A Technic for the Bacteriological Examination of Soils.

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For nearly half a century investigators have been developing bacteriological technic. Most of the "standard" methods that have resulted from past investigations are adapted to medical rather than to industrial bacteriology. The results obtained by following the average technic are only "qualitatively quantitative" for the methods used are qualitative.

Only of late has extended research been done in the field of soil bacteriology and consequently there is no standard technic for the bacteriological examination of soils. Judging from recent publications soil bacteriologists are adapting medical methods with varying successes and failures. Of late (1) a move has been made to standardize methods for the bacteriological examination of soils. Methods that are accepted as "standard" will have to be founded on the fundamental principles of physics and chemistry. Soil physics is not completely understood and the fundamental chemical changes going on in the soil have not been worked out so advances in bacteriological methods will of necessity have to be related to the development of soil physics and soil chemistry. The following article is submitted with the hope that it may bring out some applications of physical and chemical technic worthy of consideration by other investigators and may help a little in the standardization of methods of technic. These methods have been successfully followed in the Horticultural Research Chemistry and Bacteriology laboratories of the Purdue Agricultural Experiment Station during the past three years. The technic followed, while not entirely original with the authors of this paper, has greatly facilitated the manipulation of sample, media, and apparatus, without in any way impairing the accuracy of the methods used.

That part of the apparatus which differs from that used in most laboratories is described below.

Sampler for Taking Soil Sample.

For sampling the soil, the bacteriologist's soil sampler is used. This sampler is the result of an attempt on the part of the senior author to devise a piece of apparatus that would overcome the inaccuracies that occur through the employment of the usual methods of sampling. The authors have considerable data which show that differences in the aeration of soils affect the bacterial content and are hence stronger advocates than before, for the sampler. We quote as follows from the published article which deals with the bacteriologist's soil sampler.[2] "The sampler is a brass tube 11 inches

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in length and 2 inches in diameter, open at both ends. One end is sharpened to a cutting edge. This cutting edge is so made that the core of soil is cut out and the compaction of soil that is necessary in order to make room for the sampler takes place outside the tube. The cutting end is fitted with a tight fitting 2 inch brass cap. The uncapped end plugged with cotton makes the sampler complete. This sampler embodies at least four of the principles that a good sampler should have: (1) it is easily sterilized and kept sterile;

Figure I—Dilution Bottle and Mixing Spoon.

(2) it is easy to use; (3) it takes and keeps the soil sample free from contamination; (4) it is durable."

For Mixing the Soil Sample.

For mixing the soil when it is taken from the sampler a two quart granite-ware sauce pan and an aluminum spoon, tablespoon size, are used. The aluminum spoon is bent so that it will fit the mouths of the 12 ounce dilution bottles. Figure I.

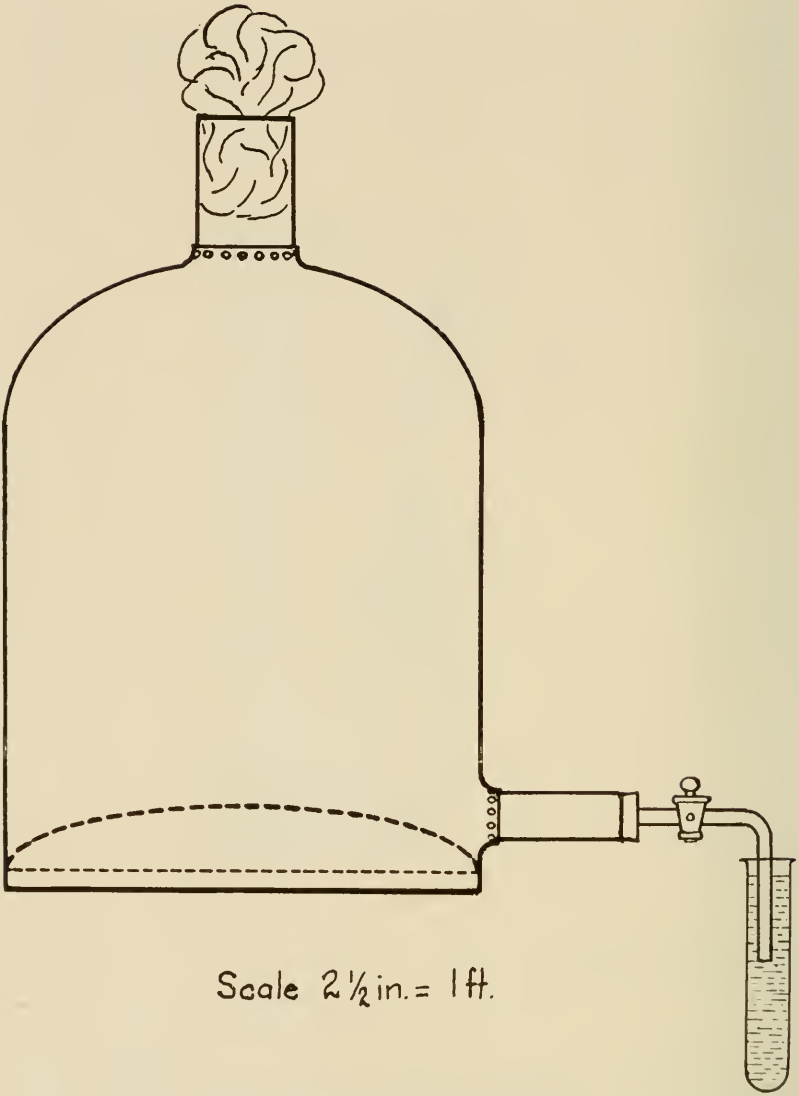

Figure 11—Container for sterile water.

Dilution and Test Bottles.

Whitall-Tatum Co's make, (Figure I—regular shape, saltmouth bottles have been found more satisfactory for making dilutions and soil extracts than erlenmeyer flasks. The sizes used are 8, 12 and 16 ounces respectively. The 8 and 12 ounce sizes being used in making dilutions and the 16 ounce size for extracting nitrates. The advantages of these bottles for making dilutions are that they stand sterilization at 200° C, can be compactly piled into the hot air sterilizer, are not as easily broken as flasks, and can be advantageously washed with a bottle brush. (The bottle breakage with us has been about one tenth what the erlenmeyer flask breakage used to be, and the bottles apparently stand sterilization as well.)

Container for Sterilizing and Keeping Sterile Water.

All water is sterilized by boiling on three successive days in an especially constructed copper boiler, lined with tin. Figure II. The boiler is an 8 gallon copper aspirator bottle having a rather large and long neck, allowing first, a large cotton plug (which permits the water to be boiled without blowing out the plug), second, the transmission of enough heat to thoroughly dry the cotton plug after boiling is over. The outlet tube is closed by a rubber stopper, through which passes a tube fitted with a glass stop-cock. The glass tube on the other side of the stop-cock contains a right angle bend. The stop-cock and outlet tube are sterilized by allowing about a pint of boiling water to run out through them each time the water is boiled, and kept sterile by keeping the end of the tube immersed in a test tube of 70 percent alcohol, or 3 per cent hydrogen peroxide.

For Incubation Tests.

One half pint jell glasses with loose fitting lids are used as containers for soil subjected to incubation tests. The jell glass is preferable to either a beaker or tumbler because it has a lid, and is to be preferred to a beaker because the soil can be removed by inverting the jell glass and dropping it with smart slap, on a hard surface.

Pipettes.

The short form volumetric pipettes, Figure III, are used for making dilutions and in taking aliquots for plating. These can be used as accurately as the regular form and apparently have the following advantages for bacteriological work: (1) are easier to handle and wash; (2) can be readily sterilized in large test tubes, as they pack in well and are not as liable to breakage; (3) are easier to fill; and (4) soil emulsions drain out more quickly and completely from them.

Sterilization by Dry Heat.

For hot air sterilization the "Lautenschläger" ovens are quite satisfactory. The large amount of sterile glassware necessary for soil work and the desirability of handling the glassware by the ovenful had led to the supplement-

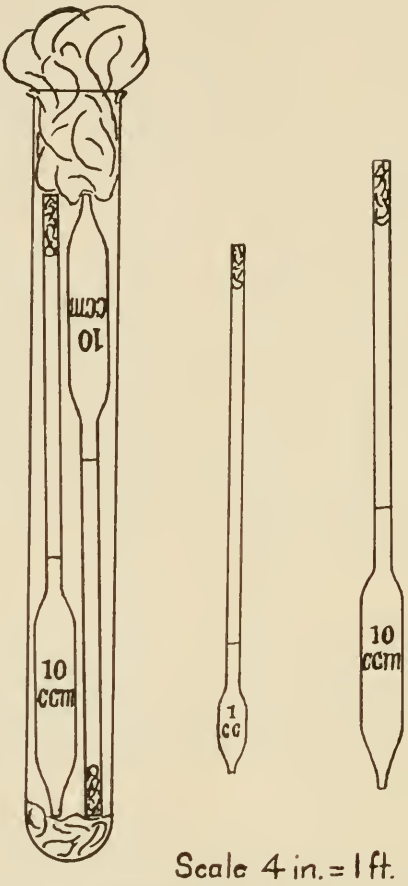

Figure III—Pipettes and pipette case.

ing of the "Lautenschläger" oven by a two burner gas plate and several detachable baking ovens. A square of asbestos is laid over each burner to diffuse the heat, and holes about one insh and a half in diameter are made in the top lining and top of each oven, to allow the moisture formed by the

burning gas to escape. It is desirable to have the holes cut in the oven so that one is not exactly above the other. By using the detachable baking ovens apparatus can be sterilized some days previous to the time the samples are taken and an oven with its contents can be set to one side while another ovenful of glassware is sterilized. This simplifies the handling of petri plates and dilution bottles as the ovens can be carried directly to the work table without handling the apparatus and risking chance contamination. This is very important where a large number of petri plates are used at one time.

Autoclave.

The American Sterilizer Company's autoclave has proven in spite of its high initial cost to be very efficient and economical for within one half hour from the time the gas is lit not only eighteen pounds pressure of steam can be generated but one load of material can be given a ten minute sterilization under eighteen pounds pressure of live steam. The automatic valve which regulates the pressure of the steam in the boiler by shutting off or turning on the gas is a feature appreciated by the investigator.

Water Bath

A water bath, is used to cool the agar media to 45°C after it has been melted in the autoclave. The media is then kept in the bath at 40°-42°C until it is poured into the petri dishes.

Absorbent Cotton

By buying in quantity absorbent cotton is as cheap as the bat cotton at retail prices. It is preferable because the fibre is cleaner and better plugs can be made from it.

Mallets.

The mallet for driving the bacteriologist's soil sampler should be of wood and not weigh over three pounds. The light mallet for use in the laboratory weighs about a half a pound and is made from very light wood.

Petri Dishes

Petri dishes 10 centimeters in diameter and 15 millimeters high have been most satisfactory. The top of the dish should not have a rim deep enough to come down to the table when the dish is placed right side up, on a table.

THE TECHNIC

The technic is given as directions for handling ten samples of soil at one time. In addition to the ordinary apparatus such as burners, autoclave, ovens and so forth being ready for use, the following apparatus must be ready

for use. No list is made here of the chemical apparatus that is necessary for nitrogen, moisture, nitrates or other chemical determinations that are to be run on each sample.

List of Apparatus Needed Before Plating is Finished

10 Bacteriologist's soil samplers capped, plugged and sterilized.
1 Driving mallet.
1 Driving head.
1 Light wooden mallet.
1 2 quart sauce pan.
1 Aluminum spoon (tablespoon size.)
11 12 ounce plugged and sterile salt mouth bottles.*
55 8 ounce plugged and sterile salt mouth bottles.
10 16 ounce salt mouth bottles with rubber stoppers.
30 One half pint jell glasses (sterile.)
1 Set Balances sensitive to 0.1 gram with a capacity of 400 grams per pan.
8 Liters of sterile water.
11 Rubber stoppers to fit 12 ounce bottles.
72 Test tubes of sterile media.
67 Sterile 10 cc. vol. short form pipettes, plugged and in cases.
24 Sterile 1 cc. vol. short form pipettes plugged and in cases.
1 Sterile 5 cc. pipette plugged and in a test tube case.
72 Sterile 100 millimeter petri dishes.
10 One pint Mason jars with rubbers.
1 200 cc. graduated cylinder, plugged and sterilized.
1 100 cc. graduated cylinder, plugged and sterilized.
2 6-inch Battery jars.

The numbers given are exactly what is needed. More apparatus is always prepared to take care of mishaps or mistakes.

Procedure

Take 10 samplers, remove the caps, rub a piece of paraffin over the outside of each just above the cutting edge (this is so the cap will slip on easily after the sample is taken), and replace the caps on the samplers. The cap and the sampler it fits should be stamped with the same number. Plug the samplers with absorbent cotton, sterilize them in hot air oven, cool and stand in a suitcase. Keep the samples upright.

Plug, with absorbent cotton, the 55 eight ounce bottles two hundred cc. graduated cylinder and one hundred cc. graduated cylinder, place them in one of the made over baking ovens and sterilize them. Plug the 11 twelve ounce bottles with absorbent cotton, weigh each to the nearest decigram,

*See note at end of article.

mark the weight on the bottle and sterilize in the same manner as the other bottles.

Weigh each of the 30 jell glasses to the nearest decigram and record the weight on the tin lid. As a rule it is advisable to sterilize the jell glasses with dry heat so as to be sure that they are free from any contaminations resulting from previous use or storage.

Plug the stems of the 10 cc. the 5 cc. and the 1 cc. pipettes with absorbent cotton. Prepare 12 inch by 1 inch test tubes by placing a mat of absorbent cotton in the bottom of each. Place the pipettes in the test tubes, plug the test tubes with cotton and sterilize in a hot air sterilizer. (Two ten cc. or four one cc. pipettes are sterilized in one test tube.)

PLATE I.
Ready to Start.

Media is made up and sterilized and distilled water for making dilutions is sterilized in the special boiler. The casein solution for ammonification, the mannite solution for nitrogen fixation and the ammonium sulphate solution for nitrification are made up and sterilized.

The light wooden mallet, a bunsen burner, the two quart saucepan, the aluminum spoon, the sixteen ounce salt mouth bottles, the weighed bottles and jell glasses, the ten one pint mason jars and the balance are arranged on the laboratory table. (Plate I.) The heavy mallet, the driving head, and the samples are taken to the field and the soil samples taken according to directions given in a previous article.[2] Eleven rubber stoppers of such size that they will fit the twelve ounce bottles are put in the inner part of a double

boiler, covered with distilled water and the double boiler placed over a bunsen burner.

Handling of Soil Sample

When the samples reached the laboratory they are set out in a row on the laboratory table where the apparatus has already been arranged. The bunsen burner is lighted and the saucepan and aluminum spoon are sterilized by passing them through the free flame several times. The pan is set down on the bench and the spoon placed inside of it. A sampler containing a sample of soil is wiped off with a towel to free it from loose soil on the outisde, the light mallet is sterilized in the free flame and placed in the sterile saucepan. The outside of the sampler is sterilized in the flame. The cap is removed from the sampler, and laid to one side The sampler is held, with the left hand, over the saucepan and struck with the mallet, the soil falls out into the pan. (We have found this the most efficient way of removing the soil from the sampler unless the soil is frozen.) The soil is now thoroughly mixed with the aluminum spoon.

Place one of the previously weighed twelve ounce bottles on one pan of the balance and then place 50 grams more than the bottle weighs on the other pan. Remove the cotton plug flame the mouth of the bottle, place the bottle on the balance pan and lay the cotton plug top down in the balance pan beside the bottle. By means of the aluminum spoon quickly introduce fifty grams of soil into the bottle, flame, then replace the cotton plug and set the bottle to one side.

Into each of three of the jell glasses weigh out one hundred grams of the soil, for conducting the physiological tests, and set the jell glasses to one side. Weigh out on a paper 50 gms. of the soil and put it in one of the 16 ounce saltmouth bottles. (This is the aliquot for the determination of nitrates present in the field soil.) Put the remainder of the sample in one of the Mason jars and seal the jar. Clean the graniteware pan and aluminum spoon. Usually all the visable soil is removed by wiping with a piece of absorbent cotton. Each of the other samples of soil is handled in the same way. The heat is started under the double boiler containing the rubber stoppers and preparations are made to melt the media and get it in the 40°C water bath. If proper preparations were made the media can be melted and prepared while the dilutions are being made.

Making Bacterial Dilutions

The sterile graduated cylinders and the tank of sterile water are placed on the laboratory table and sterile water drawn off as follows. Remove the test tube of alcohol that is over the outlet to the tank, allow about a pint of water to run out and discard it. Flame the cotton plug of the 200 cc. graduated cylinder, remove it, flame the mouth of the cylinder, and then by means of the graduated cylinder add 200 cc. of the sterile water to each of the 10 twelve

ounce bottles to which aliquots of soil were added and to the extra bottle that was prepared for a blank check. In each case the cotton plug and lip of the bottle is flamed just before the bottle is opened for the addition of the water. Now bring up the oven containing the fifty-five eight ounce bottles, take the sterile 100 cc. graduated cylinder, flame the cotton plug, remove it, flame the mouth of the cylinder and by means of the graduated cylinder place 90 cc. of sterile water in each bottle. The cotton plug closing each of the bottles is flamed just before the bottle is filled. The eight ounce bottles are arranged in five rows each eleven bottles long, running lengthwise of the bench.

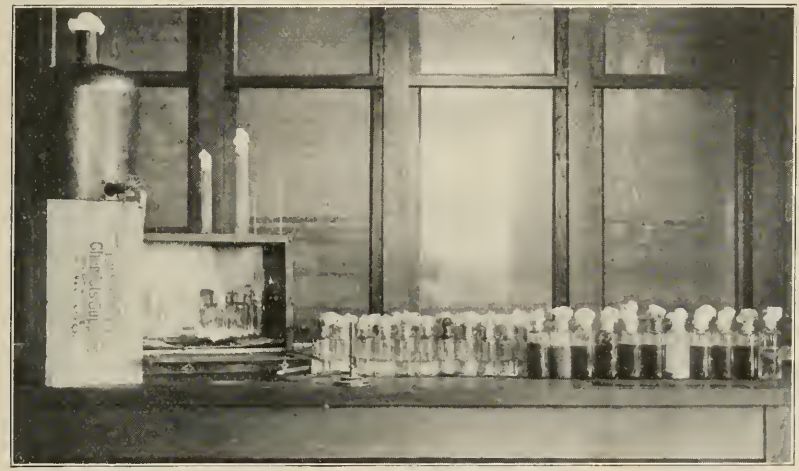

PLATE II.
Adding of sterile water to soils and dilution bottles.

The bottles containing the water and soil are set out in a row in front of the other bottles, (Plate II). The rubber stoppers that have been steaming in the double boiler are put in the bottles containing soil and water in place of the cotton plugs. Work from left to right always as it is easier and such a system prevents mistakes. The first two of the bottles in the front row are grasped, one in each hand, in such a way that the index finger presses down on the stoppers. The bottles are lifted up from the laboratory table, the hands and arms are turned so that the bottles are upside down, and the bottles are shaken vigorously for fifteen seconds and then placed back on the table. The next two bottles are picked up in the same manner, inverted, shaken for fifteen seconds and then placed back on the table. This is continued until each bottle has been shaken 10 fifteen second periods.

The pipettes, in their test tube sterilizing cases, are brought within easy reach. Commencing at the left end of the front row of bottles proceed as follows: Remove, without having the fingers come in contact with the bulb, a 10 cc. pipette from its test tube case, get the soil and water in the first bottle, (which is a 1-4 dilution of the bacteria in the field soil) thoroughly in motion by shaking and while the mixture is still in motion fill the pipette. Pick up the eight ounce dilution bottle which was directly behind the first bottle taken, flame the cotton plug and remove it. While holding the plug in the hand, blow out the 10 cc. mixture of soil and water from the pipette into the 90 cc. of water, replace the cotton plug, and set the bottle back in place.

PLATE III.
Making Bacterial Dilutions.

The pipette is dropped into a battery jar half full of water as it is hard to clean if the soil is allowed to dry on the glass. The mixture just made is a 1-40 dilution of the bacteria in the field soil. Make the 1-40 bacterial dilutions of the other samples in the same way. The bottle containing the 200 cc. of sterile water is treated exactly as though it contained soil, (Plate III.) The bottles from which the dilutions have been made are put on a tray and carried away.

The bottles containing the 1-40 bacterial dilutions are each shaken for ten seconds as vigorously as it is possible to shake them without wetting the cotton plugs. When all have been shaken start over and shake each bottle again. This is repeated until each bottle has been shaken 10 ten second periods. The procedure followed in making the 1-40 bacterial dilutions is

followed in making the 1-400 bacterial dilutions and the bottles containing 1-40 bacterial dilutions are taken away. The 1-400 dilutions of the bacteria in the field soil are shaken, in order, until each bottle has been shaken the 10 ten second periods. Following the same technic we make 1-4,000, 1-40,000 and 1-400,000 bacterial dilutions. The 1-40,000 and 1-400,000 bacterial dilutions are retained for plating while all other dilutions are discarded. The 1-400,000 bacterial dilutions are shaken exactly as though dilution were to be made from them.

PLATE IV.
Ready to pour plates.

PLATING

Thirty-six petri dishes are taken from an oven and spread out on the laboratory table. Care is taken to prevent contamination from the lifting up or sliding of the lids of the dishes. The dishes are arranged in rows of three. All dishes are labeled and numbered, each set of three being numbered to correspond to one of the soil samples. The six dishes in excess of the thirty actually needed for plating one dilution of the bacteria in the ten samples of soil are utilized as follows; three for plating the water that has been run as a check on the technic and three to plate the media alone. This enables the investigator to trace contamination to the water, air, or media, and classify the contamination accordingly. (Plate IV.)

One of the dilution bottles is taken, the cotton plug flamed, a one cc. pipette taken from a test tube case, the bottle shaken, the plug flamed and

removed, the pipette inserted and allowed to fill to the mark. The pipette is withdrawn and the cubic centimeter of solution blown out into one of the petri dishes prepared for plating that sample. Using the same technic two more one cubic centimeter aliquots are taken from the same bottle and put in the other two of the triplicate plates prepared. The bottle and the pipettes are put to one side. A tube of media is taken from the 40°C water bath, the plug removed, the mouth of the tube flamed and the media then poured into one of the petri dishes to which the aliquot of solution has been added. The dish is rotated to thoroughly mix the media and solution and to get an even layer all over the dish. In carrying out the above procedure as much care as possible is taken to prevent the plates from being contaminated from outside sources. The other two of the triplicate plates are then poured. Three plates are then made in the same way from each of the other 1-40,000 bacterial dilutions and of the media. The plates are piled in stacks of three and moved to one end of the laboratory table. The remaining thirty-six petri dishes are taken from the oven, laid out on the table, labeled, and platings made from the 1-400,000 bacterial dilutions. These plates are stacked in piles of three. The piles of plates after the agar has hardened are inverted, placed in trays and the trays are set in the 20°C incubating room. The plates are inverted because after they are poured they are less liable to contamination if inverted, and because the formation of spreaders is hindered.

Where a man works slowly or is working alone the 1-400,000 bacterial dilutions are not made until after the 1-40,000 bacterial dilution have been plated.

The jell glasses containing the one hundred gram quantities of field soil are separated into three groups, one of each of the triplicate glasses from one sample of soil being put in each group. To each glass of one set is added five cc. of ammonium sulphate solution for nitrification tests, to each glass of another set ten cc. of mannite solution for nitrogen fixation tests and to each glass of the third set ten cc. of casein solution for ammonification tests. The jell glasses are then incubated in the 20°C incubation room for the proper lengths of time.

To each of the 16 ounce bottles containing the fifty gram aliquots of soil distilled water is added and the nitrates determined. The samples in the Mason jars are analyzed, as soon as time permits, for moisture, nitrogen, and other elements desired. The moisture is necessary to put results on a dry basis and the nitrogen content is needed to base nitrogen fixation results upon.

DISCUSSION

Sterile Apparatus

The technic calls for the sterilization of samplers, of dilution bottles, of the glasses used for incubation tests and all other apparatus. From reports made recently[3] it would seem to some that sterility of apparatus for agricultural bacteriology has been over-emphasized. Some might maintain that

the samplers need not be sterile if they are clean and that the contamination in the lower dilutions from clean dilution bottles would be negligible.

As long as clean does not mean the same to all workers and just as long as we will admit that unless apparatus is sterile we do not know just how great or of what kind the contamination is—sterile utensils and glassware should be used for the crudest of tests and are absolutely necessary for investigational and research work.

Sampler

The reasons for using the sampler have already been given. The facts (1) that this apparatus samples as accurately under sod as under clean cultivation; (2) that it does not destroy the cultural practices, and (3) that it may be used with as much accuracy and safety near a tree, shrub, or bush, as in the open field are emphasized.

Field Versus Air Dry Samples

If aliquots for bacteriological analysis are taken from air dry samples a discussion of methods and a technic for sieving or grinding the air dry samples would be in place. Air dry samples cannot properly be used to determine the bacteria present in field soils unless it is proven by careful investigation that changes in moisture, in aeration, and in temperature have no effect on the bacterial content of the soil.

Size of Aliquot of Soil

A small sample of soil is not representative. Soils are not composed of equal sized particles or of particles of the same material. Granting that a given soil contained particles all of the same composition and which were non-porous, the variation in the area of the soil particles of two different chance aliquots might be as much as the area of the particles of one of the aliquots. Add the factor of different kinds of particles to that of variation in size and it is safe to say that no two samples of soil are exactly alike. If the size of the aliquot of soil taken for analysis is decreased beyond a certain point, a small proportion of the larger, rock particles must necessarily account for a larger per cent of the weight and cut down the area of the soil particles in the aliquot.

If air dry samples could be used the aliquot required to be representative would be smaller than that required from a field sample. It has not been proven advisable to use air dry samples and so aliquots of the moist field soil are used. Dr. P. E. Brown (4) takes on one hundred gram aliquots of field soil from which to make bacterial dilutions, while Dr. H. Joel Conn (5) uses one-half gram aliquots of field soil from which to make bacterial dilutions.

The authors of this paper have investigated the amount of soil to use to get representative counts and summarize their results as follows:

(1) When tests were made to determine the amount of soil necessary to get reliable checks in moisture determination on a silty clay soil, taking aliquots from the sample jars as they came from the field, it was found that it took ten gram aliquots to have the duplicates check regularly to one tenth of one per cent. The soil rarely contained particles of stone or foreign matter that could be picked out and no attempts were made to weigh out exact amounts so we were forced to conclude that to get accurate moisture results ten grams of field soil had to be used. This was when the soil contained 12% to 15% of moisture. Having made the above test, and knowing that the bacteria which are present in large numbers, are small, and must be distributed in the sample more or less relatively to the internal surface of and the composition of the sample, the authors are forced to conclude that it would take larger aliquots to get good bacteriological results than it would for good moisture results.

(2) Where the ground is covered with plants and their roots are incorporated in the surface soil, it is evident that a larger aliquot must be taken to represent the soil.

(3) When the soil is frozen it is harder to mix the samples and a large aliquot must be taken to overcome inaccuracies in attempts at mixing.

(4) A large quantity of soil must be chosen so that the same sized aliquots of all normal types of soil can be analyzed accurately. This gives a standard.

(5) Fifty grams of field soil were chosen as the quantity from which to make dilutions for the following reasons. Although smaller than the amount used by Dr. Brown it allows the counts made from duplicate and triplicate aliquots from the same field sample to check as well. The results of an extended investigation on size of aliquot of field soil to use will be published as a separate paper.

Dilution

The chemist makes dilutions of various chemical compounds and mixtures of chemical compounds. To the chemist a specified dilution is a mixture such that all aliquots taken from it by weight or by volume will be of the same composition.

Definite amounts of solids are put in solvents and the resulting clear true solutions are exact dilutions of the substances used providing no chemical reaction takes place. Solutions of acids, bases and salts are diluted with water or some other proper solvent and the amounts of materials in the more dilute true solutions bear definite relations by weight and volume to the amounts in the original more concentrated solutions. Definite amounts of a finely divided or ground material are put with definite amounts of another finely divided or ground material and thoroughly mixed. The resulting mixtures are dilutions of both materials because aliquots of mixture have the same composition and the proportions of the original substances contained,

bear definite relations to the concentration of the materials used to make the mixture.

The bacteriologist makes specified dilutions and solutions of media and salts in the same way the chemist does. Further the bacteriologist has to reduce the concentration of the bacteria in all kinds of materials so that an aliquot containing few enough bacteria to be handled with present apparatus and technic can be taken. Water is the usual diluent employed and the concentration of the bacteria has to be reduced whether the substance under examination is a gas, liquid, or solid, whether it is soluble or insoluble, miscible or non-miscible.

When the substance under bacteriological examination is a liquid the report is usually made of number of bacteria per cc when a solid of the number of bacteria per gram. When the materials are such that specified dilutions of them can be made, the bacteriological dilutions are dilutions of both the materials and the bacteria. When the materials are solids or non-miscible the bacteriological dilutions are dilutions of the bacteria only.

Bacteriologically speaking the dilution of bacteria states the number of cubic centimeters of diluent which would contain the number of bacteria in one gram or one cubic centimeter of the original material.

Throughout this paper the phrases dilution of bacteria per gram of field soil and bacterial dilution have been used. Investigators and texts agree fairly well that the bacteria in soil are intimately associated with the moisture in the soil. Explanations of the finding of living bacteria in frozen soil cluster about discussions of whether the films of moisture surrounding the soil particles are really frozen. The bacteria in soil, when dilutions are made, are diluted for they are distributed through a larger volume of water. Dilutions are based wholly on the volume of water added. Fifty grams of soil and 200 cc of water means a 1-4 bacterial dilution of the fifty grams taken, for each gram of soil has four cubic centimeters of water to give up its bacteria to. Subsequent dilutions are based on the volume of the aliquot of the lower dilution taken. The soil that makes up part of the aliquot is considered as water. So little of the field soil is soluble and such a small part of it really gets through to the high bacterial dilutions that the soil in the aliquot is usually ignored.

Basis of Dilutions

The reason that the dilutions are based on 1-4 and not on the 1-2 or 1-10 is that it has been found that on the 1-4 basis the best plates from average soil are secured from either the 1-40,000 or the 1-400,000 bacterial dilutions of the bacteria in the soil. 100 colonies on plates from the 1-40,000 bacterial dilution mean 4 million bacteria per gram of field soil while 100 colonies on plates from the 1-400,000 dilution mean 40 million bacteria per gram of field soil taken. This allows a larger variation in the bacterial content to be handled more satisfactorily with the same number of dilutions and technic than can

be handled on the 1-2, 1-200, 1-2,000, etc., or the 1-10, 1-100, 1-1,000, etc. systems of dilution. Calculations are not hard as the number of colonies divided by 25 or 2.5, according to the dilution, gives millions of bacteria per gram of field soil.

Number of Bacterial Dilutions Necessary

Results obtained where the bacterial content is low should be comparable with results obtained where the bacterial content is high, therefore a uniform system for making dilutions is advisable. Suppose the 1-40 bacterial dilution of a gravel should contain few enough bacteria to yield good plates but that it was necessary to make a 1-40,000 bacterial dilution of a sandy soil in order to get as good plates. A 1-40 dilution of the sandy soil should be made in the series of dilutions to have the results comparable for maximum errors occur in the first bacterial dilution made.

Representative aliquots are necessary if results are to approach accuracy. It takes larger aliquots of some solutions and mixtures to have the aliquots represent the solution or material under investigation than it does of others. Using a pipette graduated to .005 of a cubic centimeter, a .05 cc aliquot of a clear, dilute sodium chloride solution would be representative of the sodium chloride present in the solution. A .05 cc aliquot of a turbid solution would not be representative even if taken with the same pipette, for the suspended material would interfere with the composition of the small aliquot and affect the accuracy with which the pipette could be used. In mixtures of soil and water the soil particles vary in size, in shape, and in specific gravity. An aliquot of a soil and water mixture must be rather large to be representative (at all) of the mixture. A soil and water mixture is not homogeneous for the following reasons:

1. It is not a solution.
2. Soil is heavier than water and the particles settle out, even when the mixture is in motion because of differences in size, in shape and in specific gravity.

Errors that occur in aliquoting a soil and water mixture are:

1. Some of the material in suspension is taken as part of the aliquot when an aliquot is drawn from the moving mixture.
2. Solid material drawn as part of the aliquot clings to the walls of the pipettes, and they do not drain accurately as a result.
3. Bacteria are in, or on, the soil particles besides being in suspension so it is practically certain that not all colonies are broken up by the first shakings.

The errors enumerated above can not be entirely eliminated but are cut down when the size of the aliquot taken is increased. As a rule, in every series of bacterial dilutions there are at least two dilutions from which platings are made. One of these dilutions is ten times another, thus, for uni-

formity and so that results obtained from either higher or lower dilutions of other materials would be more comparable, every higher bacterial dilution should be ten times the one from which it is made. Thus there would naturally be two uniform systems of making dilutions of the bacteria in soil that might reasonably be employed; either 1 cc of the lower bacterial dilution and 9 cc of sterile water to make the next bacterial dilution or 10 cc of the lower bacterial dilution and 90 cc of sterile water to make the next bacterial dilution. It would be necessary in either case to make as many dilutions as ten is a factor of the highest dilution desired.

Many investigators take one cc of the first mixture made up and put it with 99 cc or 199 cc of sterile water, making the resulting bacterial dilution one hundred times, or two hundred times as great at once.

PLATE V.

To determine whether a one cc aliquot of a soil and water mixture would be as representative as a ten cc aliquot the following experiments were carried out. Dry and sieved samples were used as counts were not desired. Two acid soils were chosen, No. 1, an acid black sand and No. 2 a very acid peat. 50 grams of each soil were taken in each case, put with 200 cc of water and shaken for five minutes. A one cc portion of each mixture was taken with a one cc pipette while the mixture was still in motion and put with 99 cc of distilled water and shaken. Ten cc aliquots of the original mixtures were taken in the same manner as the one cc aliquots, put with 90 cc of water and shaken. This gave a 1-40, and 1-400 bacterial dilution of each soil. Ten cc aliquots of the 1-40 bacterial dilutions were put with 90 cc of distilled water giving 1-400 bacterial dilutions. Thus two 1-400 bacterial dilutions of each soil were made, one on the 10 cc basis and one on the 1 cc basis. Using 10 cc

aliquots of each of these bacterial dilutions and 90 cc of distilled water, 1-4,000 bacterial dilutions were made, and, following the same procedure, 1-40,000 bacterial dilutions were made.

When ammonium hydroxide is added to acid soil containing a large amount of organic matter the solution becomes dark colored in proportion as the soil is acid. One cc portions of strong ammonium hydroxide were added to each of the bacterial dilutions made and it was found that the color produced was darker in each case where the 10 cc aliquots had been taken from the original 1-4 bacterial dilutions first made up. The fact that the amounts of material soluble and insoluble in water which would react with ammonium hydroxide were different in high dilutions made when 10 cc aliquots were taken at the start from what they were when 1 cc aliquots

PLATE VI.

were taken at the start, is conclusive evidence that the results of working the two ways are not the same. Figure V shows the results using soil No. 1. The two bottles at the left are the 1-400 bacterial dilutions while those at the right are the 1-4,000 bacterial dilutions. The left hand bottle in each case shows the result of using 10 cc aliquots from the start. When tested in a colorimeter the depth of color of the solution in the left hand bottle of each set was about 1.5 times that in the corresponding bottle where a 1 cc aliquot was taken at the start.

Figure VI shows the results using soil No. 2 which was much more acid and which contained over seven times as much organic matter. Six of the bacterial dilutions made were photographed, the two at the left being the 1-400, the middle two the 1-4,000, and the two at the right the 1-40,000 bacterial dilutions. The left hand bottle of each set is the one where 10 cc

aliquots were used from the start. The colorimeter shows the depth of color in all bottles made from the 1 cc original aliquot to be about 4-5 of the depth where the 10 cc aliquot was taken at the start. Just as small pebbles form a large part of a small aliquot of soil that may be taken for bacteriological analysis and prevent the results from being representative, the variations in the solid material contained in small aliquots of soil and water mixtures cause a large error in high dilution based on these aliquots.

To determine whether the differences in color in the above tests might be due to differences in amount of the original soil carried through as part of the aliquots rather than to representativeness of the aliquots under comparison, the bacterial dilutions of two silty loam soils which happened to be at hand were examined. Ten and one cc aliquots of the 1-4, 1-40, and 1-400 bacterial dilutions were put into tared evaporating dishes, evaporated, dried in the oven at 105°C for two hours, and the residues weighed. The results obtained are given in Table 1.

TABLE 1.

Weight of soil in 10 cc and 1 cc aliquots of soil and water mixtures made up by technic under discussion.

Soil	Bacterial Dilution 1-4		Bacterial Dilution 1-40		Bacterial Dilution 1-400	
	10 cc	1 cc	10 cc	1 cc	10 cc	1 cc
A	1.139	.098	.092	.003	.004	.000
	1.191	.075	.093	.005	.003	.000
Average...	1.161	.087	.092	.004	.004	.000
B	1.185	.113	.097	.006	.003	.000
	1.246	.103	.090	.006	.004	.000
Average....	1.216	.111	.094	.006	.004	.000

The weight of soil taken in 1 cc from the 1-4 bacterial dilution represents the weight that would be in the 1-400 bacterial dilution providing the bacterial dilution was increased 100 times at once.

The weight of soil taken in the 10 cc aliquot from the 1-40 bacterial dilution represents the weight that would be in the 1-400 bacterial dilution providing the bacterial dilution is increased 10 times at the start.

1 cc aliquots would cause .087 and .111 grams of soil, respectively, to be

present in the 1-400 bacterial dilutions. 10 cc aliquots would cause .092 and .094 grams of soil respectively, to be present in the 1-400 bacterial dilutions. The variation in these figures is not 1.5 to 1.0 nor 1 to .8 and thus would not account for the variation in the ammonium hydroxide tests on the bacterial dilutions made from soils No. 1, and No. 2. As a result of these two tests it is thought that 1 cc is too small an aliquot of a water and soil mixture to be taken as representative.

To determine whether there is more uniformity between triplicate ten cc aliquots from the same bottle than there is between triplicate one cc aliquots from the same bottle, the following experiment was undertaken. All aliquots were taken from the 1-4 bacterial dilutions of the soils used. The acid peat and the acid black sand used were the same soils used in a previous test except that they were oven dried and reground in a mill. The red silty clay is a "freak" soil, so fine that it dusts, and contains a large percentage of soluble matter. The one cc aliquots were taken first and then the ten cc aliquots were taken from the same bottle. The mixture of soil and water was always in motion when aliquots were drawn.

TABLE II.

Uniformity of 10 cc and 1 cc aliquots of 1-4 bacterial dilutions of three soils.

Soil and Aliquot	Weight of soil in aliquot			Av.	Range	Range per. cc taken
	1	2	3			
Acid peat	0.127	0.105	0.119	0.117	0.022	0.022
1 cc						
10 cc	1.412	1.565	1.602	1.526	0.190	0.049
Acid black sand						
1 cc	0.065	0.082	0.065	0.071	0.017	0.017
10 cc	0.749	0.797	0.842	0.796	0.093	0.009
Red silty clay						
1 cc	0.141	0.118	0.144	0.134	0.026	0.026
10 cc	1.669	1.684	1.698	1.684	0.029	0.003

The table shows:

1. That the differences between the results obtained with triplicate 1 cc aliquots and triplicate 10 cc aliquots vary as the type of soil.

2. That the soil in the soil and water mixtures is not accurately aliquoted.

3. That variation between one cc aliquots is so great that the increasing concentration of the soil in the mixture (due to 2) does not regularly increase the amount of soil in each succeeding aliquot.

4. That the increased concentration of the soil in the soil and water mixtures increases the amount of soil taken in succeeding 10 cc aliquots.

5. That per cc of aliquot taken the 10 cc aliquots contain nearer constant amounts of a given soil in spite of 4.

The experiments to this point having shown that a ten cc aliquot is more representative of a soil and water mixture than a 1 cc aliquot the following determinations and calculations were made to find out if, in following regular systems of dilution, the soil making up part of a 10 cc aliquot would cause more or less departure from the bacterial dilution desired than the soil making up part of the 1 cc aliquot would.

In addition to using the determinations of the soil in the 10 cc and 1 cc aliquots already given, the results from five more soils were also secured. All determinations used as the basis of calculations are given in Table III. The soil in the first 10 or 1 cc aliquot taken is used as this would be the aliquot used in making bacterial dilutions.

TABLE III.

Weights of soil taken with 1 cc and 10 cc aliquots of 1-4 bacterial dilutions of soils.

Soil.	Gms. soil 10 cc aliquot	Gms. soil 1 cc aliquot	Percentage soil in 1 cc aliquot is of soil in 10 cc aliquot.
*Acid Peat	1.412	.127	8.99
Peat	0.274	.025	9.12
*Acid Black sand	0.749	.065	8.68
Black sand	0.608	.037	6.09
Black sandy loam	0.208	.016	7.69
Sandy loam	0.621	.045	7.25
Silty loam A	1.139	.098	8.60
Silty loam B	1.185	.113	9.54
Yellow tight sand	0.148	.012	8.11
*Red silty clay	1.669	.141	8.45
Average			8.25

Table IV shows the dilutions that actually would be made if uniform systems of dilution were employed. A 1 cc aliquot would be put in practice, with 9 cc of water and a 10 cc aliquot with 90 cc of water to increase the dilution ten times.

TABLE IV.

Actual bacterial dilutions made with 10 cc and 1 cc aliquots of 1-4 bacterial dilutions of ten soils.

Column headings denote bacterial dilutions desired.

*Samples very finely divided and dry.

1 cc aliquot plus 9 cc water to give next dilution versus 10 cc aliquot plus 90 cc water to give next dilution.

Soil and size of Aliquot.	Bacterial Dilution Desired.	
	1-40	1-400
Acidpeat*		
10 cc	43.72	441.1
1 cc	43.33	433.3
Peat		
10 cc	40.68	407.6
1 cc	40.69	406.9
Acid black sand		
10cc	41.08	412.0
1 cc	40.96	409.6
Black sand		
10 cc	40.88	409.6
1 cc	40.56	405.6
Black sandy loam		
10 cc	40.28	403.2
1 cc	40.24	402.4
Sandy loam		
10 cc	40.92	409.2
1 cc	40.68	406.8
Silty loam A		
10 cc	41.72	448.9
1 cc	41.48	414.8
Silty loam B		
10 cc	41.80	419.7
1 cc	41.28	412.8
Tight yelow sand		
10 cc	40.20	402.0
1 cc	40.16	401.6
Red silty clay		
10 cc	42.56	428.2
1 cc	42.12	424.2
Average 10 c	41.38	415.1
Average 1 c	41.15	411.5
Difference	0.23	4.6

*Specific gravity of peat taken as 1.5 all other soils calculated as having a specific gravity of 2.5.

In the above table the volume of soil taken in the aliquot from the 1-40 bacterial dilution is ignored when 1 cc is taken, and used as one tenth the volume taken from the 1-4 bacterial dilution when a 10 cc aliquot is taken.

The tables present evidence that the 10 cc aliquots should be taken for a chance clump of bacteria in the 1 cc aliquot first taken would cause a much greater error than the differences in bacterial dilutions calculated above.

Table V is based on the same data as Table IV. The only difference being that, as is recommended by some, the one cc aliquot is used to increase the bacterial dilution 100 times at the start.

TABLE V.

Actual Bacterial dilutions made by taking 10 cc and 1cc aliquots of 1-4 bacterial dilution.

Soil	1–40	1–400	1–4,000	1–40.000	1-400,000
Acid Peat					
10 cc	43.72	441.135	4,411.35	44,113.5	441,135.
1 cc		436.64	4,366.4	43,664.	436,640.
Difference		4.495	44.95	449.5	4,495.
Peat					
10 cc	40.68	407.6136	4,076.136	40,761.36	407,613.
1 cc		406.72	4,067.2	40,672	406,720.
Difference		.8936	8.936	89.36	893.60
Acid Black Sand					
10 cc	41.08	412.0324	4,120.324	41,203.24	412,032.4
1 cc		410.56	4,105.6	41,056.	410,560
Difference		1.4724	14.724	147.24	1,472.4
Black Sand					
10 cc	40.88	409.6176	4,096.176	40,961.76	409,617.6
1 cc		406.0	4,060.	40,600.	406,000.
Difference		3.6176	36.176	361.76	3,617.6
Black sandy loam					
10 cc	40.28	403.2028	4,032.028	40,320.28	403,202.8
1 cc		402.56	4,025.6	40,256.	402,560.
Difference		.6428	6.428	64.28	642.8
Sandy loam					
10 cc	40.92	410.0184	4,100.184	41,001.84	410,018.4
1 cc		407.24	4,072.4	40,724.	107,240.
Difference		2.7784	27.784	277.84	2,778.4
Silt Loam A					
10 cc	41.72	418.8688	4,188.688	41,886.88	418,868.8
1 cc		416.16	4,161.6	41,616	116,160.
Difference		2.8088	28.088	280.88	2,808.8

TABLE V.—Continued.

Soil	1–40	1–400	1–4,000	1–40,000	1–400,000
Silt Loam B					
10 cc	41.80	419.672	4,196.72	41,967.2	419,672.
1 cc		418.68	4,186.8	41,868.	418,680.
Difference		0.992	9 92	99.2	992.
Tight yellow sand					
10 cc	40.20	402 0	4,020.	40,200.	402,000.
1 cc		401.92	4,019 2	40,192.	401,920
Difference		0 080	0.80	8 0	80.
Red silty clay					
10 cc	42.56	428.154	4,281 54	42,815.4	428,154.
1 cc		423.28	4,232.8	42,328.	423,280.
Difference		4 874	48.74	487.4	4,874.
Av. Diff.		2 265	22 65	226.5	2,265.

This table supports the data given in Table IV. An investigation is under way to determine upon a formula for correcting for the error caused by volume of soil that is taken as part of the aliquot.

The largest error occurs in taking the first aliquot from the 1-4 bacterial dilution first made up. Calculations are given in Table VI of the effect of errors in measuring 10 cc and 1 cc aliquots on 1-40, and 1-400 bacterial dilutions.

TABLE VI.

Effect of errors in measuring on bacterial dilutions.

	Bacterial Dilutions Desired.		
	1–40	1–400	1–4000
Error—minus .01 cc			
10 cc pipette	40 04	400 4	4,004.
1 cc pipette		404 0	4,040.
Error—minus .02 cc			
10 cc pipette	40 07	400 7	4,007.
1 cc pipette		408.1	4,081.
Error—minus .03 cc			
10 cc pipette	40 11	401.1	4,011.
1 cc pipette		412.2	4,122.

TABLE VI—Continued.

	Bacterial Dilutions Desired.		
	1–40	1–400	1–4000
Error—plus .01 cc			
10 cc pipette	39.96	399.6	3,996.
1 cc pipette		396.1	3,961.
Error—plus .02 cc			
10 cc pipette	39.92	399.2	3,992.
1 cc pipette		392.2	3,922.
Error—plus .03 cc			
10 cc pipette	39.88	398.8	3,988.
1 cc pipette		388.5	3,885.

This table shows:

That the errors in measuring which might occur in using pipettes are magnified when made in taking a 1 cc aliquot.

TABLE VI IN COMPARISON TO TABLE V.

(1) Errors of .01 to .02 of a cc that may occur in using a 1 cc pipette cause a larger error in the high bacterial dilutions than the volume of ordinary soil contained in the aliquot does.

(2) That the slightly larger error caused by the volume of ordinary soil in 10 cc aliquots of soil and water mixtures is more than offset by the accuracy with which the ten cc aliquots can be measured.

ADDING WATER TO DILUTION BOTTLES.

The water is not sterilized in the dilution bottles, because:

Water is lost from the dilution bottles if it is sterilized in them, and

The amount lost varies with:

1. The autoclave.
2. The size of the load in the autoclave.
3. The variation in hardness of the cotton plugs in the bottles.
4. The position of the bottle in the autoclave.
5. The surface of liquid exposed and the amount of liquid in the bottle.

The following tests show the results of one sterilization in the autoclave. In putting the water into the eight ounce bottles used in these tests the technic was as follows: Each bottle was weighed to the nearest decigram and 99 gms., or 90 gms., as desired, in excess of the weight of the bottle was placed on the

opposite pan of the balance. 99 cc or 90 cc aliquots of distilled water were measured out by means of a 100 cc graduated cylinder and poured into each bottle. In no case was the amount of water poured in more than .35 of a gram away from that desired. Water was taken out or added so that each bottle contained the weight desired.

The bottles were sterilized for 15 minutes under 18 pounds pressure of live steam and then the pressure was reduced at the rate of one pound per minute; the door being opened 35 minutes after it was first closed.

Test 1.

Two eight ounce salt mouth bottles containing 99 grams of water and two containing 90 grams of water.

All four bottles were plugged with absorbent cotton.

The results of this test are given in Table VII.

Table VII.

Bottle No.	Wt. of H_2O put in.	Wt. after sterilization.	Losses in Weight	% Wt. lost.
1	99 gms.	95 9 gms.	3 1 gms.	3 13
2	99 gms.	95 2 gms.	3 8 gms.	3 84
3	90 gms.	86 5 gms.	3 5 gms.	3 89
4	90 gms.	86.9 gms.	3 1 gms.	3 44

Test 2.

Fourteen 8 ounce salt mouth bottles containing 90 grams of water.

Seven were plugged with absorbent cotton and seven were left unplugged. They were set in the autoclave in sets of two. The two at the rear were numbered 1 and 2, and the two nearest the door 13 and 14. Even numbers denote bottles having no plugs. Experiment conducted as Test 1. Results are given in Table VIII.

TABLE VIII.

Bottle No.	Wt. of H_2O put in.	Wt. of H_2O lost.	% of H_2O lost with plug.	% of H_2O lot without plug.
1	90	2.2	2 44	.....
2	90	6.3	.....	7 00
3	90	3.7	4 11	.....
4	90	8.3	.....	9.22
5	90	3.0	3.33	.....
6	90	8.3	.....	9.22
7	90	2.9	3.22	.....
8	90	3.7	.....	4.11
9	90	3.0	3.33	.....
10	90	8.0	.....	8.69
11	90	2.4	2 67	.....
12	90	7.6	.....	8.44
13	90	2.5	2.78	.....
14	90	5.1	.....	5.67
	Average	4.8	3 13	7.51

All of the bottles were left standing on the laboratory table for 24 hours. They were then weighed again. Those with cotton plugs had lost about 1.5 grams on standing, and those with no plugs had lost more.

If sterilization in the autoclave did not change the volume of water in the dilution bottle the practice should be discontinued in soil work as the bottles would have to stand on the laboratory tables for varying lengths of time.

Water could be more accurately added to the dilution bottles with an automatic pipette than it can be with a graduated cylinder. Automatic pipettes that can be easily sterilized and connected directly to the special tank for sterile water are being investigated, but cannot at this time be recommended to graduated cylinders which can be made absolutely sterile.

SHAKING DILUTION BOTTLES

Stress has been laid on the manner of holding and the manner of shaking the soil and water mixtures. The following make it necessary to emphasize shaking.

1. Variations in manner different individuals shake up materials.
2. Variation in the ease with which colonies of bacteria are disintegrated by shaking.
3. Variation in nature of different soils.

Rather than recommend shaking a certain length of time in a specified make of shaking machine the technic of shaking is given in such a way that any accidental variations introduced by different individuals will have little effect on the results.

In investigations with milk and food products large variations in results have been credited to differences in manner of shaking up the samples and bacterial dilutions. Dr. H. W. Conn,[6] found within the last two years that variations occurring between reports from different collaborators on the same sample of milk might be due to variations in the way in which different workers carried out given directions for shaking. The rubber stoppers are put in the first set of bottles so that they may be shaken more vigorously, as it is here that colonies ought to broken up. The number of times specified and the lengths of time given are considered sufficient to overcome variations in carrying out the technic of shaking the bacterial dilutions.

Plating

A one cc aliquot of the proper bacterial dilution is put directly into the petri plate before the media is added. This is so that all the bacteria in the aliquot may have a possible chance to develop into colonies. Care should be taken to rotate the dish sufficiently to have the bacteria evenly distributed through the media. It has been our experience to have the bacteria in one cc aliquots evenly distributed throughout the media when the bacteria in 0.1 cc aliquots were clumped together. One cc aliquots are advised both to increase the accuracy of aliquoting and to insure more representative aliquots.

Incubator

We do not feel justified in discussing incubators for this laboratory has an inside room over the vaults where the temperature does not vary over four degrees Centigrade in the course of a year.

Reports

Reports are always made of the number of bacteria or the amount of material per gram of dry soil.

Physiological Tests and Media

Three methods of studying the activities of the soil bacteria have been mentioned together with certain solutions for starting these tests. These methods of determining the physiological activities are not necessarily recommended as we have not studied them in great detail or compared them extensively with other methods. They are given to emphasize the advisability of weighing out the aliquots of soil to be used for physiological tests *at the same time the aliquot from which bacterial dilutions are to be made is weighed out.*

The media used in the laboratory has been omitted because the technic is the same whatever the agar media used.

Note. Glass stoppered bottles such as are used in milk laboratories are not used for dilution bottles. They are not even used where rubber stoppers

are later on put in the bottles for silt and clay are found to work up around the stoppers and cause them to stick unduly. (This holds true even when the proper stoppers are kept with the bottles they are made for.)

Summary.

(1) All apparatus used should be sterile.

(2) Fresh samples of soil are used for analysis.

(3) A 50 gram aliquot of fresh soil has been found as satisfactory as a 100 gram aliquot. 50 grams of fresh soil is recommended as the standard amount of soil to take.

(4) Dilutions are made of the bacteria and not of the soil.

(5) Fifty grams of fresh soil and 200 cc of sterile water are used as the basis of all dilutions.

(6) Each higher bacterial dilution should be made by taking 10 cc of the lower bacterial dilution and 90 cc of sterile water.

(7) Water should be added to dilution bottles after the water and bottles have been sterilized.

(8) Bacterial dilutions should be shaken long enough so that variations in carrying out the technic of shaking will be eliminated.

(9) A one cc aliquot of the proper dilution is used for plating. This is added direct to the petri dish.

(10) The procedures followed in preparing the apparatus, in mixing the soil sample, in making the bacterial dilutions, and in plating are given in detail.

References

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[6]Conn, W. H., Reprint 295 from the Public Health Reports, August, 1915.