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Modification of the Gelatin-Matrix Method for Enumeration of Respiring Bacterial Cells for Use with Salt-Marsh Water Samples†

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In this report I describe a modification of the gelatin-matrix method for determining the percentages of bacterial cells having active electron transport systems. This modification is one-step fluorochroming followed by use of an antioxidant solution instead of the secondary gelatin layer. The modified technique is more successfully applicable to water samples containing high densities of flocculant particulate matter, owing to the brighter green fluorescence of the cells, which contrasts with the red-orange fluorescence of the floc.

Zimmerman et al. (12) introduced a method which made it possible to microscopically detect those bacterial cells within an assemblage which were respiring (had active electron transport systems). The method involved brief incubations (<1 h) in the presence of INT [2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride]. Respiring bacterial cells reduce INT to INT-formazan, and the dark red formazan crystals are deposited on or within their cell walls. These are then detected by an acridine orange direct-count (AODC) procedure in which the cells are detected by epifluorescence microscopy and the crystals are detected by transmitted light. Tabor and Neihof (11) described a modification of the technique of Zimmerman et al. which permitted much improved detectability of small formazan crystals. The visibility of the crystals was improved by transferring sample concentrates from 0.2-μm pore-size polycarbonate filters to a film of gelatin and then coating the sample with a thin layer of gelatin. Thus, the structural background caused by the filter, which hid small crystals from view, was eliminated. In addition, the loss of formazan crystals to solution in immersion oil was circumvented, because in the gelatin-matrix method of Tabor and Neihof (11), the immersion oil does not contact the sample cells.

When I applied the gelatin-matrix method of Tabor and Neihof (11) to water samples from the salt marsh at Sapelo Island (9), I found that bacterial cells fluoresced less brightly (yellow) than in standard AODC preparations (8) in which they fluoresce bright green. Marshwater samples contain dense concentrations of clay-organic aggregates (floc particles) which fluoresce red-orange. The yellow bacterial cells of gelatin-matrix preparations were not readily visible when on or in the floc (Fig. 1A). Bacterial cells are easily visible as green inclusions in the floc in standard AODC concentrates (8). However, I did find, as Tabor and Neihof (11) had documented, that the gelatin-matrix method sharply improved the detectability of small formazan crystals. In contrast, neither the method of Harvey and Young with 0.08-μm pore-size polycarbonate filters (5) nor that of Bright and Fletcher with 0.2-μm pore-size filters seated on gelatin (1) improved the detectability of the small crystals.

I tried a variety of modifications of the technique of Tabor and Neihof (11), including the use of gelatin of two gel strengths (Bloom numbers 200 and 300), the inclusion of fluorochromes (proflavine, ethidium bromide) which had not been tested by Tabor and Neihof (11), the addition of a fluorescence-quenching agent (irgalan black) in the gelatin, modification of the acridine orange staining and destaining steps, and replacement of the secondary gelatin film with a variety of aqueous solutions (formazan is not soluble in water). I discovered a technique which yielded cells which fluoresced as brightly as those of the standard AODC method against a dark, structureless background. This technique does not include the presoaking (3 min), citrate destaining (22 min), or gelatin misting of the Tabor and Neihof (11) method. The revised method, referred to hereinafter as the GAB/INT method (for gelatin-antioxidant-irgalan black), is as follows.

Water samples are incubated with INT (final concentration, 182 μg ml^{-1}), formaldehyde fixed, and stored at 4°C for a minimum of 12 h, as described by Tabor and Neihof (11). (Note: INT will not dissolve in seawater at the concentration required for the stock solution, 2,000 μg ml^{-1} [12]; deionized or distilled water must be used.) Samples are filtered onto irgalan black-stained polycarbonate filters (pore size, 0.2 μm; Nuclepore Corp.), transferred to cover slips bearing a film of gelatin, and dried as described by Tabor and Neihof (11), with the following exceptions: (i) filters are removed from the filtration apparatus without removing the vacuum (4); (ii) filters are placed on glass slides supported above water in petri dishes immediately after filtration; (iii) gelatin of gel-strength 200 Bloom (Difco Laboratories) is used. The changes in filter handling prevent (i) excess liquid on the filter from causing the sample to be unevenly distributed on the gelatin and (ii) excessive drying of the sample on the filter, which causes bacterial cells to fluoresce orange rather than green (6). Gelatin with gel-strength 300 Bloom caused filters to stick too tightly; it also retained too much fluorochrome and prevented bacterial cells from taking up sufficient fluorochrome.

Staining is done while the filter is attached to the dried gelatin by covering the horizontal filter and cover slip with an acridine orange solution (0.01% in 2% artificial seawater [Rila Marine Mix] filtered by a 0.2-μm pore-size filter and containing 2% formaldehyde) for 10 min. The preparation is then gently rinsed in running distilled water for 1 min and dried in a desiccator under vacuum at about 25°C. The filter is removed from the gelatin by peeling (a dissecting microscope is useful here) with forceps, after being incubated while supported above water in a petri dish for 30 to 45 min. Peeling is facilitated if the water in the dish is initially warm (~40°C) and allowed to cool during incubation. A scalpel can be used to initiate separation between the filter and the slightly pliable, partially rehydrated gelatin. The cover slip is

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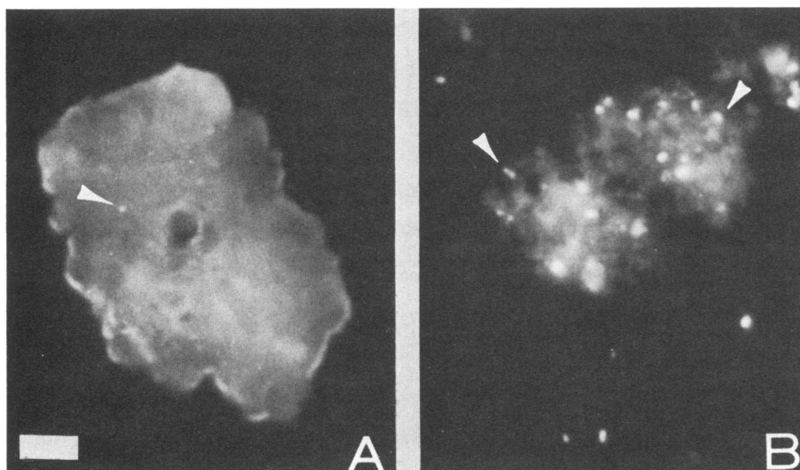

FIG. 1. Representative particles from the Duplin River sample of Table 1. (A) Gelatin-matrix preparation made according to reference 11; bacteria (pointer) faintly visible at best. (B) GAB/INT preparation as described herein; bacteria (pointers) bright green in contrast to other red-orange particulate material. Bar equals 5 μm.

then placed on a glass microscope slide, with the gelatin layer facing the slide as described by Tabor and Neihof (11 [see their Fig. 1]), except that Double Stick Tape (3M Co.) is used. (Note: P. S. Tabor [personal communication] finds that soaking in glycerin solution [11] immediately after destaining permits the peeling of the filters from dried preparations without rehydrating.)

Immediately before microscopic counting of the preparation, a solution of ascorbic acid (200 $\mu g\ ml^{-1}$) and irgalan black (10 $\mu g\ ml^{-1}$) in distilled water is added to the space between the cover slip and the slide. The bacteria are then counted by epifluorescence microscopy at a magnification of ×1,250. Zeiss epifluorescence light filter set 487709 (excitation, 440 to 490 nm; return > 520 nm) can be used with either HBO 50-W mercury or XBO 75-W xenon light sources (Osram). Bacterial cells are bright green against a black background. For observation of formazan crystals, transmitted light is used. The liquid beneath the sample layer eliminates the structural image of the filter on the gelatin, causing the crystals to be more clearly visible. For confirmation of dark spots as formazan crystals, it is helpful to remove the LP520 barrier filter from the light path so that the dark red color of formazan can be detected. It is also often helpful in clarifying the presence or absence of crystals to use magnification in excess of ×1,250. This is readily accomplished on the Zeiss standard microscope by removing the ×0.5 step-down lens at the top of the light path in the epifluorescence attachment (part 3.2 in the Zeiss operating instructions for the IV F1 Epifluorescence Condenser). The removal of this lens also permits the inclusion of the Zeiss Optovar (×1.0 to ×2.0) attachment above the epifluorescence attachment. Although the additional magnification above ×1,250 is "empty" (10), at ×2,000 detectability of formazan crystals is often enhanced. (Note: this extra magnification also enhances the detectability of dividing cells [8] and permits more accurate measuring of cells with the ocular micrometer [2].)

It is important, for accurate use of the GAB/INT method, to practice the method with samples which have been incubated without amendments, with INT alone, and with INT plus formaldehyde. This permits one to clarify for himself, under conditions peculiar to his microscope, the appearance of formazan-negative and formazan-positive cells.

Distilled water was first tried as an agent for elimination of the filter image in the gelatin. This was effective but resulted in a bright green background, and the bacterial cells, though initially bright, faded to near invisibility within 3 min. Therefore, the irgalan black was added to reduce background fluorescence, and the ascorbic acid was added to prevent fading of bacterial cell fluorescence (3).

The GAB/INT method was tested for its applicability to two salt-marsh water samples, one from a small creek and one from a tidal river (Table 1). The examination of peeled filters (11) and parallel AODC counting (8) revealed that all sample material was transferred to the gelatin for samples from both sites. Cell-associated formazan deposits ranged from distinct dark red spheroids (~0.1 μm^3) to very small, dark grains (~0.02 μm^3). The percentage of cells with detectable formazan deposits was near 30% in both samples, both for cells on or in red-fluorescing floc particles and for cells which were free of these larger floc particles. The

TABLE 1. Mean numbers (±95% confidence interval [CI]) and percentages of cells with detectable formazan crystals for two salt-marsh water samples[a]

Site	Cell type[b]	10^6 Cells ml^{-1} (CI)	%+, FC, or PC	%+ (total)
Duplin River	FC+	2.53 (±0.8)	31	33
	FC−	5.62 (±2.0)		
	PC+[c]	1.34 (±0.8)	36	
	PC−	2.37 (±1.3)		
Marsh Creek	FC+	5.48 (±1.1)	30	30
	FC−	12.69 (±2.6)		
	PC+[c]	2.3 (±1.3)	29	
	PC−	5.5 (±2.4)		

[a] Samples taken 10 August 1983 at 1400 h during low, ebbing tide; water temperature, 30°C; salinity, 24 to 26‰; see Pomeroy and Wiegert (9) for site description.

[b] FC, Cells free of red-fluorescing floc particles; PC, cells on or within these floc particles; +, with detectable formazan deposits; −, without detectable formazan. Only floc particles of width ≥5 μm were considered for these determinations.

[c] Some of the PC+ cells are false-positive cells; see text.

smallest cells observed (~0.02 μm^3) did not contain detectable formazan deposits, perhaps because the deposits were too small to be visible. Parallel samples which were fixed (2% formaldehyde) before incubation with INT revealed that no cells (of 221 examined in the two samples) which were free of floc particles bore detectable formazan deposits, but some (20 ± 9%, equivalent in the two samples) of the cells on or in floc particles in the fixed samples lay adjacent to small, dark spots in the particles. These spots may not have been formazan deposits, but they would likely be interpreted as false-positive cells in nonfixed samples. D. J. W. Moriarty (personal communication) has observed similar problems in trials of the method of Zimmerman et al. (12) with surface sediments. For conservative estimation of the percentage of respiring cells on or in particles, the total potential false-positive percentage should be subtracted from the percentage of respiring cells on or in particles for nonfixed samples. In the two present samples, this subtraction yielded percentages of respiring cells for floc-associated cells of 9 and 16% for creek and tidal river samples, respectively, as opposed to the 30 to 31% recorded for free cells (cf. reference 5).

Finally, in performing counts of formazan-positive cells with GAB/INT preparations, I observed that determinations of percent association of bacterial cells with particles can be inaccurate when performed by epifluorescence microscopy. When the same fields were compared by blue epifluorescence versus transmitted light, it could be seen that many small suspended solids were either only very faintly red fluorescent or not fluorescent at all. Thus, values for percentages of particle-associated cells reported here and elsewhere (7) may be low; i.e., they may be values for percent association with one particular type of particle (relatively brightly fluorescent), not for total percent association with all types of particles present.

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