APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1982, p. 945–953
0099-2240/82/100945-09$02.00/0

Vol. 44, No. 4

Improved Microautoradiographic Method to Determine Individual Microorganisms Active in Substrate Uptake in Natural Waters

PAUL S. TABOR* AND REX A. NEIHOF

Environmental Sciences Division, Naval Research Laboratory, Washington, D.C. 20375

Received 5 April 1982/Accepted 7 June 1982

We report a method which combines epifluorescence microscopy and microautoradiography to determine both the total number of microorganisms in natural water populations and those individual organisms active in the uptake of specific substrates. After incubation with ^{3}H-labeled substrate, the sample is filtered and, while still on the filter, mounted directly in a film of autoradiographic emulsion on a microscope slide. The microautoradiogram is processed and stained with acridine orange, and, subsequently, the filter is removed before microscopic observation. This novel preparation resulted in increased accuracy in direct counts made from the autoradiogram, improved sensitivity in the recognition of uptake-active (^{3}H-labeled) organisms, and enumeration of a significantly greater number of labeled organisms compared with corresponding samples prepared by a previously reported method.

A major need of ecological microbiology is to develop and improve methods which reflect metabolic activity of microorganisms in natural waters under conditions approaching those in situ.

A composite microbial population may be studied by nonselective methods which measure electron transport system activity, oxygen consumption, or heat production, or by selective measurements such as the uptake of radiolabeled organic substrates or the growth of culturable bacteria. Quantifying the activity of microbial populations as a whole, however, yields no information on the structure of the population in terms of the number of microorganisms and the specific fraction of the total number engaged in the measured function. Specific activity indices, introduced by Wright (14), express metabolic activity parameters per organism by dividing the activity term (V_{max}, turnover rate, or uptake rate) measured for composite microbial populations by total microscopic direct counts (DC) for samples. These indices are taken as indicators of the physiological state of bacteria in various aquatic environments. This approach represents a first approximation for the interpretation of the relationship between measured microbial activities and a structural component of the population, i.e., the number of microorganisms.

Methods are available to relate active metabolic functions to individual microorganisms specifically. These autecological approaches provide for the direct enumeration of those individual microorganisms with a specific function. Among these methods is one in which individual heterotrophic microorganisms with active substrate uptake systems are recognized directly, using radiolabeled substrate and microautoradiography (1, 7, 8, 10, 11). The distribution of microorganisms in natural aquatic systems active in the uptake of a variety of labeled substrates has been reported. Hoppe (9) has reviewed 15 microautoradiographic studies through 1977. Fry and Ramsay (4), using microautoradiography, have observed increased glucose uptake by bacteria in response to the release of dissolved organic substances by aquatic macrophytes. A correlation has been observed between the number of [^{3}H]glucose uptake-active microorganisms determined by autoradiography and the glucose uptake rate for whole aquatic populations. However, there is no correlation between these activities and numbers of colony-forming microorganisms, DC or microbial cell biomass (10). Stanley and Staley (13) have enumerated morphologically distinct organisms from the microbial population of an aeration lagoon and have calculated the quantity of substrate uptake (endogenous acetate plus [^{3}H]acetate) necessary to produce an exposed silver grain, i.e., an autoradiogram. They were able to determine kinetic parameters of acetate uptake by using autoradiography for several bacterial types in situ. The methods described

by Fliermans and Schmidt (3) and Meyer-Reil (10) combine recognition of microorganisms by immunofluorescence or fluorochrome staining, respectively, with microautoradiography to determine individual organisms with active substrate transport function and, thus, to distinguish them from organisms not active in substrate transport. A preliminary version of our microautoradiographic technique (P. S. Tabor and R. A. Neihof, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, N80, p. 186) has been modified and used by Fuhrman and Azam (5) for the determination of the portion of microorganisms in populations of marine surface waters active in thymidine uptake as a part of the development of a method to measure growth based on thymidine incorporation into DNA.

We are interested in the relationships of structure and function of microbial populations in dynamic aquatic environments such as the Chesapeake Bay, where microorganisms undergo continuous variation in their physiological state. To approach this problem, accurate enumeration of the total microbial population coupled with sensitive detection of active individual members of the population is necessary. Although the total DC and the count of the individual microorganisms engaged in active substrate transport are made from the same microautoradiographic preparation in the method developed by Meyer-Reil (10), in our hands, DC for Chesapeake Bay water samples determined from autoradiograms prepared by this method were routinely only 70% of acridine orange-stained DC (AODC) made by using the method of Hobbie et al. (6). In addition, the structure of the Nuclepore filter interfered with the recognition of microautoradiograms of a few silver grains. In the study reported here, technical problems have been solved, and increased sensitivity and improved visual clarity of the sample preparations were achieved with a new microautoradiographic method.

MATERIALS AND METHODS

Sampling. Water samples were collected at a single station in the Chesapeake Bay 4 km from Chesapeake Beach, Md. (38° 41.4′ N, 76° 30.0′ W; depth, 10 m). Water samples were drawn from 8.5 m by a hand vacuum pump through 6.4-mm-diameter sterile, particulate-free Teflon tubing. The system was flushed well before a 1-liter sample was collected in a particulate-free, sterile Teflon bottle. Water temperatures were recorded. Sample bottles were stored in an insulated container for transportation to the laboratory. Salinities were determined with a refractometer.

Processing samples for microautoradiographic determination of uptake-active microheterotrophs. Within 1 h of collection, 10-ml volumes of the sample, in duplicate, were pipetted into 30-ml amber glass bottles and amended with one of the following: [^{3}H]acetic acid (specific activity, 800 mCi mmol^{-1}) (ICN, Irvine, Calif.) to give a final activity of 0.2 μCi ml^{-1} and a final added concentration of 250 nM; a mixture of ^{3}H-amino acids (specific activity, 35.87 mCi mg^{-1}) (Amersham Corp., Arlington Heights, Ill.) to give final activities of 0.05 and 0.1 μCi ml^{-1} and final added concentrations of 1.4 and 2.8 μg liter^{-1}, respectively; or [*methyl*^{3}H]thymidine (specific activity 75 Ci mmol^{-1}) (Amersham) to give a final activity of 0.4 μCi ml^{-1} and a final added concentration of 5.3 nM. Samples were incubated for 2.5 h at in situ temperature on a shaker and subsequently fixed with Formalin (37% formaldehyde solution filtered through a 0.2-μm Nuclepore filter; final concentration, 2% Formalin). Controls to determine microorganisms or other particulates that form microautoradiograms by abiotic adsorption were prepared by fixing 10-ml sample volumes within 15 min of collection. Fixed control samples were held for 1 h at in situ temperature before labeled substrates, corresponding to the substrates used for the samples, were added as described above. Samples and controls were stored overnight at 3°C before autoradiographic preparation.

In duplicate, 1 ml of each sample was diluted in 9 ml of sterile, particulate-free artifical seawater (Sea-rite) (pH 7.4) at the salinity of the water sample and filtered onto a 0.2-μm-pore-size, 25-mm-diameter Nuclepore filter previously dyed with Iragalan Black according to the method of Hobbie et al. (6). The sample was rinsed twice by drawing two 10-ml volumes of the artificial seawater diluent through the filter.

The filtered microorganisms were either (i) mounted directly on a gelatin subbing film coating a microscope slide, after which the Nuclepore filter was removed and the slide was dipped into autoradiographic emulsion (microautoradiograms referred to as MARG-G, see Fig. 1b) or (ii) mounted directly on an autoradiographic emulsion film coating a microscope slide, after which the filter was removed (microautoradiograms referred to as MARG-E, see Fig. 2). The method of Meyer-Reil (10), in which the Nuclepore filter is mounted on a gelatin subbing film (with the filtered sample facing away from the film) and then dipped in autoradiographic emulsion (microautoradiograms referred to as MARG, see Fig. 1a), was also used, and a comparison of the three methods was made.

MARG-G preparation. An acid-cleaned slide was dipped into a membrane filtered (0.22-μm pore size; Millipore Corp.) 5% gelatin (Fisher Scientific Co., Fair Lawn, N.J.)–0.05% KCr $(SO_4)_2 \cdot 12H_2O$ subbing solution prepared according to the method of Meyer-Reil (10), and then the back of the slide was wiped clean and the slide was drained vertically for 20 s to produce a thin gelatin film. The filter retaining the sample was cut in half, and one filter half was applied to the slide with the filtered sample in direct contact with the gelatin (Fig. 1b). The slide was immediately placed on a cold surface to solidify and, subsequently, was dried thoroughly in a desiccator over silica gel. The slide was immersed in a 1% aqueous glycerin solution for 1 min; the gelatin film was allowed to dry completely; and the Nuclepore filter was gently peeled off, leaving the sample mounted in the film. Alternatively, the filter was removed from the sample after the preparation was rehumidified in an atmosphere of 70% relative humidity (in a plastic glove bag). To check the completeness of retention of microorganisms by the gelatin film, the removed filter halves were stained with

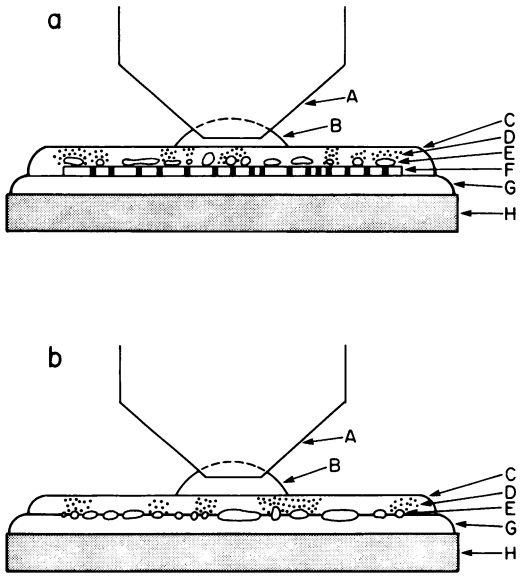
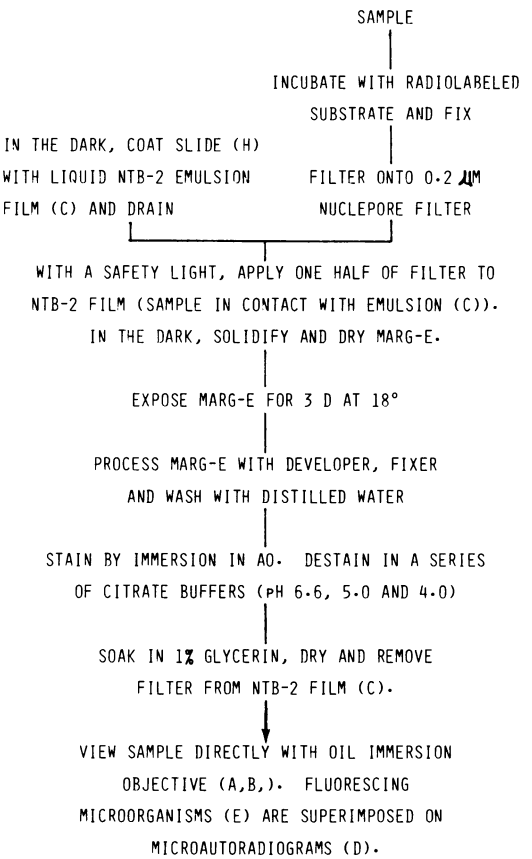


FIG. 1. Schematic representation of the processed and stained microautoradiograms prepared (a) by the method of Meyer-Reil (10) (referred to as MARG) and (b) by the microautoradiographic preparation in which filtered organisms are mounted on a gelatin film, the Nuclepore filter is removed, and the mounted sample is coated with a film of autoradiographic emulsion, processed, and stained with AO (referred to as MARG-G). A, oil immersion objective; B, immersion oil; C, developed autoradiographic emulsion; D, silver grains; E, microorganisms; F, Nuclepore filter; G, gelatin film; H, microscope slide.



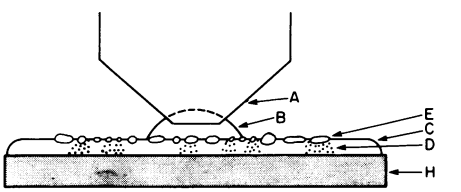


FIG. 2. Protocol for preparation of aquatic samples and schematic representation of the microautoradiogram in which filtered organisms are mounted directly on the autoradiographic emulsion and the Nuclepore filter is removed before viewing. In these preparations (referred to as MARGE-E), uptake-active organisms stained with AO are superimposed on the clusters of silver grains.

acridine orange (AO) (Eastman-Kodak, Rochester, N.Y.) by the method of Hobbie et al. (6) and examined by epifluorescence microscopy for any remaining microorganisms. The gelatin film was again thoroughly dried. To prepare autoradiograms, the procedure of Meyer-Reil (10) was used to coat the samples mounted in the gelatin film with autoradiographic emulsion (Fig. 1b). After the autoradiographic emulsion was solidified on a cold surface, the MARG-G were placed in test tube racks and exposed in the dark for 3 days at 18°C under vacuum over silica gel.

MARG-E preparation. A summary of the protocol used for MARG-E preparation is presented in Fig. 2. The sample was filtered, and one-half of the filter was temporarily attached by the corner of the filter to an acid-cleaned slide by a droplet (approximately 1 μl) of glycerin near the top of the slide, with the sample facing away from the slide. In a darkroom with the aid of a safety light operated by a foot switch, the filter was removed from the slide and held with a forceps. In total darkness, the slide was dipped in diluted NTB-2 autoradiographic emulsion (Eastman-Kodak) (1 part emulsion to 2 parts distilled water) held at 43°C and drained for 20 s. With the momentary aid of the safety light, the filter was applied to the slide with the filtered sample toward the emulsion film (Fig. 2). The back of the slide was wiped clean of emulsion, and the slide was set on a cold surface which was kept in an isolated, light-tight drawer. MARG-E were exposed under vacuum as described above for MARG-G. MARG-E were also prepared for additional filtered samples by using Ilford L-4 autoradiographic emulsion (Polysciences, Warrington, Pa.) (1 part L-4 diluted with 1 part distilled water) held at 43°C.

Developing and staining of MARG-G and MARG-E. Microautoradiograms prepared with NTB-2 were

processed according to the procedure of Meyer-Reil (10). Microautoradiograms prepared with Ilford L-4 were developed for 4 min in 1 part Kodak D-19 diluted with 1 part distilled water, rinsed in distilled water for 30 s, fixed in full-strength Kodak fixer for 4 min, washed in distilled water for 3 min, and dried.

Thoroughly dried MARG-G and MARG-E were stained by using, essentially, the procedure of Meyer-Reil (10). After a 3-min presoak in citrate buffer (pH 6.6), the samples were stained with AO (40 mg/100 ml of citrate buffer [pH 6.6]) for 7 min, destained for 6 min each in buffers at pH 6.6 and 5.0 and for 10 min in buffer at pH 4.0, and rinsed for 1 min in distilled water. Destaining was satisfactory when no visible traces of AO remained in the developed emulsion or gelatin. After destaining of MARG-E, each slide was dipped in 1% glycerin for 1 min to facilitate the removal of the filter. After complete drying, the outline of the filter was traced on the back of the slide, and the filter was gently peeled away from the emulsion film. Alternatively, as described above for MARG-G, the filters were removed after the MARG-E were rehumidified. A schematic diagram of the final preparation is shown in Fig. 2. The filter from each of the MARG-E also was examined by epifluorescence microscopy to determine whether any cells had been removed from the emulsion.

Determination of AODC. The AODC was determined for each radiolabeled sample and corresponding control, in duplicate, by the method of Hobbie et al. (6).

Microscopy. Microautoradiograms prepared by all three methods, viz., MARG, MARG-G, and MARG-E, were examined with a Zeiss Standard 14 microscope (Zeiss Planachromat 100/1.35 objective, KPL 1.25/20 eyepieces) by epifluorescence and transmitted light. Incident illumination for fluorescence microscopy was provided by an XBO 150-W xenon burner with a red suppression filter (BG 38) and a Calflex heat filter. Zeiss filter combination 487709 BI was used for samples stained with AO. Isolated, individual silver grains or clusters of silver grains, not necessarily associated with fluorescing cells, were enumerated for microautoradiograms of controls and samples and are referred to as microautoradiographic counts (MC). An isolated silver grain was equated with a cluster of grains as 1 MC. DC and MC were made with epifluorescence illumination using transmitted light alternately or simultaneously by partially blocking the transmitted light source. Cells were counted within the field of a graticule (14 by 14 divisions) which corresponded to an area of 70 by 70 μm on the sample. More than 400 cells were counted for each sample.

DC and MC were made for two filtered samples from each 10-ml sample. The coefficient of variance (standard deviation × 100 divided by the mean) of the counts within a sample was <10% for all samples. By using the *t*-test, no significant difference at the 5% significance level was found for counts of duplicate 10-ml samples treated in the same manner. The *t*-test was used to determine whether differences between sample treatments were significant at the 1% significance level.

Microautoradiograms were photographed with an Olympus (model PM-10AD) system and a large-format adapter (3.25 by 4.25 in.) [ca. 8.26 by 10.80 cm]) attached to the Zeiss Standard 14 microscope. Polaroid type 667 film (ASA 3,000) was used, and, for photography under epifluorescence illumination, exposure times were 4.5 to 5.5 s.

Supplemental studies of fluorochromes for staining organisms in MARG-E. MARG-E prepared for a 30 October 1980 Chesapeake Bay sample were stained with the DNA-binding fluorochromes mithramycin (Sigma Chemical Co., St. Louis, Mo.), 4′,6-diamidino-2-phenylindole (DAPI) (Polysciences), and Hoechst 33258 (American Hoechst Co., Sommerville, N.J.) in addition to AO. Sets of replicate filtered samples were prepared. After the filter was removed, the samples were stained by applying 30-μl volumes of fluorochromes directly to filtered organisms mounted in the developed autoradiographic emulsion. A cover glass (22 by 22 mm) was applied to spread the staining solution. Samples were stained with mithramycin at concentrations of 5.0, 50, and 200 μg ml^{-1} and incubated for 20 min at 25 or 35°C in moist air to avoid drying of the staining solution. The concentrations of DAPI were 1.43 and 8.6 μM, and the samples were stained for 0.25 or 1 h at 25°C. The concentrations of Hoechst 33258 used were 10, 20, and 100 μM, and the samples were stained for 0.5 or 1 h at 35°C.

For epifluorescence microscopy, the Zeiss filter combination 487709 BI was used with the samples stained with mithramycin. The Calflex filter was removed, and Zeiss filter combination 487702 was used with DAPI- (2) and Hoechst 33258-stained samples. Also, a Zeiss Neofluar 100/1.30 objective was used to view DAPI- and Hoechst 33258-stained samples (12).

The DC for each MARG-E was determined, and the nonspecific staining and the amount of background fluorescence were noted for each fluorochrome at each concentration used. The results were compared with those obtained for the corresponding MARG-E made from the second filter half stained with AO. The *t*-test was used to indicate significant differences (1% significance level, $n = 4$, for each comparison) in the DC obtained by the different staining techniques.

RESULTS AND DISCUSSION

DC determined for samples prepared by three microautoradiographic methods. DC and counts of substrate uptake-active organisms were determined from the microautoradiographic preparations described above. Therefore, DC obtained from the microautoradiograms and AODC of the samples were compared to determine the accuracy of DC. AODC determined for September 1980 radiolabeled samples (incubated for 2.5 h) and AODC of corresponding controls (fixed immediately) were not significantly different and had an average of 14.9×10^6 cells ml^{-1} ($n = 12$). DC for samples prepared for microautoradiography by the method of Meyer-Reil (10) (MARG) were 57 to 81% of corresponding AODC. The Nuclepore filter included in the MARG created a background fluorescence higher than that of sample preparations which did not include the filter (MARG-G and MARG-E) (Fig. 1 and 2). This nonspecific fluorescence reduced the contrast of fluorescing organisms and resulted in

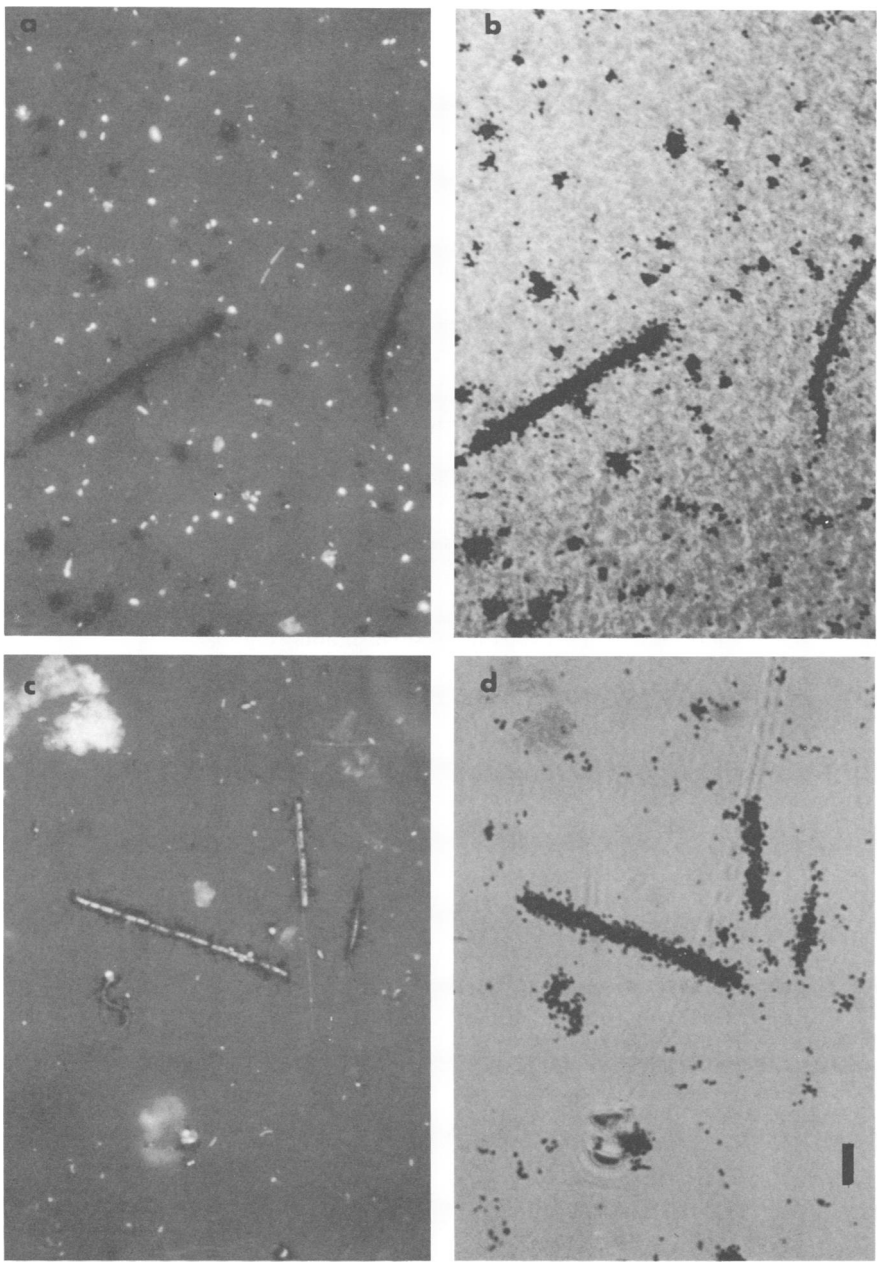


FIG. 3. Photomicrographs of microautoradiograms prepared for September 1980 Chesapeake Bay water samples amended with ^{3}H-amino acids (0.05 μCi ml^{-1}) and incubated for 2.5 h. (a) MARG (prepared by the method of Meyer-Reil [10]) photographed with epifluorescence illumination. (b) MARG [same microscopic field as in (a)] photographed with transmitted illumination. (c) MARG-E (see Fig. 2) photographed with epifluorescence illumination. (d) MARG-E [same microscopic field as in (c)] photographed with transmitted illumination. Fluorescing organisms are bright; silver grains are black. The filter structure of the Nuclepore filter is apparent in the background of the MARG (a and b), whereas the background of the MARG-E (c and d) is structureless. Opaque silver grains mask fluorescing organisms in MARG, whereas in MARG-E fluorescing organisms are visible because they are superimposed on the silver grains. Bar = 5 μm.

TABLE 1. DC and MC of a Chesapeake Bay water sample[a]: comparison of techniques for preparation of microautoradiograms

Substrate	MARG[b]		MARG-G		MARG-E		AODC
	MC[c]	DC[c]	MC	DC	MC	DC	
Acetate (0.2 μCi ml^{-1})							
Control	0.4	8.6	1.4	14.3	0.1	14.2	14.5
Sample	5.0	8.5	7.0	11.9	6.9	14.0	14.5
Amino acids (0.05 μCi ml^{-1})							
Control	0.9	12.1	1.1	14.2	0.08	14.4	14.5
Sample	7.1	9.3	7.7	12.5	7.5	14.7	14.9
Thymidine (0.4 μCi ml^{-1})							
Control	0.1	12.0	0.2	13.6	0.1	14.7	15.1
Sample	10.7	11.4	11.9	13.9	12.0	16.4	15.9

[a] Chesapeake Bay water sample collected from an 8.5-m depth on 21 September 1980 (water temperature, 24.6°C; salinity, 18.1‰).
[b] Prepared according to the method of Meyer-Reil (10).
[c] Expressed as $\times 10^6$ counts ml^{-1}.

decreased DC. Also, individual pores of the Nuclepore filter fluoresced as discrete, bright points of light and interfered with the recognition of fluorescing organisms of <0.4 μm (Fig. 3).

The filters could not be successfully stripped away from completely dried gelatin film (MARG-G) or autoradiographic emulsion (MARG-E). However, the filters were readily removed after the film was immersed in 1% glycerin for 1 min and then dried or after the film equilibrated in an atmosphere of 70% relative humidity. Since the filters were removed from the MARG-E after the preparations were stained, it was convenient to include the glycerin-dipping step after the destaining procedure. A microscopic examination of filters removed from MARG-G and MARG-E showed that microorganisms had been quantitatively removed from the filters by the stripping technique. A comparison of DC of MARG-E and AODC of the same samples confirmed that the microorganisms were quantitatively retained on the emulsion film through autoradiographic processing and staining.

DC for MARG and MARG-G of the controls were greater than DC for the corresponding MARG and MARG-G prepared for the samples (Table 1). In these autoradiographic preparations, fluorescing microorganisms were situated beneath the film of autoradiographic emulsion (Fig. 1, 3). For MARG-E of radiolabeled samples, a much larger fraction of the MC associated with fluorescing microorganisms was found than was found for MARG or MARG-G. DC for MARG-E of the samples were significantly greater than DC for corresponding MARG or MARG-G (Table 1). Because organisms were superimposed on the autoradiographic emulsion in the preparation of MARG-E (Fig. 2), fluorescence of the cells was not masked by the opaque silver grains (Fig. 3).

MC determined for samples prepared by three microautoradiographic methods. MC determined for corresponding MARG-G and MARG-E of the samples were not significantly different. However, MC for MARG of the samples were significantly less than MC determined for MARG-G and MARG-E of the corresponding samples. Under transmitted light, the Nuclepore filter included in the autoradiograms prepared according to the method of Meyer-Reil (10) created a highly structured background that interfered with recognition of single or small clusters of silver grains (Fig. 3). The similarity in size of the filter pore structure (0.2 μm) and the silver grains of NTB-2 (0.2 μm) and L-4 (0.14 μm) emulsions made it difficult to discern silver grains.

MC determined for MARG and MARG-G of the controls were routinely <20 per microscope field, or <10% of MC of the labeled samples. MC for MARG-E of the controls were less than MC for the other autoradiographic preparations; they were routinely <2 per field, or <1% of MC for MARG-E of the samples (Table 1). MARG-E were less complicated preparations and involved fewer manipulative steps in their preparation. We believe that this was responsible for the reduced numbers of background silver grains formed.

DC determined for the MARG-E of samples incubated with radiolabeled amino acids and especially, thymidine were larger than DC of the corresponding controls (Table 1). Individual silver grains were easily recognized under trans-

mitted light. Then, by focusing on these grains under epifluorescence illumination, additional fluorescing microorganisms of approximately 0.2 μm were recognized. Thus, the increase in the DC was due to a more thorough microscopic examination of samples as a consequence of the greater MC.

Samples reported in this study were incubated with radiolabeled substrates for 2.5 h. For additional samples collected in September and October 1980 and incubated for 4 h, labeled microorganisms produced microautoradiograms that extended to adjacent cells. Because of this overlap, it was not possible to discern whether these adjacent organisms were specifically associated with silver grains. Significantly lower MC were found for MARG-E prepared for October samples that had been incubated for 2.0 h, compared with corresponding samples incubated for 2.5 h ($n = 8$).

MARG-E were prepared with both L-4 and NTB-2 autoradiographic emulsions, and no significant difference in the MC between corresponding MARG-E was found ($n = 4$). Microautoradiograms of labeled organisms prepared with L-4 were not as dense as corresponding microautoradiograms prepared with NTB-2, and small (0.2 μm) fluorescing cells were more easily recognized with L-4 emulsion. However, L-4 was more sensitive to handling, and MARG-E prepared with L-4 emulsion that was melted twice had a noticeably higher background level of silver grains than did MARG-E prepared with NTB-2 melted twice. The results presented in Tables 1 and 2 are those from microautoradiograms prepared with NTB-2.

Staining. The DNA-binding fluorochromes mithramycin, DAPI, and Hoechst 33258 were investigated to determine their ability to specifically stain the filtered sample. A fluorochrome staining only microorganisms would eliminate the need to destain the film of gelatin or autoradiographic emulsion, which was necessary when AO was used.

MARG-E stained with 50 μg of mithramycin ml^{-1} had bright fluorescing microorganisms and dark backgrounds. Samples fixed with ethanol and stained with 50 μg of mithramycin ml^{-1} for 0.5 h fluoresced more intensely than did Formalin-fixed samples. However, the fluorescence of the microorganisms faded under epifluorescence illumination, and within 2 min few cells were detected in the exposed field. The fluorescence of the microorganisms on MARG-E stained with 5 μg of mithramycin ml^{-1} faded still more rapidly. Staining with 200 μg of mithramycin ml^{-1} resulted in a highly fluorescent background that reduced the contrast of fluorescing microorganisms. Microorganisms on MARG-E stained directly with 8.6 μM DAPI fluoresced brightly, and the background fluorescence of the developed photographic emulsion was low (dark blue). Fluorescence of the samples stained with 1.43 μM DAPI was less intense, and the background was completely dark. For us, the large difference in the intensity of background light when viewing MARG-E alternately under epifluorescence illumination and with transmitted light made observation of the samples stained with 1.43 μM DAPI more difficult than that of the samples with a low background fluorescence. Samples stained with 10 μM Hoechst 33258 fluoresced with less intensity than samples stained with DAPI. Higher background fluorescence was observed with 20 μM Hoechst 33258. Particulates with white-yellow fluorescence, not recognized as microbial cells, were stained with 20 and 200 μM Hoechst 33258. The quality of samples stained with DAPI, in terms of intensity of fluorescence of organisms, level of background fluorescence, and nonspecific staining, was much less sensitive to small changes in the concentration of the fluorochrome than was that of samples stained with Hoechst 33258.

DC and MC determined for MARG-E stained with the four fluorochromes and AODC determined for all samples are presented in Table 2. DC of AO-stained samples were significantly greater than DC determined for corresponding samples stained with DAPI (8.6 μM), Hoechst 33258 (10 μM), or mithramycin (50 μg ml^{-1}) (Table 2) ($n = 4$). Thus, AO was the most effective fluorochrome for staining the Chesapeake Bay samples used in this study. Nonmicrobial particulates in the samples which were stained with AO were destained during the procedure to destain the gelatin and autoradiographic emulsion. DAPI gave consistent staining results and may be useful for staining samples with high concentrations of particulates, such as sediments and detritus, because nonmicrobial particulates were not stained. In this study, DC for MARG-E stained with DAPI had an average of 63% of DC of corresponding samples stained with AO (Table 2). MC of DAPI-stained MARG-E and MC of the corresponding AO-stained MARG-E were in agreement.

The DC for the MARG-E of the October 1980 sample amended with amino acids and stained with AO was significantly greater than DC determined for the other MARG-E stained with AO and AODC, which were similar to each other (Table 2) ($n = 4$). The MC for the MARG-E of the sample amended with amino acids was also greater than the MC for any other AO-stained MARG-E. As discussed above, the increase in the DC was recognized as a consequence of an increase in the MC.

The MC of Hoechst 33258- and mithramycin-

TABLE 2. DC and MC of MARG-E prepared for a Chesapeake Bay water sample[a]: comparison of fluorochromes used to stain MARG-E

Substrate	AO		DAPI		Hoechst 33258		Mithramycin		AODC
	MC[b]	DC[b]	MC	DC	MC	DC	MC	DC	
Amino acids (0.05 μCi ml^{-1})									
Control	0.2	9.2	<0.04	8.1	ND	ND	ND	ND	9.4
Sample	5.3	11.6	5.0	8.1	2.7	6.0	3.7	6.8	9.5
Thymidine (0.4 μCi ml^{-1})									
Control	<0.04	9.8	ND[c]	ND	ND	ND	<0.04	5.7	9.2
Sample	1.8	9.4	1.4	5.1	2.2	3.1	2.0	3.8	9.6

[a] Water sample collected from an 8.5-m depth on 30 October 1980 (water temperature, 13.6°C; salinity, 18.4 ‰).
[b] Expressed as $\times 10^6$ counts ml^{-1}.
[c] ND, Not determined.

stained MARG-E for the sample amended with amino acids was significantly less than the MC of AO-stained MARG-E. Difficulties in the microscopic observation of samples stained with the two former fluorochromes, described above, resulted in lower counts. The lower DC determined for the thymidine sample stained with mithramycin, compared with the control, was due to fading of the fluorescence of the organisms during the time required to simultaneously make the DC and the MC (>1 min).

Evaluation of MARG-E. Mounting the filtered sample on the photographic emulsion (MARG-E) has eliminated problems of interference caused by the filter and of silver grains masking fluorescing organisms (Fig. 3). DC for MARG-E were found to be equivalent to AODC of corresponding samples. In fact, DC were greater than AODC for samples with large MC (the September 1980 thymidine-labeled sample and the October 1980 amino acids-labeled sample). The increased DC was not an artifact, as only fluorescing microbial bodies with sharp, continuous cell outlines, as described by Meyer-Reil (10), were enumerated. Silver grains of MARG-E stained with AO occasionally had a low-intensity green fluorescence which faded rapidly under epifluorescence illumination. However, all microorganisms fluoresced red-orange by the AO-staining technique and were well distinguished from silver grains. Silver grains were closely examined for associated fluorescing microorganisms under epifluorescence illumination, and small and dimly fluorescing microorganisms were recognized as a result.

Nanomolar substrate additions, a 2.5-h incubation time, and in situ incubation temperatures were used to minimize any modification of the environmental conditions of the Chesapeake Bay samples processed for microautoradiographic determination of microheterotrophs. Determined from DC and MC for MARG-E of samples corrected for MC of the controls, 49, 50, and 73% of the total microorganisms in the September 1980 Chesapeake Bay sample were involved in active uptake of acetate, amino acids, and thymidine, respectively. Notably, the number of uptake-active organisms was very dependent on the specific activity of the substrate in the sample. For example, the percentage of the total DC of the September sample forming microautoradiograms with the addition of amino acids to a final activity twice that of the sample reported in Table 1 (0.1 μCi ml^{-1} and 2.8 μg ml^{-1}) was 94%.

Due, in part, to technical and standardization problems, microautoradiography has been criticized as having only qualitative usefulness and has not been generally conceived to be a quantitative tool (14). However, with the use of sensitive autoradiographic emulsions and a high-efficiency microautoradiographic method, the minimum quantity of accumulated radioactivity per organism required to produce an autoradiogram can be calculated by the use of standard radioactive sources of a size comparable to that of microbial cells found in natural waters. When an aquatic sample is amended with radiolabeled substrate and incubated for a short period of time, an individual microorganism of the microbial population must have a minimum uptake rate to become sufficiently labeled to produce a microautoradiogram. From enumeration of microorganisms with minimum uptake rates at each of a number of levels of specific activity, the distribution of the microbial population active in the uptake of a specific substrate over the range of minimum rates of uptake can be determined. A >twofold difference in the number of uptake-active organisms (19 to 49% of the DC) as determined from MC of MARG-E was found for Chesapeake Bay samples collected during March 1981 and amended with tracer additions of methionine ranging in activity from 0.05 to

0.30 μCi ml^{-1}. However, a different distribution of the numbers of uptake-active microorganisms was found for both April 1981 and May 1981 samples amended in the same manner (P. S. Tabor and R. A. Neihof, manuscript in preparation). Stanley and Staley (13) have used a similar approach to show a distribution of acetate uptake rates for individual bacteria of the same morphological type and a difference in uptake rates among different types in the microbial population of a pulp mill waste lagoon. Thus, unique information on the metabolic activity of the microbial population is gained. In addition, it is possible to observe relationships between structural parameters of the population (e.g., numbers of microorganisms, morphological types, or biomass) and measured microbial activity. Such information would be useful in determination of the diversity of a microbial population, viz., the range of uptake rates found for individuals in the population, and as an indicator of the physiological state of populations.

The method described here for direct determination of microheterotrophs involved in substrate uptake is sensitive and yields reproducible results. The method is applicable where it is desired to enumerate and describe the total microbial population and those individual microorganisms with active substrate transport systems.

ACKNOWLEDGMENTS

P.S.T. is the recipient of a National Academy of Sciences/National Research Council research associateship at the Naval Research Laboratory, Washington, D.C.

LITERATURE CITED

1. **Brock, M. L., and T. D. Brock.** 1968. The application of micro-autoradiographic techniques to ecological studies. Mitt. Int. Ver. Theor. Angew. Limnol. **15**.
2. **Coleman, A.** 1980. Enhanced detection of bacteria in natural environments by fluorochrome staining of DNA. Limnol. Oceanogr. **25**:948–951.
3. **Fliermans, C. B., and E. L. Schmidt.** 1975. Autoradiography and immunofluorescence combined for autecological study of a single cell activity with *Nitrobacter* as a model system. Appl. Microbiol. **30**:676–684.
4. **Fry, J. C., and J. J. Ramsay.** 1977. Changes in the activity of epiphytic bacteria of *Elodea canadensis* and *Chara vulgaris* following treatment with the herbicide, paraquat. Limnol. Oceanogr. **22**:556–562.
5. **Fuhrman, J. A., and F. Azam.** 1982. Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: evaluation and field results. Mar. Biol. **66**:109–120.
6. **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.
7. **Hoppe, H.-G.** 1976. Determination and properties of actively metabolizing heterotrophic bacteria in the sea, investigated by means of microautoradiography. Mar. Biol. **36**:291–302.
8. **Hoppe, H.-G.** 1977. Analysis of actively metabolizing bacterial populations with the autoradiographic method, p. 179–197. *In* G. Rheinheimer (ed.), Microbial ecology of a brackish environment. Springer-Verlag, New York.
9. **Hoppe, H.-G.** 1978. Relations between active bacteria and heterotrophic potential in the sea. Neth. J. Sea Res. **12**:78–98.
10. **Meyer-Reil, L.-A.** 1978. Autoradiography and epifluorescence microscopy combined for the determination of number and spectrum of actively metabolizing bacteria in natural waters. Appl. Environ. Microbiol. **36**:506–512.
11. **Munro, A. L., and T. D. Brock.** 1968. Distinction between bacterial and algal utilization of soluble substances in the sea. J. Gen. Microbiol. **51**:35–42.
12. **Porter, K. G., and Y. S. Feig.** 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. **25**:943–948.
13. **Stanley, P. M., and J. T. Staley.** 1977. Acetate uptake by aquatic bacterial communities measured by autoradiography and filterable radioactivity. Limnol. Oceanogr. **22**:26–37.
14. **Wright, R. T.** 1978. Measurement and significance of specific activity in the heterotrophic bacteria of natural waters. Appl. Environ. Microbiol. **36**:297–305.