J. Milk Food Technol. Vol. 38, No. 9, Pages 540-545 (September, 1975)

Understanding and Teaching the Most Probable Number Technique[1]

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(Received for publication Febraury 10, 1975)

ABSTRACT

The most probable number (MPN) technique is extensively used in food microbiology. However, because statistics involved often are cumbersome and it is difficult to obtain some of the widely scattered literature, many individuals are not able to delve into the subject to gain a thorough understanding of the method. To overcome these inherent difficulties, this review and discussion was prepared as an introduction and aid in presenting the MPN method to students and individuals working in food microbiology.

BACKGROUND

As we surveyed the literature dating back to Phelps' 1908 method (*14*) for calculating numbers of *Bacillus coli* (*Escherichia coli*) in dilution tests and continuing up to Parnows' 1972 publication (*13*) dealing with computer programs and the Most Probable Number (MPN), it was obvious that considerable time and effort has been spent in examining the statistical theory behind the method for determining bacterial densities by the tube dilution technique, i.e., the MPN method. What we propose to discuss is a teaching approach that will lend itself to the presentation of pertinent theory and data dealing with this subject to food microbiology oriented individuals. It is hoped that such an approach will enable us to convey both the advantages and disadvantages of this widely used method. We do not want to overlook the complexities of the statistics involved but, rather, we would like to suggest that the student not be overburdened with calculations that are not fundamental to understanding, using, and interpreting the MPN as a laboratory method.

It must be remembered that the mathematics of the MPN method are based on probability statistics and that results are directly related to the frequency of occurrence of a series of positive results most likely to occur when given numbers of bacteria are present in a sample.

Phelps (*14*) suggested that the number of *E. coli* in water samples be expressed as the reciprocal of the smallest portion of the sample in a geometric series of dilutions which gives a positive test. This method assumes that the dilutions of the sample are such that the final few dilutions give negative results. The method becomes confusing when "skips" occur in the sequence of dilutions and when more than one observation is obtained from a given sample.

A plate count can be converted directly into a number that corresponds to the most probable number of bacteria and this number is a linear function of the plate count. But, with a multiple tube result, the most probable number of bacteria is a logarithmic function of this result and further calculations are necessary to convert the results into the MPN value.

McCrady (*12*) was the first to discuss in depth the numerical interpretation of fermentation tube results in terms of both actual numbers and precision. In fact, the MPN tables he developed are still in use today. McCrady also emphasized the need to use many tubes, e.g., 10 per dilution to obtain meaningful (precise) results for accurate interpretation.

Since McCrady's early report, numerous authors have attempted to modify this cumbersome method of estimating bacterial densities. Greenwood and Yule (*5*), Wolman and Weaver (*19*), Stein (*17*), and Reed (*15*) emphasized the need to use large numbers of replicate tubes within series of dilutions to obtain accurate data. Halvorson and Ziegler (*6, 7, 8, 9*) and Halvorson and Moeglein (*10*) published articles dealing with the application of statistics to problems in bacteriology. In these articles not only was the role of large numbers of replicate tubes discussed, but also the effects of the nature of the bacterial population were stressed, in regard to experimental accuracy. All these workers pointed out that much of the previous work in this area dealt with estimates of bacterial densities determined by inoculating aliquots of a single dilution into a number of replicate tubes, and that such estimates did not yield accurate data. Greenwood and Yule (*5*) were given credit for first describing the general case of using several dilutions inoculated into a series of tubes to make such estimates. Halvorson and Ziegler (*7*) showed that when three effective dilutions are used to determine the bacterial population, the accuracy is independent of the number of organisms, and dependent only on the number of tubes per dilution. This is in contrast to the case in which only one dilution is used, the reason being that deviation from the mode (most probable value) is narrowed as more tubes are used. That is, the probability of a more accurate assessment of the bacterial density is

[1]*Florida Agricultural Experiment Stations Journal Series No. 5785.*

increased as the number of tubes is increased. In 1935, Halvorson and Ziegler (*9*) published a comparison of the multiple tube method (dilution method), the plate count, and the direct microscopic count. They found that, as a rule, the dilution method gave higher values for bacterial populations than did the plate count method. The direct count method gave the same value as the plating and dilution methods only when used on cultures before they had entered the death phase, indicating that viability is another variable that must be considered when evaluating bacterial densities.

The following formula for the estimation of the MPN was given by Thomas (*18*):

$$\text{M.P.N.} = \frac{\text{Number of positive tubes}}{\sqrt{(\text{Number of ml of sample in negative tubes}) \times (\text{Number of ml of sample in all tubes})}}$$

This formula is not restricted as to number of tubes and dilutions used. The author noted, however, that where increased precision is desired, a corresponding increase in the number of tubes tested is necessary. In addition, if tubes in the lowest dilution are all positive, the omission of this lowest dilution from the computation will improve the agreement between the formula and table values.

McCarthy et al. (*11*) demonstrated a substantial mathematical bias in MPN values relative to plate counts. With the agar plate count method as the control estimator, 10 replicate MPN's indicated an average bias of + 29%, + 10%, + 6%, and − 4% when compared to the plate counts' arithmetic average, geometric mean, median, and harmonic mean, respectively. The precision of 10 replicate plate counts was at least three times that of replicate MPN values. Perhaps Woodward (*20*) summed it up the best: "The lack of precision of MPN estimates of bacterial densities is generally recognized—at least by those who perform these tests."

With the foregoing discussion serving as a broad overview of the MPN method, let us then get to some definite features of the method that need to be related to the food microbiology student who may someday find himself or herself in a quality control or regulatory laboratory working with the MPN.

BASIC ASSUMPTIONS

Several assumptions are involved with the MPN as a method. First, it is assumed that organisms are distributed randomly throughout the sample. This means that an organism can be found in any portion of the sample and that clumping or attracting and/or repelling forces do not exist. This is important when one considers the various types of foods that are examined in most laboratories, especially when the sample is comprised of particulate matter. Second, one has to assume that each aliquot from the liquid will exhibit growth whenever such an aliquot contains one or more organisms and said aliquot is incubated in an "appropriate" growth medium. Freedom from contamination of supplies and equipment is also assumed, as is appropriate technical expertise.

APPLICATIONS

Disadvantages often associated with the MPN include time, space, and material considerations. However, the advantages of the MPN technique allow numerous useful applications: (*a*) Estimates can be made of a population using any number of dilutions—just as with the plate count method. (*b*) Accuracy can be adjusted by increasing the number of tubes per dilution—this consideration has been previously discussed. (*c*) Sample size can be quite large—this is becoming more important as limitations of the membrane filter technique become more widely known (*4, 16*). (*d*) Sensitivity and recovery with the MPN is generally better at low population levels than with the plate count (*2*)—the reason for this being that it is possible to use larger sample volumes than with the plate count method. (*e*) Recovery may be better in liquid than solid media, depending on the particular sample involved (*6*). (*f*) Materials can be prepared in advance and readily used under field conditions. (*g*) If the appropriate medium is available, estimates can be made of any organism(s).

USING MPN TABLES

Practically speaking, the most widely used form of the MPN involves three-tube and five-tube (per dilution) analyses for coliforms. Once the actual experimental manipulations of samples and materials are completed, regardless of the type of MPN analysis, tubes go into an appropriate incubator. After a prescribed incubation period, observations are recorded, further analyses may be conducted and then the appropriate MPN table is sought. The reader is referred to the 1960 edition of *Standard Methods for the Examination of Water and Wastewater* (*1*) for exhaustive tabular 5-tube MPN values and confidence limits. Some unusual sample volume combinations and their appropriate confidence limits are shown in Table 1; Table 2 presents MPN values and confidence limits for 3-tube series. Examples of MPN results and their interpretation are given in Table 3.

TABLE 1.[a] *MPN and 95 per cent confidence limits for various combinations of positive results in the following series:*
A—five 10-ml tubes
B—five 10-ml, five 1-ml & five 0.1-ml tubes
C—five 10-ml, one 1-ml & one 0.1-ml tubes
D—one 50-ml & five 10-ml tubes
E—one 50-ml, five 10-ml & five 1-ml tubes
F—five 50-ml, five 10-ml & five 1-ml tubes

	No. of Positive Tubes out of: Five 10-ml Tubes	MPN per 100 ml	Limits of MPN Lower	Upper
A	0	0	0	6.0
	1	2.2	0.1	12.6
	2	5.1	0.5	19.2
	3	9.2	1.6	29.4
	4	16.0	3.3	52.9
	5	∞	8.0	∞

TABLE 1. (*Continued*).

B

Five 10-ml Tubes	Five 1-ml Tubes	Five 0.1-ml Tubes	MPN per 100 ml	Limits of MPN Lower	Limits of MPN Upper
0	0	1	2	<0.5	7
0	0	2	4	<0.5	11
0	1	0	2	<0.5	7
0	1	1	4	<0.5	11
0	1	2	6	<0.5	15
0	2	0	4	<0.5	11
0	2	1	6	<0.5	15
0	3	0	6	<0.5	15
1	0	0	2	<0.5	7
1	0	1	4	<0.5	11
1	0	2	6	<0.5	15
1	0	3	8	1	19
1	1	0	4	<0.5	11
1	1	1	6	<0.5	15
1	1	2	8	1	19
1	2	0	6	<0.5	15
1	2	1	8	1	19
1	2	2	10	2	23
1	3	0	8	1	19
1	3	1	10	2	23
1	4	0	11	2	25
2	0	0	5	<0.5	13
2	0	1	7	1	17
2	0	2	9	2	21
2	0	3	12	3	28
2	1	0	7	1	17
2	1	1	9	2	21
2	1	2	12	3	28
2	2	0	9	2	21
2	2	1	12	3	28
2	2	2	14	4	34
2	3	0	12	3	28
2	3	1	14	4	34
2	4	0	15	4	37
3	0	0	8	1	19
3	0	1	11	2	25
3	0	2	13	3	31
3	1	0	11	2	25
3	1	1	14	4	34
3	1	2	17	5	46
3	1	3	20	6	60
3	2	0	14	4	34
3	2	1	17	5	46
3	2	2	20	6	60
3	3	0	17	5	46
3	3	1	21	7	63
3	4	0	21	7	63
3	4	1	24	8	72
3	5	0	25	8	75
4	0	0	13	3	31
4	0	1	17	5	46
4	0	2	21	7	63
4	0	3	25	8	75
4	1	0	17	5	46
4	1	1	21	7	63
4	1	2	26	9	78
4	2	0	22	7	67
4	2	1	26	9	78
4	2	2	32	11	91
4	3	0	27	9	80
4	3	1	33	11	93
4	3	2	39	13	106
4	4	0	34	12	96
4	4	1	40	14	108
4	5	0	41	14	110
4	5	1	48	16	124
5	0	0	23	7	70
5	0	1	31	11	89
5	0	2	43	15	114
5	0	3	58	19	144
5	0	4	76	24	180
5	1	0	33	11	93
5	1	1	46	16	120
5	1	2	63	21	154
5	1	3	84	26	197
5	2	0	49	17	126
5	2	1	70	23	168
5	2	2	94	28	219
5	2	3	120	33	281
5	2	4	148	38	366
5	2	5	177	44	515
5	3	0	79	25	187
5	3	1	109	31	253
5	3	2	141	37	343
5	3	3	175	44	503
5	3	4	212	53	669
5	3	5	253	77	788
5	4	0	130	35	302
5	4	1	172	43	486
5	4	2	221	57	698
5	4	3	278	90	849
5	4	4	345	117	999
5	4	5	426	145	1,161
5	5	0	240	68	754
5	5	1	348	118	1,005
5	5	2	542	180	1,405

C

Five 10-ml Tubes	One 1-ml Tube	One 0.1-ml Tube	MPN per 100 ml	Limits of MPN Lower	Limits of MPN Upper
0	0	0	0		5.9
0	1	0	2	0.050	13
1	0	0	2.2	0.050	13
1	1	0	4.4	0.52	14
2	0	0	5	0.54	19
2	1	0	7.6	1.5	19
3	0	0	8.8	1.6	29
3	1	0	12	3.1	30
4	0	0	15	3.3	46
4	0	1	20	5.9	48
4	1	0	21	6.0	53
5	0	0	38	6.4	330
5	0	1	96	12	370
5	1	0	240	12	3,700
5	1	1		88	

D

One 50-ml Tube	Five 10-ml Tubes	MPN per 100 ml	Limits of MPN Lower	Limits of MPN Upper
0	1	1	<0.5	4
0	2	2	<0.5	6
0	3	4	<0.5	11
0	4	5	1	13
1	0	2	<0.5	6
1	1	3	<0.5	9
1	2	6	1	15
1	3	9	2	21
1	4	16	4	40

E

One 50-ml Tube	Five 10-ml Tubes	Five 1.ml Tubes	MPN per 100 ml	Limits of MPN Lower	Limits of MPN Upper
0	0	1	1	<0.5	4
0	0	2	2	<0.5	6
0	1	0	1	<0.5	4
0	1	1	2	<0.5	6
0	1	2	3	<0.5	8
0	2	0	2	<0.5	6
0	2	1	3	<0.5	8
0	2	2	4	<0.5	11
0	3	0	3	<0.5	8
0	3	1	5	<0.5	13
0	4	0	5	<0.5	13

TABLE 1. (*Continued*).

	1	0	0	1	<0.5	4
	1	0	1	3	<0.5	8
	1	0	2	4	<0.5	11
	1	0	3	6	<0.5	15
	1	1	0	3	<0.5	8
	1	1	1	5	<0.5	13
	1	1	2	7	1	17
	1	1	3	9	2	21
	1	2	0	5	<0.5	13
	1	2	1	7	1	17
	1	2	2	10	3	23
	1	2	3	12	3	28
	1	3	0	8	2	19
	1	3	1	11	3	26
	1	3	2	14	4	34
	1	3	3	18	5	53
	1	3	4	21	6	66
	1	4	0	13	4	31
	1	4	1	17	5	47
	1	4	2	22	7	69
	1	4	3	28	9	85
	1	4	4	35	12	101
	1	4	5	43	15	117
	1	5	0	24	8	75
	1	5	1	35	12	101
	1	5	2	54	18	138
	1	5	3	92	27	217
	1	5	4	161	39	<450

	Five 50-ml Tubes	Five 10-ml Tubes	Five 1-ml Tubes	MPN per 100 ml	Limits of MPN	
					Lower	Upper
F	0	0	1	1	<0.5	2
	0	0	2	1	<0.5	2
	0	1	0	1	<0.5	2
	0	1	1	1	<0.5	2
	0	1	2	1	<0.5	2
	0	2	0	1	<0.5	2
	0	2	1	1	<0.5	2
	0	3	0	1	<0.5	2
	1	0	0	1	<0.5	2
	1	0	1	1	<0.5	2
	1	0	2	1	<0.5	2
	1	0	3	2	<0.5	4
	1	1	0	1	<0.5	2
	1	1	1	1	<0.5	2
	1	1	2	2	<0.5	4
	1	2	0	1	<0.5	2
	1	2	1	2	<0.5	4
	1	2	2	2	<0.5	4
	1	3	0	2	<0.5	4
	1	3	1	2	<0.5	4
	1	4	0	2	<0.5	4
	2	0	0	1	<0.5	2
	2	0	1	1	<0.5	2
	2	0	2	2	<0.5	4
	2	0	3	2	<0.5	4
	2	1	0	1	<0.5	2
	2	1	1	2	<0.5	4
	2	1	2	2	<0.5	4
	2	2	0	2	<0.5	4
	2	2	1	2	<0.5	4
	2	2	2	3	1	7
	2	3	0	2	<0.5	4
	2	3	1	3	1	7
	2	4	0	3	1	7
	3	0	0	2	<0.5	4
	3	0	1	2	<0.5	4
	3	0	2	2	<0.5	4
	3	1	0	2	<0.5	4
	3	1	1	2	<0.5	4
	3	1	2	3	1	7
	3	1	3	4	1	9
	3	2	0	3	1	7
	3	2	1	3	1	7
	3	2	2	4	1	9
	3	3	0	3	1	7
	3	3	1	4	1	9
	3	4	0	4	1	9
	3	4	1	4	1	9
	3	5	0	5	2	12
	4	0	0	2	<0.5	4
	4	0	1	3	1	7
	4	0	2	3	1	7
	4	0	3	4	1	9
	4	1	0	3	1	7
	4	1	1	4	1	9
	4	1	2	4	1	9
	4	2	0	4	1	9
	4	2	1	4	1	9
	4	2	2	5	2	12
	4	3	0	5	2	12
	4	3	1	5	2	12
	4	3	2	6	2	14
	4	4	0	6	2	14
	4	4	1	7	3	17
	4	5	0	7	3	17
	4	5	1	8	3	19
	5	0	0	4	1	9
	5	0	1	4	1	9
	5	0	2	6	2	14
	5	0	3	7	3	17
	5	0	4	8	3	19
	5	1	0	5	2	12
	5	1	1	6	2	14
	5	1	2	7	3	17
	5	1	3	9	3	21
	5	2	0	6	2	14
	5	2	1	8	3	19
	5	2	2	10	4	23
	5	2	3	12	4	28
	5	2	4	15	5	37
	5	2	5	18	6	53
	5	3	0	9	3	21
	5	3	1	11	4	26
	5	3	2	14	5	34
	5	3	3	18	6	53
	5	3	4	21	7	66
	5	3	5	25	8	78
	5	4	0	13	5	31
	5	4	1	17	6	47
	5	4	2	22	7	70
	5	4	3	28	9	85
	5	4	4	35	11	101
	5	4	5	43	14	118
	5	5	0	24	8	75
	5	5	1	35	11	101
	5	5	2	54	18	140
	5	5	3	92	27	218
	5	5	4	161	39	424

[a]This table taken from *Standard Methods for the Examination of Water and Wastewater* (*1*).

At this point it is questionable as to whether or not one needs to be able to derive the formula that is responsible for the tabular MPN values. We are going under the assumption that it is not necessary. It is important that one be able to read the table properly and to understand the significance of the results. Assuming that the tabulated numbers are correctly computed, the next consideration should be an appreciation of the "confidence interval" surrounding a given value in this table. That is, the tabular MPN value really represents a

TABLE 2.[a] *MPN and 95 per cent confidence limits for various combinations of positive results using three tubes each with volumes of 10, 1, 0.1 ML**

No. of Positive Tubes out of:			MPN per 100 ml	Limits of MPN	
Three 10-ml Tubes	Three 1-ml Tubes	Three 0.1-ml Tubes		Lower	Upper
0	0	0		0	
0	0	1	3		9
0	0	2	6		
0	0	3	9		
0	1	0	3	0.085	13
0	1	1	6.1		
0	1	2	9.2		
0	1	3	12		
0	2	0	6.2		
0	2	1	9.3		
0	2	2	12		
0	2	3	16		
0	3	0	9.4		
0	3	1	13		
0	3	2	16		
0	3	3	19		
1	0	0	3.6	0.085	20
1	0	1	7.2	0.87	21
1	0	2	11		
1	0	3	15		
1	1	0	7.3	0.88	23
1	1	1	11		
1	1	2	15		
1	1	3	19		
1	2	0	11	2.7	36
1	2	1	15		
1	2	2	20		
1	2	3	24		
1	3	0	16		
1	3	1	20		
1	3	2	24		
1	3	3	29		
2	0	0	9.1	1.0	36
2	0	1	14	2.7	37
2	0	2	20		
2	0	3	26		
2	1	0	15	2.8	44
2	1	1	20		
2	1	2	27		
2	1	3	34		
2	2	0	21	3.5	47
2	2	1	28		
2	2	2	35		
2	2	3	42		
2	3	0	29		
2	3	1	36		
2	3	2	44		
2	3	3	53		
3	0	0	23	3.5	120
3	0	1	39	6.9	130
3	0	2	64		
3	0	3	95		
3	1	0	43	7.1	210
3	1	1	75	14	230
3	1	2	120	30	380
3	1	3	160		
3	2	0	93	15	380
3	2	1	150	30	440
3	2	2	210	35	470
3	2	3	290		
3	3	0	240	36	1,300
3	3	1	460	71	2,400
3	3	2	1,100	150	4,800
3	3	3		460	

* For values not given, *approximate* lower and upper limits may be estimated as 21 per cent of the MPN for the lower and 395 per cent for the upper. The confidence limits given are more exact calculations for the tube results most likely to be encountered. The results for which confidence limits are not given my be expected to be less than 1 per cent of the results commonly observed.

[a]This table taken from *Standard Methods for the Examination of Water and Wastewater* (*1*).

TABLE 3. *Examples of determining MPN estimates*

Example	Dilution of sample 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	Reported positive tubes	MPN (org/gram)
a	3/3*	2/3	0/3	0/3	0/3	3-2-0	93
b	3/3	3/3	3/3	1/3	0/3	3-1-0	43,000
c	3/3	2/3	2/3	0/3	0/3	3-2-2	210
d	3/3	3/3	0/3	1/3	0/3	3-0-1	3,900
e	3/3	3/3	3/3	3/3	3/3	3-3-3	<1,100,000
f	3/3	0/3	1/3	0/3	0/3	3-0-1	39
g	3/3	2/3	1/3	1/3	0/3	3-2-2	210
h	3/3	3/3	2/3	2/3	1/3	3-2-3	290
i	2/3	2/3	2/3	2/3	0/3	2-2-2	350
j	0/3	1/3	0/3	0/3	0/3	0-1-0	3

$$\frac{\text{* Numerator}}{\text{Denominator}} = \frac{\text{Number of positive tubes}}{\text{Number of tubes inoculated}}$$

Directions for use of MPN table (3-tube). When more than three dilutions are used in a series of dilutions, the results from only three of these dilutions are used in determining the MPN value. Select the highest dilution in which all three tubes are positive and the next two higher dilutions (examples: a,b,c,d,e,f). When a positive result is noted in a dilution higher than the three selected according to the rule, it should be added to the result for the highest dilution chosen (examples: g,h). If more than three dilutions are made and none of the dilutions show three positive tubes, use the data which incorporate the positve tubes that are present (example: i). For those cases in which the numbers of microorganisms are very low (example: j), incorporate the positive result such that it is represented by the middle dilution if possible. The MPN value for 0-1-0 or 1-0-0 is nearly identical and well within published confidence limits.

range and not an absolute value. MPN estimates are often credited with a precision they do not live up to.

Confidence limits for MPN estimates can be computed on the basis of a logarithmically normal distribution. As the MPN estimate is biased (as we have previously discussed), these confidence limits are not symmetric about the MPN estimate. Woodward (*20*) published tables of confidence limits for three- and five-tube multiple dilution assays and pointed out that for a three-tube test the 95% confidence limits cover a 33-fold range from approximately 14 to 458% of the actual MPN estimate. For a five-tube multiple dilution test, the 95% confidence limits cover a 13-fold range from approximately 24 to 324% of the MPN. It is highly doubtful that a student would have occasion to compute confidence limits, but they should understand that such limits exist.

Another point that should be made clear to students just becoming familiar with MPN tables is that you really need only one "master" table. That is to say, once the sample volume per tube is established, it's just a matter of moving the decimal point for the MPN value that corresponds to the dilution being reported.

TEACHING APPROACH

In teaching the application of the MPN method, we try to progress through a series of experiments which enable the student to grasp both the positive and negative

aspects of the method. A lecture approach using much of the previous discussion as background is given first. Then, in the laboratory, a water sample is analyzed using lactose broth rather than lauryl sulfate tryptose broth so that an "estimation of total numbers" from growth-positive tubes can be made as well as for presumptive coliforms (gas-positive tubes). Then the rationale for transfer of gas-positive tubes into Brilliant Green broth is given and a discussion of reading the tubes is presented. Final calculations are made and an understanding of the confidence limits to be placed on the results is stressed. A discussion of using the tables, with the possibilities of unusual combinations of sample volumes [Table 1 (*1*)], is presented and possible applications are discussed. The value of the MPN method in survey work, screening for low populations of organisms, and using large sample volumes is stressed.

Then a more involved experiment, quantification of low numbers of enteropathogenic *E. coli* from a meat sample, is done. Emphasis is placed on the care required in marking all tubes, plates, and individual isolates, so that following serological identification of the isolates, results may be related back to the original sample dilutions. As the course progresses, working with staphylococci, enterococci, and other organisms, the method becomes second nature to the student.

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