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THE ACCURACY OF THE PLATING METHOD OF ESTIMATING THE DENSITY OF BACTERIAL POPULATIONS

Author's Note (CMS 4.324a)

Starting with a purely empirical examination of the precision obtained in estimates of the numbers of soil bacteria, the authors were led to examine the properties of the small samples of the Poisson series, and to recognise that the statistic χ^2 supplies an index of dispersion for sets of parallel plates by which their homogeneity may readily be examined. This new tool is then applied to examine the circumstances in which aberrant or exceptional counts have been found to arise, in data from Rothamsted and elsewhere.

THE ACCURACY OF THE PLATING METHOD OF ESTIMATING THE DENSITY OF BACTERIAL POPULATIONS

WITH PARTICULAR REFERENCE TO THE USE OF THORNTON'S AGAR MEDIUM WITH SOIL SAMPLES

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(With 2 Text-figures)

1. INTRODUCTION

THE accuracy of the estimates of bacterial density, in samples of soil, water, or other material, obtained by the plating method, is only one of many points which arise in the interpretation of bacterial counts. The full interpretation of such data would include a consideration of the divers species that occur on the culture media, and of the forms in which they exist in the soil. The partial or total exclusion of certain forms, such as anaerobes, that require special cultural conditions, must also be considered in a full examination of such data, for a single medium supplies, necessarily, but a single aspect, however comprehensive, of the bacterial flora of the soil. Questions too, as to what is to be considered as the unit of enumeration—the individual organism as it exists in the soil, or possibly groups of such organisms adhering to single particles of soil, and undetached by the processes of sampling and dilution—whatever their importance may be, are not the object of the present investigation.

For if all these inquiries could be answered with certainty and precision it would still remain to be discovered with what accuracy the numerical estimate of bacterial density, obtained from a single set of plates, represented the actual bacterial density in the sample, and in the material from which the sample was drawn.

The question of *accuracy*, therefore, unlike the other elements in the interpretation of bacterial count data, is primarily a statistical question

and may be thrown into the characteristic statistical form of the estimation of a population from a sample. Only in peculiarly favourable cases, however, as will be seen more clearly below, could we rely upon an *a priori* mathematical solution.

2. THE PLATING METHOD

The plate method of counting soil bacteria is an adaptation of the plate counting technique, developed by Koch in 1881, applied to the special conditions of soil bacteria.

The process in general consists in making a suspension of a known mass of soil in a known volume of salt solution, and in diluting this suspension to a known degree. The bacterial numbers in this diluted suspension are estimated by plating a known volume in a nutrient gel medium and counting the colonies that develope on the plate. An estimate of the bacterial numbers in the original soil is then made by a simple calculation, the mass of soil taken and the degree of dilution being known.

There are great variations in the details of the method as employed by various workers. These differences concern all the stages in the process and also the nature of the gel medium used in plating. An idea of the extent of this lack of standardisation may be gathered from a paper by Z. N. Wyant[16] in which a number of the variations in technique used by different workers has been collected from the literature.

As an example illustrating the process, however, the technique used at Rothamsted and employed by Cutler in the bacterial count work discussed below, will be described.

Ten grams of the soil sample are placed in 250 gm. of sterile saline solution and shaken for four minutes to obtain a suspension of the soil. 1 c.c. of this suspension is placed in 99 c.c. of sterile saline solution and shaken for one minute to ensure a uniform distribution of the contained organisms. 1 c.c. of this second dilution is placed in another 99 c.c. of saline and shaken for one minute.

Every cubic centimetre of this final dilution will then contain $\frac{1}{250000}$ grams of the original soil sample.

One c.c. of this dilution is then delivered into each of five petri dishes and mixed with an agar medium. After incubation the bacterial colonies on each plate are counted, and the mean of the five parallel counts taken. From this the bacterial numbers per gram of soil are estimated.

The bacterial numbers obtained by the plating method do not represent the total bacterial content of the soil. This is clear from the fact

that on no single medium will all the physiological groups of soil bacteria develope. In using this method, however, it is hoped to obtain a standard of bacterial density by which two or more soil samples can be compared. To obtain this result from the method a careful standardisation of the whole technique is essential, in order that those sources of error that cannot at present be eliminated, such as the failure of some organisms to develope on the plates, may be rendered so uniform as to affect the count in a constant manner.

This standardisation must comprise both (*a*) the manipulative portion of the technique involved in making the dilutions, and (*b*) the composition of the medium employed in plating.

In applying results obtained by the method it is necessary to have an estimate of its degree of accuracy, and in order to improve it, some knowledge must be obtained as to which stages in the process are the chief causes of the variation in results.

For the results of the plating method to have their highest possible accuracy, very severe conditions would have to be fulfilled. An imaginary experiment will perhaps serve to make the conditions clear.

If a 10 gm. sample of soil were diluted down to a dilution of 1 gm. in 250,000 c.c., enough material would be provided for $2\frac{1}{2}$ million plates. The result of such an experiment would be of the highest possible accuracy, if one could assume that

(I) Each plate offers the same facilities for development.

(II) The development of any organism is independent of other organisms present.

(III) Development results in only one visible colony.

Since in practice only a few plates are prepared, two additional conditions are involved in the sampling theory.

(IV) Each plate has an equal chance of receiving any organism.

(V) The organisms are distributed independently.

The fulfilment of the first, fourth and fifth conditions depends upon the perfection of the technique employed. The second and third conditions depend definitely on the nature of the organisms, and are only matters of technique in so far as this term may be employed for the choice or elaboration of a medium upon which the organisms, which it is desired to study, fulfil those conditions, and which excludes the interference of those which would fail to do so.

These conditions can to some extent be tested independently. Thus, in a short experiment, where a single batch of medium is used, it is to be expected that the medium in each plate will offer the same facilities

for development (Condition I). In a long experiment, however, where a number of different batches of medium are used, this will be the case only if the medium can be accurately reproduced, if, that is, different batches of medium, prepared independently, give significantly the same results. This reproducibility has been confirmed for Thornton's agar medium (Thornton, 1922(11)).

Again condition (IV) would fail if from any cause the dilution was carried out in an irregular manner. This may be tested directly by carrying through the whole dilution process independently with different portions of the same sample. The following experiment is an example of such a test.

Four portions of a sample of Barnfield soil, simultaneously analysed by four different workers (Aug. 14, 1921), gave the following counts:

Table I

Plate	Portion			
	A	B	C	D
1	26	28	31	37
2	30	33	26	32
3	30	32	28	32
4	29	26	32	30
5	32	27	31	26
Mean	29·4	29·2	29·6	31·4

The four sets of plates are indistinguishable from random samples from a single population. The variance estimated as from a single sample of 20 is 8·52, actually less than the mean value for the variance within each set, 9·15. An equivalent test is provided by the correlation between different plates of the same set; this is $-·089 \pm ·108$, negative and quite insignificant. In spite of the fact that the different plates of the same set agree very closely, the variation between the four means is quite insignificant.

Table II

Plate	Portion			
	I	II	III	IV
1	72	74	78	69
2	69	72	74	67
3	63	70	70	66
4	59	69	58	64
5	59	66	58	62
6	53	58	56	58
7	51	52	56	54
Mean	60·86	65·86	64·28	62·86

Equally close is the agreement between the sets of seven plates prepared from four parallel series of dilutions (June 22, 1922), shown in Table II. No trace of differentiation is observable, and the four sets must be regarded as random samples from a single population.

On certain occasions the same point is established by the analysis of simultaneous samples from the same field. An agreement in such cases shows the uniformity in bacterial density of the portion of the field sampled; it also serves to show that no significant differences are introduced by variations in the process of dilution. Thus four simultaneous samples from Broadbalk (Aug. 14, 1921) gave the following counts.

Table III

Plate	Sample			
	I	II	III	IV
1	38	45	43	27
2	32	40	34	41
3	52	45	52	35
4	32	31	55	36
5	40	43	38	45
Mean	38·8	40·8	44·4	36·8

From the whole set of 20 the variance is 56·27, from the four sets of 5, 56·97, not a significantly greater value. The correlations between plates of the same group is + ·014 ± ·108, an insignificant positive value. By the most sensitive tests possible, no differentiation is observable.

There is thus reason to claim that the manipulative technique can be so efficiently standardised that no significant variations in it are detectable, having regard to the variance that occurs between the colony numbers developing on parallel plates from a single final dilution.

Our attention is thus drawn to this variance between parallel plates, which may be due solely to the chance distribution of organisms within the final dilution, or may in addition be influenced by the mutual interference between organisms on the plates, or by the failure of certain organisms to develop into single discrete colonies.

It is therefore necessary, in interpreting the results of the counting technique, to discover the relative importance of these influences, on the colony numbers, and on the variance between them. It is on the experimental evidence as to the actual nature of this variance between parallel plates that our further conclusions will be based.

Nevertheless, the two questions of the reproducibility of the medium and of the equivalence of results obtained by independent series of

dilutions made from a single sample, are here insisted upon, because failure in either of these two points would not necessarily affect the agreement between parallel platings, from the same final dilution, which is studied below.

3. THE POISSON SERIES

It was shown by Poisson(1) in 1837, that if a large number of individuals, N, are each exposed independently to a very small risk of an event of which the probability of occurrence in any instance is p, then the number of occurrences, x, in any trial will be distributed according to a definite law, sometimes called the Law of Small Numbers. The distribution of x is found to depend on a single parameter

$$m = pN,$$

in such a way that the probability that the number of occurrences shall be x is given by the formula

$$e^{-m} \frac{m^x}{x!}.$$

It should be noted that x is always a whole number, while m may be fractional; the mean value of x is equal to m, and when m is large the distribution, except for its essential discontinuity, resembles a normal distribution, having its mean at m and the *variance* (the square of the standard deviation) also equal to m.

The importance of the Poisson series in modern statistics was brought out by "Student"(2) in 1907[1], in discussing the accuracy of counting yeast cells with the haemocytometer. Since the chance of any given yeast cell settling upon any given square of the haemocytometer is extremely small, while the number of cells is correspondingly great, "Student" arrived independently at the Poisson formula, as a theoretical result under technically perfect conditions. He was able to show that, in some instances, counts of 400 squares agreed with the theoretical

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[1] The Poisson Series had been successfully applied by von Bortkiewicz to the annual number of deaths from horse-kick in a number of Prussian Army Corps(10). Miss Whitaker's criticism(8) of this application is entirely vitiated by her neglect of the variation of random samples.

H. Bateman (1910)(9) arrived at the formula for the Poisson Series, as the distribution of the number of a particles, emitted by a film of polonium, which strike a sensitised screen in successive equal intervals of time. The formula was used by Rutherford and Geiger to test the independence of simultaneous emissions. The distribution of 2608 counts shows a general agreement with expectation, though there are discrepancies not easily to be explained by chance. The observations are certainly not adequate, as these authors suggest, as "a method of testing the laws of probability."

distribution, and that when this is the case the accuracy of the count is known with precision and depends only on the number of cells counted[1].

The ideal conditions for bacterial counts made by the dilution method, are closely parallel to those found necessary in the case of the haemocytometer. The chief practical difference lies in the fact that instead of 400 squares with only a few yeast cells in each, we have some five plates with perhaps 200 colonies apiece. The agreement of the results with the theoretical distribution cannot, therefore, be demonstrated from a single count. Under ideal conditions the data would consist of a number of small samples from different Poisson series. For this reason as soon as it was suspected that this ideal condition might have been realized in practice, a special investigation of the nature of such samples was undertaken, owing to the importance of demonstrating the substantial fulfilment of the severe conditions laid down in the previous section.

4. PRELIMINARY REDUCTION OF CUTLER'S DATA

When the question of the accuracy of the bacterial counting technique was discussed between the present authors in the spring of 1921, it was decided that the daily observations of bacterial numbers then being carried out at Rothamsted by Cutler would afford a valuable opportunity of studying the variance between parallel plates and its causes. In this choice our investigation was more than fortunate, for no other series of bacterial counts known to us, of which many have been examined, would have gone so far in clearing up the obscurities of the subject.

In conjunction with daily estimations of soil protozoa carried out at Rothamsted from July 1920, daily counts of bacteria were also made in the protozoological laboratory (Cutler(17)). The dilution technique used in this work has been described above. Plates were incubated at 18° C., and counted after five and seven days, the seven day counts only are considered here. Throughout the work the agar medium recently elaborated by Thornton(11) was used. The data thus supply an extensive test of this medium under routine conditions.

When the statistical examination of these data was commenced it was not anticipated that any clear relationship with the Poisson distribution would be obtained; the reduction was designed to determine empirically the relation between the mean bacterial number calculated from any set of plates, and the variability of that set about the mean. Knowing this relation, a probable error could be assigned to each value.

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[1] Valuable tables of the Poisson Series have been prepared by H. E. Soper(7).

Two statistics were calculated from each set of plates. If x stand for the number of colonies on each plate, and n for the number of plates, the necessary statistics were:

the *mean*
$$\bar{x} = \frac{1}{n} S(x),$$

and the *variance*
$$v = \frac{1}{n-1} S(x - \bar{x})^2,$$

where S stands for summation.

The values of v, being the estimates of the variance from small samples, were inevitably affected by large sampling errors, which depended upon the number of plates. The whole body of four-plate sets was therefore divided into groups, according to the value of $\bar{x}$. Thus for the two groups of four-plate sets having a mean number of colonies 65–75 and 75–85, the following values of v were obtained:

Table IV			Table V		
65–75			75–85		
Set No.	$\bar{x}$	v	Set No.	$\bar{x}$	v
29	69·75	65·58	59	77·00	78·00
33	73·50	27·00	89	76·75	142·91
51	68·75	312·25	97	84·75	144·25
60	71·50	401·67	105	84·50	56·33
128	73·75	60·91	149	79·50	77·67
164	72·75	146·25	169	84·50	123·67
227	67·50	27·67	240	82·25	8·91
241	68·75	8·91	273	84·50	48·33
249	67·25	7·58	301	84·25	73·91
263	73·25	112·58	Mean		83·78
272	72·75	52·91			
330	70·00	55·33			
Mean of 12		106·55			
Mean of 10		56·47			

Two facts are apparent from these results (1) the variability of v is so great that accurate values are not obtained from the means of about 10 values; (2) the difficulty of estimating the variance for given values of $\bar{x}$ is still further increased by the occurrence of occasional very large values of v. The values of v in sets 51 and 60 in Table IV are much greater than the other 10 values in the same group. The values of the means obtained by excluding and including these high values are given at the foot of the table.

The first difficulty could be overcome by fitting to the actual values obtained a smooth curve representing the mean v for given $\bar{x}$; before

doing so, however, it was thought advisable to exclude as far as possible the exceptional large values. As a rough criterion it was decided to exclude those values which exceeded by more than threefold the mean value of the group. In the larger groups this criterion acted well; in the smaller groups, such as occurred for high and low values of $\bar{x}$, it was necessarily inconclusive, even when account was taken of neighbouring groups. The curve fitting was therefore confined to the region in which the data appeared to be sufficiently abundant.
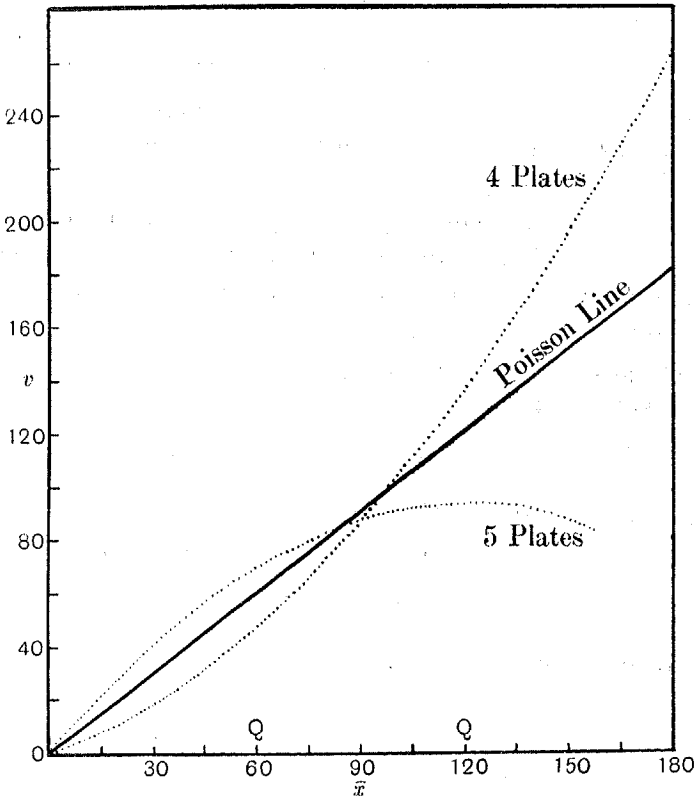

Fig. 1. Smooth curves fitted to Cutler's data.

Curves of the form
$$v = A\bar{x} + B\bar{x}^2$$
(where A and B are two constants determined from the data) were fitted to the four-plate data from $\bar{x} = 0$ to $\bar{x} = 180$, and to the five-plate data from 0 to 160; the curves obtained are shown in Fig. 1.

The straight line, $v = \bar{x}$, represents the relation between the variance and the mean in the Poisson Series. The curves evidently tend to cling closely to this line, especially in the region (60–120) where the data are most abundant. The curves strongly suggested that the departures in

these data from the Poisson samples were not, as had been expected, *systematic*, but were due to the *sporadic* occurrence of exceptional sets; the curvature in the smooth curves being perhaps largely due to the crudity of the criterion employed in excluding the exceptions. This view impressed the authors with the necessity of studying the distribution of small random samples from the Poisson Series, with the double object of devising a valid criterion for the recognition of exceptions, and of testing accurately whether or not the remainder were in reality such random samples.

5. SMALL SAMPLES OF THE POISSON SERIES

The study of small samples, essential as it is to the development of adequate statistical methods, has hitherto been practically confined to the normal curve and surface. The following investigation may serve to show, that by taking account of the fundamental properties of those statistics which are derived by the method of Maximum Likelihood, the sampling problems of even discontinuous distributions admit of material simplification.

In a sample from a Poisson Series, the chance of any observation having the value of x is

$$e^{-m} \frac{m^x}{x!},$$

where m is the parameter of the series.

Hence the chance of observing a given series of values $x_1, x_2 \ldots x_n$ is

$$\Delta f = e^{-nm} \frac{m^{n\bar{x}}}{x_1! \, x_2! \ldots x_n!}.$$

If we estimate m from such a sample by the method of maximum likelihood, we have

$$\frac{\partial}{\partial m} (\log \Delta f) = -n + \frac{n\bar{x}}{m} = 0,$$

so that $\bar{x}$ is the most likely value of m, and in consequence, as Fisher has recently shown[3], it may satisfy the criterion of sufficiency, in which case the distribution of any other statistic, for a given value of $\bar{x}$, must be independent of m.

That this is so may be proved directly; for

$$e^{-nm} \frac{m^{n\bar{x}}}{x_1! \, x_2! \ldots x_n!}$$

may be put into the form

$$e^{-nm} \frac{(nm)^{n\bar{x}}}{(n\bar{x})!} \cdot \frac{(n\bar{x})!}{n^{n\bar{x}} x_1! \, x_2! \ldots x_n!},$$

the first factor represents the chance of obtaining a given value of $\bar{x}$, and the second, which does not involve m, gives the chance that the sample shall show any particular partition of the total, once the total is fixed. The distribution of any statistic which depends upon this partition, must therefore be independent of m, once $\bar{x}$ is fixed. The problem of the distribution of v is therefore susceptible of the great simplification, that we need only consider its distribution for given values of $\bar{x}$, and that this distribution is wholly independent of m.

The distribution of this, or any other, statistic, which depends upon a partition of an integer, must necessarily be discontinuous; when, however, $\bar{x}$ is large, even for small values of n, the number of possible values of v becomes sufficiently great for its distribution to be represented by a frequency curve. This procedure is the more advantageous in that. by the choice of a new statistic, which shall replace v, we can throw the distribution into a form independent of $\bar{x}$, whereas the actual partitions possible in the neighbourhood of equipartition, will necessarily change with the fractional part of $\bar{x}$.

The frequency with which any given partition of the total, $n\bar{x}$, occurs, is in fact the frequency with which any given series of values are obtained when the total is distributed at random into n cells, the expectation in each being $\bar{x}$. It is well known that when this is the case, the statistic

$$\chi^2 = \frac{1}{\bar{x}} S (x - \bar{x})^2 = (n - 1) \frac{v}{\bar{x}}$$

measures the departure of the sample from equipartition, being equivalent mathematically to Pearson's test of agreement between observation and expectation. The distribution of $\frac{1}{2}\chi^2$ is well represented by a smooth curve independent of $\bar{x}$ of the form (Pearson's Type 3)

$$df = \frac{1}{\frac{n - 3}{2}!} t^{\frac{n-3}{2}} e^{-t} dt,$$

and the frequency with which χ^2 exceeds successive integral values, has been tabulated by Elderton (4, 1902 and 5, 1914) for values of n from 0 to 30.

We are therefore in a position to test whether the conditions which lead to the Poisson Series are in fact fulfilled in any given body of bacterial data for which the counts on individual plates are known; it is only necessary to calculate the above index of dispersion (χ^2) from each set of parallel plates, and to determine whether the distribution of this

index is or is not in accordance with the distribution predicted from Elderton's tables, when

$$\chi^2 = \frac{1}{\bar{x}} S \, (x - \bar{x})^2$$

and $$n' = n.$$

The statistic χ^2 thus supplies an index of dispersion for sets of parallel plates. If the bacterial counts conform to the Poisson distribution the average value of χ^2 will be one less than the number of plates. For sufficiently numerous sets of plates the agreement may be tested more exactly by the use of Elderton's Tables.

6. THE χ^2 INDEX OF DISPERSION APPLIED TO CUTLER'S DATA

The values of χ^2 obtained from the sets of four parallel plates, grouped according to the value of the mean, are shown in Table VI.

Table VI

χ^2

$\bar{x}$	·5	1·5	2·5	3·5	4·5	5·5	6·5	7·5	8·5	9·5	10·5	> 11	Total
20	2	—	—	—	—	—	—	—	—	—	—	—	2
30	1	—	—	—	—	—	—	—	—	—	—	—	1
40	2	—	2	—	2	1	—	—	—	—	—	—	7
50	—	1	2	5	2	1	1	—	—	—	—	—	12
60	5	3	2	—	—	—	—	2	—	—	—	12·3	13
70	3	2	4	—	1	—	1	—	—	—	—	13·6, 16·9	13
80	1	1·5	2·5	1	1	2	—	—	—	—	—	—	9
90	2	3	1	3	1	—	—	—	—	1	—	—	11
100	1	3	3·5	—	—	—	—	1	1	—	—	14·8, 24·5	11·5
110	—	2·5	1	1	—	—	—	—	—	—	—	—	4·5
120	2	—	—	1	—	—	1	—	—	—	—	15·1	5
130	—	—	1	·5	—	1	—	—	—	—	1	14·2	4·5
140	—	—	2	1·5	1	1	—	—	—	—	—	—	5·5
150	3	1	5	1	—	—	—	—	—	—	—	—	10
160	6	1	—	—	—	—	—	—	—	—	—	—	7
170	1	—	—	1	—	—	—	—	1	—	—	17·5	4
180	4	1	1	—	—	—	—	—	1	—	1	24·0, 13·9	10
190	1	—	·5	—	—	—	—	—	—	—	—	—	1·5
200	2	1	·5	—	—	—	—	—	—	1	—	15·8	5·5
210	1	1	—	1	—	—	—	—	—	—	—	—	3
220	—	—	1	—	—	—	—	1	—	—	—	12·2, 16·8	4
230	—	—	—	—	1	—	1	—	—	—	—	21·4	3
240	—	—	1	—	2	1	—	—	—	—	—	—	4
250	1	—	—	—	—	—	—	—	—	—	—	—	1
260	1	—	—	—	—	—	—	—	—	—	—	11·4	2
270	—	1	—	—	—	—	—	—	—	—	—	29·1	2
	39	22	30	16	11	6	5	4	3	2	2	16	156

No obvious relationships are observable between the value of χ^2 and that of $\bar{x}$. There is indeed an excess of the exceptionally large values of

χ^2 (> 11) among the higher values of $\bar{x}$, but this on investigation proved to be completely accounted for by the epidemic character of the occurrences of these large values, which we shall demonstrate below (see Fig. 2). The longest and most severe epidemic occurred during a period (Oct.–Dec.) when the bacterial numbers were generally high. Within this period no sensible association is apparent.

Confining attention therefore to the distribution of χ^2, irrespective of the mean number of colonies counted, it is clear that the sets with exceptionally large variations, which interfered with the preliminary reduction of the data, are now distinguishable as those with high values of χ^2. If the sets were random samples of Poisson Series, it appears from Elderton's Tables that only 3 per cent. of the observed values should exceed 9. It is clear that there is here a group which must be excluded in considering the agreement of the remainder with the theoretical distribution. If this were the only irregularity in the observed numbers we should therefore compare them with a theoretical series having the same total below 9. As it is there is also some irregularity visible at the beginning of the series, suggesting that there is also an excess of unduly small values of χ^2. For this reason we shall base our comparison on the total observed between 1 and 9, as is shown in Table VII.

Table VII

Comparison of observed and expected distribution of χ^2, 4-plate data.

χ^2	Expected m	Observed $m + x$	Difference x	$\dfrac{x^2}{m}$	
·5	24·97	39	+ 14·03		
1·5	28·76	22	− 6·76	1·589	
2·5	22·72	30	+ 7·28	2·333	
3·5	16·36	16	− ·36	·008	
4·5	11·27	11	− ·27	·006	·136
5·5	7·56	6	− 1·56	·322	
6·5	4·99	5	+ ·01	·000	
7·5	3·25	4	+ ·75	·173	·266
8·5	2·10	3	+ ·90	·386	
over 9	3·68	20	$\chi^2 = 4\cdot817,\ 4\cdot324$		
Total	125·66	156	$P = \cdot682,\ \cdot232$		

Within the range from 1 to 9, the agreement of the observed with the expected values is striking. When tested in eight groups, the probability of obtaining a worse fit by chance from perfectly normal data is ·682,

and even when grouped in the most unfavourable manner, by throwing together consecutive positive and negative residuals, a method suggested by Mr Udny Yule, the probability is still ·232. There is therefore no significant deviation of those values from expectation.

Of those above 9, we may anticipate that some three or four will be normal values and the remainder exceptions. It is of course impossible to separate these with absolute certainty. In discussing the evidence for epidemics we shall assume that the four values below 11 are normal and that the remainder are exceptions. When, however, the fact of the epidemic incidence of those exceptional values is taken into account, it appears that the two between 10 and 11 are among the relatively few "normal" sets occurring in an epidemic period and are therefore probably exceptions, while the two between 9 and 10, and possibly also the value at 11·4, are for the same reason probably normal.

It is thus possible to separate this class of exceptions from the remaining data with some degree of certainty and to study them individually, but this is not possible for the exceptionally invariable sets. All that we can do here is to show that the evidence for their real existence is stronger than appears in Table VII. If we subdivide the region of the first two groups of that table somewhat more closely we obtain

<div align="center">Table VIII.</div>

χ	Expected	Observed
0		
	11·82	21
·75		
	9·97	12
·95		
	12·56	17
1·15		
	14·15	9
1·35		

the excess of numbers is most clearly marked in the group of smallest values, and is possibly though not certainly confined to the region.

These conclusions are independently confirmed by the sets of five parallel plates. In Table IX is shown a comparison of the observed distribution with that expected, on the basis of the total observed between 2 and 11.

The agreement with expectation in the range from 2 to 11 is perfectly satisfactory; when tested in the 9 unit groups, the possibility of obtaining

a worse fit by chance from normal data is ·765. Grouping together the consecutive positive and negative errors, it only falls to ·571. There is again no significant deviation of the distribution in this range from expectation.

Table IX

Comparison of observed and expected distribution of χ^2, 5-plate data

χ^2	Expected m	Observed $m+x$	Difference x	$\dfrac{x^2}{m}$	
·5	10·94	25	+ 14·06		
1·5	21·10	27	+ 5·90		
2·5	21·58	24	+ 2·92	·271 }	·402
3·5	18·41	20	+ 1·59	·137 }	
4·5	14·39	12	− 2·39	·397	
5·5	10·69	11	+ ·31	·009 }	·146
6·5	7·67	9	+ 1·33	·231 }	
7·5	5·37	5	− ·37	·025 }	1·826
8·5	3·70	0	− 3·70	3·700 }	
9·5	2·51	3	+ ·49	·096 }	·157
10·5	1·68	2	+ ·32	·061 }	
over 11	3·22	18		$\chi^2 = 4\cdot927,\ 2\cdot938$	
Total	121·26	156		$P =\ ·765,\ ·571$	

Of the values above 11, three lie between 12 and 13, and in discussing the evidence for epidemics we shall assume that these are normal sets, and that all those above 13 are exceptions. When we take the evidence of epidemic incidence into account, it is found that the only four sets above 13 which might reasonably be considered normal all occur in epidemic periods, and that the same is true of one out of the three between 12 and 13. This therefore (No. 160, see Fig. 2) is probably also an exception.

The conclusions to be drawn from the 4-plate and from the 5-plate data, thus confirm each other at every point. In both groups the sets having exceptionally high variability may be identified in almost every case with certainty. The majority of both groups, about 124 of the 4-plate sets, and about 117 of the 5-plate sets, are evidently true samples of the Poisson Series. Both groups show an excess of cases of small variability, but it is not possible to specify the actual sets affected by this; it is evident that this cause, like that which produces high variability, is sporadic and not systematic in its action; it affects a certain number of sets in a definite manner, leaving the majority unaffected. This effect, whatever be its nature, is more clearly brought out in the

5-plate than in the 4-plate sets, possibly because the sets of five plates make possible a closer scrutiny into the exactitude of the agreement between the observed sets, and samples from a Poisson Series.

For the same reason the 50 sets of three plates cannot be expected to provide much additional information. The seven exceptionally high values stand out perfectly clearly; the lowest is 9·2, a value which would be exceeded by only one normal sample (of 3) in 100. The next highest values 5·4 and 6·4, would not be suspect save for their occurrence in December; they will be treated as normal.

Since the 3-plate sets are relatively scanty, we can best test their agreement with theory by dividing the theoretical distribution of 43 values at its quintiles, so that the expectation is the same in each group. We then have

Table X. Sets of three plates

$$\chi^2 = 1\cdot77 \qquad P = \cdot775$$

χ^2	Expected m	Observed $m + x$	x^2
0			
	8·6	8	·36
·4464			
	8·6	6	6·76
1·0216			
	8·6	11	5·76
1·8326			
	8·6	8	·36
3·2190			
	8·6	10	1·96
Total	43	43	15·20

The agreement with expectation is excellent, and the sets of three plates bear out the conclusions derived from the sets of four and five plates, save that here there is no visible excess of low values of χ^2.

It appears therefore that out of the 362 sets of plates examined the majority represent true samples from the Poisson Series, such as would be the case if the biological and technical difficulties of the bacterial count method as applied to soil had been completely surmounted. Forty sets, which can be identified almost with certainty, are affected by some cause or causes which greatly increase the variability between th plates, while probably a smaller number, including apparently non of the 3-plate sets, are affected by a second cause of error, which reduces the variability between the plates.