraformaldehyde for the direct counts. The area of each wavelength scan was calculated for all water samples.

Precision

To measure the precision of the fluorescence spectrophotometer, water samples were collected from the shipyard at San Diego Bay, Harbor Island and Shelter Island. All three samples were 0.45 µm syringe filtered to remove large particles and eukaryotic organisms. One microlitre of 10 000× SYBR Gold was added to 1 ml of each seawater Sample. The three different water samples were measured three separate times for their total fluorescence using the Hitachi f4500 and counted three separate times using epifluorescent microscopy.

DNase I treatments

Two microlitres of 6475 units ml⁻¹ DNase I (Sigma; St Louis, MO) was added to 1 ml of water sample and incubated for 5 min at room temperatures. Samples for this experiment were measured on the Gemini fluorometer. The relative fluorescence was estimated by calculating the area under the emission spectrum. Four replicates were measured per sample and averages were calculated.

Effects of preservatives

To test the effects of different preservatives on the SYBR Gold bulk fluorescence method, water samples collected from three different sites were stored at 4°C, -20°C, treated with 2% paraformaldehyde, or 0.1% sodium azide. Fluorescence was measured at 0, 24, 48 h and 1 week after preservation using the Gemini fluorometer. The relative fluorescence was estimated by calculating the area under the emission spectrum. Four replicates were measured per sample and averages were calculated.

Quantity of relative fluorescence in actively growing versus dormant cells

Vibrio parahaemolyticus was used as a model to test the effects of cells with high DNA/RNA content versus low DNA/RNA content on our bulk fluorescence methods. An overnight culture of *V. parahaemolyticus* was grown at 37°C in LB

broth. Three different dilutions of overnight were added to three flasks containing 250 ml of 0.2 µm filtered autoclaved seawater. Cells were grown at room temperature with aeration for 12 h (high DNA/RNA content cells) and for 72 h (low DNA/RNA content cells). Subsamples were taken, dilutions were made and cells were measured for their fluorescence on the Gemini fluorometer using the methods previously described. One millilitre of each sample dilution was fixed in 2% paraformaldehyde and counted using epifluorescent microscopy.

Preparation and measurement of samples using 'recommended' conditions

Four freshwater and seven seawater samples were collected from the San Diego area. Water samples were 0.45 µm filtered (Sample) and 0.02 µm filtered (Blank) as previously described. Two microlitres of 6475 units ml⁻¹ DNase I was added to 1 ml of both filter fractions per sample and incubated for 5 min. One microlitre of 10 000× SYBR Gold was added and water samples were measured using the Gemini fluorometer as previously described. The RFUs were generated by calculating the area under the emission spectrum and subtracting the background fluorescence generated from the Blank measurement. One millilitre of each 0.45 µm filtered water sample was fixed in 2% paraformaldehyde and counted using epifluorescent microscopy.

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