A STATISTICAL INQUIRY INTO METHODS FOR ESTIMATING NUMBERS OF RHIZOBIA[1]

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INTRODUCTION

The importance of accurate methods for the estimation of bacterial numbers is apparent to any worker in bacteriology. The successful application of this science to practical problems was made possible only after development of methods which enabled census-taking of the bacterial population. The dairy industry, food products, sewage disposal, and water supply furnish examples of bacterial control placed on a firm foundation through the development and application of suitable methods for the enumeration of microörganisms. Of equal importance are counting methods to the research student engaged in more theoretical problems. Interpretation of data concerned with problems in metabolism and physiology of organisms can undoubtedly be made with greater assurance if the mass of cells which took part in the transformations is accurately known.

Most investigations are made with organisms whose characteristics are well known and counts made by either the plate or the direct methods are accepted without question. Such a procedure is probably not objectionable since the methods used have been thoroughly tested by numerous workers and found to be adequate and entirely trustworthy in the hands of a careful technician. However, certain organisms possess peculiarities in reproduction which tend to introduce errors in the ordinary counting methods

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unless especial care is taken to eliminate these. In such cases it is advantageous to inquire into the accuracy of the methods employed. Examples of this latter type are the members of the genus *Rhizobium*, or root nodule bacteria. Due to their high gum production, which renders difficult proper distribution in dilutions, as well as to other little understood characteristics, e.g., the life cycle, attempts to enumerate these organisms by the plate count often give highly erratic results. Werkman (1927), comments, "Plate counts were abandoned since any strain of *Azotobacter* or *Rhizobium* forms some gum and plate counts were found unreliable." His lack of faith in the results of the plate count is no doubt shared by numerous other workers. The necessity of determining both viable and total counts in connection with certain research problems in this laboratory led to an investigation of methods that are commonly used to enumerate these bacteria. The purpose of this work was to establish the accuracy of the methods now employed and was not an attempt to develop a more reliable procedure. A problem of this nature is essentially one involving the origin of errors or deviations. In general, these can be classified as follows: (1) Those arising from non-uniformity or carelessness in technique; (2) those arising from failure to provide optimum conditions for the growth of the organism; (3) those due to chance. In a viable count the first two sources of errors are minimized in so far as possible by choice of medium, standardization of procedure, etc. In the case of direct counts only the first and third operate. Hence, if counts are made under carefully controlled conditions so as to eliminate sources 1 and 2 the deviations noted should be only those due to chance. If we compare the deviations or errors noted with those expected from chance alone and find that the expectancy is exceeded, we must conclude that our technique does not provide the proper conditions for results of sufficient accuracy. If the difference between errors noted and those due to chance is very large, the sources of errors should be eliminated if possible, or failing in this, the method should be used with full cognizance of the errors involved.

EXPERIMENTAL

The plate count

Method. The estimation of bacteria by a plate count is the usual method employed in attempting to count viable bacteria. This method is based on the assumption that every living cell or aggregate of cells is capable of producing a colony if placed in a "suitable medium." However, it is not always possible to elaborate a medium that will satisfy the assumption. The depth of agar, choice of substrate, and the more complex effects of association tend to introduce errors in this method. Under properly controlled conditions and especially with a pure culture, these errors are fairly constant, so that the method is adequate for comparable counts. It must be realized, however, that every living cell is not capable of reproduction; consequently the count really represents cells or aggregates that reproduce and not total viable organisms. Rubner (1904), in his work on the energetics of alcohol fermentation observed that the ability of yeast to metabolize and to form colonies on a malt agar plate were quite distinct. He says "Lebensfähigkeit und Wachstumsfähigkeit sind zwei Dinge die man auseinanderhalten muss."

Choice of substrate is a delicate problem that often is decided by personal likes and dislikes. In this laboratory it has been found that Fred and Waksman's medium 79 (1928) gives the most constant counts with *Rhizobium* and it was therefore employed in this work:

Agar	15	grams
Mannitol	10	grams
K_2HPO_4	0.5	gram
$MgSO_4 \cdot 7H_2O$	0.2	gram
NaCl	0.1	gram
Yeast water	100	cc.
Water	900	cc.

The yeast water is prepared by steaming 100 grams of fresh yeast (starch free) with 900 cc. of tap water for three hours, allowing to settle for several days, then centrifuging to remove débris. It contains about 1 mgm. of nitrogen per cubic centimeter. Other

workers have used soil-extract agar, plant-extracts agar, etc., with success but we have not been able to find these media advantageous in our work. This is not intended as a criticism of these media, since success with a more or less suitable medium depends to a great extent on familiarity with methods of preparation and experience in duplication.

In each experiment a sufficient quantity of the above medium was prepared to last throughout the test. Ten cubic centimeters were measured with a pipette into tubes, sterilized, and these tubes melted by placing in a steamer for 15 minutes before pouring the plates each day. The plates were chosen for uniformity of size so that the same depth of agar was obtained in each plate. This point is important in counting the *Rhizobia* because of their peculiar sensitiveness to changes in oxygen tension relations. Another factor which must be carefully controlled is the temperature of the agar at pouring since the organisms are very sensitive to heat, Alicante (1926). A special water bath was used which kept the tubes of agar to be poured at 42° ± 1°C.

The water blanks were accurately measured in 9 and 99 cc. portions. The former were placed in test-tubes and the latter in 6 oz. bottles so as to avoid undue losses in sterilization. Ordinarily tap water was used in the blanks but in a few experiments physiological salt solution was used. Accurate 1 cc. Mohr pipettes were used for all dilutions and these were made at room temperature, about 25°C. In order to insure distribution of the organism, sterile glass beads were placed in the flask used for growing the organism and the flask shaken 25 times before a sample was withdrawn for counting. The first dilution bottle was also provided with sterile beads for breaking up aggregates of bacteria. All dilutions were shaken 25 times before a sample was withdrawn.

Statistical treatment. If a standard technique made all errors other than those arising from chance constant, the method would be satisfactory since usually the counts are wanted for comparative results; absolute numbers, while desirable, are not actually essential. Fisher, Thornton, and MacKenzie (1922) and Fisher (1930) have shown that the conditions laid down in the Poissan Exponential Summation are fulfilled in a plate count made under

ideal conditions. These conditions are (1) the probability (p) that a given organism from a large population falls in a certain plate shall be very small; (2) the number (n) of organisms exposed to this probability shall be very large. With similar assumptions Poissan showed that the probability that the number of occurrences shall be x is given by the expression $e^{-m} \frac{m^x}{x!}$. x is always a whole number (as the colonies per plates), while m is the mean value of x (average of all plates). For large values of m, the distribution is essentially normal with a standard deviation equal to $\sqrt{m}$. Now in order to test whether the variations in plate counts are due to those arising from random sampling it would be necessary to have a large number of plates, say 100, of each dilution and to compare the distribution of the number of colonies per plate with that predicted by the Poissan law. In actual practice we have only 3 to 5 plates of each dilution, hence such a comparison would be useless, due to the small number of samples. However, Fisher and associates have shown that for small samples of a Poissan series an index of dispersion (measure of variation) can be calculated from the sum of the squares of the deviations from the mean, divided by the mean. This statistic is called Chi square and expressed mathematically it is

$$X^2 = \frac{\Sigma (x - \bar{x})^2}{\bar{x}}*$$

* Mathematically this is equivalent to Pearson's "Goodness of Fit" test.

where x is the individual value observed, e.g., colonies per plate, $\bar{x}$ is the mean value (average of all plates, and Σ denotes summation. The advantage of this statistic over others that might be used, e.g., standard deviation, variance, etc., is that its distribution is independent of the mean value ($\bar{x}$); hence a number of small samples from a series of parallel platings can be used to test whether these samples are taken from the theoretical Poissan distributions. This is made possible through the fact that the values of X^2 calculated as above will be distributed in a known manner if the samples are from a Poissan series. Tables by El-

derton (1902) give the number of times X^2 will exceed successive integral values for values of n from 0 to 30, where n is one less than the number of parallel plates. Fisher, *et al.* (1922), have shown that soil counts made under standardized conditions have a distribution of X^2 which is very close to the theoretical one and confirm the view that the bacterial counts on the parallel plates vary in the same way as small samples from Poissan Series. In some cases the distribution of X^2 departed from the theoretical but on investigation it was found that (1) occurrence of certain organisms which reduced colony development or (2) deficiencies in the medium, gave rise to (1) excessive and (2) subnormal variation in parallel plates. This led to the observed abnormal departure from the theoretical in the distribution of X^2. The authors conclude, "Any significant departure from the theoretical distribution is a sign that the mean may be wholly unreliable."

Experiment I. Three-plate data. In view of the analysis of Fisher, Thornton, and MacKenzie of different sets of data concerned with counts of variable bacterial flora, it would be expected that the distribution of X^2 from counts made on a pure culture should show a very good agreement with the theoretical distribution. In this case the selection of media to suit the organism is simplified and errors arising from antagonistic effects of one type of organism on another are eliminated.

In the course of experiments dealing with the growth of *Rh. trifolii* on different sources of nitrogen it was noticed that parallel plates (two) often showed wide variation so that the mean was highly untrustworthy. In order to secure more reliable data it was decided to standardize the technique as much as possible and to make three parallel plates instead of two. In work of this kind there is a limit to the number of parallel plates that can be made because of the time factor involved. In a laboratory test in which a comparison of three or four types of treatment are being investigated, e.g., effect of the sources of nitrogen on the growth of an organism, it would not be feasible to make parallel platings of five or more since the time involved would affect the counts to such an extent that the variability would not be decreased by the added number of plates. In order that the find-

ings of Fisher *et al.*, might be applied to our actual laboratory practice it was decided that three or four plate data should be examined. After preliminary experiments concerned with composition of medium, water blanks, temperature, etc. the technique described under *Methods* was adopted. The plates were incubated at 28°C. and counted after 5 and 10 days but only the 10-day count is considered here.

Following the standardization of the technique, data for about 100 sets of 3 plates were gathered. Upon calculation of the X^2's

TABLE 1

Comparison of observed distribution of X^2 with theoretical for three plates

X^{2*}	THEORETICAL m	OBSERVED x	$x - m$	$\frac{(x-m)^2}{m}$
0.00 -0.438	12	12	0	
0.438-0.973	12	11	−1	0.083
0.973-1.663	12	7	−5	2.083
1.663-2.636	12	13	1	0.083
2.636-4.300	12	14	2	0.333
4.300-	12	15	3	0.750
Totals........	72	72		X^2 = 3.332† P = 0.65

*These values of X^2 represent the distribution of this statistic in the data examined.

†This X^2 is the total of $\frac{(x-m)^2}{m}$ for all cells and is the value used to test the goodness of fit of the observed data with the theoretical. See Fischer (1922, 1930).

for these data, it was found that there was a great excess of large values of this statistic, in other words, the data showed an excessive variance. Upon investigation of these large values of X^2's it was found that most of them arose from counts of the following type: 159, 162, 103; 174, 160, 107; 73, 69, 54. It will be noticed that two of the plates do not show unduly wide variation, but the third plate is so much smaller than the other two that the variance and hence X^2 becomes very large. These cases caused a fairly large number of X^2 figures to have values from 10 to 30. Now in 100 samples with $n = 3$, there should be only one sample

with X^2 greater than 10. Accordingly the data were examined and those sets eliminated which showed a value of X^2 greater than 10 and *in which this value was due to the large deviation of one plate.* The distribution of X^2 from the data after this elimination is shown in table 1. The test of agreement was made by the Pearson test of *Goodness of Fit* (Fisher, 1930) and the result shows that 65 times out of a 100, one would expect a worse fit due to chance alone—a very good agreement.

Experiment II. Four-plate data. From the results with the 3-plate data, there were indications that the erratic results noted with two parallel plates were due to factors that sometimes caused

TABLE 2

Comparison of observed distribution of X^2 with theoretical for four plates

X^2	THEORETICAL m	OBSERVED x	$x - m$	$\frac{(x-m)^2}{m}$
0.5	8.94	6	−2.94	0.97
1.5	10.29	14	3.71	1.34
2.5	8.14	8	−0.14	0.02
3.5	5.85	3	−2.85	1.39
4.5	4.05	6	1.95	0.94
6.5	4.50	4	−0.50	0.06
>6.5	3.23	4	0.77	0.18
Totals........	45	45	X^2 = 4.90 P = 0.56	

a certain plate to show an abnormal deviation from the mean of the population. With two plates it is impossible to judge the erratic plate; with three, one might select that plate which departs most markedly from the average of the other two. However, this is somewhat arbitrary and might give rise to bias if a large number of plates in a given series are unusually variable. In order to determine if these erratic plates were the exception rather than the rule, a series of 5 plates were made, then one plate eliminated from each count so that a 4-plate series resulted. If there was any one plate in the 5 replicates that showed marked deviation from the others this was eliminated, otherwise the elimination was left to chance by throwing out the third plate counted. It

was necessary to eliminate by choice in 18 of the 45 sets and as noted in experiment I, the majority (14) were plates that gave a much smaller count than the other four parallels. The distribution of X^2 for this set of data is given in table 2. In table 1 the distribution was made in sextiles, i.e., the expected number in each cell is the same. In table 2 the distribution of X^2 is given in integral values grouping the values at the extremes so that the expected number in each cell is three or more. While 45 samples

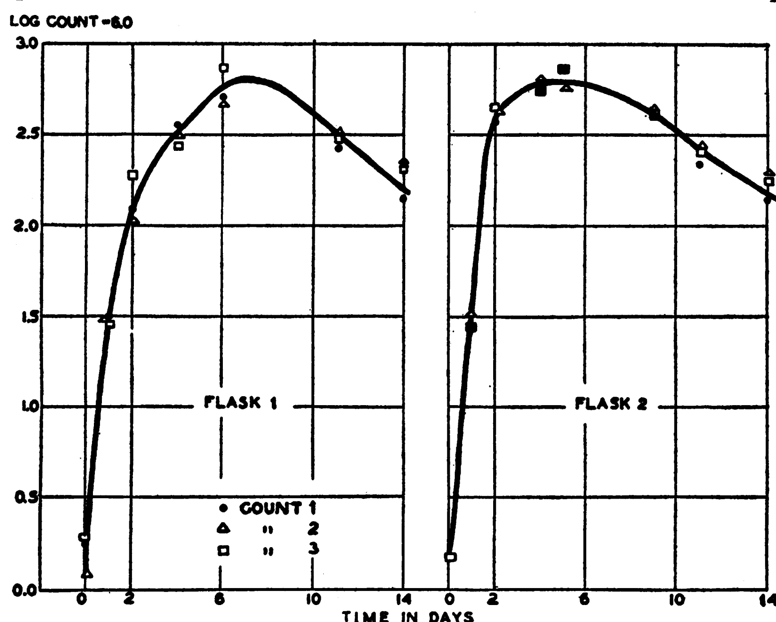

CHART 1. GROWTH CURVES OF *Rhizobium trifolii* IN MEDIUM 79

are hardly sufficient to make a through test of the distribution, the probability found (0.56) that the deviation noted arose from sampling is indicative that the distribution of values tends toward the expected ones.

The data discussed above were counts made periodically on the growth of *Rhizobium trifolii*, Culture 200, in Medium 79. Chart 1 shows two of the growth curves made from the counts. In this particular experiment duplicate flasks were taken and 3 counts made on each flask after varying number of days. These counts

were made with three parallel plates and in the case of those sets of three that had one plate markedly different from the other two the average of the two plates that showed the least variation was taken as the correct count. The distance of the average of the individual counts from the "best" line is a measure of the variability among the triplicate counts. These growth curves are quite similar to those found for other organisms, exhibiting a

TABLE 3

Comparison of counts of Rhizobium trifolii culture 209 in replicate flasks

	AGE						
	0 day	1 day	2 days	3 days	5 days	7 days	10 days
Experiment I. Three plates							
Flask 1	0.31*	19.0	170	253	257		
Flask 2	0.21	14.7	125	239	227		
Experiment II. Three plates							
Flask 1	0.66	19.3	173	490	463		
Flask 2	0.38	46.0	309	508	464		
Experiment III. Four plates							
Flask 1	7.2	23.3	297	337	229 (?)	335	280
Flask 2	1.7	4.8	78	118	160	187	188
Flask 3	2.4	11.0	128	148	349	267	271
Flask 4	0.8	68.3		229	450	495	458

* All counts expressed as millions per cubic centimeter.

period of logarithmic increase during the first two days, a phase of negative growth acceleration during the second to fourth days, a brief period of maximum growth followed by a period of "death" that approaches the logarithmic. The growth curves in the two flasks were quite similar in this experiment but this is not always the case as shown in table 3. In a comparison of growth on different media this variability of replicate flasks would have to be taken into consideration in interpretation of the data.

The direct count

Method. The direct count of bacteria by means of a haemocytometer was first made in 1898 when Winterberg (1898) made use of the apparatus devised by Lyons and Thoma for estimation of blood corpuscles. The Petroff-Hausser counter has been developed especially for the counting of bacteria by this method; its chief advantage is the decreased depth of the chamber (0.02 mm.) so that it is not necessary to move the microscope up and down in order to locate the bacteria in different levels of the counting chamber. The direct count offers many obvious advantages over plate counts if knowledge of the total number of bacteria present rather than viable bacteria will suffice. In many experiments this information is that desired and a direct count is of much more value than a plate count. However, it has the disadvantage that each bacterium per square on the Petroff-Hausser counter represents 20,000,000 organisms per cubic centimeter of the sample and therefore can be used to advantage only when the number of organisms present is very high. In the experiments reported below, most of the counts were made with the dark field. Some were made with the light field in which case the 1 cc. of the diluted culture was mixed with 1 or 2 cc. of Meissner solution. The dark field work is preferred as the counting is easier on the eyes and it is less difficult to distinguish the bacteria from occasional débris. The counts were made on 48 to 120 hours cultures in liquid medium, with and without carbohydrate, and on suspensions of the organism from agar slants. In the latter case the growth was washed with sterile water into 100 cc. volumetric flasks, then further diluted according to the numbers present. Beads were used in all cases to break up clumps.

Statistical treatment. "Student" (1907) from theoretical consideration of the count of yeast cells in a haemocytometer arrived at a distribution of the counts per unit area that followed the Poissan Exponential Summation. He showed that under favorable conditions this theoretical distribution was actually realized in practice. He further pointed out that the standard deviation of the count was $\sqrt{m}$; hence the accuracy of any count was pro-

portional to the total number counted. "Student's" counts were made with the purpose of verifying Poissan's law rather than to test the accuracy of the count and his technique was not exactly

TABLE 4

Comparison of theoretical distribution with observed when counting Rhizobium trifolii in Petroff-Hausser counter

NUMBER PER SQUARE	THEORETICAL m	OBSERVED x	$x - m$	$\frac{(x - m)^2}{m}$	$\frac{(x - m)^2}{m}$ (GROUPING)
			I. Mean—5.16		
<2	14.28	16	+1.72	0.21	0.21
2	30.77	25	−5.77	1.08	0.88 (2–3)
3	52.81	50	−2.81	0.15	
4	67.98	73	+5.02	0.37	0.71 (4–5)
5	70.01	75	+4.99	0.36	
6	60.10	57	−3.10	0.16	0.16
7	44.22	49	+4.78	0.52	0.52
8	26.46	23	−3.46	0.45	0.45
9	16.30	18	+1.70	0.18	0.18
10	8.40	5	−3.40	1.37	1.37
>10	6.65	9	+2.35	0.83	0.83
Totals......	400	400	$X^2 = 5.68$, $P = 0.77$		$X^2 = 5.31$, $P = 0.62$
			II. Mean—2.50		
0	32.83	34	+1.17	0.04	0.04
1	82.08	68	−14.08	2.41	2.41
2	102.60	112	+9.60	0.90	1.60 (2–4)
3	85.50	94	+8.50	0.84	
4	53.44	55	+1.56	0.05	
5	26.72	21	−5.72	1.22	1.22
6	11.12	12	+0.88	0.07	0.07
>6	5.62	4	−1.67	0.49	0.49
Totals......	400	400	$X^2 = 6.02$, $P = 0.43$		$X^2 = 4.83$, $P = 0.30$

that followed in actual laboratory practice. For example, he fixed the yeast cells in gelatin under the cover slip so as to prevent movement of the cells during the counting. Since under the

more or less ideal conditions obtained by "Student," it was shown that the distribution of organisms per unit area followed Poissan's law, a good test of the accuracy of a laboratory count can be made by comparison of the theoretical distribution given by Poissan's Exponential Summation with that actually obtained. This was done on a large number of cultures of rhizobia. Due to gum formation, clumping might be expected to interfere with the accuracy of the count, but in actual counting it was found that clumps were not frequent and were easily identified by their large size. In making the counts an effort was made to estimate the number of organisms in each clump and, if a sample showed a large number of these, to discard the count. The observed distributions were compared with the theoretical by means of Pearson's *Goodness of Fit* test. The table given by Fisher (1930) was used to determine P, *i.e.*, the probability of a worse fit through chance; in this table n is two less than the total number of the various frequencies compared. For example in table 4, Part I, eleven frequencies are compared in the ungrouped data hence the table is entered with $n = 9$.

Experiment I. Six counts were made on *Rhizobium trifolii*, culture 209, in which the entire 400 small squares were counted and the distribution of organisms per square compared with the theoretical value given by the expansion:

$$Ne^{-m}\left(1 + m + \frac{m^2}{2!} + \frac{m^3}{3!} \cdots\right)$$

where N equals total squares counted and m equals mean number of organism per square. The table of Soper (1914) was used to calculate the theoretical distribution. Table 4 gives results of two typical counts. The probabilities that the deviations noted arose from chance, 0.77 and 0.43, indicate that the observed distributions followed the theoretical Poissan law, hence the accuracy of the counts made under these conditions can be immediately determined since the standard deviation of the mean of this distribution is equal to $\sqrt{\frac{m}{N}}$. Thus in Part I, the mean number of organisms per square was 5.16. This has a standard deviation of

$\sqrt{\frac{5.16}{400}} = 0.1135$. If we assume that the true mean is within plus or minus three times the standard deviation of the observed mean, we know that the true mean is between the limits 4.82 and 5.60 and the maximum error in the observed mean is 6.6 per cent.

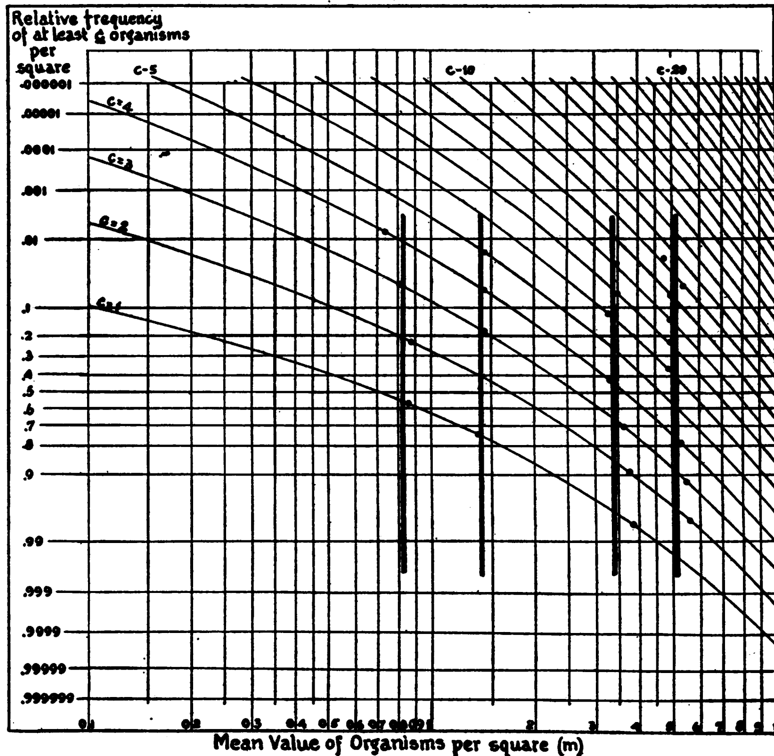

CHART 2. COMPARISON OF OBSERVED DISTRIBUTIONS OF *Rhizobium trifolii* WITH THEORETICAL GIVEN BY POISSAN EXPONENTIAL SUMMATION

Even when grouped in an unfavorable manner by placing together consecutive positive and negative residuals (x-m) the probabilities are such as would be expected from perfectly normal data. Thorndyke (1926) has shown that if the *relative frequency* of obtaining at least c occurrences in data from a Poissan distribution is plotted against c on a special graph paper[2] which she describes,

[2] Arithmetic Probability Paper No. 3127. Designed by Hazen, Whipple and Fuller. Codex Book Company, Inc., New York.

the points should follow a straight line drawn from the number on the base which gives the average number of occurrences (m). Thus, in order to test any data to determine whether they were drawn from a population with a Poissan distribution it is only necessary to plot the relative frequency of at least c occurrences against c and observe whether the points so located follow the proper straight line. For details of the construction of this graph paper and the reasons why these points must lie on a straight line for a Poissan distribution, the reader is referred to the original article. Data from four more counts of 400 squares were tested in this way as shown in chart 2. The fit to the straight line is satisfactory in every case, especially the points in the center where the data are more reliable.

These results indicate that under laboratory conditions counts made with the Petroff-Hausser bacteria counter followed the theoretical distribution satisfactorily and therefore confirmed the accuracy and reliability of counts obtained by this method.

Experiment II. In actual practice it is not feasible to count the entire 400 squares as the added accuracy obtained would not repay for the time required. In subsequent work counts were made of 144 small squares (9 large ones) distributed symetrically over the total area of 400 mm. Dilutions were made so that the average number per small square was between 4 and 5. This average number was found to lead to distributions most readily counted and since a total of about 600 organisms are counted the result is probably accurate within 8 per cent which is sufficient for most routine work. Fifty tests counting 144 squares were made and the observed distributions compared with the theoretical ones. The organisms used were *Rhizobium trifolii, Rhizobium meliloti, Rhizobium japonicum,* and *Rhizobium leguminosarum.* These were grown in liquid medium with and without added carbohydrate, on agar slants and on agar in 16 oz. bottles. The probabilities found that the deviations noted arose from random sampling were grouped as follows:

15 samples had probabilities between 0.00 and 0.20
6 samples had probabilities between 0.20 and 0.40
10 samples had probabilities between 0.40 and 0.60

10 samples had probabilities between 0.60 and 0.80
9 samples had probabilities between 0.80 and 1.00

Since the expectation in each group is 10 it can readily be seen that the agreement with theory of the 50 distributions examined

TABLE 5

Effect of dilution

	DILUTION	COUNT	$\frac{x - \bar{x}}{\text{S.D.}} = t$*
		$\times 10^{-6}$	
Culture 209	1:1	121	1.42
		144	3.66
	1:2	89	1.68
		101	0.51
	1:3	80	2.55
		106	0.03
	1:4	103	0.32
Mean		106.3	
Standard deviation		10.3	
Culture 29:16	1:20	270	3.32
		312	1.01
	1:10	374	2.39
		366	1.95
Mean		330.5	
Standard deviation		18.20	
Culture 29:16	1:8	550	0.55
		528	0.40
	1:10	513	1.05
		558	0.89
Mean		537.3	
Standard deviation		23.18	

* x = observed count.
$\bar{x}$ = mean of counts.

is fairly close to that expected. If the observed probabilities are grouped in 4 series the agreement with expectation is even closer. This agreement was confirmed by summing the X^2's and the n's used in making the individual tests for goodness of fit. Fisher

(1930) shows that in this case if $\sqrt{2x^2} - \sqrt{2n-1}$ is less than 2.00 the data may be considered normal. For the 50 counts made, this difference was +0.57; hence the data are entirely satisfactory. It can be concluded on the basis of these 50 counts that, even in routine work, the theoretical distributions are achieved closely enough to establish confidence in the accuracy of the counts made.

Experiment III. The effect of dilution and the checks to be expected on duplicate counts were next investigated. Three cultures were taken and duplicate counts made at different dilutions; the mean of all the counts and the standard deviation were calculated. The statistic, t = deviation from mean of individual counts divided by the standard deviation was then determined. If this is less than 3.0 the counts may be considered to be unaffected by dilution. Table 5 shows that only 2 out of 15 tested indicated abnormal variation in count due to dilution.

The checks on duplicate samples were determined in a similar manner. Twelve sets of duplicates and two sets of triplicate counts were examined and the value of t was determined. Of the 18 values of t so calculated, 9 were less than 1.00; 5 were between 1.00 and 1.50; and 4 were between 1.50 and 2.00; one was 3.04.

Other methods of counting

A few experiments were made in which direct counts were obtained by the Fries method (1921). However, this method did not prove satisfactory since the counts obtained were erratic and in a large number of cases they were less than the corresponding plate count, an entirely unexpected result. Winslow (1905) reports a similar finding; he found a disappearance of 100,000,000 bacteria per cubic centimeter within four hours, which he concluded to be due to loss of staining property. With rhizobia another possibility may be suggested. Examination of slides reveals rod-shaped bacteria with rounded granular bodies staining heavily. Always in the same fields are numerous spherical bodies the size of those contained within the rods, staining similarly. At the time of examination, there was considerable doubt as to whether or not these granular bodies should be included in the count; but

owing to extreme difficulties in drawing lines of distinction between them and precipitates of dyes and medium and dirt particles on the slide, it was deemed best not to include them. That these granular bodies may have been released from the banded cells during the course of vigorous shaking, has been suggested as a possible explanation for their presence on the slides; it may also be an explanation for the increased plate counts over the Fries counts, if we may assume that the granules are cell fragments or stages in the life cycle capable of reproduction.

The dilution method for estimation of viable bacteria was also tried. One cubic centimeter of each dilution was added to 5 tubes of litmus milk and to 5 tubes of Medium 79. Readings of the tubes were made after 10 days and the most probable number of organisms determined from McCrady's tables (1918). There was little difficulty in determining growth in either the litmus or the yeast-water mannitol solution but the results obtained indicated that some factor was present that interferred with the accuracy of the counts. For example most of the significant figures were of the type *500.* It would appear that the fundamental assumption of this method, i.e., that one cell can initiate growth is not always fulfilled with this organism. It is more likely that fairly large numbers of organisms must be present in order that growth can start in which case all the tubes are positive, but as soon as the number of organisms become smaller than some critical value, growth is not initiated. Such a condition would interfere with the variations in growth due only to a chance distribution which is the basis of the dilution method. The work of Allyn and Baldwin (1930) on the dependence of growth on the oxidation-reduction character of substrate lends support to the view that initiation of growth may be a function of population rather than of individual cells. This may also be a factor in the case of erratic plate counts.

SUMMARY

Plate counts of members of the genus *Rhizobium* are very erratic unless especial care is taken to maintain a standardized technique. Even if the latter is made uniform, the variability among individ-

ual plates is often larger than that attributable to random sampling. In such cases the abnormal variability usually results from one plate in a series, rather than from uniform variation among the replicates.

This variability can be partially eliminated by an increase in the number of parallel plates. Also, if three or more plates are made in parallel, the elimination of any one plate that is markedly different from its replicates can be made without bias. Sets of three and four plate data examined after elimination of any plate that was decidedly at variance with its replicates indicated that the variations noted in the remaining plates could have arisen by chance alone.

The cause of this abnormal variation was not investigated but other experiments and observations indicated that (1) stages in the life cycle and (2) unique growth-initiation requirements of the individual cells or stages, especially in regard to the oxidation-reduction character of the medium, may be factors involved.

In any study requiring a viable count the use of as many parallel plates as possible without introducing an undue time factor is desirable. In all cases at least three replicates are advisable.

Direct counts of rhizobia by means of a Petroff-Hausser counting chamber can be made under laboratory conditions in such a manner that the variance is that due to chance alone. In this case the distribution of the cells per unit area follows the Poissan Exponential Summation, and the accuracy of the count will be dependent solely on the total number of organism counted. The standard deviation of such a count is equal to $\sqrt{m}$ and the standard deviation of the mean $= \sqrt{\frac{m}{N}}$ where m is the mean number of organism per unit area, and N is the total squares counted.

The direct count with the Petroff-Hausser or similar bacteria counting chambers proved to be the most accurate of all the methods investigated and its use whenever possible is advised.

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