

Use of Nuclepore Filters for Counting Bacteria by Fluorescence Microscopy

J. E. HOBBIE,* R. J. DALEY, AND S. JASPER

Marine Biological Laboratory, Woods Hole, Massachusetts 02543, and Canada Center for Inland Waters,
West Vancouver, British Columbia, Canada*

Received for publication 8 October 1976

Polycarbonate Nuclepore filters are better than cellulose filters for the direct counting of bacteria because they have uniform pore size and a flat surface that retains all of the bacteria on top of the filter. Although cellulose filters also retain all of the bacteria, many are trapped inside the filter where they cannot be counted. Before use, the Nuclepore filters must be dyed with irgalan black to eliminate autofluorescence. Direct counts of bacteria in lake and ocean waters are twice as high with Nuclepore filters as with cellulose filters.

The direct-count method using a fluorescent dye and the epifluorescent microscope to count aquatic bacteria has been widely adopted. It is far from a routine procedure, however, and many different dyes, membrane filters, and microscopes have been tested. We report here modifications of the technique that allow the use of Nuclepore filters.

The criteria for a successful direct-counting technique are simple; all the bacteria must be retained by the filter, all the bacteria must be visible at the filter surface, and the staining and optical conditions must produce high contrast between the bacteria and the background. The problems in evaluating counting techniques are that almost all methods give numbers of some sort and all methods work very well with bacterial cultures where the cells are usually large and stain well. In spite of these problems, recent advances in microscopy (dichroic mirrors, improved filters, cheaper lamps) and membrane filters (dyed cellulose) have resulted in several techniques that give similar results with bacteria from nature (e.g., Fliermans et al. [4] with fluorescein isothiocyanate, Jones and Simon [6] with euchrysin, and Francisco et al. [5] and Daley and Hobbie [2] with acridine orange). However, it is well known that the cellulose membrane filters have a very rough surface (1, 8), so we have attempted to improve the technique by using Nuclepore filters. Because these are quite fluorescent, a staining procedure was developed to reduce this fluorescence. Previous methods of eliminating this problem with Nuclepore filters were very time consuming (11) or did not give good contrast (6).

Two microscopes gave good results. The Leitz Ortholux was fitted with a Ploem illuminator,

an HBO-200 lamp, a heat filter, a BG-38 exciter filter, one or two KP 490 exciter filters, a TK 510 beam splitter, and a K 510 barrier filter. (The recently introduced Ploemopak filter system [1] is even better.) The Zeiss Standard 18 microscope was fitted with an IV FL epifluorescence condenser, a 100-W halogen lamp, a 455-500 band-pass filter, a 510 beam splitter, and an LP 528 barrier filter (it is one-third the cost of the Leitz).

Nuclepore filters (0.2- μ m pore size, 25-mm diameter) were stained before use by soaking for 2 to 24 h in a solution of 2 g of irgalan black in 1 liter of 2% acetic acid. The dye (Chemical Index, acid black 107) is available from Ciba-Geigy Corp., Dyestuffs and Chemicals Division, Greensboro, N.C., and from Union Color and Chemical, Boston, Mass. The dye does not need to be filtered before use, and the filters are individually rinsed by dipping several times in clear water and used immediately. They may also be dried on absorbent paper and stored.

Unfortunately, some batches of Nuclepore filters are partially hydrophobic. This is easily noticed during routine counting as large areas of the filter will be void of cells. To avoid this, several drops of a 0.5% solution of a surfactant (Wayfos, Phillip A Hunt Chemical Corp., East Providence, R.I.) are placed above and below the filter, and the vacuum is briefly applied to wet the filter. This is done immediately before the sample is filtered and should be carried out rapidly to avoid destaining. If possible, the surfactant should not be used as it will increase background fluorescence in some types of waters. It is vital that the bacteria are examined while still moist, so the filters should be counted within an hour or so after preparation. Preservation of the sample with an aldehyde

works for several weeks (2) in fresh and seawater.

The sample is stained with 0.01% (final concentration) acridine orange (AO). It is convenient to place a 2.0-ml subsample in a small test tube and add 0.2 ml of 0.1% AO (in distilled water), incubate for 1 or 2 min, and add to the filter tower. The filter is placed on top of a cellulose filter (any type) or a piece of fine nylon netting to give better distribution of the vacuum (0.8 atm).

After the last drops of water have passed through the filter, the damp filter is placed on a microscope slide, and immersion oil, a cover slip, and oil are placed on top. This preparation will last for some hours at room temperature, or longer at 0°C. The numbers of bacteria per milliliter were estimated from a count of at least 10 randomly chosen microscope fields. A total of at least 200 bacteria were counted. An eyepiece micrometer disk is used to delineate a portion of the field for the actual counting.

A number of precautions should be taken for good results. First, the AO solutions as well as any water used for dilution of the sample may contain many bacteria (distilled water especially). These should be filtered through 0.2- μ m cellulose or Nuclepore filters each day. To avoid this, the solutions may be preserved for weeks with 2% formaldehyde (final concentration). Blanks of AO plus any dilution water must be run each day. Second, at least 2 ml of fluid should pass through the 25-mm-diameter filter to give a random distribution of cells. Ideally, 2 ml of the sample water is used, but dilutions can be made if necessary. Third, immersion oil can cause problems. Some brands are fluorescent, some will remove stain from the filter, and some will not mix with others. Fresh Cargille immersion oil works well. Finally, bacteria numbers can change soon after the samples are collected, so preservation with an aldehyde may be necessary (2). We use 2% formaldehyde (final concentration).

At a final concentration of 0.01% AO, about 95% of the bacteria fluoresce green and the remainder red or yellow. Other organic particles have a weak red fluorescence. After the preserved water sample is heated to the boiling point in a water bath and then stained, the bacteria all fluoresce red. Strong acids, drying, or organic solvents have the same effect. Although red-fluorescing bacteria can be counted, bacteria are easier to count when they fluoresce green because they are brighter and the contrast is greater. The 0.01% AO concentration usually gives this color for most of the bacteria, but we have found that the actual amount of dye in the AO varies somewhat between manu-

facturers; therefore, other concentrations should be tested.

Most of the bacteria in freshwater, sediments, or soils are quite small, 0.3 to 0.7 μ m in diameter. In one lake, 56% were retained on 0.4- μ m filters and 99% on 0.2- μ m filters (Table 1). Many more were visible on Nuclepore filters than on cellulose filters, but there is no fixed relationship as the percentage of very small bacteria will vary from water mass to water mass. For example, the ratio of the count on cellulose filters to the count on Nuclepore filters ranged from 0.45 to 0.57 in the upper 10 m of Loon Lake and was 0.38 and 0.33 at 25 and 50 m (Fig. 1). In the surface waters of a pond, a reservoir, and an estuary (Burrard Inlet), the ratio was 0.42 to 0.56 (Table 2). When the filtrate that passed through the cellulose filter was passed through a Nuclepore filter, less than 1% of the original number of cells was found (Table 2); therefore, the cellulose filter is trapping the bacteria, but some of them are held inside where they cannot be seen.

Bacteria in the oceans are equally small. Ferguson and Rublee (3) found that most were cocci of 0.5 μ m or less in diameter; our observations agree. Oceanic bacteria are also quite abundant, and 6.3×10^6 /ml were counted in rich coastal waters off southern Africa (Hobbie, unpublished data). In deeper waters off Africa, 1.6×10^6 /ml were found at the surface and 34×10^3 /ml at a depth of 4,200 m. In view of the large number of adenosine 5'-triphosphate measurements that have been carried out with glass-fiber filters, it is noteworthy that between 57 and 69% of the bacteria in waters off Africa passed through a Whatman GF/C filter.

It is very important to have good contrast between the weakly staining bacteria and the Nuclepore background. Both the irgalan black dye and the correct optical filters enhance this contrast. We find that a filter combination that passes blue light from 455 to 490 nm reduces autofluorescence a great deal. The Zeiss filter cube is so efficient that the cheap and conven-

TABLE 1. *Bacteria numbers in Loon Lake, August 1975, counted on Nuclepore filters of different pore sizes^a*

Filter (μ m)	10^5 cells/ml	% of count on 0.1- μ m filter
3.0	0.6	7
1.0	1.1	13
0.4	4.6	56
0.2	8.1	99
0.1	8.2	100

^a At least 400 cells were counted, so the 95% confidence interval is the mean \pm 10%.

ient 100-W halogen lamp may be used. The Leitz filters apparently still need the more powerful Hg lamps, but both microscopes give excellent results. R. T. Wright (personal communication) has also had good results with an American Optical Co. microscope.

When the concentration of the AO is kept relatively low, the dye appears to interact with the nuclear material of bacteria in exactly the same way that AO interacts with mammalian nucleic acids (e.g., 7, 10). Thus, bacteria grown at high growth rates in batch or continuous culture will fluoresce red-orange due to the predominant ribonucleic acid (RNA). The random coil of the RNA allows so many AO molecules to attach and interact that the AO fluoresces as a dimer. In contrast, inactive bacteria have mostly deoxyribonucleic acid (DNA) and fluoresce green; the rigid structure of the double helix allows fewer AO molecules to attach, they do not interact, and the AO fluoresces as a monomer. In living bacteria, the DNA fluorescence is always present but is sometimes masked by the great amounts of RNA. Fixation with aldehyde does not change the nucleic acids, so the green and red distinction will continue after cell death. However, when the bacteria are heated, the double-stranded DNA

TABLE 2. Comparison of bacterial numbers on cellulose (Sartorius) and Nuclepore filters^a

Sample site	10 ⁵ cells/ml ^b	
	Sartorius (0.45 μm)	Nuclepore (0.2 μm)
Burrard Inlet		
Filter 1	8.6	14.8
Filter 2	8.4	19.0
Filter 3	7.6	16.6
Filter 4	9.6	17.2
Mean (n = 4)	8.6	16.8
Sartorius filtrate		0.15
Capilano Reservoir		
Filter 1	3.8	6.8
Filter 2	4.1	7.1
Filter 3	4.2	7.8
Mean (n = 3)	4.0	7.2
Ambleside Pond		
Filter 1	16.0	38.0
Filter 2	17.1	41.1

^a The 95% confidence limit on all individual counts is the mean ± 10%.

^b Corrected for control samples containing no sample (usually 0.2 × 10⁵ to 0.14 × 10⁵ cells/ml).

breaks down to single-stranded DNA and a red fluorescence results (7). Recombination is prevented by the presence of an aldehyde. Thus, both the most active and the dead bacteria will fluoresce red with this concentration of AO. A green fluorescence, the most common occurrence in nature, implies inactivity or very slow growth. When both green and red fluorescence are present, this appears yellow to the eye. These observations may well lead to a microspectrophotometric technique for looking at the ratio of RNA to DNA in individual cells from nature. This could give information about the amount and location of bacterial activity.

Filtration onto the Nuclepore filter appears to be the best way to make a direct count of bacteria. This method has been verified by a comparison of counts of bacteria on a Nuclepore filter with this method, with the scanning electron microscope (1), and with carbon replica and transmitted electron microscope techniques (9). In addition, the *Limulus* lysate method for detecting lipopolysaccharides (9) agreed very well with the AO direct counts of bacteria in ocean waters from off Africa (Watson, personal communication). This agreement with other methods increases the certainty that the fluorescent particles we count are really bacteria rather than detritus.

This research was supported by National Science Foundation grant OPP-7512951 and Environment Canada (Kamloops Lake Study).

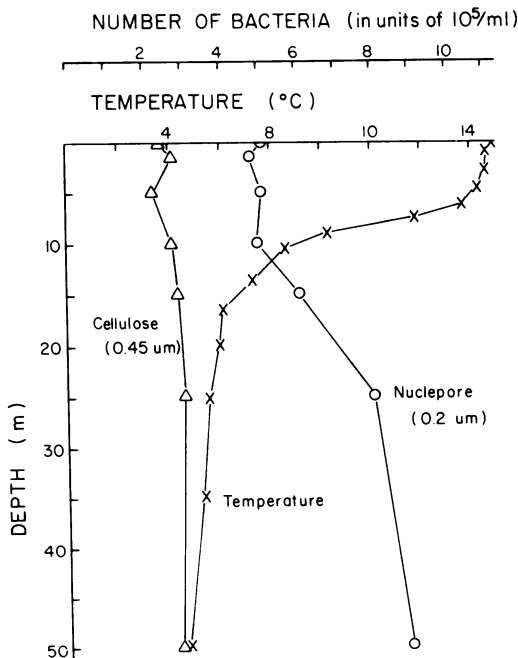


FIG. 1. Temperature and numbers of bacteria at various depths in Loon Lake, B.C., in September 1975. The bacteria were counted on Nuclepore filters (0.2-μm pore size) or Sartorius (cellulose) filters (0.45-μm pore size).

LITERATURE CITED

1. Bowden, W. B. 1977. Comparison of two direct-count techniques for enumerating aquatic bacteria. *Appl. Environ. Microbiol.* 33:1229-1232.
2. Daley, R. J., and J. E. Hobbie. 1975. Direct counts of aquatic bacteria by a modified epi-fluorescent technique. *Limnol. Oceanogr.* 20:875-882.
3. Ferguson, R. L., and P. Rublee. 1976. Contribution of bacteria to the standing crop of coastal plankton. *Limnol. Oceanogr.* 21:141-145.
4. Fliermans, C. B., P. Schneider Cain, and E. L. Schmidt. 1975. Direct measurement of bacterial stratification in Minnesota lakes. *Arch. Hydrobiol.* 76:248-255.
5. Francisco, D. E., R. A. Mah, and A. C. Rabin. 1973. Acridine orange epi-fluorescent technique for counting bacteria in natural waters. *Trans. Am. Microsc. Soc.* 92:416-421.
6. Jones, J. G., and B. M. Simon. 1975. An investigation of errors in direct counts of aquatic bacteria by epi-fluorescence microscopy, with reference to a new method for dyeing membrane filters. *J. Appl. Bacteriol.* 39:1-13.
7. Rigler, R., Jr. 1966. Microfluorometric characterization of intracellular nucleic acids and nucleoproteins by acridine orange. *Acta Physiol. Scand.* 67(Suppl. 267):1-122.
8. Todd, R. L., and T. J. Kerr. 1972. Scanning electron microscopy of microbial cells on membrane filters. *Appl. Microbiol.* 23:1160-1162.
9. Watson, S. W., T. J. Novitsky, H. L. Quinby, and F. W. Valois. 1977. Determination of bacterial number and biomass in the marine environment. *Appl. Environ. Microbiol.* 33:940-954.
10. Yamabe, S. 1973. Binding of acridine orange with DNA. *Arch. Biochem. Biophys.* 154:19-27.
11. Zimmerman, R., and L. Meyer-Reil. 1974. A new method for fluorescence staining of bacterial populations on membrane filters. *Kiel. Meeresforsch.* 30:24-27.