APPLIED MICROBIOLOGY, Feb. 1968, p. 330-334
Vol. 16, No. 2

Printed in U.S.A.

Plate-Dilution Frequency Technique for Assay of Microbial Ecology[1]

R. F. HARRIS AND L. E. SOMMERS
Department of Soils, University of Wisconsin, Madison, Wisconsin 53706

Received for publication 29 September 1967

The plate-dilution frequency technique described facilitates simultaneous enumeration of a wide range of physiologically different microorganisms in complex systems with a precision comparable to dilution tube (most probable number) methods. Replicate microsamples are inoculated from each member of a dilution series onto areas delineated on plates of prepoured solid media; the plates are incubated, and the occurrence of growth or specific biochemical transformation is recorded for each inoculated area. Microbial enumeration is accomplished by reference to appropriate tables. Details of the experimental procedures are described, and tables are presented from which microbial numbers with 95% confidence limits can be obtained and compared for significant difference directly for 10-fold and 4-fold dilution series. Results of experiments in which microbial populations were estimated simultaneously by the plate-dilution frequency and conventional plate count methods are compared. The potential of the technique for broad-spectrum microbial assay is also discussed.

Elucidation of the types and numbers of organisms constituting mixed microbial populations is restricted mainly by the time and labor inherent in existing procedures for microbial analysis of complex systems. The plate-dilution frequency technique described in this paper combines many of the advantages of the conventional pour-plate and dilution tube (most probable number) methods, and reduces the time and materials involved in the initial preparation, inoculation, and data recording of large numbers of plates and tubes. The accuracy is comparable to the dilution tube method, in that both methods are based essentially on the "most probable number" concept (2) and the final numerical result is a function of a dilution series rather than one member of the series.

MATERIALS AND METHODS

In essence, the technique consists of inoculating replicate microsamples from each member of a dilution series onto areas delineated on plates of prepoured solid media and recording the presence or absence of growth or of specific biochemical transformation in each area after incubation of the plates for an appropriate length of time. The number of specific organisms or physiological groups of organisms present in the original sample is determined by reference to appropriate tables.

[1] Published with the approval of the Director, Wisconsin Agricultural Experiment Station, Madison.

The basic procedure found applicable to most systems involves the inoculation of eight replicates of each of six dilutions of a 10-fold or 4-fold dilution series onto three plates.

Preparation of plates. Petri plates of solid media are poured on a level surface, allowed to harden overnight, and then partially dried (e.g., 1 hr at 60 C for plastic disposable plates). Eight approximately equidistant circular areas are marked lightly in each half of the plate with a sterile cork borer (1-cm diameter); equidistance is easier to achieve with bisected and quadrisected plates (e.g., Falcon no. 1003, 1009) than with nondivided plates. The plates are stored in plastic bags and checked for contamination before use.

Inoculation of plates. Proceeding from the highest dilution of a 10-fold or 4-fold dilution series of six tubes, a 0.1-ml pipette graduated in hundredths is used to deliver eight replicate 0.01-ml samples from each dilution to the center of the appropriate prescribed circles on the medium. The fact that 0.01 ml is less than one drop assures that delivery of this amount can be accomplished accurately, since the 0.01 ml will not be released until contact is made between the tip of the pipette and the solid medium surface. Prior to delivery, the pipette is rinsed at least three times, and the external surface of the pipette tip is finally wiped clear of water drops on the sterile inside of the dilution tube. With minimal practice and experience, eight replicate 0.01-ml samples can be delivered accurately in the correct locations on the medium from one filling of the 0.1-ml pipette. The samples normally sink into the predried medium within minutes, but as a precaution against movement out of the delineated areas the inoculations should be carried out on a level

TABLE 1. *Enumeration of microorganisms based on the number of positive responses in plates inoculated with a 10-fold or 4-fold dilution series*

Positive responses in plates inoculated with 8 replicate 0.01-ml samples from 6 dilution levels							Organisms per ml of suspension at dilution level 1[a]					
Total no.	No. at each level[b]						10-fold series			4-fold series		
	1	2	3	4	5	6	No. (d)	log d	log d/ 0.2743	No. (d)	log d	log d/0.2128
4	4	0	0	0	0	0	58	1.760	6.416	46	1.660	7.801
5	5	0	0	0	0	0	78	1.892	6.898	60	1.776	8.346
6	6	0	0	0	0	0	102	2.010	7.328	76	1.879	8.830
7	7	0	0	0	0	0	133	2.125	7.747	94	1.975	9.281
8	8	0	0	0	0	0	173	2.237	8.155	115	2.061	9.685
9	8	1	0	0	0	0	228	2.357	8.593	139	2.142	10.066
10	8	2	0	0	0	0	306	2.485	9.059	166	2.221	10.437
11	8	3	0	0	0	0	422	2.625	9.570	200	2.300	10.808
12	8	4	0	0	0	0	581	2.764	10.077	238	2.377	11.170
13	8	5	0	0	0	0	780	2.892	10.543	286	2.456	11.541
14	8	6	0	0	0	0	1,020	3.010	10.973	341	2.533	11.903
15	8	7	0	0	0	0	1,330	3.125	11.393	409	2.612	12.274
16	8	8	0	0	0	0	1,730	3.237	11.801	489	2.689	12.636
17	8	8	1	0	0	0	2,280	3.357	12.238	585	2.767	13.003
18	8	8	2	0	0	0	3,060	3.485	12.705	698	2.844	13.365
19	8	8	3	0	0	0	4,220	3.625	13.215	834	2.921	13.727
20	8	8	4	0	0	0	5,810	3.764	13.722	995	2.998	14.088
21	8	8	5	0	0	0.	7,800	3.892	14.189	1,200	3.079	14.469
22	8	8	6	0	0	0	10,200	4.010	14.619	1,430	3.156	14.831
23	8	8	7	0	0	0	13,300	4.125	15.038	1,720	3.235	15.202
24	8	8	8	0	0	0	17,300	4.237	15.447	2,050	3.312	15.564
25	8	8	8	1	0	0	22,800	4.357	15.884	2,460	3.391	15.935
26	8	8	8	2	0	0	30,600	4.485	16.351	2,940	3.468	16.297
27	8	8	8	3	0	0	42,200	4.625	16.861	3,520	3.547	16.668
28	8	8	8	4	0	0	58,100	4.764	17.368	4,220	3.625	17.035
29	8	8	8	5	0	0	78,000	4.892	17.834	5,060	3.704	17.406
30	8	8	8	6	0	0	102,000	5.010	18.265	6,070	3.783	17.777
31	8	8	8	7	0	0	133,000	5.125	18.684	7,300	3.863	18.153
32	8	8	8	8	0	0	173,000	5.239	19.100	8,770	3.943	18.529
33	8	8.	8	8	1	0	229,000	5.359	19.537	10,600	4.024	18.910
34	8	8	8	8	2	0	309,000	5.490	20.014	12,700	4.104	19.286
35	8	8	8	8	3	0	428,000	5.631	20.529	15,400	4.186	19.671
36	8	8	8	8	4	0	590,000	5.771	21.039	18,500	4.268	20.056
37	8	8	8	8	5	0	796,000	5.901	21.513	22,500	4.352	20.451
38	8	8	8	8	6	0	1,050,000	6.021	21.950	27,300	4.436	20.846
39	8	8	8	8	7	0	1,370,000	6.137	22.373	33,300	4.523	21.255
40	8	8	8	8	8	0	1,800,000	6.256	22.807	40,900	4.612	21.673
41	8	8	8	8	8	1	2,450,000	6.388	23.288	50,700	4.705	22.110
42	8	8	8	8	8	2	3,360,000	6.526	23.791	63,000	4.799	22.552
43	8	8	8	8	8	3	4,820,000	6.683	24.364	78,900	4.897	23.012
44	8	8	8	8	8	4	6,950,000	6.842	24.943	100,000	5.000	23.496

[a] The 95% confidence limits are log ($d \pm 0.3921$) and log ($d \pm 0.3042$) for the 10-fold and 4-fold series, respectively. If (log d_1/0.2743) − (log d_2/0.2743) (10-fold) or (log d_1/ 0.2128) − (log d_2/0.2128) (4-fold) is >2.000, then d_1 and d_2 are significantly different.

[b] These columns are included for convenience of reference. Since d is computed from the total number of positive responses irrespective of their distribution at each dilution level, sequences not represented in the table can be readily obtained by reference to the total number column.

surface. Experimentally, the procedure is comparable in many respects to the surface drop method, for which Badger and Pankhurst (1) demonstrated that the numbers of organisms in drops released from one filling of a pipette are highly reprodu cible.

Incubation and enumeration. The plates are incubated under the desired environmental conditions and positive responses at each dilution are noted. Microbial enumeration is achieved by consulting Table 1 to obtain an estimate of the number of orga-

TABLE 2. *Microbial numbers determined by the plate-dilution frequency and conventional plate count methods*

Microorganism	Microbial numbers (log)		
	Plate-dilution frequency		Plate count[a]
	No.	Range (95% confidence limits)	No.
Bacillus cereus var. *mycoides*	7.125	6.733–7.517	7.029
B. thuringiensis[b]	6.444	6.052–6.836	6.413
Escherichia coli	8.631	8.239–9.023	8.415
Penicillium sp.	7.137	6.745–7.529	7.043
Pseudomonas aeruginosa	8.359	7.967–8.751	8.076
Sarcina lutea	8.631	8.239–9.023	8.760
Serratia marcescens	9.239	8.847–9.631	9.505
Soil suspension			
Total fungi	4.631	4.239–5.023	4.519
Mucoraceous fungi	3.359	2.967–3.751	3.301
Total bacteria	6.239	5.847–6.631	6.342

[a] The same media and 10-fold dilution series were used for the plate count as for the plate-dilution frequency enumeration. Samples (0.1 ml) were spread over the surface of the plates to achieve uniform aeration conditions; numbers are the average of five replicates of the dilution giving 30 to 200 colonies per plate.

[b] Average of 12 tests on independent but similar systems. Plate count numbers (log) varied from 5.903 to 6.435 and were all within the corresponding plate-dilution frequency ranges.

nisms present in the most concentrated dilution of the dilution series. This table was derived from Table $VIII_2$ of Fisher and Yates (3), in which the estimate of $\log d$ (d = number of organisms) is based on the total numbers of positive and negative growth responses observed. As noted by Cochran (2), although this is not really a table of the most probable number, it appears to give results as precise as the most probable number method.

Derivations. Confidence limits are calculated as described by Fisher and Yates (3):

$$V_d = (\log 2 \log a)/n$$
$$S_d = \sqrt{V_d}$$
$$95\% \text{ confidence limits} = \log (d \pm t_{0.05} S_d)$$

where V_d is the variance of the mean of $\log d$, S_d is the standard error of $\log d$, d is the estimated organism density, a is the dilution ratio, n is the number of replicates per dilution, and t is Student's t derived from the normal probability tables. Thus, for a 10-fold dilution ratio:

$$V_d = (\log\ 2 \log 10)/8 = 0.03763$$
$$S_d = 0.1940$$
$$95\% \text{ confidence interval} = \log (d \pm 0.3921)$$
$$\text{or} = d/2.47 \text{ to } 2.47\, d$$

and for a fourfold dilution ratio

$$V_d = 0.02265$$
$$S_d = 0.1505$$
$$95\% \text{ confidence interval} = \log (d \pm 0.3042)$$
$$\text{or} = d/2.02 \text{ to } 2.02\, d$$

The significance of the difference between two estimated numbers d_1 and d_2 is tested by computing t (2):

$$t = (\log d_1 - \log d_2)/\sqrt{V_{d_1} + V_{d_2}}$$

Thus, for a 10-fold dilution ratio,

$$t = (\log d_1 - \log d_2)/0.2743$$

If $(\log d_1/0.2743) - (\log d_2/0.2743)$ is > 2.000 (tabular $t_{0.05}$), then d_1 and d_2 are significantly different. For a fourfold dilution ratio, if $(\log d_1/0.2188) - (\log d_2/0.2128)$ is > 2.000, then d_1 and d_2 are significantly different.

Values for $\log d/0.2743$ and $\log d/0.2128$ are included in Table 1 to facilitate direct determination of significant differences.

RESULTS AND DISCUSSION

As shown in Table 2, microbial numbers determined from 10-fold dilution series by the plate-dilution technique were comparable to corresponding numbers obtained by conventional plate counts. In practice, the more sensitive (Table 1) fourfold dilution series is preferable unless the numbers of specific organisms in the microbial system to be assayed cover a very wide and unpredictable range. The range for the 4-fold dilution is 46 to 100,000, and for the 10-fold dilution is 57 to 6,950,000 per ml of suspension; where higher numbers of organisms are anticipated, additional dilutions may be inserted before the dilution level designated as 1 in the series.

The simultaneous assay of many different types of organisms in complex systems is feasible, even under mobile laboratory conditions, because of the simplicity of the experimental procedure: enumeration of each microbial type requires only three prepoured plates; inoculation of diverse media can be achieved rapidly; incubation of the inoculated plates presents minimal storage problems; and data recording of the number of positive samples requires little effort compared with the time-consuming counting of individual colonies associated with conventional plate-count methods.

In addition to conserving time and labor and simplifying data-recording, the technique provides a potentially much broader scope than many other microbial assay methods. Because each microsample is inoculated on a well-defined area, the subsequent colonies can be scanned and examined in detail microscopically with relative ease. Thus, positive growth can be easily discerned, and, in addition, the development of

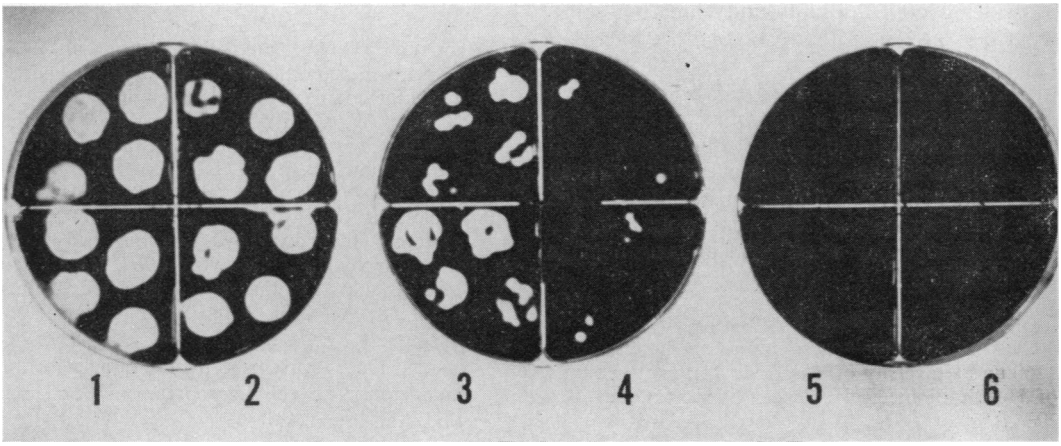

FIG. 1. *Appearance of plates inoculated with a fourfold dilution series of starch-hydrolyzing organisms The plates were flooded with Lugols iodine to delineate areas of starch hydrolysis. Proceeding from dilution level 1 to 6, the numbers of positive responses are 8, 8, 8, 4, 0, 0 (28 total), which is equivalent to 4,220 organisms per ml of dilution level 1 (Table 1).*

ecological relationships and microbial successions in the inoculated areas can be examined as a function of incubation time if desired. Possible contamination of the plates in other areas is readily recognizable and can be removed if necessary, leaving the rest of the plate unchanged. Similarly, once a positive response is observed, the appropriate areas can be removed (e.g., with a sterile 1.5-cm cork borer) and the plugs of media can be discarded or kept for further investigation of the organisms growing on them. The ability to eliminate spreading or antagonistic organisms and to allow each microsample to develop independently circumvents the restrictions on media richness and incubation time inherent in normal plate-count methods. However to reduce the necessity for extensive removal of spreading or rapidly sporulating colonies, it is preferable with certain systems (e.g., rich media incubated for long time periods) to use more plates and inoculate the microsamples farther apart; it is for this reason also that bisected and quadrisected plates are generally preferable to nondivided plates.

The plate and media conservation aspect of the technique facilitate the inclusion of incubation conditions and media not commonly used in other microbial assay methods. For example, silica gel can now be prepared (4; Sommers and Harris, *in press*) in quantities sufficient for its widescale application to microbial enumeration by the plate-dilution frequency technique. In addition to its applicability to autotroph determination, silica gel can also be used for unambiguous enumeration of heterotrophic organisms on a carbon nutrition basis, since appreciable growth on silica gel, unlike that on agar (5), can only result from the utilization of specific organic compounds as sole sources of carbon.

In a manner comparable to the dilution tube method, each microsample may be considered as a biochemical system and can be tested for the occurrence of specific biochemical transformations. Certain transformations, such as the dissolution of insoluble carbonates and phosphates, can be observed directly as a halo around the microsample. Enumeration of organisms causing other types of transformations may require the addition of chemicals to test for the absence of the specific substrate or the presence of its degradation products in the immediate vicinity of the microsample. The appearance of plates used for enumerating starch-hydrolyzing organisms is shown in Fig. 1. In practice, testing of the highest dilution plates would be delayed until colony growth was comparable to that produced by the more concentrated inocula. Alternatively, each microsample can be removed from the plate after small pieces of media cut from the periphery of the inoculated area have yielded positive responses; this approach is particularly appropriate for systems in which the enzymes or degradation products move rapidly through the medium to produce, if left unchecked, a uniformly positive response throughout the plate.

Results of experiments cited above and others still in progress indicate that the plate-dilution frequency technique can be applied successfully to the broad-spectrum assay of physiologically different microorganisms in complex systems with

a greater overall flexibility and scope but somewhat less precision than comparable microbial enumeration methods. It is probable that for many ecological circumstances the precision is sufficient to differentiate microbial levels likely to be of practical significance. Furthermore, for experiments requiring greater accuracy, the technique may still be useful for preliminary evaluation and screening of microbial systems to improve the efficiency of subsequent more detailed investigations.

ACKNOWLEDGMENTS

This investigation was supported by Hatch Project 1360 and University of Wisconsin National Institutes of Health Biomedical Sciences Support Grant 144-7034.

LITERATURE CITED

1. BADGER, E. H. M., AND E. S. PANKHURST. 1960. Experiments on the accuracy of surface drop bacterial counts. J. Appl. Bacteriol. 23:28–36.
2. COCHRAN, W. G. 1950. Estimation of bacterial densities by means of the "most probable number." Biometrics 6:105–116.
3. FISHER, R. A., AND F. YATES. 1953. Statistical tables for biological, agricultural and medical research. Oliver and Boyd, London.
4. FUNK, B., AND T. A. KRULWICH. 1964. Preparation of clear silica gels that can be streaked. J. Bacteriol. 88:1200–1201.
5. MARSHALL, K. C., J. S. WHITESIDE, AND M. ALEXANDER. 1960. Problems in the use of agar for the enumeration of soil microorganisms. Soil Sci. Soc. Am. Proc. 24:61–62.