Copyright ©

Aus dem Institut für Meereskunde an der Universität Kiel

A new method for fluorescence staining of bacterial populations on membrane filters

By Reinhard Zimmermann and Lutz-Arend Meyer-Reil

Eine neue Methode zur Fluoreszenzfärbung von Bakterienpopulationen auf Membranfiltern (Zusammenfassung): Dieser Beitrag beschreibt und diskutiert eine neue Methode zur fluoreszenzmikroskopischen Untersuchung von Bakterien auf Nuclepore-Membranfiltern. Die durch Filtration angereicherten Keime werden mit einer Akridinorange-Lösung gefärbt. Anschließend werden die Filter mit Isopropanol und Xylol entfärbt. Diese Methode ermöglicht es, auch kleine Bakterien zu erkennen und sie von Detritus zu unterscheiden.

(Summary): This article describes and discusses a new method for the fluorescence microscopic examination of bacteria on Nuclepore membrane filters. The bacteria, which have been concentrated by filtration, are stained with a solution of acridine orange. Then the filters are destained with isopropyl alkohol and xylene. This method makes it possible to recognize small bacteria and to distinguish them from detritus.

In order to evaluate the role of bacteria in the metabolic processes of aquatic ecosystems, data on the qualitative and quantitative composition of the bacterial populations are important. For this purpose there are indirect (Hopton et. al., 1972) and direct methods. Among the latter are, for example, the direct introduction of the sample material onto amicroscopic slide, or the filtering of water samples through membrane filters. The membrane filter method is preferable because, by means of concentrating the bacterial cells, a rapid examination of samples which have a low bacterial population is possible. The sample is then evaluated microscopically.

Staining with diachromes (Ehrlich, 1955; Jannasch, 1958; Sorokin and Overbeck, 1972), as we were also able to confirm in our own investigations, resulted in the following problems: detecting small cells (especially in oligotrophic waters), distinguishing them from detritus, and differentiating single cells in aggregates and as growth upon detritus (Jannasch and Jones, 1959; Jannasch, 1965; Kunicka-Goldfinger, 1973). An alternative method is staining with fluorochromes. In this process the water sample is either stained and then filtered onto membrane filters (Jannasch, 1954), or else the cells are directly stained on the filters (Deufel, 1959; Schwantes, 1971). Our research showed neither method to be advantageous. The membrane filters with a cellulose base, which were used by the authors cited, exhibit a disturbing background fluorescence. In using these relatively thick filters with their spongy structure and large inner surface area, a thorough washing with solvents is necessary, which leads to a rapid fading of the small cells. During the symposium: "Detritus and its role in aquatic ecosystems" in Pallanza 1972, Madsen (1972), Wiebe and Pomeroy (1972) mentioned the problems concerned with the fluorescence-microscopic counting of bacteria. Using Nuclepore membrane filters, we have been able to overcome the main difficulties.

This article describes a simple method for fluorescence staining of bacteria on membrane filters. The investigations were carried out with water of different degrees of trophication from the Kiel Bight.

The water samples which were obtained by means of the ZoBell-sampling apparatus were fixed with freshly filtered 2% formalin. 1—3 ml of the samples were taken and suspended in 10 ml of sterile-filtered water from the same station. Filters from Nuclepore. Pleasanton Ca., (diameter 25 mm, pore size 0,2 µ) and a filtration apparatus from the Schleicher-Schüll Company (Selectron GV 025) were used. For a thorough wetting of the filter, we recommend the treatment of the porous-glass plate with a surface active agent before filtration. For staining we used the following fluorochromes: Acridine orange (Merck), auramine O (Chroma), berberine sulfate (Fluka), fluoresceinisothiocyanate (Merck) and fluolite XNR (ICI); (STRUGGER, 1949; BABIUK and PAUL, 1969; PEBERDY and BUCKLEY, 1973). Acridine orange was preferable because an intensive stain was obtained and the fluorescence lasted a long time. We compared different acridine orange concentrations in phosphate buffer pH 6,7 (STRUGGER, 1949) with different staining times. Optimal results were obtained with an acridine orange concentration of 1 : 10000 and a staining time of 3 min. At first the filters were stained by floating in a petri dish containing the dye solution. A more favourable method consisted of placing the filter in the filter apparatus and staining it from above with the previously filtered staining solution. Destaining was made with iso-propyl alkohol and xylene (SCHWANTES, 1971) in the filtering apparatus. Then the filter was removed and air-dried. For the microscopic examination (Zeiss Universal, condensor for epifluorescence III RS, blue-excitation: BG 12 (2 ×), Fl 500, barrier filter 50, Neofluar 100, 12,5 × ocular) the filters were rendered transparent with a mixture of cinnamaldehyde and eugenol (2:1). Using this mixture as a mounting medium, the following effect is observed: After adding the mixture, the detritus loses much of its colour whereas the bacteria remain nearly unchanged and retain their full fluorescence. Thereby the bacteria can easily be detected on solid particles as well as within flocculent detritus. The filter background is dark and completely homogeneous.

A more detailed description of these experiments, and a comparison of direct counts obtained by the fluorescence-microscopic method and by a scanning electron microscope ist the subject of a dissertation which will soon be published (ZIMMERMANN).

Although the length of time for staning and destaining can be variable, we recommend on the basis of our own investigations the following procedure:

Place the filter in the filter apparatus and stain by adding 1 ml of acridine orange solution (1 : 10000; pH = 6,7) onto the filter for 3 min.

Remove the dyeing-solution by suction (vacuum pressure 0,2 atm.)

Equalize the pressure and add 3 ml of iso-propyl alkohol onto the filter.

Remove iso-propyl alkohol after 2 min and equalize pressure again.

Add 1 ml of xylene.

Remove by suction after 30 sec.

Take off the filter and let dry.

Colouring and decolouring solutions are to be filtered particle-free before use.

The following table shows the relationship between the number of bacteria obtained by direct count and by plate count in a horizontal profile of the Kiel Bight. With increasing distance from the shore, the counts obtained by the direct method clearly show a smaller decrease than that of colony-forming units (poured-plate method with ZoBell agar).

Table

Relationship between the number of bacteria obtained by direct count and by plate count on ZoBell-agar.

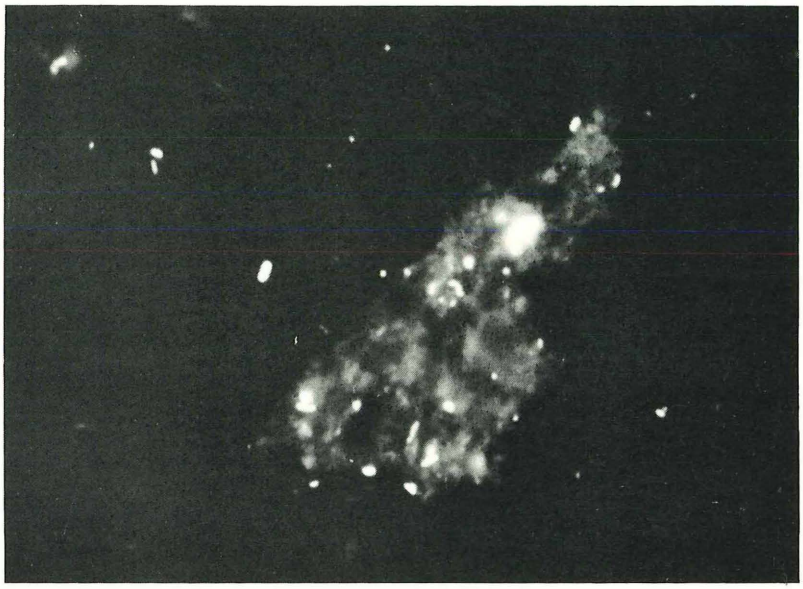
Method	Stations 1—5: increasing distance from the shore					
	1	2	3	4	5	
Direct count	1,97	1,43	0,78	0,95	0,51	$\times 10^6$
%*)	100	73	40	48	26	
Plate count	6,00	5,18	1,65	0,17	0,22	$\times 10^3$
%*)	100	86	26	3	3	

*) Station with the highest count of bacteria = 100%

Fig. 1 a, b, is an example of the bacterial colonisation of detritus. By representing the object in different planes (optical sectioning), a good differentiation of the bacteria is possible: thin rods, short rods (some of which are dividing) and coccoid forms. The smallest bacteria have a diameter of about 0.2 μ; however, an evaluation of their exact form is difficult due to the limited resolving power of the microscope. These coccoid forms are actually bacteria as it is confirmed by the occurrence of micro-colonies of cells of the same size. Whereas the majority of the bacteria fluoresce orange, a small number show a green fluorescence of lower contrast. They are, however, easily recognizable due to their clear contours. The microscopic image is characterized by a long lasting fluorescence, even of the smallest bacteria (under blue-light for a minimum of 15 min without change). Unfixed samples show a fluorescence of poorer contrast. Therefore fixing the samples with formalin is recommended. Further experiments are necessary to determine how form and size of the cells are influenced by formalin fixation and staining.

The method described is intended to aid in a more thorough study of series of ecological problems, such as the number of bacteria in the aquatic habitat, the bacterial biomass, the growth rate and differentiation of the bacterial population.

Tafel 1 (zu R. Zimmermann u. L.-Arend Meyer-Reil)



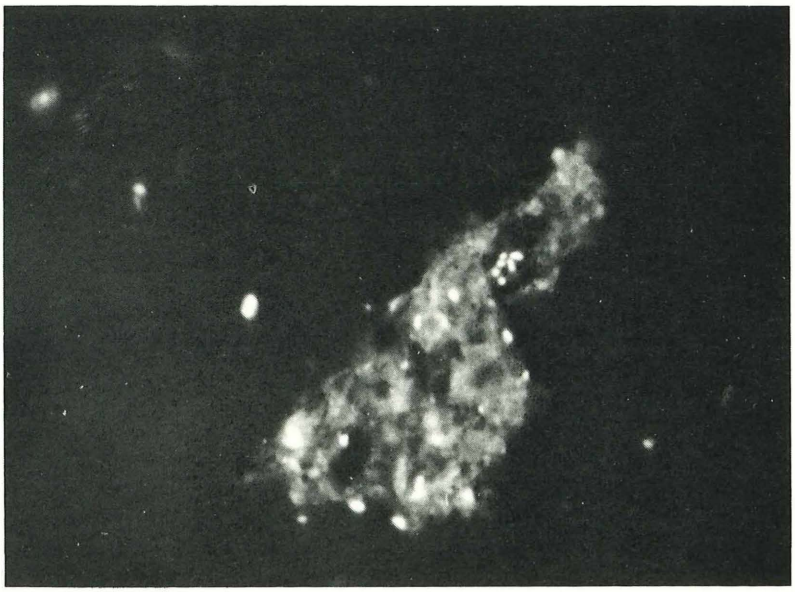


Fig. 1a, b: Bacteria adhering to detritus. Fluorescence staining with acridine orange on membrane filters. For details see text. (× 3000)

Bibliography

Babiuk, L.A. and E.A. Paul (1969): The use of fluoresceinisothiocyanate in the determination of the bacterial biomass of grassland soil. Can. J. Microbiol. 16, 57—62.

Deufel, J. (1959): Zählung von Wasserbakterien auf Membranfiltern mittels Fluoreszenzmikroskopie. Naturwiss. 46, 654.

Ehrlich, R. (1955): Technique for microscopic count of microorganisms directly on membrane filters. J. Bacteriol. 70, 265—268.

Hopton, J. W., U. Melchiorri-Santolini and Y. J. Sorokin (1972): Enumeration of viable cells of microorganisms by plate count technique. In: Techniques for the assessment of microbial production and decomposition in fresh waters. IBP-handbook 23, ed. Y. I. Sorokin and H. Kadota, 59—64.

Jannasch, H. W. (1954): Kurze Mitteilung zur Anwendung der Fluoreszenzmikroskopie bei bakteriologischen Wasseruntersuchungen. Ber. limnol. Flußst. Freudenthal 6, 60.

Jannasch, H. W. (1958): Studies on planktonic bacteria by means of a direct membrane filter method. J. Gen. Microbiol. 18, 425—430.

Jannasch, H. W. and G. E. Jones (1959): Bacterial populations in see water as determined by different methods of enumeration. Limnol. Oceanogr. 4, 128—139.

Jannasch, H. W. (1965): Biological significance of bacterial counts in aquatic environments. Coll. Repr. Woods Hole Oceanogr. Inst. 1490, 127—131.

Kunicka-Goldfinger, W. (1973): An attempt to measure the growth of indigenous aquatic bacteria by the technique of semicontinuous cultures on membrane filters. In: Bull. Ecol. Res. Comm (Stockholm) 17, 311—315, ed. T. Rosswall.

Madsen, B. L. (1972): Detritus on stones in small streams. Mem. Ist. Ital. Idrobiol. 29 Suppl., 385—403, ed. Melchiorri-Santolini, U. and J. W. Hopton.

Peberdy, J. F. and C. E. Buckley (1973): Adsorption of fluorescent brighteners by regenerating protoplasts of Aspergillus nidulans. J. Gen. Microbiol. 74, 281—288.

Schwantes, H. O. (1971): Mikroskopischer Nachweis von Mikroorganismen auf Membranfiltern nach Filtration aus Flüssigkeiten mit Hilfe der Akridinorange-Fluoreszenz. Microsc. Acta 70, 236—238.

Sorokin, Y. I. and J. Overbeck (1972): Direct microscopic counting of microorganisms. In: Techniques for the assessment of microbial production and decomposition in fresh waters. IPB-handbook 23, 44—47, ed. Y. I. Sorokin and H. Kadota.

Strugger, S. (1949): Fluoreszenzmikroskopie und Mikrobiologie. Hannover: M. und H. Schaper.

Wiebe, W. J. and L. R. Pomeroy (1972): Microorganisms and their association with aggregates and detritus in the sea: a microscopic study. Mem. Ist. Ital. Idrobiol. 29. Suppl. 325—352, ed. Melchiorri-Santolini, U. and J. W. Hopton.