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Hobbie J E, Daley R J & Jasper S. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. *Appl. Environ. Microbiol.* 33:1225-8, 1977.
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A method was developed for the direct counting of bacteria in natural waters. Bacteria were rapidly stained with acridine orange, filtered onto a Nuclepore filter, and counted with epifluorescence microscopy. [The *SCI*® indicates that this paper has been cited in over 915 publications, making it the most-cited paper from this journal.]

How to Count a Wild Bacterium

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One goal of ecologists is to understand the flux of elements and energy through food webs, but the microbes of natural waters are particularly difficult to study. They are less abundant ($\sim 10^6$ ml^{-1}) and much smaller (a diameter of 0.2-0.6 μm) than bacteria growing in laboratory cultures; only 1 percent or less grow on agar plates. Russian scientists concentrated the plankton bacteria onto filters and stained them for contrast, but the bacteria still appeared as tiny dots at the limit of resolution of the light microscope. Acridine orange staining of their DNA helped distinguish bacteria from other particles but worked only for the very abundant soil bacteria.

A part of the answer to the problem of aquatic bacteria came during a phone call to Thomas Brock—he suggested trying the epifluorescence microscope, in those times a rather rare and expensive research microscope. With this, the illuminating light passes down through the lens so there is no need to clear the filter. A graduate student at UNC, Don Francisco, had encouraging results[1] from lab and reservoir samples, but the microscope's owner refused to allow the scope to be taken out on a ship so we could not make measurements at sea. In the middle 1970s, these microscopes became more widely available, much cheaper, and better.

The rest of the answer was a drastic improvement in the filters used for concentrating the bacteria. The solution was the newly developed Nuclepore filter manufactured from a flat polycarbonate sheet with etched holes. Bacteria were held on the surface of these filters, unlike the thick Millipore-type, but the filters also absorbed too much stain. Ralph J. Daley, a postdoc, worked with the manufacturer and found a masking stain. He and Steve Jasper tested the method in a variety of fresh and marine waters when Ralph moved to Canada.

Once perfected, the technique became absurdly easy. The bacteria glow with a green light against a dark background like stars in the sky. Because the bacteria are the source of the light, they appear as sharply outlined images despite their extremely small size. Immersion oil is placed directly on top of the moist bacteria, so there is no layer of water and no movement of the microbes. For this reason, bacteria can be counted on shipboard without antivibration tables. The samples may also be preserved with formalin and counted months later. One sample may be counted in less than 10 minutes.

Not only are bacteria now recognized as the most abundant marine organism, but most of the biomass of the oceans is bacterial—this latter is true only because algae are restricted to the upper 100 m of the lighted waters while bacteria are found throughout the water column, which averages 3,900 m in the ocean.

But these direct measurements of numbers and mass of microbes give only a partial picture of the flux of energy and elements through the microbial part of the food web. We also need to know how fast the bacteria are growing. The direct count technique has allowed the calibration of two other recent methods that utilize the incorporation of ^{3}H-labeled thymidine[2] or leucine[3] as a measure of bacterial growth. These measures of growth lead to questions of control of the bacterial populations, and here too the direct count method is used for measurements of the rate of feeding of small flagellates in the ocean.[4]

The article is widely cited because the technique of direct counting it describes is fundamental to any measurement of the role of bacteria in aquatic food webs. No easier method has so far been discovered, although the filters are now prestained and other fluorescent dyes are often used.

1. **Francisco D E, Mah R A & Rabin A C.** Acridine orange epi-fluorescent technique for counting bacteria in natural waters. *Trans. Amer. Microsc. Soc.* 92:416-21, 1973. (Cited 60 times.)
2. **Fuhrman J A & Azam F.** Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: evaluation and field results. *Mar. Biol.* 66:109-20, 1982. (Cited 270 times.)
3. **Simon M & Azam F.** Protein content and protein synthesis rates of planktonic marine bacteria. *Mar. Ecol.—Progr. Ser.* 51:201-13, 1989.
4. **Hagstrom A, Azam F, Andersson A, Wikner J & Rassoulzadegan F.** Microbial loop in an oligotrophic pelagic marine ecosystem: possible roles of cyanobacteria and nanoflagellates in the organic fluxes. *Mar. Ecol.—Progr. Ser.* 49:171-8, 1988.

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