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The use of DAPI for identifying and counting aquatic microflora[1]

Abstract—A highly specific and sensitive fluorescing DNA stain, 4′6-diamidino-2-phenylindole (DAPI) was compared with acridine orange (AO) for counting aquatic microflora. Use of DAPI improved visualization and counting of <1-μm bacteria and blue-green algae in seston-rich samples and extended sample storage to at least 24 weeks.

Small bacteria and blue-green algae may be major contributors to autotrophic and heterotrophic processes in planktonic systems; however, their detection and enumeration are often difficult. Direct counts of natural microflora collected on membrane filters and stained with a fluorescing dye have yielded higher numbers than other techniques and are considered the most reliable methods available (*see* Daley 1979). When excited with light at the appropriate wavelengths, bound dyes produce a fluorescent glow which allows cells below the limit of resolution of light microscopy (generally <1 μm) to be visualized and distinguished from other particles, although their shapes and structures cannot be seen. Acridine orange (AO) binds specifically with DNA and RNA under controlled conditions and, when excited with light at a wavelength of 436 or 490

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nm, the pure DNA-AO complex fluoresces green and the RNA-AO complex red (Ris and Plaut 1962; Wittekind 1972).

Several difficulties are inherent in the use of AO for direct counts. It is difficult to distinguish bacteria from nonliving particles such as clays, detritus, or colloids which may pick up the stain and may also autofluoresce. We observed red- and orange-fluorescing detritus and colloidal flocs on unstained slides of lake water. This autofluorescence of nonliving matter can mask the autofluorescence of algal chlorophyll and the fluorescence of AO-stained bacterial cells that are diagnostic in epifluorescence and fluorometric techniques. This problem is intensified in waters rich in suspended particulate matter. The standard AO method also requires that slides be prepared from samples within 2 weeks of collection and counted while still moist (Daley and Hobbie 1975; Hobbie et al. 1977). We describe a method which allows independent confirmation of AO identifications and counts, provides more accurate counts of bacteria in seston-rich waters, and increases the time allowed for sample storage and counting.

The fluorescing stain 4′6-diamidino-2-phenylindole (DAPI) can be used with modifications of the standard AO technique for epifluorescent counts. DAPI is a highly specific stain for DNA under a wide range of conditions. It is used as a cytochemical probe for nuclear, mitochondrial, and chloroplast DNA (Williamson and Fennell 1975; James and Jope 1978) and for DNA present at previously undetectable levels (Hajduk 1976). When excited with light at a wavelength of 365 nm, the DNA-DAPI complex fluoresces a bright blue at or at >390 nm, while unbound DAPI and DAPI bound to non-DNA material may fluoresce a weak yellow. Bacteria can, therefore, be easily distinguished from other particulate matter.

The purpose of our study was to develop a technique for using DAPI to visualize planktonic microflora, to compare it to the AO method, and to provide an independent confirmation of identifications and counts of bacteria, blue-greens, and algae. We thank W. Cosgrove, R. Meagher, M. Pace, L. Pomeroy, R. E. Hodson, C. Cavanaugh, J. Hobbie, J. G. Jones, and W. Wiebe for aid and comments.

A modification of the direct count method (Francisco et al. 1973; Zimmermann and Meyer-Reil 1974; Jones 1974; Daley and Hobbie 1975; Watson et al. 1977) was used. Samples were processed in a darkened still air hood to reduce exposure to airborne contamination and light. All reagents were filtered (0.22-μm Millipore) when made and again just before use. All glassware was acid-washed (10% nitric) and rinsed with deionized (5×) and filtered distilled (2×) water. Twenty-milliliter samples fixed with 1 ml of filtered Formalin (37% formaldehyde) were stored at 4°C in the dark in 20-ml glass scintillation vials with Teflon-lined caps. To provide a dark background for improved visibility of fluorescing bacteria, we stained Nuclepore filters (25-mm diam, 0.2-μm pore size) by flotation in a Petri dish containing an Irgalan Black (Baker) solution (2 $g \cdot liter^{-1}$ plus 20 ml of acetic acid) for 12 h or more. Before use, the filters were rinsed in filtered, distilled water. We wanted even distribution of the sample, so filters were used wet and placed on top of a damp backing filter (Millipore, 25-mm diam, 0.45-μm pore size) in a small (25 mm) glass filter holder.

The fluorescing stain 3,6-tetramethyl diaminoacridine (acridine orange, AO: Fisher Sci.) was diluted to 0.1 $mg \cdot ml^{-1}$ with sterile, filtered, distilled water and stored at 4°C for several months. A concentrated stock solution (1.0 $mg \cdot ml^{-1}$) for DAPI (Boehringer) was stored indefinitely at 0°C. This was diluted to 0.1 $\mu g \cdot ml^{-1}$ before use and was stable at 4°C for several weeks (Williamson and Fennel 1975). Dilute stain solutions were filtered and 0.2 ml added with an automatic pipette (Pipetman: Gilson) to 2.0 ml of whole or diluted sample in the filter apparatus. Final stain concentrations were 10 $\mu g \cdot ml^{-1}$ of AO and 0.01 $\mu g \cdot ml^{-1}$ of DAPI. The stain and sample were al-

lowed to stand for 2 min for AO and 5 min or longer for DAPI and then drawn through the filter with a vacuum pump (Millipore) at 178 mm (7 inches) of Hg. The vacuum was removed immediately after the subsample passed through the filters. The damp Nuclepore filter was then removed and placed on a drop of low fluorescing immersion oil (Cargille type B) on a large microscope slide (50 × 75 mm); a drop of oil was added, and the filter was covered with a round 25-mm No. 1 coverslip. The technique can also be used with glutaraldehyde- and Formalin-preserved planktonic marine samples (L. R. Pomeroy pers. comm.) and freshwater and marine sediment samples (N. Phillips pers. comm.). Other concentrations of DAPI ranging from 0.05 to 0.10 $\mu g \cdot ml^{-1}$ have been used successfully (J. Hobbie pers. comm.; A. Coleman pers. comm.), as has DAPI from Sigma Chemical Corporation.

We used a Zeiss Universal microscope equipped with a 75-W W2 DC Xenon light system and vertical illuminator with BG38 and KG1 red-free filters; a Zeiss standard microscope with 100-W HBO mercury light source can also be used. For AO counts, we used a G436 exciter filter, FT 510 dichromatic beam splitter, LP 515 barrier filter (filter set: 48 77 07) and Planachromat 100/1.25 oil with iris objective, for DAPI a BP 365/10 exciter filter, FT 390 chromatic beam splitter, LP 395 barrier filter (filter set: 47 77 01) and Neofluar 100/1.30 oil objective. The Neofluar objective without flat-field correction must be used for DAPI fluorescence because the lens coatings of the optically superior Planachromat absorb in the excitation range of the DAPI filter set. Other combinations of optical systems may produce absorbance or autofluorescence within the microscope system (L. R. Pomeroy pers. comm). Photomicrographs were taken with Ektachrome 400 film in a Zeiss MC 63 photomicrographic camera. All bacterial cells in a Whipple grid field (71.5 × 71.5 μm) were counted at 1,250×. In general, we counted 50 fields per slide for comparison of methods and 10 fields per slide for field data. At least 1,000 cells were counted for each sample as attached, unattached, filamentous, and coccoid cells. Natural bacteria were collected with a 5-liter PVC Niskin bottle from Lake Oglethorpe, a eutrophic manmade Georgia lake (30 ha, Z_m = 8.5 m). To determine the minimum time required for staining, we stained samples for 2, 5, and 30 min, and 2 and 24 h with DAPI. Stain was applied in the filter apparatus for 2 and 5 min and to samples stored in vials at 4°C for 0.5, 2, and 24 h. The stored samples were allowed to stand in the filter apparatus for 5 min before filtration. All samples were then prepared as above and counted.

Table 1. Comparison of total counts of natural bacteria (27 January 1979) stained for various intervals with DAPI. Data reported as mean ± SE for 50 fields (n = 50). No significant differences were found as determined by ANOVA.

Time	Cell counts
5 min	98.62 ± 2.99
30 min	102.98 ± 2.81
2 h	100.58 ± 2.37
24 h	97.72 ± 3.22

We compared the two stains for visibility by observation and photomicrography. To test the stability of the stains under light excitation in the microscope, we observed the slides and took the photomicrographs at 0-, 1-, 3-, 5-, 7-, and 10-min intervals. We also made a comparison of natural and cultured bacteria stained by the standard AO method, with a 2-min minimum staining time (Hobbie et al. 1977), and those stained with DAPI for 5 min and 24 h. Finally, we tested shelf storage stability of prepared slides by comparing two sets of stained slides. We stored AO and DAPI slides in the dark at 4°C and at room temperature (24°C). At weekly intervals we counted 25 fields per slide.

The minimum period needed to expose the sample to DAPI for effective visualization and counting is 5 min; there was no significant improvement in counting ease or accuracy after 5 min (Tables 1, 2). Exposure for 2 min, the standard time for AO staining, was not enough to

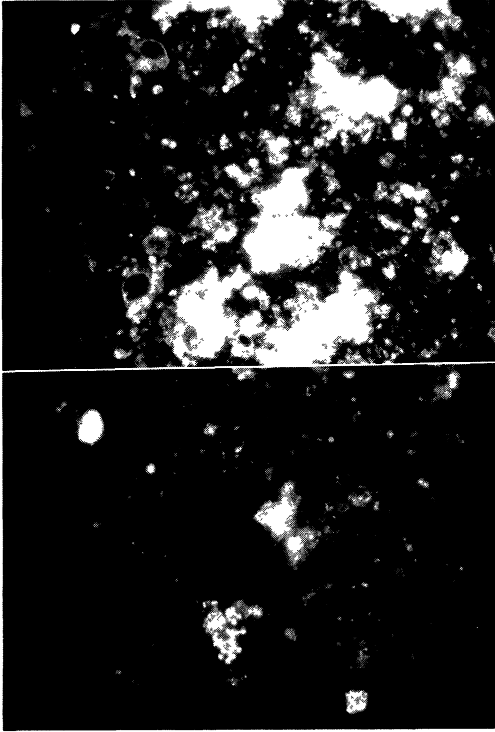

Fig. 1. Fluorescence photomicrographs of AO-stained natural water sample (top) showing fluorescing clay and detritus particles. DAPI-stained sample (bottom) showing bacteria, colloids, the green alga *Crucigenia*, and a blue-green algal colony (3,125×). Concentrations of chlorophyll which autofluoresce red with AO excitation wavelengths appear a brighter blue than the rest of the cell stained with DAPI.

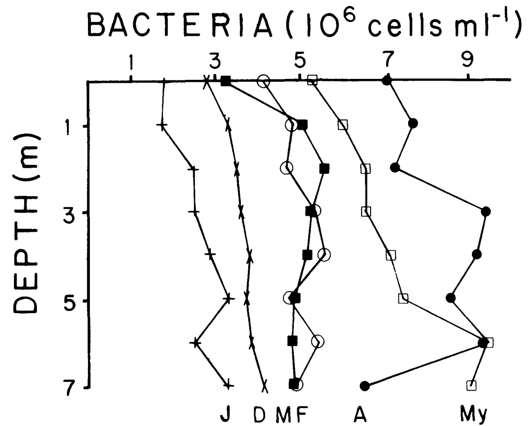

Fig. 2. Depth and seasonal changes in bacterial abundance in samples collected at monthly intervals from Lake Oglethorpe. Error bars contained within data points as drawn. Counts were as high as 3×10^7 cells·cc^{-1} in late summer.

provide visible fluorescence with DAPI. DAPI fluorescence remained stable under excitation with our light system for up to 3 min while AO stain faded visibly after 1 min, necessitating rapid counting and making photomicrography difficult. Excitation at 490 nm with a quartz-halogen light source gives greater AO stability (J. Hobbie pers. comm).

We found no significant difference between AO and DAPI counts of total, coccoid, filamentous, attached or unattached natural bacteria collected during an unproductive and relatively seston-free period, 8 January 1979 (Table 2). Under these sampling conditions DAPI provided an independent confirmation of identifications made with AO. During seston-rich periods, clays, colloids, and detritus clutter the field and produce a reddish-orange background which makes accurate AO counting impossible, but the bright blue DAPI-stained bacteria are highly visible against a black background (Fig. 1). Other particles are not visible or are a pale yellow which enhances the visibility of DAPI fluorescence.

Table 2. Comparison of counts of natural bacteria (8 January 1979) stained with AO for 2 min and DAPI for 5 min and 24 h. AO color differences were counted. Data reported as mean ± SE for 50 fields ($n = 50$). No significant differences within categories were found as determined by ANOVA.

Categories	AO (2 min)	DAPI (5 min)	DAPI (24 h)
Coccoid	33.10 ± 1.17	30.72 ± 1.03	36.72 ± 1.14
Filamentous	10.42 ± 0.94	12.06 ± 0.73	7.92 ± 0.64
Unattached	40.26 ± 1.51	40.04 ± 1.21	32.88 ± 1.22
Attached	3.26 ± 0.56	2.74 ± 0.46	3.86 ± 0.65
Total	43.52 ± 1.59	42.78 ± 1.19	36.72 ± 1.14

Table 3. Counts of natural bacteria on slides stained with AO and stored at 4°C and room temperature. Data reported as mean ± SE (n = 25).

Weeks	Storage temp (°C)	Red cells	Green cells	Total cells
0	4	31.80 ± 2.71	135.68 ± 4.96	167.48 ± 4.83
	24	31.48 ± 3.09	136.83 ± 3.91	168.31 ± 4.99
1	4	15.04 ± 1.50*	134.80 ± 6.83	149.84 ± 7.35*
	24	3.36 ± 0.62*	91.92 ± 3.58*	95.28 ± 3.46*
2	4	13.48 ± 1.78*	117.60 ± 5.88*	131.08 ± 7.23*
	24	4.28 ± 0.73*	88.56 ± 4.84*	92.84 ± 5.09*
3	4	6.24 ± 0.42*	104.36 ± 5.06*	110.60 ± 5.14*
	24	3.60 ± 0.52*	64.24 ± 2.44*	67.84 ± 2.56*

* Significantly different from original count at that storage temperature as determined by t-test ($P < 0.05$).

After 1 week of storage in the dark at 4° and 24°C, counts of bacteria on AO slides were significantly lower than original counts (Table 3). Bacterial counts on DAPI slides decreased significantly within a week when stored in the dark at 24°C (Table 4), but remained consistent for at least 24 weeks at 4°C (series b). Storage time of samples can, therefore, be increased if slides are prepared within 2 weeks of collecting the sample and stored at 4°C. This also reduces storage space, and the slides can be counted or examined later.

Total cell counts in lake water collected at monthly intervals from December 1978 through May 1979 are shown in Fig. 2. At all depths and times, >90% of the cells are coccoid and unattached to particles. Standard error bars are contained within the points as drawn, indicating a high degree of accuracy of the counting method when applied to natural samples.

Table 4. Counts of natural bacteria on slides stained with DAPI, stored at 4°C and at room temperature. Slides in series "a" counted weekly for 2 weeks. Slides in series "b" counted weekly until a significant reduction in countable cells was found. Data reported as means ± SE.

Weeks	Series	n	Storage temp (°C) 4	24
0	a	50	98.62 ± 2.99	99.30 ± 2.88
	b	25	166.80 ± 5.52	168.08 ± 5.78
1	a	50	98.10 ± 2.10	49.66 ± 1.65*
	b	25	170.28 ± 4.01	110.22 ± 4.13*
2	a	50	99.12 ± 2.02	44.80 ± 1.33*
	b	25	163.12 ± 4.25	109.56 ± 1.97*
4	b	25	169.04 ± 4.14	109.40 ± 2.53*
6	b	25	163.44 ± 4.22	105.00 ± 2.34*
12	b	25	161.96 ± 2.48	—
24	b	25	159.24 ± 3.77	—
36	b	25	151.80 ± 3.78*	—

* Significantly different from original count in series as determined by t-test ($P < 0.05$).

A method was developed for using the highly specific and sensitive fluorescing DNA stain, DAPI, for counting natural microflora. It provides an independent confirmation of identifications of bacteria, cyanobacteria, blue-green algae, and algae made with the standard AO technique. The bright blue fluorescence improves visualization of cells and accuracy of counting especially in eutrophic and seston-rich waters. Under these conditions autofluorescence and uptake of AO by particles and colloids produce a reddish-orange background that masks bacterial cells. DAPI cell counts are consistent even with variations in handling time. The time allowed to count a slide and between collection and counting of the sample is greatly increased over the standard AO method. Even in eutrophic seston-rich water, 90% of the naturally occurring bacteria were found to be coccoid and unattached.

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Enhanced detection of bacteria in natural environments by fluorochrome staining of DNA[1]

Abstract—The DNA-staining fluorochromes 4′6-diamidino-2-phenylindole (DAPI) and mithramycin, when used in conjunction with epi-illumination fluorescence microscopy, readily reveal the presence of bacteria, thus simplifying the detection of bacterial contaminants in allegedly axenic laboratory cultures of eucaryote cells. Both gram-negative and gram-positive cells are stained, and aqueous solutions of the dyes penetrate living as well as fixed cells. The use of an epi-illumination system for fluorescence microscopy makes it possible to scan the microflora epiphytic on material collected from freshwater or marine environments, even when the thickness of the material renders both phase and Nomarski optics inadequate.

In recent years two fluorochromes have become available which are particularly useful in localizing DNA. These are the trypanocide 4′6-diamidino-2-phenylindole (DAPI) and the fungal antibiotic mithramycin. Both are readily soluble in water, can be used as stains throughout the pH range 4 to 8, and bind almost exclusively to native DNA (Ward et al. 1965; Chrissman and Tobey 1974; Lin et al. 1977). The DNA-dye complex has a fluorescence greatly enhanced over that of the unbound dye molecule, so that nuclei can be observed clearly in cells still bathed in the dye solution, and the intensity of the fluorescence is such that even very small aggregates of DNA can be detected easily. In the combination of these characteristics, the new fluorochromes represent a considerable improvement over such dyes as acridine orange. As a consequence, DAPI has already become a standard tool for detecting the presence of pleuropneumonia-like organisms in

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