

MICROBIOLOGICAL ANALYSIS OF SOIL AS AN INDEX OF SOIL FERTILITY: II. METHODS OF THE STUDY OF NUMBERS OF MICROÖRGANISMS IN THE SOIL¹

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INTRODUCTORY

In taking up the study of bacterial numbers in the soil as the first function for the characterization of the bacteriological condition of the soil, one should keep in mind that this is done not because it is the most important function, nor because it is known to be the most representative function, but for several other reasons. Following the determination of bacterial numbers in the soil by Koch in 1881 with the introduction of the gelatin plate, the first attempt was then made to make a thorough study of bacteria in the soil. With the possible exception of the decomposition of nitrogenous organic matter (ammonification) and nitrification, more work has been done in the study of numbers of bacteria in the soil as influenced by various factors, than of any other soil bacteriological function. This is a simple function, readily placed on a quantitative basis and does not have the complex qualitative character of the most other functions, which have not yet been placed fully on a quantitative basis. Our methods for determining bacterial numbers in the soil are well worked out, the limitations involved are well recognized and the variability factor can be readily calculated.

A historical review of the occurrence and distribution of bacterial numbers in the soil is found in the work of Voorhees and Lipman (17, p. 10-12), Löhns (11) and various papers dealing with the subject Brown (2), Conn (4), Waksman (18), etc.

The determination of numbers of microörganisms in the soil has not been looked upon as of prime importance in the study of its bacteriological condition. The results obtained have been very variable, non-uniform and not very promising for the interpretation of soil fertility phenomena. A lack of confidence has been felt on the part of even the trained bacteriologist, who has recognized the limitations of the methods and found himself unable to correlate the results obtained by the determination of numbers of microörganisms

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in the soil with its crop producing power. This has led bacteriologists (Remy, 14) to state that the number of bacteria in any soil has but a very limited diagnostic value in ascertaining its fertility. This statement of Remy was based merely upon the fact that the number of colonies of microorganisms on the plate shows no direct relationship to the ammonifying, nitrifying or denitrifying power of the soil. It remains to be proved however, whether these last mentioned physiological activities are of diagnostic value. This untrustworthiness was also pointed out by Löhnis (10) who called attention to a difference in a small number of efficient bacteria and a large number of organisms possessing only a slight efficiency. He further points to the determination of bacteriological numbers in the soil as "rather worthless and spirit-destroying countings." In a later publication (12), Löhnis emphasizes this fact by stating that "all further investigations have shown, without any exception, that no definite relationship between the total number of bacteria and fertility of corresponding soil is recognized." Even as late as 1921, we find such a striking statement as "a quantitative bacteriological analysis of soil for total numbers of microorganisms has but a comparatively small significance as compared with that for the estimation of numbers in one or more physiological groups" (Northrup-Wyant, 13). Such a severe criticism of the value of determining the total numbers of microorganisms in the soil has been justified by the great variability in bacterial numbers reported by various soil bacteriologists and the lack of correlation between the numbers and the physiological activities of specific groups of microorganisms in the soil which are assumed to be of great importance to soil fertility (Remy, 14).

However, not all investigators have reached such negative conclusions as to the value of determining bacterial numbers in the soil. We may but refer to the work of Russell and Appleyard (16), who found the curves for bacterial numbers, nitrate content and carbon dioxide in the soil to be sufficiently similar to justify the view that all the phenomena are related.

The physiological activities of soil microorganisms will be taken up in the following papers, while here, we will limit ourselves only to numbers. A study of the variability and methods of mathematical interpretation of bacterial numbers has been reported in the previous paper (19) in this series. In this latter paper the author has endeavored to show that the variability and lack of correlation mentioned above are due to lack of uniformity in the methods, inaccurate methods, and changing soil flora. Nonuniformity of methods is made even worse by the fact that the details of the technic used by the various investigators are so widely divergent and that the data obtained even from one soil by the various methods are incomparable. Each investigator is, in the words of Northrup-Wyant (13), "a law unto himself in so far as the technic used in quantitative bacteriological soil analysis is concerned." Great variability in the numbers obtained on the same soil by the same investigator may also be due to the fact that the results are not checked sufficiently and the probable errors are too great for any accurate scientific work. This has been well recognized by various bacteriologists such as Chester (3).

METHODS

Although the methods used in a quantitative bacteriological analysis of soil have been reviewed in several of the more recent publications, as pointed out above, the various limitations of the methods have not been studied sufficiently, so that the ground is only incompletely covered. To determine just how much weight should be attached to the methods by which a particular soil phenomenon (function) is measured in comparison with the other soil microbiological activities (functions) in the building up of a system of soil bacteriology, a study of methods is of prime importance.

The various methods used for determining quantitatively the microörganisms in the soil can be classified into 3 groups:

1. Dilution method
2. Plate method
3. Direct counting method

The dilution method can be utilized not only for the determination of the total soil flora but also for the study of specific physiological groups, utilizing differential media. But the use of this method in routine bacteriological soil analysis is too cumbersome, involving a number of dilutions and cultures for each soil, and then only approximate results would be obtained, particularly in view of the fact that the selective action of the medium would be as manifest as in the case of the plate method, since not all organisms would develop on any one medium. The microscopic method suggested by Conn (5) gives promising results, but has not yet been developed to a sufficient extent to warrant any definite conclusions. The great difference between the plate and microscopic counts in normal soil is due to organisms which cannot be grown on plates. In the case of those organisms that develop on plates, the plate count will be nearly as high or even higher than the microscopic count, according to Conn. If we keep in mind, therefore, the limitations of the plate method, we will find it quite satisfactory for our work, until the direct counting method is developed sufficiently to warrant its exclusive use.

The plate method has been the one most commonly employed by bacteriologists, and has also been used in the present investigations. It is an indirect method, since we do not count the organisms directly, only the colonies that are produced on the plate. It is assumed that every bacterium, actinomyces, fungus spore or hypha develop into a colony. This can be justified only when the organisms are well separated from the soil into the diluting fluid, when the medium is favorable for the development of all these organisms, and when the temperature, oxygen supply and period of incubation are favorable. We can never obtain the ideal conditions but we can work out our technic so as to approach them as near as possible. The plate method has various limitations. The strict anaerobic microörganisms are excluded as well as the important groups of nitrifying, non-symbiotic nitrogen-fixing bacteria, sulfur-

oxidizing bacteria, to some extent the denitrifying, symbiotic nitrogen-fixing, pectin- and cellulose-decomposing bacteria. Then, of course, the algae and protozoa are eliminated. A further limitation is the fact that high dilutions are necessarily used, so that if groups of microorganisms are determined on the plate, those organisms occurring only in small numbers will give a rather inaccurate count. However, the fact that various important groups of soil microorganisms do not develop on the plate does not detract very much from the value of the method since it holds true for all soils, the microorganisms that do develop on the plate are constant soil forms, and no one method, with the possible exception of the direct microscopic method, will allow a study of all soil microorganisms. Therefore, the plate method within defined limits, will serve as a certain measure of the quantitative bacterial flora. The soil microorganisms that develop on the plate include the fungi, the actinomycetes and those bacteria which are concerned in the decomposition of organic matter in the soil, assimilation and certain transformations of minerals as well as other not sufficiently studied activities.

The composition of the medium is one of the most important factors in the determination of numbers of microorganisms in the soil by the plate method. It must be of definite chemical composition (synthetic) and must allow the development of the greatest number of microorganisms.

Both gelatin and agar media are usually employed in the plate method. Gelatin was the first solid medium suggested by Koch for the study of pathogenic bacteria and was also used for the study of soil bacteria by the same investigator. The advantages of agar media over the gelatin are several, chief among which is the fact that agar can be kept at higher temperatures than gelatin, that the gelatin-liquefying microorganisms do not interfere with accurate counts, particularly after a long incubation period which allows all microorganisms to develop. Agar media can be prepared of an exact chemical composition. This cannot be said of gelatin which is in itself a nutrient for various microorganisms. For qualitative work, gelatin media no doubt present certain advantages as in the case of the separation of bacteria into liquefying and non-liquefying groups. But even this separation is of doubtful value and may give different results under different conditions, since, under the best of conditions, it is a qualitative rather than a quantitative distinction. In the case of actinomycetes, for example, we find that they nearly all liquefy the gelatin, some in 3-4 days and some in 40-50 days.

Similar disadvantages are found in the case of the nutrient agar used chiefly by the earlier soil bacteriologists. The introduction of media of exact chemical composition (Lipman and Brown, 9 and Fischer, 6) was an important step in the standardization of the plate method.

A comparative study of the various synthetic media to be used for the determination of numbers of soil microorganisms, has been given by Brown (2) and by Conn (4). The medium used in the present work is a modification of Brown's albumen agar:

$K_2 H PO_4$	0.5 gm.
$Mg SO_4$	0.2 gm.
Dextrose.....	10.0 gm.
Powdered egg-albumen.....	0.25 gm.
$Fe_2 (SO_4)_3$	Trace
Agar.....	15.0 gm.
Distilled water.....	1000.0 cc.

All the ingredients, with the exception of the powdered egg-albumen are dissolved in boiling water, then filtered; the powdered egg-albumen is placed in a beaker and suspended by means of a stirring rod in a little distilled water, a drop of phenolphthalein is then added and sufficient 0.1 *N* NaOH is added from a burette until a distinct pink color is obtained. The egg-albumen is thus transformed into sodium albuminate, which is then added to the filtered agar and stirred in thoroughly. If the sodium albuminate solution has a few undissolved particles, it can be filtered first through a piece of filter paper. The use of sodium albuminate does away with the coagulation of albumen when added to the hot agar and allows the use of a standard product. The medium is tubed, in 10-cc. portions, and sterilized in the autoclave for 15 minutes at 15 lbs. pressure. The medium does not have to be freshly made up for each determination. The sterile tubed medium under cover for two months did not deteriorate so long as there was no appreciable drying out.

SOIL SAMPLING

It has been assumed that the same soil type, under the same conditions of treatment, contains at the same depth the same number of bacteria, and that soils differing in one way or another differ also in their bacterial activities (Hiltner and Störmer (8)). Due allowance must be made, however, to the natural variability of the soil itself. Where numerous samples are taken and thoroughly mixed, then carefully sampled, the danger is not so great as where only one or two samples are taken. By comparing the results obtained from various samples taken from the same uniform soil and treated alike, important variations have been obtained as pointed out in the previous paper of this series (19). For the actual field studies, four or five samples were taken from various parts of the plot, each sample being a composite of 3-4 borings, which were then thoroughly mixed.

Since the bacterial numbers are not the same at the various soil depths (18), the depth of sampling should be carefully considered. In taking samples for this study about $\frac{1}{4}$ inch of the surface was scraped away with a clean spatula and samples taken by means of a sampling tube to a depth of 6-6 $\frac{1}{2}$ inches. The samples were taken into sterile containers, and counts made as soon as possible.

DILUTIONS AND PLATING

The first dilution was made as follows: 5 or 10 gm. of soil were added to a 250-cc. Erlenmeyer flask containing 100 cc. of sterile water; the flask was then

shaken for exactly 5 minutes; this gave a dilution of 1:10 or 1:20. Of the first dilution 1 cc. was then taken out, without allowing the soil to settle, and transferred into a flask with 99 cc. of sterile water, giving dilutions of 1:1000 or 1:2000. Higher dilutions than the last were obtained by adding 1 or more cc. of the last dilutions to corresponding amounts of water with each new dilution. The final dilution was such as to allow 30–200 colonies to develop on the plate. The flask is shaken for about 30 seconds. One cc. of the final dilution is then transferred by means of fresh, sterile 1-cc. pipettes into Petri dishes and the cooled agar is added. Eight to ten plates were used for each count. The plates were then incubated at 25°C. for various periods of time and all the colonies were counted with the naked eye.

The importance of using a comparatively large amount of soil to make the first dilution and making that dilution comparatively low, like 1:10 or 1:20, has been recognized by the earlier bacteriologists (Remy '14) as well as in the more recent investigations (Northrup-Wyant '13), since a more representative soil sample is thus obtained. Too large an amount of soil, as used in some cases, to give too low a dilution like 1:2, is also objectionable since a thorough mixture of the soil and water is difficult. It is entirely possible to obtain a thorough suspension of bacteria in water, since it has already been demonstrated by Hiltner and Störmer (8) that, on sufficient shaking, all bacteria are washed off the soil particles which remain almost sterile.

There is small need of calling attention to the importance of not allowing the soil to stand in contact with the water for more than the few minutes necessary for the manipulations of shaking, diluting and plating out. A longer period will lead to an appreciable decrease in numbers, due to plasmolysis of microorganisms as pointed out by Hiltner and Störmer (8). An increase in numbers may be obtained only in special instances, as in the case of frozen soil, air-dry soil and subsoil.

INCUBATION AND COUNTS

Various periods of incubation were used in the preliminary experiments, these finally led to the adoption of a 7-days incubation at 25°C. A shorter period of incubation will not allow a full development of all microorganisms and a proper differentiation between the bacteria and actinomycetes.

The numbers of microorganisms were estimated in the preliminary experiments on the basis of soil dried in a electric oven at 100°C. to constant weight. However this method of calculating the data is hardly logical. Microorganisms usually decrease in number with a decrease in the water-content below the optimum. In this connection the author does not fully agree with the more recent workers (Northrup-Wyant, 13) in this field who calculated the bacterial numbers only on the basis of a dried soil. In a soil which is almost dry, the numbers, which are small at that, are increased only very little by figuring back to an air-dry basis. In the same soil at a much higher moisture content, it is found that the addition of moisture did not serve merely

to dilute the soil, but had a stimulating effect upon the development of the bacteria, and it would hardly be advisable to multiply the numbers further to bring them to an arbitrary dry basis. The dry soil in itself does not signify anything, for the numbers depend on the relative moisture content as one of the important factors. Multiplying the numbers, to allow for the moisture, would be equivalent to doing the same calculation twice over.

To be scientifically accurate and have a basis for comparison, we should change the numbers in such a manner, by multiplying it by a moisture factor, so as to reduce those with a high moisture and increase those with a low moisture content. However, before such a factor has been found, the author agrees with Hiltner and Störmer (8) that the results should be reported per gram of moist soil (or even dry soil), giving also the moisture content of the particular soil as well as its optimum moisture (65-70 per cent of its water-holding capacity). The soil reaction, the author feels, should also be reported.

Perhaps, when our data are more complete, we may be able to calculate the potential bacterial activities from the soil type, its water content, reaction, nitrogen and carbon content, etc.

INFLUENCE OF MEDIUM

Preliminary work was carried out with the purpose of demonstrating the influence of the composition of the medium, reaction of medium, temperature of incubation, final dilution, etc., upon the numbers of microörganisms in the soil. The results are reported in tables 1-7.

Composition

Casein agar was prepared in a similar way to the egg-albumen agar except for replacing the egg-albumen by casein. Sodium asparaginate was made up according to directions given by Conn (4). Soil extract agar and urea nitrate agar were made according to directions given by Fred (7). The soil used for these preliminary studies was a greenhouse soil rich in organic matter, having an optimum moisture of 30 per cent and a reaction equivalent to pH 6.2. The fungi were not counted in these preliminary experiments, while under bacterial numbers, both bacteria and actinomycetes are included.

When the media of different composition are compared (table 1) the albumen agar, casein agar and soil extract agar are found to give the highest numbers. The last medium, although giving the largest numbers of all, has to be eliminated due to the fact that it is not standard in composition. The choice was then between the albumen and casein agar. The first was selected in spite of the fact that, in this experiment, it gave somewhat lower numbers than the casein agar. Albumen agar has been used by the writer for several years and has always given excellent results and stood out well in comparison with any other synthetic medium tested; it is also readily prepared and is of an exact chemical composition. Egg-albumen, of course, is not a pure protein,

but since it is used in the powdered form, it is always readily duplicated. This medium was, therefore, selected for further work.*

TABLE 1
*Influence of composition of medium on bacterial numbers**

PLATE NUMBER	ALBUMEN AGAR	CASEIN AGAR	SOIL EXTRACT AGAR	SODIUM ASPARAGINATE AGAR	UREA NITRATE AGAR
	<i>colonies</i>	<i>colonies</i>	<i>colonies</i>	<i>colonies</i>	<i>colonies</i>
1	86	107	109	78	62
2	106	119	134	72	43
3	93	96	103	67	54
4	78	103	128	94	57
5	84	114	113	66	56
6	102	133	136	76	48
7	85	112	142	83	38
8	94	101	131	87	47
9	74	122	98	69	55
10	89	109	118	72	53
Mean	89.1 \pm 2.23	111.6 \pm 2.33	121.2 \pm 3.22	76.4 \pm 1.91	51.3 \pm 1.52
σ	10.44 \pm 1.57	10.97 \pm 1.65	15.10 \pm 2.27	8.97 \pm 1.35	7.16 \pm 1.08
C. V.	11.7 \pm 1.7 %	9.6 \pm 1.5 %	12.5 \pm 1.9 %	11.9 \pm 1.8 %	14.0 \pm 2.1 %
Em	2.5 %	2.1 %	2.6 %	2.5 %	2.96 %

* Plates incubated at 25°C. for 7 days; the figures designate the number of all the colonies on the plate except the fungi. Dilution 1:200,000.

Temperature and period of incubation

The data presented in table 2 show the influence of temperature and period of incubation upon the bacterial numbers found in a soil. When the plates are incubated at room temperature, practically no colonies developed in 2 days, while in 12 days not all the colonies seemed to have developed as yet, since more than twice as many colonies have been found in 12 days than in 5 days. By incubating the plates at 37°, not all the microorganisms are found to develop into colonies and the plates dry up on prolonged incubation. A temperature of 25 to 27°C. proved to be the most favorable, one or two degrees either way having little influence with a long period of incubation. The plates should certainly be incubated longer than even 5 days. Further experiments along this line have shown that, at 25°, there is very little increase in numbers

* After this study was completed, the author in coöperation with Dr. Fred of the University of Wisconsin (20) suggested, as definite uniform media for the determination of total numbers of microorganisms in the soil, a modification of the albumen agar given above and casein agar. The modification of the albumen agar consists in reducing the amount of dextrose from 10 to 1 gm. per liter, so as to prevent the development of spreading colonies. The casein agar is the same as the albumen agar, only 1 gm. of purified casein dissolved in 8 cc. of 0.1 N NaOH is substituted for the egg-albumen. However, the albumen agar used in the studies reported in this as well as in the following paper, was of the composition reported in the text above.

TABLE 2
*Influence of temperature and period of incubation on bacterial numbers**

PLATE NUMBER	ROOM TEMPERATURE				25°C.				37°C.			
	Incubation				Incubation				Incubation			
	2 days	5 days	12 days		2 days	5 days	12 days		2 days	5 days	12 days	
1	colonies	colonies	colonies		colonies	colonies	colonies		colonies	colonies	colonies	
2	No growth	76	152		38	120	164		28	102	123	
3		70	169		42	84	158		24	123	126	
4		58	128		35	105	172		16	126	104	
5		78	125		47	139	191		31	104	134	
6		56	130		48	118	197		26	92	79	
7		73	143		44	128	183		21	83	67	
8		53	88		43	126	152		24	76		
9		61	154		56	142	168		18			
10		64	134		39	81	179		25			
		48	164		40	116	161					
Mean		63.7 ± 2.18	138.7 ± 6.0		43.2 ± 1.28	115.9 ± 4.39	172.5 ± 3.14		23.9 ± 0.9	98.6 ± 4.93		
σ		10.22 ± 2.28	23.47 ± 3.55		6.01 ± 1.34	20.65 ± 3.11	14.72 ± 2.21		4.26 ± 0.95	23.14 ± 5.17		
C. V.		16.0 ± 3.6 %	17.6 ± 2.5 %		13.9 ± 3.1 %	17.8 ± 2.7 %	8.5 %		17.8 ± 4.0 %	23.5 ± 5.2 %		
Em		3.42%	3.6 %		2.96%	3.79%	1.82%		3.77%	5.0 %		

* The numbers represent all the colonies on the plate, except the fungi. Dilution 1:100,000.

above 7 days, so that this period has been decided upon for future work. In this connection, attention should be called to the fact that Conn also found a seven-day period, at a temperature of 25°, sufficient since he seldom found any appreciable increase on further incubation.

The futility of short incubation periods (2–3 days), sometimes even at room temperature, is thereby made clear.

Reaction of medium

A slight acidity (+0.5–+0.25) has usually been recommended as the optimum reaction of the medium used for the 'bacteriological analysis of soils. Several portions of egg-albumen agar were adjusted to definite hydrogen-ion concentrations by means of 1.0*N* NaOH and 1.0*N* H₂SO₄ solutions and used for the plating out of bacterial numbers.

TABLE 3
*Influence of reaction of medium upon the growth of bacteria on the plate**

PLATE NUMBER	pH = 5.2	pH = 6.4	pH = 6.8	pH = 7.2	pH = 7.6
	<i>colonies</i>	<i>colonies</i>	<i>colonies</i>	<i>colonies</i>	<i>colonies</i>
1	62	77	63	53	44
2	56	105	66	66	42
3	52	83	91	41	38
4	89	69	79	64	34
5	86	76	87	56	41
6	47	97	83	45	43
7	96	92	88	58	45
8	51	89	97	51	39
9	57	86	61	44	24
10	69	78	76	48	36
Mean	66.5 ± 3.76.	85.2 ± 2.31	79.1 ± 2.64	52.6 ± 1.83	38.6 ± 1.32
σ	17.68 ± 2.66	10.87 ± 1.64	12.41 ± 1.87	8.6 ± 1.29	6.22 ± 0.94
C. V.	26.6 ± 4.0 %	12.7 ± 1.9 %	15.7 ± 2.4 %	16.3 ± 2.4 %	16.1 ± 2.4 %
Em	5.65%	2.7 %	3.34%	3.5 %	3.4 %

* Dilution 1:200,000. Plates incubated 12 days at 25°. All colonies, exclusive of fungi, are reported.

The data presented in table 3 point definitely to the fact that a reaction of the medium equivalent to an hydrogen-ion concentration of about pH 6.4 is best. With more acid media, there is a decrease in bacterial numbers accompanied by a greater overgrowth of fungi. When the medium is made less acid there is also a drop in numbers, particularly above the neutral point, so that, at a pH 7.6, there are already less than a half as many colonies of bacteria developing than at pH 6.4. A reaction of about pH 6.5 is therefore, best. This happens to be the reaction of the egg-albumen agar when prepared according to the directions given above.

Method of preparing the dilutions

It seems to be generally agreed that the number of colonies to be allowed per plate should be between 30 and 200, for agar plates (Breed and Dotherer, 1), or a narrower limit 50 to 150 for gelatin plates (Conn, 4). However, where the incubation is at 25° for 7 days, there is danger of fungi overgrowing the plates. A plate badly overgrown with fungi, particularly in the case of certain *Mucorales*, should be discarded and not considered in the final count. With the medium used, there is very little danger of bacterial spreaders.

The next two experiments deal with the method of preparing and time of shaking the soil with water in preparing the first dilution (table 4) and influence of the final dilution (table 5).

TABLE 4
*Influence of stirring and time of shaking upon bacterial numbers**

PLATE NUMBER	SOIL STIRRED IN MORTAR	SHAKEN 1 MINUTE	SHAKEN 5 MINUTES	SHAKEN 10 MINUTES
	<i>colonies</i>	<i>colonies</i>	<i>colonies</i>	<i>colonies</i>
1	144	104	158	144
2	168	114	168	168
3	170	125	162	171
4	153	136	184	155
5	156	113	162	147
6	178	122	181	158
7	149	131	151	175
8	153	116	174	168
9	171	119	189	167
10	158	108	179	151
Mean	160.0 \pm 2.3	118.8 \pm 2.11	170.8 \pm 2.65	160.4 \pm 2.31
σ	11.07 \pm 2.70	9.91 \pm 2.22	12.49 \pm 1.88	10.83 \pm 2.42
C. V.	6.9%	8.3%	7.3%	6.8%
Em	1.4%	1.77%	1.55%	1.44%

* Plates incubated 7 days at 25°C. Dilution 1:100,000. All colonies except fungi are reported.

The stirring referred to in the first column of table 4 was done by thoroughly stirring 5 gm. of soil in sterile water, in a sterile mortar for 5 minutes, pouring off the supernatant liquid into a sterile flask, stirring residue again for 2-3 minutes with a fresh portion of sterile water, and so forth, until only a few grains of pure sand remained. The shaking referred to in the other three columns was done by shaking 5 gm. of soil in 100 cc. of sterile water and withdrawing samples after 1, 5, and 10 minutes for the further dilutions. The final dilution for this experiment was 1:100,000. The results reported in table 5 were obtained by shaking the original dilution 5 minutes, and making the final dilutions 1 to 20,000, 50,000, 100,000, 200,000, 500,000 and 1,000,000.

The results obtained in table 4 justify, without further discussion, the conclusion that 5 minutes shaking is sufficient for suspending all the bacteria in

TABLE 5
*Influence of final dilution upon the bacterial numbers**

PLATE NUMBER	DILUTION, 1:20,000		DILUTION, 1:50,000	
	Incubation		Incubation	
	3 days	7 days	3 days	7 days
1	192	Too numerous to count	127	156
2	238		124	146
3	262		148	172
4	206		143	179
5	227		108	124
6	231		113	138
7	214		137	149
8	199		132	167
9	223		141	153
10	211		117	133
Mean	220.3 \pm 4.10		129.0 \pm 2.88	152.7 \pm 3.73
σ	19.25 \pm 2.93		13.51 \pm 2.03	17.53 \pm 2.64
C.V.	8.7 %		10.5 \pm 1.6 %	11.5 \pm 1.7 %
Em	1.86%		2.23%	2.44%
Average number of Bacteria per gm. of wet soil	4,403,000		6,450,000	7,635,000

PLATE NUMBER	DILUTION, 1:100,000		DILUTION, 1:200,000	
	Incubation		Incubation	
	3 days	7 days	3 days	7 days
1	76	113	29	59
2	65	106	28	62
3	56	103	30	61
4	74	112	27	69
5	62	142	38	73
6	59	135	24	64
7	67	129	33	68
8	51	127	31	56
9	63	118	41	61
10	69	131	36	74
Mean	64.2 \pm 1.64	121.6 \pm 2.81	31.7 \pm 1.12	64.7 \pm 1.28
σ	7.76 \pm 1.17	13.18 \pm 1.99	5.29 \pm 0.79	6.04 \pm 0.91
C. V.	12.1 \pm 1.8 %	10.8 \pm 1.5 %	16.6 \pm 2.5%	9.3 %
Em	2.55%	2.31%	3.53%	2.0 %
Average number of Bacteria per gm. of wet soil	6,420,000	12,160,000	6,340,000	12,940,000

* Numbers of all microorganisms, except fungi, are given.

TABLE 5—*Continued*

PLATE NUMBER	DILUTION, 1:500,000		DILUTION, 1:1,000,000	
	Incubation		Incubation	
	3 days	7 days	3 days	7 days
1	14	18	4	11
2	19	26	4	17
3	18	22	9	19
4	13	21	5	13
5	13	19	5	14
6	13	18	6	17
7	17	23	4	11
8	16	21	7	13
9	17	25	5	9
10	21	29	9	12
Mean	16.1 \pm 0.60	22.2 \pm 0.77	5.8 \pm 0.41	12.6 \pm 0.76
σ	2.81 \pm 0.42	3.62 \pm 0.55	1.93 \pm 0.29	3.53 \pm 0.53
C. V.	17.4 \pm 2.6 %	16.3 \pm 2.5 %	33.3 \pm 5.0%	28.0 \pm 4.2 %
Em	3.73%	3.47%	7.07%	6.03%
Average number of Bacteria per gm. of wet soil	8,050,000	11,100,000	5,800,000	12,600,000

the water. One minute is insufficient, while a period greater than 5 minutes proves to be injurious.

When the various dilutions are compared, it is found that both too low dilutions and too high dilutions give unfavorable results. With the low dilutions too many colonies develop on the plates and it is impossible to determine accurately even the number of colonies that have developed. With too many colonies on the plate, many microörganisms, particularly those that develop only late, fail to develop at all.

It is interesting to note that, while at 1:20,000 dilution the colonies were, at 7 days, so numerous that no accurate count could be made, particularly due to overgrowth of fungi and that with the 1:50,000 dilution, an accurate count was made, but the numbers of organisms obtained are much less than with the higher dilutions. This simply indicates that, with too many colonies on the plate, many organisms simply fail to develop. Another disadvantage of the too low dilutions is the fact that it is difficult to make an accurate differentiation, under these conditions, between bacterial and actinomyces colonies. Of course the advantage of the low dilution lies in the smaller error obtained, but this can be obviated by the use of a larger number of plates for the count.

In the case of too high dilutions, like those of 1:500,000 and 1:1,000,000, there is apt to be not only greater variability with a greater error involved and the actual elimination of many specific types but the actual count may be smaller.

In the case of the two highest dilutions used in this experiment, the number of colonies developing on the plate was below 30 and although in this case there was plenty of room for development, the count was less, even with a 7-days incubation period, than with the optimum dilution (1:100,000 and 1:200,000). This, chiefly, is the reason why the number of colonies allowed per plate has been usually recommended as between 40 and 200. The optimum dilution for ordinary field soils is from 1:100,000 to 1:200,000. For poor sandy soils, a lower dilution may have to be used; for heavily manured soils or green-house soils, higher dilutions should be used.

The results reported here are directly opposed to the claim of Rossi (15) that the number of microorganisms present in the soil depends entirely on the dilution and increases with the higher dilutions. That is true only within certain narrow limits (below the optimum dilution).

TABLE 6

*The use of tap water and salt solution (0.85 per cent NaCl) as diluents for making bacterial counts**

PLATE NUMBER	TAP WATER		SALT SOLUTION	
	All colonies except fungi	Actinomycetes	All colonies except fungi	Actinomycetes
	<i>colonies</i>	<i>colonies</i>	<i>colonies</i>	<i>colonies</i>
1	91	24	71	22
2	83	20	72	23
3	68	22	76	26
4	62	19	64	19
5	71	21	59	26
6	79	18	71	16
7	86	26	56	21
8	78	21	61	22
9	85	27	62	16
10	82	23	76	23
Mean	78.5 \pm 1.91	22.1 \pm 0.62	66.7 \pm 1.54	21.4 \pm 0.75
σ	8.98 \pm 1.35	2.92 \pm 0.63	7.25 \pm 1.09	2.53 \pm 0.53
C. V.	11.4 \pm 1.7	13.2 \pm 1.9 %	10.8 \pm 1.6 %	16.5 \pm 2.5
Em	2.43%	2.81%	2.31%	2.5%

* Dilution 1:200,000. Plates incubated 7 days at 25°C.

DILUENT

Ordinary sterile tap water is commonly used in making dilutions. The use of bouillon or sugar solution has not been found beneficial, while a solution of 0.4 per cent NaCl and 0.4 per cent KCl has actually been found injurious (Hiltner and Stormer, 8). Ordinary sterile tap water was compared with saline (0.85 per cent NaCl) solution with the results presented in table 6.

No advantage is obtained from the use of salt solution over ordinary tap water. If anything, there is an injurious effect due to the use of the salt solution, confirming the results of Hiltner and Störmer, who used a mixture

of sodium and potassium chlorides. No appreciable difference has been found in the number of actinomycetes with both diluents. The use of distilled water for making dilutions should be condemned, since plasmolysis will readily take place.

SUMMARY

With these preliminary experiments in mind, we can now establish some of the important points to be observed in the determination of bacterial numbers in the soil.

1. A medium of standard composition should be used, containing no peptone, meat extract, soil extract or similar material, which would vary greatly in composition. In addition to the necessary minerals and carbohydrate, the medium should contain a definite nitrogen source, like asparagine, purified casein or powdered egg-albumen.

2. The final reaction of the medium should be about pH 6.2-6.8, with an optimum at pH 6.5.

3. Sterile tap water should be used for making the dilution.

4. The soil should be shaken uniformly, for 5 minutes, in preparing the first dilution.

5. The original dilution should be 1:20 or 1:10, high enough to give a ready distribution of the bacteria, and low enough to allow a representative sample to be taken. The further dilutions should be uniform, preferably 1:10 or 1:100. The final dilution should be made in such a manner, as to give 40-200 colonies of microorganisms excluding fungi, per plate.

Where a count of soil fungi is wanted, special acid media should be used having a pH 4.0 (like raisin agar and special synthetic agar (Waksman, 19), which due to their nature, do not allow any development of actinomycetes or bacteria, so that a low dilution (one fiftieth to one two-hundredth of that used for bacteria) can be used. This, combined with a short incubation period will allow a count of fungi, involving a comparatively low probable error.

6. At least 3-5 samples, composite if possible, should be taken from each soil examined for each determination.

7. At least 6-10 plates should be used in plating out each sample. These last two points are important where we want to work out the variability of numbers of microorganisms, and reduce these to a mathematical standard.

8. The plates should be incubated at 25°C. for at least 7 days or at room temperature for at least 14 days, the first to be preferred due to uniform temperature.

9. Plates badly overgrown with fungi, particularly in case of certain *Mucorales*, should be discarded from the counts.

10. The numbers should be computed on the basis of wet soil or soil dried to constant weight, in each case stating the moisture-content and the moisture-holding capacity, or optimum moisture, of the particular soil.

11. The most probable error of the counts should not be greater than 2.0 to 2.5 per cent for each soil, and not above 3.0 per cent for each soil sample.

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