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## DAPI direct counting underestimates bacterial abundances and average cell size compared to AO direct counting

**Abstract**—We compared estimates of bacterial abundance and of average bacterial cell size based on direct counts of bacterioplankton cells stained with acridine orange (AO) or 4'-6-diamidino-2-phenylidole (DAPI). Estimates of numbers  $\text{ml}^{-1}$  of bacteria stained with DAPI were on average 70% (range 57–82%) of bacterial counts made with AO as the stain. Cell volumes estimated from DAPI-stained cells were even less, on average 59% (range 34–80%) of those determined from measurement of AO-stained cells. The combination of lower DAPI values for both cell numbers and cell volumes resulted in overall underestimation of total bacterial biovolume  $\text{liter}^{-1}$ . DAPI-determined total biovolumes ranged from 25 to 61% of biovolume values obtained via counts and measurements of AO-stained cells in the two data sets. These results can be ascribed to the differential staining of cell components by DAPI and AO. We recommend that DAPI staining be used with caution for estimating bacterial standing stock parameters.

The ability to accurately estimate bacterial abundance and standing stock biomass in fresh and marine waters, via epifluorescence microscopic inspection of bacterioplankton cells stained with a fluorochrome, revolutionized the field of aquatic microbial ecology. Fuhrman (1981) compared the use of epifluorescence microscopy and electron microscopy for determining bacterial cell size and concluded that the former method was preferable due to severe cell shrinkage during preparation of bacterial samples for electron microscopy. Two of the most widely used fluorochromes for staining bacteria are acridine orange (AO) (Zimmerman and Meyer-Reil 1974; Hobbie et al. 1977) and 4'-6-diamidino-2-phenylidole (DAPI) (Porter and Feig 1980). AO binds to both DNA and RNA and fluoresces apple-green when excited with blue light (Kasten 1981). In practice, AO appears to also stain other structures in the cell (e.g. the cell wall). DAPI fluoresces blue upon excitation with ultraviolet

light only when complexed with double-stranded DNA (Porter and Feig 1980; Sieracki et al. 1985). Based on these differences in staining, it is reasonable to suppose that cells stained with DAPI would look smaller in size than those stained with AO, which would lead to underestimation of average cell biovolume when DAPI is used.

We tested this idea by comparing cell counts and measurements of average cell size for bacterioplankton stained with either DAPI or AO. Seawater samples used for the comparisons were taken from both an inshore estuary and a coastal station 8 km off the Oregon coast. Our results indicated that not only did DAPI staining result in smaller average cell size, as expected, but also in significantly lower average cell abundance, compared to values obtained with AO staining.

Water samples were taken in September 1990. Duplicate surface samples were collected from six discrete locations along a transect down the South Slough estuary (43°18'–20'N, 124°19'W), which drains into Coos Bay on the southern Oregon coast. The sampling took place 1 h after high slack; salinity, measured by refractometer, was 22‰ at the upper station (Sta. 1) and ranged from 31 to 33‰ at the five lower stations. A second set of duplicate samples was collected offshore at station NH-5 (44°39'N, 124°11'N) on the Newport hydrographic sampling line, 8 km west of Yaquina Bay, at depths of 0, 8, 12, 20, 25, and 40 m. Samples were fixed with 3% (final concn) formaldehyde and stored in polyethylene vials at 5°C before staining and microscopic analysis, which took place within a week of sample collection.

Subsamples (1 ml) of each duplicate water sample were stained with either AO, following the method of Kirchman et al. (1982), or with DAPI, according to a procedure modified from Porter and Feig (1980). AO samples were stained with 1 ml of 0.01% AO solution (final concn, 50  $\mu\text{g ml}^{-1}$ ) for 1 min, then filtered. DAPI samples were stained with 20  $\mu\text{l}$  of 1 mg

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ml<sup>-1</sup> DAPI stock solution (25 µg ml<sup>-1</sup> final concn) for 7 min, then filtered. One milliliter of 0.2-µm-filtered seawater was added to DAPI samples before staining to raise the total volume to 2 ml, equal to the volume in the AO procedure, which ensured more even distribution of cells onto the filter. Stock solutions of both AO and of DAPI were prefiltered through 0.2 µm before use to minimize background counts. Stained samples were passed through 0.2-µm black Nuclepore polycarbonate filters mounted onto prewetted 0.8-µm Nuclepore Membrafil cellulosic filters. Sample filters were removed from the filtration tower under vacuum to prevent cells from floating off the filter and then mounted onto glass slides with a drop of Cargille type A immersion oil on the slide beneath the filter and a drop of immersion oil on top of the filter beneath the cover slip.

A few selected samples were doubled-stained; 1-ml subsamples were prestained with DAPI as described above, filtered, and then stained with 1 ml of AO solution. The filters were mounted onto slides as described above, and individual cells were examined to determine whether all cells that appeared stained with AO were also stained with DAPI. For each prepared slide, counts were made of the number of bacterial cells within an ocular grid in 10 randomly selected fields (magnification, 1,250×). A blank value determined from AO or DAPI staining of 1-ml of 0.2-µm-filtered seawater (on average, <5% of sample counts) was subtracted from the average cell count per grid before calculation of abundance ml<sup>-1</sup>. Microscopy was performed with a Zeiss Universal epifluorescence microscope equipped with a 75-W xenon lamp. AO counts were made with Zeiss blue light filter set 47 77 09 (BP 450-490 excitation filter, FT 510 beam splitter, and LP 520 barrier filter) and DAPI counts with Zeiss UV filter set 47 77 02 (G 365 excitation filter, FT 395 beam splitter, and LP 420 barrier filter).

Photomicrographs (Kodak TMAX print film, ASA 400) were made of five additional randomly selected fields from one of each set of duplicate slides of subsamples stained with AO or DAPI. Cell volumes were determined from negative images of the bacteria projected onto a screen, resulting in a final enlargement

factor of 5,000×, as described by Lee and Fuhrman (1987). For each subsample, the average bacterial cell volume was determined from 100 to 150 individually measured cells. Total bacterial biovolume (µl liter<sup>-1</sup>) was calculated for each water sample as the product of the average cell abundance and volume.

The level of significance of differences between cell counts done with the two staining procedures was tested via the Wilcoxon non-parametric ranks test and a two-way ANOVA with the Statgraphics statistical package (Statistics Graphics Co.). Level of significance of differences between average cell biovolumes determined from AO- vs. DAPI-stained samples was determined with a two-way ANOVA.

DAPI cell counts were lower than AO cell counts for all of the samples in both the South Slough and the NH-5 data sets (Tables 1 and 2). DAPI counts varied from 59 to 80% of AO counts (avg, 74%) for the South Slough samples and from 57 to 82% (avg, 67%) of AO counts for the NH-5 samples. The differences between bacterial abundance values determined by the two methods were significant at the level of  $\alpha = 0.05$  according to the Wilcoxon rank test and  $\alpha = 0.001$  according to the multi-way ANOVA.

The discrepancy between DAPI- and AO-prepared samples was even greater for the cell volume determinations (Tables 1 and 2). Measurement of DAPI-stained cells resulted in cell volumes 45–80% (avg, 61%) smaller than those determined from AO-stained cells for the South Slough samples and 34–77% (avg, 58%) smaller than those determined from AO-stained cells for the NH-5 samples. The differences between cell volumes of DAPI- vs. AO-stained cells were also highly significant: at the level of  $\alpha = 0.05$  in the Wilcoxon rank test; at the  $\alpha = 0.001$  level according to results of the two-way ANOVA.

Estimation of total bacterial biovolume (µl liter<sup>-1</sup>) in each sample with the average cell abundance and volume values for either DAPI or AO samples compounded the differences between the two methods. Total biovolume values determined with DAPI-stained cells were 34–61% of biovolume values determined with AO-stained cells. The average DAPI:AP biovolume ratio was 0.45 for South Slough samples and 0.38 for NH-5 samples.

Table 1. Estimates of bacterioplankton abundance ( $10^6$  cells  $ml^{-1}$ ) and average cell volume ( $\mu m^3$ ) made with subsamples stained with AO or DAPI, and DAPI values as a percentage of AO values, for samples taken in the South Slough estuary. Mean values  $\pm 1$  SD.

South Slough Sta. No.	Bacterial abundance			Avg cell volume		
	AO	DAPI	DAPI/AO	AO	DAPI	DAPI/AO
1	7.25 $\pm$ 0.03	5.81 $\pm$ 0.75	80	0.122 $\pm$ 0.12	0.093 $\pm$ 0.10	76
2	7.25 $\pm$ 0.36	5.40 $\pm$ 1.03	74	0.115 $\pm$ 0.09	0.071 $\pm$ 0.04	59
3	5.46 $\pm$ 0.04	3.21 $\pm$ 0.28	59	0.142 $\pm$ 0.13	0.094 $\pm$ 0.06	50
4	4.39 $\pm$ 0.04	3.26 $\pm$ 0.70	74	0.117 $\pm$ 0.08	0.094 $\pm$ 0.06	80
5	4.03 $\pm$ 0.10	3.23 $\pm$ 0.27	80	0.169 $\pm$ 0.15	0.078 $\pm$ 0.06	45
6	3.92 $\pm$ 0.38	2.98 $\pm$ 0.20	76	0.163 $\pm$ 0.15	0.086 $\pm$ 0.05	53
Mean DAPI/AO			74 $\pm$ 8			61 $\pm$ 14

Inspection of several samples that had been double-stained with both DAPI and AO revealed that, on average, 14% of bacterial cells that were brightly stained with AO had no apparent DAPI fluorescence associated with them.

Both AO and DAPI direct count methods are routinely used to enumerate, and in some cases also to size, bacteria in freshwater and marine environments. In addition, DAPI is the preferred stain for bacterial cells visualized by image analysis systems (Sieracki et al. 1985; Estep et al. 1986); DAPI-stained cells do not fade quickly as do cells stained with AO or other fluorochromes, and DAPI is highly specific for bacteria, i.e. there is very little non-specific staining of detrital material by DAPI as there is with AO (Sieracki et al. 1985).

We found, as expected, lower values for average cell size of DAPI- vs. AO-stained bacterial cells (Tables 1 and 2). Interestingly, although there was an order of magnitude difference in bacterial abundance values between the estuarine and offshore water sam-

ples, the average cell sizes of bacteria in samples from the two areas were similar (Tables 1 and 2). The bacterial biovolumes reported here were larger than the typical cell size ( $0.05$ – $0.10 \mu m^3$ ) previously reported in coastal marine waters (Ferguson and Rublee 1976; Ammerman et al. 1984; Newell et al. 1988; Gonzalez et al. 1990). In this study, samples were taken during the summer upwelling season on the northwest U.S. coast. Phytoplankton biomass in the euphotic zone was  $2$ – $10 \mu g$  Chl *a* liter $^{-1}$  in late summer of 1990 (M. L. Dickson unpubl. data). The relatively large average cell size of bacteria found in September may thus have resulted from abundant bacterial substrate due to upwelling-induced phytoplankton blooms. During winter when upwellings are absent along the Oregon coast, average bacterial cell volumes in coastal seawater, determined with the same AO-staining procedure as in this study, were as low as  $0.05 \mu m^3$  (Suzuki unpubl. data).

We also consistently found variable, but significant, deficits in cell counts for DAPI- compared to AO-stained cells (Tables 1 and 2).

Table 2. As Table 1, but for samples taken from six depths at station NH-5, 8 km off the Oregon coast.

Sta. NH-5 depth, m	Bacterial abundance			Avg cell volume		
	AO	DAPI	DAPI/AO	AO	DAPI	DAPI/AO
0	0.50 $\pm$ 0.09	0.31 $\pm$ 0.09	62	0.160 $\pm$ 0.12	0.105 $\pm$ 0.08	66
8	1.53 $\pm$ 0.02	1.13 $\pm$ 0.02	74	0.154 $\pm$ 0.11	0.053 $\pm$ 0.04	34
12	0.82 $\pm$ 0.00	0.67 $\pm$ 0.01	82	0.169 $\pm$ 0.12	0.087 $\pm$ 0.05	52
20	0.92 $\pm$ 0.00	0.60 $\pm$ 0.03	66	0.111 $\pm$ 0.06	0.085 $\pm$ 0.05	77
25	0.78 $\pm$ 0.04	0.49 $\pm$ 0.02	63	0.141 $\pm$ 0.10	0.075 $\pm$ 0.05	53
40	0.55 $\pm$ 0.00	0.32 $\pm$ 0.08	57	0.130 $\pm$ 0.09	0.080 $\pm$ 0.07	62
Mean DAPI/AO			67 $\pm$ 9			57 $\pm$ 14

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Previous reports in which the two counting methods have been compared have yielded equivocal results. In their original description of the DAPI direct count method, Porter and Feig (1980) found no statistical differences in bacterial counts between DAPI- and AO-staining methods; however, total counts for cells stained with DAPI for 24 h averaged 16% lower compared to counts of cells freshly stained with AO. Newell et al. (1986) reported that DAPI sometimes yielded lower cell counts than did AO for samples of estuarine water and suggested that this might be generally true for seawater as opposed to freshwater.

We cannot explain the lower DAPI cell counts reported here. Inspection of water samples double-stained with both fluorochromes revealed that some particles which were indistinguishable from other AO-stained bacterial cells showed no DAPI fluorescence when excited by UV light. One possible explanation might be that a portion of in situ bacterial cells do not have sufficient DNA to be detectable by the DAPI-staining procedure. This idea is supported by Moyer and Morita (1989) who observed that when a marine vibrio was starved for 3 months, DNA per bacterial cell dropped to between 4.2 and 8.3% of the original amount. An alternate explanation could be related to reports that a large fraction of bacteria in natural waters is infected with viruses (Proctor and Fuhrman 1990; Bratbak et al. 1990). Perhaps some AO-stained particles that resemble intact bacteria are cells emptied of DNA due to viral lysis.

Our results comparing the two staining methods suggest that estimation of bacterial abundance and average cell size with DAPI should be done with caution. In particular, estimates of total bacterial biovolume or biomass per liter made via a combination of enumeration and sizing of DAPI-stained cell may be > 50% lower than estimates with AO-stained cells. We therefore recommend that sizing of bacterial cells be done with the AO method. When abundances of bacteria are routinely estimated with DAPI, some counts should also be made with AO to determine the degree of underestimation of DAPI-stained bacterial numbers. It would be of interest to determine why some AO-stained cells in natural samples apparently cannot be visualized with DAPI.

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## Effect of UV-B radiation and humic substances on growth and motility of *Gyrodinium aureolum*

**Abstract**—We investigated the effect of ultraviolet-B radiation (280–320 nm) on the growth and motility of the marine dinoflagellate *Gyrodinium aureolum* in the presence of humic substances. The specific growth rate after 7 d of the UV-B<sub>BE</sub> (biologically effective radiation) exposure (2.15 kJ m<sup>-2</sup>d<sup>-1</sup>) decreased by 92% compared to the control. The growth rate for UV-B treated cells increased by 18, 26, and 10% when cells were cultured in medium enriched with humic substances (1.7, 4.2, and 8.3 mg liter<sup>-1</sup>). However, the growth rate was lower at the highest humic substance than at the two lower concentrations. When humic substances were used as a filter to shade the organisms from UV-B radiation without substantial changes in the amount of white light, instead of being added to the medium, the growth rate increased with the concentration of humic substances. UV-B radiation had little or no effect on the motility of *G. aureolum*, whether humic substances were added or not. Phototactic orientation of the UV-B-treated cells was significantly weaker than the control to actinic white light (400–700 nm, 47 m<sup>-2</sup>). The presence of humic substances during treatment with UV-B radiation reduced the inhibition of the positive phototaxis in *G. aureolum*.

The stratospheric ozone layer protects organisms on earth from ultraviolet-C (190–280 nm) and high levels of ultraviolet-B radiation (280–320 nm). The increased UV-B radiation reaching the earth's surface as a result of chlorofluorocarbons (CFCs)-dependent ozone depletion could have negative effects on living

organisms. Smith et al. (1992) measured a minimum of 6–12% reduction in primary production associated with O<sub>3</sub> depletion in October 1990 over the Southern Ocean. Since marine phytoplankton are responsible for half of the CO<sub>2</sub> fixation on earth and since these organisms form the first step in the marine food web, increased radiation could have negative ecological and economical effects.

Increased acidification of soils and lakes can also affect living organisms. Decreasing pH of soil and water often leads to precipitation of metals. Agricultural activity could cause humic substances to leach from the soil and end up in coastal waters (R. C. Petersen pers. comm.). Humic substances in natural waters differ widely with time and place. Rivers on the Swedish west coast have concentrations of 2.3–7.1 mg liter<sup>-1</sup> and the Laholm Bay into which many of these rivers discharge has 1.4 mg liter<sup>-1</sup> of humic substances (Granéli et al. 1985). Values from the Gdansk Bay (Baltic Sea) are between 1 and 3 mg liter<sup>-1</sup>. Laboratory and field experiments have demonstrated that humic substances affect growth productivity and nutrition in marine phytoplankton. Although the nature of humic substances is not so well understood, some important characteristics are that they can act as chelators and ion exchange resins and affect the nutrient dynamics of surface waters (Petersen et al. 1987). Humic substances can form complexes with metals and increase or decrease their toxicity. The toxicity of humic substances is due to their

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