APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1977, p. 675-680 Vol. 33, No. 3
 Printed in U.S.A.

Microtechnique for Most-Probable-Number Analysis

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Received for publication 20 September 1976

A microtechnique based on the most-probable-number (MPN) method has been developed for the enumeration of the ammonium-oxidizing population in soil samples. An MPN table for a research design ([8 by 12] i.e., 12 dilutions, 8 replicates per dilution) is presented. A correlation of 0.68 was found between MPNs determined by the microtechnique and the standard tube technique. Higher MPNs were obtained with the microtechnique with increased accuracy in endpoint determinations being a possible cause. Considerable savings of time, space, equipment, and reagents are observed using this method. The microtechnique described may be adapted to other microbial populations using various types of media and endpoint determinations.

Interest is increasing in the quantification of microbial processes, such as nitrification and denitrification, in natural and manipulated ecosystems. In such studies, direct determinations of microbial activity may be extremely difficult, and indirect measurements must be relied upon. Such determinations usually involve the enumeration of microorganisms having the potential to carry out a certain biochemical reaction.

The most-probable-number (MPN) technique is one means of determining the potential activity of a microbial population and has been used by Alexander (1-3), Focht and Joseph (10), Smith et al. (21), and Todd et al. (22). Standard methods of MPN analysis as used by these investigators have proved time consuming and tedious.

Various microsystems have been developed to alleviate these problems. These microsystems originated for such uses as enumeration of viable cells in bacterial cultures (6, 9), as a quick means of determining acid and gas production (12), for the study of heat destruction and bacterial spores (4), and for IMViC tests (12). Microsystem techniques have also been developed for microbial analysis of food (11). Curtis et al. (7) describe an MPN microsystem for assaying nitrifiers in water and sediment samples. Serial dilutions were made in test tubes, and aliquots were transferred to the microwells of a 25-compartment "repli-plate" for incubation and assay using the MPN tables described by Alexander (2). Darbyshire et al. (8) developed a micromethod for estimating the MPN of total bacterial and protozoan populations in soil using an 8 by 12 plate (12 dilutions, 8 replicates per dilution). In lieu of an MPN table, a description of the statistical method, based on the Poisson distribution, used to compute the MPNs was provided.

In this communication we describe a microanalysis system designed for both serial dilution and incubation. An MPN table designed for use with this system in enumerating microbial populations is provided. Results of a direct comparison between the microsystem and the standard tube method for estimating the ammonium-oxidizing microbial population are also presented.

MATERIALS AND METHODS

Microtiter system. A 0.05-ml aliquot of media is placed into each of the 8 by 12 wells of a sterile microplate. Aliquots of the soil suspension (0.05 ml) to be tested are pipetted into each of the first eight wells. Serial dilutions are then performed by using flame-sterilized loops calibrated to deliver 0.05 ml (Fig. 1). The loops are placed into the eight previously inoculated wells, rotated rapidly, and then moved to the next eight wells where they are again rapidly rotated. This process is continued until serial dilutions have been carried out across the plate. The result is 12 twofold serial dilutions with eight replicates at each dilution.

Nitrification method. Ammonium-calcium carbonate medium as described by Alexander and Clark (3) is placed in the wells. After inoculation and the performance of serial dilutions, the plates are covered with polypropylene tape and incubated for 3 weeks, as recommended by Alexander and Clark (3). However, conflicting reports exist concerning the optimum incubation time for ammonia- and nitrite-oxidizing microorganisms (7, 18). At the end of the incubation period carried out at room temperature each plate is scored by adding an indi-

cator (0.2 g of diphenylamine in 100 ml of concentrated H_2SO_4) to test for the presence of nitrate and/or nitrite (19). A blue color reaction indicates that these end products have formed, and the well is scored positive. The absence of a blue color is scored negative (Fig. 2).

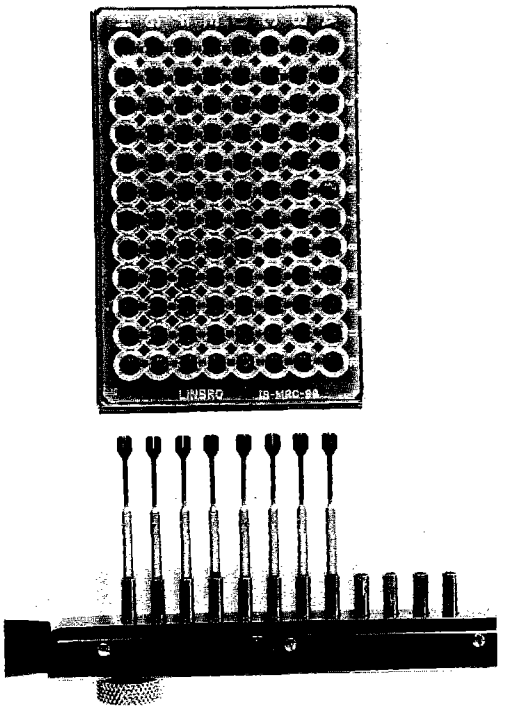

FIG. 1. *A microplate with dilution loops.*

MPN table. The statistical basis for the MPN technique is well established (5, 14, 15). For any combination of positive readings in a set of serial dilutions, it is possible to calculate the MPN estimate of population size, the standard error of the MPN, and its probability of occurrence. Table 1 presents MPN values and standard errors for a twofold dilution series with eight tubes per dilution. The codes (p_1, p_2, p_3) represent the number of positive tubes in three successive dilutions, where p_1 corresponds either to the highest dilution at which all tubes gave positive readings, or to the dilution showing the highest number of positive tubes.

Table 1 was prepared following de Man (17) and Parnow (20). For each possible code, from (0 0 1) to (n_1 n_2 n_3 −1), where n_j is the number of tubes in the j^{th} dilution, the MPN value was calculated. The standard error and the probability of occurrence of the MPN were also calculated. Then, for that MPN value, the probability of obtaining all codes from (0 0 0) to (n_1 n_2 n_3) were successively calculated and ordered from largest to smallest. These probabilities do not represent the probabilities of obtaining the given MPN, but rather the probability of obtaining the various codes, assuming that the MPN is the true population size. If the code in question had the largest probability of all codes of the MPN, then the MPN result was accepted. If not, then the code in question was consecutively compared with all codes in order of decreasing probabilities. If the code was encountered before the sum of the probabilities was greater than or equal to 0.95, it was accepted; otherwise, it was rejected. This prevents the investigator

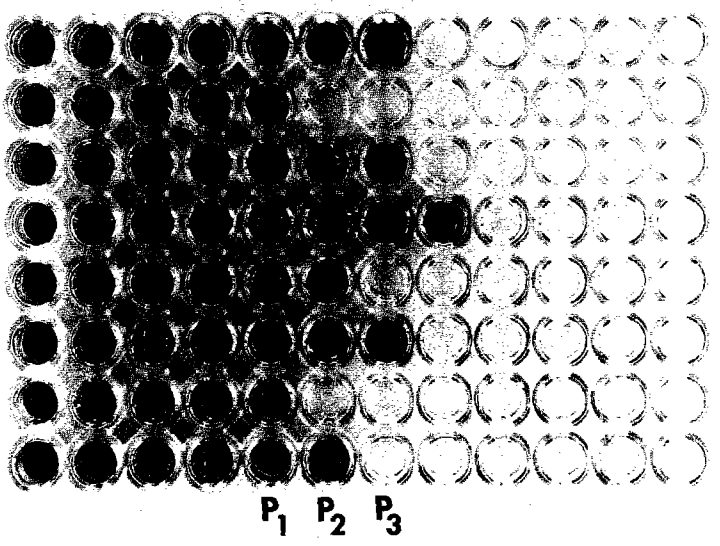

FIG. 2. *The result of a nitrification test. Wells 1–12 represent twofold serial dilutions, and rows A–H represent replicates.*

TABLE 1. *Most probable numbers*[a]

p_1	p_2	p_3	MPN	Standard error	p_1	p_2	p_3	MPN	Standard error
1	0	0	0.037	1.000	6	1	4	0.570	0.311
2	0	0	0.077	0.708	6	1	5	0.635	0.299
2	0	1	0.117	0.578	6	2	0	0.397	0.361
2	1	0	0.118	0.578	6	2	1	0.455	0.342
2	1	1	0.160	0.501	6	2	2	0.515	0.326
3	0	0	0.121	0.579	6	2	3	0.579	0.312
3	0	1	0.163	0.502	6	2	4	0.645	0.300
3	0	2	0.206	0.449	6	2	5	0.715	0.289
3	1	0	0.165	0.502	6	3	0	0.462	0.342
3	1	1	0.208	0.499	6	3	1	0.524	0.326
3	1	2	0.253	0.411	6	3	2	0.589	0.312
3	2	0	0.210	0.450	6	3	3	0.657	0.301
3	2	1	0.256	0.411	6	3	4	0.728	0.290
3	2	2	0.302	0.381	6	3	5	0.803	0.281
4	0	0	0.168	0.502	6	4	0	0.533	0.327
4	0	1	0.213	0.450	6	4	1	0.599	0.313
4	0	2	0.259	0.411	6	4	2	0.668	0.301
4	0	3	0.306	0.382	6	4	3	0.742	0.291
4	1	0	0.216	0.450	6	4	4	0.818	0.282
4	1	1	0.262	0.412	6	4	5	0.901	0.275
4	1	2	0.310	0.382	6	5	0	0.608	0.314
4	1	3	0.360	0.358	6	5	1	0.680	0.302
4	2	0	0.265	0.412	6	5	2	0.755	0.292
4	2	1	0.314	0.382	6	5	3	0.835	0.283
4	2	2	0.365	0.358	6	5	4	0.919	0.276
4	2	3	0.416	0.338	6	5	5	0.101	0.269
4	3	0	0.318	0.382	7	0	0	0.347	0.386
4	3	1	0.369	0.358	7	0	1	0.404	0.362
4	3	2	0.422	0.339	7	0	2	0.463	0.343
4	3	3	0.477	0.322	7	0	3	0.525	0.327
5	0	0	0.221	0.451	7	1	0	0.410	0.363
5	0	1	0.269	0.412	7	1	1	0.470	0.343
5	0	2	0.319	0.383	7	1	2	0.534	0.327
5	0	3	0.370	0.359	7	1	3	0.601	0.314
5	0	4	0.423	0.339	7	1	4	0.671	0.302
5	1	0	0.272	0.413	7	1	5	0.746	0.292
5	1	1	0.323	0.383	7	2	0	0.478	0.343
5	1	2	0.375	0.359	7	2	1	0.543	0.328
5	1	3	0.429	0.340	7	2	2	0.612	0.315
5	1	4	0.484	0.323	7	2	3	0.684	0.303
5	2	0	0.327	0.383	7	2	4	0.760	0.293
5	2	1	0.380	0.360	7	2	5	0.841	0.284
5	2	2	0.435	0.340	7	2	6	0.926	0.277
5	2	3	0.492	0.323	7	3	0	0.552	0.329
5	2	4	0.550	0.309	7	3	1	0.623	0.315
5	3	0	0.385	0.360	7	3	2	0.696	0.304
5	3	1	0.441	0.340	7	3	3	0.775	0.294
5	3	2	0.499	0.324	7	3	4	0.858	0.286
5	3	3	0.559	0.310	7	3	5	0.947	0.278
5	3	4	0.622	0.298	7	3	6	1.042	0.272
5	4	0	0.447	0.341	7	4	0	0.634	0.316
5	4	1	0.507	0.324	7	4	1	0.710	0.305
5	4	2	0.568	0.310	7	4	2	0.790	0.295
5	4	3	0.632	0.298	7	4	3	0.877	0.287
5	4	4	0.699	0.288	7	4	4	0.969	0.280
6	0	0	0.280	0.414	7	4	5	1.068	0.273
6	0	1	0.332	0.384	7	4	6	1.176	0.268
6	0	2	0.386	0.360	7	5	0	0.724	0.306
6	0	3	0.442	0.341	7	5	1	0.808	0.296
6	0	4	0.500	0.324	7	5	2	0.896	0.288
6	1	0	0.336	0.384	7	5	3	0.992	0.281
6	1	1	0.391	0.361	7	5	4	1.096	0.275
6	1	2	0.449	0.341	7	5	5	1.209	0.270
6	1	3	0.508	0.325	7	5	6	1.332	0.266

TABLE 1—*Continued*

p_1	p_2	p_3	MPN	Standard error	p_1	p_2	p_3	MPN	Standard error
7	6	0	0.825	0.298	8	4	3	1.054	0.287
7	6	1	0.917	0.290	8	4	4	1.171	0.282
7	6	2	1.018	0.283	8	4	5	1.301	0.278
7	6	3	1.126	0.278	8	4	6	1.444	0.274
7	6	4	1.244	0.273	8	4	7	1.607	0.272
7	6	5	1.376	0.269	8	5	0	0.870	0.302
7	6	6	1.523	0.266	8	5	1	0.972	0.295
8	1	0	0.495	0.346	8	5	2	1.085	0.289
8	1	1	0.564	0.330	8	5	3	1.208	0.284
8	1	2	0.637	0.317	8	5	4	1.346	0.281
8	1	3	0.714	0.306	8	5	5	1.500	0.278
8	1	4	0.796	0.296	8	5	6	1.679	0.277
8	2	0	0.574	0.331	8	5	7	1.886	0.277
8	2	1	0.648	0.318	8	6	0	0.999	0.297
8	2	2	0.728	0.307	8	6	1	1.117	0.292
8	2	3	0.812	0.297	8	6	2	1.248	0.288
8	2	4	0.903	0.289	8	6	3	1.396	0.285
8	2	5	1.001	0.283	8	6	4	1.565	0.283
8	2	6	1.106	0.277	8	6	5	1.760	0.283
8	3	0	0.662	0.319	8	6	6	1.993	0.285
8	3	1	0.744	0.308	8	6	7	2.279	0.288
8	3	2	0.831	0.299	8	7	0	1.153	0.295
8	3	3	0.925	0.291	8	7	1	1.293	0.291
8	3	4	1.027	0.285	8	7	2	1.452	0.289
8	3	5	1.138	0.279	8	7	3	1.636	0.289
8	3	6	1.259	0.275	8	7	4	1.855	0.291
8	3	7	1.395	0.271	8	7	5	2.124	0.294
8	4	0	0.759	0.310	8	7	6	2.465	0.301
8	4	1	0.850	0.301	8	7	7	2.921	0.312
8	4	2	0.947	0.293					

[a] p_1, Number of positive wells in the least concentrated dilution in which either all of the wells are positive or the greatest number are positive; p_2 and p_3, number of positive wells in the next two higher dilutions, respectively.

from accepting statistically improbable results.

Table 1 presents only those codes found to be statistically acceptable, as described above. Also, those codes not operationally feasible are excluded from Table 1. For example, if 8, 8, 8, 7, and 4 positive tubes were found in a twofold dilution series, then the sample would be scored as 8, 7, 4 rather than 8, 8, 7. Also, if the number of positive tubes in the three highest dilutions of a series were 8, 8, and 7, it would indicate that the dilution series used was not adequate for estimating the population size.

A program written for a Control Data Corporation Cyber 70/74 computer system was used to construct Table 1. It will calculate MPN values for any serial dilution series with any number of tubes per dilution, not necessarily all equal. The table presented lists three codes (p_1, p_2, p_3). However, the program has the capacity to determine any number of codes from p_1 to p_{12}. Copies of the program may be obtained from the authors.

MPN determination. A typical test result of enumerating ammonium-oxidizing bacteria in a sample is depicted in Fig. 2. Microwells containing dark-colored solutions are positive tests for nitrate-nitrite. Values for p_1, p_2, and p_3 are determined as follows: p_1 is the number of positive wells in the least-concentrated dilution in which either all the wells are positive or the greatest number are positive, and p_2 and p_3 are the number of positive wells in the next two higher dilutions, respectively. In Fig. 2, p_1 5 8, p_2 = 6, and p_3 = 4. These values give an MPN of 1.565 (Table 1). The MPN value is then multiplied by the dilution factor for p_2, in this case 64, to obtain a value of 100.16. To obtain the MPN in 1 ml of the original inoculum, this number is multiplied by 20 (20 × 100.16 = 2,003.2). Corrections must also be made for initial dilution factors and moisture content of samples.

RESULTS

The microplate technique was tested in a comparison with the standard tube technique as used by Alexander and Clark (3). Soil samples were collected from three experimental watersheds at the Coweeta Hydrologic Laboratory in southwestern North Carolina. Samples were taken from a watershed vegetated by an oak-hickory forest (watershed 18), a watershed which was planted in Eastern white pine in

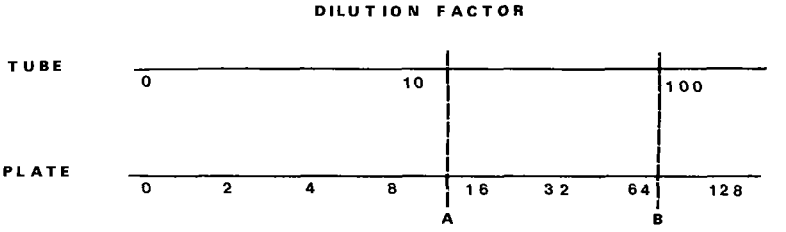

FIG. 3. *Theoretical considerations in comparing endpoint determinations for the tube (10-fold dilutions) and microplate (2-fold dilutions) techniques. The dashed lines represent two population sizes (A and B).*

1956 (watershed 17), and a watershed in the 10th year of successional regrowth after the herbiciding of all vegetation in 1966 (watershed 6). More complete watershed descriptions are provided elsewhere (16).

Three soil suspensions were prepared from each sample, and the MPN of nitrifying organisms in each suspension was determined by both the microplate and by the tube method. Results are given in Table 2. In both methods the highest number of nitrifiers was found on the successional, grass-covered watershed. Numbers of nitrifiers on the hardwood watershed were higher than those on the pine watershed in the suspensions analyzed by the microplate technique, but lower in the standard tube analysis. A correlation of 0.68 was obtained between the two methods of analysis, which is statistically significant at the 90% level.

TABLE 2. *Mean nitrifiers per gram of dry soil ± standard error in soil from three watersheds*[a]

Watershed	Mean nitrifiers/g of dry soil ± standard error	
	Microplate	Tube
Hardwood	2,009 ± 705	32± 4
Pine	1,178 ± 82	269± 47
Grass	3,261 ± 48	2,737± 0

[a] The mean represents three determinations of each sample.

DISCUSSION

The microplate technique gave consistently higher MPNs than did the tube technique (Table 2). Increased accuracy in endpoint determination in the twofold serial dilution of the microplate technique as opposed to determination in the 10-fold dilutions of the tube technique may account for this discrepancy. Consider the hypothetical situation depicted in Fig. 3. The tube technique would give an MPN of 10 for both A and B, since by the next serial dilution of 100-fold both populations would have been diluted to extinction. However, the microplate technique would give an MPN of 8 for population A and 64 for B. Cochran (5) discussed similar aspects of the comparison of 2-fold and 10-fold dilution series.

Reasons for the reversed order of the MPNs on the pine and hardwood watersheds by the two different techniques remain obscure. The extremely large standard error for the MPN estimate on the hardwood watershed using the microplate technique may suggest that one of the suspensions for this sample gave anomalous results. An increased sample size may result in qualitatively similar results by the two techniques. In any case, Table 2 reveals a fairly good agreement between the two procedures, granting the potentially more accurate endpoint determinations in the 1:2 dilutions of the microplate method as discussed above.

Advantages of the described MPN technique are the ease with which large numbers of samples may be processed, increased precision, improved sample replication (the microplate technique gives eight replicates per sample as compared with five for the tube technique), and savings in equipment, reagents, and incubation space. For the past year, this technique has been used in our laboratory for assessing the ammonia-oxidizing bacterial populations in soil samples from a variety of systems. The technique has also been used for denitrifiers and for enumerating separate populations of ammonia and nitrite oxidizers. Adaptations to other microbial populations are possible using various types of media and endpoint determinations.

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