APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1978, p. 506-512
0099-2240/78/0036-0506$02.00/0

Vol. 36, No. 3
Printed in U.S.A.

Autoradiography and Epifluorescence Microscopy Combined for the Determination of Number and Spectrum of Actively Metabolizing Bacteria in Natural Waters†

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Received for publication 30 March 1978

A technique is described for the determination of bacterial numbers and the spectrum of actively metabolizing cells on the same microscopic preparation by a combined autoradiography/epifluorescence microscopy technique. Natural bacterial populations incubated with [^{3}H]glucose were filtered onto 0.2-μm Nuclepore polycarbonate membranes. The filters were cut into quarters and fixed on the surface of glass slides, coated with NTB-2 nuclear track emulsion (Kodak), and exposed to the radiation. After processing, the autoradiographs were stained with acridine orange. A combination of overstaining on the slightly alkaline side and gradual destaining on the acid side of neutrality gave the best results. Epifluorescence microscopy revealed bright-orange fluorescent cells with dark-silver grains associated against a greenish-to-grayish background. Based on the standardization curves, detection of actually metabolizing cells was optimal when cells were incubated with 1 to 5 μCi of [^{3}H]glucose per ml of sample for 4 h and the autoradiographs were exposed to NTB-2 emulsion at 7°C for 3 days. In water samples taken immediately above sandy sediments at beaches of the Kiel Fjord and the Kiel Bight (Baltic Sea, FRG), between 2.3 and 56.2% (average, 31.3%) of the total number of bacteria were actually metabolizing cells. Spearman rank correlation analysis revealed significant interrelationships between the number of active bacteria and the actual uptake rate of glucose.

Ecological studies of the role of microorganisms in aquatic systems are limited by the number and validity of methods available. Although each of the methods has its own importance, the data obtained with different methods are difficult to compare and to interpret. Only a very limited number of bacteria actually occurring are capable of growth on agar plates. Epifluorescence microscopy permits information on the bacterial standing crop (number, biomass), which does not necessarily reflect bacterial activity. The same holds true for chemical methods (ATP, DNA, lipopolysaccharide) for determining standing crop. The results obtained from uptake studies of organic solutes (tracer technique) provide information on the uptake of the total bacterial population, regardless of the spectrum of actually metabolizing cells.

In addition to the above-mentioned techniques, autoradiography becomes a useful tool in ecological studies, since it enables investigators to relate activity to individual cells (2, 14). The problems involved in grain density autoradiography have been pointed out recently (7). Determination of the number of actually metabolizing cells by spot counting (6, 12) is limited by the difficulty of relating spots to individual cells. Single silver grains or small groups of grains often regarded as "background" seem to be an additional problem. As an alternative, autoradiographs of either smears or filtered samples were stained with traditional dyes (4, 11, 13). The identification of cells and their relation to silver grains becomes extremely difficult in some cases. By staining autoradiographs with fluorescent antibodies (immunofluorescence), much better microscopic preparations can be obtained (5).

Epifluorescence microscopy of samples filtered onto Nuclepore polycarbonate membranes and stained with acridine orange permits a reliable picture of number and spectrum of natural bacterial populations (16). This paper deals with the coupling of autoradiography and epifluorescence microscopy to determine which organisms are metabolizing in an ecological situation.

† Publication no. 209 of the Joint Research Program at Kiel University (Sonderforschungsbereich 95 der Deutschen Forschungsgemeinschaft).

MATERIALS AND METHODS

Sample preparation. Water samples were taken in sterile glass flasks immediately above sandy sediments of beaches of the Kiel Fjord and the Kiel Bight (Table 1) during a joint research program (in situ water temperatures, 18 to 20°C; incubation at in situ temperature). Standard curves were run with water samples taken from the inner part of the Kiel Fjord (in situ water temperature, 5 to 8°C; incubation temperature, 10°C).

Duplicate 7-ml water samples were incubated in 50-ml bottles (Sovirel) with the addition of 35 μl of D-[2-^{3}H(N)]glucose (5 μCi/ml; specific activity, 10 to 20 Ci/mmol; New England Nuclear) and aerated by shaking (100 rpm). After 4 h of incubation at a temperature within 1 to 2°C of the in situ water temperature, the samples were fixed with 35 μl of concentrated Formalin (35%). In pilot experiments this concentration was found to be sufficient to stop uptake. Portions of the fixed samples were filtered by applying low vacuum (0.2 kPa/cm^2) through 0.2-μm Nuclepore polycarbonate membranes (shiny side up; filters prestained in 2 g of Irgalan Black per liter in 2% acetic acid; Watson, personal communication) and rinsed 10 times with a total of 100 ml of filter-sterilized 1% sodium chloride solution. The filters were then dried and cut into quarters. Chromic acid-washed glass slides were dipped into filtered, prewarmed (45°C) subbing solution (2.5 g of gelatin, 0.025 g of chrome alum, 50 ml of double-distilled water). After draining off the excess gelatin, the gelatin was wiped off the back side, and the filter quarters were fixed on the coated slides. Prior to drying, the slides were placed horizontally on a cold metal tray to solidify the gelatin.

Autoradiographic preparations. Kodak NTB-2 nuclear track emulsion (diluted to a concentration of 1:3 with filtered double-distilled water; stored at 4°C) was heated to 43°C in a constant-temperature water bath for 45 min. The emulsion was gently poured into a dipping jar (beaker, 20-ml content) and checked for a uniform emulsion free of bubbles (14). Slides were immersed in the emulsion in a reproducible manner. The excess emulsion was allowed to drain off, and the back of the slides was carefully wiped clean. The slides were then placed on a cold metal tray for 20 min to gel the emulsion, after which the slides were transferred to a dry tray for a further 1 h. The slides were then mounted on a rack, dried overnight at room temperature in the presence of dried silica gel, placed in black plastic boxes (containing silica gel), sealed, and exposed at 7°C for 3 days. The time at which the boxes were sealed was regarded as zero time. Each dipping procedure included a slide with a blank filter through which double-distilled water instead of the sample was filtered as a control for the quality of the emulsion. All manipulations involving NTB-2 emulsion were performed in total darkness.

Processing the autoradiographs. The slides were developed for 1 min in Kodak D-19 developer diluted to a final concentration of 1:3 with double-distilled water, fixed for 4 min in 30% sodium thiosulfate, washed in tap water, and air dried. All solutions were held at 23°C (5).

Staining. Dried slides were soaked for 5 min in citrate buffer (pH 6.6) and stained through the developed and fixed emulsion for 20 min with acridine orange (Merck no. 1333; concentration, 1:2,500 in citrate buffer, pH 6.6). The slides were destained by immersing in citrate buffer of gradually decreasing pH values (6.6, 5, 4) and rinsed with double-distilled water. The destaining procedure must be controlled visually with regard to a uniform destaining.

Microscopic analysis. The dried slides were viewed with a drop of Cargille immersion oil (type A) by epifluorescence microscopy (Zeiss Universal microscope; BG 12, ×2; FL 500; BF 50; epifluorescence condensor III RS; Osram HBO 200). Microphotographs were taken with a Zeiss CS-matic camera, using Agfapan Professional 100 film (ASA 100; exposure

TABLE 1. *Microbiological variables measured in water samples taken above sandy sediments at beaches of the Kiel Fjord and the Kiel Bight (4 to 13 July 1977)*[a]

	Station	Colony-forming units/ml × 10^3 (plate counts)	Total no. of bacteria/ml × 10^5 (direct counts)	Total biomass (mg/ml, × 10^{-5})	No. of active bacteria/ml × 10^5 (autoradiography)	Active bacteria (% of total no. of bacteria)	Actual uptake rate of glucose (μg/ml per h, × 10^{-3})
A	Hindenburgufer	261	41.7	50.2	19.8	47.5	18.9
A′	Mönkeberg	188	57.8	65.4	32.5	56.2	23.5
B	Falckenstein	81.4	26.5	32.1	11.0	41.5	11.9
B′	Möltenort	187	64.2	62.4	29.9	46.6	10.3
C	Strande	433	68.8	74.2	3.1	4.5	2.6
C′	Laboe	98.2	67.2	70.9	23.8	35.4	6.6
D	D.-Nienhof	3.4	52.2	58.0	1.2	2.3	1.2
D′	Stein	3.1	51.4	40.9	6.1	11.9	7.8
E	Surendorf	5.5	39.4	43.6	16.6	42.1	6.9
E′	Heidkate	2.9	65.8	60.1	19.2	29.2	4.8
F	Krusendorf	7.4	56.1	60.8	28.3	50.5	3.8
F′	Schönberg	1.7	50.0	44.7	3.9	7.8	5.2

[a] A to F mark stations at the west side; A′ to F′ are corresponding stations at the east side of the Kiel Fjord and the Kiel Bight, respectively. Stations A/A′ are located at the inner part, B/B′ at the center part, and C/C′ at the outer part of the Kiel Fjord. Stations D/D′, E/E′, and F/F′ are located at the Kiel Bight.

time, 45 s). The microscopic image displays a greenish-to-grayish background with orange or green fluorescent cells and dark-silver grains, both in similar focal planes. Only bodies with distinct fluorescence, clear outline, and recognizable bacterial shape were regarded as bacterial cells. The question of lysis of cells during filtration cannot be answered as yet. Fluorescent cell debris (obviously cell envelopes) were recognized very seldom. However, in some samples with a relatively high number of active cells, silver-grain aggregations of bacterial shape and size were observed, which might be a visible expression of cell lysis. Actually metabolizing cells are characterized by silver grains above or attached to the cells. Even if the cells are heavily coated with silver grains, the bright-orange fluorescence remains visible. No attempt was made to count single silver grains. At least 400 cells were counted, chosen at approximately even intervals between the periphery and the center of the filter. The analysis comprises the total number of cells and total biomass (8) and the number of active cells and background silver grains on duplicate filters and on the controls. For the final analysis, the number of "active" cells in the controls (normally less than 1% of the total number of cells) was subtracted from the number of active cells in the samples. A standard error of 11.8% was calculated for counting eight parallel filters. Generally, the total number of cells counted on the autoradiography/epifluorescence microscopy preparations was in agreement with the total number of cells counted on Nuclepore membranes prepared for the normal enumeration procedure (10). The detection and differentiation of active cells is limited by the problems of fluorescence microscopy in general. At certain seasons the differentiation between bacterial and algal cells may cause problems, which may be significant if transfer studies of isotopically labeled substrate between trophic levels are desired. Other limitations are the differentiation between bacteria and detritus and the subjectivity in the counting procedure.

RESULTS AND DISCUSSION

Filter preparation. Although different glues were tried to fix the filter quarters onto the glass slides, only gelatin gave satisfactory results. The disadvantages with other glues were either an inherent background fluorescence (glues with silicone basis) or an indistinct microscopic image (nail polish), obviously due to differences in the refraction index caused by the layer of nail polish between the focal plane and the objective. By using Ulrich adhesive (2), yellow-orange fluorescent spots could be observed, which might be aggregations of silicate. Different filter systems were tried, but only Nuclepore polycarbonate membranes were acceptable. Cellulose ester membranes with their spongy structure trap bacterial cells in all filter layers. The association between bacteria and silver grains became difficult, because the inherent background fluorescence interfered mainly with the recognition of smaller cells. Thorough destaining of the filter background resulted in destaining of smaller cells. It should be mentioned that difficulties were observed with the distribution of cells on the filter surface. To our knowledge, the manufacturing procedure of Nuclepore filters had been changed several times. This resulted in uneven wettable batches of filters rolling up after filtration. It is therefore recommended to check the filters before use according to even wettability.

Staining procedure. The stain can be introduced before applying the emulsion (prestaining) or after processing the emulsion (poststaining). In prestaining possible loss and/or translocation of radioactivity are two of the major problems that must be considered (14). Cells prestained with acridine orange lost their fluorescence during the subsequent procedures, although the background fluorescence was considerably reduced. The loss of fluorescence is mainly due to the removal of acridine orange from the cells by gelatin, which could be demonstrated by coating prestained filters with gelatin. Staining through the undeveloped and unfixed emulsion after exposure worked well as far as the fluorescence of the cells and the background was concerned, but one should be aware of possible interactions between the undeveloped emulsion and the staining solutions. Because of the limitations mentioned above, poststaining is preferable. To obtain optimal results, different buffer systems, acridine orange concentrations, and destaining techniques were tried. Generally, the application of black Nuclepore membranes (prestained in Irgalan Black; see Materials and Methods) is recommended. After exposure, photographic processing, and staining with acridine orange, untreated filters displayed a cloudy light-green background, which darkened only after prolonged illumination, thus leading to a fading of the fluorescence of the cells. However, filters prestained with Irgalan Black should be thoroughly washed in double-distilled water prior to filtration to avoid unbound residues of stain on the filter surface. Staining of the autoradiographs with acridine orange dissolved in phosphate buffer (pH 6.6, 5, or 4) was not suitable because of the dark-reddish filter background, which interfered with the fluorescence of the cells. Acridine orange dissolved in double-distilled water gave similar unsatisfactory results.

A significant improvement was obtained when autoradiographs were stained with acridine orange dissolved in citrate buffer (pH 6.6). Bright-orange fluorescent cells and dark-silver grains are easily detectable against a greenish back-

ground, which darkens to gray with time. Staining at lower pH values (5, 4, or 3) led to a light-greenish background which interfered with the fluorescence of the cells, decreasing with decreasing pH values. Prior to staining, the slides were immersed for 5 min in citrate buffer (6.6), which seemed to make the staining of the cells less variable. Optimal results were obtained by preparing a 1:2,500 solution of acridine orange in citrate buffer. Lower concentrations (1:5,000; 1:10,000) were sufficient to stain the cells but resulted in a more rapid fading. Higher concentrations of acridine orange (up to 1:1,000) caused a grossly overstained background, which darkened only after prolonged illumination, thus leading to a fading of the fluorescence of the cells. If a sufficient concentration of acridine orange is used, the period of staining (between 10 and 20 min) does not seem to influence the quality of the microscopic image to any degree. Treatment of the specimen with citrate buffer of decreasing pH values (6.6, 5, 4) washes out the excess of stain from the background without influencing the fluorescence of the cells. Subsequent rinsing of the slides with double-distilled water seems to increase the contrast between the fluorescence of the cells and the background. Generally, a combination of overstaining on the slightly alkaline side and gradual destaining on the acid side of neutrality is recommended. Other fluorescent dyes (such as fluorescein isothiocyanate) were not applied to the autoradiographic technique. From former experiments, acridine orange was found to be superior to fluorescein isothiocyanate because of its brighter fluorescence, which is necessary for analyzing autoradiographs.

Standardization. The method requires the standardization of both substrate uptake (influence of incubation time and substrate concentration) and exposure of the autoradiographs. Standard curves were repeated at least once with similar results. Because of the relatively short incubation periods (see below) and the low decomposition rate of [^{3}H]glucose (radiochemical specifications; New England Nuclear), the problem of possible exchange of tritium can be regarded as not significant.

In activity studies short incubation periods require the addition of relatively high substrate concentrations, whereas lower substrate concentrations can be applied in combination with extended incubation periods. However, high substrate concentrations might increase the natural concentration considerably, possibly leading to the induction of transport systems. With extended incubation periods, the "natural" environment enclosed in the incubation bottles will change rapidly. The effect of different substrate concentrations and incubation periods on the percentage of labeled bacteria is demonstrated in Fig. 1. Autoradiographs were exposed for 3 days at 7°C. By applying high substrate concentrations (5 and 10 μCi/ml of sample; corresponding to 24 and 48 μg of C per liter, respectively), cells were maximally labeled within 2 to 4 h, whereas at 1 μCi/ml (corresponding to 4.8 μg of C per liter) 4 to 6 h of incubation was required. However, incubation at high substrate concentration causes an increase in the number of background silver grains.

The effect of different substrate concentrations and exposure periods on the percentage of labeled cells is shown in Fig. 2. The water sample was incubated with 1, 5, and 10 μCi/ml for 4 h. Parallel filters were exposed to the radiation for different periods (0 to 3 days). Although high substrate concentrations enhance early labeling of the cells, the maximum percentage of labeled cells obtained after 2 to 3 days of exposure is similar for the different substrate concentrations added. However, the number of background silver grains increases significantly with high substrate concentration (10 μCi/ml).

Most autoradiographs were exposed at 4°C (4, 6, 13, 14) or even at room temperature (2, 5). Brock and Brock (2) mentioned the use of scintillator fluid to detect relatively weak amounts of radioactivity after short incubation periods. From the work of Durie and Salmon (3) it becomes obvious that use of a scintillator and exposure at low temperature (−85°C) greatly enhance early labeling of the cells. Based on this information, different filter treatments and exposure temperatures were tried (Fig. 3). A water sample was incubated with 5 μCi/ml for 4 h. Parallel filters were treated after coating with

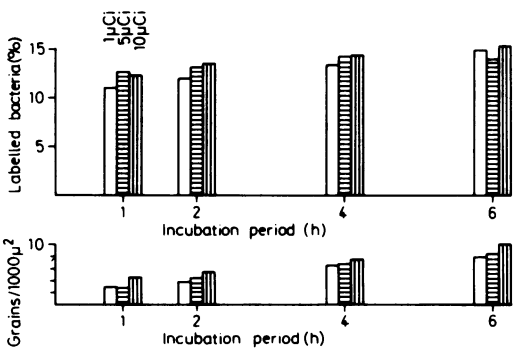

FIG. 1. *Standardization of autoradiographic technique. Effect of different [^{3}H]glucose concentrations and incubation periods on percentage of labeled bacteria (above) and on background level (below). Autoradiographs were exposed for 3 days at 7°C.*

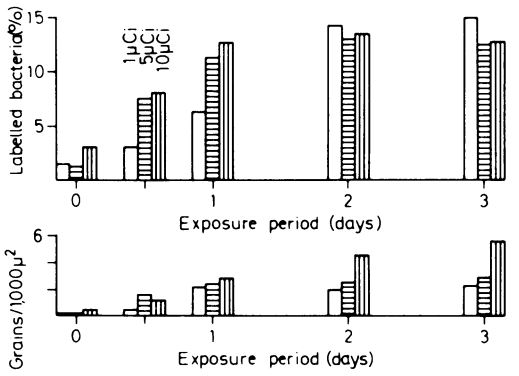

FIG. 2. *Standardization of autoradiographic technique. Effect of different [^{3}H]glucose concentrations and exposure periods on percentage of labeled bacteria (above) and on background level (below). The water sample was incubated with different [^{3}H]-glucose concentrations for 4 h.*

emulsion in the dark with and without scintillator (2) and exposed at 7 and −25°C, respectively. By applying the scintillator, the fluorescence of the background remains unchanged. No stimulatory effect of either temperature or scintillator on the percentage of labeled bacteria could be observed. However, the lower temperature significantly reduced the number of background silver grains. Since treatment of the filters with scintillator introduces another step into the procedure, the exposure of untreated filters is preferable.

From the standardization curves, a 2- to 4-h incubation period with 1 to 5 µCi of [^{3}H]glucose per ml of sample added can be recommended, after which the autoradiographs can be exposed for 3 days at 7°C.

Application to ecological studies. During a joint research program, the method was applied to water samples taken immediately above sandy sediments at beaches of the Kiel Fjord and the Kiel Bight (Table 1). Depending upon location, between 2.3 and 56.2% of the total number of cells were actually metabolizing (cells with associated silver grains) with regard to [^{3}H]-glucose uptake (absolute values: between 1.2×10^5 and 32.5×10^5 cells per ml). The average percentage of active cells (31%) agrees with the 29% [^{14}C]glucose active cells reported by Hoppe (6). Generally, the number of active cells decreased from the inner part to the outer part of the Kiel Fjord (stations A/A′ to D/D′). At those stations located at the Kiel Bight (stations E/E′ and F/F′), the number of active cells generally increased (Table 1). This might be dependent on differences in the bacterial populations and their substrate preference.

For the purpose of this paper, the relationship between the number of actually metabolizing cells and a few "key" variables will be discussed very briefly. A detailed report on the interrelationships between all of the variables measured is scheduled to appear separately (L.-A. Meyer-Reil and M. Bölter, manuscript in preparation). The number of colony-forming units varied by a factor of 200, with highest values in the inner part of the Kiel Fjord. In contrast, total bacterial number and biomass showed only small variations (maximally by a factor of 2). Parallel to the variations in the number of actually metabolizing cells, the actual uptake rate of glucose (determined according to a method described by Meyer-Reil [9]) varied by a factor of 20. Again, high values were found in the inner part and lower values were found towards the outer part of the Kiel Fjord.

Spearman rank correlation analysis (15) revealed significant correlations between the bacterial biomass and bacterial numbers and colony-forming units (Table 2). These interrelationships could be expected from previous investigations (1, 10). For the evaluation of the method described, the significant correlation between the number of actually metabolizing cells and the actual uptake rate of glucose should be pointed out. This means that the total amount of glucose taken up by an unknown number of cells (tracer technique) can be related directly to

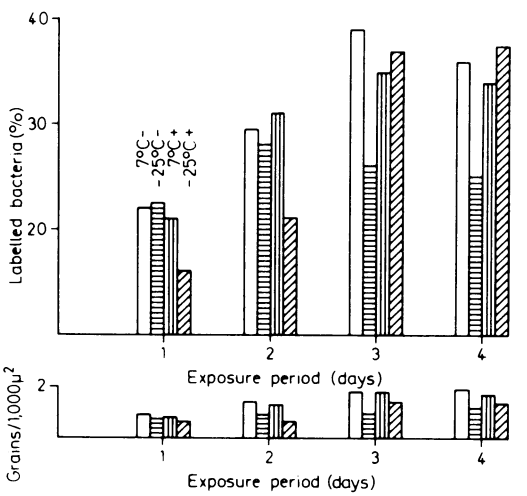

FIG. 3. *Standardization of autoradiographic technique. Effect of different exposure temperatures (7°C, −25°C) and autoradiograph treatments (+, with scintillator fluid; −, without scintillator fluid) on percentage of labeled bacteria (above) and on background level (below). The water sample was incubated with 5 µCi of [^{3}H]glucose per ml for 4 h.*

the number of actually metabolizing individual cells (autoradiography/epifluorescence microscopy technique). The absence of a significant correlation between standing crop variables (colony-forming units, total number of cells and biomass) and activity variables (actual uptake rate of glucose, number of actually metabolizing cells) in this study is not surprising, since bacterial standing crop does not necessarily reflect metabolic activity (10).

Figure 4 presents photomicrographs of the autoradiographs obtained. Unfortunately, the photomicrographs presented in black and white do not reflect the quality and the information content of the colored microscopic images showing bright-orange fluorescent cells and dark-silver grains. From the microscopic observations it becomes obvious that uptake of [^{3}H]glucose is spread over the total spectrum of the natural bacterial population. Rods and curved cells, as well as small cocci (0.2 to 0.4 μm in diameter), take part in the uptake of [^{3}H]glucose. Filamentous cells, which are typical for samples taken at those locations in early summer (8), accounted for a maximum of 20% of the total number of cells. In comparison with nonfilamentous cells, filamentous cells were heavily coated with silver grains (Fig. 4). Therefore, the filamentous cells can be expected to account for much higher percentages of the total actual uptake rates than the nonfilamentous cells. Generally, the number of background silver grains was significantly higher in the samples than in the controls. This means that bacterial activity causes an increase in the number of background silver grains, possibly due to the release of highly labeled substances into the water.

TABLE 2. *Spearman rank correlation matrix for microbiological variables measured in water samples taken above sandy sediments at beaches of the Kiel Fjord and the Kiel Bight*[a]

Parameter	CFU	C	B	AC	AUp
CFU	1.00	0.29	0.55[b]	0.38	0.36
C		1.00	0.90[c]	0.21	−0.39
B			1.00	0.37	−0.25
AC				1.00	0.56[b]
AUp					1.00

[a] CFU, Colony-forming units; C, number of bacteria; B, bacterial biomass; AC, number of active bacteria; AUp, actual uptake rate of glucose.
[b] Significant at the 0.05 level (rank correlation).
[c] Significant at the 0.001 level (rank correlation).

The method described enables the determination of standing crop variables (bacterial number, biomass) and the spectrum of actually metabolizing cells on the same autoradiographic/epifluorescence preparation. Since activity can be related directly to individual cells, the method is a useful tool for other studies in addition to ecological studies.

ACKNOWLEDGMENTS

The initial development of the technique described in this paper was developed in the laboratory of R. Y. Morita, Oregon State University, Corvallis. I greatly appreciate the comments

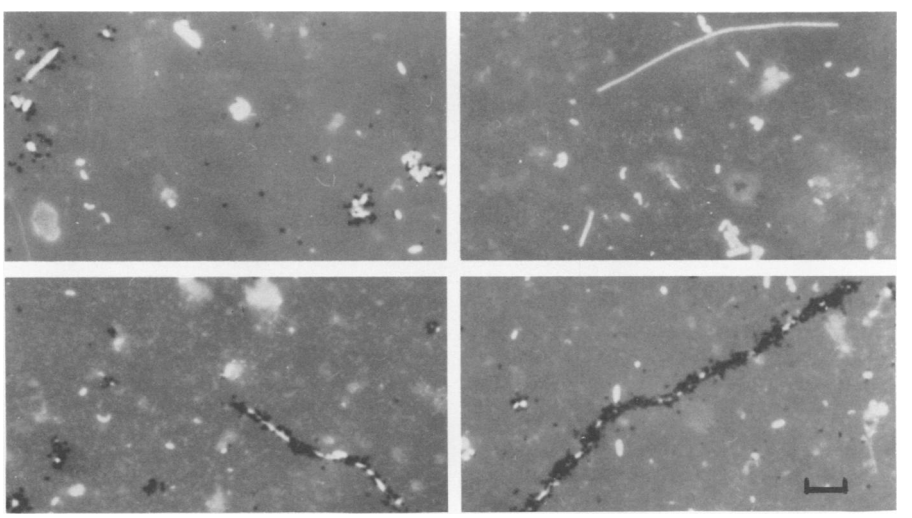

FIG. 4. *Combined technique of autoradiography and epifluorescence microscopy. The photomicrographs show the spectrum of unlabeled and labeled (with associated silver grains) bacterial cells of a natural water sample incubated with 5 μCi of [^{3}H]glucose per ml for 4 h. In comparison with nonfilamentous cells (upper left), filamentous cells (lower left, lower right) are heavily coated with silver grains. The control (fixed with Formalin before labeling) displays only unlabeled cells (upper right). Bar = 2 μm.*

of R. Y. Morita on the manuscript and thank W. Schmidt for valuable technical assistance.

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