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Comparison of Two Direct-Count Techniques for Enumerating Aquatic Bacteria

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Planktonic bacteria from an estuary were concentrated on membrane filters and counted with both a scanning electron microscope and an epi-illuminated fluorescent microscope. Counts on 0.2-μm Nuclepore filters (polycarbonate) were significantly higher ($P < 0.001$) than counts on 0.2-μm Sartorius filters (cellulose). In contrast, there was not a statistically significant difference between the two techniques when Nuclepore filters were used ($0.5 < P < 0.9$). The average cell volume from this study area was 0.047 μm^3. The estimated number of bacteria ranged from 10^6 to 10^7 bacteria per ml, representing from 4 to 40 mg of C per m^3.

Several reports have been published on the advantage of using a scanning electron microscope (SEM) and Nuclepore filters to view microbes and detritus (8, 9, 15, 16). It is not yet known, however, if the SEM can be used to estimate bacterial numbers and biomass quantitatively or if these estimates agree with those from other methods.

Aquatic bacteria concentrated on membrane filters were counted using an extension of the SEM technique developed by Paerl and Shimp (15). These counts were compared to counts determined using the acridine orange direct-count (AODC) technique of Hobbie et al. (11).

A quantitative SEM technique is dependent on two important assumptions. The first is that a 0.2-μm Nuclepore filter can remove all of the bacteria from a water sample, and the second is that bacteria can be distinguished from nonliving particles with an SEM. The unique features of Nuclepore filters have been discussed elsewhere (7, 17). The problems of identifying bacteria from microscope fields or micrographs are discussed by Wiebe and Pomeroy (18).

Surface samples were taken from an estuary (the Newport River in North Carolina) bordered by extensive *Spartina* marshes. Subsamples for the AODC technique were concentrated on 25-mm-diameter, 0.2-μm, stained Nuclepore or black Sartorius filters using a membrane filter assembly (Millipore Corp.). Each sample filter was placed over an underfilter which had been soaked thoroughly with a surfactant (WAYFOS, Phillip A. Hunt Corp., East Providence, R.I.) to prevent clumping of particles. The bacteria on damp filters were viewed with an Ortholux epi-illuminated microscope. Details of the method are given in Daley and Hobbie (4) and Hobbie et al. (11).

For the SEM technique, subsamples were concentrated on 13-mm-diameter, 0.2-μm Nuclepore filters using the base of a 13-mm-diameter Swinnex filter holder (Millipore Corp.) fitted with a top made from a short section of 1-cm (ID) glass tubing. A small volume of 3% glutaraldehyde (E. M. grade, Polysciences, Inc.) was added to the subsample before it completely passed through the filter. Then each sample filter was placed in an aluminum-foil boat and covered by a clean 0.4-μm Nuclepore filter to reduce the possibility of contamination or loss of bacteria in subsequent steps (12). Each boat (with sample and cover filter) was soaked in the dark in filtered 3% glutaraldehyde at 4°C for 1 to 3 h. Cell volumes were increased by 5% to correct for the 3 to 5% shrinkage caused by glutaraldehyde (K. Muse, personal communication).

Next, the boats were transferred to a filtered buffer solution of cacodylic acid and HCl (0.2 M) with 5% (wt/vol) sucrose to stabilize cell membranes. The boats were left in this buffer wash for about 24 h. Detailed descriptions of the fixative and buffer are contained in Hayat (10). Residual water in the samples was removed by dehydration in water-ethanol and ethanol-Freon solutions, followed by critical-point drying with Freon (1, 2). The boats were rinsed for 15 min in solutions of 30, 50, 75, 95, and 100% pure ethanol in distilled water, followed by solutions of 30, 50, 75, 95, and 100% Freon 113 in pure ethanol (3). After dehydration, the boats were critical-point dried with Freon 13.

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Viewing was performed with a ETEC model U-2 scanning electron microscope at a 0° angle and ×2,000 to ×10,000 magnification. First, the boats were disassembled and the dried filters, their cover filters, and blank control filters were mounted with Scotch Double-Stick tape to metal stubs. Each filter was then coated with a 20- to 30-nm layer of gold. Estimates of bacterial numbers and size were taken from micrographs of randomly selected fields on the filters, using Polaroid type 55 P/N or type 52 film. SEM micrographs of bacteria on membrane filters have been published before (17). The only substantial difference between these micrographs and mine are that, because I used natural water samples, detrital material is present and the bacteria are smaller.

Three bacterial-counting treatments were compared as follows: (i) the SEM with 0.2-μm Nuclepore filters; (ii) the AODC with 0.2-μm stained Nuclepore filters; and (iii) the AODC with a 0.2-μm black Sartorius filters (Table 1). An analysis of variance (Table 2) indicates that there is a significant difference between the treatments ($P < 0.001$). In addition, there was a significant difference between the days on which the experiments were run ($P < 0.001$) although there appears to be no interaction between the day and treatment effects ($0.25 < P < 0.50$), indicating that these results are valid for any given day.

The method-filter treatments were compared using an approximate t test of means made necessary by the heterogeneity in sample sizes and variances. The results still strongly support the hypothesis that there is a significant difference between the SEM-Nuclepore and AODC-Sartorius techniques ($P < 0.001$) but that this difference cannot be demonstrated if Nuclepore filters are used for both techniques ($0.5 < P < 0.9$). It can be concluded, therefore, that any difference between the SEM and AODC techniques can be attributed to the type of filter used.

TABLE 1. *Means of the cell counts from the method-filter experiment*[a]

Day	Treatment	Mean/filter (×10⁶/ml)	Fields/filter
18 Dec. 75 (3.82)	SEM-0.2-μm Nuclepore (4.34)	3.22	12
		5.62	6
		4.97	9
	AODC-0.2-μm Nuclepore (4.81)	4.71	20
		4.69	20
		5.03	20
	AODC-0.2-μm Sartorius (2.00)	2.62	20
		1.37	20
10 Feb. 76 (2.19)	SEM-0.2-μm Nuclepore (3.30)	3.24	10
		4.34	9
		2.89	10
		2.76	8
	AODC-0.2-μm Nuclepore (2.92)	2.70	20
		2.74	20
		3.32	20
	AODC-0.2-μm Sartorius (0.78)	0.96	20
		0.65	20
		0.73	20

[a] The weighted mean cell count for the day and treatment levels is indicated in parentheses.

TABLE 2. *Summary of the analysis of variance of the method-filter comparison*[a]

Source of variation	df	MS[a]	F
Experiment	17	→ 38.00	34.23[b]
Days	1	186.18 ←	34.54[c]
Treatment	2	192.68 ←	35.75[c]
Days × treatments	2	4.93 ←	0.91[d]
Replication	12	→ 5.39 —	4.86[b]
Error	266	— 1.11	
Total	283		

[a] The brackets indicate the approximate mean square (MS) comparisons of the stated F values. A treatment is defined as the combination of a specific method with a specific type of filter.

[b] $P < 0.0001$.

[c] $P < 0.001$.

[d] Not significant ($0.25 < P < 0.50$).

A total of 158 cells were measured from SEM micrographs of samples on Nuclepore filters to estimate the average cell volume of the population. The average cell volume and 90% confidence interval of 68 rods was 0.061 ± 0.021 μm^3. The cell volume and 90% confidence interval of 90 cocci was 0.036 ± 0.014 μm^3. The weighted cell volume and 90% confidence interval of the 158 cells measured was 0.047 ± 0.012 μm^3. Over the entire study, population estimates ranged from 10^6 to 10^7 bacteria per ml. Assuming that the average density of a bacterium is 1.07 g/ml (5) with a dry weight-to-wet weight ratio of 0.23 (13, 14) and a carbon-to-dry weight ratio of 0.344 (6), then given 10^6 to 10^7 bacteria per ml whose average cell volume is 0.047 μm^3, the average bacterial biomass will range from 4 to 40 mg of C per m^3. The carbon-to-dry weight ratio used here is low compared to that usually quoted (0.45 to 0.50) but is based on bacterial carbon content measurements of cultures from my sample site (Ferguson and Murdoch [1973] as quoted by Ferguson and Rublee [6]).

There can be little doubt that Nuclepore filters are superior to cellulose ester filters as a background and support for bacteria to be viewed with an SEM. In addition, they have excellent retention, as indicated by Azam and Hodson (manuscript in preparation), who found no indication of heterotrophic activity, adenosine 5′-triphosphate, chlorophyll a, or ^{14}C photosynthesis in the filtrates from oceanic water that had been filtered through 0.2-μm Nuclepore filters. They did measure significant bac-

terial heterotrophic activity in filtrates obtained using Nuclepore filters with pores as small as 0.4 μm in diameter. The error in biomass caused by ignoring any bacteria small enough to pass through a 0.2-μm-diameter pore is probably very small, since the volume—and hence the biomass—of a coccus, for example, decreases with the cube of the diameter.

However, there is some doubt that a direct-count technique can be completely objective, because it is often difficult to distinguish bacteria from nonliving particulate material in natural water samples. Wiebe and Pomeroy (18) discuss this problem with reference to phase-contrast microscopy; however, it was not evident before this investigation whether the problem of subjectivity could be minimized by the resolution and depth of field of an SEM. I used shape and texture as two important criteria to identify bacteria, as did Wiebe and Pomeroy (18). The particles that were obviously bacteria had round shapes and smooth surface textures. Nonliving particles appeared to consist of small fragments of plant material, a few animal fragments, suspended sediment, and other irregularly shaped particles. Bacteria were seldom seen attached to particles. Although I found that subjectivity cannot be eliminated by using SEM, I believe that the data reported are accurate considering the variation within a population of aquatic bacteria and are sufficient to make meaningful comparisons between techniques or populations.

It is encouraging that a wide variety of techniques utilizing several modes of information produce similar population estimates. Watson et al. (19) show that numbers estimated from carbon replica transmission electron microscopy and from measurement of lipopolysaccharides in bacterial cell walls agree well with estimates from the AODC technique. The present study shows that the AODC and SEM techniques agree well as long as 0.2-μm Nuclepore filters are used. It is unlikely that these uniform conclusions would result from such diverse techniques unless the bacterial population estimates are accurate.

Although the population estimates obtained using these techniques appear to be in agreement, there is some discrepancy in the cell volume estimates between techniques as well as within a technique. There are threefold differences in the cell volume measured with the lipopolysaccharide technique on two samples taken a week apart by Watson et al. (19) and a two- to sixfold difference between the cell volume reported here and those reported by Watson et al. These data suggest that bacterial volume and biomass may vary drastically in time and space. If so, then cell volume must be measured routinely to get accurate biomass data, a task for which the SEM and carbon replica transmission electron microscopy techniques are too time consuming.

In summary, the important findings are: (i) that it is possible to use the SEM as a quantitative counting tool; (ii) the number of bacteria-like forms counted agrees closely with the fluorescing particles (bacteria) counted using the AODC technique; and (iii) because of this favorable comparison the latter technique is preferable for routine ecological sampling. In spite of the fact that SEM is a powerful viewing instrument, the AODC technique can be used in its place to make population estimates with negligible loss in accuracy, a possible gain in precision (more samples can be processed in less time, thus reducing the confidence intervals), and a considerable savings of time and money. On the other hand, total bacterial biomass may best be estimated using the lipopolysaccharide technique of Watson et al. (19) because it is less time consuming and less subject to investigator bias than the other techniques mentioned here, where each cell must be measured exactly.

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