

statistical aspects of the microbiological analysis of foods

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CHAPTER 8

ERRORS ASSOCIATED WITH DILUTION COUNT AND PRESENCE/ABSENCE METHODS

In its simplest form, the dilution count method consists of serial dilution of the initial food homogenate (or culture) followed by inoculation of an appropriate volume of each dilution into one or more tubes of culture medium (agar or broth). Following incubation, the number of tubes showing evidence of growth (e.g. colonial growth in agar; turbidity in broth; production of acid and gas in MacConkey's Bile Salt broth in the test for presumptive coliforms, etc.) are recorded. The cultures showing no evidence of growth are assumed not to have received a viable inoculum ($q = 1$), i.e. to have received < 1 viable cell or cell aggregate; an assumption which may not always be correct. Similarly, any culture showing growth is assumed to have received at least one viable propagule ($p = 1$). If each viable propagule is assumed to be capable of producing a growth response, then the proportion ($P_{x=0}$) of cultures at any one dilution which receive no viable inoculum after inoculation with a mean inoculum level of m propagules is given by the first value of the Poisson expansion, i.e.:

$$P_{(x=0)} = e^{-m} \quad (\text{Table 3.2})$$

Similarly, the proportion becoming turbid:

$$P_{(x>1)} = 1 - P_{(x=0)} = 1 - e^{-m}$$

8.1 Tube dilution series and Most Probable Number counts

When more than one culture is inoculated at each dilution level, and several dilution levels are inoculated, one would expect a graded response with all tubes positive at the lowest dilution and none, or only a few tubes, positive at the highest dilution tested. This is illustrated in Table 8.1 for dilutions from 10^{-3} to 10^{-8} of culture initially containing 2×10^5 viable cells/ml, and 10, 5 or 1 replicate tubes at each dilution level.

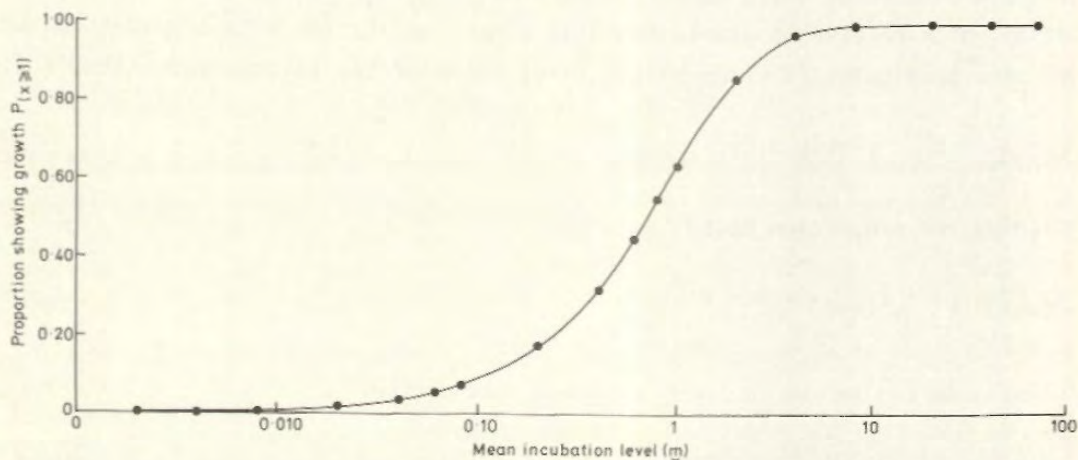
Figure 8.1 illustrates the shape of the expected dose-response curve, based on Poisson distribution. It has been pointed out by several workers (e.g. Eisenhardt and Wilson, 1943) that the response curve may be less steep. This could arise through systematic technical error (Meynell and Meynell, 1965) and as a result of contagious distribution, reflecting clumping, sublethal damage, etc.

TABLE 8.1

EXPECTED PROPORTIONAL RESPONSE IN A MULTIPLE-TUBE DILUTION SERIES.
INITIAL INOCULUM CONTAINS 2×10^5 PROPAGULES/ml; DILUTION FACTOR = 10

Dilution tested	Mean inoculum level/tube* (m)	Expected proportion tubes		Expected no. tubes positive for		
		positive ($P_{x \geq 1}$)	negative ($P_{x=0}$)	1 tubes inoculated	5 tubes inoculated	10 tubes inoculated
10 ⁻³	200	0.999	0.001	1	5	10
10 ⁻⁴	20	0.999	0.001	1	5	10
10 ⁻⁵	2	0.865	0.135	1	4	9
10 ⁻⁶	0.2	0.181	0.819	0	1	2
10 ⁻⁷	0.02	0.020	0.980	0	0	0
10 ⁻⁸	0.002	0.002	0.998	0	0	0

* Assuming 1 ml of dilution added to medium in each tube.



8.1 Expected proportion of cultures giving positive response for various mean inoculum levels (m)

8.2 Single tube dilution tests

If only one tube is inoculated at any given dilution level, only two responses are possible, i.e. negative ($P_{x=0}$) or positive ($P_{x \geq 1}$). Where presence or absence tests (P/A) are used at various dilution levels, it is not uncommon for "skips" to occur, e.g.

Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Response	+	+	+	-	+

The probability for a negative response (i.e. less than one "viable propagule/tube") is dependent upon the level of organisms in the original sample. For instance, if the above series were derived from a sample containing (say) 5×10^3 viable propagules/ml, the probability for the observed results at the 10^{-4} and 10^{-5} dilutions (assuming 1 ml inoculum) would be 0.62 and 0.05, respectively. Hence, the result obtained on the 10^{-4} dilution might be expected to occur in 61 tests out of 100 (i.e. 6 in 10) and that on the 10^{-5} dilution in 5 tests out of 100 (i.e. 1 in 20). Neither of these results would be improbable. However, if the original inoculum contained only 1×10^3 viable propagules, the relative frequencies for the observed results in the 10^{-4} and 10^{-5} dilutions would be 0.90 and < 0.01 , respectively. Hence, the observed result at 10^{-4} dilution could be expected to occur in 9 tests out of 10, but that at the 10^{-5} dilution might reasonably be expected only in one out of more than 100 tests. Such a result would be improbable, but not impossible. The probabilities for such an observation are listed for various values of m in Table 8.2, from which it can be seen that a "skip" could occur by chance at least once in every 20 tests for mean inoculum levels between 0.5 and 3 viable propagules/culture at the 10^{-5} dilution. When such skips occur, the analyst is not able to decide whether the observed result could have been expected to occur merely by chance (i.e. $P > 0.05$) or whether the probability of its occurrence is so remote that the result should be ignored.

Calculation of the initial contamination level using data derived from a single-tube assay, at best, can give only an approximate indication, as illustrated in Ex. 8.1. However, it should be noted that the occurrence of a "skip" can reduce the range of the 95% confidence limits (Ex. 8.1.B) compared with the sharp demarcation shown in Ex. 8.1.A. Such results should therefore always be recorded, though in general it is dubious whether it is justifiable to attempt to derive probable contamination levels for single-tube dilution (P/A) tests.

It should be noted that the calculation of these confidence limits assumes Poisson distribution (which may not be correct) and ignores any effects of dilution or other technical errors. In the single-tube, as in multiple-tube series (§ 8.3.2) the confidence limits for the results can be improved by reducing the dilution interval (i.e. 1 in 2 dilution is better than 1 in 4, which is better than 1 in 10), since the response curve obtained would be smoothed out to some extent (e.g. the 95% confidence limits for an initial dilution of 10^{-3} followed by sequential 2-fold dilutions, positive at 2^1 and 2^2 but negative at 2^3 and 2^4 would be 200 to 12,000 propagules/unit of original sample, c.f. Ex. 8.1.A.

TABLE 8.2

PROBABILITIES OF OCCURRENCE OF "SKIPS" IN A SINGLE-TUBE DILUTION SERIES FOR VARIOUS INOCULA LEVELS

Mean inoculum level in dilution $10^{-z}^{\dagger}$ ($\bar{m}$)	Probability* of occurrence of	
	negative result ($P_{x=0}$) at dilution $10^{-z}^{\dagger}$	positive result ($P_{x>1}$) at dilution $10^{-(z+1)}^{\dagger}$
0.01	0.99	<< 0.01
0.05	0.95	0.01
0.1	0.90	0.01
0.2	0.82	0.02
0.3	0.74	0.03
0.4	0.67	0.04
----- **		
0.5	0.61 (ca. 1 in 2)	0.05 (1 in 20)
0.6	0.55	0.06
0.7	0.50	0.07
0.8	0.45	0.08
0.9	0.41	0.09
1.0	0.37	0.10
2.0	0.14	0.18
3.0	0.05 (1 in 20)	0.26 (1 in 4)
----- **		
4.0	0.02	0.33
5.0	< 0.01	0.40
30.0	<< 0.01	0.95

* To 2 significant places.

** 95% confidence limits for both events to occur.

$^{\dagger} 10^{-z}$ = dilution level e.g. 10^{-1} ; $10^{-(z+1)}$ = next dilution level, e.g. 10^{-2} .

Example 8.1. Calculation of initial contamination level from a single-tube dilution series

Assume 10-fold serial dilutions, with 1 ml inoculum from each dilution and the following results obtained:

Dilution	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Response in series: (A)	+	+	-	-	-
(B)	+	+	-	+	-

(A) series: the presumed initial contamination level lies between 1×10^3 and 1×10^4 propagules/ml of original sample. But from Table 8.2 the probability that a positive result $P_{(x>1)} = 1 - P_{(x=0)}$ will occur in at least 1 in 20 tests at dilution 10^{-z} (i.e. 10^{-3} in this example) is associated with a mean inoculum level ranging upwards from 0.05 propagules/unit volume of dilution (10^{-z}). The 1 in 20 chance of a negative result $P_{(x=0)}$ at the $10^{-(z+1)}$ dilution ranges downwards from a mean inoculum level of 3 propagules/unit volume of dilution $10^{-(z+1)}$, i.e. < 30 propagules/unit volume of dilution 10^{-z} .

Hence, although the apparent contamination level is 1000- 10,000 propagules per unit of original sample, the 95% confidence limits range from 0.05×10^3 (i.e. 50) to 30×10^3 (i.e. 30,000). Therefore result (A) indicates, with 95% confidence, that the contamination level lies between 50 and 30,000 viable propagules/unit of original sample!

(B) series: the apparent contamination level is either $> 10^3$ and $< 10^4$ or $> 10^5$ and $< 10^6$ propagules, (i.e. between 10^3 and 10^6 propagules/unit of original sample.) Applying the same approach as that applied for the (A) series, the 95% limits for the count of $10^3 - 10^4$ are 50 - 30,000 propagules, whilst the limits for the count being $10^5 - 10^6$ are 5,000 - 3,000,000 propagules. The likelihood of the combinations occurring in at least 1 in 20 tests is limited so that the most probable contamination level (95% confidence limit) lies between 5,000 and 30,000 propagules/unit of original sample. These limits are shown (**) in Table 8.2.

8.3 Multiple-tube dilution series

8.3.1 Multiple tubes at a single dilution level

If more than one tube is inoculated at a given dilution level, either all tubes show a positive or negative reaction (when numbers of organisms inoculated are very high or very low, respectively), or a graded response is achieved with some tubes remaining sterile whilst others exhibit growth (see last 2 columns, Table 8.1). For a single dilution level, it may be shown that the probability for no organisms (i.e. < 1 fertile propagule) occurring in a sample of volume v when the true density = m organisms/unit volume is given by $p = e^{-vm}$. If n samples each of volume v are tested and, of these, s samples are sterile, then the proportion of sterile samples (s/n) provides an estimate of p . Hence:

$$\hat{p} = s/n = e^{-vm} \quad \text{and}$$

$$m = -(1/v) \ln (s/n) = -(2.303/v) \log (s/n)$$

where $\ln$ and $\log$ are logarithms to bases e and 10 , respectively. The value m is the most probable number ($\hat{m}$) of organisms per ml.

If the probability that a sample is "sterile" is p_0 , then the probability that s/n samples are sterile is given by the binomial expansion as

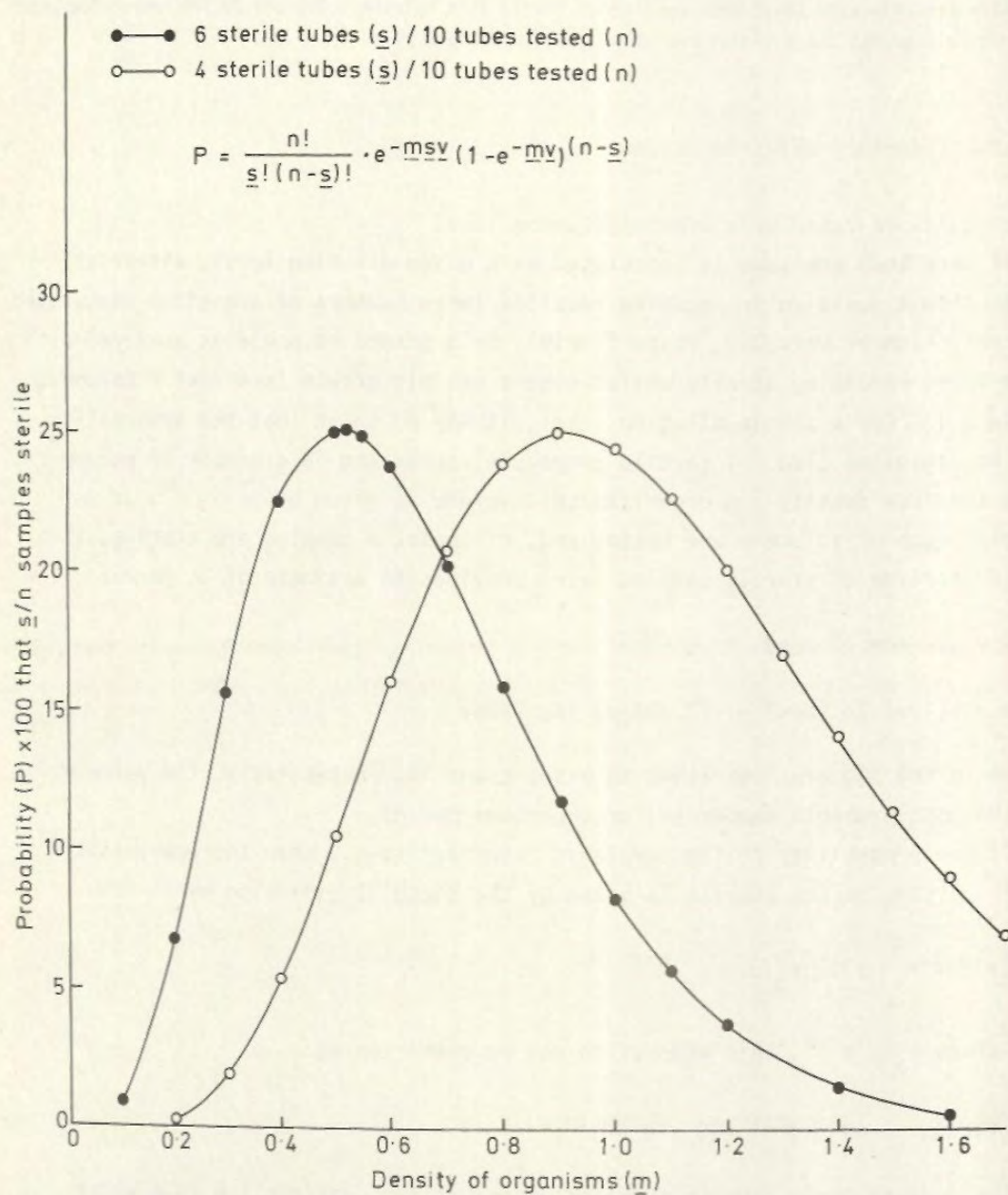
$$[n!/s!(n-s)!] p_0^s (1-p_0)^{n-s}$$

but since $p_0 = e^{-vm}$, this expression may be rewritten as:

$$[n!/s!(n-s)!] e^{-vms} (1 - e^{-vm})^{(n-s)}$$

The derived terms from this expansion permit the construction of a curve showing probability (p) against density of organisms (m). This is illustrated (Fig. 8.2) for two values of s/n and assuming $v = 1$ ml; when $s/n = 0.6$, the

most probable number (MPN) of organisms inoculated ($\hat{m}$) = 0.51 propagules/ml; when $s/n = 0.4$, MPN ($\hat{m}$) = 0.92 propagules/ml. These values are the same as those derived using $\hat{m} = -(1/v)\ln(s/n)$. From the shapes of the curves it is clear that many other densities of organisms could also give the observed ratios (s/n) of 0.4 or 0.6, though with lower probability.



8.2 Probability distribution for multiple-tube dilution test at single dilution level

8.3.2 Multiple-tubes at several dilution levels

In practice, more than one dilution level is necessary since the precision of the MPN is very poor when the number of organisms inoculated is likely to give either all positive or all negative results. When all replicate tests are positive, maximum probability occurs when m is infinite, so that the estimated density ($\hat{m}$) is also infinite. When all results are negative, the estimated density is zero at maximum probability. Thus, a single dilution is of value only if the numbers of organisms inoculated (mv) is chosen so that some tests give a positive and others a negative result.

When more than one dilution level is used, an estimate of population density ($\hat{m}$) can be derived for each dilution level. In a series with inoculum volume v_i and s_i tubes sterile out of n_i tubes tested, the estimated density of organisms for the i th dilution is given by:

$$\hat{m}_i = -(2.303/v_i) \log (s_i/n_i)$$

However, since the best estimate for each dilution will vary in precision, it is not possible to take the arithmetic mean of the estimates for each dilution level in order to derive an overall estimate. Two primary methods exist for deriving the best estimate of numbers.

8.3.2.1 Stevens' method for multiple dilution levels. Fischer and Yates (1954) published Tables based on the mean fertile level (x) and the mean sterile level (y) where x = number of positive results at all dilution levels divided by the number of cultures (n) at each level, and y = number of levels minus x . Using the same notation as before (i.e. s_i is the number of sterile cultures (negative results) out of n_i tubes at the i th dilution level), then

$$x = \Sigma [(n_1 - s_1) + (n_2 - s_2) + \dots + (n_i - s_i)]/n$$

and

$$y = i - x$$

One then enters the appropriate Table (Table 8.3) for values of x or y according to the number of dilution levels and the dilution ratio in order to determine a factor K . The estimated number of organisms is then given by:

$$\log \lambda = x \log \alpha - K$$

where λ = estimate of number of organisms ($= \hat{m}$) and α = dilution ratio. Hence for 10-fold dilutions:

$$\log \lambda = x - K$$

TABLE 8.3

K VALUES FOR DETERMINING THE DENSITY OF ORGANISMS BY THE DILUTION METHOD
(from Fisher & Yates, 1954)

Value of x	Value of K for			10-fold dilutions*	
	4-fold dilutions with			(> 3 levels)	
	4 levels	5	> 6	$x \leq 1$	$x > 1$
0.0	-	-	-		0.763
0.1	-	-	-		0.768
0.2	-	-	-		0.768
0.3	-	-	-		0.760
0.4	0.704	0.706	0.707	0.761	0.747
0.5	-	-	-	0.740	0.736
0.6	0.615	0.617	0.618	0.733	0.733
0.7	-	-	-	0.736	0.736
0.8	0.573	0.576	0.577	0.744	0.749
0.9	-	-	-	0.753	0.753
1.0	0.555	0.558	0.559	0.763	0.763
1.5	0.545	0.551	0.553		
2.0	0.537	0.548	0.551		
2.5	-	0.545	0.552		

* When $x > 1$ enter Table with decimal part of x only.

The variance of the estimate of $\log \lambda$ is given by:

$$1/n (\log 2) (\log \alpha)$$

Hence for 10-fold dilutions:

$$s_{\log \lambda}^2 = 1/n (\log 2) = 0.301/n$$

This method is applicable to 2-, 4- and 10-fold dilution ratios with not less than 4, 4 or 3 dilution levels, respectively. Unfortunately, the method does not discriminate in any way between valid and invalid series of results (see § 8.3.2.2 below).

8.3.2.2 Most probable number method for multiple dilution levels. The most probable number method is an extension of the concept used above (§ 8.3.1) for multiple tubes at a single level. Assuming that for i dilution levels we have the following proportions of sterile cultures:

$$s_1/n_1, s_2/n_2, s_3/n_3 \dots s_i/n_i$$

then the probability that these events should all happen at once can be derived from the product of the probabilities that each individual event would occur. As before, a graph of the probability against m shows a single maximum, and the value of m at that maximum probability is taken as the Most Probable Number ($\hat{m}$).

As pointed out by Cochran (1950), the value of $\hat{m}$ cannot be written down explicitly, but can be derived from the equation:

$$\begin{aligned} s_1 v_1 + s_2 v_2 + s_3 v_3 + \dots + s_i v_i = & [(n_1 - s_1) v_1 e^{-v_1 m} / (1 - e^{-v_1 m})] + \\ & + [(n_2 - s_2) v_2 e^{-v_2 m} / (1 - e^{-v_2 m})] + [(n_3 - s_3) v_3 e^{-v_3 m} / (1 - e^{-v_3 m})] + \dots + \\ & + [(n_i - s_i) v_i e^{-v_i m} / (1 - e^{-v_i m})] \end{aligned}$$

Methods for solving this equation by iteration have been given by Halvorson and Ziegler (1933) and Finney (1947). More recently De Man (1975, 1983) used computer techniques to derive the composite probability distribution curves for various combinations of results, dilution levels and culture numbers.

For routine purposes, standard Tables can be used to derive the most probable number of organisms in a sample. Such Tables, cited in the publications of Woodward (1957), DHSS (1969), AOAC (1975), APHA (1976), ICMSF (1978), do not always provide data on the confidence limits which can be applied to the MPN values and in some instances include many unacceptable and improbable results (i.e. nonsense codons; De Man, 1975).

Both Woodward (1957) and De Man (1975) have pointed out that the occurrence of many improbable results indicates some malfunction in the performance of the MPN tests. As an extreme example De Man (1975) cites the case of a result 0, 0, 10 positive results from a 10-tube 3-dilution series ($10^0 - 10^{-2}$). The MPN for such a result can be calculated as 0.9 propagules/ml with 95% confidence limits of 0.5 - 1.7. However, the probability of obtaining such a result is only 0.17×10^{-24} when the mean inoculum level is 0.9 organisms/ml. Furthermore, it should be noted also that the method of Stevens (§ 8.3.2.1) would give the same estimate of the number of organisms ($\lambda = 1.73$; 95% CL = 0.78 - 3.85) for this codon (0-0-10) as for the codons 0-10-0 and 10-0-0; however, only the latter would be an acceptable result and the MPN according to De Man (1975) would be 2.3 with 95% CL of 1.2 - 5.8.

In order to assess whether an improbable result has been obtained, it is possible to test the goodness of fit of observed results with expected results using the χ^2 test. However, Moran (1954 a,b; 1958) has proposed a more severe

test which is applicable, however, only when the extreme dilutions give approximately 0% and 100% positive results. Moran's test is performed by calculating a value T_{obs} , which is given by:

$$T_{\text{obs}} = \sum f_m (n - f_m)$$

where f_m = the number of positive results at each dilution and n is the total number of tubes at each dilution. The expected value of T (T_{exp}) and the standard error of T (SE_T) are then obtained from Table (8.4) and a value M is derived for the significance of the difference between T_{obs} and T_{exp} , viz:

$$M = (T_{\text{obs}} - T_{\text{exp}}) / SE_T$$

Values of $M > 1.645$ or > 2.326 occur by chance with probabilities of < 0.05 and < 0.01 respectively. The use of this method is illustrated in Ex. 8.2.

TABLE 8.4

EXPECTED VALUES OF T (T_{exp}) AND STANDARD ERROR OF T (SE_T) FOR MORAN'S TEST

n	T_{exp}			SE_T		
	2*	4*	10*	2*	4*	10*
5	20	10	6.02	5.69	4.02	3.12
6	30	15	9.03	7.63	5.40	4.19
7	42	21	12.64	9.75	6.89	5.35
8	56	28	16.86	12.02	8.50	6.60
9	72	36	21.67	14.46	10.22	7.93
10	90	45	27.09	17.03	12.04	9.34
11	110	55	33.11	19.74	13.96	10.83
12	132	66	39.74	22.58	15.97	12.39
13	156	78	46.96	25.55	18.07	14.02
14	182	91	54.79	28.63	20.24	15.71
15	210	105	63.22	31.83	22.51	17.74
16	240	120	72.25	35.14	24.85	19.28
17	272	136	81.88	38.56	27.27	21.16
18	306	153	92.12	42.07	29.75	23.08
19	342	171	102.95	45.70	32.31	25.08
20	380	190	114.39	49.41	34.94	27.11
30	870	435	261.90	91.53	64.72	50.22
40	1560	780	469.61	141.49	100.05	77.63

n = number of tubes inoculated from each dilution.

* Dilution factor.

Example 8.2. Use of Moran's test for validity of multiple-tube dilution counts

Assume the following results obtained in a multiple-tube dilution assay, using 10 tubes at each dilution level:

Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
No. tubes positive (A):	10	10	8	4	0
(B):	10	9	7	5	0

For series (A):

$$\begin{aligned}
 T_{\text{obs}} &= 10(10 - 10) + 10(10 - 10) + 8(10 - 8) + 4(10 - 4) + 0(10 - 0) \\
 &= 0 + 0 + 16 + 24 + 0 \\
 &= 40
 \end{aligned}$$

From Table 8.4:

$$T_{\text{exp}} = 27.09 \text{ and } SE_T = 9.34$$

then:

$$M_A = (40 - 27.09)/9.34 = 1.38$$

Since $M_A < 1.45$, the observed results for series (A) do not differ significantly ($P > 0.05$) from those expected and the series is valid for a MPN calculation.

For series (B):

$$\begin{aligned}
 T_{\text{obs}} &= 10(10 - 10) + 9(10 - 9) + 7(10 - 7) + 5(10 - 5) + 0(10 - 0) \\
 &= 0 + 9 + 21 + 25 + 0 \\
 &= 54
 \end{aligned}$$

From Table 8.4:

$$T_{\text{exp}} = 27.09 \text{ and } SE_T = 9.34$$

then

$$M_B = (54 - 27.09)/9.34 = 2.88$$

Since $M_B > 2.326$, the observed number of positive tubes differs significantly ($p < 0.01$) from the expected number and therefore series (B) provides an improbable result from which to calculate a MPN.

Cochran (1950) pointed out that in planning an MPN test the following decisions are required: (1) the range of volumes to be tested; (2) the dilution factor to be used; and (3) how many tests to be inoculated at each dilution level. In planning such a test, the aim is to obtain equal precision across a number of possible levels of cell density, i.e. the ratio between the standard error and the true density should be unity. When the density of organisms is expected to be reasonably constant (e.g. in analysis of a water supply of known

quality) it is possible to select inoculum levels such that the lowest dilution volume (i.e. largest volume of inoculum) should contain at least one propagule and that the highest dilution (i.e. smallest volume) should contain not more than two propagules. However, when the upper density of organisms nears the limit of the test for the dilutions chosen, the probability of obtaining all positive cultures is greater for a small number of replicates at each dilution level than for a large number of replicates. Hence, it is safer to use the rule that the maximum number should not exceed 1 propagule per volume of the highest dilution. This can be done by "guesstimating" the limits ($\hat{m}_L$ and $\hat{m}_H$) between which the true density would be expected to lie; then $V_H > 1/\hat{m}_H$ and $V_L < 1/\hat{m}_L$; where V_H = largest volume (or volume at lowest dilution level) and V_L = smallest volume (or volume at highest dilution level).

For example, if the probable true density (m) lies between 10 and 800 propagules/ml, the largest volume to be inoculated per test should not exceed 1/10 ml (0.1 ml of original sample or 1 ml of the 10^{-1} dilution) and the smallest volume should not exceed 1/800 (0.00125 ml original sample or 1.25 ml of the 10^{-3} dilution). In practice, this range could be covered either by using 1 ml per test of each of three 10-fold dilutions (i.e. 1/10, 1/100 and 1/1000) or by using 1 ml per test of (say) one 10-fold and four 4-fold dilutions (1/10, 1/40, 1/160, 1/640, 1/2560).

The choice of dilution ratio affects precision significantly over the range of dilution ratios between two and ten only if the total number of tests is not kept constant. However, use of the lower dilution ratio results in the precision being kept more constant throughout the range of densities tested (i.e. $1/V_H - 1/V_L$). This is illustrated in Fig. 8.3 for dilution ratios of two and ten.

The standard error of the MPN can be derived (Cochran, 1950) from the following equation (assuming a cell density between $1/V_H$ and $1/V_L$, a limited number of tests at each dilution and a dilution ratio of five or less):

$$SE_{\log \hat{m}} = 0.55 \sqrt{(\log a)/n}$$

where a = dilution ratio and n = number tests at each dilution. For 10-fold dilutions, where the standard error may peak at the value for $\hat{m}$,

$$SE_{\log \hat{m}} = 0.58 \sqrt{\log a/n} = 0.58 \sqrt{1/n}$$

Values for various levels of a and n , together with the factors for deriving standard errors and 95% confidence limits are given in Table 8.5.

TABLE 8.5

STANDARD ERROR OF $\log \hat{m}$ AND FACTOR FOR CONFIDENCE LIMITS (COCHRAN, 1950)

No. of samples per dilution n	SE($\log_{10} \hat{m}$) for dilution ratio (α)				Factor* for 95% confidence limits with dilution ratio (α)			
	2	4	5	10	2	4	5	10
1	0.301	0.427	0.460	0.580	4.00	7.14	8.32	14.45
2	0.213	0.302	0.325	0.410	2.67	4.00	4.47	6.61
3	0.174	0.246	0.265	0.335	2.23	3.10	3.39	4.68
4	0.150	0.214	0.230	0.290	2.00	2.68	2.88	3.80
5	0.135	0.191	0.206	0.259	1.86	2.41	2.58	3.30
6	0.123	0.174	0.188	0.237	1.76	2.23	2.38	2.98
7	0.114	0.161	0.174	0.219	1.69	2.10	2.23	2.74
8	0.107	0.151	0.163	0.205	1.64	2.00	2.12	2.57
9	0.100	0.142	0.153	0.193	1.58	1.92	2.02	2.43
10	0.095	0.135	0.145	0.183	1.55	1.86	1.95	2.32

* In deriving the 95% confidence limits (CL) the MPN should be multiplied or divided by the appropriate factor. For example if the MPN for a 5-tube dilution series with a 10-fold dilution ratio is 22 propagules/ml, the 95% CL are given by (22×3.30) and $(22/3.30)$ i.e. 72.5 - 6.7.

The standard error increases (precision decreases) on either side where the likelihood of all positive or all negative results increases. The precision is greatest when approximately equal numbers of positive and negative results are most likely to occur. The optimum mean inoculum (about 1.6 propagules/culture) is more likely to be missed by chance in a ten-fold than in a two-fold dilution series, hence the graph for ten-fold dilutions fluctuates more than does that for two-fold dilutions (Fig. 8.3).

These CL assume that the logarithm of the MPN is normally distributed and differ from those cited by De Man (1975), who derived CL from the computer-printed histograms of the distribution functions for different combinations of tube numbers and positive results. However, De Man's Tables (Tables 8.6 and 8.7) cover only 10-fold dilution ratios; hence the data in Table 8.4 at least allow some confidence limits to be assigned to MPNs derived from other dilution series.

TABLE 8.6

MPN TABLE FOR 3 x 1, 3 x 0.1, AND 3 x 0.01 g (ml) (DE MAN, 1983)

Number of positive results			MPN	Category when No. of tests					Confidence limits			
				1	2	3	5	10	> 95%	> 95%	> 99%	> 99%
0	0	0	< 0.30						0.00	0.94	0.00	1.40
0	0	1	0.30	3	2	2	2	1	0.01	0.95	0.00	1.40
0	1	0	0.30	2	1	1	1	1	0.01	1.00	0.00	1.60
0	1	1	0.61	0	3	3	3	3	0.12	1.70	0.05	2.50
0	2	0	0.62	3	2	2	2	1	0.12	1.70	0.05	2.50
0	3	0	0.94	0	0	0	0	3	0.35	3.50	0.18	4.60
1	0	0	0.36	1	1	1	1	1	0.02	1.70	0.01	2.50
1	0	1	0.72	2	2	1	1	1	0.12	1.70	0.05	2.50
1	0	2	1.1	0	0	0	3	3	0.4	3.5	0.2	4.6
1	1	0	0.74	1	1	1	1	1	0.13	2.00	0.06	2.70
1	1	1	1.1	3	3	2	2	2	0.4	3.5	0.2	4.6
1	2	0	1.1	2	2	1	1	1	0.4	3.5	0.2	4.6
1	2	1	1.5	3	3	3	3	2	0.5	3.8	0.2	5.2
1	3	0	1.6	3	3	3	3	2	0.5	3.8	0.2	5.2
2	0	0	0.92	1	1	1	1	1	0.15	3.50	0.07	4.60
2	0	1	1.4	2	1	1	1	1	0.4	3.5	0.2	4.6
2	0	2	2.0	0	3	3	3	3	0.5	3.8	0.2	5.2
2	1	0	1.5	1	1	1	1	1	0.4	3.8	0.2	5.2
2	1	1	2.0	2	2	1	1	1	0.5	3.8	0.2	5.2
2	1	2	2.7	0	3	3	3	3	0.9	9.4	0.5	14.2
2	2	0	2.1	1	1	1	1	1	0.5	4.0	0.2	5.6
2	2	1	2.8	3	2	2	2	1	0.9	9.4	0.5	14.2
2	2	2	3.5	0	0	0	0	3	0.9	9.4	0.5	14.2
2	3	0	2.9	3	2	2	2	1	0.9	9.4	0.5	14.2
2	3	1	3.6	0	3	3	3	3	0.9	9.4	0.5	14.2
3	0	0	2.3	1	1	1	1	1	0.5	9.4	0.3	14.2
3	0	1	3.8	1	1	1	1	1	0.9	10.4	0.5	15.7
3	0	2	6.4	3	3	2	2	2	1.6	18.1	1.0	25.0
3	1	0	4.3	1	1	1	1	1	0.9	18.1	0.5	25.0
3	1	1	7.5	1	1	1	1	1	1.7	19.9	1.1	27.0
3	1	2	12	3	2	2	2	1	3	36	2	44
3	1	3	16	0	0	0	3	3	3	38	2	52
3	2	0	9.3	1	1	1	1	1	1.8	36.0	1.2	43.0
3	2	1	15	1	1	1	1	1	3	38	2	52
3	2	2	21	2	1	1	1	1	3	40	2	56
3	2	3	29	3	3	3	2	2	9	99	5	152
3	3	0	24	1	1	1	1	1	4	99	3	152
3	3	1	46	1	1	1	1	1	9	198	5	283
3	3	2	110	1	1	1	1	1	20	400	10	570
3	3	3	> 110									

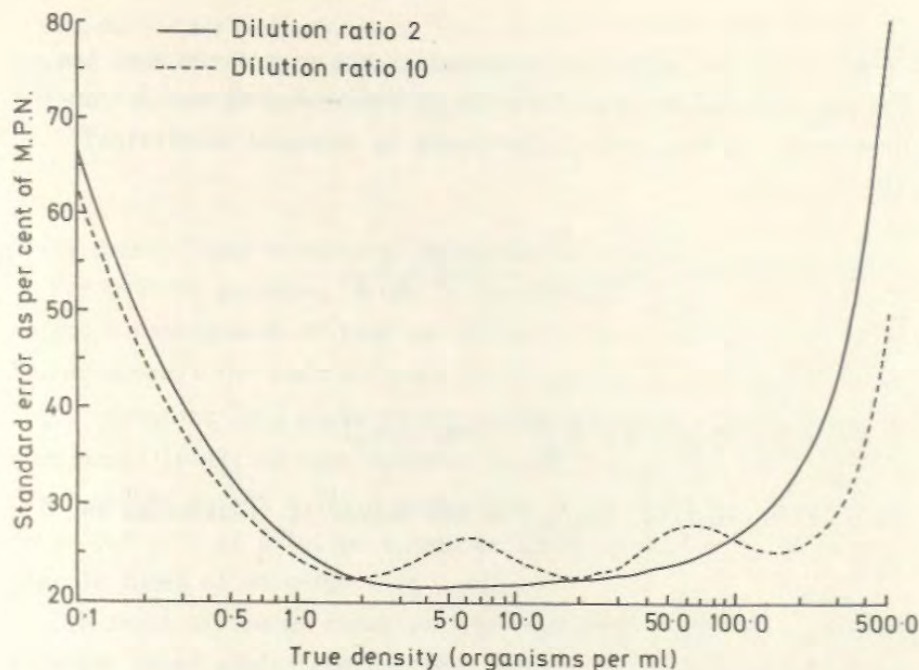
TABLE 8.7

MPN TABLE FOR 5 x 1, 5 x 0.1, AND 5 x 0.01 g (ml) (DE MAN, 1983)

Number of positive results			MPN	Category* when No. of tests					Confidence limits			
				1	2	3	5	10	≥ 95%	≥ 95%	≥ 99%	≥ 99%
0	0	0	< 0.18						0.00	0.65	0.00	0.93
0	0	1	0.18	2	2	2	1	1	0.00	0.65	0.00	0.93
0	1	0	0.18	1	1	1	1	1	0.01	0.65	0.00	0.93
0	1	1	0.36	3	3	3	2	2	0.07	0.99	0.02	1.40
0	2	0	0.37	3	2	2	2	1	0.07	0.99	0.02	1.40
0	2	1	0.55	0	0	0	3	3	0.17	1.40	0.09	2.10
0	3	0	0.56	0	3	3	3	3	0.17	1.40	0.09	2.10
1	0	0	0.20	1	1	1	1	1	0.02	0.99	0.01	1.40
1	0	1	0.40	2	1	1	1	1	0.07	1.00	0.02	1.40
1	0	2	0.60	0	0	3	3	3	0.17	1.40	0.09	2.10
1	1	0	0.40	1	1	1	1	1	0.07	1.10	0.03	1.40
1	1	1	0.61	3	2	2	2	1	0.17	1.40	0.09	2.10
1	1	2	0.81	0	0	0	0	3	0.33	2.20	0.20	2.80
1	2	0	0.61	2	1	1	1	1	0.18	1.40	0.09	2.10
1	2	1	0.82	3	3	3	3	2	0.35	2.20	0.20	2.80
1	3	0	0.83	3	3	3	3	2	0.33	2.20	0.20	2.80
1	3	1	1.0	0	0	0	0	3	0.3	2.2	0.2	2.8
1	4	0	1.1	0	0	0	0	3	0.3	2.2	0.2	2.8
2	0	0	0.45	1	1	1	1	1	0.08	1.40	0.04	2.10
2	0	1	0.68	2	1	1	1	1	0.18	1.50	0.09	2.10
2	0	2	0.91	0	3	3	3	3	0.33	2.20	0.20	2.80
2	1	0	0.68	1	1	1	1	1	0.19	1.70	0.10	2.30
2	1	1	0.92	2	2	1	1	1	0.33	2.20	0.20	2.80
2	1	2	1.2	0	0	3	3	3	0.4	2.5	0.2	3.4
2	2	0	0.93	1	1	1	1	1	0.34	2.20	0.20	2.80
2	2	1	1.2	3	3	2	2	2	0.4	2.5	0.2	3.4
2	2	2	1.4	0	0	0	0	3	0.6	3.4	0.4	4.4
2	3	0	1.2	3	2	2	2	1	0.4	2.5	0.2	3.4
2	3	1	1.4	0	3	3	3	3	0.6	3.4	0.4	4.4
2	4	0	1.5	0	3	3	3	3	0.6	3.4	0.4	4.4
3	0	0	0.78	1	1	1	1	1	0.21	2.20	0.12	2.80
3	0	1	1.1	1	1	1	1	1	0.4	2.2	0.2	2.9
3	0	2	1.3	3	3	3	2	2	0.6	3.4	0.4	4.4
3	1	0	1.1	1	1	1	1	1	0.4	2.5	0.2	3.4
3	1	1	1.4	2	1	1	1	1	0.6	3.4	0.4	4.4
3	1	2	1.7	3	3	3	3	2	0.6	3.4	0.4	4.4
3	2	0	1.4	1	1	1	1	1	0.6	3.4	0.4	4.4
3	2	1	1.7	2	2	2	1	1	0.7	3.9	0.5	5.1
3	2	2	2.0	0	3	3	3	3	0.7	3.9	0.5	5.2
3	3	0	1.7	2	2	1	1	1	0.7	3.9	0.5	5.2
3	3	1	2.1	3	3	3	2	2	0.7	3.9	0.5	5.2
3	3	2	2.4	0	0	0	3	3	1.0	6.6	0.7	9.4
3	4	0	2.1	3	3	2	2	2	0.7	4.0	0.5	5.2
3	4	1	2.4	0	3	3	3	3	1.0	6.6	0.7	9.4
3	5	0	2.5	0	0	0	3	3	1.0	6.6	0.7	9.4
4	0	0	1.3	1	1	1	1	1	0.4	3.4	0.3	4.4

TABLE 8.7 (continued)

Number of positive results			MPN	Category* when No. of tests					Confidence limits			
				1	2	3	5	10	≥95%	≥95%	≥99%	≥99%
4	0	1	1.7	1	1	1	1	1	0.6	3.4	0.4	4.4
4	0	2	2.1	3	2	2	2	2	0.7	3.9	0.5	5.2
4	0	3	2.5	0	0	0	0	3	1.0	6.6	0.7	9.4
4	1	0	1.7	1	1	1	1	1	0.6	3.9	0.4	5.1
4	1	1	2.1	1	1	1	1	1	0.7	4.1	0.5	5.3
4	1	2	2.6	3	3	2	2	2	1.0	6.6	0.7	9.4
4	1	3	3.1	0	0	0	0	3	1.0	6.6	0.7	9.4
4	2	0	2.2	1	1	1	1	1	0.7	4.8	0.5	6.1
4	2	1	2.6	2	1	1	1	1	1.0	6.6	0.7	9.4
4	2	2	3.2	3	3	3	2	2	1.0	6.6	0.7	9.4
4	2	3	3.8	0	0	0	0	3	1.3	10.0	0.9	14.7
4	3	0	2.7	1	1	1	1	1	1.0	6.6	0.7	9.4
4	3	1	3.3	2	2	1	1	1	1.0	6.6	0.7	9.4
4	3	2	3.9	3	3	3	3	2	1.3	10.0	0.9	14.7
4	4	0	3.4	2	2	1	1	1	1.3	10.0	0.9	14.7
4	4	1	4.0	3	3	2	2	2	1.3	10.0	0.9	14.7
4	4	2	4.7	0	0	0	3	3	1.4	11.3	0.9	14.7
4	5	0	4.1	3	3	3	3	2	1.3	10.0	0.9	14.7
4	5	1	4.8	0	0	3	3	3	1.4	11.3	0.9	14.7
5	0	0	2.3	1	1	1	1	1	0.7	6.6	0.5	9.4
5	0	1	3.1	1	1	1	1	1	1.0	6.6	0.7	9.4
5	0	2	4.3	3	2	2	2	1	1.3	10.0	0.9	14.7
5	0	3	5.8	0	0	0	3	3	2.1	14.9	1.4	20.0
5	1	0	3.3	1	1	1	1	1	1.0	10.0	0.7	14.7
5	1	1	4.6	1	1	1	1	1	1.4	11.3	0.9	14.7
5	1	2	6.3	2	2	1	1	1	2.1	14.9	1.4	20.0
5	1	3	8.4	3	3	3	3	2	3.4	22.0	2.1	27.0
5	2	0	4.9	1	1	1	1	1	1.5	14.9	0.9	20.0
5	2	1	7.0	1	1	1	1	1	2.2	16.8	1.4	23.0
5	2	2	9.4	2	2	1	1	1	3.4	22.0	2.1	28.0
5	2	3	12	3	3	2	2	2	3	24	2	32
5	2	4	15	0	0	0	0	3	6	35	4	45
5	3	0	7.9	1	1	1	1	1	2.3	22.0	1.5	27.0
5	3	1	11	1	1	1	1	1	3	24	2	32
5	3	2	14	1	1	1	1	1	5	35	3	45
5	3	3	17	3	2	2	2	1	7	39	4	51
5	3	4	21	3	3	3	3	2	7	39	4	51
5	4	0	13	1	1	1	1	1	3	35	3	45
5	4	1	17	1	1	1	1	1	6	39	4	51
5	4	2	22	1	1	1	1	1	7	44	4	57
5	4	3	28	2	1	1	1	1	10	70	6	92
5	4	4	35	2	2	2	1	1	10	70	6	92
5	4	5	43	0	0	3	3	3	15	106	9	150
5	5	0	24	1	1	1	1	1	7	70	4	92
5	5	1	35	1	1	1	1	1	10	106	6	150
5	5	2	54	1	1	1	1	1	15	166	10	223
5	5	3	92	1	1	1	1	1	23	253	15	338
5	5	4	160	1	1	1	1	1	40	460	20	620
5	5	5	> 160									



8.3 Composition of dilution ratios 2 and 10. (From Cochran, 1950)

1: When the number of bacteria in the sample is equal to the MPN found, the result is one of those that have the greatest chance of being obtained. There is only at most 5% chance of obtaining a result that is less likely than the least likely one in this category.

2: When the number of bacteria in the sample is equal to the MPN found, the result is one of those that have less chance of being obtained than even the least likely one in category 1, but there is only at most 1% chance of obtaining a result that is less likely than the least likely one in this category.

3: When the number of bacteria in the sample is equal to the MPN found, the result is one of those that have less chance of being obtained than even the least likely one in category 2, but there is only at most 0.1% chance of obtaining a result that is less likely than the least likely one in this category.

0: When the number of bacteria in the sample is equal to the MPN found, the result is one of those that have less chance of being obtained than even the least likely one in category 3. There is only a chance of 0.1% to obtain a result in this category, without anything being wrong.

Before starting testing it should be decided which category will be acceptable, i.e., only 1, 1 and 2 or even 1, 2 and 3. When the decision to be taken on the basis of the result is of great importance, only category 1 or at most 1 and 2 results should be accepted. Category 0 results should be considered with great suspicion.

Caution: The confidence limits given in the tables are meant only to provide some idea about the influence of statistical variations on results. There will always also be other sources of variation, which may sometimes even be more important.

8.3.3 Differences between MPN values

From time to time it may be necessary to establish the significance of the difference between two MPN estimations. This can be determined using the combined standard errors for the two MPNs by reference to standard statistical tables of Students "t", viz:

$$t = (\log \hat{m}_1 - \log \hat{m}_2) / 0.55 \sqrt{(\log a_1/n_1) + (\log a_2/n_2)}$$

or:

$$t = (\log \hat{m}_1 - \log \hat{m}_2) / 0.58 \sqrt{(\log a_1/n_1) + (\log a_2/n_2)}$$

when dilution ratio (a) is 10-fold. The use of the method is illustrated in Ex. 8.3.

Example 8.3. Calculation of Most Probable Numbers for two dilution series and the significance of the differences between the MPN estimates

Assume: series (A), 10-fold dilutions with 5 tubes per dilution (each inoculated with 1 ml of dilution) giving the following results:

Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Result	5 + 0-	2 + 3-	1 + 4-	0 + 5-

Then from Table 8.7, MPN = $7 \times 10 = 70$ propagules/ml, with 95% confidence limits of 22 - 168/ml.

Assume: series (B), 10-fold dilutions, with 3 tubes per dilution (each inoculated with 1 ml of each dilution) giving the following results:

Dilution	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Result	3 + 0-	3 + 0-	0 + 3-	0 + 3-

The MPN from Table 8.7 is 170 propagules/ml using codon 3-3-0 with 95% CL of 70 - 390/ml.

These two test series provide MPNs of 70 and 170 propagules/ml but, although different, the 95% CLs of the MPNs overlap. The significance of the difference is tested by:

$$t = (\log \hat{m}_1 - \log \hat{m}_2) / 0.58 \sqrt{(\log a_1)/n_1 + (\log a_2)/n_2}$$

where:

$\hat{m}_1$ and $\hat{m}_2$ = MPNs for series (A) and (B), respectively;

$a_1 = a_2$ = dilution ratio = 10;

n_1 and n_2 = number of tubes for series (A) 5, and (B) 3, respectively.

Then:

$$\begin{aligned} t &= (\log 170 - \log 70) / 0.58 \sqrt{(\log 10)/5 + (\log 10)/3} \\ &= (2.2304 - 1.8451) / 0.58 \sqrt{(0.2 + 0.33)} = 0.3853 / 0.4236 = 0.9097 \end{aligned}$$

From Pearson and Hartley's (1966) Table 12, for $v = (5 + 3 - 2) = 6$ degrees of freedom, the observed value of t would be expected to occur on about 4 occasions in 10 ($p \approx 0.40$). Hence, the difference between these observed MPN values could occur by chance and is not significant at the 5% level.

8.3.4 Special applications of multiple-tube dilution tests

For control purposes it may be required to make a decision to accept or reject a consignment of food on the basis of a MPN test (e.g. for *Enterobacteriaceae*). For such purposes it is possible to construct an operating characteristics (OC) curve (§ 5.2.3) for acceptance or rejection at specific confidence levels of tube dilution counts. The best control system would be based on the use of a limit permitting "acceptance of the 'lot' if less than c tubes out of n at dilution i show evidence of growth" (i.e. if the number of sterile tubes at dilution i is $> \sigma_i$).

The shape of the OC curve will be dependent upon the values c and n for the dilution level giving the critical density of propagules. On the assumption that $\log n$ is normally distributed when n is large, the steepest part of the OC curve will occur at the 50% acceptance point (approximate value of $c = 0.2 n$). Lower percentage acceptance would be associated with $c < 0.2 n$ and vice versa. The appropriate choice of c will therefore lie in the range $0 - 0.5 n$, assuming that the cumulative binomial distribution is a valid model.

8.4 Presence or absence tests based on single or multiple samples

In the analysis of foods for low numbers of pathogenic organisms (e.g. salmonellae), it is normal to inoculate one or more samples into a suitable selective or enrichment medium prior to streaking on to a diagnostic medium to determine whether or not the specific organism sought is present. Apart from purely technical errors, presence/absence tests are subject to a number of statistical considerations concerning the distribution of organisms.

A quality control procedure based on this concept has been proposed by Aspinall and Kilsby (1979). In their scheme a critical value (c) is chosen such that when: (1) the cell density exceeds c the probability of rejection is at least 0.95; and (2) when the cell density is less than $0.2 c$, the probability of acceptance is 0.95.

For a critical value (c) of 10^{x+1} g the material is diluted $10^{(z-1)}$ -fold and 0.5 ml of this suspension is inoculated into each of two tubes of culture medium. A further 10-fold dilution of the suspension (i.e. a 10^{-z} -fold dilution) is then prepared and 0.5 ml is added to each of seven tubes of media. Following incubation, the MPN and confidence limits can be derived from the data in Table 8.8;

note that the CL cited are twice those quoted in the original paper of Aspinall and Kilsby (1979) (Kilsby and Aspinall, personal communication, 1983). If all tubes give a positive response, the material is rejected; if a result is obtained which is not shown in Table 8.8 the test must be repeated. The scheme is illustrated in Ex. 8.4.

TABLE 8.8

ESTIMATES OF MPN/g (MODIFIED FROM ASPINALL AND KILSBY: 1979)

Numbers of positive tubes at dilution		MPN/g $\times 10^{-(z-1)}$	95% Confidence limits	
$10^{(z-1)}$	10^z		lower $\times 10^{-(z-1)}$	upper $\times 10^{-(z-1)}$
0	0	< 0.76 (0)	-	2.8
0	1	0.76	0.014	2.8
1	0	0.92	0.038	4.8
1	1	1.9	0.32	6.4
2	0	2.7	0.48	10.8
1	2	3.0	1.34	6.8
2	1	4.6	0.90	16.2
2	2	7.4	1.92	22.0
2	3	11.0	2.8	30.0
2	4	17.0	4.0	40.0
2	5	25.0	6.8	58.0
2	6	39.0	11.2	98.0
2	7	> 39 (∞)	19.6	∞

Example 8.4. Use of Multiple Tube MPN for Quality Acceptance/Rejection Purposes

Suppose that for a food product the quality specification requires rejection of product containing more than 1000 organisms/g. Then $c = 10^3 = 10^{(z+1)}$, hence $z = 2$.

Prepare product dilutions of $10^{-(z-1)}$ (i.e. 10^{-1}) and 10^{-z} (i.e. 10^{-2}) and inoculate 0.5 ml of the 10^{-1} dilution into each of 2 tubes and 0.5 ml of the 10^{-2} dilution into each of 7 tubes of broth. Following incubation, examine for evidence of growth in the tubes of media.

Suppose the following results are obtained on three lots of product:

Product	Result at dilution of		MPN/g*	Decision
	10^{-1}	10^{-2}		
A	2+	7+	$> 39 \times 10^1 = > 390$	Reject lot
B	1+	5+	Not calculable	Repeat test
C	0+	1+	$0.76 \times 10^1 = 7.6$	Accept lot

* From Table 8.8.

A potential problem in the application of the methods as described is that if all nine tubes give positive results (i.e. 2+, 7+) the MPN cannot be calculated, yet requires that the product be rejected. However, if only eight positive tubes occur (i.e. 2+, 6+) the product would be accepted with an MPN of 390/g (Table 8.8). If, as in Ex. 8.4, the critical level (α) is an MPN of 1000/g, the cut-off of 390/g would appear to be somewhat low. However, the (2,7) scheme of Aspinall and Kilsby (1979) is much less likely to reject satisfactory material than is a 3 level, 3 tube (3,3,3) scheme such as is frequently used for control purposes (ICMSF, 1978).

8.4.1 Quantification based on relative occurrence of defectives

In Chap. 5, the use of the binomial distribution to describe the operating characteristics (OC) curve for sample plans was described. The binomial relationship describing the probability of occurrence of defective samples can be used also to quantify the probable level of occurrence of organisms in the lot of food under test.

On the assumption that the organisms in a "lot" of food are distributed randomly and that a number (n) of samples is tested, then the probability (P) of no defective test samples (i.e. no positive tests) is given by:

$$P = 1 - [1 - (d/100)]^n$$

where d = actual % defectives in the lot. This will be true provided that n is a small fraction (< 25%) of the total lot. The equation may be rearranged to give the % defectives for a given probability and number of samples:

$$d = 100 (1 - \sqrt[n]{1 - P})$$

Knowing the incidence of defective samples (d) and the size of each sample (W) it is then possible to derive the maximum contamination level (C) for the lot:

$$\begin{aligned} C &= (d/100) (1/W) \text{ organisms/g at probability level } P \\ &= [1 - \sqrt[n]{1 - P}]/W \end{aligned}$$

This calculation is illustrated below in Ex. 8.5. Derived values for the maximum incidence of contamination at $P = 0.95$ and $P = 0.99$, assuming a sample size of 25 g, are presented in Table 8.9 for various sample sizes (n).

From the data in Table 8.9, it can be seen that in testing for salmonellae, a negative test result on a single sample (i.e. $n = 1$) of 25 g will merely indicate, on average, that on 19 occasions out of 20 the lot will contain < 38 salmonellae/kg. Increasing the number of samples tested to 10 or 20 increases

the confidence for freedom from salmonellae since, on average, on 19 occasions out of 20 the lot would be expected to contain < 11 or < 6 salmonellae/kg, respectively, if all test results were negative. The data also indicate on average that on 99 occasions out of 100, the maximum contamination levels would correspond to 40, 15 or 9 salmonellae/kg, respectively, for 1, 10 or 20 samples tested and found to be "negative".

TABLE 8.9

PERCENTAGE DEFECTIVES AND MAXIMUM CONTAMINATION LEVELS ASSUMING RANDOM DISTRIBUTION OF ORGANISMS

No. samples tested n	Maximum % defectives* at		Maximum no. organisms/kg*† at	
	P = 0.95	P = 0.99	P = 0.95	P = 0.99
1	95	99.0	38	40
2	77.6	90.0	31	36
3	63.2	78.5	26	32
4	52.7	68.4	21	28
5	45.1	60.2	18	24
10	25.9	36.9	11	15
20	13.9	20.6	6	9
30	9.5	14.2	4	6
50	5.8	8.8	3	4
100	3.0	4.5	2	2

* Assuming no positives detected.

† Assuming sample size = 25 g; rounded up to the nearest whole number.

Quantification based on positive results can be derived as the most probable number (MPN) (§ 8.3.1 above) for multiple tests at a single dilution. Hence, using $\hat{m} = -(1/v) \ln(s/n)$, a single positive from 10 tests each of 25 g provides a MPN of 4.2 organisms (e.g. salmonellae)/kg. This value is well below the maximum number which would be expected at $P = 0.95$ if all tests had been negative but the 95% confidence limits of the MPN range from < 1 to > 60 salmonellae/kg

8.4.2 Effect of subculture volumes and incubation times

When samples are tested for presence/absence of specific microbes, the analytical procedure may involve the use of pre-enrichment and selective enrichment stages.

Different methodological details are recommended by different authorities. Using the recommendations of ICMSF (1978) for salmonellas testing, the initial sample is dispersed in 9 volumes of pre-enrichment medium, and after a suitable incubation period 1 ml is transferred to 10 ml of each of two selective enrichment media. Thereafter, one 5 mm loopful (about 0.02 ml) for each selective enrichment is streaked on to one plate of each of three selective diagnostic media and, after incubation, selected colonies are picked for typing.

Hence, three dilution stages occur. The first stage results in a 10-fold dilution of the sample. Organisms inoculated with the sample will grow during incubation, but the number growing will depend upon (a) the initial contamination level and the dispersion both of the organism being sought and of competitive organisms; (b) the condition of the organisms (i.e. whether cells have been "stressed" by the environmental conditions in the food); (c) the population doubling time for the organism sought in the conditions of the test (i.e. culture medium composition, incubation temperature and time).

The second and third stages (selective enrichment) will be affected similarly, but in the third stage the individual propagules will be required to form discrete colonies on the diagnostic medium. The fourth stage (picking of colonies) is considered below (§ 8.4.3).

Clearly, the possibilities for isolation of specific organisms will depend not only on the initial incidence of contamination but also on the manner in which the test is done. A too short period of pre-enrichment may result in no recovery of stressed organisms, thereby reducing the statistical probability of transferring resuscitated organisms to the enrichment broth. Too long a period in a non-selective pre-enrichment may provide opportunity for overgrowth by competitive organisms, leading to suppression of the organism sought. A hypothetical example of the effects of incubation time is shown in Ex. 8.6 and 8.7 and in Fig. 8.4.

Example 8.5. Calculation of maximum probable contamination level for presence/absence tests

Assume 10 x 25 g samples are tested for salmonellae and all tests are negative. What are the maximum contamination levels at probabilities of 95% and 99%. Using the formula:

$$d = 100 (1 - \sqrt[n]{1 - P}), \text{ for (A): } P = 0.95 \text{ and (B): } P = 0.99:$$

$$\begin{aligned} \text{(A) } d &= 100 (1 - \sqrt[10]{1 - 0.95}) = 100 (1 - \sqrt[10]{0.05}) \\ &= 100 (1 - 0.741) \\ &= 25.89\% \end{aligned}$$

Hence, at 95% probability, maximum contamination of the lot by salmonellae = $C_A = 25.89/(100 \times 25) = 10.4$ salmonellae/kg.

$$(B) \text{cl} = 100 (1 - \frac{10}{\sqrt{0.01}}) = 36.90\%$$

Hence at 99% probability, the maximum contamination of the lot by salmonellae = 14.8 salmonellae/kg = c_B
 $c_B = 36.9 \times 1000 / (100 \times 25)$.

Example 8.6. Effect of incubation time in pre-enrichment medium on numbers of salmonellae for subculture.

Assume: (1) 25 g dry food sample inoculated into 225 ml nutrient broth for pre-enrichment; (2) initial contamination level = x salmonellae/g; (3) length of lag phase (L) for resuscitation = 6 h; (4) population doubling time (T_d) = 30 min = 0.5 h, at the appropriate incubation temperature; (5) synchronous growth occurs.

Using:

$$N = N_0 2^{(T-L)/T_d}$$

where:

N = no. salmonellae/ml after time T (h)

N_0 = no. salmonellae/ml initially

L = length of lag phase (h)

and

T_d = doubling time (h)

Then since:

$$N_0 = 25 x / 250 = 0.1 x$$

$$L = 6 \text{ h}$$

and

$$T_d = 0.5 \text{ h}$$

After:

$$6 \text{ h}, N = 0.1 x 2^{(6-6)/0.5} = 0.1 x 2^0 = 0.1 x$$

$$9 \text{ h}, N = 0.1 x 2^{(9-6)/0.5} = 0.1 x 2^6 = 6.4 x$$

$$12 \text{ h}, N = 0.1 x 2^{(12-6)/0.5} = 0.1 x 2^{12} = 410 x$$

$$24 \text{ h}, N = 0.1 x 2^{(24-6)/0.5} = 0.1 x 2^{36} = 6.9 x \times 10^9$$

Hence, the expected number of organisms likely to be transferred in an inoculum of pre-enrichment medium and the probability of transferring at least 1 viable organism, assuming random distribution when $x = 0.1$ (i.e. 10 organisms/100 g) is

Incubation time (h)*	No. organisms** in 1 ml pre-enrichment medium	Probability of > 1 organism** in	
		1 ml	10 ml
6	0.01	< 0.01	0.095
9	0.64	0.47	> 0.99
12	41.0	> 0.99	> 0.99
18	1.7×10^5	> 0.99	> 0.99
24	6.9×10^8	> 0.99	> 0.99

* In pre-enrichment, assuming conditions as above.
 ** Assuming $x = 0.1$ salmonellae/g food sample

Once sufficient growth has occurred in pre-enrichment for at least 3 viable salmonellae/ml medium, the probability of transferring at least one viable organism in an inoculum of 1 ml or more will be > 0.95 , assuming Poisson distribution, with $\hat{m} = 3$: $P(x=0) = 0.05$, therefore $P(x>1) = 0.95$. However, at lower numbers, the chances of transferring at least 1 viable organism will be affected not only by the initial contamination level (x) but also by the volume of medium inoculated into the enrichment culture. In methods which permit only resuscitation, not growth, during pre-enrichment, it is essential to use the entire pre-enrichment culture to inoculate double-strength enrichment medium (Hall, 1975), although selectivity may then be affected by the food constituents.

Obviously the level of salmonellae in the pre-enrichment culture after any given incubation period will depend upon the cultural conditions used, the initial contamination level, the extent of sublethal cell damage and the relative numbers and types of competitive organisms in the sample.

8.4.2.1 Compositing of samples. When a number of samples is to be tested it is sometimes recommended that the samples be composited in order to reduce the work load on the laboratory. Several methods of compositing can be undertaken:

(1) Combine all samples at pre-enrichment stage; i.e. for 10 x 25 g samples inoculate all 250 g directly into 2250 ml of pre-enrichment medium. After incubation, carry out tests on the same basis as for a single sample (i.e. inoculate 10 ml into 100 ml enrichment medium).

(2) Prepare 10 pre-enrichment series (i.e. one for each 25 g sample) and after incubation composite equal volumes of each pre-enrichment medium (e.g. 10 x 1 ml) to inoculate the 100 ml enrichment broth.

(3) A combination of (1) and (2) above where, say, 5 samples are composited at pre-enrichment stage and aliquots of the duplicate pre-enrichment media are composited at the stage of inoculating the enrichment medium.

(4) Composite all 10 samples and then test one (or rarely two) 25 g aliquots from the bulk composited samples.

Methods (1), (2) and (3) provide the same degree of statistical confidence as would be attained by testing the 10 samples separately, in so far as a negative result indicates no sample contamination and therefore < 11 organisms/kg (at $P = 0.95$). However, if a positive result is obtained, it is not possible to estimate (even approximately) the degree of contamination. Method (2), at least in theory, permits the tests to be repeated on stored pre-enrichment cultures

so that some indication of the proportion of contaminated samples can be obtained. Method (3) is a compromise between Methods (1) and (2).

Method (4) is based on a fallacious concept, since by compositing the original sample and testing only one aliquot of the composite sample, the results will provide no more information about the lot than would testing of a single sample of, say, 25 g. It is totally incorrect to interpret a negative result as indicating (at $P = 0.95$) a maximum contamination level of 11 organisms/kg, which would have been given by 10 negative results.

8.4.3 Selection of colonies for identification

After development of isolated colonies on the surface of the selective-diagnostic medium, it is necessary to select for further identification colonies showing typical morphology. The chances of isolation of specific organisms from an almost pure culture will of course be high. However, when mixed cultures are present and some of the organisms of different types have apparently similar colonial morphologies, then several colonies may need to be picked. In some laboratory manuals (Hall, 1975) no reference is made to the number of colonies to pick; others (ISO, 1979) recommend selection of the square root of the number of "typical" colonies, whilst ICMSF (1978) recommend picking of at least two typical colonies and ISO (1978) recommends picking five typical colonies.

It can be shown that the chance of picking any one colony of the type sought is dependent upon the number (n) of the specific type relative to the total number (N). Since N is not large the relationship is governed by the hypergeometric distribution. Table 8.10 shows the number of colonies of the type sought (e.g. *E. coli*) which must be present in order to select, with a given statistical probability, at least one colony of the type sought from among a population of otherwise typical colonies. For instance, if the true incidence of *E. coli* is 50% of the colonies having "typical" coliform morphology, then if one picks 5 colonies out of a population of 30 colonies, the chance of picking at least one *E. coli* is slightly greater than 95% ($p > 0.95$). If, however, the true incidence is only 10% (i.e. 3 *E. coli*/30 colonies) then the chance that at least one *E. coli* colony is included in the sample of 5 colonies is somewhat less than 50% ($P < 0.50$).

Example 8.7. Statistical Probability of Transferring Salmonellae during Enrichment and Isolation.

Assume:

- (1) initial mean contamination level = x salmonellae/g sample;
- (2) pre-enrichment for 12 h (as Ex. 8.6), yielding $410 x$ salmonellae/ml;
- (3) 1 ml pre-enrichment medium inoculated into 10 ml selective enrichment medium;
- (4) lag time (L) in enrichment medium = 4 h; doubling time = 60 min = 1 h;
- (5) enrichment period 18 or 24 h and
- (6) 0.02 ml (1 loopful) inoculated onto diagnostic medium.

Then, from Ex. 8.6, after 12 h pre-enrichment the medium would contain $410 x$ salmonellae/ml. Since pre-enrichment is diluted 1 in 10 into selective enrichment medium the initial level of salmonellae would be $410 x/10 = 41 x/\text{ml} = N_0^1$

Then after 18 h, assuming synchronous growth and other factors as above:

$$N = 41x \cdot 2^{(18-4)/1} = 41x \cdot 2^{14} \approx 6.7x \times 10^5 \text{ salmonellae/ml}$$

Similarly, after 24 h:

$$N = (41x) \cdot 2^{(24-4)/1} = 41x \cdot 2^{20} \approx 4.3x \times 10^7 \text{ salmonellae/ml}$$

Now if initial contamination level (x) = 0.04 (i.e. 1 salmonella/25 g sample), a 0.02-ml loop would be expected to transfer 536 or 34,400 salmonellae after 18 and 24 h incubation, respectively. Hence the probability of transferring at least one viable salmonellae to the isolation medium would be $\gg 0.99$.

However, had the initial mean inoculum level after 6 h pre-enrichment, with recovery but no growth, been only 0.01 x (Ex. 8.6, 6 h incubation), then for $x = 0.04/\text{ml}$ the expected numbers transferred with a 0.02 ml loop after 18 h and 24 h enrichment culture would be about 0.01 and 0.84 salmonellae, respectively. Hence, the probability of transferring at least one viable salmonella would be < 0.01 and < 0.50 , respectively.

It is essential, therefore, to ensure that an adequate inoculum is transferred from the pre-enrichment culture; otherwise the probability of detecting salmonellae will be low. These effects are illustrated diagrammatically in Fig. 8.4.

Example 8.8. Probability for selection of a specific colony from a diagnostic plate culture

What is the probability for picking at least one *E. coli* colony from a diagnostic plate containing 30 typical coliform colonies if numbers of non-*E. coli* colonies are (a) 10 and (b) 20?

Since the population is finite and (relatively) small, removal of any one colony will reduce the residual number from which to select another colony. Hence, the hypergeometric distribution is the most appropriate. The probability of selecting no *E. coli* colonies is described by the equation:

$$P_{(x=0)} = [a! (N - n)!] / [(a - n)! N!]$$

where:

N = number of typical coliform colonies

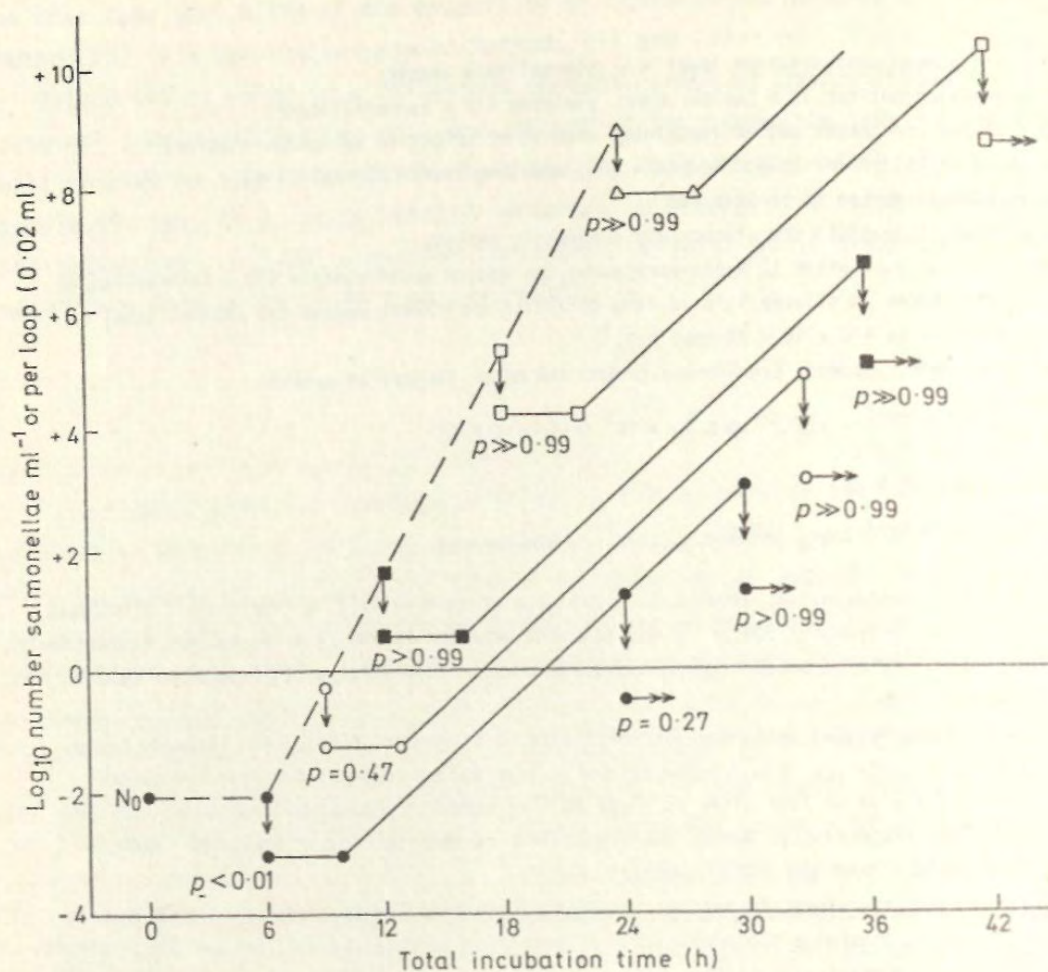
n = number of colonies picked for identification (= integer of $\sqrt{N}$)

and,

a = number of non-*E. coli* colonies on the plate.

Since:

$$P_{(x>1)} = 1 - P_{(x=0)}$$



8.4 Growth of *Salmonella* in pre-enrichment (---) and enrichment (—) cultures

then;

$$P_{(x \geq 1)} = 1 - [a! (N - n)! / (a - n)! N!]$$

which simplifies to:

$$P_{x \geq 1} = [a (a - 1) (a - 2) \dots (a - n + 1) / N (N - 1) (N - 2) \dots (N - n + 1)]$$

Hence, for $N = 30$, $n = 5$ (i.e. integer of $\sqrt{30}$):

(a) For $a = 10$;

$$\begin{aligned} P_{(x \geq 1)} &= 1 - [(10)(9)(8)(7)(6) / (30)(29)(28)(27)(26)] \\ &= 1 - 0.001768 \\ &= 0.9982 \end{aligned}$$

TABLE 8.10

STATISTICAL PROBABILITY FOR PICKING 1 OR MORE SPECIFIC ORGANISMS FROM AMONGST A NUMBER OF "TYPICAL" COLONIES ON A DIAGNOSTIC PLATE

No. of "typical" colonies (<i>N</i>)	No. of colonies picked* (<i>n</i>)	No. and % of colonies of specific organism required on culture plate**			
		<i>P</i> = 0.50	<i>P</i> = 0.90	<i>P</i> = 0.95	<i>P</i> = 0.99
30	5	4 (13)	11 (37)	13 (43)	17 (57)
40	6	5 (12)	12 (30)	15 (38)	21 (53)
55	7	5 (9)	15 (27)	19 (35)	26 (47)
70	8	6 (9)	17 (24)	21 (30)	30 (43)
90	9	7 (8)	20 (22)	25 (28)	35 (39)
110	10	8 (7)	22 (20)	28 (25)	39 (35)
135	11	8 (6)	25 (19)	31 (23)	45 (33)
150	12	9 (6)	26 (17)	32 (21)	47 (31)

* Based on sample size = integer of $\sqrt{N}$.

** To provide the stated probability (*P*) for picking at least one specific organism from the *N* typical colonies on the plate.

(b) For $\alpha = 20$;

$$\begin{aligned} P_{(x \geq 1)} &= 1 - [(20)(19)(18)(17)(16)/(30)(29)(28)(27)(26)] \\ &= 1 - 0.108795 \\ &= 0.8912 \end{aligned}$$

Hence, if 1/3 typical colonies are non-*E. coli* there is a better than 99% chance, on average, that at least one *E. coli* colony will be picked in a sample of size 5 from a population of 30 typical colonies. However, if 2/3 of the typical colonies are coliforms other than *E. coli*, then the chance of selecting at least one *E. coli* in a sample of size 5 would be slightly less than 90%.