APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1981, p. 1132-1138
0099-2240/81/051132-07$02.00/0

Vol. 41, No. 5

Comparison of Two Direct-Count Methods for Determining Metabolizing Bacteria in Freshwater†

JAMES S. MAKI AND CHARLES C. REMSEN*

Center for Great Lakes Studies, University of Wisconsin-Milwaukee, Milwaukee, Wisconsin 53201

Received 17 October 1980/Accepted 2 March 1981

Planktonic bacteria collected from several freshwater environments and cultured bacteria were used to compare two methods for determining the numbers of metabolizing bacteria. The methods used were (i) reduction of 2-(ρ-iodophenyl)-3-(ρ-nitrophenyl)-5-phenyl tetrazolium chloride 2-(ρ-iodophenyl)-3-(ρ-nitrophenyl)-5-phenyl to tetrazolium chloride-formazan and (ii) elongation of cells by using yeast extract and nalidixic acid. No statistically significant difference was found between methods in determining metabolizing bacteria, although significant differences ($P < 0.05$) were found when comparing numbers of total bacteria. A combination of the two methods yielded significant changes, both positive and negative, in the numbers of metabolizing bacteria.

The accurate enumeration of aquatic bacteria has long been a challenge to aquatic microbiologists. For example, bacterial numbers determined by plate count methods have been shown to be much smaller than those determined by a direct-count method (11). It is fairly certain that not all bacteria present in aquatic systems are metabolically active (20, 23); therefore, determination of a normalized percentage of metabolically active cells to total counts would be of use to microbial ecologists interested in determining the influence of microorganisms on a given aquatic environment.

Presently, one of the most widely used direct-count methods employs epifluorescence microscopy with acridine orange as a stain. Since its adaptation (1, 5), this method has undergone changes in methodology and in the interpretation of results (4, 8, 12, 14, 18, 29). Hobbie et al. (8) reported that dead and actively metabolizing bacteria would fluoresce red after staining with acridine orange because of a predominance of single-stranded nucleic acids (single-stranded deoxyribonucleic acid and ribonucleic acid, respectively), whereas inactive bacteria would fluoresce green because of a predominance of double-stranded deoxyribonucleic acid. Jones (12) considered the reported appearance of different colors as much a reflection of concentration and of contact time with the fluorochrome as of the viability of the organism at the moment of staining. Despite the controversy, however, bacteria counts obtained by this technique have compared well with those obtained by other methods (2, 18, 24). More recent techniques proposed to help determine bacterial activity in relation to total numbers have included using concurrent uptake and respiration studies with labeled compounds (22, 25) and directly using autoradiography to determine active cell numbers (19).

Two recently developed methods allow direct determination of actively metabolizing bacteria from filtered samples. The first involves the reduction of 2-(ρ-iodophenyl)-3-(ρ-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to INT-formazan crystals by active bacterial electron transport systems (28). Electron transport system activity is a characteristic of living cells and has been measured with tetrazolium salts (10, 21). INT acts as an H^+ acceptor and builds optically dense, stable formazan crystals (10, 28) which can be observed with light microscopy (28). The second uses a preincubation of the sample with a combination of yeast extract and nalidixic acid (YE + NA) (16). This technique is based on the specific inhibition of deoxyribonucleic acid synthesis by NA (6, 7). Active cells unable to divide because of lack of deoxyribonucleic acid synthesis will continue to grow and become elongated. This paper describes the comparison and combination of these two methods in which filtered bacterial samples collected from several freshwater environments were used.

MATERIALS AND METHODS

Water samples collected for analysis from several water bodies having a wide range of trophic status and depth were stored and later analyzed in the laboratory (Table 1). Each sample was split into subsamples and processed as follows.

In test 1, a 10-ml subsample from each depth was

† Contribution 215, Center for Great Lakes Studies, The University of Wisconsin-Milwaukee, Milwaukee, WI 53201.

TABLE 1. *Sample location, trophic status, depth, and method of collection*

Sample location and trophic status	Depth	Method of collection	Storage	Time before treatment
Trout Lake[a], Vilas County, Wis. (mesotrophic-oligotrophic)	2 m 8 m	Van Dorn bottle	Acid-washed opaque plastic bottle	20 min
Lake Michigan 43°11'18"N, 87°41'10"W (oligotrophic)	0 m 20 m 90 m	Niskin bag sampler	Sterile glass jars 4°C	1 h and 45 min
Bog pond no. 1[b], Cedarburg, Wis. (eutrophic)	20 cm	Sterile hand pump	Sterile glass jars	15 min
Milwaukee Harbor (eutrophic)	0 m	Acid-cleaned plastic bucket	N/A[c]	Immediately

[a] A description of the lake was given by Juday and Birge (15).

[b] A description of the pond was given by S. C. Danos (M.S. thesis, University of Wisconsin-Milwaukee, Milwaukee, 1980).

[c] N/A, Not applicable.

placed in a sterile screw-capped test tube and fixed with 0.1 ml of 40% formaldehyde.

In test 2, a 10-ml subsample from each depth was placed in a sterile screw-capped test tube, and 1 ml of 0.2% INT dye (J. T. Baker Chemical Co., Phillipsburg, N.J.) was added. Samples were incubated in the dark for 20 min (28) at 20°C. The reaction was stopped with 0.1 ml of 40% formaldehyde.

In test 3, duplicate 100-ml samples from each depth were enriched with 0.025% (wt/vol) YE and 0.002% (wt/vol) NA (16) and incubated in 250-ml sterile screw-capped glass bottles for 6 h in the dark at 20°C. After incubation, samples were fixed with 40% formaldehyde (final concentration, 2%).

In test 4, duplicate 100-ml samples from each depth were placed in sterile 250-ml screw-capped glass bottles and enriched with both (i) 0.025% (wt/vol) YE and 0.002% (wt/vol) NA and (ii) 10 ml of 0.2% INT dye. Samples were incubated and fixed as in test 3.

In test 5, duplicate 100-ml samples from each depth were placed in sterile 250-ml screw-capped glass bottles, and 10 ml of 0.2% INT dye was added. Samples were incubated and fixed as in test 3.

In test 6, duplicate 100-ml samples from each depth were placed in sterile 250-ml screw-capped glass bottles and incubated and fixed as in test 3.

All samples were incubated at 20°C after Kogure et al. (16) for YE + NA to eliminate temperature as a variable.

To further examine the two techniques, a pure culture of an unidentified gram-negative rod, isolated from the hypolimnion of Lake Michigan and grown in nutrient broth (Difco Laboratories, Detroit, Mich.) at 20°C, was subjected to similar sets of tests, and the observations were used for comparison. Test tubes containing nutrient broth were inoculated 48 h before tests began. Small samples of the broth were added to autoclaved and filter-sterilized water (Gelman filter, 0.2-μm pore size) and treated as in tests 1, 2, 3, and 4.

A time course experiment with INT and incubation periods of up to 6 h was also performed. Water was collected from a pond and placed in an aquarium in the laboratory. A subsample (500 ml) containing a natural population of bacteria was taken from the aquarium and placed in a sterile flask containing a stirring bar. A 30-ml amount of 0.2% INT dye was added, and the sample was mixed and then incubated at 20°C. Samples of 10 ml were removed at the following times and fixed with 0.1 ml of filtered (0.2-μm pore size) 40% formaldehyde: 0, 1, 2, 10, and 30 min and 1, 2, 4, and 6 h. After fixation, samples were stored in the dark at 4°C for approximately 2 weeks until preparation for direct counts.

Microscopy. For epifluorescence microscopy, Nuclepore polycarbonate filters (25-mm diameter, 0.2-μm pore size) were stained with Sudan black B (28). To counter hydrophobic areas on the filters, several drops of a 0.5% solution of Wayfos surfactant (Phillip A. Hunt Chemical Corp., East Providence, R.I.) were placed below the filter just before filtering of the sample.

Samples were stained with a 0.01% final concentration of acridine orange (8). A 1-ml amount of each sample was mixed with 1 ml of dilution water and 0.2 ml of 0.1% acridine orange in a test tube and incubated for 2 min before being added to the filter column. The test tube was rinsed with 2 ml of filtered (Nuclepore filter, 0.2-μm pore size [see below]) water, which was then added to the filter column. To ensure their sterility, the dilution and rinsing water and the acridine orange were filtered by drawing the liquids into graduated syringes, attaching an autoclaved Swinnex-13 filter holder (Millipore Corp., Bedford, Mass.) containing a 13-mm-diameter Nuclepore filter (0.2-μm pore size), and forcing the liquid through the filter directly into the test tube. After filtering, the filter was removed from the column and placed on a glass slide. A drop of nonfluorescing immersion oil (Cargille, Cedar Grove, N.J.) was placed on top, and a cover slip was added.

Counts were made with a Zeiss standard 18 microscope with Neofluar objectives and adapted for epifluorescence (HBO 50 light source, G436 exciter filter, PL 520 barrier filter, and FT 510 beam splitter). The

total number of bacteria per milliliter was estimated from a count of at least 10 randomly chosen microscope fields under oil immersion (×1,000 for Trout Lake samples only; ×1,500 for others). At least 200 bacteria were counted from each filter. Actively metabolizing bacteria were determined by two methods. For those samples where INT was used, metabolizing bacteria reduced INT to INT-formazan, forming optically dense intracellular deposits (28). In the samples where YE and NA were used, this combination caused actively metabolizing bacteria to either elongate or fatten, and these were counted by epifluorescence (16). The actively metabolizing bacteria were determined from the same fields as the total counts. When INT and YE + NA were combined, the numbers of respiring bacteria from each test were counted separately. All counts are expressed as bacteria per milliliter.

For statistical analyses, duplicates were combined. Data were analyzed with a one-way analysis of variance (ANOVA), using a Hewlett-Packard 9825A computer and a taped program (Statistics, vol. 2, part no. 09825-15014). Data indicating a significant difference ($P < 0.05$) were further analyzed by the Newman-Keuls multiple-range test (26).

RESULTS

Observations. Observations of metabolizing cells identified by the INT-formazan method showed cells to have one or several round dark-red formazan deposits as previously described (28). These deposits were not often in the same focal plane as the outlines of the cell, so an up-and-down focus action was used in combination with bright-field transmitted light and incident fluorescent light.

After 20-min incubation periods, there was no difficulty in observing both the formazan deposits and the fluorescing bacteria in both natural samples and cultured cells. However, after incubations of 6 h, transmitted light revealed nonfluorescing bacterial shapes apparently containing large amounts of formazan in the samples collected from the lakes and pond. There was no difficulty in observing formazan deposits in cultured cells even after 6 h; this was probably due to the naturally large size of cultured bacteria. Nonfluorescing bacterial shapes were not included in counts of total numbers of metabolizing bacteria, which may account for the differences found when samples were incubated with INT for 6 h.

The time course of INT labeling was examined in a 6-h experiment (Fig. 1). Again, nonfluorescing bacterial shapes were not counted, and a slight decrease in total counts was observed over 6 h. Metabolizing bacteria showed an initial rise and then a slight decrease. These results suggest that after long incubations increasing accumulations of formazan deposits may totally block out fluorescence in cells.

Observations of metabolizing cells determined by the YE + NA method (in natural samples from the lakes and pond) indicated that these cells were elongated or fattened and fluorescing red-orange as described previously (16). With cultured cells, however, elongation and enlargement were not readily apparent. Most cells did not appear much larger than normal

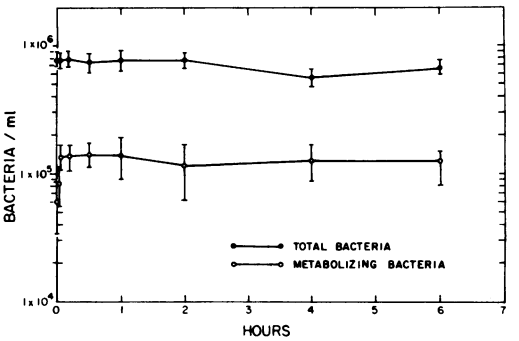

FIG. 1. *Effect of prolonged exposure of INT on numbers of both total and metabolizing bacteria. Bars denote one standard deviation.*

The INT was combined with YE + NA in the hope that elongation and fattening of the cells by YE + NA would aid in the identification of INT-formazan cells. Most cells that appeared enlarged in natural samples contained easily observed formazan deposits. Most cells with formazan deposits appeared enlarged. However, cells were observed which may have been enlarged but did not contain formazan deposits, as well as cells with formazan deposits which did not appear enlarged. Similar results were observed with cultured cells.

Measurements. The numbers of total and metabolizing bacteria in water collected from all stations are given in Table 2.

(i) Trout Lake. Determinations of total bacteria from tests 1 through 6 at 2 m were similar, and ANOVA indicated that no difference existed among tests ($F = 0.80$; $P > 0.05$). ANOVA for metabolizing bacteria at 2 m indicated a significant difference among tests ($F = 2.54$; $P < 0.05$). Significantly different means were found for tests 4 (YE + NA) and 5 (INT) (Neuman-Keuls multiple-range test, $P < 0.05$).

ANOVA for total bacteria from 8 m indicated a significant difference ($F = 9.76$; $P < 0.05$). Test 4 was significantly different from all other tests, and a further difference was found between means of tests 6 and 3 ($P < 0.05$). ANOVA for metabolizing bacteria from 8 m indicated a difference among tests ($F = 2.45$; $P < 0.05$). The significantly different test means were test 5 (INT) and tests 2 (INT) and 4 (YE + NA) ($P < 0.05$).

(ii) Lake Michigan. ANOVA for total bac-

TABLE 2. *Sample location, depth, and direct counts of total and actively metabolizing bacteria*

Station, date, and depth (m)	(1)[a] Direct count ($\times 10^6$)	(2) INT			(3) YE + NA			(4) INT + (YE + NA)					(5) INT (6 h)			(6) Direct count (6 h) ($\times 10^6$)
		INT active count ($\times 10^4$)	Total count ($\times 10^6$)	% INT active	YE + NA active count ($\times 10^4$)	Total count ($\times 10^6$)	% YE + NA active	INT active count ($\times 10^4$)	YE + NA active count ($\times 10^4$)	Total count ($\times 10^6$)	% INT active	% YE + NA active	INT active count ($\times 10^4$)	Total count ($\times 10^6$)	% INT active	
Trout Lake, 10/19/79																
2	1.09	9.10	1.14	7.9	8.60	1.01	8.5	9.20	11.10	1.06	8.7	10.5	5.70	1.09	5.2	1.05
8	1.02	7.90	1.02	7.7	6.80	0.98	6.9	6.50	7.20	0.86	7.5	8.4	5.20	1.07	4.8	1.08
Lake Michigan, 6/4/80																
0	0.58	6.06	0.47	12.9	6.93	0.74	9.3	3.04	3.04	0.32	9.5	9.5	6.85	0.57	12.0	0.60
20	0.58	6.26	0.46	13.6	4.18	0.43	9.7	4.41	6.06	0.84	5.2	7.2	8.42	0.71	11.8	0.57
90	0.48	6.26	0.51	12.3	5.78	0.81	7.1	2.65	2.86	0.29	9.1	9.8	5.59	0.38	14.7	0.57
Bog Pond, 7/4/80, 0.2	1.72	9.82	1.27	7.7	9.52	2.22	4.3	6.46	5.08	1.06	6.1	4.8	10.87	1.30	8.3	1.93
Milwaukee Harbor, 8/3/80, 0	1.69	17.60	1.68	10.5	13.40	1.84	7.3	13.30	11.70	2.17	6.1	5.4	16.80	1.83	9.2	1.59

[a] Test numbers.

teria from 0 m indicated a significant difference among tests ($F = 15.98$; $P < 0.05$). Tests 3 and 4 were significantly different from all other tests ($P < 0.05$). Test 2 was significantly different from test 6 ($P < 0.05$). ANOVA for metabolizing bacteria from 0 m indicated a significant difference ($F = 19.66$; $P < 0.05$). Tests 2 (INT), 3 (YE + NA), and 5 (INT) were significantly different from tests 4 (INT) and 4 (YE + NA).

ANOVA for total bacteria from 20 m indicated a significant difference ($F = 19.49$; $P < 0.05$). Tests 4 and 5 were significantly different from tests 2 and 3 ($P < 0.05$). ANOVA for metabolizing bacteria from 20 m indicated a significant difference ($F = 6.94$; $P < 0.05$). Test 5 (INT) was significantly different from all other tests ($P < 0.05$).

At 90 m ANOVA for total bacteria indicated a significant difference ($F = 31.80$; $P < 0.05$). Tests 3 and 4 were found to be significantly different from all other tests ($P < 0.05$). Tests 1, 2, and 6 were found to be significantly different from test 5 ($P < 0.05$). ANOVA for metabolizing bacteria showed a significant difference ($F = 12.69$; $P < 0.05$). Significantly different were tests 2 (INT), 3 (YE + NA), and 4 (INT) and tests 4 (INT) and 4 (YE + NA) ($P < 0.05$).

(iii) Bog pond. ANOVA for total bacteria from the pond showed a significant difference ($F = 40.65$; $P < 0.05$). Tests 3 and 6 were significantly different from tests 1, 2, 4, and 5 ($P < 0.05$). Test 1 was also found to be significantly different from tests 2 and 5. ANOVA for metabolizing bacteria from the pond indicated a significant difference ($F = 10.68$; $P < 0.05$). Tests 2 (INT), 3 (YE + NA), and 5 (INT) were significantly different from tests 4 (INT) and 4 (YE + NA) ($P < 0.05$).

(iv) Milwaukee Harbor. ANOVA for total bacteria from the harbor sample indicated a significant difference ($F = 4.72$; $P < 0.05$). Test 4 was significantly different from all other tests ($P < 0.05$). ANOVA for metabolizing bacteria from the harbor indicated no significant differences among tests ($F = 1.76$; $P > 0.05$).

DISCUSSION

The INT method assumes that intracellular INT reduction and the formation of optically dense formazan deposits occur exclusively in living cells; thus, the presence or absence of visible formazan spots in cells of bacteria and blue-green algae (cyanobacteria) is, generally, a function of respiratory intensity (28). Zimmermann et al. (28) showed that fixation before the addition of INT prevents cells from forming formazan deposits, indicating that only living cells take up INT to form deposits. By using

increasing concentrations of formaldehyde, which reduced the amount of respiration, they also found that the number of cells with formazan deposits was reduced. Finally, electron transport system activity in bacteria has recently been shown to be reasonably correlated to respiration (3), supporting the belief that the reduction of INT to formazan by electron transport systems is a function of respiration.

The comparison of the INT method to the YE + NA method in this study involved some changes in the INT technique as described by Zimmermann et al. (28). These changes included an incubation at 20°C (same as YE + NA) and the use of filters with a 0.2-μm pore size instead of a 0.1-μm pore size. Zimmermann et al. (28) used the smaller pore size because it reduced visible filter structure when transmitted light was used. Some bacteria may pass through a filter with a 0.2-μm pore size (27); however, Zimmermann et al. (28) stated that this loss can be ignored except in extremely oligotrophic waters since cells of 1.6 to 2.4 μm in freshwater account for the highest proportion of metabolizing bacteria.

In freshwater environments, the proportion of metabolizing bacteria ranges from 5 to 36% of the total direct count (28). In this study, the proportion of metabolizing bacteria in Trout Lake was between 7 and 8% of the total, in Lake Michigan it was between 12 and 13%, in the bog pond it was about 8%, and in the harbor sample it was about 10%. All of these lie well within the range previously reported. Since formazan deposits may be small, the use of fine optics is important for resolving them (13). Zimmermann et al. (28) used a magnification of ×1560, and in the present study the deposits were seen at magnifications of ×1,000 and ×1,500.

After samples were incubated for 6 h with INT, it was noticed that nonfluorescing particles of bacterial shape were present. Since they did not fluoresce, they were not counted as bacteria. This may account for the differences found between tests 4 and 5 and other tests for both total and metabolizing bacteria. This apparent blockage of fluorescence in cells was also observed by Zimmermann et al. (28), but they counted the shapes as bacteria when they were recognized in bright-field microscopy. In this study, no nonfluorescing bacterial shapes were observed after a 20-min incubation in INT; after 3 h, metabolizing bacteria declined. A similar decrease was observed in total numbers of bacteria for these samples; thus, the percentage of total metabolizing bacteria remained approximately the same. Apparently, long periods of incubation increase the number of cells which block fluorescence by accumulation of formazan deposits. This causes reduced numbers of total and metabolizing bacteria if nonfluorescing bacterial shapes are not counted.

One problem inherent in the INT technique relates to the size of bacteria. Very small bacteria are difficult to see to begin with, and the resolution of formazan deposits in such bacteria is even more difficult. In addition, fluorescence may be masked by these formazan deposits; in this case the cells would not be counted. To ensure the best results possible, the use of fine optical equipment is important. Zimmermann et al. (28) had no problems recognizing formazan deposits within bacterial cell sizes greater than 0.4 μm.

The YE + NA technique (16) assumes that metabolizing bacteria use YE to grow and, more importantly, that NA prevents cells from dividing, causing them to elongate. NA inhibits deoxyribonucleic acid synthesis, while ribonucleic acid synthesis and protein synthesis continue (6, 7). Kogure et al. (16) have shown that NA suppresses cell division during exponential growth but that some starved bacteria enlarge after incubation with YE. This indicates that YE will be used by bacteria for growth and that division should be prevented by NA.

However, Kogure et al. (16) reported that the growth of natural bacterial populations may not be completely suppressed by NA. They attributed this to the growth of NA-resistant bacteria. This may explain the differences of test 3 total counts in all of the samples tested in the present study. In a more recent paper, Kogure et al. (17) reported that the YE + NA technique includes both actively growing cells and dormant cells which have responded to added YE during the 6-h incubation. They felt that elongated cell numbers probably overlapped the metabolizing cells determined by autoradiographic methods (9, 19) and concluded that the YE + NA technique may be successfully applied to various aquatic environments to count living bacteria. Since ribonucleic acid synthesis is not stopped by NA (6), the reddish color reported by Kogure et al. (16, 17) in metabolizing bacteria found by the YE + NA method is probably due to the predominance of ribonucleic acid in metabolizing cells (8).

In their observations in the open ocean, Kogure et al. (16), using the YE + NA method, found metabolizing bacteria to be 5 to 10% of the total direct count. The present results indicate a range of 4.3 to 10% of the total direct count, which lies fairly well within the range above. In their more recent paper, Kogure et al. (17) found in eutrophic waters ranges of 1.5 to 39.8% of the

total direct count to be metabolizing bacteria, and in offshore oligotrophic waters 0.7 to 7.9% were apparently metabolizing.

A comparison of the two techniques reveals no significant differences between counts of metabolizing bacteria. However, significant differences were found between total counts of the two methods. Total counts by the YE + NA method were higher than total counts by the INT method, but in most of these cases the INT total counts were statistically the same as test 1 total counts (water fixed immediately). This leads us to believe that there may be NA-resistant bacteria which continue to proliferate. Similar observations were also made by Kogure et al. (16).

Jones (13) reported some success in combining the INT and YE + NA techniques, but unfortunately listed no data. In the present study, combining the two techniques did not seem to improve results for either technique. In some cases, the numbers of metabolizing bacteria from the combined methods were slightly higher than counts from the tests performed individually and, in several, significantly lower. The apparently enlarged bacteria in natural samples and in cultured bacteria without formazan deposits may not have actually been enlarged cells. In cultured samples they appeared to fluoresce green rather than red-orange. Zimmermann et al. (28) suggested that bacteria lacking formazan spots may actually be in a state of activity below the level of detectability. Bacteria which contained formazan deposits but did not appear enlarged in natural samples may have actually responded to the YE + NA, but were so small to begin with that they actually did not appear enlarged. Because of this and because of the size problems mentioned previously, we recommend that investigators planning to use either of these techniques do preliminary experiments with both cultured and natural samples of varying morphology and trophic status.

In conclusion, data from these experiments support the suggestion by others (20, 23) that not all bacteria present in aquatic systems are actively metabolizing and may be in a state of dormancy. Furthermore, this study indicates that it is possible to compare numbers of metabolizing bacteria when INT and YE + NA are used individually. However, we recommend that a small sample of water be fixed without additions to compare total counts for each method since we found differences in total counts among tests. The strengths of both methods are the relative ease with which they are performed and the fact that data on total and metabolizing bacteria are available in a matter of hours. Autoradiographic techniques, on the other hand, are technically difficult, may be limited by the substrate used, and usually take days before providing data on metabolizing bacteria (9, 19). In addition, it is often difficult to interpret autoradiographic data obtained from natural systems, where responses vary and grain deposits are not always superimposed on metabolizing cells.

Combining INT and YE + NA did not provide a significant increase in estimates of numbers of metabolizing bacteria compared with either method used alone. However, further comparisons of the two techniques, individually and combined, may be needed. Determining seasonal distributions of metabolizing bacteria by both methods in lakes of different trophic status would be an important step in this direction.

ACKNOWLEDGMENTS

We thank the staffs of both the University of Wisconsin Trout Lake Biological Station and the University of Wisconsin-Milwaukee Field Station for their assistance during this study. We also thank S. C. Danos for his aid during sample collection and the crew of the *R/V Neeskay*. Special thanks are extended to A. S. Brooks, G. J. Warren, D. B. Seale, and M. E. Boraas for their interest and for their stimulating discussions during the preparation of the manuscript. The technical assistance of D. Welch, J. Flores, and R. Ristic is deeply appreciated.

This work was supported in part by the University of Wisconsin Sea Grant Institute under a grant from the Sea Grant Office, National Oceanic and Atmospheric Administration, U.S. Department of Commerce, by the State of Wisconsin, and by the Center for Great Lakes Studies.

LITERATURE CITED

1. **Bell, J. B., and B. J. Dutka.** 1972. Bacterial densities by fluorescent microscopy, p. 15–20. *In* Proceedings of the fifteenth Conference on Great Lakes Research. International Association for Great Lakes Research, Ann Arbor, Mich.
2. **Bowden, W. B.** 1977. Comparison of two direct-count techniques for enumerating aquatic bacteria. Appl. Environ. Microbiol. **33**:1229–1232.
3. **Christensen, J. P., T. G. Owens, A. H. Devol, and T. T. Packard.** 1980. Respiration and physiological state in marine bacteria. Mar. Biol. **55**:267–276.
4. **Daley, R. J., and J. E. Hobbie.** 1975. Direct counts of aquatic bacteria by a modified epifluorescence technique. Limnol. Oceanogr. **20**:875–882.
5. **Francisco, D. E., R. A. Mah, and A. C. Rabin.** 1973. Acridine orange-epifluorescence technique for counting bacteria in natural waters. Trans. Am. Microsc. Soc. **92**:416–421.
6. **Goss, W. A., W. H. Deitz, and T. M. Cook.** 1964. Mechanism of action of nalidixic acid on *Escherichia coli*. J. Bacteriol. **88**:1112–1118.
7. **Goss, W. A., W. H. Deitz, and T. M. Cook.** 1965. Mechanism of action of nalidixic acid on *Escherichia coli*. II. Inhibition of deoxyribonucleic acid synthesis. J. Bacteriol. **89**:1068–1074.
8. **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.

9. **Hoppe, H. G.** 1976. Determination and properties of actively metabolizing bacteria in the sea, investigated by means of micro-autoradiography. Mar. Biol. **36**:291–302.
10. **Iturriaga, R., and G. Rheinheimer.** 1975. Eine einfache Methode zur Auszählung von Bakterien mit aktivem Elektronentransportsystem in Wasser- und Sedimentproben. Kiel. Meeresforsch. **31**:83–86.
11. **Jannasch, H. W., and G. E. Jones.** 1959. Bacterial populations in sea water as determined by different methods of enumeration. Limnol. Oceanogr. **4**:128–139.
12. **Jones, J. G.** 1974. Some observations on direct counts of freshwater bacteria obrained with a fluorescence microscope. Limnol. Oceanogr. **19**:540–543.
13. **Jones, J. G.** 1979. A guide to methods for estimating microbial numbers and biomass in freshwater. Scientific publication no. 39. Freshwater Biological Association, Ambleside.
14. **Jones, J. G., and B. M. Simon.** 1975. An investigation of errors in direct counts of aquatic bacteria by epifluorescence microscopy, with reference to a new method for dyeing membrane filters. J. Appl. Bacteriol. **39**:317–329.
15. **Juday, C., and E. A. Birge.** 1941. Hydrography and morphometry of some northern Wisconsin lakes. Trans. Wis. Acad. Sci. Arts Lett. **33**:21–72.
16. **Kogure, K., U. Simidu, and N. Taga.** 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. **25**:415–420.
17. **Kogure, K., U. Simidu, and N. Taga.** 1980. Distribution of viable marine bacteria in neritic seawater around Japan. Can. J. Microbiol. **26**:318–323.
18. **Larsson, K., C. Weilbull, and G. Cronberg.** 1978. Comparison of light and electron microscopic determinations of the number of bacteria and algae in lake water. Appl. Environ. Microbiol. **35**:397–404.
19. **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.
20. **Novitsky, J. A., and R. Y. Morita.** 1977. Survival of a psychrophilic marine vibrio under long-term nutrient starvation. Appl. Environ. Microbiol. **33**:635–641.
21. **Packard, T. T., M. L. Healy, and F. A. Richards.** 1971. Vertical distribution of the activity of the respiratory electron transport system in marine plankton. Limnol. Oceanogr. **16**:60–70.
22. **Ramsay, A. J.** 1978. Direct counts of bacteria by a modified acridine orange method in relation to their heterotrophic activity. N. Z. J. Mar. Freshwater Res. **12**:265–269.
23. **Stevenson, L. H.** 1978. A case for bacteria dormancy in aquatic systems. Microb. Ecol. **4**:127–133.
24. **Watson, S. W., T. J. Novitsky, H. L. Quinby, and F. W. Valois.** 1977. Determination of bacterial number and biomass in the marine environment. Appl. Environ. Microbiol. **33**:940–946.
25. **Wright, R. T.** 1978. Measurement and significance of specific activity in the heterotrophic bacteria of natural waters. Appl. Environ. Microbiol. **36**:297–305.
26. **Zar, J. H.** 1974. Biostatistical analysis. Prentice-Hall, Inc. Englewood Cliffs, N.J.
27. **Zimmermann, R.** 1977. Estimation of bacterial number and biomass by epifluorescence microscopy and scanning electron microscopy, p. 103–120. *In* G. Rheinheimer (ed.), Microbial ecology of a brackish water environment. Ecological studies, vol. 25. Springer Verlag, New York.
28. **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.
29. **Zimmermann, R., and L.-A. Meyer-Reil.** 1974. A new method for fluorescence staining of bacterial populations on membrane filters. Keil. Meeresforsch. **30**:24–27.