A Technique for the Quantitative Estimation of Soil Micro-organisms

By P. C. T. JONES and J. E. MOLLISON
Soil Microbiology Department, Rothamsted Experimental Station, Harpenden, Herts

With a Statistical Note by

M. H. QUENOUILLE
Statistical Department, Rothamsted Experimental Station

SUMMARY: Soil micro-organisms have been counted by a new technique whose essential feature is the suspension of measured amounts of soil in a molten agar gel from which small drops are removed and allowed to solidify as thin films on a haemocytometer slide of known depth. The instantaneous gelation of the agar ensures the fixation of the soil constituents in their original distribution. The films are dried and stained in a solution of acetic-aniline blue and permanent preparations made by subsequent dehydration in ethanol and mounting in euparal. If the suspension is of known dilution, since films of a definite volume contain a known quantity of soil, differential counts of a measured area of film will yield a quantitative estimate of soil micro-organisms.

The distribution of bacteria per microscopic field was found to be complex. The frequencies of bacterial colonies and of pieces of fungal mycelium form a Poisson series; those of the number of bacteria per colony form a logarithmic series; and those of the total number of bacteria per field fall into a negative binomial distribution.

The method appears to be capable of modification by the use of selective nutrient media for determining the quality of the microflora and possibly the percentage viability of the organisms present.

Since interest was originally aroused in the quantitative and qualitative estimation of the soil microflora, various methods have been developed to this end. It was early recognized that dilution plate counts, while giving consistent estimates of some soil micro-organisms and therefore having some value for comparing different soil samples, fail to give the total numbers, since the various media employed are selective in their action. Moreover, though the method has one great advantage in that living organisms only are counted, doubt always exists as to whether colonies of bacteria develop from one or more organisms and fungal hyphae from fragments of mycelium or from spores. On these considerations attention was turned to microscopic techniques of direct examination of soil.

Stained smears of soil as used by several workers, notably Conn and Winogradsky, give little accurate information of actual numbers owing to the difficulty of estimating the exact quantity of soil examined. Winogradsky (1925) attempted to estimate this quantity by the increase in weight of the microscope slide on which the smear had been fixed, while Conn (1918) sought to obviate the difficulty by spreading 10 mm.^{3} of soil suspension of known

dilution over a sq.cm. of a microscope slide. However, it is impossible by this means to ensure an even distribution of particles and organisms throughout such smears, so that the counts give an inaccurate estimate of the soil population.

A definite advance is seen in the method of Thornton & Gray (1934), in which a known quantity of soil is added to a counted suspension of indigo particles of a size and density approximately similar to those of bacteria. Small drops of this mixture are placed on a slide, dried, and then stained with erythrosin. During the drying of a drop of soil suspension, surface-tension forces will alter the distribution of contained particles, but it is assumed that indigo particles and organisms will be affected similarly. From the ratio of numbers of bacteria to indigo particles in a definite number of microscopic fields, it is possible to estimate the numbers of bacteria/g. of soil.

In applying this method, it was thought that a blue stain would give better background contrast than the red or violet acid dyes previously used. Agar films were made and stained by aniline blue lactophenol. This proved to be an excellent bacterial as well as fungal stain. However, this entailed the use of a contrasting 'ratio' particle of which many were prepared but none proved satisfactory for various reasons.

A homogeneous distribution of both organisms and soil particles could be obtained in a soil suspension in agar and in thin films subsequently prepared from it. The viscosity of the agar prevented soil flocculation, and its instantaneous gelation prevented the disturbance of the particles by surface-tension forces during drying; and a count of a known volume of soil suspension could be made when the films were prepared on a haemocytometer slide of known depth. Counts from such films gave evidence that a random distribution of organisms had been achieved and the need for adding a counted suspension of particles was thus obviated, since the number of organisms/g. of soil could now be derived directly from counts from the agar film of organisms alone.

During subsequent work with this aniline blue-lactophenol staining method it was found that the quality of the staining was variable, although the same procedure was followed on all occasions. Moreover, the stain gradually faded if the preparations were kept for several weeks. The impermanent nature of the lactophenol mounts was also unsatisfactory. Dehydrating the films in ethanol after staining and mounting in Canada balsam proved unsatisfactory, for differentiation was poor and balsam proved to have too low a refractive index for satisfactory resolution. The expedient of drying the film on to the slide was adopted after measurements had shown that the film did not shrink laterally when dried, and so the lateral distribution of particles and organisms remained unaltered, though the film had dried to a negligible thickness.

A solution of aniline blue in aqueous phenol and acetic acid was used by Maneval (1936) for staining fungal mycelium, using lactophenol as a mountant. The authors found that clear differentiation as well as permanent preparations of soil films could be obtained by the use of this stain with subsequent differentiation and washing in ethanol and finally mounting in euparal. The technique finally adopted was as follows.

Description of the method

The method involves counting the organisms in a number of microscopic fields selected at random and calculating the total volume represented by the microscopic field observed. The latter is estimated by multiplying the area of the microscopic fields by the depth of the haemocytometer slide on which the films are prepared. This gives the volume of soil suspension observed. Hence, if the initial dilution of soil in agar be known, the number of organisms per g. of soil can be readily calculated by multiplying by a conversion factor, which is much simplified if the initial quantity of soil be so adjusted as to give a simple fraction of 1 g. of soil in the total number of microscopic fields observed. For observation under oil immersion the authors found that 0·05 μg. per 20 fields ensured a satisfactory density of organisms and soil particles per field. If the volume of 20 fields of 0·1 mm. depth be x ml. for a particular eyepiece-objective combination, then 0·05 μg./x of soil must be added per ml. of agar. Hence the average number of bacteria per 20 fields $\times 2 \times 10^7$ gives the number/g. soil. (For the authors' microscope the appropriate wt. of soil was 2·58 g.)

The soil sample is first sifted through a 2 mm. sieve and the required quantity weighed out, placed in a small crucible with 5 ml. of sterile distilled water and thoroughly ground up with a glass rod. The resultant suspension is then poured off into a 100 ml. sterile flask. The sediment is washed in 5 ml. sterile distilled water and the suspended matter poured off into the same flask. With care only the heavier sand fraction then remains in the crucible, but the procedure may be repeated if necessary. The soil suspension is then made up to 50 ml. with 1·5 % agar, previously filtered hot through a No. 1 Whatman filter-paper, sterilized and kept at a temperature high enough to prevent gelation. After the flask has been shaken vigorously and left for 5 sec. to allow sedimentation of the heaviest sand grains it is ready for use and should be used almost immediately, otherwise certain thermophilic organisms multiply rapidly, giving an inaccurate picture of the numbers and kinds of organisms present. A sample is pipetted from immediately under the surface of the suspension on to the platform of a haemocytometer slide of 0·1 mm. depth, immediately covered by a cover-slip and allowed to solidify. With a slide of 0·02 mm. depth large mineral particles caused films of greater thickness to form and hence give variable counts. A slide 0·1 mm. deep with a lower dilution of soil is preferable. The slide is then immersed in sterile distilled water and the cover-slip removed. Surplus agar, which has solidified in the moat of the haemocytometer slide, may be removed by running a sharp scalpel round the central platform; or, if a circular haemocytometer is used, the thick agar rim may be efficiently removed by a cork-borer. By agitation of the slide in the water the film is gently floated on to an ordinary microscope slide and allowed to dry. It is essential to use distilled water for this purpose to prevent precipitation on drying of salts normally dissolved in tap water. The films should be dried slowly at room temperature, as attempts to speed up drying generally result in their splitting.

One type of haemocytometer slide has parallel transverse grooves, which isolate a central rectangle ground to a lower level. The authors found it more convenient to form a square in the centre of this rectangle by grinding two grooves at right angles across it (see Fig. 1).

The dried films are then immersed for 1 hr. in the following stain: phenol (5 % aqueous) 15 ml.; aniline blue W.S. (1 % aqueous) 1 ml.; glacial acetic acid 4 ml.; filtered about 1 hr. after preparation. Other sulphonated triphenyl rosaniline dyes were tested and in general proved satisfactory. Since a standard

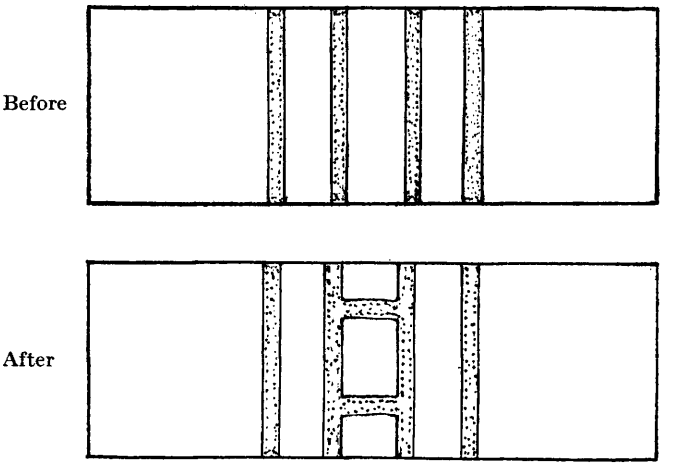

Fig. 1. Modification of haemocytometer.

dyestuff seemed desirable, British Drug Houses Ltd. water-soluble aniline blue was adopted. The specificity of the rosaniline dyes for basiphilic protoplasm was demonstrated by similar staining results with acid fuchsin.

The films are rapidly washed and dehydrated in 95 % ethanol, and permanent preparations made by mounting in euparal (Flatters and Garnett Ltd., Manchester).

For bacterial counts it is best to delimit the area of the microscope field by inserting in the eyepiece a glass disk ruled with a 2 mm. square.

Twenty random fields are then counted under oil immersion on each of four replicate slides, when multiplication by the appropriate conversion factor will give the number of organisms/g. of soil.

Verification of the method

It was thought desirable to test whether counts made by this method gave a true estimate of the total soil population. Accordingly, film counts were made of a soil sample previously sterilized and also of a replicate sample to which a known number of a particular species of bacterium was added from a counted suspension. The results in Table 1 show that the numbers of bacteria 'recovered' represent a very high percentage of those added.

The accuracy of this method depends on a uniform distribution of soil

organisms throughout the suspension and subsequently in the agar film in order that this film may be a valid sample of the population under investigation. It depends also on the possibility of so standardizing the technique of preparation and counting that similar results may be obtained by different workers.

Table 1. *'Recovery' of counted suspensions of bacteria added to sterilized soil (millions/g. soil)*

No. added	No. in sterile soil + added counted suspension	No. in sterile soil alone	No. of bacteria counted	'Recovery' (%)
6440	6205	42	6163	95·7
1642	2760	1144	1616	98·4

Since there are many sources of variation inherent in this method, it is necessary to determine when variation between counts becomes significant, i.e. when the difference between counts may be assigned to differing soil populations rather than to experimental errors.

Errors may be ascribed to:

(1) heterogeneity of the agar-soil suspension;
(2) variation between aliquot fractions of the soil sample;
(3) variation between replicate films made from the same sample of soil;
(4) variation in observation between different observers; and
(5) indifferent staining and poor optical arrangements.

In order to investigate the differences in the microflora due to different manurial treatments and at the same time to provide data for the statistical analysis of these possible sources of error, counts were made of samples of soil from three of the 'classical' Barnfield plots. These have had consistent manurial treatment for the past 100 years. The plot numbers and treatment are 8–0, no manure; 1–0, farmyard manure; and 4–A, complete minerals. Samples from these three plots are designated A, B and C respectively. Sample C was divided into two parts C_1 and C_2. In Table 2 the count made on sample A is set out in detail showing the total number of bacteria observed in each of 20 microscopic fields. The total number of bacteria per field are analysed into their constituent colonies, the numbers per colony being shown.

Only the number of bacterial colonies and total number of bacteria per slide are given in Table 3 of the counts made on samples A, B, C_1 and C_2. The four counts made on replicate slides for each sample will be seen to be reasonably consistent.

From consideration of previous techniques of dilution counting ('Student', 1907), it was at first thought that the numbers of organisms observed per field should fall in a Poisson series. The required condition for a Poisson distribution is that the probability of an event occurring is exceedingly low, but the number of trials is so large that the total number of events occurring reaches an observable total. Suppose any microscope field to be divided into units of area comparable in size to a bacterium, there will then be an exceedingly large

Table 2. *Count of bacteria from sample of soil from Barnfield, plot A*

Slide		No. of microscope field																				Total no. of bacteria/ slide
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
1	No. of bacteria within colonies	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	
		7	1	1	1	1	1	1	5	1	1	5	2	1	.	1	2	5	2	1	.	
		.	.	2	.	2	1	6	.	1	4	.	2	2	.	6	6	.	.	1	.	
		.	.	.	.	4	.	.	.	4	4	.	3	.	.	7	.	.	.	3	.	
		.	.	.	.	6	.	.	.	.	.	.	.	.	.	.	.	.	.	4	.	
	Total no. of bacteria/field	8	2	4	2	14	3	8	6	7	10	6	8	4	1	15	10	6	3	10	1	128
2	No. of bacteria within colonies	1	1	2	1	1	2	.	1	1	1	1	1	2	1	1	1	1	1	1	1	
		7	2	7	2	.	.	.	3	1	1	1	1	8	1	2	2	1	1	1	1	
		.	2	.	.	.	.	.	.	1	.	.	2	11	.	2	.	1	2	1	1	
		.	2	.	.	.	.	.	.	2	.	.	8	.	.	.	.	1	4	.	2	
		.	4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	7	.	.	
	Total no. of bacteria/field	8	11	9	3	1	2	0	4	5	2	2	12	21	2	5	3	6	15	3	5	119
3	No. of bacteria within colonies	1	1	1	1	2	1	1	1	.	1	2	1	1	.	1	2	1	.	1	1	
		2	1	2	1	3	.	2	1	.	1	3	1	1	.	1	2	1	.	2	3	
		.	1	5	4	3	.	6	2	.	.	.	2	1	.	.	7	1	.	2	8	
		.	1	.	.	.	.	.	.	.	.	.	.	2	.	.	.	1	.	2	13	
		.	3	.	.	.	.	.	.	.	.	.	.	2	.	.	.	2	.	.	.	
		.	.	.	.	.	.	.	.	.	.	.	.	4	.	.	.	.	.	.	.	
	Total no. of bacteria/field	3	7	8	6	8	1	9	4	0	2	5	5	11	0	2	11	6	0	7	25	120
4	No. of bacteria within colonies	1	1	4	1	.	1	1	1	1	1	.	1	2	1	2	1	1	1	.	1	
		1	1	.	.	.	1	1	3	1	2	.	1	2	2	.	1	1	2	.	1	
		2	.	.	.	.	1	4	4	.	3	.	1	.	.	.	2	3	.	.	1	
		2	.	.	.	.	.	.	.	.	.	.	2	.	.	.	2	.	.	.	1	
		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	8	.	.	.	2	
		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	
		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	
		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	4	
	Total no. of bacteria/field	6	2	4	1	0	3	6	8	2	6	0	5	4	3	2	14	5	3	0	14	88

number of such areas. If the observation of each of these areas in turn represents a trial and the occurrence of a bacterium in any one of them an event, then the probability of such an event occurring is exceedingly low, since the total number of bacteria per field is small.

Table 3. *Count of bacteria in four samples of soil from Barnfield*

	No. of colonies/20 fields					χ^2 of colonies, $n=19$			
Slide	A	B	C_1	C_2		A	B	C_1	C_2
1	57	128	60	55		7·17	8·60	18·00	15·90
2	54	112	67	68		18·80	15·86	19·80	6·70
3	54	131	49	65		19·33	12·60	16·71	12·80
4	49	104	47	48		28·10	14·50	16·40	17·80
Av. no./slide	53·50	118·75	55·75	59	Total χ^2 on 4 slides $n=79$	72·55	56·68	81·12	55·52
Slide	Total no. of bacteria/20 fields					χ^2 of total no. of bacteria, $n=19$			
1	128	263	130	98		48·56	41·49	41·60	66·90
2	119	220	142	149		90·58	59·09	46·45	57·52
3	120	307	132	130		91·00	62·71	213·69	34·15
4	88	294	140	84		67·91	213·41	95·43	38·62
Av. no./slide	113·75	271	136	115·25					
No. of bacteria in millions/g.	2275	5420	2720	2305					

If this be true, the statistic χ^2, calculated as $\chi^2=\frac{S(x-\bar{x})^2}{\bar{x}}$ (where $x=$ individual count of bacteria per field and $\bar{x}=$ mean value of each count and S implies summation), should be approximately equal to the number of degrees of freedom of the system (Fisher, 1946). From Table 3 it will be seen that the values of χ^2 thus obtained (the number of degrees of freedom, $n=19$) invalidate the assumption of a Poisson distribution.

However, when χ^2 was calculated on a 'recovery' count (see Table 4) agreement with the expectation of a Poisson series was obtained. In the latter case discrete organisms only were present, whereas in normal soils colonies of bacteria of various sizes occur, and it is clear that the distribution of bacteria in colonies provides a disturbing factor. There will thus obviously be a distribution within colonies to be taken into consideration on counts of normal soils. In order to assess the significance of the mean it becomes necessary to show that the distribution of colonies is Poisson, to determine further the type of distribution within colonies and hence to determine the distribution of total numbers of bacteria per field. Using the statistic χ^2 calculated as above, it was found that the distribution of colonies fitted a Poisson series. In Table 3 it will be seen that the values of χ^2 for counts on the individual slides on the whole approach the number of degrees of freedom, in this case 19. The values of χ^2 calculated on the slides grouped together with 79 degrees of freedom are also given and again do not deviate significantly from this value. In Table 7 the goodness of fit is demonstrated.

It then remained to determine the distribution within colonies. In discussion with Mr Quenouille it was suggested that these numbers might be distributed

in the form of the logarithmic series investigated by Fisher, Corbet & Williams (1943) (see Statistical Note). In this the probability of colonies containing 1, 2, 3, ..., organisms are expressed by the successive terms of the logarithmic expansion for $-\log_e(1-x)$, i.e. $-\log_e(1-x)=x+\frac{x^2}{2}+\frac{x^3}{3}+\ldots+\frac{x^n}{n}+\ldots$. Since total probability must by definition be 1, the successive probabilities for 1, 2, 3, ... organisms will become αx, $\alpha\frac{x^2}{2}$, $\alpha\frac{x^3}{3}$ ... where $\alpha=\frac{1}{-\log_e(1-x)}$, and the relative frequencies may be obtained by multiplying each probability by the total number of colonies.

Table 4. *'Recovery' of counted suspension of bacteria added to sterilized soil*

Counted by two workers, M. and J.

		No. of microscope field																					
Slide		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Total	$\chi^2(n=19)$
1	M.	5	5	4	3	2	3	3	2	3	1	5	2	7	3	5	3	12	4	4	5	81	25·91
	J.	4	5	3	3	3	3	4	3	3	1	6	2	8	4	5	3	10	4	4	4	82	18·97
2	M.	6	4	3	5	2	11	6	6	12	5	5	7	4	6	7	5	6	3	8	2	113	22·40
	J.	5	3	3	6	3	12	5	5	10	3	7	8	2	6	6	5	6	2	6	3	106	31·73
3	M.	10	4	5	4	2	8	7	1	6	3	6	6	5	5	6	4	6	5	5	4	102	14·86
	J.	9	3	5	5	3	8	7	1	6	3	5	6	5	5	8	4	5	5	5	4	102	13·68
4	M.	3	5	6	6	4	3	3	9	6	9	5	6	5	4	2	6	1	2	3	5	93	18·61
	J.	1	5	4	7	4	5	2	9	5	9	5	5	7	3	3	6	3	3	4	6	96	17·75

In Table 8 a logarithmic series is fitted to the frequency distribution within colonies in each sample with Goodness of Fit tested by χ^2; and an excellent fit is obtained. That is, the distribution of colonies per field forms a Poisson series, and the distribution within colonies forms a logarithmic series.

The combined distribution, i.e. the distribution of total numbers of bacteria per slide (see Statistical Note) is proved to be in the form of a negative binomial expansion. Using this theoretical prediction the fit of this latter distribution to the total number of frequencies was tested by χ^2 (see Table 9) and again the hypothesis was verified.

Thus the total numbers per slide are seen to fit a negative binomial distribution; the parameters of which may be calculated, the standard error estimated, and the significance of the mean determined.

Thus from the counts of the three plots from Barnfield examined (Table 3) it appears that the microflora is more numerous in the plot treated with farmyard manure, whereas no significant difference may be observed between the plots to which minerals or nothing have been added. This has been borne out by several other unanalysed counts. Comparative counts by Thornton's ratio method gave slightly lower figures.

The method has also been used in an attempt to estimate the quantity of fungal mycelium in soil. The technique gives excellent staining of mycelium, but the estimation of its quantity is difficult owing to the small amount present and to the considerable variation in length of the fragments. In order to count

Table 5. *Count of pieces of mycelium in sample of soil from Barnfield plot A*

Slide		No. of microscope field 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Total
1	No. of pieces of mycelium	1	1	3	2	.	.	.	.	1	2	.	1	1	1	.	.	.	.	.	1	14
	Total length in μ/field	25·6	25·6	512	115·2	.	.	.	.	25·6	70·4	.	25·6	57·6	76·8	.	.	.	.	.	51·2	985·6
2	No. of pieces of mycelium	2	.	.	.	.	.	1	.	1	.	1	1	.	1	.	1	.	.	.	.	8
	Total length in μ/field	128	.	.	.	.	.	25·6	.	51·2	.	115·2	64·0	.	32·0	.	76·8	.	.	.	.	416·0
3	No. of pieces of mycelium	.	.	.	1	1	1	2	2	.	1	1	.	.	.	.	2	.	.	1	.	12
	Total length in μ/field	.	.	.	102·4	19·2	25·6	281·6	172·8	.	44·8	204·8	.	.	.	.	422·4	.	.	96	.	1369·6
4	No. of pieces of mycelium	.	1	.	.	.	.	1	1	1	.	1	.	1	.	.	.	1	.	.	.	7
	Total length in μ/field	.	320	.	.	.	.	128	38·4	64·0	.	38·4	.	51·2	.	.	.	115·2	.	.	.	755·2

a sufficient number of fragments, it is necessary to examine the entire field of a low power objective, for which a new conversion factor must be calculated in order to express the quantity/g. Using a 2/3 objective and a ×8 eyepiece, only fungal mycelium is resolved, the finer actinomycete filaments being just below the limits of visibility. As for bacterial counts, 20 microscope fields on each of four replicate slides were counted. A measuring scale was inserted in the eyepiece, and the individual pieces measured, the quantity of mycelium being expressed as total length/g. soil.

Table 6. *Count of pieces of mycelium from three samples of soil from Barnfield*

	Total no. of pieces of mycelium per 20 fields			Total length (μ.) of mycelium per 20 fields			χ^2 of total no. of pieces of mycelium per 20 fields		
Slide	A	B	C_1	A	B	C_1			
1	14	12	8	985·60	947·20	614·40	20·29	28·00	12·00
2	8	15	6	416·00	1139·20	449·20	17·00	23·66	20·67
3	12	12	6	1369·60	1011·20	256·00	18·00	18·00	14·00
4	7	16	7	755·20	1152·00	358·40	13·00	21·50	13·00
Av. no./ slide	10·25	13·75	6·75	Av. length/ slide	881·60	1062·40	432·00		

The results of such an estimation made on the same slides as those used for the bacterial counts are set out in Tables 5 and 6. In Table 5 the detailed observations on sample A are given; in Table 6 only the total counts for each of the four slides of the three samples. The distribution of pieces of fungal mycelium like that of bacterial colonies fell into a Poisson series. The value of $\chi^2 = \left[\frac{S(x-\bar{x})^2}{\bar{x}}\right]$, shows close approximation to the number of degrees of freedom (Table 6).

The nature of the soil microflora

The soil bacteria in films made according to this method are largely coccoid and adherent to the humic matter, few or none being attached to mineral particles (Pl. 1). These organisms may be in the form of large zoogloeal colonies (Pl. 1, fig. 1) or may consist of smaller clumps or single individuals (Pl. 1, figs. 2–7). Frequently groups of large cocci resembling Azotobacter are seen. Long rods have been but rarely observed in fresh soil. Staining varies in intensity, colonies generally being more deeply stained than discrete organisms, but this has not yet been correlated with viability.

The same variable staining is seen with fungal mycelium, where, on the other hand, there is strong evidence of correlation of intensity of staining with viability. Progressive loss of the protoplasm from the hyphae, due either to decomposition or to its migration to the hyphal tip, can be frequently observed (Pl. 1, figs. 8–10); indeed most of the hyphal fragments appear to lack organized contents. Such hyphae are stained purple in contrast with the deep blue coloration of those filled with protoplasm. This was confirmed by inoculating

sterilized soil with fungal mycelium, allowing it to incubate for several days and making films from a sample of this soil. On these films only deeply stained fungal fragments were seen. On the whole, in normal soils mycelium is scanty and because of its filamentous nature and very variable length is not amenable to accurate statistics, though useful comparative results may be obtained. There were (Table 6) significantly fewer pieces of mycelium present on the plot with minerals than on the plots receiving farmyard manure and no manure.

Lengths of well-stained mycelium frequently have humic material adherent to their walls, probably through secreted mucilage (Pl. 2, fig. 4). This may have an important bearing on the formation of soil crumbs. Few fungal spores are seen. Fibres may be distinguished from hyphae by their lack of staining and their polarization colours under crossed nicols. Other plant tissue absorbs but little dye and at most has a greenish hue. Stained nematodes are sometimes seen and what are thought to be earthworm setae can be distinguished from fragments of mycelium by their tapering apices.

DISCUSSION

The counting method is presented as a new and more accurate technique for the estimation of quantity of bacteria and fungi, particularly for comparative analyses of different field soils. It is applicable not only to field samples but also to soil samples undergoing experimental treatment. It has, for example, been successfully used for observing the development of fungi in compacted blocks of soil mixed with resins, in the course of an investigation carried out for the Road Research Board D.S.I.R. (see Pl. 2, figs. 4–6). We also suggest the possibility of its wider application with slight modification. By using nutrient agar instead of plain agar for the soil dilutions and by incubating the soil films for several days in a damp chamber before drying and staining, excellent preparations of developing bacterial colonies (Pl. 2, figs. 1 and 3) and of actinomycete colonies, which stain beautifully, have been obtained (Pl. 2, fig. 2). By using various nutrient agar media in this way much information could be obtained about the specific nutritional groups of micro-organisms in the soil and of the relative frequency of dead and living organisms, inability to determine which is at present the chief disadvantage of direct counting methods as opposed to plate counts.

The direct method of examination and counting may well be applicable to solid environments other than soil, such as faeces, or sewage sludge. Moreover, soil films made in agar might be examined by a petrological microscope to identify the chief mineral elements in the soils and to estimate their relative abundance. A similar statistical analysis could be applied to this data, for a Poisson distribution of the rare minerals is to be expected.

This work was carried out during the course of an investigation on the decomposition of resins for the Road Research Laboratory by one author (P. C. T. J.), and in the course of an investigation for the Agricultural Research Council by the other (J. E. M.).

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Statistical Note

By M. H. QUENOUILLE

Distribution of colonies per field—Poisson series

For a series of observations, the value of χ^2, calculated from $\frac{S(x-\bar{x})^2}{\bar{x}}$ can be used to test the hypothesis that the observations are distributed in a Poisson series. This statistic does not necessarily expose any systematic deviation from the Poisson series, but a comparison of the observed distribution of colonies per field with the theoretical distribution expected from a Poisson series shows (Table 7) no systematic deviation.

Table 7. *Poisson series fitted to colony counts*

No. of colonies	Sample A		Sample C_1		Sample C_2	
	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
0	7	5·5	0	4·9	4	4·2
1	8	14·7	20	13·7	9	12·4
2	24	19·7	24	19·1	16	18·2
3	20	17·6	12	17·8	23	17·9
4	11	11·8	9	12·4	17	13·2
5	8	6·3	8	6·9	8	7·8
6 and over	2	4·4	7	5·2	3	6·1
Totals	80	80·0	80	80·0	80	80·0
	$\chi^2_{(5)}=9{\cdot}10$		$\chi^2_{(5)}=12{\cdot}68$		$\chi^2_{(5)}=5{\cdot}33$	
	$m=2{\cdot}68\pm18$		$m=2{\cdot}79\pm10$		$m=2{\cdot}95\pm19$	

No. of colonies	Sample B		No. of colonies	Samples A and C	
	Obs.	Calc.		Obs.	Calc.
0–3	10	12·5	0	11	14·6
4	10	10·9	1	37	40·9
5	16	13·0	2	64	57·2
6	12	12·8	3	55	53·4
7	16	10·9	4	37	37·4
8	6	8·1	5	24	20·9
9 and over	10	11·8	6	12	15·6
Totals	80	80		240	240·0
	$\chi^2_{(5)}=5{\cdot}18$			$\chi^2_{(5)}=3{\cdot}41$	
	$m=5{\cdot}94\pm27$			$m=2{\cdot}80\pm11$	

Distribution of bacteria per colony—logarithmic series

On the following assumptions, which seem reasonably likely, the distribution of colonies consisting of 1, 2, 3, 4, ... n individuals is given by the successive terms of the logarithmic series—$\log_e(1-x)=x+\frac{x^2}{2}+\frac{x^3}{3}+\frac{x^4}{4}+\ldots+\frac{x^n}{n}+$, with $x=\frac{y-pz}{qyz}$.

(i) In a fixed interval in time, the probability of a colony of size n becoming a colony of size $(n+1)$ is proportional to ny^n, i.e. is proportional to the size of the colony times a damping factor, which could be taken as unity.

Table 8. *Logarithmic series fitted to counts of bacteria per colony*

Bacteria per colony	Sample A		Sample C_1		Sample C_2	
	Obs.	Calc.	Obs.	Calc.	Obs.	Calc.
1	116	116·9	103	112·5	140	136·5
2	53	43·4	47	44·5	46	48·0
3	11	21·5	26	23·5	20	22·5
4	13	12·0	16	14·0	12	11·9
5	4	7·1	15	8·9	7	6·7
6	5	4·4	6	5·9	6	3·9
7 and over	12	8·7	10	13·7	5	6·5
Totals	214	214·0	223	223·0	236	236·0
	$\chi^2_{(5)}=8·81$		$\chi^2_{(5)}=6·54$		$\chi^2_{(5)}=1·94$	
	$x=0·743\pm0·026$		$x=0·794\pm0·022$		$x=0·704\pm0·027$	

Bacteria per colony	Sample B		Samples A and C	
	Obs.	Calc.	Obs.	Calc.
1	243	249·3	359	362·1
2	111	96·0	146	136·1
3	39	49·3	57	68·3
4	32	28·5	41	38·5
5	15	17·5	26	23·2
6	11	11·1	17	14·5
7 and over	24	23·3	27	30·3
Totals	475	475·0	673	673·0
	$\chi^2_{(5)}=5·46$		$\chi^2_{(5)}=3·91$	
	$x=0·770\pm0·016$		$x=0·752\pm0·014$	

(ii) In a fixed interval in time, the probability of a colony of size n breaking into two or more colonies is proportional to nz^n, i.e. is proportional to the size of the colony times an increasing factor, which could be taken as unity.

(iii) The probability that the break-up of a colony of size n will produce a colony of size m is proportional to

$$^{(n-2)}C_{(m-1)}\cdot p^{(m-1)}\cdot q^{(n-m-1)},$$

where $(p+q)=1$. This is the binomial probability that the division of n balls into at least two sets will result in a particular set having m balls in it.

(iv) The distribution is stable, i.e. it is not altering to any extent with time.

Comparison of the observed distribution with the theoretical (Table 8) shows no systematic deviation.

Distribution of bacteria per field—negative binomial series

If we assume both the Poisson and logarithmic series to represent the observed distributions very closely, then the expected distribution of bacteria per field may be shown to be successive coefficients of the negative binomial $(1-x)^{\beta}(1-xt)^{-\beta}$.

As yet there is insufficient data to test this distribution adequately. However, since no significant difference has been observed between samples A, C_1 and C_2 (see next section), these samples have been combined.

Table 9. *Negative binomial series fitted to bacterial counts*

	Samples A and C		
No. of bacteria	Obs.	Max. likelihood fit	Calc. fit
0	11	13·0	14·6
1	17	21·0	22·0
2	31	24·6	24·8
3	24	25·4	24·7
4	29	24·2	23·4
5	18	22·0	21·1
6	19	19·4	18·5
7	16	16·7	15·9
8	13	14·1	13·5
9	17	11·7	11·3
10	6	9·6	9·3
11	8	7·8	7·6
12 and over	31	30·5	33·3
Totals	240	240·0	240·0
		$\chi^2_{(10)}=9{\cdot}08$	$\chi^2_{(10)}=9{\cdot}66$
		$\beta=2{\cdot}2$	$\beta=2{\cdot}0$
		$x=0{\cdot}735$	$x=0{\cdot}752$

The negative binomial can be fitted by the method of maximum likelihood, or alternatively by using the observed values of m and x to calculate the parameter β. The fitted series for both methods are given in Table 9, where it is seen that both methods, in this case, give adequate fits to the observed figures.

Use of analysis of variance

(*a*) *On counts of colonies*

The analysis of variance can be used to test the technique used in counting colonies. A comparison of the variations between slides and the variations between counts on the same slide will indicate whether the variation between slides and the variation between replicate films made from the same sample of soil is likely to be large. We can also compare the samples C_1 and C_2 to investigate whether the variation between samples of the same soil is large.

Before carrying out the analysis of variance, the square roots of the counts were taken to normalize the data. Under this transformation, we will expect the mean square between counts on the same slide to have a value tending to 1/4, and any large deviation from this value can only be due to some fault in the

technique. From the analysis of variance of the counts of colonies (Table 10) it is seen that the variation due to the technique is small, and furthermore that, although there is no significant difference between samples A and C, there is a large difference between sample B and the other samples.

Table 10. *Analysis of variance of colony counts*

	Degrees of freedom	Sum of squares	Mean square
Sample B *v.* other samples	1	39·816	—
Sample A *v.* samples C	1	0·479	—
Sample C_1 *v.* sample C_2	1	0·047	—
Between slides of the same sample	12	4·828	0·402
Between counts of the same slide	304	79·089	0·260
Totals	319	124·259	

(*b*) *On counts of bacteria*

The same technique can be used on the counts of bacteria. The mean square between counts on the same slide will, in this case, tend to the value $\frac{1}{4(1-x)}$, i.e. approximately 1. The analysis of variance is given in Table 11, the conclusions being the same as above.

Table 11. *Analysis of variance of bacterial counts*

	Degrees of freedom	Sum of squares	Mean square
Sample B *v.* other samples	1	95·622	—
Sample A *v.* samples C	1	1·832	—
Sample C_1 *v.* sample C_2	1	1·543	—
Between slides of the same sample	12	14·409	1·201
Between counts of the same slide	304	305·352	1·004
Totals	319	418·758	

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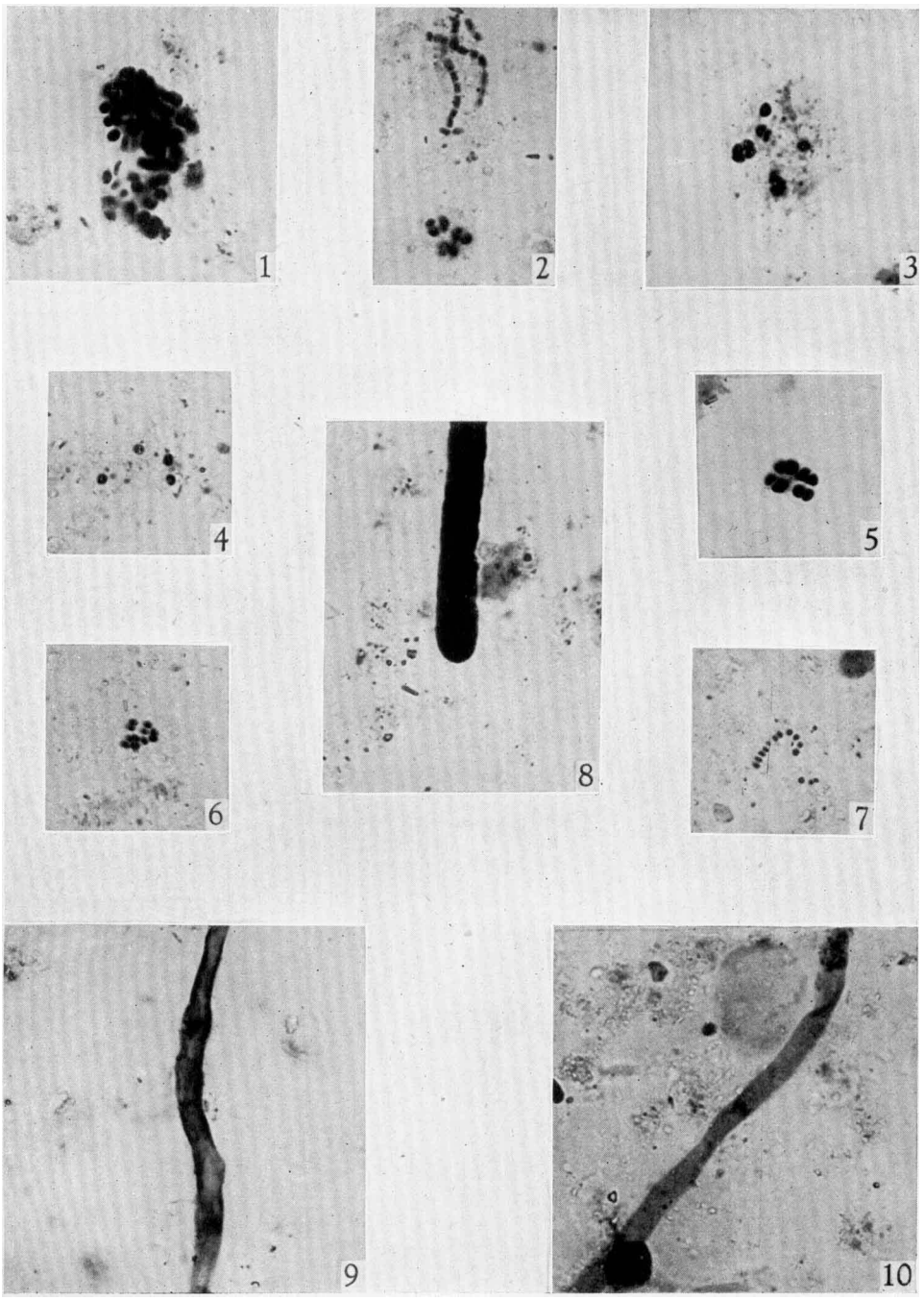
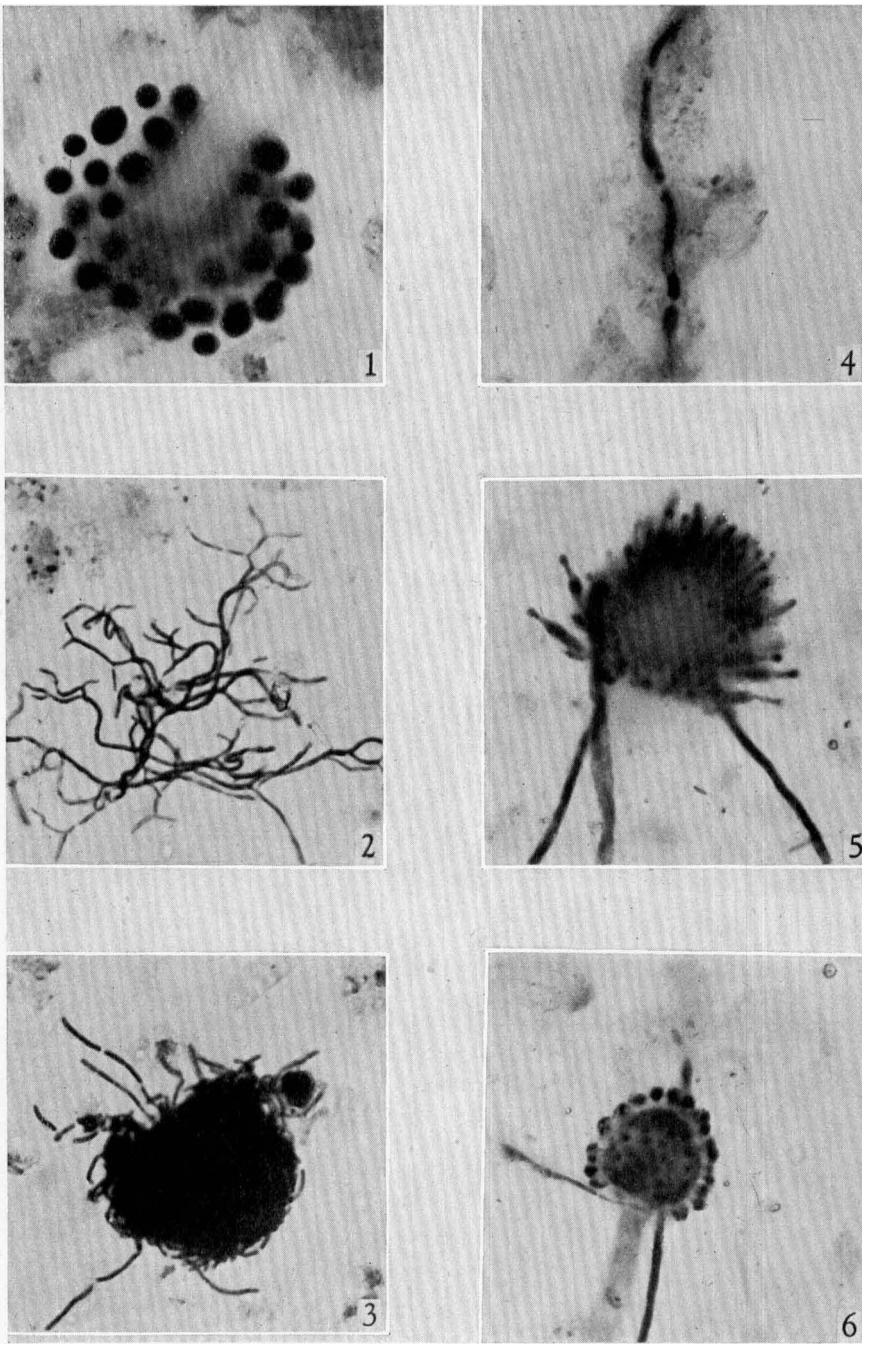


Figs. 1–10

P. C. T. Jones and J. E. Mollison—A technique for the quantitative estimation of soil micro-organisms. Plate 1



Figs. 1–6

P. C. T. Jones and J. E. Mollison—A technique for the quantitative estimation of soil micro-organisms. Plate 2

EXPLANATION OF PLATES

PLATE 1

Figs. 1–7 from Barnfield manured plot. Figs. 8–10 from Broadbalk manured plot. Magnification × 1000.

Fig. 1. Large zoogloeal colony.
Fig. 2. Small mucoid colony of cocci, and chains of rods.
Fig. 3. Colonies of cocci with adherent humic material.
Fig. 4. Single bacterial cells.
Fig. 5. Mucoid colony of large cocci.
Fig. 6. Colony of small cocci.
Fig. 7. Chain of cocci.
Fig. 8. Hyphal tip—Intense staining of preparation.
Fig. 9. Strand of mycelium with lysis or migration of protoplasmic contents.
Fig. 10. Hypha with only cell wall remaining (purplish staining of preparation).

PLATE 2

Figs. 1–3. Nutrient agar film of allotment soil incubated 24 hr. Figs. 4–6. Film of crushed block of Harmondsworth brick-earth +1 % Rosin. Magnification × 1000.

Fig. 1. Colony of large capsulated cocci with mineral crystal.
Fig. 2. Young actinomycete colony showing branching and fragmenting filaments.
Fig. 3. Young colony of rod-shaped bacteria.
Fig. 4. Fungus mycelium with adherent humus.
Fig. 5. *Penicillium* sp. conidiophore.
Fig. 6. *Aspergillus* sp. conidiophore.

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