APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1983, p. 1263–1267
0099-2240/83/121263-05$02.00/0

Vol. 46, No. 6

Malachite Green-INT (MINT) Method for Determining Active Bacteria in Sewage

R. J. DUTTON, G. BITTON,* AND B. KOOPMAN

Laboratory of Environmental Microbiology, Department of Environmental Engineering Sciences, University of Florida, Gainesville, Florida 32611

Received 17 June 1983/Accepted 26 September 1983

A membrane filtration method was developed to determine the proportion of active (respiring) bacteria at various stages of sewage treatment. Samples were incubated in the presence of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) and, after fixation, passed through membrane filters. Filters were counterstained with malachite green and then were examined by bright-field microscopy. The contrast between bacteria and the filter background was greatly improved by drying and then clearing the filter before counterstaining. By this method, it was found that active bacterial fractions in raw sewage, settled sewage, and secondary effluent were 40, 29, and 58%, respectively, whereas the proportion of respiring bacteria in chlorinated secondary effluent was 6.1%. The active bacterial fraction of activated sludge was found to be 16%. The proposed method represents a significant improvement in speed and simplicity over existing methods for determining active bacteria in sewage.

It is now generally accepted that standard plate counts are inadequate for determining numbers of viable bacteria in water samples (5, 8, 11, 12). New approaches to measuring the viability of bacterial populations have used direct microscopic counting of stained cells on membrane filters. Investigators have combined fluorochrome staining with nalidixic acid (NA) incubation (14, 15, 18), autoradiography (3, 16), and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (INT) reduction (8, 15, 21, 23). These methods all involve epifluorescence microscopy and subsequent light microscopy to enumerate the active bacterial fraction.

The NA incubation procedure (14) is based on the specific inhibition of DNA synthesis and concomitant enlargement of actively respiring bacterial cells in the presence of yeast extract (YE). It may be insensitive owing to the specific nature of added substrate or presence of NA-resistant bacteria (14, 15, 18). Autoradiography (16) is a complicated procedure and may also be substrate specific. INT reduction is the basis of a direct microscopic technique developed by Zimmermann et al. (23). INT acts as a hydrogen acceptor. The reducing power generated by active bacterial electron transport systems forms, through dehydrogenase-catalyzed reactions, water-insoluble, red, INT-formazan crystals in the cells.

By using INT reduction as a measure of metabolic activity, it was found that active bacterial fractions ranged from 4 to 61% in the marine environment (21, 22) and from 5 to 36% in the freshwater environment (23). Similar measurements on sewage effluents are unavailable, and in fact, relatively few measurements of active bacterial fractions in sewage by any method have been made. Fry and Zia (7) found a value of 21% for raw sewage based on counts of microcolonies formed on agar-coated glass slides. Pike (19) used standard plate counts to determine active bacterial fractions and reported values of 1.1% for settled sewage and 2.1% for secondary effluent.

This study involved the use of a malachite green-INT (MINT) method for counting total and respiring bacterial cells from sewage directly on membrane filters by using bright-field microscopy. A variation of the MINT method in which glass slides were substituted for membrane filters was proposed earlier by Bitton and Koopman (1) as a means of assessing the viability of filamentous bacteria in activated sludge and has subsequently been applied successfully to predict the dosage of chlorine required to control filamentous bulking of activated sludge (14a). As described here, the MINT method combines INT and malachite green to stain cells on an oil-treated membrane filter. The oil interface prevents the filter from taking up an excessive amount of malachite green while still allowing cells to be adequately counterstained for good contrast with INT-formazan crystals (G. Bitton, R. J. Dutton, and J. A. Foran, Stain Technol., in press).

MATERIALS AND METHODS

Sampling. Samples were taken from the sewage treatment plant of the University of Florida, Gainesville, Fla. Samples were collected in sterile glass bottles and processed within 30 min of collection. All diluents and stains used in sample processing were filter-sterilized with 0.2-μm-pore-size membrane filters.

INT reduction assays. Samples were incubated with INT according to one of two protocols. In the first protocol, triplicate 100-ml subsamples were amended with INT by the method of Zimmermann et al. (23). In the second, triplicate 100-ml subsamples were amended with INT, NA, and YE by the method of Kogure et al. (14) and Maki and Remsen (15). After incubation periods of 30 min with INT alone or 6 h with INT, NA, and YE, samples were fixed with 37% Formalin (final concentration, 2.0%). Preserved samples were stored for 2 days or less in the dark at 4°C.

Filter preparation for microscopy. Filters used for counting were 25 mm in diameter. Treated samples were homogenized for 1 min with a high-speed Waring blender and then were diluted as necessary before filtration. At least 4 ml of diluted sample was passed through each filter to give a uniform distribution of cells.

Two general types of membrane filters were used. Cellulosic filters (0.1 μm pore size, type VC, or 0.2 μm pore size, type GS; Millipore Corp., Bedford, Mass.) were prepared for total and INT-active counts by the MINT method as shown in Fig. 1. Nucleation-track polycarbonate filters (0.2 μm pore size; Nuclepore Corp., Pleasanton, Calif.) were prepared for epifluorescence direct counts by the method of Hobbie et al. (9) or for total and INT-active counts by a modified version of the gelatin matrix method of Tabor and Neihof (21). In the latter technique, 0.1% (wt/vol) aqueous malachite green (MC/B, Norwood, Ohio) was substituted for acridine orange. This alteration allowed both total and INT-active bacteria to be counted by bright-field microscopy in a manner analagous to that of the MINT method.

Plate counts. Casitone-glycerol-yeast medium was used by the method of Pike et al. (20). Samples were homogenized for 1 min with a high-speed Waring blender before plating.

Microscopy. Counts of bacteria on filters prepared by the MINT and modified gelatin matrix methods were made under bright-field illumination by using a Leitz SM compound microscope at a magnification of ×1,600. This magnification facilitated detection of INT-formazan crystals in smaller cells. Epifluorescence direct counts were made with a Leitz LM-LUX fluorescence microscope (HBO 200W, VB 390-490 exciter filter, 515 barrier filter, RKP 510 beam splitter). Bacteria were enumerated with a graticulated eyepiece at a magnification of ×1,250. In all counts, at least 250 cells were enumerated in 6 to 10 randomly chosen fields per filter. Counts were performed within 1 h of completing filter preparations.

Statistical analysis of bacterial counts. Statistical analysis of direct count methods suggests that the optimal scheme for sampling uses six or seven fields per filter and one filter per subsample (13). This sampling scheme was generally followed throughout the present study. Comparisons of results from different tests were made, as appropriate, by the *t* test, one-way analysis of variance, or the Duncan multiple range test (release no. 79.6, SAS Institute, Inc., Cary, N.C.).

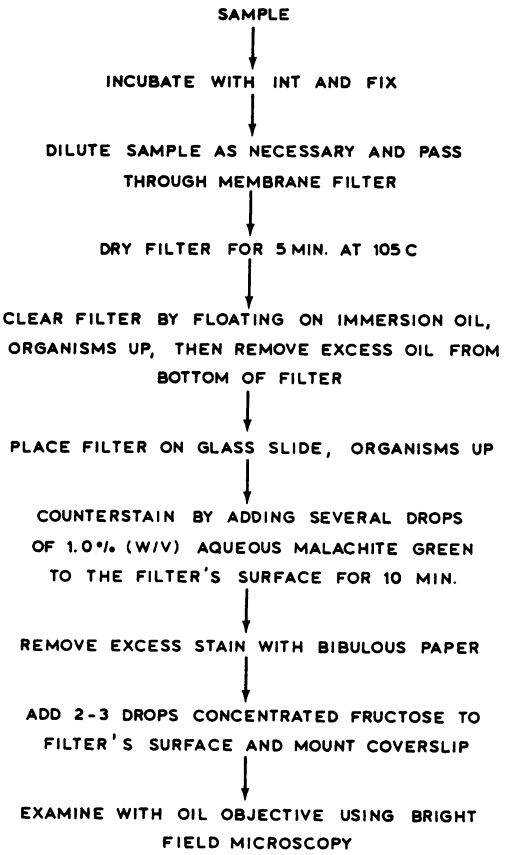

FIG. 1. Flow diagram for the MINT method.

RESULTS AND DISCUSSION

Staining. An important aspect of the MINT method is the ability to observe both bacterial cells and intracellular INT-formazan crystals simultaneously on the same microscopic preparation with only bright-field illumination (Fig. 2). To achieve the optimal color contrast, the use of a wide range of stains was studied. Many stains (crystal violet, methylene blue, carbol fuchsin, etc.) may be useful in determining total bacterial numbers. Only malachite green, however, demonstrated the essential feature of enabling simultaneous microscopic viewing of both active (green with red INT-formazan crystals present) and inactive (appearing green only) bacteria.

INT-formazan dissolution. In vitro enzymatic studies have demonstrated that INT-formazan is solubilized in Triton X-100, Tween 20, and gelatin (17). INT-formazan is also lipid soluble, as evidenced by the gradual disappearance of INT-

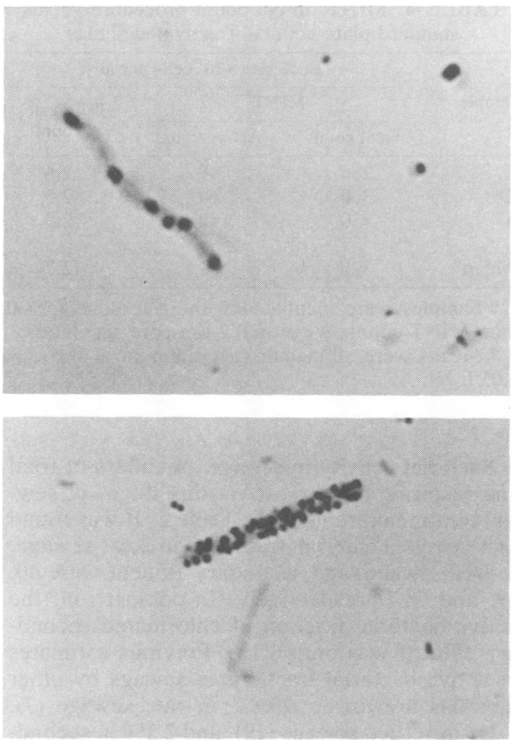

FIG. 2. INT-formazan crystals in sewage bacteria.

formazan crystals from specimens in contact with immersion oil (10, 21, 23). We found that immersion oil (Cargille type A) dissolved 25% of the INT-formazan crystals in *Pseudomonas fluorescens* within 60 min of its application. Cedar, paraffin, castor, vegetable, and mineral oils yielded similar results. Nonlipid fluids approximating the refractive index of immersion oil ($n_D^{23°C}$ = 1.515) include glycerol and saturated sugar solutions. However, microscopic preparations with glycerol or saturated sucrose solution were found to extract the malachite green counterstain within 2 min. Tabor and Neihof (21) have observed previously that glycerol and sucrose can extract acridine orange. A concentrated fructose solution (Natural Sales Co., Pittsburgh, Pa.) did not extract malachite green substantially. Furthermore, by lightly dehydrating the fructose solution (gentle boiling, 10% loss by weight) before use, we have achieved reproducible total and INT-active counts from sealed microscopic preparations in contact with fructose after storage for up to 2 months in the dark at room temperature.

Incubation procedure. In preliminary studies, two incubation techniques were compared. One technique involved a 30-min incubation in the presence of INT alone (23); the other involved a 6-h incubation in the presence of INT, NA, and YE (15). A comparison of the two procedures reveals a significant difference in total counts (higher with INT + NA + YE) but no significant difference between numbers of respiring bacteria (Table 1). These results corroborate those of Maki and Remsen (15), who concluded that combining INT with NA and YE during incubation, in comparison to incubation with INT alone, increased total counts of bacteria but left counts of INT-active bacteria unchanged.

Kogure et al. (14) have shown that NA inhibits cell division, whereas respiration continues in the presence of YE, causing cell enlargement to occur. This phenomenon facilitates the viewing of very small bacteria that are common in nutrient-poor waters. A possible drawback of this method, however, is the growth and reproduction of NA-resistant bacteria (14, 15). Also, results of Peele and Colwell (18) indicate that counts of enlarged cells may depend on incubation conditions, bacterial species composition, and substrate type and concentration. Thus, the validity of cell enlargement as an indicator of viability is questionable. In this study, all INT-active bacteria were enumerated without regard to cell enlargement. Results shown here (Table 1) and elsewhere (15) suggest that combining INT with NA and YE does not artificially elevate bacterial activity.

Membrane filters. A possible problem with the cellulosic filters used as part of the MINT technique is their open, colloidal structure, which can allow smaller cells to become embedded in the filter so deeply that they are obscured from view (2, 6, 9). Nucleation-track filters are made of a dense, polycarbonate film into which precisely defined cylindrical holes have been etched, thus minimizing cell penetration. Unfortunately, this type of structure imposes a granular background in microscopic preparations, and this background can be confused with INT-formazan crystals. The gelatin matrix technique of Tabor and Neihof (21) overcomes this difficulty by transferring cells from the polycarbonate filter onto a clear gelatin matrix. Avoidance of

TABLE 1. Effect of incubation procedure on direct counts of total and active bacteria in settled sewage by the MINT method[a]

Procedure	Mean count ($\times 10^7$ cells per ml)		% Active
	Total[b]	Active	
INT + NA + YE, 6 h	9.0	3.1	34
INT alone, 30 min	6.1	2.0	33

[a] Counts were on 0.1-μm-pore-size filters.
[b] Means were significantly different at the α = 0.05 level.

TABLE 2. Comparison of cellulosic membrane filters and nucleation-track polycarbonate membrane filters for total bacterial counts on settled sewage[a]

Sample	Bacteria ($\times 10^7$ cells per ml) on filters:	
	Cellulosic	Polycarbonate
1	8.6	9.1
2	9.8	9.9
3	8.9	9.2
Mean[b]	9.1	9.4

[a] Cellulosic filters (0.2 μm pore size) were prepared by the MINT method. Samples were incubated for 6 h with INT, NA, and YE. Nucleation-track polycarbonate filters (0.2 μm pore size) were prepared by the modified gelatin matrix method.

[b] Means were not significantly different at the $\alpha = 0.05$ level.

this additional procedure is desirable to simplify and speed counting.

Total bacterial counts obtained with cellulosic filters (via MINT) were compared with total counts obtained with nucleation-track polycarbonate filters (via epifluorescence and modified gelatin matrix). Results (Tables 2 and 3) indicate that with the pore sizes employed, the use of cellulosic filters did not pose a significant disadvantage. Other investigators (4, 12) have arrived at similar conclusions.

MINT versus plate counts. Numbers of total and viable bacteria in activated sludge are given in Table 4. MINT counts were significantly higher than counts derived from spread plates incubated for 6 days, indicating that plating procedures may significantly underestimate the viability of activated sludge. The active bacterial fraction of activated sludge as estimated by the MINT method was 16%, whereas Pike (19) obtained values of 0.83 to 1.7% with plate counts.

TABLE 3. Total bacterial numbers in secondary effluent determined by epifluorescence direct and MINT counts[a]

Sample	Bacteria ($\times 10^7$ cells per ml)	
	MINT	Epifluorescence
1	0.91	0.89
2	0.77	0.74
3	0.86	1.06
Mean[b]	0.85	0.89

[a] Samples were incubated for 6 h with INT, NA, and YE. MINT counts were on 0.1-μm-pore-size filters. Epifluorescence counts were on 0.2-μm-pore-size filters.

[b] Means were not significantly different at the $\alpha = 0.05$ level.

TABLE 4. MINT[a] direct count procedure versus standard plate counts of activated sludge

Sample	Bacteria ($\times 10^7$ cells per ml)		
	MINT		Plate viable count
	Total count	Active count	
1	280	49	2.6
2	360	64	2.5
3	330	40	2.9
Mean[b]	320	51	2.7

[a] Samples were incubated with INT alone for 30 min. MINT counts were on 0.2-μm-pore-size filters.

[b] Means were all significantly different at the $\alpha = 0.05$ level.

Bacterial activity in sewage. Numbers of total and respiring bacteria at various stages of sewage treatment are given in Table 5. It was found that active bacterial fractions in raw sewage, settled sewage, and secondary effluent were 40, 29, and 58%, respectively. In comparison, the active bacterial fraction of chlorinated secondary effluent was only 6.1%. Previous estimates of active bacterial fractions in sewage by other methods are lower: 20.8% in raw sewage (7), 1.1% in settled sewage (19), and 2.1% in secondary effluent (19).

A new method was developed whereby INT-active bacteria can be enumerated by bright-field microscopy with cellulosic membrane filters. This technique compared favorably with methods employing nucleation-track polycarbonate membrane filters in enumerating total sewage bacteria. An essential feature of the MINT method is that filters are cleared with oil before counterstaining. This simple modification results in a better contrast between bacteria and the surface of the filter. The use of fructose circumvents the problem of INT-formazan dissolution in immersion oil. The MINT method is simple, inexpensive, and possibly applicable to a wide range of waters. The only alteration re-

TABLE 5. Direct counts of total and active bacteria by the MINT method at various stages of sewage treatment[a]

Sample	Mean count ($\times 10^7$ cells per ml)		% Active
	Total	Active	
Raw sewage	11	4.4	40
Settled sewage	9.2	2.7	29
Secondary effluent	0.45	0.26	58
Chlorinated secondary effluent	0.95	0.058	6.1

[a] Samples were incubated for 6 h with INT, NA, and YE. Counts were on 0.1-μm-pore-size filters.

quired for marine waters or buffered solutions would be an adequate rinse of the filter surface with deionized filter-sterilized water before counterstaining. This method is currently being tested with samples from lakes which are in different trophic states and in which cell sizes may be substantially smaller than those encountered in sewage.

ACKNOWLEDGMENTS

This study was partially funded by the Engineering and Industrial Experiment Station, University of Florida, Gainesville, Fla., and by grant R 809402-01-0 from the U.S. Environmental Protection Agency. We thank the Millipore Corp. for providing samples of type VC filters.

LITERATURE CITED

1. **Bitton, G., and B. Koopman.** 1982. Tetrazolium reduction-malachite green method for assessing the viability of filamentous bacteria in activated sludge. Appl. Environ. Microbiol. **43**:964–966.
2. **Bowden, W. B.** 1977. Comparison of two direct-count techniques for enumerating aquatic bacteria. Appl. Environ. Microbiol. **33**:1229–1232.
3. **Bright, J. J., and M. Fletcher.** 1983. Amino acid assimilation and electron transport system activity in attached and free-living marine bacteria. Appl. Environ. Microbiol. **45**:818–825.
4. **Brock, T. D.** 1983. Membrane filtration: a user's guide and reference manual. Science Tech., Inc., Madison, Wis.
5. **Buck, J. D.** 1979. The plate count in aquatic microbiology, p. 7–18. *In* J. W. Costerton and R. R. Colwell (ed.), Native aquatic bacteria: enumeration, activity and ecology, STP 695. American Society for Testing and Materials, Philadelphia.
6. **Daley, R. J.** 1979. Direct epifluorescence enumeration of native aquatic bacteria: uses, limitations, and comparative accuracy, p. 29–45. *In* J. W. Costerton and R. R. Colwell (ed.), Native aquatic bacteria: enumeration, activity and ecology, STP 695. American Society for Testing and Materials, Philadelphia.
7. **Fry, J. C., and T. Zia.** 1982. Viability of heterotrophic bacteria in freshwater. J. Gen. Microbiol. **128**:2841–2850.
8. **Herson, D. S., and K. H. Baker.** 1982. New approaches to measuring total bacterial populations in water supplies. J. Am. Water Works Assoc. **74**:537–539.
9. **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.
10. **Horobin, R. W.** 1980. Structure-staining relationships in histochemistry and biological staining. J. Microsc. **119**:345–355.
11. **Jannasch, H. W., and G. E. Jones.** 1959. Bacterial populations in sea water by different methods of enumeration. Limnol. Oceanogr. **4**:128–139.
12. **Jones, J. G.** 1974. Some observations on direct counts of freshwater bacteria obtained with a fluorescence microscope. Limnol. Oceanogr. **19**:540–543.
13. **Kirchman, D., J. Sigda, R. Kapuscinski, and R. Mitchell.** 1982. Statistical analysis of the direct count method for enumerating bacteria. Appl. Environ. Microbiol. **44**:376–382.
14. **Kogure, K., U. Simidu, and N. Taga.** 1979. A tentative direct microscopic method of counting living bacteria. Can. J. Microbiol. **25**:415–420.

14a. **Logue, C., B. Koopman, and G. Bitton.** 1983. INT-reduction assays and control of sludge bulking. J. Environ. Eng. **109**:915–923.

15. **Maki, J. S., and C. C. Remsen.** 1981. Comparison of two direct-count methods for determining metabolizing bacteria in freshwater. Appl. Environ. Microbiol. **41**:1132–1138.
16. **Meyer-Reil, L.-A.** 1978. Autoradiography and epifluorescence microscopy combined for determination of number and spectrum of actively metabolizing bacteria in natural waters. Appl. Environ. Microbiol. **36**:506–572.
17. **Mollering, H., A. W. Wahlefield, and G. Michal.** 1974. Visualization of NAD(P)-dependent reactions, p. 136–143. *In* H. U. Bergmeyer (ed.), Methods of enzymatic analysis, vol. 1. Academic Press, Inc. (London), Ltd., London.
18. **Peele, E. R., and R. R. Colwell.** 1981. Application of a direct microscopic method for enumeration of substrate-responsive marine bacteria. Can. J. Microbiol. **27**:1071–1075.
19. **Pike, E. B.** 1975. Aerobic bacteria, p. 1–63. *In* C. R. Curds and H. A. Hawkes (ed.), Ecological aspects of used water treatment, vol. 1. Academic Press, Inc. (London) Ltd., London.
20. **Pike, E. B., E. G. Carrington, and P. A. Ashburner.** 1972. An evaluation of procedures for enumerating bacteria in activated sludge. J. Appl. Bacteriol. **35**:309–321.
21. **Tabor, P. S., and R. A. Neihof.** 1982. Improved method for determination of respiring individual microorganisms in natural waters. Appl. Environ. Microbiol. **43**:1249–1255.
22. **Van Es, F. B., and L. A. Meyer-Reil.** 1982. Biomass and metabolic activity of heterotrophic marine bacteria, p. 111–170. *In* K. C. Marshall (ed.), Advances in microbial ecology, vol. 6. Plenum Publishing Corp., New York.
23. **Zimmermann, R., R. Iturriaga, and J. Becker-Birck.** 1978. Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. Appl. Environ. Microbiol. **36**:926–935.