APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1986, p. 664–667
0099-2240/86/030664-04$02.00/0

Vol. 51, No. 3

Comparison of Acridine Orange, Acriflavine, and Bisbenzimide Stains for Enumeration of Bacteria in Clear and Humic Waters

IRINA BERGSTRÖM,[1] ANNE HEINÄNEN,[2] AND KALEVI SALONEN[2]*

Department of General Microbiology, University of Helsinki, SF-00280 Helsinki,[1] and Lammi Biological Station, University of Helsinki, SF-16900 Lammi,[2] Finland

Received 1 July 1985/Accepted 27 October 1985

In highly humic water, acridine orange precipitated with dissolved humic matter, resulting in such bright background fluorescence that no bacteria could be seen. With bisbenzimide staining, a similar precipitate was nonfluorescent but obscured many cells. An acriflavine staining method proved useful and reproducible both in clear and in humic waters. Fading of fluorescence was not a problem, and stained samples could be stored after preparation. The fluorescence of cells stained with acriflavine was weaker than that with acridine orange, making counting extremely small cells slightly more difficult with the former stain.

The acridine orange (AO) epifluorescence counting method for aquatic bacteria (3) was originally developed for seawater, which is chemically rather uniform. In inland waters, water chemistry is different and highly variable, and therefore the methods developed for marine waters often require refinement and modification. In studying humic lakes, we have found microscopic enumeration of bacteria to be difficult, because humic substances interfere strongly with the commonly used AO fluorochrome. In this study we looked for stains and staining methods which would allow easy and reproducible enumeration of bacteria in humic waters.

We selected three fluorochromes for the experiments: AO (G. T. Gurr Ltd., London, England), acriflavine (AF; Chroma Gesellschaft, Stuttgart, Federal Republic of Germany), and bisbenzimide (BBI; dye 33258, Riedel de Haen AG, Hannover, Federal Republic of Germany). The water samples were fixed with prefiltered (pore size, 0.22 μm; Millipore GS; Millipore Corp., Bedford, Mass.) formaldehyde to a final concentration of about 2% (vol/vol). We first determined the most suitable staining time and concentration of stain for each stain and test water. Using the optimized procedures, we then compared the results obtained with different stains in two lake waters with highly contrasting concentrations of dissolved humic substances (Lake Nimetön: color, 180 mg of Pt liter^{-1}; Lake Syrjänalunen: color, below 5 mg of Pt liter^{-1}). The test samples were taken soon after the spring maximum of phytoplankton primary production.

In the method of Hobbie et al. (3), AO is added to the sample, and after being stained the bacteria are filtered onto Nuclepore membranes. We found this method worked well in waters with a low concentration of dissolved humic substances, but with high humic concentrations, AO precipitated with humic substances and led to extremely bright background fluorescence so that no cells could be seen. This problem could be largely avoided by staining the bacteria after collection on the filter. Cells were concentrated onto black (3) 25-mm-diameter Nuclepore filters under a vacuum of 200 mm Hg (2.67×10^4 Pa). To obtain random distribution of cells on the filter but avoid too high densities of bacteria, all samples were diluted with prefiltered deionized water (5). Immediately after filtration, AO solution was injected onto a Millipore GS filter (pore size, 0.22 μm) with a syringe. After the appropriate staining time, the AO solution was sucked through the filter, and the vacuum was released. To avoid drying, a drop of nonfluorescent immersion oil was added to the filter while still on the supporting plate of the filtering device. The filter was then immediately removed, set on a slide, and covered with a cover glass. When the filters were allowed to dry, the fluorescence of cells was weaker, as also observed by Daley and Hobbie (2). We tested several immersion oils, of which the Nikon oil had the lowest background fluorescence and was therefore used in the experiments. Of the other oils, Cargille type A was practically equivalent to Nikon oil. The use of Leitz, Zeiss, and Cargille type df or ff oils resulted in less satisfactory background fluorescence.

AF is a DNA-specific stain which has been applied to eucaryotic cells (1). We employed a simplified method more suitable for use in diverse waters. We omitted acid hydrolysis, and therefore both DNA and RNA contribute to cell staining. It is also possible that cells fixed in aldehyde for 2 weeks or more may exhibit nonspecific cytoplasmic staining (1) as a result of reactions of aldehyde with unsaturated fatty acids. Our method yielded as intense fluorescence as that in the procedure used by Crissman et al. (1) but was easier to use and did not produce any nonfluorescent precipitate, such as that produced in our trials with the unmodified method. In our method, AF was simply diluted with deionized water, and the staining procedure was as for the AO method except that the samples were diluted with prefiltered citrate-hydrochloric acid buffer (pH 4). The best results were obtained when filters were allowed to dry before the addition of immersion oil.

For BBI staining (6), the prefiltered stain solution was added to water samples which were incubated in test tubes in a water bath at 37°C. After incubation, the cells were filtered onto unstained Nuclepore filters (pore size, 0.2 μm). We found no improvement in the detection of small cells when using black filters. The filters were allowed to dry before the immersion oil was added.

For microscopic examination, we used a Nikon Optiphot epifluorescence microscope with an Osram HBO 100 W

* Corresponding author.

TABLE 1. Characteristics of optical filters

Stain	Block type	Excitation filter (nm)	Dichroic mirror (nm)	Barrier filter (nm)
AO	B 12	480/20	510	515 IF
AF	B 20	450–490	510	520
BBI	UV	330–380	400	420K

mercury lamp and a 100×, N.A. 1.30 glycerol immersion objective. Total magnification was ×1,250. We used the sets of optical filters described in Table 1.

For AF staining, the combination with the broader excitation band (430 to 490 nm) in some cases gave better resolution of small cells than the combination used in this study. Instead, the blue violet combination (excitation, 400 to 440 nm; dichroic mirror, 455 nm; and barrier filter, 470 nm) was clearly less satisfactory than the blue combination B 20. We counted bacteria in 20 random fields (about 200 cells per filter) in the middle region of the filter. For testing the effect of the concentration of the stain and staining time, we counted bacteria on only one filter, but for the comparison of the optimized methods we made complete counts on five replicate filters.

In water samples from clear (Fig. 1C) and humic (Fig. 1F) lakes, the concentration of 0.5 mM AO in most cases (one exception) yielded the highest counts of bacteria. An AO solution ten times more dilute resulted in weak fluorescence and hence the lowest counts. With 5 mM AO, the counts were also lower than with 0.5 mM AO, probably owing to increased background fluorescence and increased difficulty in counting the smaller bacteria. With 0.5 mM AO, the staining time did not seem critical. In humic water, a 3-min staining gave the highest count, but the difference between the counts after 10 min was not statistically significant.

With AF staining, the 1 and 10 mM concentrations yielded about the same counts. The results indicate that with AF the concentration of stain is not as critical as with AO. With 0.1 mM AF, the fluorescence of bacteria was weak, making the detection of small cells difficult and resulting in lower counts. Increased background fluorescence also affected counting of cells stained with 10 mM AF. In clear water, staining time had practically no effect on the results, but in humic water at least 3 min was necessary to obtain maximum counts. Our later experiences with extremely humic water (color above 550 mg of Pt liter^{-1}) confirmed that humic waters required longer staining. In this case, 10-min staining was necessary.

In humic water, the BBI solution formed a yellow precipitate which was clearly visible (without a microscope) at all concentrations. The precipitate was composed of small particles or cloudiness. Although the particles did not fluoresce markedly, they made counting of very small and faint cells difficult and prone to decisions which may differ between different persons and equipment. When the volume of sample filtered was increased, the precipitate covered more bacteria to such an extent that their fluorescence could not be seen. Similar precipitates also developed in a sample prefiltered through a Millipore filter (pore size, 0.22 μm).

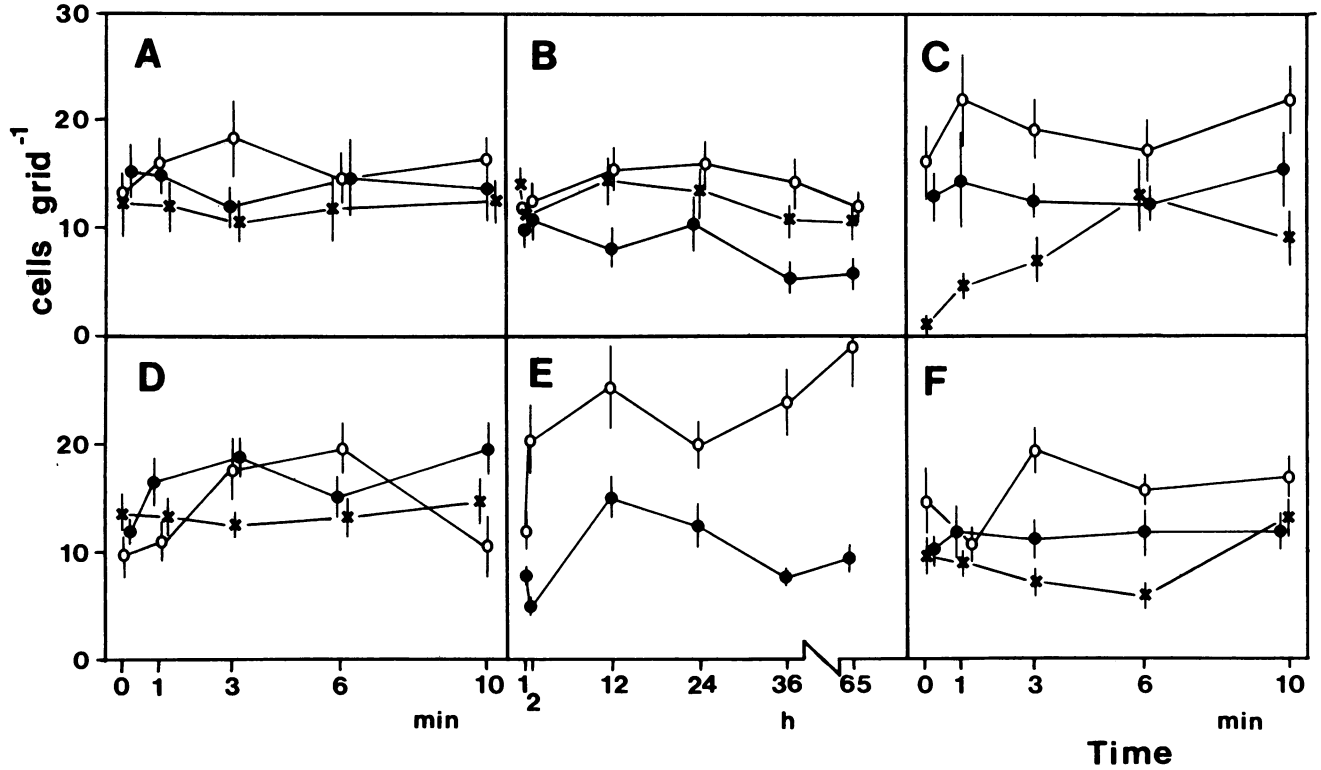

FIG. 1. Effect of staining time and concentration of stain on counts of bacteria with AF (A and D), BBI (B and E), and AO (C and F) staining in clear (A, B, and C) and humic (D, E, and F) waters. The bars represent 95% confidence limits of the mean. (A and D) Symbols: ×, 0.1 mM AF; ○, 1 mM AF; ●, 10 mM AF. (B and E) Symbols: ×, 1 μM BBI; ○, 10 μM BBI; ●, 100 μM BBI. (C and F) Symbols: ×, 0.01 mM AO; ○, 0.5 mM AO; ●, 5 mM AO.

Even in clear water samples the intensity of the fluorescence of BBI-stained bacteria was too weak to allow satisfactory counting of very small cells.

In clear water samples, counts with 1 mM AF and 10 μM BBI staining were similar, but counts with 1 mM AO were significantly higher. In humic water, 0.5 mM AO and 1 mM AF yielded similar results. The counts in 10 μM BBI were about two times higher, which was probably due to BBI precipitate. We conducted an additional test to compare the three methods with respect to optimized stain concentrations and staining times (AO: 0.5 mM, 3 min; AF: 1 mM, 3 min; BBI: 10 μM, 12 h). Practically no cells were found in blank preparations. The reproducibility of all methods was good, varying between 9.9 and 15.5% of the mean within a single count of 20 fields. In clear water the results of AF and AO staining were similar. The cell density counted with BBI staining was only 65% of that found with the other methods (Table 2) and the difference was statistically significant (Student's t test; $P < 0.01$). In humic water, the AO method gave slightly higher results ($P < 0.05$) than the AF method. This difference may have resulted from counting of the smallest bacteria.

Altogether, AO staining had a tendency to yield slightly higher numbers of bacteria than AF staining. The difference may be due to more intensive fluorescence of AO-stained cells. Bright fluorescence also allowed the use of narrow-band excitation, which clearly reduced the background fluorescence. On the other hand, the AO method has some disadvantages. AO is not strictly DNA specific (1), and it is uncertain whether all counted particles are bacteria. According to Paul (6), the AO method may overestimate the number of bacteria because of autofluorescent particles, but in our samples we could not detect such particles. At room temperature the fluorescence of AO also faded so rapidly that preparations had to be examined immediately after staining. However, maybe the most important disadvantage of AO staining is its sensitivity to variations both in the quality of test water and in small details of the staining procedure. For example, correct wetness of the filter is important but difficult to standardize. Further, there are marked differences between AO stains of different manufacturers (2, 4).

In contrast to the AF method with acid hydrolysis, our simple AF staining method is, like the AO method, not strictly DNA specific. AF staining is not as sensitive to small changes in the procedure and it is also compatible with humic substances. From our experience, AF staining consistently produces good preparations, while with AO staining the quality even of replicate preparations may vary. Drying the filter before addition of the immersion oil may be one reason for better reproducibility in the AF procedure, but this may also be a disadvantage. Drying of filters may lead to shrinkage of cells and hence to underestimates of cell biomass (9). In practical work, the ability to store AF preparations in darkness is useful.

In BBI staining, the number of bacteria detected in our humic water sample still increased after the recommended (6) 1-h staining time. We do not know whether this was due to more intensive staining of bacteria or to the appearance of interfering particles. In any case, the intensity of the fluorescence of BBI-stained bacteria was too weak to allow for reliable counting. The method seems particularly unsuitable for use in humic waters.

Because the size of bacteria in natural waters is generally very small, counting cells near the limit of resolution of the light microscope is a formidable problem. Subjective discrimination and the quality of the optical system become highly important. Therefore, it might be reasonable to enumerate particles above a size limit near 0.3 μm separately to reduce the proportion of cells that are prone to subjective counting. Results should then be both more reproducible and comparable between different equipment and persons than when counts include the smallest cells. This larger cell fraction frequently encompasses most of the bacterial biomass (7, 8). If necessary, water passing through 0.3-μm pores could be refiltered through filters with 0.2- or 0.1-μm pores for counting of smaller cells. In this way, counting might be easier and the effect of possibly critical fading of the fluorescence of small cells can be reduced, because of the shorter counting time. Moreover, large bright cells would not mask the fluorescence of very small ones. In addition, the measurement of linear dimensions of such small cells by light microscopy is very unreliable. More realistic mean cell volumes might be obtained simply by using a theoretical mean volume for a narrow size fraction, for example, between 0.2 and 0.3 μm. For the smallest cell fraction, truly specific staining is extremely important to differentiate between cells and other fluorescing particles.

TABLE 2. Comparison of densities of bacteria counted by different methods

Method	Density (10^6 cells/ml) of bacteria from Lake (type)[a]	
	Syrjänalunen (clear)	Nimetön (humic)
AO	2.1 ± 0.33	12.9 ± 1.4
AF	2.1 ± 0.26	10.6 ± 1.1
BBI	1.3 ± 0.30	—[b]

[a] Each mean is based on five replicates (each representing counts from 20 fields of view).
[b] —, Not done.

In conclusion, the staining of bacteria in humic water for epifluorescence microscopy has many inherent methodological difficulties, such as formation of precipitates and high background fluorescence. Under such conditions, the simple AF staining method seems to best allow for counting of bacteria with reasonable reproducibility. However, we are still far from unequivocal counting of bacteria, and one of the most important challenges is to solve the problems of counting small cells near the resolution limit of light microscopy.

We thank R. Jones and P. Ross for checking the language for our manuscript.

This study was supported by the Maj and Tor Nessling Foundation, the Academy of Finland, and the Emil Aaltonen Foundation.

LITERATURE CITED

1. **Crissman, H. A., P. F. Mullaney, and J. A. Steinkamp.** 1975. Methods and applications of flow systems for analysis and sorting of mammalian cells. Methods Cell Biol. **9**:180–246.
2. **Daley, R. J., and J. E. Hobbie.** 1975. Direct counts of aquatic bacteria by a modified epifluorescence technique. Limnol. Oceanogr. **20**:875–882.
3. **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.
4. **Jones, J. G.** 1974. Some observations on direct counts of freshwater bacteria obtained with a fluorescence microscope. Limnol. Oceanogr. **19**:540–543.
5. **Jones, J. G., and B. M. Simon.** 1975. An investigation of errors in direct counts of aquatic bacteria by epifluorescence microscopy, with reference to a new method for dyeing membrane filters. J. Appl. Bacteriol. **39**:317–329.

6. **Paul, J. H.** 1982. Use of Hoechst dyes 33258 and 33342 for enumeration of attached and planktonic bacteria. Appl. Environ. Microbiol. **43**:939–944.
7. **Salonen, K.** 1977. The estimation of bacterioplankton numbers and biomass by phase contrast microscopy. Ann. Bot. Fenn. **14**:25–28.
8. **Salonen, K.** 1981. The ecosystem of the oligotrophic lake Pääjärvi. 2. Bacterioplankton. Verh. Int. Verein Limnol. **21**: 448–453.
9. **Straskrabova, V., and J. Komarkova.** 1979. Seasonal changes of bacterioplankton in a reservoir related to algae. I. Numbers and biomass. Int. Rev. Gesamten Hydrobiol. **64**:285–302.