Bermuda Biological Station For Research, Inc.
Bermuda Atlantic Time-series Study

Chapter 17. Determination of Bacterioplankton Abundance

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Modified from: Hobbie *et al.* (1977), Porter and Feig (1980)

1.0 Scope and field of application

This procedure describes a method for the determination of the abundance of bacteria in seawater using acridine orange or DAPI (4,6-Diamidino-2-phenylindole). The assay is appropriate for measuring oceanic bacterial abundance (10^7-10^9 bacteria kg^{-1}). Both of the common staining techniques are described below. Some scientists use modifications of these techniques. New techniques of flow cytometry are emerging but are not described here. Scientists who employ this or other methods to measure bacterial abundance should make themselves aware of the current and historical issues that surround these techniques and make appropriate decisions about specific methodologies for their application based on the scientific requirements and constraints of their individual programs.

2.0 Definition

Bacterial abundance is given in terms of the number of bacterial cells kg^{-1} seawater.

3.0 Principle of Analysis

Bacteria are preserved, stained with either acridine orange or DAPI and concentrated onto a membrane filter. This causes the individual bacteria cells to fluoresce green (using acridine orange) or bluish white (using DAPI) under blue or ultraviolet excitation respectively on an epifluorescence microscope. The individual cells are counted in fields of view of known area and the concentration of bacteria in the original sample is calculated.

4.0 Apparatus

4.1 Any high quality epifluorescence microscope equipped with objectives specifically designed for fluorescence work at <400nm with immersion oil. The numerical aperture of the objective should be high and the focal plane should be constant across the entire field of vision. Total magnification (objective, eye pieces and auxiliary magnifiers) should be at least 1000x.

4.2 A blue filter set (blue excitation 450-490 nm, dichromatic beam splitter 510 nm, barrier filter 520 nm) is used with acridine orange.

4.3 An ultraviolet filter set (ultraviolet excitation 365 nm, dichromatic beam splitter 395 nm, barrier filter 420 nm) is used with DAPI.

5.0 Reagents

5.1 *Glutaraldehyde*: 25%, Grade II (Sigma)

5.2 *Acridine Orange*: 80% dye content (Sigma)

5.3 *DAPI (4,6-Diamidino-2-phenylindole)*: (Sigma)

6.0 Sampling

Samples (90 ml) are measured into a graduated cylinder and then transferred into 125 ml high-density polyethylene bottles. Immediately following collection the samples are preserved in 10 ml of 25% glutaraldehyde and capped and swirled. They are then allowed to stand for 10 minutes at room temperature, after which they are gently swirled and placed in the dark at 4° C for storage. The amount of water to be filtered is a function of expected cell number. Following slide preparation, samples should be examined to ensure the proper number of cells (25-100 per field) (Kirchman *et al.*, 1982) and distribution over the field.

Samples should be processed, stained and filtered as soon as possible (within 2-3 days) after sampling to avoid loss of bacterial numbers (Turley and Hughs, 1992).

7.0 Procedures

7.1 *Acridine Orange*: A sample volume necessary to yield approximately 100 cells per field of view (total volume > 2 ml) is combined with 0.05% acridine orange (Sigma, 80% dye content) to a final concentration of 0.005% and filtered at <100 mm Hg onto a 0.2 µm, Irgalan Black stained Nuclepore polycarbonate filter (Hobbie *et al.*, 1977). Uniform cell distribution is obtained by prewetting the ground glass base of the filtration apparatus prior to placement of the wet polycarbonate membrane. After filtration, the Nuclepore filter is immediately mounted while still damp on a slide using Resolve brand immersion oil. The stained bacterial cells can be accurately counted up to one year after preparation if the slides are stored frozen and in the dark.

7.2 *DAPI*: A sample volume necessary to yield 25-100 cells per field of view (Kirchman et al., 1982) is filtered onto a 0.2 µm Nuclepore filter prestained with Irgalan Black. After filtration, the filter is covered with approximately 1 ml of the DAPI solution (50 µg/ml), and left to stain in the dark. Some researchers choose to add 0.3-0.4 ml of a 1 mg/ml DAPI solution to the sample when all but 3-4 ml have filtered. After 3 minutes the DAPI is filtered off and the dry Nuclepore filter is immediately mounted on a slide using Zeiss brand immersion oil 518C (n_e=1.518). The stained filters are stored frozen at -20°C in sealed slide boxes which are also sealed in zip-lock bags.

7.3 Kirchman et al. (1982) recommend a minimum of 7 fields per filter to be counted per sample. Bacteria are distinguished by distinct morphologies which brightly fluoresce; fluorescing images less than 0.2 µm in diameter are disregarded. An eyepiece of known area should be used during enumeration.

8.0 Calculation and expression of results:

$$Bacterial\ Abundance\ (cells \cdot l^{-1}) = \frac{C_f \cdot R}{F_s}$$

Where:

C_f = mean number of cells per field

R = (active area of filter)/(area of field counted)[2]

F_s = volume of water filtered (liters)

8.1 The units of kg^{-1} can be obtained by dividing the calculated bacterial abundance by the density of the seawater.

9.0 Quality control

Accurate measurements of sample filtered and preservative added is important for accurate estimates.

Accurate, repeatable enumeration of bacterial cells by eye requires experience as well as a good microscope. New enumerators should train by counting the same samples as an experienced microscopist until reliable and consistent results are obtained. Periodic

[2] Note that the active area of filter through which the water passed is not the outer diameter of the filter. It is equivalent to the inner diameter of the bottom of the filter tower used for that filter.

exchange of samples among different microscopists is useful for maintaining data integrity.

Counts may be calibrated by adding fluorescent microspheres to samples prior to counting. These are available in a variety of sizes, 0.4-2.0 μm and fluorescence properties from Duke Scientific Corporation, Box 50005, Palo Alto, CA 94303 USA; tel 800-334-3883.

There is no absolute standard for bacterial counts. Replicate samples drawn from a single OTE bottle and prepared and counted in parallel should agree to within +/- 15% over the entire range of abundances encountered if the samples are prepared correctly. The precision of the estimate declines if too few or too many cells are concentrated on the filter. See Kirchman *et al* (1982) for a discussion of subsampling and statistical treatments.

10.0 References

Hobbie, J.E., R.J. Daley and S. Jasper. (1977). Use of Nuclepore filters for counting bacteria by fluorescence microscopy. *Appl Environ. Microbiol.***33**: 1225-1228.

Kirchman, D.L., J. Sigda, R. Kapuscinski, and R. Mitchell. (1982). Statistical analysis of the direct count method for enumerating bacteria. *Appl. Environ. Microbiol.* **44**:376-382.

Porter, K.G. and Y.S. Feig. (1980). The use of DAPI for identifying and counting aquatic microflora. *Limnol. Oceanogr.* **25**:943-948.

Turley, C.M and D.J. Hughes. (1992). Effects of storage on direct estimates of bacterial numbers in preserved seawater samples. *Deep-Sea Res.* **39**: 375-394.