

Note

Quantitative difference in bacterial abundance determined with each protocol for SYBR Green I and 4'6-diamidino-2-phenylindole (DAPI) methods

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Abstract: Bacterial abundance determined with SYBR Green I and 4'6-diamidino-2-phenylindole (DAPI) methods were compared using the protocol for each method employing epifluorescence microscopy. This comparison, using seawater samples that were collected from Sagami Bay, revealed that the abundance determined with the SYBR Green I method was on average 1.6 fold greater than that with the DAPI method. A model 2 regression analysis showed the following relationship:
 $\text{Log SYBR Green I} = -0.238 + 1.08 \text{ Log DAPI}$ ($r^2=0.85$, $n=68$, $p<0.0001$). These results suggest that when the method is switched between the SYBR Green I and DAPI methods, the difference in bacterial abundance determined by the two methods should be considered for a quantitatively consistent assessment of the bacterial biomass in the ocean.

Key words: abundance, bacteria, 4'6-diamidino-2-phenylindole (DAPI), epifluorescence microscopy, SYBR Green I

Bacterial biomass has been estimated mostly based on carbon conversion factors for a single bacterial cell (Lee & Fuhrman 1987, Fukuda et al. 1998) and abundance determined by epifluorescence microscopy. With the standard microscopy technique, bacteria stained with fluorochrome are collected on a Nuclepore filter (pore size, 0.2 μm) and then counted; acridine orange (AO) or 4'6-diamidino-2-phenylindole (DAPI) has been used for staining in the last few decades (Hobbie et al. 1977, Porter & Feig 1980). On the other hand, SYBR Green I staining is used for enumerating bacteria as well as viruses (e.g. Hewson et al. 2004, Winter et al. 2005, Bongiorni et al. 2005). Staining with SYBR Green I following filtration through 0.02 μm pore-size Anodisc filters recommended by Noble & Fuhrman (1998) has methodological advantages in that bacteria and viruses stained with this reagent produce intensive brightness and that abundance of these two microbes can be determined simultaneously on one filter. Since both bacteria and viruses are important components of the marine food web (Fuhrman 1999, Suttle 2005), the SYBR Green I procedure has been widely adopted for studies of planktonic ecology employing epifluorescence microscopy.

In spite of the widespread use of SYBR Green I stain, there are little comparative data on bacterial abundance estimated using this method and the conventional AO and DAPI methods. Noble & Fuhrman (1998) reported that information on bacterial abundance obtained with the SYBR Green I method was statistically indistinguishable from that gained with the AO method using the original protocol for each method, but to our knowledge, such a comparison has not been done using counts obtained with the DAPI method. Thus, a comparison of data on abundance estimated using the SYBR Green I and DAPI methods would provide a better understanding of the characteristics of data obtained by the former method. Results of such a comparison would be of assistance in interpreting data when one of these two methods for determining bacterial abundance is not used consistently.

In the present study, a comparison of enumeration of bacterial abundance between SYBR Green I and DAPI methods has been done by following two procedures using natural seawater samples. Firstly, to determine if there was a difference in bacterial abundance estimated by the two methods that did not originate from an error in measurement, glass slides for each method were prepared in quadruplicate from each sample, and abundance was determined by each method. Secondly, for examination of the relational relationship between bacterial abundance estimated by the two methods, a single glass slide for each method was prepared from each sample among those

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that were collected during various seasons and ocean depths, and abundance was determined by each method.

Seawater samples were collected using Niskin bottles at depths between 0 and 100 m from November 2000 to August 2005 at a site 2 km off the Manazuru Peninsula in Sagami Bay, Japan ($35^{\circ}09'N$, $139^{\circ}10'E$). Polypropylene tubes (50 ml, Corning, Inc.) were washed with the sample seawater 3 times and then filled. All collected samples were fixed with formaldehyde at a final concentration of 0.74% immediately after collection, and stored at $4^{\circ}C$ in the dark for later analysis.

Bacterial abundance was determined using the DAPI staining protocol (Porter & Feig 1980) in which seawater was stained with DAPI (final concentration, $1.0 \mu\text{g mL}^{-1}$) for 20 minutes, and then filtered onto a $0.2 \mu\text{m}$ pore size polycarbonate Nuclepore filter (Whatman Int., Ltd.). All of the filters used for the DAPI method were prestained with iргalan black to reduce background fluorescence. Bacterial abundance from samples stained with SYBR Green I (Invitrogen Corp.) was also determined using the protocol of Noble & Fuhrman (1998). Samples were filtered through an Anodisc filter, $0.02 \mu\text{m}$ pore size (Whatman Int., Ltd.), and then the filter was stained with SYBR Green I (final dilution 2.5×10^{-3}) in the dark for 15 minutes. Glass slides were prepared with either anti-fade mounting reagent, 50% glycerol, 50% phosphate buffered saline with 0.1% *p*-phenylenediamine (GPP), or the commercially available anti-fade mounting reagent, SlowFade Light (Invitrogen Corp.). An Axioskop 2 plus epifluorescence microscope equipped with an HBO 100 mercury lamp (Carl Zeiss, Inc.) was used for enumerating the bacteria and viruses on filters mounted on glass slides and covered with a coverslip. Blue (excitation at 353 to 377 nm, 395 nm cutoff, emission at 515 to 565 nm) and UV (excitation at 353 to 377 nm, 395 nm cutoff, emission at >397 nm) optical filter sets were used when counts were done under microscopy using the SYBR and DAPI methods, respectively. For the SYBR Green I method, virus particles were distinctly shaped 'pinpricks' and fluoresced bright green, and bacterial cells could be distinguished from viruses based on their relative size and brightness (Noble & Fuhrman 1998). A minimum of 20 randomly selected fields were examined, producing a count of at least 400 bacterial cells. Each count in a microscopic field was done within 10 to 15 seconds

from the commencement of excitation.

Comparisons of bacterial abundance between SYBR Green I and DAPI methods were made using the following two counting conditions. Firstly, glass slides were made in quadruplicate for each method when enumeration of bacteria was done from samples that were collected at a depth of 10 m from November 2004 to August 2005 ($n=6$). These slides were prepared within 1 hour after fixation for 30 minutes, and then counts were done immediately. GPP was used for the preparation of slides for the SYBR Green I method. Secondly, a single glass slide was prepared under each condition for each method; samples collected at depths between 0 and 100 m from November 2000 to March 2003 ($n=68$) and stored at $4^{\circ}C$ were used. These slides were prepared within 2 days after the beginning of storage, and then counts were made immediately. The anti-fade mountant, SlowFade Light, was used for the preparation of slides for the SYBR Green I method.

Statistical analysis was performed to investigate the difference in bacterial abundance estimated by the SYBR Green I and DAPI methods, in which quadruplet glass slides were prepared under each condition of each method, by the two-sided Student's *t*-test. To investigate the difference in bacterial abundance between SYBR Green I and DAPI methods when a single glass slide was prepared under each condition, the two-sided paired Student's *t*-test was performed. All data on bacterial abundance obtained from the single glass slide determinations were log-transformed and tested for distribution normality using the Kolmogorov-Smirnov test, followed by a model 1 regression analysis. We also performed a model 2 regression analysis using the same data.

SYBR Green I-to-DAPI ratios of bacterial abundance from 6 samples ranged from 1.0 to 1.8 when glass slides were prepared in quadruplicate for each method (Table 1); the average value was 1.4 ± 0.3 . There was a significant difference in abundance obtained with each method in 5 out of 6 samples ($p < 0.05$). These results indicate that bacterial abundance determined with the SYBR Green I method was equivalent to or exceeded that determined with the DAPI method.

SYBR Green I-to-DAPI ratios of bacterial abundance ranged from 1.0 to 2.6 (average \pm SD, 1.6 ± 0.4) when a single glass slide was prepared for each method (Fig. 1). These SYBR

Table 1. Bacterial abundance determined with SYBR Green I and DAPI methods from natural seawater samples. Values in parenthesis are standard deviation of the abundance with each method. Significance level of difference is shown by probabilities: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Two-sided Student's *t*-test). No asterisk was shown when the probability was more than 0.05.

Sampling date	Bacterial abundance ($\times 10^6 \text{ cells mL}^{-1}$)		Ratio
	SYBR Green I	DAPI	
18 November, 2004	2.3 (0.2)	2.0 (0.1)	1.2*
17 December, 2004	1.0 (0.1)	0.97 (0.05)	1.0
18 January, 2005	0.96 (0.06)	0.78 (0.11)	1.2*
20 May, 2005	1.6 (0.0)	0.95 (0.07)	1.7***
19 June, 2005	1.8 (0.1)	0.97 (0.07)	1.8***
11 August, 2005	2.6 (0.2)	1.8 (0.1)	1.4***
Average SYBR Green I/DAPI			1.4 ± 0.3

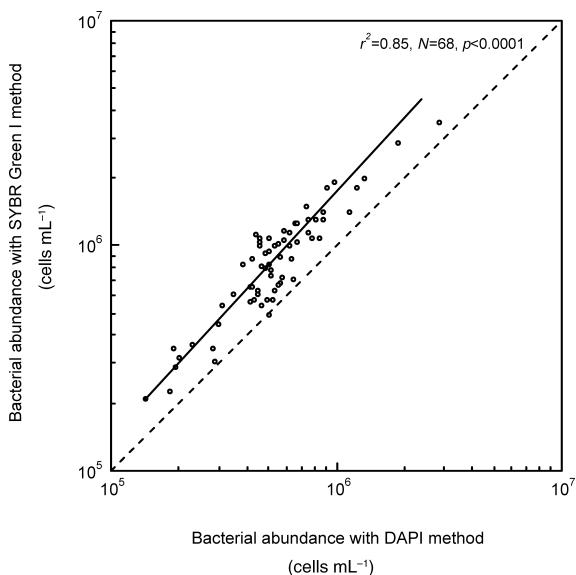


Fig. 1. Relationship between bacterial abundance determined with the DAPI and SYBR Green I methods. Solid line represents a model 2 regression equation derived from all the data used in the comparison. Dotted line represents the assumption that abundance determined by the two methods is identical.

Green I-to-DAPI ratios agreed with those ratios obtained from the former determination in which slides were prepared in quadruplicate (1.0 to 1.8), showing a consistency in results between the two counting conditions. Abundance estimated with the SYBR Green I method was significantly higher than that estimated with the DAPI method on the whole ($p<0.0001$). A model 1 regression analysis showed the following relationship:

$$\text{Log SYBR Green I} = 0.24 + 0.99 \text{ Log DAPI} \\ (r^2=0.85, n=68, p<0.0001). \quad (1)$$

The log-transformed data for both SYBR Green I and DAPI were normally distributed ($p>0.1$). The 95% confidential intervals (CI) of the slope and y -intercept were ± 0.10 and 0.59, respectively. On the other hand, the following relationship was derived when a model 2 regression analysis was applied:

$$\text{Log SYBR Green I} = -0.24 + 1.08 \text{ Log DAPI} \\ (r^2=0.85, n=68, p<0.0001). \quad (2)$$

The slope of the equation derived from a model 2 regression analysis did not differ significantly from the slope value of 1, when the 95% CI of the slope derived from the model 1 regression analysis was applied (Sokal & Rohlf 1994).

The result of our comparison between the SYBR Green I and DAPI methods differed from that of the previous comparison between the SYBR Green I and AO methods, which showed that the abundance between the two methods was statistically indistinguishable (Noble & Fuhrman 1998). The different results should be due to the stains used (DAPI and AO) if we simply interpret the results of the two comparisons. In fact, bacterial abundance determined with the AO method is significantly higher than that determined with the DAPI

method (Suzuki et al. 1993, Newell et al. 1988). Suzuki et al. (1993) reported that DAPI staining generally resulted in smaller bacterial cell size than the AO staining and that 14% of bacterial cells were brightly stained with AO but not stained with DAPI, since AO binds to both DNA and RNA but DAPI binds only to DNA. Indeed, bacterial cells stained with DAPI were generally smaller than those stained with SYBR Green I under epifluorescence microscopy when a few samples were double-stained with the two fluorochromes (results not shown). As SYBR Green I binds to double- and single-stranded DNA and RNA (Tuma et al. 1999), this binding specificity to nucleic acids is basically the same as that of AO. Thus, our results showing the difference in bacterial abundance determined with the SYBR Green I and DAPI methods is possibly due to different binding specificities of the stains.

The other possible factor influencing the observed quantitative difference in bacterial abundance is the difference in the filters used between the SYBR Green I and DAPI methods: 0.02 μm pore size Anodisc filters and 0.2 μm pore size Nuclepore filters were used for the former and latter methods, respectively. Because approximately 10% of the total abundance of bacterial cells, called ultramicrobacteria, pass through 0.2 μm pore size Nuclepore filters (Zimmermann 1977, Li & Dikie 1985), the 0.02 μm pore size filter potentially retains a greater number of bacterial cells than the 0.2 μm pore size filter. However, the more important factor appears to be filter type rather than pore size. Jones et al. (1989) reported that bacterial abundance on the 0.2 μm pore size Anopore filter (renamed Anodisc filter) was 21 to 33% higher than that on the Nuclepore filter of the same pore size when pond water samples were stained with AO. Similarly, from seawater samples, up to 39% of higher bacterial abundance has been determined on the former filter than the latter filter (Hara et al. 1996). Such high retention efficiency of the Anodisc filter is likely due to the surface charge and/or fine sieve-like morphology (Jones et al. 1989). In addition, bacterial abundance determined between 0.2 and 0.02 μm pore size Anodisc filters is statistically indistinguishable (Hara et al. 1996, Shibata et al. 2006). Considering these findings, the retention efficiency of bacterial cells on Anodisc filters can be regarded as being high regardless of pore size; hence, the observed higher bacterial abundance obtained with the SYBR Green I method than with the DAPI method could be due to the difference in filter type as well as pore size.

Our comparisons revealed the characteristics of data obtained with the two methods: bacterial abundance determined with the SYBR Green I method generally exceeded that determined with the DAPI method, and the relationship of observed abundance between the two methods followed Equation (2). These results suggest that when the method for determining bacterial abundance is switched between the SYBR Green I and DAPI methods, the possibility for a difference in results on abundance between the two methods should be considered for a consistent assessment of the dynamics of the bacterial biomass in the ocean. When bacterial abundance is determined with either of the two methods, Equation (2) can be used to ap-

proximate the abundance that would be determined by the other method. Although we have addressed the relational characteristics of data between the two methods, the mechanism producing the quantitative difference was not clarified in the present study. To identify the factors that produced the difference in abundance between the SYBR Green I and DAPI methods, further investigation considering variables mainly focused on fluorochrome, filter type, and filter pore size between the two methods is needed in the future.

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References

- Bongiorni L, Magagnini M, Armeni M, Noble R, Danovaro R (2005) Viral production, decay rates, and life strategies along a trophic gradient in the north Adriatic Sea. *Appl Environ Microbiol* 71: 6644–6650.
- Fuhrman JA (1999) Marine viruses and their biogeochemical and ecological effects. *Nature* 339: 541–548.
- Fukuda R, Ogawa H, Nagata T, Koike I (1998) Direct determination of carbon and nitrogen contents of natural bacterial assemblage in marine environments. *Appl Environ Microbiol* 64: 3352–3358.
- Hara S, Koike I, Terauchi K, Kamiya H, Tanoue E (1996) Abundance of viruses in deep oceanic waters. *Mar Ecol Prog Ser* 145: 269–277.
- Hewson I, Govil SR, Capone DG, Carpenter EJ, Fuhrman JA (2004) Evidence of *Trichodesmium* viral lysis and potential significance for biogeochemical cycling in the oligotrophic ocean. *Aquat Microb Ecol* 36: 1–8.
- Hobbie JE, Daley RJ, Jasper S (1977) Use of Nuclepore filters for counting bacteria by fluorescence microscopy. *Appl Environ Microbiol* 33: 1225–1228.
- Jones SE, Ditner SA, Freeman C, Whitaker CJ, Lock MA (1989) Comparison of a new inorganic membrane filter (Anopore) with a track-etched polycarbonate membrane filter (Nuclepore) for direct counting of bacteria. *Appl Environ Microbiol* 55: 529–531.
- Lee S, Fuhrman JA (1987) Relationships between biovolume and biomass of naturally derived marine bacterioplankton. *Appl Environ Microbiol* 53: 1298–1303.
- Li WKW, Dickie PM (1985) Growth of bacteria in seawater filtered through 0.2 µm Nuclepore membranes: implications for dilution experiments. *Mar Ecol Prog Ser* 26: 245–252.
- Newell SY, Fallon RD, Sherr BF, Sherr EB (1988) Mesoscale temporal variation in bacterial standing crop, percent active cells, productivity, and output in a salt-marsh estuary. *Int Ver Theor Angew Limnol Verh.* 23: 1839–1845.
- Noble RT, Fuhrman JA (1998) Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria. *Aquat Microb Ecol* 14: 113–118.
- Porter KG, Feig YS (1980) The use of DAPI for identifying and counting aquatic microflora. *Limnol Oceanogr* 25: 943–948.
- Shibata A, Goto Y, Saito H, Kikuchi T, Toda T, Taguchi S (2006) Comparison of SYBR Green I and SYBR Gold stains for enumerating bacteria and viruses by epifluorescence microscopy. *Aquat Microb Ecol* 43: 223–231.
- Sokal RF, Rohlf J (1994) Biometry: the principles and practice of statistics in biological research, 3rd ed. (Eds Freeman WH and Co., New York, pp. 887.
- Suttle CA (2005) Viruses in the sea. *Nature* 437: 356–361.
- Suzuki MT, Sherr BF, Sherr EB (1993) DAPI direct counting underestimates bacterial abundances and average cell size compared to AO direct counting. *Limnol Oceanogr* 38: 1566–1570.
- Tuma RS, Beaudet MP, Jim X, Jones LJ, Cheung C, Yue SV, Singer L (1999) Characterization of SYBR Gold nucleic acid gel stain: A dye optimized for use with 300-nm ultraviolet transilluminators. *Anal Biochem* 268: 278–288.
- Winter C, Smit A, Herndl GJ, Weinbauer MG (2005) Linking bacterial richness with viral abundance and prokaryotic activity. *Limnol Oceanogr* 50: 968–977.
- Zimmermann R (1977) Estimation of bacterial number and biomass by epifluorescence microscopy and scanning electron microscopy. In: *Microbial ecology of a brackish water environment* (Eds Rheinheimer G) Springer, Berlin, pp. 103–120.