Differential Counting in Mixed Cultures with Coulter Counters

J. F. DRAKE and H. M. TSUCHIYA

Department of Chemical Engineering and Materials Science and Department of Microbiology, University of Minnesota, Minneapolis, Minnesota 55455

Received for publication 15 February 1973

A critical comparison of Coulter, viable, and microscope counts for several mixed cultures of microorganisms has been made. This investigation shows that Coulter counting can provide reliable estimates of microbial numbers in mixed cultures. Precautions and limitations of Coulter counting in mixed cultures are discussed.

Studies on mixed cultures of microorganisms require differential counting of the component organisms. A variety of techniques can be used depending upon the particular system studied. Our experience with mixed cultures of more than one pair of microbial forms has shown that the Coulter counter can be used to count microorganisms in mixtures; however, certain precautions must be followed and its limitations must be borne in mind.

This paper reports some of the details of preparing samples and counting mixed cultures with the Coulter counter, or instruments similar to it. An appreciation of the potential of the conventional Coulter counters for mixed culture studies should prove useful to workers who do not have access to more elaborate instrumentation.

Numerous descriptions of the theory and operation of the Coulter counter have been given and the capabilities and limitations of its use in particle counting are well documented. An excellent discussion pertaining to microorganisms is given by Kubitschek (3). Counting is based on the simultaneous passage of a conducting fluid and current through a small aperture. The aperture serves as a resistance element in a measuring circuit; the passage of a suspended particle momentarily blocks a portion of the aperture and changes its resistance. The magnitude of the resistance pulse is a measure of the volume of the particle. The pulse height is proportional, or nearly so, to the volume of the particle (3) under suitable conditions. As particles of different volumes pass through the aperture, a series of pulses are generated. The pulse heights correspond to the volume distribution of the suspended particles. The Coulter counter has threshold dials that may be set for counting only those pulses of a certain magnitude. By varying the threshold settings, particles within limits of a given volume range can be selectively counted.

For counting purposes, a mixed culture may be viewed as a series of overlapping volume distributions. If the mixed culture is composed of organisms which differ in size to such an extent that their volume distributions do not overlap appreciably, they can then be counted with a conventional Coulter counter. The apertures and threshold settings can be chosen so that the pulse-height distributions of the component organisms can be isolated.

If appreciable overlapping of the size distributions occurs, the individual distributions can be resolved only by using techniques that give an undistorted volume distribution and by assuming a reasonable shape of the component distributions. By using a multichannel pulse-height analyser with special apertures (3), hydrodynamic focusing (7), or electronic shaping of the peaks (2), volume distributions of microorganisms can be obtained with very good resolution.

Commercially available apertures are designed for counting rather than sizing; the pulses generated by them do not correspond to the exact volume distribution of the particles (2). This distorted pulse-height distribution is related, however, to the actual volume distribution; it can be used for differential counting but with loss of some resolution. This loss is not a serious objection since the application of the Coulter counter to mixed cultures is not limited so much by instrument resolution as by biological considerations. These may involve changes in both the mean size and the shape of microbial volume distributions as environmental
conditions change. Moreover debris or nonviable cells may occur. Thus, those microorganisms differing considerably in size can be adequately resolved under a variety of environmental conditions. In addition, the precise total particle count provided by the Coulter counter must be interpreted with due regard for the biological phenomena involved.

MATERIALS AND METHODS

Organisms and media. Media and culture conditions have been previously described for *Escherichia coli* B/r and *Dictyostelium discoideum* strain NC-4 (9), *Azotobacter vinelandii* OP and *Tetrahymena pyriformis* W (1), and *Lactobacillus casei* NRRL B-1445 and *Saccharomyces cerevisiae* NRRL Y-967 (4).

Methods. Cell counting was carried out with Coulter model A, B, or ZB counter using either a 30- or 100-µm orifice. Samples were diluted to give bacterial counts of 10,000 to 30,000 counts in a count-on volume of 0.05 ml with the 30-µm aperture. The 95% confidence limits for total particle count are 2 to 5% of the mean of four counts of a single sample for counts in this range. For protozoa counts, 0.5 to 1 ml of culture was diluted to 20 or 50 ml of saline. Counts of protozoa were in the range of 10 to 10,000 counts per 0.5 ml.

To adequately resolve small cells with the 30-µm aperture, the matching switch of the Coulter counter was set to a position which gave a maximum signal to noise ratio. This setting was found empirically by observing the pulse-height distribution of small particles on the instrument’s oscilloscope display at different matching switch settings. With our instruments and saline, the matching switch was set at 32L on the model B and 40K on the model ZB. Both instruments were operated at maximum gain.

Setting the matching switch for maximum signal to noise usually results in a mismatching of the impedance of the aperture and the instrument preamplifier. The increased sensitivity is accompanied by an increased sensitivity to electrical interference. Therefore, the sample counting stand must be carefully shielded. This was accomplished by enclosing the sample stand in a cabinet of 0.25-inch (0.63-cm) aluminum sheet; the instrument, stand, and the shield were grounded to a common ground. Inadequate grounding can be a source of interference along with transient noise from nearby refrigeration units or relays operating on the same electrical line as the counter.

The threshold scale of the counter was calibrated on a volume basis using mono-disperse latex spheres (Dow Chemical, Midland, Mich.).

A standard hemacytometer was used for microscope counts of *Dictyostelium*. For *Tetrahymena*, the protozoa in a capillary of known volume (Drummond Microcaps, Drummond Scientific Co., Broomall, Pa.) were counted under an ×60-dissecting microscope. Viable counts of bacteria were made either by the capillary tube method of Schoon (6) or by plate counts with 10 replicate plates per count using the drop plate method (5). Plaque counts of *Dictyostelium* were made on Bonner’s agar with an *E. coli* B/r lawn (8).

Samples for Coulter counting were diluted in 0.6% NaCl containing 0.02% disodium ethylenediaminetetraacetate dihydrate as an inhibitor of microbial growth. Background particles were removed by filtration through membrane filters (Type HA, Millipore Corp., Bedford, Mass.). Careful rinsing of both the receiving and storage flasks gave saline with a total background of 200 to 600 counts per 0.05 ml using a 30-µm aperture and the most sensitive instrument settings. Methods of continuous culture have been reported previously (9).

RESULTS AND DISCUSSION

On the basis of size, the microorganisms studied in our laboratory fall into three categories: (i) bacteria such as *E. coli*, *Lactobacillus casei*, or *Bacillus licheniformis* with cell volumes of 0.3 to 2 µm³; (ii) large bacteria and yeasts (*Azobacter*, *Saccharomyces cerevisiae*) with cell volumes of 2 to 100 µm³; (iii) protozoa (*Dictyostelium*, *Tetrahymena*, *Colpoda*) with volumes of 300 to 20,000 µm³. Differential counts in mixed cultures made up of a member from each group can be made with the Coulter counter.

The smaller bacteria approach the limits of resolution of a 30-µm aperture; larger bacteria and yeast overlap the volume range of 30- and 100-µm apertures. The protozoa are best counted with 100 µm or larger apertures.

The rigid cell walls of bacteria and yeasts minimize volume changes, or lysis, after dilution into fluid with osmotic properties different from that of the culture media. However, chains, or clumps of cells, involution forms, or yeasts in the process of rapid budding will have volumes many times the mean cell size and may appear as counts in an interval assigned to a larger microorganism. On the other hand, protozoa are sensitive to osmotic shocks and lysis may occur after dilution. Lysis may be minimized by matching the dilution fluid and growth media with respect to osmotic properties. Also, debris may accumulate during normal protozoan growth as a result of excretion of undigested food materials or cell death. The debris often contain particles of the size of bacteria. Frequent microscope examination of mixed cultures is necessary to avoid errors due to these causes.

Motile protozoa may cease movement after dilution, especially if preservatives are used in the diluent. Such protozoa settle out rapidly and must be stirred frequently during counting to avoid a low count. The rapid settling of larger particles can be helpful when bacterial counts are made in the presence of protozoa. Since
bacteria settle relatively slowly, allowing the sample to stand for a few minutes allows the protozoa to settle out and minimizes plugging of the 30-μm aperture.

The counting time for a specific volume of sample should be checked frequently during a count. Minor plugging may not seriously affect the total count but can significantly alter the pulse-height distribution. Under fixed conditions, the counting time should be constant and reproducible. Increased counting times indicate plugging of the aperture, whereas decreased counting times indicate leaks in the vacuum system.

The use of the Coulter counter for counts of a pure culture of E. coli B/r is shown in Table 1. A steady-state chemostat operating at 25°C with a holding time of 7.6 h was sampled periodically for viable and Coulter counts. Both methods give essentially the same value for cell number. The mean cell size of E. coli under these conditions is 0.25 μm² with the smaller cells lying close to the limits of resolution of a 30-μm aperture.

The viable and total count in continuous culture may not be the same even through a steady state is obtained. Table 2 shows the difference in viable and Coulter count for S. cerevisiae when grown with L. casei in continuous cultures (4). Viability of the yeast is only about 55% in these experiments. Both viable and Coulter counts for L. casei, on the other hand, were essentially the same; turbidity and pH were constant.

Application of the Coulter counter to counts of mixed cultures must be verified by an independent method of counting under a variety of conditions. The validity of the Coulter count was assumed and the counts were checked during various stages of mixed culture experiments. Table 3 shows the results for the pair D. discoideum-E. coli. In continuous culture, the two populations oscillate in a prey-predator relationship (9). The Coulter counts at several different points in the cycle were compared with viable and microscope counts made at similar time intervals. Neither plate counts nor hemacytometer counts were fully satisfactory as a basis for determining the accuracy of Coulter counts; both methods are subject to rather large systematic and random errors (5).

The Coulter count agrees reasonably well with the viable count at the lower end of the range for the population of D. discoideum and at the higher end for the population of E. coli.

### Table 1. Comparison of Coulter and viable counts for pure culture of E. coli

| Sample (day) | Coulter count (x 10⁶/ml) | Viable count (x 10⁶/ml) |
|--------------|--------------------------|-------------------------|
| 6            | 15.3                     | 13.5                    |
| 7            | 13.9                     | 9.0                    |
| 8            | 15.2                     | 16.0                    |
| 9            | 15.4                     | 14.7                    |
| 10           | 14.1                     | 15.5                    |
| 11           | 15.0                     | 14.5                    |
| 12           | 15.3                     | 14.6                    |
| 13           | 16.7                     | 14.1                    |
| 14           | 15.2                     | 15.7                    |
Mean          | 15.1 x 10⁴                | 14.8 x 10⁴              |
Variance      | ±0.65                    | ±0.72                   |

*Samples were taken daily from continuous culture for Coulter and viable counts. The chemostat was operated for 5 days at a constant flow rate before sampling was begun. Holding time of 7.8 h with minimal medium containing 0.49 mg of glucose per ml as the limiting substrate. The temperature was 25°C.

### Table 2. Difference in viable and Coulter counts of S. cerevisiae in continuous culture with L. casei

| Glucose (g per liter in feed) | Viable (x 10⁶) | Coulter (x 10⁶) | Viable count/Coulter count |
|-------------------------------|---------------|----------------|---------------------------|
| 0.25                          | 0.13          | 0.24           | 0.54                      |
| 1.75                          | 1.0           | 1.6            | 0.63                      |
| 4.0                           | 2.0           | 4.2            | 0.49                      |
| 6.0                           | 3.8           | 6.4            | 0.60                      |

*Continuous cultures were at a holding time of 16 h with glucose as the limiting substrate. Data are the average of steady-state values at each level of glucose. Data from Megee et al. (4).

### Table 3. Comparison of Coulter with viable and visual counts for mixed culture

| Sample | Amoebae (x 10⁶) | Bacteria (x 10⁶) |
|--------|----------------|-----------------|
|        | Coulter | Viable | Microscope | Coulter | Viable |
| 1      | 7.1     | 7.3    | 6.7        | 1.59    | 1.30   |
| 2      | 4.4     | 4.5    | 4.5        | 0.56    | 0.43   |
| 3      | 3.4     | 3.1    | 1.9        | 1.27    | 1.25   |
| 4      | 2.8     | 2.4    | 2.4        | 0.34    | 0.29   |
| 5      | 3.0     | 1.9    | 2.4        | 5.60    | 4.9    |
| 6      | 0.82    | 0.70   | 1.3        | 4.13    | 3.83   |
| 7      | 0.69    | 0.10   | 14.3       | 14.8    |       |
| 8      | 0.51    | 0.48   | 15.2       | 16.9    |       |
| 9      | 0.09    | 0.53   | 1.22       | 1.16    |       |
| 10     | 0.03    | 0.021  | 2.90       | 8.78    |       |
| 11     | 0.02    | 0.011  | 1.21       | 0.93    |       |

*Mixed cultures of D. discoideum and E. coli in chemostats were sampled simultaneously for amoebae and bacteria by several methods. Data are representative of the results over a range of population levels in several cultures.
with the other estimates of the populations. The greatest discrepancies are noted at low population densities. When the bacterial levels are low, the amoebae numbers are high and starvation of the predator is beginning. Although losses of amoebae due to lysis are small, the debris can give rise to apparent "bacterial" counts that are too high. The intact amoebae do not contribute any counts as such to the bacterial count over the threshold intervals assigned to E. coli; however, the passage of large particles through the 30-μm orifice may cause transient instabilities and increase the noise level of the system.

Similarly, low numbers of the amoebae are overestimated by the Coulter count. A portion of this difference may be due to losses in the viable count. During the initial portion of their minima, the amoebae are vacuolated and osmotically fragile due to starvation. Such amoebae may lyse during the preparation of plates for plaque counts and accentuate the difference between viable and total counts.

Data of the type shown in Fig. 1 for a three-member population would be difficult, if not impossible, for a single investigator to obtain by using microscope or viable counting procedures. If the experiments extend over several days and involve several cultures, Coulter counting becomes the only feasible method.

The threshold must be chosen carefully to distinguish E. coli from Azotobacter since the two distributions may overlap somewhat. Mean cell volumes of Azotobacter vary over a wide range depending upon culture conditions. Stationary phase nitrogen-fixing cells may have mean volumes of 1 μm³ or less. Rapidly growing cells may reach mean volumes of at least 10 μm³. In minimal medium with ammonia, the mean cell volume is 4 to 6 μm³. The overlap of Azotobacter and E. coli volume distributions is minimal; Fig. 2 shows the pulse-height distributions of the mixed cultures. Very small Azotobacter cells may overlap with E. coli and cannot be adequately resolved by conventional Coulter counters (7). However, under most culture conditions, the estimate of the population by Coulter counting is adequate. The increase in accuracy gained by viable counts does not justify the additional time and labor. If viable counts are required, the Coulter count provides a useful estimate of the population for determining the appropriate dilution that gives an optimal number of colonies per plate.

Data of the type shown in Fig. 2 should be obtained in order to assess the feasibility of the Coulter counter for differential counting of organisms with closely spaced pulse-height distributions. The pulse-height distribution becomes increasingly skewed toward higher thresholds as the total particle count increases. This increases the degree of overlap and errors can arise if low numbers of the larger organism are counted in the presence of high numbers of the smaller organism.

**Fig. 2.** Pulse-height distributions for mixtures of E. coli B/r and A. vinelandii. Distribution of pulse heights with a 30-μm aperture of a 1:1 mixture of E. coli and A. vinelandii at different growth phases. Mixture 1, stationary phase (O); mixture 2, exponential phase (Δ). Threshold of 100 corresponds to a volume of 1.5 μm³.
The *Tetrahymena* are easily distinguished from the bacteria. Their large volume, about $10^4 \mu m^3$, separates them clearly from bacteria; the only difficulty in counting arises from the low number densities encountered in most experiments. Background levels with the 100-μm aperture at the settings used to count these protozoa are very low, usually 0 to 2 counts per ml; counts of 5 to 10 organisms per ml are significant if a sufficient number of counts are accumulated by repeated counting of the suspension. Reliable counts of *Tetrahymena* at the level of 100 cells per ml of the original culture can be made using a 1-ml sample diluted to 20 ml of saline. The microscope and Coulter counts for *Tetrahymena* agree well but microscope counting is tedious and difficult for the motile ciliates at low population densities. Attempts to count *Paramecium caudatum* and *Paramecium multimicronucleatum* with the Coulter counter were unsuccessful; there appeared to be an avoidance of the aperture by the protozoa.

Similar procedures have been used to verify the use of the Coulter counter for reliable differential counts in mixed cultures of *Lactobacillus casei*-Saccharomyces cerevisiae (4), and Colpoda steinii-S. cerevisiae-E. coli. Provided the investigator is aware of the limitations and is willing to perform the necessary checks and controls, the Coulter counter can be used to provide estimates of microbial numbers with greater ease than with either viable or microscope counting.

ACKNOWLEDGMENTS

This investigation was supported in part by National Science Foundation grant GI-28874XI, by Public Health Service grant 16692 from the National Institute of General Medical Services, and by U.S. Department of Agriculture grant 85690. J. F. Drake was a trainee of Public Health Service training grant AI-0090-01 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Jost, J. L., J. F. Drake, A. G. Fredrickson, and H. M. Tsuchiya. 1973. Interactions of *Tetrahymena pyriformis*, *Escherichia coli*, Azotobacter vinelandii, and glucose in a minimal medium. J. Bacteriol. 113:834-840.
2. Harvey, R. J., and A. G. Marr. 1966. Measurement of size distributions of bacterial cells. J. Bacteriol. 92:805-811.
3. Kubitschek, H. E. 1969. Counting and sizing microorganisms with the Coulter counter, p. 593-610. In R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 1. Academic Press Inc., New York.
4. Megee, R. D., J. F. Drake, A. G. Fredrickson, and H. M. Tsuchiya. 1972. Studies in intermicrobial symbiosis. Saccharomyces cerevisiae and Lactobacillus casei. Can. J. Microbiol. 18:1733-1742.
5. Postgate, J. R. 1969. Viable counts and viability, p. 611-628. In R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 1. Academic Press Inc., New York.
6. Schoon, D. J., J. F. Drake, A. G. Fredrickson, and H. M. Tsuchiya. 1970. Automated counting of microbial colonies. Appl. Microbiol. 20:815-820.
7. Shuler, M. L., R. Aris, and H. M. Tsuchiya. 1972. Hydrodynamic focusing and electronic cell-sizing techniques. Appl. Microbiol. 24:384-388.
8. Sussman, M. 1981. Origin of cellular heterogeneity in the slime molds, *Dictyosteliaceae*. J. Exp. Zool. 118:407-416.
9. Tsuchiya, H. M., J. F. Drake, J. L. Jost, and A. G. Fredrickson. 1972. Predator-prey interactions of *Dictyostelium discoideum* and *Escherichia coli* in continuous culture. J. Bacteriol. 118:1147-1153.