PREVENTION OF ANTIBIOSIS AND LIBERATION OF RETAINED ORGANISMS AS IMPORTANT FACTORS IN THE COMPARATIVE PLATE COUNT OF BACTERIA IN SOILS

by

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Part I.

PREVENTION OF ANTIBIOSIS (SPREADING) AS AN IMPORTANT FACTOR IN THE COMPARATIVE PLATE COUNT OF BACTERIA IN SOILS

INTRODUCTION

Soil microbiologists have realized for sometime that the number of organisms per gram of soil as determined by the plate method depends upon the composition of the medium. Media containing greatly reduced organic sources of nitrogen such as beef extract and peptone found in nutrient agar were discarded in preference to synthetic media, whose nitrogen compounds were either inorganic salts or amino acids. The former media allowed the rapid development of aerobic spore-formers, which spread over the plate and inhibited the growth of the numerous but slow growing bacteria (17).

Among the verious synthetic media that have been suggested are Fischer's soil extract agar (7), Lipman and Brown's synthetic agar (9), casein agar (1), urea ammonium nitrate agar (4), albumin agar (1), and Temple's .1% peptone medium (15). Although the synthetic media are all equally favorable to the development of aerobic heterotrophic bacteria, and the results obtained with these media are always higher, they do not always prevent spreading. This increase in count accompanied by decreased spreading is probably due to a reduction of the antibiotic effect of these spreading organisms.

In 1922 Thornton reported that bacterial counts were impossible on such a medium as Lipman and Brown's synthetic agar, which contained peptone but no beef extract, because a great deal of spreading still took place (16). He also reported that less spreading occurred on Conn's synthetic agar (3), which contained asparagine as the source of nitrogen. In the same paper, he investigated the mechanism of spreading and found that it was the result of the orientation of cells at right angles with each other and to their active motility. By drying sterile nutrient agar plates in a 30° C. incubator for different periods of time (2 days to 14 days) and then making spot inoculations with a spreading Bacillus, he found that the amount of spreading was a function of the quantity of free moisture in the agar. The plates that had been incubated two weeks previous to inoculation had no spreading while the organism exhibited profuse spreading on plates that had been incubated but 48 hours before inoculation.

Thornton concluded that there were no practical methods yet devised for controlling spreading on glucose nutrient agar to make quantitative bacterial analyses of soils; so he advised a synthetic medium which contained mannitol as a source of energy and asparagine as a source of nitrogen. Asparagine-mannitol agar reduces spreading to a minimum and has been used by some soil microbiologists as a

medium for obtaining an estimate of the numbers of organisms in soils (18).

In spite of the fact that glucose nutrient agar has been discarded by the majority of soil microbiologists in preference to synthetic media for making quantitative bacterial analyses of soils, it is still referred to as a "routine medium" and used in many laboratories (6). Its limitation in soil bacteriology is in the fact that the spreading organisms develop rapidly, and the antibiosis produced inhibits the growth of many of the non spore-forming bacteria. However, it has been used to determine the number of organisms in water since its recommendation by the American Public Health Association in 1917 (14). It has been found to be excellently suited for routine examination of water wherever comparative results were sought and is predominately the most generally used culture medium employed in the bacteriological laboratory (6). Nutrient agar is also recommended in the Standard Methods of Milk Analysis (13). The early objections to its non-uniformity by some bacteriologists have been met by the availability on the market of standardized beef extract and peptone, whose composition does not vary widely (6). Reliability of the results of the medium depend upon the constant composition of the peptone and the beef extract.

As a medium for many species of bacteria, glucose nutrient agar is superior to synthetic media since there are many kinds of amino acids in the former whereas mannitol-asparaginate agar contains but one. Not all bacteria can synthesize protoplasm from a single amino acid.

The purpose of this investigation was to determine whether a plating method could be developed for the use of glucose nutrient agar to make quantitative bacterial analyses of soils. It was believed that glucose nutrient agar would yield higher counts in comparison to a synthetic medium such as Thornton's sodium asparginate agar if the spreading of the aerobic spore-formers could be controlled.

EXPERIMENTAL

Procedure

Five types of plates were used in an effort to control mechanically the spreading of Bacilli on glucose agar plates. These types were designated as:

1. sandwiched, plain plates
2. clay top plates
3. clay top plus $CaCl_2$, plates
4. clay top, sandwiched plates
5. clay top plus $CaCl_2$, sandwiched plates

It was believed that if the organisms could be made to develop within the agar, rapid spreading would not take place and more organisms would develop since antibiosis would be lessened.

The sandwiched, plain plates were prepared by pouring a sterile layer of nutrient agar on the bottom of a sterile petri dish, allowing it to solidify, then mixing 1 cc of an appropriate soil dilution with sterile agar and allowing this layer to solidity before pouring the top layer of sterile agar.

The clay-top plates were petri dishes whose lids were made of porous clay, glazed only on the outside.

Clay top plus $CaCl_2$, plates contained additional absorptive properties for moisture because the lids were treated for five minutes

with a saturated solution of $CaCl_2$ before sterilizing.

The clay top, sandwiched plates (4) were a combination of (1) and (2), while the clay top plus $CaCl_2$, sandwiched plates (5) were a combination of (1) and (3).

Two controls were:

1. plain plates - glucose nutrient agar
2. plain plates - asparaginate-mannitol agar (Thornton's medium)

Each of two soils was plated out by the five methods plus the two controls. One of these soils, designated as R, was a Gray Brown Podosol, and the other, designated as W, was a Prairie soil. The pH of the R soil was 7.1; the pH of the W soil was 7.0. Dilutions of 1 to 10,000 were used in plating for both soils. Several series counts were made for each soil to check results. A series constituted the counts on seven plates made from a 10 gram sample of soil after two days incubation at 30° C.

Results

In Table I the figures given in the brackets are means of individual series. The underscored mean with its probable error is the arithmetic average for all determinations made with that particular type of plating. In Figure 1, the mean with its range (plus or minus 3x probable error) is given for all determinations.

With the use of three times the probable error, the odds are 22 to 1 against the occurrence of the true mean outside this range.

The results in Table I and Figure 1 show that the type of plating, giving the highest bacterial counts for both the R and W soils was the clay top plus $CaCl_2$, sandwiched plates. Spreading was inhibited on the surface layer of the agar by the absorption of free moisture from this layer.

All the plain plates contained spreaders, but individual series varied as to amount of spreading. This explains the wide differences in the mean count found in the plain plates, especially in the R soil.

Sandwiched, plain plates had little effect upon the control of spreaders, for the spore-formers grew vertically to the surface and then spread horizontally, sometimes covering the entire plate. It was usually more difficult to make a count with these plates than with plain plates.

The absorptive properties of the clay top plates controlled spreading to some extent, but the addition of $CaCl_2$ to the top without sandwiching resulted in lower counts since too much moisture was absorbed from the agar before the bacteria began to develop. After two days incubation some of the medium of the clay top $CaCl_2$, plates was almost completely dehydrated.

TOTAL BACTERIAL COUNT OF R AND W SOILS AS EFFECTED BY VARIOUS ATTEMPTS TO CONTROL SPREADING (ANTIBIOSIS)

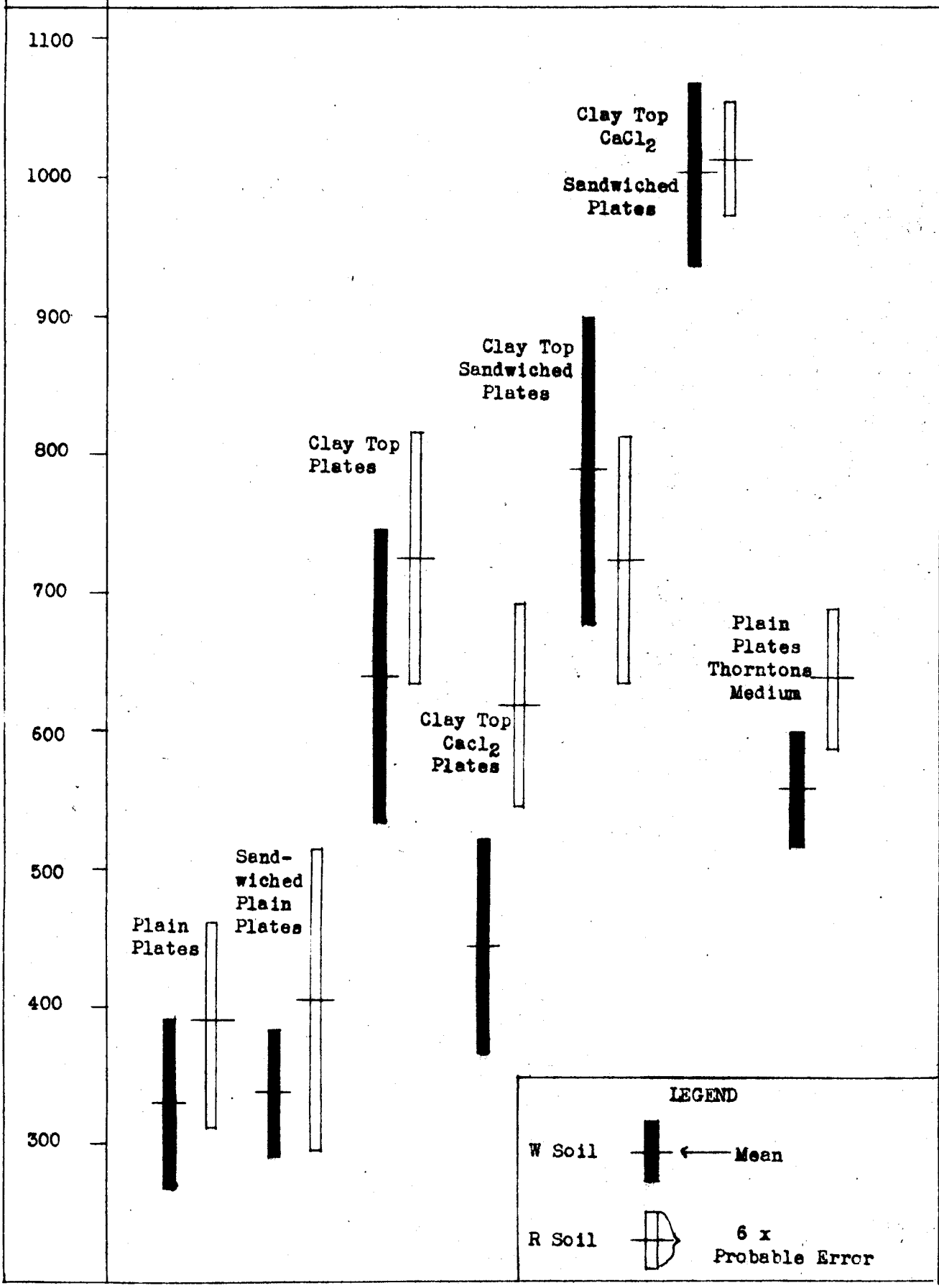
Soil	Sandwiched Plain Plates	Clay Top Plates	Clay Top $CaCl_2$ Plates	Clay Top Sandwiched Plates	Clay Top $CaCl_2$ Sandwiched Plates	Plain Plates	Plain Plates Thorntons Medium
W	(344,000 374,000 317,000)	(395,000 683,000 713,000)	(458,000 431,000)	(853,000 948,000 430,000)	(800,000 1,152,000 902,000 1,248,000 1,183,000 692,000 1,184,000 714,000)	(228,000 564,000 296,000 284,000 261,000)	(521,000 417,000 644,000 587,000)
	342,000 ±15,000	642,000 ±34,000	445,000 ±24,000	790,000 ±39,000	1,003,000 ±22,000	338,000 ±21,000	563,000 ±14,000
R	(421,000 530,000 158,000)	(386,000 647,000 670,000)	(556,000 685,000)	(880,000 759,000 275,000)	(1,358,000 1,090,000 1,091,000 1,000,000 900,000 907,000 1,074,000)	(154,000 686,000 304,000 436,000 311,000)	(613,000 617,000 624,000 691,000 691,000)
	405,000 ±34,000	726,000 ±31,000	621,000 ±24,000	726,000 ±31,000	1,016,000 ±16,000	390,000 ±25,000	640,000 ±15,000

Legend

1. Figures in brackets represent means of individual series of seven plates.
2. Underlined figures represent means for all determinations plus or minus probable error.

Per Gram x 1000

GRAPHIC REPRESENTATION OF TOTAL BACTERIAL COUNTS OF R AND W SOILS AS EFFECTED BY VARIOUS ATTEMPTS TO CONTROL SPREADING (ANTIBIOSIS)



Spreading on the clay top, sandwiched plates was quite frequent. Apparently the absorptive properties of the clay top is not efficient unless $CaCl_2$ is added.

A mean whose probable error is greater than 3 percent is not reliable (18). The only procedures which gave reliable means were the clay top plus $CaCl_2$, sandwiched plates and plain plates with Thornton's medium, asparaginate-mannitol agar. The results on the synthetic medium, however, are much lower than those on the organic medium, indicating that all soil bacteria may not be able to utilize sodium asparaginate as a source of nitrogen in the synthesis of their protoplasm.

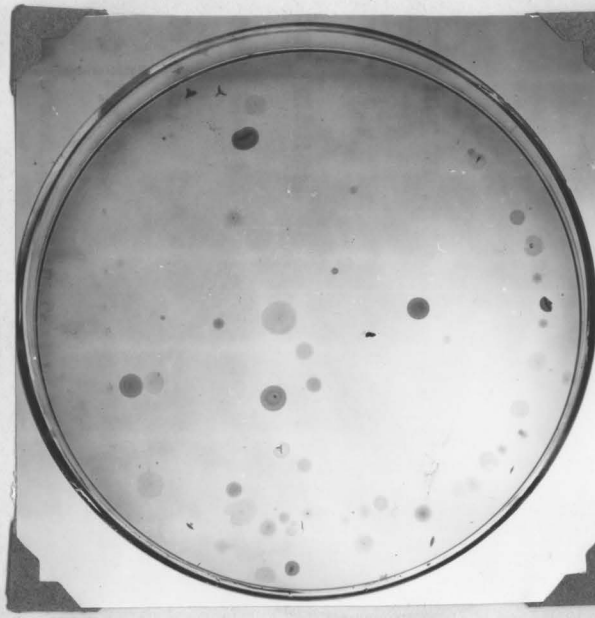
Plates on pages 11 and 12 show spreading on typical plates of the plain plate method with glucose nutrient agar, and the inhibition of spreading on glucose nutrient agar with the clay top plus $CaCl_2$, sandwiched technique. Inhibition of spreading with Thornton's medium, a synthetic medium is also shown.

PLATE I

ILLUSTRATIONS AND TOTAL BACTERIAL COUNTS OF THREE METHODS OF PLATING ON W SOIL

Glucose Nutrient Agar
Plain Plates
(230,000)
296,000

Glucose Nutrient Agar
Clay Top $CaCl_2$ Sandwiched Technic
(1,040,000)
1,152,000

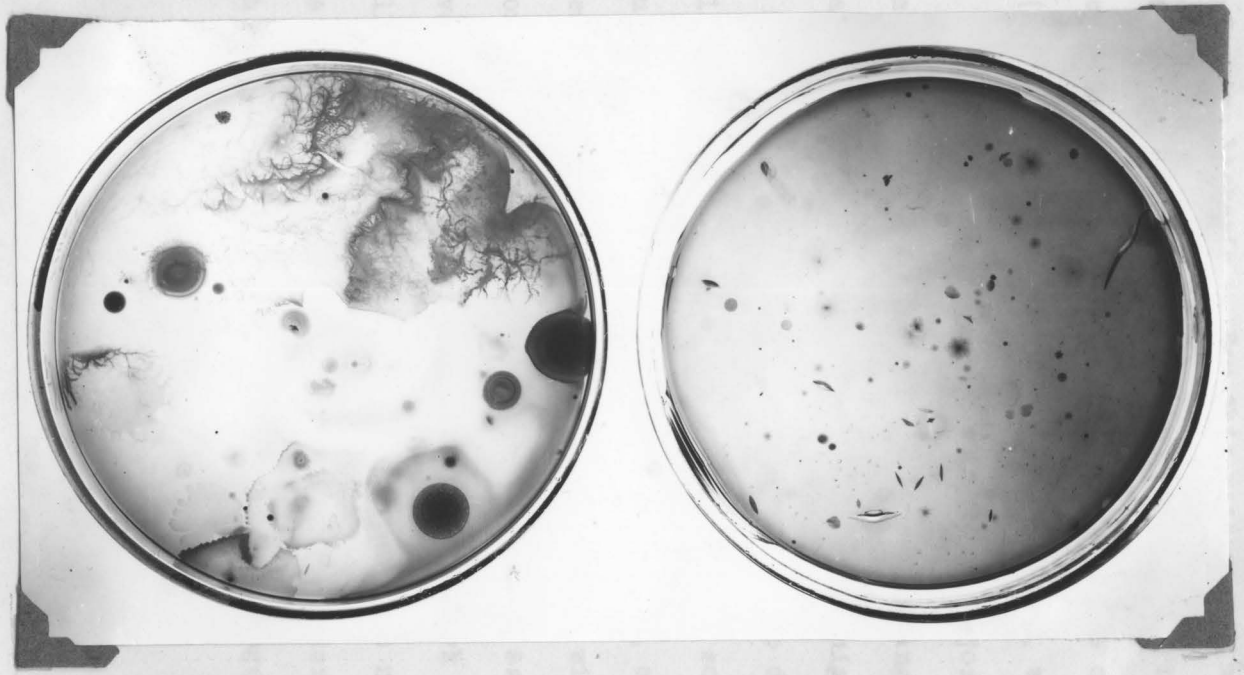


Thornton's Medium
Plain Plates
(710,000)
644,000

Legend: Figure in parenthesis is the count on the photographed plate x 10,000. Underlined figure is the mean x 10,000 of the series of seven plates, one of which is shown in the picture.

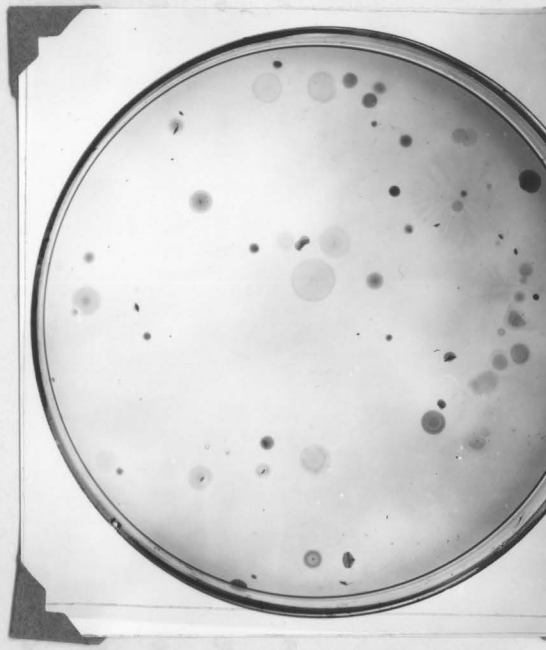
PLATE II

ILLUSTRATIONS AND TOTAL BACTERIAL COUNTS OF THREE METHODS OF PLATING ON R SOIL



Glucose Nutrient Agar
Plain Plates
(250,000)
304,000

Glucose Nutrient Agar
Clay Top $CaCl_2$ Sandwiched Technic
(1,117,000)
1,074,000



Thornton's Medium
Plain Plates
(680,000)
691,000

Legend: Figure in parenthesis is the count on the photographed plate x 10,000.
Underlined figure is the mean x 10,000 of the series of seven plates,
one of which is shown in the picture.

SUMMARY

Glucose nutrient agar, because it allows the profuse spreading of aerobic spore-formers, has been discarded by the majority of soil microbiologists as a medium for estimating the number of bacteria per gram of soil. The antibiosis produced by the rapid growing Bacilli inhibit the growth of many of the slower growing non spore-forming bacteria. For this reason soil microbiologists use synthetic media which inhibit the spreading of the bacilli.

Because the synthetic media contain but one or two sources of nitrogen (ammonium salts, nitrates, or asparagin), which is equstionable as a universal source of nitrogen for the majority of bacteria in soil, a plate method which inhibits the spreading on glucose nutrient agar might yield as high results if not higher than the synthetic media. The purpose of this work was to devise a method of controlling spreading on glucose nutrient agar so that this medium could be used in making bacterial counts of soils, and to compare the soil counts obtained on glucose nutrient agar with the counts obtained on a synthetic medium, Thornton's sodium asparaginate agar.

Five types of plates were tried in this work for the purpose of controlling the spreading of bacilli on glucose nutrient agar.

The effectiveness of the control of "spreaders" depended upon the growth of the organisms in a middle layer of agar between two

layers of sterile agar. This type of plating was designated as sandwiching. Unless the moisture on the top layer was absorbed, the bacilli grew vertically to this layer with subsequent development and spreading. The clay top plus $CaCl_2$, sandwiched plate was the most efficient in the prevention of the spreading of bacilli. This was due to keeping the surface of the top layer moisture free because the porous clay top was saturated with a hygroscopic agent, $CaCl_2$. The absorption of the moisture from the top layer apparently did not produce any ill effects of dehydration upon the organisms, which developed in the middle layer. Colonies of sufficient size for counting developed before dehydration seriously affected their growth.

CONCLUSIONS

The following conclusions were drawn from the results obtained in this investigation:

1. The spreading of bacilli can be controlled on glucose nutrient agar by sandwiching the layer of agar containing the organisms between two layers of sterile agar and dehydrating the top layer with porous clay top plates to which $CaCl_2$ has been added. Antibiosis is thus minimized so that glucose nutrient agar will give reliable results in plate counts of soils.

2. The count on glucose nutrient agar with the clay top plus $CaCl_2$, sandwiched technique increased the count 2.5 times on one soil and three times on another over glucose nutrient agar plain plates (the regular plating method).

3. Although the results on two soils with Thornton's medium, one of the synthetic media recently proposed for the control of spreading, showed a prevention of spreading and were greater than those on glucose nutrient agar plain plates (standard technique), they were much less than the counts on glucose nutrient agar where the clay top plus $CaCl_2$, sandwiched method was used.

4. The lower counts with Thornton's medium than with the clay top plus $CaCl_2$, sandwiched plates (glucose nutrient agar)

indicates that the prevention of spreading alone does not yield the greatest number of microorganisms, but the source of nitrogen is almost important. In this respect glucose nutrient glucose nutrient agar is superior to sodium asparaginate agar. A departure from the standard medium (glucose nutrient agar) is therefore not justified.

5. Spreading of aerobic spore-formers, while fairly universal in soil plating, does not necessarily occur to the same extent. Many soils show this characteristic excessively while others show it to only a slight degree. The elimination of the antibiotic effect is therefore desirable to enable one to obtain comparable counts on different soils.

PART II

LIBERATION OF ADSORBED OR RETAINED ORGANISMS AS AN IMPORTANT FACTOR IN THE COMPARATIVE PLATE COUNT OF BACTERIA IN SOILS

INTRODUCTION

In the comparative plate counts of soils it is possible that adsorption or retention of microorganisms on the soil particles might materially influence the accuracy of the comparison. This would be particularly true if different soils adsorbed or retained organisms to different degrees, i.e., two soils might have the same number of organisms per gram but might give widely different plate counts if the organisms of one were held to the soil particles by adsorption or retained among flocculated soil particles more than in the other soil. An erroneous comparison of the activity of microorganisms in the two soils might result.

The soil is regarded as a mineral framework, the particles of which are coated with a jelly-like layer of organic and inorganic material present in a colloidal condition (18). The microorganisms are found in both the colloidal material and the soil solution (18). The bacteria found in the colloidal layer may be either embedded or adsorbed and under such conditions, the soil has a marked influence upon the chemical activities of the bacteria (2). Adsorption decreases the activity of bacteria as measured by the rate of liberation of Co_2.

The percentage adsorption varies with different species of bacteria. Bacillus mycoides, Serratia marscescens, and Staphylococcus aureus are adsorbed rapidly and completely (80 - 98%); other bacteria such as Escherichia coli are only weakly adsorbed (10 - 20%) (18).

Different soil fractions adsorb bacteria to different degrees, the finer fractions having a much greater adsorptive capacity than the coarser ones (5). Novgrudsrii reported adsorption was greatest with the highest silt content and was less the larger the sand content regardless of particle size (10). Others have reported that adsorption of bacteria is inversely proportioned to the concentration of bacterial cells (12).

The pH of the soil is one of the most important factors effecting adsorption of bacteria (10). For each organism there exists a definite pH of maximum adsorption. Soil adsorption is reversible and may be varied by shaking the soils in solutions of various hydrogen-ion concentrations (10). It is possible that shaking the soils in solutions of different pH's might vary the adsorption of bacteria; but because there is probably no universal pH in different soil types for the maximum adsorption of all bacterial, results indicating an arbitrary pH for maximum liberation of organisms might be misleading.

Peel (11) found that when two widely different New York soils were placed in contact with a suspension of negatively charged bacteria, the bacterial cells are to a large extent removed from the suspension

and become attached to the soil particles. Adsorption seemed to depend on the base predominating in the soil exchange complex. Peel concluded that the adsorption of bacteria by soil is due to the attraction of unlike electric charges. His data also indicated that the ability of a soil to adsorb bacteria may be modified by changing the base predominating in the exchange complex.

Peel found that when the chlorides of certain metals were used to saturate these soils, the percentage adsorption varied from 0 to 94 percent. The percent of adsorption was least with sodium, potassium, and lithium, and greatest with manganese, iron, calcium, and aluminum. It was found that when a soil sample was treated with 0.5 molar solution of the chloride of the desired base, dispersed for twenty minutes, filtered and washed three times with sterile distilled water to remove excess ions, the number of bacteria leached from the soil in the presence of a large excess of ions was somewhat less than in the presence of water only. After the excess ions were removed by leaching with water, the base with which the soil exchange complex was saturated had a very decided effect upon the numbers of bacteria passing out in the leachate. The validity of Peel's interpretations of his data will be discussed later in the section headed "Discussion".

Since the usual procedure in making quantitative bacterial determinations of soils is to shake the soil suspension for five

minutes in sterile water before making subsequent dilutions, it is doubtful whether such a procedure uniformily releases the many bacteria that are adsorbed on or retained in the colloidal complex. Winogradsky reported in 1924 that not all bacteria were removed from heavy organic soils by three washings (19).

The purpose of this investigation was to determine whether shaking a soil suspension in a .5 M solution of NaCl followed by distilled water washings (deflocculation) will increase the total bacterial count as determined by the glucose nutrient agar plate method, modified to prevent spreading of aerobic sporeformers. Since it is quite probable that bacteria are adsorbed or retained on different soils to different degrees because of the varying composition of these soils, any method which would minimize the effect of adsorption of the bacteria and flocculation of the soil particles would give figures that could be compared with greater accuracy.

EXPERIMENTAL

Procedure

Liberation of bacteria from adsorption on soil particles by an electrolyte and from flocculated soil particles was accomplished as follows: A ten-gram sample of soil was shaken for five minutes in 100 c.c. of 2.9 percent NaCl (0.5 molar) solution; the heavy soil particles were allowed to settle before the solution was drawn off into a sterile flask. The soil was then washed four times with sterile distilled water with one minute shakings between washings, so that the final dilution in the flask was 1 to 500. Subsequent dilutions were made to 1 to 10,000 for plating. The pH of the sterile water used for washing was 6.8.

Bacterial counts were made on all plates after two days incubation at 30° C. The medium used was glucose, nutrient agar with the clay top plus $CaCl_2$, sandwiched technique.

The soils used in these experiments were designated as R, W, Clay, Sandy Loam, Rich Loam soil, Poor Leached Loam, and Sub Sod. Two types of controls were used to check the NaCl technique with the R and W soil: (1) soil shaken in distilled water plus four washings (2) soil shaken in distilled water without washings (standard procedure). Since the differences between the two controls on the R and W soils were negligible in one case (R soil) and slightly different in the

other (W soil), only one control (soil shaken in distilled water plus four washings) was used with the other soils.

Results

Results on the R and W soil are shown in Table II and Figure 2. In the table each figure within a bracket represents the mean of a series of plates from one 10-gram sample of soil. The underlined mean plus the probable error was calculated from the total individual counts made on these soils and does not represent the mean of the several series. The unshaded fractions of the bars in Figure 1 represent the range of each mean or plus or minus three times the probable error (odds of 22 to 1 against the occurrence of the true mean outside this range).

The bacterial count on the W soil when shaken in a 0.5 molar solution of NaCl is 1.7 times as great as the control, but the count on the R soil when shaken in 0.5 molar NaCl is only 1.1 times as great as the control. A few organisms were apparently liberated by washings with the W soil, as the mean count on the control with washings is 21 percent greater than on the control without washings. The differences between the counts on the two types of controls with the R soil, however, are not great enough to be significant.

The most interesting feature of these results is the fact that the ratio of the counts on the controls (with washings) is $\frac{W}{R} = 1.18$, but the ratio of the counts with the NaCl treatment

TABLE II.

TOTAL BACTERIAL COUNT OF R AND W SOILS. LIBERATION OF ORGANISMS BY 0.5 MOLAR NACL

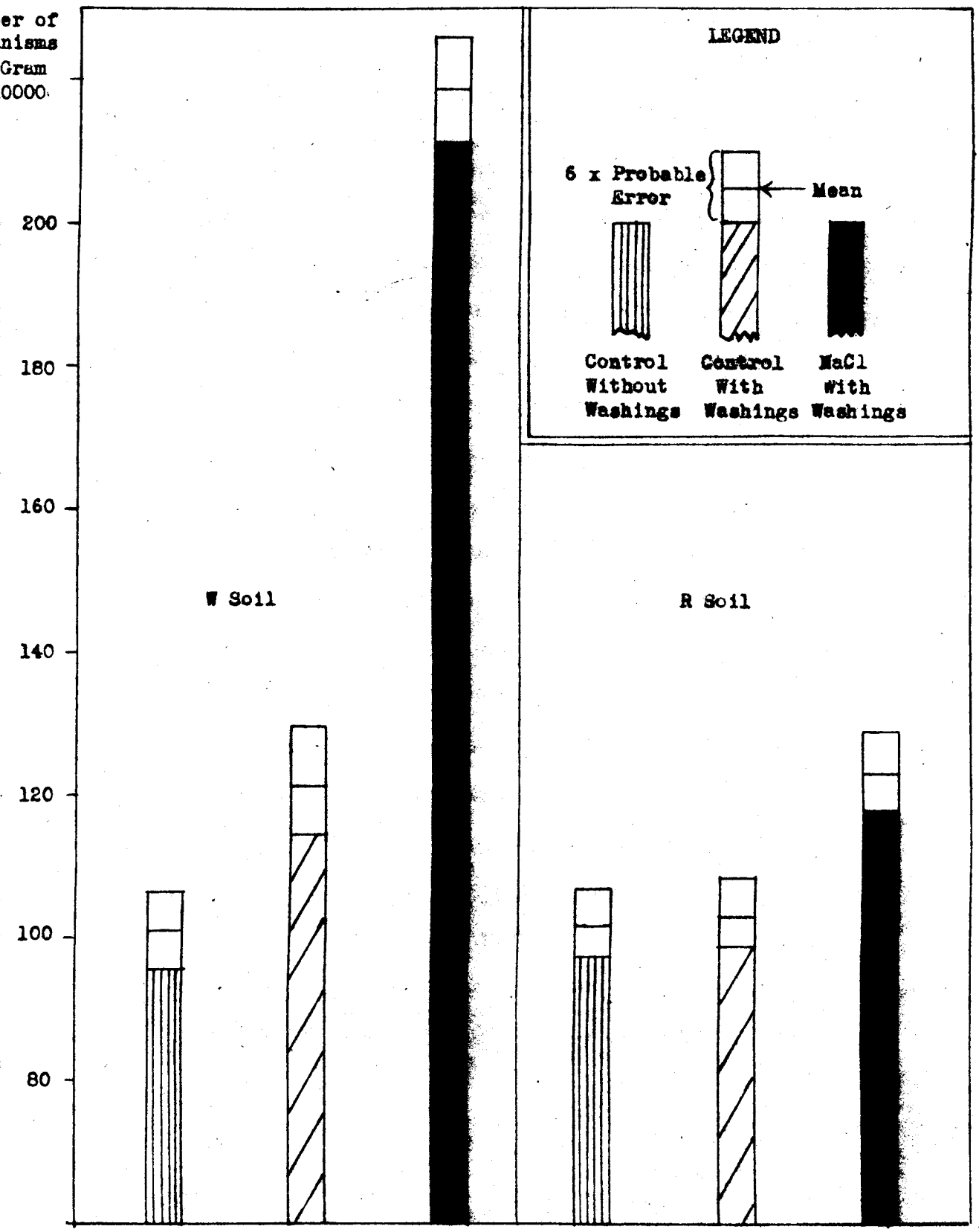
Soil	1. Control Without Washings	2. Control With Washings	Percent Increase Over Control 1	0.5 Molar NaCl with Washings	Percent Increase Over Control 1	Percent Increase Over Control 2
W	800,000 1,152,000 902,000 1,248,000 903,000 1,183,000 692,000	1,256,000 1,331,000 1,336,000 1,241,000 638,000		2,264,000 1,973,000 2,226,000 2,331,000 2,208,000 2,128,000		
	1,005,000 ±22,000	1,219,000 ±25,000	21	2,192,000 ±25,000	119	81
R	1,358,000 1,090,000 1,090,000 1,100,000 900,000 907,000	962,000 996,000 978,000 1,111,000 1,074,000		1,012,000 963,000 1,340,000 1,078,000 1,121,000 1,177,000		
	1,016,000 ±16,000	1,030,000 ±18,000	1	1,123,000 ±20,000	10	9

Legend

1. Figures in brackets represent means of individual series of seven plates.
2. Underlined figures represent means for all determinations plus or minus probable error.

Figure 2.

COMPARISON OF THE EXTENT OF LIBERATION OF BACTERIA FROM TWO SOILS BY NaCl TREATMENT



is $\frac{W}{R}$ = 1.95. These results indicate that adsorption or retention of bacteria is not the same for all soils. The R soil probably had but very few bacteria adsorbed on or in its colloidal complex, hence the count is not raised appreciably when the soil is shaken in NaCl (10 percent increase); the count on the W soil, however, is nearly doubled with the NaCl treatment (119 percent increase), indicating that nearly one-half of the organisms capable of developing on glucose nutrient agar are not released with the standard shaking procedure. Because the percentage of adsorbed or retained organisms varies with the soil, the usual method of shaking soils in sterile water, therefore, does not always give a true picture of the relative number of organisms in that soil.

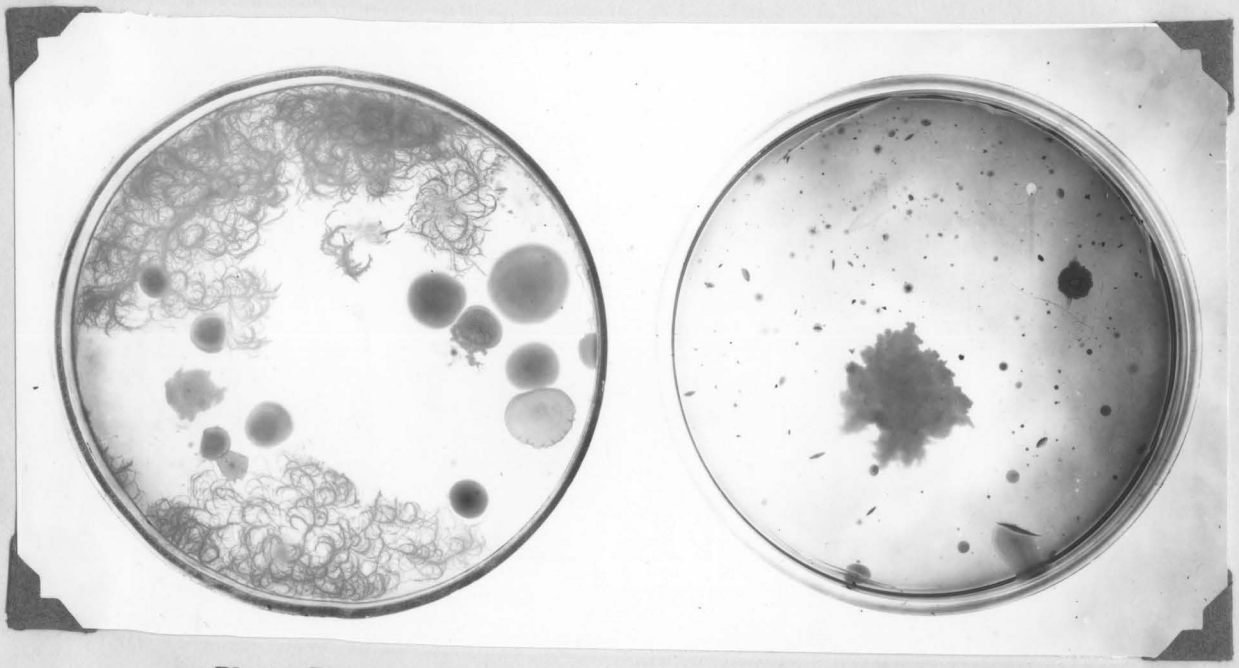
An experiment was also undertaken to determine whether the increase in numbers of bacteria due to the NaCl technique could be detected on glucose nutrient agar, plain plates (standard method of plating) where aerobic spore-formers would be allowed to spread freely. Plates III and IV on pages 27 and 28 show that the antibiosis produced by the spreading of the bacilli inhibits so many organisms that the increase in numbers due to liberation of bacteria could never have been detected by this method. On the same pages are shown representative results with the clay top plus $CaCl_2$, sandwiched technique which was used in the foregoing experiments. Underneath

each picture is given the count on the plate and an underlined arithmetic average, which is the mean for the series of seven plates.

To check the results obtained in Table I with soils R and W, several other soils were examined to determine to what extent adsorption or retention may vary in different soils. While the data obtained (Table III and Figure 2) are not as extensive as for the R and W soils, they do nevertheless support the conclusions reached with these first two soils with the use of NaCl. The percentage increase over the control (with washings) with these other soils was as follows: Clay, 75 percent; Sandy Loam, 169 percent; Rich Loam Soil, 83 percent; Poor Leached Loam, 257 percent; Sub Sod, 153 percent.

PLATE III

ILLUSTRATIONS OF TWO METHODS OF PLATING WITH GLUCOSE NUTRIENT AGAR ON W SOIL
(SHAKEN IN 0.5 MOLAR NaCl SOLUTION)



Plain Plate Method

(28,000)
<u>43,000</u>

Clay Top $CaCl_2$ Sandwiched Method

(2,500,000)
<u>1,973,000</u>

Legend: Figure in parenthesis is the count on the photographed plate x 10,000.

Underlined figure is the mean x 10,000 of seven plates, one of which is shown in the picture.

PLATE IV

ILLUSTRATIONS OF TWO METHODS OF PLATING WITH GLUCOSE NUTRIENT AGAR ON R SOIL
(SHAKEN IN 0.5 MOLAR NaCl SOLUTION)

Plain Plate Method	Clay Top $CaCl_2$ Sandwiched Method
(410,000)	(950,000)
510,000	978,000

Legend: Figure in parenthesis is the count on the photographed plate x 10,000.

Underlined figure is the mean x 10,000 of seven plates, one of which is shown in the picture.

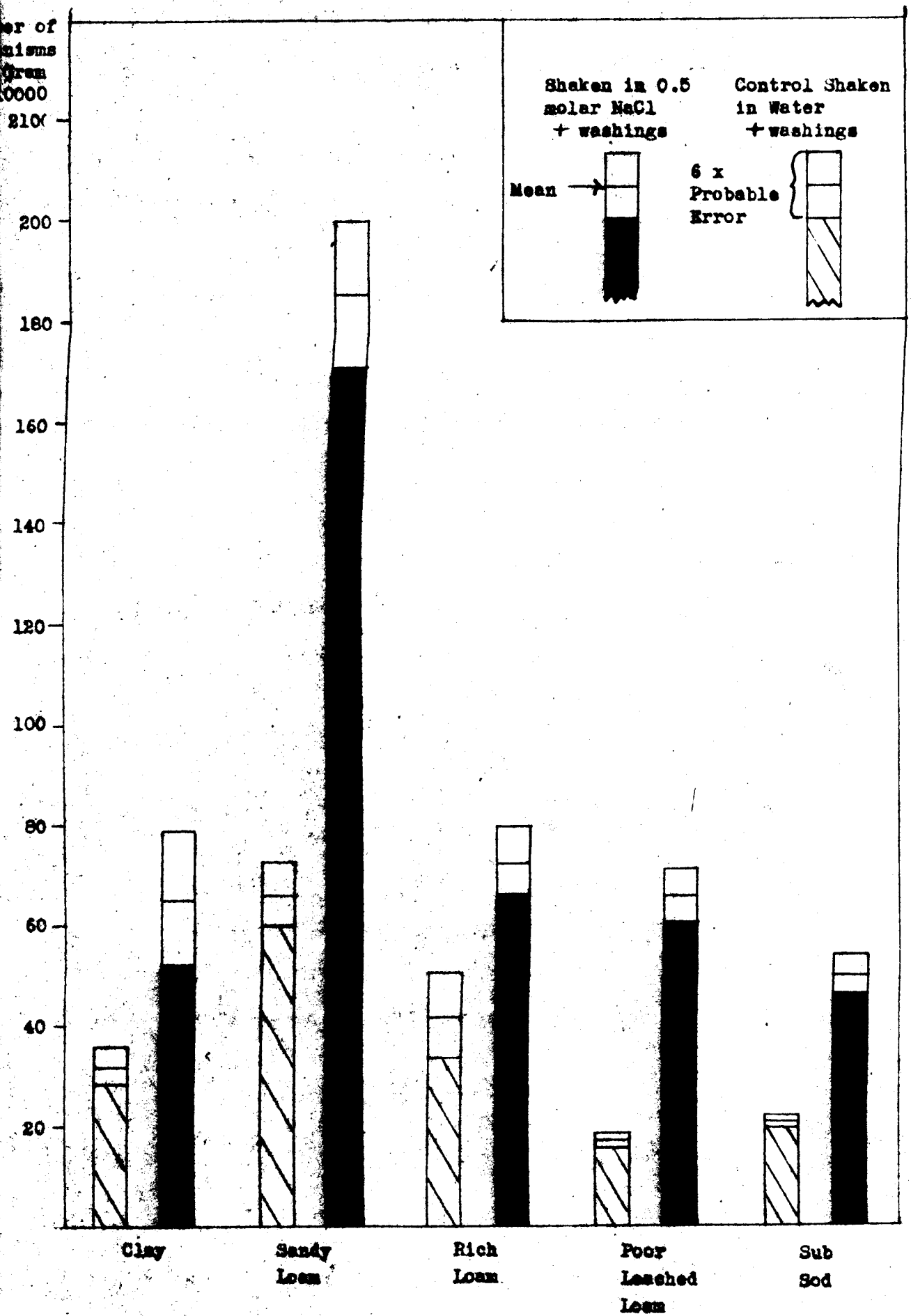
THE TOTAL BACTERIAL COUNT OF VARIOUS SOILS AS EFFECTED BY NaCl TREATMENT

Soil	Control Shaken in Water Washings	Shaken in 0.5 Molar Washings	Percent Increase Over Control
Clay	(390,000 380,000 394,000) 389,000 ±31,000	(455,000 678,000 892,000) 681,000 ±67,000	75
Sandy Loam	(695,000 584,000 793,000) 697,000 ±30,000	(1,866,000 1,925,000 1,845,000) 1,877,000 ±73,000	169
Rich Loam	(478,000 418,000) 430,000 ±43,000	(817,000 757,000) 787,000 ±36,000	83
Poor Leached Loam	190,000 ±6,000	679,000 ±27,000	257
Sub Sod	215,000 ±6,000	544,000 ±20,000	153

Legend

1. Figures in brackets represent means of individual series of seven plates.

2. Underlined figures represent means for all determinations plus or minus probable error.


er of
nisms
gram
0000
210
200
180
160
140
120
100
80
60
40
20
Shaken in 0.5 molar NaCl + washings
Control Shaken in Water + washings
Mean
6 x Probable Error
Clay
Sandy Loam
Rich Loam
Poor Leached Loam
Sub Sod

DISCUSSION

The bio-physical phenomenon of the adhesion of bacteria to soil particles has been given little consideration in the United States (11). The European workers (8), (12), as well as Peel (11), in this country have placed emphasis upon the attraction of unlike electric charges to explain this phenomenon.

Peel's results indicated that the chloride with which the base-exchange of a soil was saturated modified the amount of adsorption of Azotobacter cells (charged negatively) after the soil was leached with water. His conclusion, however, that the removal of cells by a soil is entirely due to the attraction of unlike charges (negatively charged bacteria and positively charged inorganic colloids) is not in accord with the fact that Na^{+}, Li^{+}, NH_4^{+} and K^{+} showed the greatest amount of liberation while Al^{+++}, Fe^{+++}, and Mn^{++} showed the least because all of these ions are charged positively. Peel offered no explanation as to why NaCl is very effective in the prevention of bacterial adsorption in the soil. He suggests, however, that Mn may be toxic to bacteria.

The explanation which Peel suggests (the attraction of unlike charges) may be an important factor, but the facts also point to another more plausible theory. It is possible that NaCl is effective

in releasing organisms from the soil due to deflocculation of soil particles. That is, bacteria are held mechanically within aggregates of soil particles in flocculated soils and are relased when the particles of the aggregates are dispersed (deflocculation).

If any one of the several cholorides of Na^{+}, Ca^{++}, NH_4^{+}, Mg^{++}, Al^{+++}, etc. be added to a soil in sufficient quantities to saturate the base exchange, the charge on the colloidal particles would be neutralized and the particles would agglutinate due to forces of cohesion. In the flocculating process, bacterial cells would be removed from the soil solution. In the presence of excess Ca^{++}, leaching of the soil with water would remove all soluble salts (mostly chlorides). With the removal of the chlorides, Ca^{++} would remain in the base exchange complex because the sulphates, carbonates, and phosphates of calcium are insoluble, thus the soil would remain flocculated.

Na^{+}, NH_4^{+}, Li^{+} and K^{+} saturated soils, however, would cause deflocculation as soon as the soil was leached, because all of their salts are soluble. Thus these cations would be removed from the base exchange, and the colloidal particles would resume their negative charge, and become dispersed (deflocculation). Any bacteria held within the aggregate of soil particles would then be released. The fact that Peel found that the base predominating in the exchange complex had no decided effect on the number of organisms released

from the soil until the soil was leached indicates that flocculation may play an important part in removing bacteria from the soil solution.

An excess of any cation in the soil would first result in the flocculation of soil particles. As long as Cl ions are present also, leaching would remove most of the cations including Ca , but continued leaching would remove all of the Cl ions. Thus Ca would remain in the base exchange (flocculated soils) because the other salts of Ca^{++}, i.e., $CaSO_4$, $Ca_2(PO_4)_3$, $Ca(NO_3)_2$, are insoluble. Na^{+}, Li^{+}, and NH_4^{+}, however, would be removed from the base exchange (deflocculation soils) even after Cl^{-} ions were removed because their salts are soluble.

The photomicrographs in Peel's paper (11) also indicate that flocculation and deflocculation determine the number of bacteria in the soil solution. These photomicrographs of soils treated with various chlorides with subsequent leachings show few bacteria and large clumps of soil particles per field where Fe^{+++} and Al^{+++} dominated the base exchange. Suspensions from Na^{+} treated soils show essentially the same thing before leaching. After leaching they show very small soil particles and many bacteria per field.

SUMMARY

The bacteria of the soil are found in both the soil solution and the colloidal material which surrounds the individual soil particles; and they are probably retained among flocculated soil particles as well. Many of the organisms found with the colloidal complex are adsorbed due to the attraction of unlike electric charges (11) or other physical chemical phenomena so that it is within reason to assume that these organisms are not liberated with the standard procedure of shaking the soil in distilled water for five minutes in preparation of dilutions for bacterial plate counts. Peel (11) found that the percentage of "adsorption" of a pure culture of bacteria in two widely different New York soils could be varied by saturating the base exchange complex with the chloride of various metals. The least amount of "adsorption" occurred when the soil complex was saturated with NaCl and the soil washed three times to remove the excessive ions. The greatest amount of adsorbed-bacteria was released in the third washing.

The purpose of this work was to determine the effect on the total count of shaking soils in a 0.5 molar solution of NaCl plus several washings. Because a bacterial count of a soil is a relative figure and has little meaning in itself unless compared with results from other soils, the value of liberating microorganisms from the

colloidal complex in making quantitative bacterial analyses of soils would depend upon their being retained in different percentages in different soils. That is, nothing would be gained by liberation of organisms for the bacterial count of soil "x" where the count was increased from one million to two million if the same procedure increased the count on soil "y" from one-half million to one million and soil "z" from three million to six million. (100 percent increase). If, however, the liberation were percentagely different in different soils, a truer comparative picture of bacterial activity in these soils would be obtained by liberating the organisms not counted in the standard procedure.

Two sets of counts were made on several soils in these experiments:

(1) Soil shaken in 0.5 molar solution of NaCl plus four washings.

(2) Soil shaken in distilled water plus four washings (control).

On two soils designated as R and W a second control was used (soil shaken in distilled water without washings). Because the differences on the controls were slightly higher with the washing technique in one case (W soil) and insignificant with the other, only one control (soil shaken in distilled water plus four washings) was used with the other five soils. The results were checked several times on the

majority of the soils used in these experiments. The percentage increase in the total bacterial count due to liberation of organisms by saturating the base exchange with NaCl plus washings (deflocculation) varied from 9 percent to 257 percent.

Conclusions

From the results of this work the following conclusions were drawn:

1. By shaking soils in 0.5 molar NaCl, the total bacterial count is increased slightly in some soils and greatly in others over those shaken in water alone.

2. Total bacterial counts obtained by shaking soils in distilled water (standard method) does not give a true picture of the numbers of bacteria in several soils since it does not take into account the different percentages of retained organisms.

3. Counts obtained from shaking soils in NaCl are more significant than those from the standard procedure because the former include both "adsorbed" organisms, whose percentage varies from soil to soil, and "unadsorbed" organisms.

4. It was suggested that this "adsorption" may be not only true adsorption but the retention of bacteria among flocculated soil particles. This retention among flocculated soil particles may be a very important factor when soil dilutions are made for plate counts.

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