



**A PRACTICAL MANUAL OF  
SOIL MICROBIOLOGY  
LABORATORY METHODS**

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Section 1

INTRODUCTION

## THE PLACE OF SOIL MICROBIOLOGY IN AGRICULTURE

According to the best estimates, it took from the beginning of time until about the year 1830 A.D. for the human population in the world to reach the 1,000 million mark. It took only 100 years more to multiply to 2,000 million. By the year 2,000, the prediction is that there will be six and a quarter thousand million inhabitants on the earth. These people will have to be fed.

While research may hasten the advent of synthetic food manufacture or the use of new methods such as algae culture, the production of food will be chiefly dependent on the soil and the farmer who tills it. Soil, therefore, is a necessary and increasingly valuable resource of mankind. We can no longer afford to waste its fertility, but must apply all our knowledge to preserve its productivity. Soil, according to a simple definition, is that part of the earth's surface which is changed by climate and vegetation. It can also be defined as a bio-organic-mineral complex of the loose surface layer of the earth's crust. The mineral and organic part of soil are easily seen and familiar to all, if for no other reason than that they are easily seen. The bio-part of soil, except for some larger forms of life, is not visible to the naked eye and thus is relatively unknown.

Soil microbiology is the science that deals with the millions of very small forms of life found in the soil. One ordinary teaspoonful of fertile moist soil contains more living micro-organisms than there are people in the world i.e. more than 2,000 million. Another way of saying this is to state that one hectare/foot (one hectare to a depth of 30 cm) contains between 2 and 10 metric tons of living micro-organisms. This soil population consists mainly of bacteria, actinomycetes, fungi, protozoa and algae. Individually these creatures range in size from the barely visible, to forms that can only be seen with the microscope. Soil micro-organisms are in a constant struggle for existence, not as chance inhabitants of the soil, but as a basic part of it. Without micro-organisms soil would be an inactive geological mass incapable of supporting plant life, and without plant life, animal life would not be possible.

Soil microbes have some amazing characteristics not common to the higher forms of life. First among these is their widespread nature. The majority of forms are found generally all over the earth. In some places they may be inactive because of climatic conditions, but with slight adjustments in temperature, moisture and organic matter content, even the soils of the frozen tundra or the hottest desert quickly contain much microscopic life. Another feature of soil microbes is their diversity. They live and carry on their life processes in temperatures ranging from just above the freezing point to over 60°C. They live in the moisture contained in the soil pores. Severe drying reduces their numbers and activity, but does not completely eliminate them. Many forms have the ability to enter a stage where they are not active during severe conditions. The population may be reduced, but when conditions become favorable the normal numbers are quickly restored. Most forms, especially among the bacteria, have marvellous adaptability; they can adjust themselves relatively quickly and permanently to new conditions.

To understand the importance of soil micro-organisms one must realize that one of their main activities in nature is breaking down complex organic matter into simple chemical compounds. Life is divided roughly into plants and animals, and most soil micro-organisms occupy an intermediate position between these two groups. Plants use simple mineral compounds plus carbon-dioxide from the air and energy from sunlight to manufacture proteins, carbohydrates and fats. Animals use these complex compounds or other animals for food materials. With the exception of that used for food and destroyed otherwise, organic matter eventually is returned to the soil where it is decomposed by soil micro-organisms. Thus, there is an endless cycle of synthesis and destruction, but a dynamic equilibrium is maintained with soil micro-organisms continually breaking down the organic matter built up by living systems.



Another important role of certain soil micro-organisms is their ability to use (or "fix") atmospheric nitrogen. The atmosphere above each acre of soil contains about 35,000 tons, or 70,000 pounds, of free nitrogen. Except for the nitrogen used in making chemical fertilizers and the small amounts fixed by electrical storms, only certain soil micro-organisms and certain algae have the ability to use this potential reserve and put it into a form available to plants. According to a survey made in the United States of America in 1953, 2.1 million metric tons of nitrogen are fixed from the air annually by legume bacteria.

Still another example of a vital part played by soil micro-organisms is the return of carbon-dioxide to the atmosphere. As noted before, chlorophyll-bearing plants using energy from sunlight convert atmospheric carbon-dioxide, water and simple mineral nutrients into complex carbohydrates, proteins and fats. The atmosphere contains 2,199 million kilograms of carbon-dioxide. The vegetation of the whole earth is estimated to require an annual supply of 90,000 million kilograms of CO<sub>2</sub>. Thus, if the CO<sub>2</sub> of the atmosphere were not replenished it would be exhausted in about 30 years. Soil micro-organisms are one of the major agents of replacement. They decompose organic matter into simple mineral compounds, water and carbon-dioxide, releasing the CO<sub>2</sub> to the atmosphere where plants use it again. In general one hectare plow layer of soil of average fertility liberates 15 tons of CO<sub>2</sub> during the growing season in temperate zones. This same volume is required by cultivated plants to produce 50 tons of green vegetable matter. Soil micro-organisms generating CO<sub>2</sub> moreover help to make mineral nutrients available. Carbonic acid formed from water and CO<sub>2</sub> dissolves new supplies of soil minerals.

Depending on local conditions, soil micro-organisms are responsible for increases and decreases in the availability of plant nutrients. Certainly when large amounts of mature cereal straws are returned to the soil, micro-organisms use nitrogen and other nutrients during the initial decomposition process, leaving little or none for plant use. This is a temporary condition but if it happens at a crucial stage of crop growth, the plants will be starved for nitrogen and yields will be reduced.

Soil micro-organisms in the rhizosphere (the soil in intimate contact with plant roots) appear to influence the plant in many ways that are not well understood. It is known that microbiological activity is greater in the rhizosphere than in the adjacent soil. In some species of plants this activity is greater in the diseased plants than healthy plants.

Soil micro-organisms often have important effects on soil structure. They may produce chemical substances which bind soil particles together affecting soil permeability, water holding capacity and tilth as well as retarding wind and water erosion. This effect is closely related to the decomposition of organic matter in soils because the number of micro-organisms in soil is directly dependent on the presence of organic matter. Soils in a good physical condition generally are associated with large numbers of micro-organisms.

The evolution of soil microbiology dates only from the mid-nineteenth century. To some extent it has been the step-child of soil science. Because of the nature of the subject, it is little understood by the soil husbandry-man. Attention to soil microbiology has seldom been planned in a national way; instead development has been the result of the interest and work of a few dedicated scientists. Most of our soil microbiological knowledge has been developed in the temperate zones. Tropical areas have been badly neglected. Many developing countries pay little attention to the subject. However, now that national planning is being applied to the soil resources of most nations, soil microbiology should be included.

The tropical areas, in particular, require the development of soil microbiological studies and practices. In humid tropical areas many of the soil microbiological processes are greatly accelerated. They go on continuously and are not halted by cold winter periods. Therefore, while the principles probably remain the same, the practices developed in temperate zones cannot always be applied to the tropics. The plant material

being returned to the soil differs for example. Most importantly, building up of organic matter is very difficult in the tropics. Instead it is a question of the timing of organic matter applications which needs to be determined.

Algae are capable of fixing large amounts of nitrogen particularly under water-flooded cropping such as rice. Ways must be developed to harness this group of micro-organisms and take practical advantage of this valuable characteristic.

Hundreds of species of legumes flourish in the tropics but little is known of practical ways to use the symbiotic nitrogen fixing bacteria with these plants. Concentrated and prolonged research is required to determine the species of both legume and bacteria that act in symbiosis to fix the maximum amount of nitrogen annually.

These are some of the uses of soil microbiology and problems associated with them. Every person concerned with agricultural crop production should insure that full use is made of the benefits to be gained from proper management of soil micro-organisms. The very basis of a fertile soil depends on these microscopic forms of life, and our food supplies are directly dependent on their activities.

### THE METHODOLOGY AND MATERIALS OF SOIL MICROBIOLOGY

The detection and isolation of soil micro-organisms and the evaluation of their physiological functions in relation to the soil economy constitute the major work of soil microbiologists. To assess the overall microbial activities in the soil, or to determine the density of population of various microbial groups involved in these activities, a number of methods have been developed. Among these are:

1. The direct method - The examination of soil directly, under a microscope by various techniques.
2. The dilution plate method - This is based on the assumption that each living micro-organism, when incubated on a suitable medium, will grow into a colony visible to the unaided eye.
3. The measure of the by-products of microbial activity - Certain end products of microbial growth such as carbon-dioxide, nitrate, or ammonia are indicators of the extent and importance of micro-organisms in a soil system.
4. The measure of microbiological activity by the rate of disappearance of a known compound added to the soil - Use is made of oxygen uptake, decomposition of organic matter or the rate of destruction of some specific compound added to soil.

All methods have certain advantages and disadvantages. Those outlined in the manual represent various experimental approaches to the more important aspects of the subject matter of soil microbiology. The experimental approaches are simple, selected and designed to demonstrate techniques and principles but not advanced research methods. An agricultural scientist or a technician without formal training in the microbiology of soils must be alert to the interesting and important facets of soil science exposed in the methods. He must not be overly impressed with the fact that most of the methods lack precision, and lack reproducibility. The soil itself is exceedingly complex; the dynamic and vastly complicated biological forms that colonize the soil are no less complex. It is a small wonder that the methodology of soil microbiology is imperfect. It is large wonder that organisms so small can have so great an impact on all properties of soil, including that of crop production.

The selection of soils for soil microbiology demonstrations should be approached thoughtfully by the agricultural scientist who plans to apply the methods of this manual. Soils that are of most interest to local problems will probably be most instructive, even though their properties may not be the best to illustrate a particular microbial group or process. When possible, local soils that differ in properties should be included in the soil microbiology experiments. Information that is available on the properties of the soils and their cropping histories contributes to the significance of the experiments.

Composite samples from a soil plot, field, or special site, are more desirable than a single sample. A clean trowel or shovel should be used to collect each composite sample. For most purposes the surface soil is sampled between 0 and 6 inches depth after the uppermost centimeter or so is scraped away. Composite samples are combined in a collection vessel.

The handling and storage of soils for soil microbiology work involves one extremely important precaution. Maintain the soil samples in a moist condition, and never allow the soil to dry completely. If wet soil is collected allow it to dry at ambient temperatures to a moist state so that it may be handled conveniently and will maintain desirable physical features. Fresh soil samples may be sieved by passage through a 2-3 mm mesh screen. Sieved soil samples are best stored in polyethylene plastic bags, tightly closed to avoid drying. Such samples should not be refrigerated, but may be stored at about field temperatures or slightly below. Soils high in fresh organic residues should be used shortly after collection, and not stored.

#### LABORATORY FACILITIES FOR SOIL MICROBIOLOGY

Since soil microbiology is important to agricultural production, it should be a part of every soil research program with separate well-planned quarters if possible, and with the basic equipment that is necessary for reliable accurate work. It should be reasonably free from dust and other sources and contamination.

In planning a soil microbiology laboratory, if resources are limited, it is good economy to start with enough space to permit later expansion and further acquisition of equipment. Since a large amount of the equipment is also common to soil chemistry, these two activities can adjoin each other and share certain facilities. A soil microbiology laboratory should have the following basic areas:

1. A soil preparation room where the moist soil samples are mixed, sieved, and stored for future use or reference. This facility should be separate from the main laboratory, but it should not become dusty.
2. A store room for storing chemicals, glassware and equipment.
3. A main general laboratory work area, including microscope benches and microscope storage facilities.
4. A separate ventilated laboratory for strictly chemical determinations equipped to handle corrosive fumes and solutions.
5. A relatively small airtight room for aseptic work with at least one glass windowed working cabinet equipped with ultraviolet light.
6. Adequate steam, gas or electricity to operate an autoclave, and sterilizing oven.
7. Refrigeration equipment for storage of cultures, media, and biological supplies.

There is no set floor plan for a soil microbiology laboratory but advice from an experienced microbiologist would be helpful in the planning.

Cleanliness is very necessary to do high grade microbiological work, hence the interior should be finished with materials that are smooth and easily cleaned. The floors should be free of cracks and holes. Spaces difficult to reach and clean should be eliminated. The room chosen for aseptic work should be capable of being sealed off with airtight windows and doors. Generally speaking, the strictly microbiological working areas should be separated from the chemical work that is essential to many microbiological determinations.

Eventually, every soil microbiology laboratory should have greenhouse space. A separate greenhouse is ideal but a section of an existing unit is satisfactory provided it can be sealed off. Here again special precautions about cleanliness are essential, especially if pure cultures are involved.

Section 2

THE SOIL POPULATION - ANIMALS AND ALGAE



## METHOD 1

### OCCURRENCE OF ARTHROPODS IN SOIL

The living portion of any soil includes small animals that spend all or part of their existence in the soil. In some soils the numbers of such forms may be exceedingly high and evidence of their presence is readily apparent. This is the case generally in forest soils both temperate and tropic, in soils with rapidly decomposing organic matter, and in soils where arthropod mounds occur. The arthropods - springtails, myriapods, collembola, termites, beetles, mites, ants, flies and many others - affect the soil environment in many ways. The organic matter they take in is partially digested, mixed with the microflora of the gut, and expelled in greatly modified form. Their action in feeding results in channels in the soil, mixing of the soil, formation of aggregates, and physical transport of soil micro-organisms.

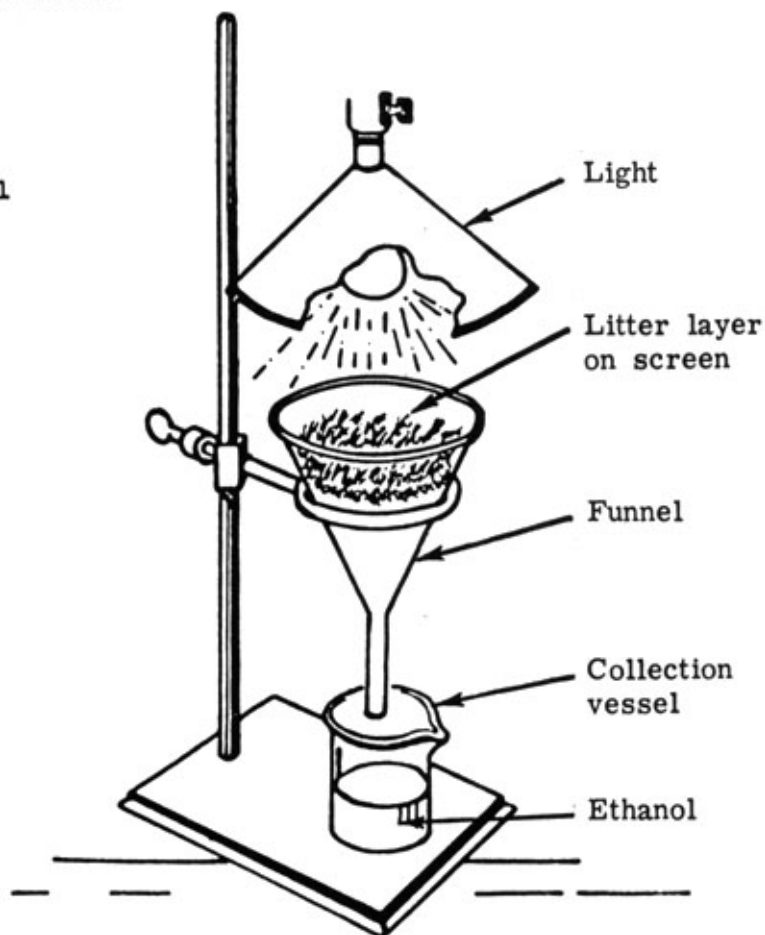
One commonly used method for obtaining substantial numbers of small animals from soil was described by Berlese in 1905. The method and apparatus, essentially as described by Berlese, are both simple and effective.

#### MATERIALS REQUIRED

Litter layer and forest soil with leaves, twigs, and partially decomposed organic matter, freshly collected and not dried.  
Wire screen, 6-8 mesh for funnel.  
Ring stand and ring clamp.  
Funnel, 15 cm diameter, 60 degree angle.  
Lamp, 25-40 watt with reflector shade.  
Ethanol, 70%.  
Beaker, 50-100 ml.

#### PROCEDURE

1. Assemble the Berlese funnel following Fig. 1



2. Place the fresh forest litter on the screen within the funnel in a layer 3-4 cm thick. Partially fill the beaker with 70% ethanol and place it beneath the stem of the funnel to collect the specimens. The stem of the funnel should not touch the alcohol.

3. Position the lamp above the litter layer and turn it on. Observe the heating effect of the lamp carefully for several hours and adjust distance between the lamp and litter as seems necessary. The lamp must not be so close that drying is so rapid as to kill the animals or that the dried material might ignite; nor should the lamp be so far above that the drying is inadequate to drive the specimens downward.

4. Operate the Berlese funnel for a period of 24-30 hours without disturbing the soil. Examine the contents of the collecting vessel after this time, using a watch glass or petri dish and a low power microscope. Sketch the specimens collected and identify with the help of the key in the volume SOIL ZOOLOGY, D.K. Kevan, editor, 1955.

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#### METHOD 2

##### NEMATODES IN SOIL

The round, non-segmented worms known as nematodes are found in soils wherever the pore spaces are large enough, and wherever they find adequate moisture. The numbers and varieties of nematodes are greatest in wet or moist soils of high organic content. Soil forms are generally 0.5 to 2 mm in length, and concentrate in the vicinity of plant roots. Many species are parasitic on plants so that considerable attention has been directed to soil nematodes because of their economic importance. Free living forms are common in the soil too, and these may feed on organic material or on soil bacteria and fungi. The funnel method proposed by Baermann in 1917 is useful to extract nematodes from soil to allow for their further examination (Oostenbrink, 1960).

#### MATERIALS REQUIRED

- Soil samples, moist and freshly collected, sieved but not air dried (organic or rhizosphere soil is best).
- Wire screen about 1 mm mesh.
- Ring clamps and stands.
- Pinch clamps and rubber tubing.
- Cheesecloth.
- Rubber bands or string.
- Beakers, 50 ml.
- Funnels, 15 cm diameter, 60 degree angle.

## PROCEDURE

1. Set up an apparatus as illustrated in Fig. 2 below for each soil to be tested:

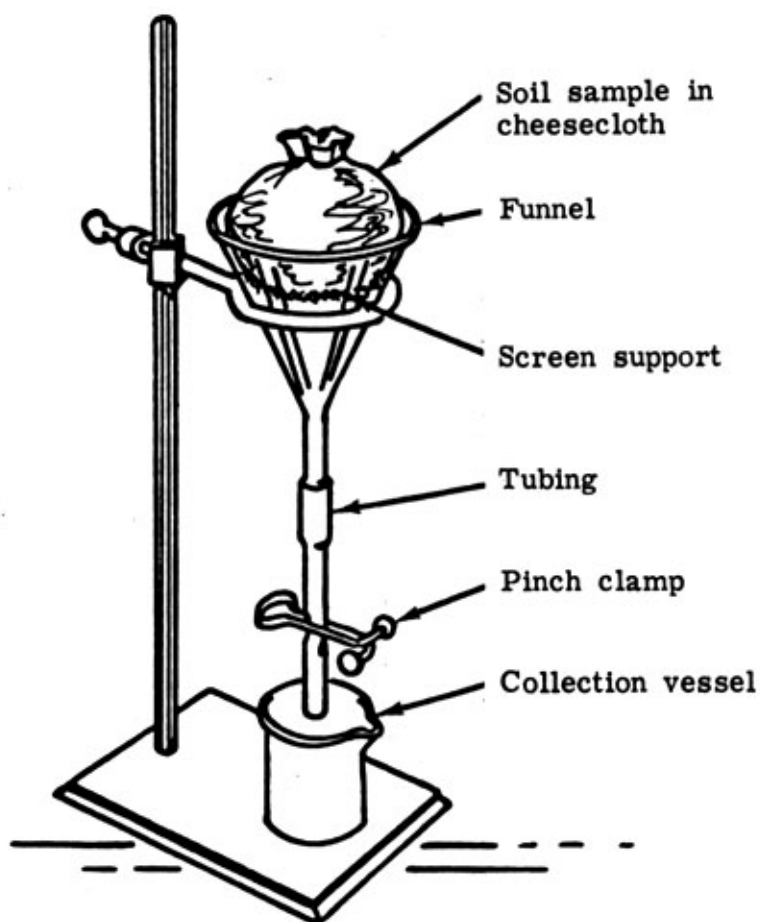


Fig. 2

2. Place 300-500 grams of soil in double thickness of cheesecloth and tie it closed with string or rubber bands.

3. Position the bag of soil on a piece of screen about halfway down the taper of the funnel, with the tied part of the bag on top. Close off the tubing attached to the funnel stem by means of the pinch clamp. Add water to the funnel, lifting the soil as necessary to allow the water to reach a level at which the soil is fully saturated.

4. Hold the moisture level so that the soil remains saturated for a minimum of 24 hours. During this time some nematodes will emerge from the soil and will sink down the funnel to the pinch clamp.

5. Collect worms trapped above the clamp by opening the pinch clamp fully, but momentarily, so as to obtain about 5-10 ml of the water in the collection beaker. Examine the nematodes under low power magnification after transferring the contents of the beaker to a watch glass or petri dish half.

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## METHOD 3

### EXAMINATION OF SOIL PROTOZOA

Many members of the phylum Protozoa inhabit the soil. Their abundance, nature, and activity depend on the extent to which the soil environment provides an adequate moisture phase in the pore spaces, and an adequate supply of food in the form of organic matter and bacteria. A density of hundreds of thousands per gram of soil may be observed under highly favorable conditions, but such conditions usually are short lived, and a large proportion of the protozoa readily form inactive cysts as the soil dries or as the food supply declines. Soil protozoa are grouped as flagellates, ciliates, or pseudopods (amoebae) according to their means of locomotion. Other classes of the Protozoa are not found in terrestrial environments.

### MATERIALS REQUIRED

- Soil samples.
- Nutrient agar, semi-solid, 100 ml/flask in 250 ml flasks. Beef extract, 3 g; peptone, 5 g; agar, 0.5 g; distilled water, 1,000 ml.
- Mannitol-soil extract-asparagine semi-solid agar in flasks as above.
- Mannitol, 5 g;  $K_2HPO_4$ , 1.0 g; asparagine, 0.1 g; agar, 0.5 g; tap water, 500 ml; (soil extract 500 ml, add 1 liter of tapwater to 1 kilogram fertile soil, autoclave 20 minutes, flocculate colloids with 0.5 g  $CaCO_3$  and filter).
- Sterile pipettes, 1.0 ml, three for each soil initially, several additional pipettes, for examination of culture plates after incubation.
- Methyl cellulose, 10 percent solution.
- Aqueous solution of neutral red dye, 1/10,000.

### PROCEDURE

1. Melt the nutrient semi-solid agar and the mannitol-soil extract asparagine semi-solid agar by placing the flasks in a boiling water bath for about 10 minutes. Allow the media to cool to a temperature of about 42°C and pour 4 plates of each medium (total 8) for each soil. Pour about 15 ml of medium into each petri plate.

2. Weigh out 10 grams of moist soil and place this into a 95 ml water blank. The result is a 1/10 dilution of the soil. Shake vigorously for 4-5 minutes, allow the largest particles to settle for a few seconds, withdraw 1.0 ml of the suspension and add this to a 9 ml water blank (now a 1/100 dilution). Before discarding the pipette withdraw 2 additional 1.0 ml aliquots to inoculate the surface of a nutrient agar plate and a mannitol-soil extract-asparagine plate. Label these plates to indicate the medium, soil, and dilution (1/10).

3. Continue the dilution series by shaking the 1/100 dilution well. Transfer a 1.0 ml aliquot to a second 9 ml pipette water blank to yield a 1/1,000 dilution, and then inoculate a plate of each medium with the same pipette and the 1/100 dilution. Label.

4. Similarly prepare the 1/10,000 dilution and the 1/1,000 plates, and finally plate the 1/10,000 tube as the terminal dilution.

5. Incubate the plates right side up in a high humidity box or bell jar.

6. Examine the plates every day or two over a period of about a week. Look at the surface of the plates with a microscope using the low power objective. When there is evidence of some of the larger protozoa on the plates, withdraw portions of the liquid films from the plates with a sterile pipette and place this material on a glass microscope slide to permit closer examination at higher magnifications. Place the liquid inside a small ring of methyl cellulose positioned on the glass slide, and press a cover slip against the top of the ring. Protozoa inside the ring will be slowed down in their movement by the increased viscosity due to the methyl cellulose, and can then be observed more easily through the cover slip. Addition of a drop of neutral red dye to the liquid material in the methyl cellulose may be helpful in the examination of some preparations. The dye penetrates the protozoa and provides some intracellular staining.

7. Note the abundance of various kinds of soil protozoa as a function of soil, medium, dilution, and time of incubation. Make sketches of some characteristic types.

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#### METHOD 4

##### EXAMINATION AND ESTIMATION OF SOIL ALGAE BY THE MOST PROBABLE NUMBER METHOD

Some algae are present in practically all surface soils, but it is difficult to assess their abundance and importance. The greatest number and the greatest variety of soil algae occur at the very surface of moist soils where the photosynthetic activities of the algae are favored. Thus in certain tropical soils where light intensity is high and where agricultural practice requires a high water table, as in rice paddy soils, the algae are exceedingly important. The nutrients assembled in the algae by photosynthesis eventually are released to higher plants when the algae die, and since some algae can fix atmospheric nitrogen as well as atmospheric carbon, their contribution is



of particular significance. These blue-green, nitrogen-fixing algae thus provide a free supply of an otherwise expensive and easily limiting major fertilizer element. In a few other special terrestrial environments algae may play an equally prominent role. In highly eroded and wasted soils the algae may initiate the slow process of soil repair by introducing organic matter by means of their photosynthetic processes. And in semi-arid soils the algae crust may comprise the major source of plant nutrients.

Soil algae exist both in unicellular and in filamentous forms, and some species appear capable of limited non-photosynthetic development in soil not penetrated by light. These features and the lack of adequate attention to the study of soil algae contribute to difficulties in estimating their numbers. Dilution techniques with the soil dilutions cultured in selective liquid media are usually necessary. The selectivity of the media is achieved by the omission of organic carbon so that only photosynthetic forms may develop during incubation in the light. Enumeration is achieved by arranging the culture media to conform to the experimental design of the most "probable number" test.

#### MATERIALS REQUIRED

Fresh soil samples of known or determined moisture content.

90 ml, 95 ml, and 99 ml sterile water blanks.

1.0 ml and 10.0 ml pipettes, sterile.

Medium a - modified Bristol's solution for enumeration of total algae.

30 tubes per soil, 10 ml in each tube:  $\text{NaNO}_3$ , 0.25 g;  $\text{CaCl}_2$ , 0.025 g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.075 g;  $\text{K}_2\text{HPO}_4$ , 0.075 g;  $\text{KH}_2\text{PO}_4$ , 0.018 g;  $\text{NaCl}$ , 0.025 g;  $\text{FeCl}_3$ , 0.005 g; distilled water, 1,000 ml.

Medium b - nitrogen free solution for enumeration of nitrogen-fixing algae; 30 tubes per soil, 10 ml in each tube: modified Bristol's (above) but omit  $\text{NaNO}_3$ .

Test tube racks.

Balance.

Microscope.

Microscope slides and cover glasses.

#### PROCEDURE

1. Prepare soil dilutions as follows:

Add 10 grams of fresh soil to a 95 ml water blank and shake this 1/10 dilution thoroughly for five minutes. Transfer 1.0 ml of the 1/10 dilution to a 99 ml water blank and label this as the 1/1,000 dilution. Transfer 10.0 ml of the shaken 1/1,000 dilution to a 90 ml water blank and label this as the 1/10,000 dilution. Make a final dilution of 1/100,000 by transferring 10.0 ml of the shaken 1/10,000 dilution to a 90 ml water blank.

2. Set up "most probable number" test to estimate the abundance of (1) total algae as cultivatable in the modified Bristol's solutions (medium a), and (2) nitrogen-fixing algae as cultivatable in the nitrogen free solution (medium b). The most probable number test may be set up in various ways. The table of probability values to be used in this application is generally useful for estimations of other kinds of soil micro-organisms as well. It is based on the use of 1.0 ml inoculation volumes of three consecutive ten-fold dilutions, and on 10 replicate inoculations.

3. Use the 1/10, 1/100, and 1/1,000 dilutions to estimate numbers of nitrogen-fixing algae by transferring 1.0 ml samples from each dilution into each of 10 replicate tubes of medium b. There will be a total of 30 culture tubes inoculated. Similarly use the 1/1,000 and 1/10,000 and 1/100,000 dilutions to estimate the numbers of total algae by transferring 1.0 ml samples from each dilution into each of 10 replicate tubes of medium a. For certain soils unusually high in algae, or for those unusually low, it may

be necessary to use dilutions other than those specified above. Whenever the same dilution is to be transferred to more than one culture medium, use the same pipette.

4. Incubate all tubes in a greenhouse or exposed to light on a window ledge for a period of about one month. Make periodic observations during incubation to note the development of green pigmented growth forms.

5. Final observations shall include both an estimate of numbers of algae per gram of soil, and a qualitative examination of the various kinds of algae occurring in the culture tubes. Estimate numbers by means of the probability table for the most probable number procedure.

#### THE MOST PROBABLE NUMBER METHOD

a) Count and record the number of tubes at each dilution that showed evidence of algae growth.

b) Consult the probability table. Note that the column listed as "Code" is made up of 3 numbers. These numbers represent the numbers of positive values (in this case the number of algae-positive tubes) at each of the three successive dilutions. If, for example, the data record 10 algae-positive tubes at the 1/1,000 dilution, 7 at the 1/10,000 dilution, and 1 at the 1/100,000 dilution, the code is then 10-7-1.

c) Find the code corresponding to the experimental results in the table, and look at the "X" value listed opposite this code. The x value represents the most probable number of organisms added at the second dilution as calculated according to probability theory for that particular set of results. For the example in (b) a code of 10-7-1 gives an x value of 1.16. According to this statistical table then, the most probable number of algae present in the 1.0 ml of the 1/10,000 dilution used as the inoculum was 1.16 or:

algae per gram of original moist soil =  $10,000 \times 1.16 = 11,600$ .

d) The final column in the table is headed "P". The value listed in column P reflects the statistical reliability of the set of data (code) obtained. P indicates the number of times the same code might be expected for each 100 times the test was repeated on the same sample, assuming an infinite number of tests.

The x-value of 1.16 corresponds to a P value of 10.01. This states that if this experiment were repeated an infinite number of times on this same sample, the identical result would appear 10.01 percent of the time.

6. Calculate the most probable number of algae for each soil and for each medium based on dry weight of soil. Examine a number of tubes that gave good evidence of algae development. Withdraw a few drops of pigmented solution, transfer to a glass microscope slide, cover with a cover slip and examine under the microscope. Note pigmentation, morphology, and relative abundance of various forms. Make sketches.

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PROBABILITY TABLE

MOST PROBABLE NUMBER METHOD

Code			X	P	Code			X	P	Code			X	P
10	10	10	100.0	100.0	10	10	0	2.40	3.4	10	9	0	1.70	5.30
10	10	9	23.0	38.7	10	9	9	6.07	0.01	10	8	7	3.10	0.01
10	10	8	16.2	30.2	10	9	8	5.26	0.04	10	8	6	2.78	0.14
10	10	7	12.0	26.2	10	9	7	4.58	0.27	10	8	5	2.49	0.56
10	10	6	9.18	25.0	10	9	6	3.98	0.85	10	8	4	2.21	1.64
10	10	5	7.02	24.2	10	9	5	3.46	2.25	10	8	3	1.96	4.50
10	10	4	5.42	23.4	10	9	4	2.98	4.80	10	8	2	1.71	8.46
10	10	3	4.28	21.7	10	9	3	2.63	8.57	10	8	1	1.50	10.83
10	10	2	3.49	17.3	10	9	2	2.28	11.77	10	8	0	1.30	7.21
10	10	1	2.75	10.2	10	9	1	1.97	11.02	10	7	6	2.19	0.01
10	7	5	1.95	0.20	10	6	3	1.25	0.59	10	4	5	1.073	0.01
10	7	4	1.74	0.80	10	6	2	1.09	2.73	10	4	4	0.943	0.07
10	7	3	1.53	2.57	10	6	1	0.933	8.42	10	4	3	0.818	0.48
10	7	2	1.33	6.17	10	6	0	0.792	10.73	10	4	2	0.700	2.26
10	7	1	1.16	10.01	10	5	5	1.30	0.02	10	4	1	0.589	7.97
10	7	0	1.01	9.33	10	5	4	1.15	0.14	10	4	0	0.493	14.99
10	6	6	1.75	0.01	10	5	3	1.02	0.91	10	3	4	0.773	0.02
10	6	5	1.57	0.08	10	5	2	0.872	3.30	10	3	3	0.662	0.24
10	6	4	1.41	0.11	10	5	1	0.742	8.94	10	3	2	0.561	1.60
10	6	3	1.25	0.59	10	5	0	0.622	12.68	10	3	1	0.474	6.93
10	3	0	.399	17.67	10	0	2	.314	0.15	9	5	1	.372	0.86
10	2	4	.631	0.01	10	0	2	.268	1.44	9	5	0	.334	1.78
10	2	3	.534	0.12	10	0	0	.231	7.95	9	4	3	.408	0.02
10	2	2	.456	0.99	9	8	0	.499	0.03	9	4	2	.365	0.22
10	2	1	.388	5.30	9	7	1	.488	0.02	9	4	1	.324	1.13
10	2	0	.329	18.14	9	7	0	.435	0.17	9	4	0	.290	3.58
10	1	3	.442	0.04	9	6	2	.474	0.06	9	3	3	.362	0.04
10	1	2	.376	0.60	9	6	1	.425	0.23	9	3	2	.324	0.26
10	1	1	.317	3.71	9	6	0	.381	0.56	9	3	1	.288	1.77
10	1	0	.275	15.14	9	5	2	.416	0.10	9	3	0	.255	6.23
9	2	3	.316	0.03	8	6	0	.270	0.11	8	2	2	.210	0.14
9	2	2	.284	0.26	8	5	1	.267	0.11	8	2	1	.188	1.24
9	2	1	.253	2.19	8	5	0	.242	0.46	8	2	0	.169	7.12
9	2	0	.223	9.53	8	4	2	.266	0.04	8	1	2	.187	0.11
9	1	2	.249	0.19	8	4	1	.240	0.33	8	1	1	.166	1.46
9	1	1	.221	1.84	8	4	0	.217	1.47	8	1	0	.147	9.31
9	1	0	.193	9.03	8	3	2	.239	0.06	8	0	2	.166	0.06
9	0	2	.217	0.07	8	3	1	.214	0.77	8	0	1	.146	0.88
9	0	1	.191	0.79	8	3	0	.193	3.73	8	0	0	.128	6.41
9	0	0	.164	5.02	8	2	3	.233	0.01	7	6	0	.212	0.02
7	5	1	.209	0.08	7	2	0	.133	5.49	6	4	0	.139	0.32
7	5	0	.191	0.16	7	1	2	.149	0.08	6	3	2	.153	0.01
7	4	2	.208	0.01	7	1	1	.132	1.10	6	3	1	.138	0.20
7	4	1	.188	0.12	7	1	0	.116	8.98	6	3	0	.123	1.35
7	4	0	.171	0.68	7	0	2	.132	0.03	6	2	2	.137	0.03
7	3	2	.188	0.03	7	0	1	.116	0.80	6	2	1	.122	0.45
7	3	1	.169	0.35	7	0	0	.101	7.92	6	2	0	.107	4.12
7	3	0	.152	2.44	6	5	0	.155	0.08	6	1	2	.121	0.06
7	3	0	.167	0.04	6	4	2	.171	0.00	6	1	1	.106	0.87
7	2	1	.150	0.78	6	4	1	.155	0.04	6	1	0	.092	8.73
6	0	2	.106	0.04	5	2	0	.086	3.36	4	3	0	.080	0.43
6	0	2	.092	0.83	5	1	2	.099	0.03	4	2	1	.080	0.16
6	0	0	.078	9.77	5	1	1	.086	0.58	4	2	0	.068	2.16
5	5	0	.126	0.04	5	1	0	.073	8.28	4	1	2	.080	0.01
5	4	1	.127	0.02	5	0	2	.085	0.01	4	1	1	.068	0.50
5	4	0	.114	0.14	5	0	1	.072	0.79	4	1	0	.056	7.21
5	3	1	.113	0.08	5	0	0	.060	12.10	4	0	2	.067	0.02
5	3	0	.100	0.79	4	5	0	.106	0.02	4	0	1	.056	0.76
5	2	2	.113	0.01	4	4	0	.093	0.06	4	0	0	.045	14.62
5	2	1	.099	0.28	4	3	1	.092	0.03	3	4	0	.076	0.04
3	3	2	.086	0.00	3	0	0	.032	18.64	1	2	1	.038	0.02
3	3	1	.075	0.02	2	4	0	.062	0.02	1	2	0	.029	0.53
3	3	0	.064	0.23	2	3	0	.051	0.11	1	1	1	.028	0.12
3	2	1	.064	0.08	2	2	0	.041	3.95	1	1	0	.019	4.76
3	2	0	.053	1.53	2	1	1	.040	0.02	1	0	1	.019	0.34
3	1	2	.064	0.01	2	1	0	.030	6.01	1	0	0	.110	34.58
3	1	1	.053	0.31	2	0	2	.040	0.01	0	2	0	.018	0.21
3	1	0	.043	7.00	2	0	1	.030	0.41	0	1	1	.018	0.04
3	0	2	.052	0.02	2	0	0	.020	24.13	0	1	0	.009	3.05
3	0	1	.042	0.51	1	3	0	.038	0.04	0	0	1	.009	0.26

Section 3

THE SOIL POPULATION - BACTERIA AND FUNGI

## METHOD 5

### QUALITATIVE EXAMINATION OF SOIL MICROFLORA BY THE BURIED SLIDE METHOD

One of the most interesting methods for the qualitative examination of soil was proposed in 1930 by Cholodny. The method is known as the "buried slide" or the "contact slide" technique. It consists simply of allowing a clean microscope slide to incubate in contact with soil. During incubation soil particles adhere to the slide surface and micro-organisms develop on the glass surface. The method can be applied to the examination of soil in the field or in the laboratory. When properly prepared for microscopic examination contact slides will provide a wealth of interesting information, and will disclose some of the natural orientations and arrangements of the micro-organisms, and some of their interrelationships with each other and with soil particles. It is likely that the introduction of the glass slide into the soil and the presence of a continuous glass surface for microbial development may result in a somewhat distorted picture of the microbial environment. While it is difficult to evaluate these limitations, it is clear that the technique has been extremely useful; a particularly helpful guide to the effective use of the contact slide approach is to be found in the well illustrated paper by Starkey (1938).

#### MATERIALS REQUIRED

Soil.

Containers, glass, 5-10 cm diameter x 8-10 high with loosely fitted covers.

Clean glass microscope slides.

Organic amendment for soil treatment (peptone or legume meals, or some other high-nitrogen material).

Spatula or sharp knife to make slit in soil.

Beakers, 100-ml.

Phenolic rose bengal stain - rose bengal, 1.0 g;  $\text{CaCl}_2$ , 0.3 g; add both to 100 ml of 5% aqueous solution of phenol.

Microscope complete with oil immersion lens.

#### PROCEDURE

1. Weigh out 100 grams of soil for each container if soil is to be studied in the laboratory. To some of the containers add 1 percent organic amendment and mix well with the soil. Moisten soil to approximately the moisture equivalent. If soil is to be used in the field choose locations that will remain moist.

2. Make a narrow slit in the soil with a spatula, insert a clean glass slide vertically with about 20-30 mm exposed above the soil. Label stains. Cover soil loosely to prevent drying.

3. Recover slides from soil at convenient intervals after about 1 week of incubation.

4. As each slide is recovered take care to leave one side with attached soil undisturbed. Remove the larger soil particles from the undisturbed face by gentle flooding with water, and clean the other side. Allow the slide to air dry.

5. Place the slides across the top of a beaker of boiling water. Flood the undisturbed face with phenolic rose bengal for 10 minutes. Be careful to add stain as necessary during this time so that the stain does not dry.

6. Wash the slide thoroughly to remove excess stain. After drying examine the slide with the high dry microscope objective in place. Examine further with the oil immersion lens without use of a cover slip.



7. Compare the microscopic appearance of contact slides taken from treated and untreated soils. Detect bacteria and microbial filaments on the basis of morphology and staining. Micro-organisms should stain a deep pink or red color, mineral particles will be unstained, and inert organic matter will appear mostly yellow, unstained, or a light pink.

8. Observe the abundance of microbial cells, characteristic formations, qualitative differences related to soil treatment or incubation time, and the relationships of micro-organisms to each other and to soil particles.

#### REFERENCES AND ADDITIONAL READING

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- Stevenson, I.L. 1956. Antibiotic activity of actinomycetes in soil as demonstrated by direct observation techniques. J. GEN. MICROBIOL. 15:372.

#### METHOD 6

##### ENUMERATION OF SOIL BACTERIA BY DIRECT MICROSCOPIC COUNT

A direct microscopic count of the bacteria in soils by a method similar to one already in use for milk was first proposed by H.J. Conn in 1918. Little interest was aroused until the method was adopted by Winogradsky around 1925, and it was his studies that called attention to the useful aspects of the technique.

The method involves placing a known volume of an infusion of soil in a dilute colloidal fixative and spreading this in an even film over a measured area on a glass microscope slide. After an appropriate staining procedure (with erythrosin or rose bengal) the preparation should show the bacteria colored a deep pink or red, the mineral particles uncolored, and some of the inert organic matter light pink, but mostly either yellow or unstained. Micro-organisms are counted using an oil immersion lens calibrated so that the area of the microscopic field is known. Bacterial counts of soil based on this and similar methods are usually very high as compared to data derived from plate counts. This is because there are many more bacteria in soil than will develop on dilution plates. But the direct count does not give a completely reliable estimate either, even in the hands of an experienced technician. Living bacteria cannot be distinguished from dead, many bacteria are hidden behind the surfaces visible in the microscopic field, and it is often difficult to distinguish bacteria from inert particles.

#### MATERIALS REQUIRED

Soil taken from field sites or soil samples previously treated with about 0.5-1.0 percent organic amendment.  
Pipettes calibrated to deliver 0.01 ml.

Microscope.

Microscope stage micrometer calibrated in 0.1 mm and 0.01 mm divisions.

Microscope slides, thoroughly cleaned (1 for each soil sample).

Fixative solution (sterilized, or freshly prepared non sterile) - 9.5 ml of either 0.04 percent agar, or 0.015 percent gelatin.

Steam bath (beaker will do) with wire support to hold slides over the steam.

Acetic acid, 40 percent solution.

Phenolic rose bengal (Method 5).

PROCEDURE

1. Weigh out 1.0 gram of soil and place in a tube containing 9.5 ml of fixative solution. Repeat for each soil to be examined.
2. Mark off 2 areas, 1 square centimeter each, on the same scrupulously clean face at one end of a microscope slide.
3. Shake soil-fixative tubes well for 4-5 minutes, allow about 1 minute for larger particles to settle, then transfer 0.01 ml of the suspension to a slide by means of a pipette.
4. Spread 0.01 ml of soil dilution evenly over each 1 sq. cm area.
5. Dry the smears over a bath of boiling water.
6. Immerse the slides in 40% acetic acid for about 2 minutes. Wash off excess acid and stain with phenolic rose bengal over a boiling water bath for about 8 minutes. Add stain as necessary to avoid any drying on the slide.
7. Measure the diameter of the microscopic field for the oil immersion objective, using a stage micrometer. Note the number of the microscope so calibrated so that you may return to the same instrument if necessary. Place the micrometer slide on the microscope stage and examine the small area of the slide that has been calibrated in 0.1 mm and 0.01 mm graduations. Then place the oil immersion lens in position and measure the diameter of the field by counting the graduations visible across the field when focused on the stage micrometer scale.
8. Calculate the area of each microscope field, and then determine how many such fields there are in the 1 sq. cm. area. Based on the 1/10 dilution of the soil and the use of 0.01 ml volume of that dilution, a dilution factor of 1,000 results.
9. Count the number of bacteria appearing in each of 10 fields selected at random, for each of the duplicate smears. Record the counts for each field, and determine the average number of bacteria per field. Observe carefully the various types of microorganisms present, their distribution in the microscope field, and any effects of soil treatment.
10. Calculate the number of bacteria per gram of soil for each soil sample studied:
  - (a) Area of field =  $\pi r^2$  - If diameter of field as measured under the oil immersion objective is 0.163 mm, then field radius is 0.0815 mm, and: area =  $3.1416 (0.0815)^2 = 0.0209 \text{ mm}^2$ .
  - (b) Number of fields in 1 sq. cm:  
 $1 \text{ sq. cm} = 100 \text{ mm}^2$ ;  
 $\frac{100}{0.0209} = 4784 \text{ fields in 1 sq. cm.}$

(c) Number of bacteria per gram of soil:

Average count/field  $\times$  field/cm<sup>2</sup>  $\times$  dilution factor = bacteria/gram of moist soil.

#### REFERENCES AND ADDITIONAL READING

- Conn, H.J. 1918. The microscopic study of bacteria and fungi in the soil. N.Y. AGRIC. EXP. STA. TECH. BULL. 64.
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#### METHOD 7

##### DIRECT COUNT OF SOIL MICRO-ORGANISMS BY THE HAEMOCYTOMETER METHOD

The direct microscopic count of bacteria in soil, the technique of H.J. Conn, was described in Method 6. The Conn technique involves spreading a known volume of soil dilution over a known area on a glass slide, staining, and counting the bacteria observed; however soil dilutions are difficult to distribute evenly on the glass slide so that this and other technical difficulties limit the accuracy of the method.

Some of the difficulties of the Conn technique have been overcome in a procedure described by Jones and Mollison in 1948. A soil suspension is made and distributed in molten agar. Small drops are removed and allowed to solidify as thin films on a haemocytometer slide of known depth. The agar matrix solidifies at once and fixes soil constituents in place. The agar films are transferred to a microscope slide, dried, and stained, and the organisms in a number of fields are counted. Since the depth of the agar film is determined by the known depth of the haemocytometer well, and since the area of the microscopic field can be determined for a given microscope and for different lens combinations, the volume of the agar film represented in the microscopic field may be calculated. If the dilution of the soil suspension is known, then the amount of soil represented in that volume of agar film seen under the microscope may be calculated; thus the bacterial count may be related quantitatively to the amount of soil.

#### MATERIALS REQUIRED

Soil sample, passed through a 2 mm sieve, fresh and only partially dried, moisture content determined.

Balance.

Porcelain crucible, 15 ml.

Glass rod, 10 mm length.

Sterile flask, 100 ml.

Haemocytometer slide, 0.1 mm depth preferred.

Cover glasses.

Pipettes, 5 ml.

Microscope slides.

Aniline blue dye (5% aqueous phenol, 15 ml; 1% aqueous aniline blue W.S., 1 ml; glacial acetic acid, 4 ml; filter about 1 hour after preparation).

Alcohol, ethyl, 95%.

Sterile distilled water.

Agar solution, 1.5%; sterilized, filtered while hot, and held in a water bath 42-45C degrees.

Microscope and stage micrometer to measure diameters of microscope fields.

#### PROCEDURE

1. Weigh out between 2.50 and 3.00 grams of soil and correct this weight to the oven dry basis. Place the soil in a small crucible previously flamed and cooled. Add 5 ml of sterile water and mix thoroughly with a sterile glass rod.
  2. Pour the suspension into a dry sterile 100 ml flask allowing the heavier sand particles to remain in the crucible. Add a second 5 ml of sterile water, resuspend the sediment remaining in the crucible then pour the suspended material into the same flask.
  3. Equilibrate the pooled suspension for a few minutes in a water bath at 42-45°C., then make up to 50 ml volume by adding the agar solution also tempered at 42-45°C.
  4. Shake the soil-agar suspension vigorously for 2-3 minutes, allow to stand 5 seconds and then withdraw at once a few drops of suspension with a warm pipette. Transfer the suspension to the recessed well of a haemocytometer slide so as to completely fill the well. Cover the well of the slide with a cover slip, pressing it down gently to displace the excess and insure a uniform depth of agar suspension.
  5. When the agar has solidified thoroughly after about a half hour, the slide is immersed in cold sterile distilled water and the cover slip is removed. The agar film formed on the haemocytometer platform is cut free with a scalpel and floated off by gentle agitation. The film is transferred carefully to an ordinary microscope slide and allowed to dry slowly at room temperature so as to avoid cracking and splitting of the film.
  6. The dried films are then immersed for 1 hour in the aniline blue stain. After washing briefly and rapidly with water, the preparations are dried in the air, and then examined under low, high dry, and oil immersion lens.
  7. Count 20 random fields or each of 4 replicate slides, using oil immersion.
  8. Calculate the number of bacteria per gram of dry soil:  
Count per gram = 
$$\frac{\text{number per field} \times \text{fields per ml} \times \text{ml of soil suspension}}{\text{weight of dry soil in suspension}}$$
- where:
- (a) Number per field = average count based on 20 fields.
  - (b) Fields per milliliter = number field-volumes in each milliliter of suspension, or the reciprocal of the volume represented by 1 microscope field.  
Volume represented by 1 field =  $\pi$  (radius of field in cm)<sup>2</sup>  
(depth of field in cm.).
  - (c) Milliliters of soil suspension = volume to which soil is diluted.  
In this case 50 ml.
  - (d) Weight of soil = number of grams of dry soil placed in suspension.

Example:

If 2.5 grams dry soil are suspended in 50 ml agar, and counts are made with oil immersion lens so that the diameter of the field measures 0.16 mm., and an average count of 12 bacteria per field results, then:

$$12 \left( \frac{1}{\frac{3.14 (0.008)^2 (0.01)}{2.5}} \right) 50 = 440 \text{ millions/gram}$$

REFERENCES AND ADDITIONAL READING

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Skinner, F.A., Jones, P.C.T., and Mollison, J.E. 1952. A comparison of a direct and a plate counting technique for the quantitative estimation of soil micro-organisms, J. GEN. MICROBIOL. 6:261.

METHOD 8

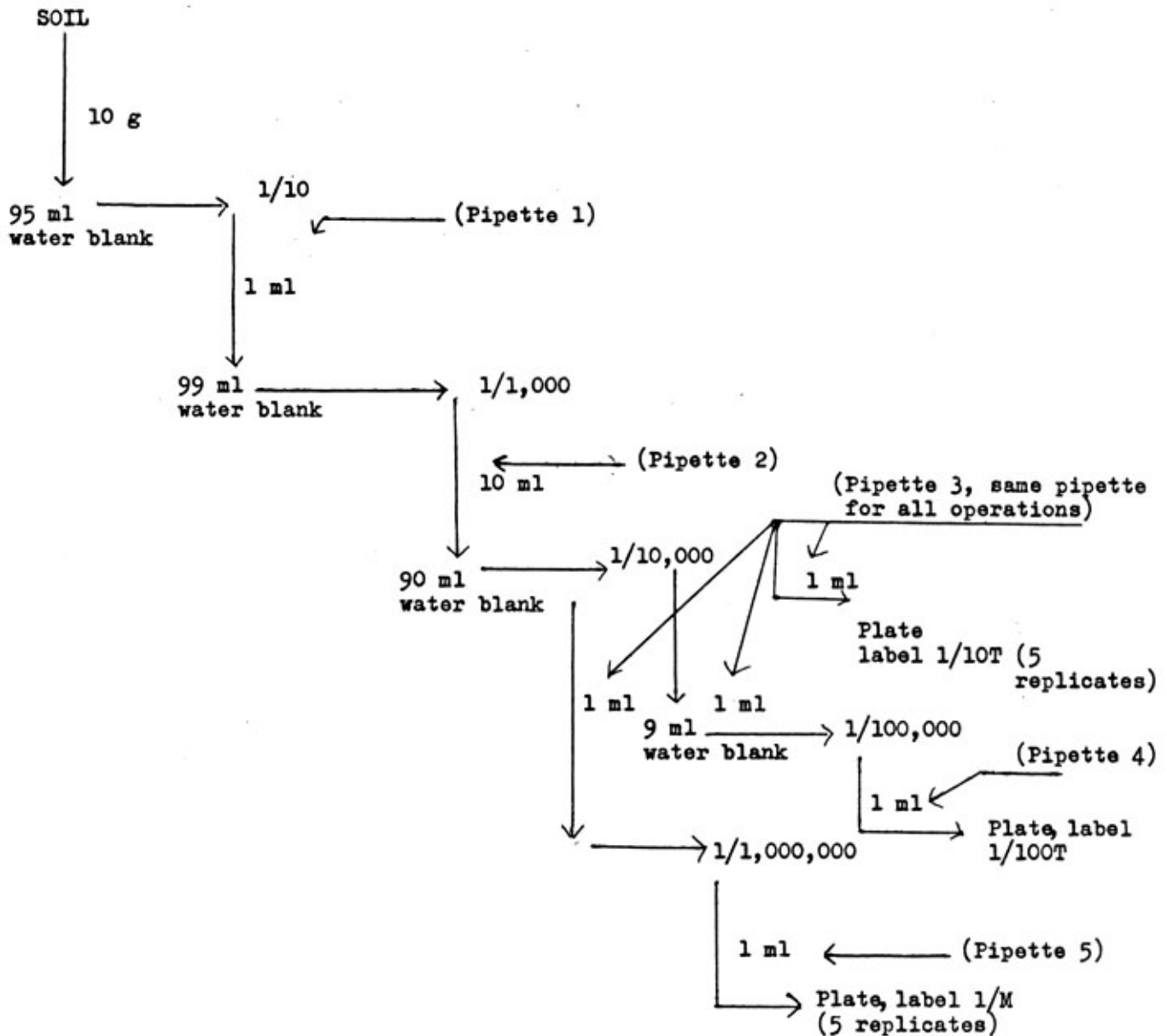
BACTERIA AND ACTINOMYCETES AS ESTIMATED BY THE DILUTION PLATE METHOD

The dilution plate procedure has been used extensively as a means to estimate the numbers of living micro-organisms in soil. The method involves the introduction of a known amount of soil into a known amount of sterile diluent, followed by dispersion to break up clumps of bacteria. Subsequent quantitative serial dilution of this original dilution, together with shaking to attain dispersion at each dilution, eventually gives some concentration of micro-organisms which is convenient to count. Counting is accomplished by transferring a known amount of an appropriate dilution (usually 1 ml) to the bottom of a sterile petri dish; molten and partially cooled agar culture medium is then poured into the dish and is mixed thoroughly with the diluted soil inoculum. After solidification the plate is incubated. Each colony that develops during incubation is considered to arise from a single cell present in the inoculum. By counting the colonies and considering the dilution factor, the number of micro-organisms present in the original soil sample may be calculated easily. In practice, a range of dilutions is plated in order to select that dilution most suitable for counting.

The dilution plate method is most accurate when applied to a pure culture of a bacterium known to be capable of growth on the plating medium used. It is least accurate when applied to the problem of enumerating the diverse microflora of so complex a natural environment as soil. In soil the population is both complex and difficult to disperse; the net result is that only a small fraction of the bacteria present in the soil will appear as colonies on the dilution plates. The selectivity of the method is inherent because no one medium or no group of media will permit growth of all the soil forms. With intentional emphasis on this selectivity, special selective media may be used with dilution plating to encourage the development of certain physiological groups of soil bacteria only. One such selective plating approach is used to estimate the numbers of soil actinomycetes. Actinomycetes comprise a group of filamentous bacteria, and special attention is directed to the actinomycetes because they are a large and important segment of the soil population.



DIAGRAM PLAN OF DILUTION PLATING PROCEDURE



MATERIALS REQUIRED

Soil samples with moisture content determined.  
Balance, with sensitivity to  $\pm 0.05$  mg.  
Sterile water blanks; tap water measured into screw cap bottles or test tube and autoclaved

- 9 ml - 1 for each soil sample
- 95 ml - 1 for each soil sample
- 90 ml - 1 for each soil sample
- 99 ml - 2 for each soil sample.

Sterile pipettes - 4 to deliver 1 ml, 1 to deliver 10 ml for each soil sample.  
Sterile petri dishes - 6 for each dilution plated (5 replicate + 1 uninoculated control).

Soil extract agar (for bacteria) in tubes, about 15 ml per tube, 1 tube for each petri dish: Glucose, 1.0 g;  $K_2HPO_4$ , 0.5 g; agar, 15 g; soil extract (Method 3), 100 ml; distilled or tap water, 900 ml.

Nutrient agar (for bacteria) in test tubes as for soil extract agar (Method 3 but with 1.5 percent agar).

Caseinate agar (for actinomycetes) in test tubes as for soil extract agar: Sodium Caseinate, 0.2 g;  $K_2HPO_4$ , 0.5 g;  $MgSO_4 \cdot 7H_2O$ , 0.2 g;  $FeCl_3$ , 0.01 g; agar, 15 g; distilled water, 1,000 ml; pH adjusted to 6.5 - 7.0.

### PROCEDURE

1. Weigh out 10 grams of soil and suspend this in a 95 ml water blank to obtain a dilution of 1:10 (10 grams of soil has a volume of about 5 cc). Shake suspension vigorously for 5 minutes.

2. Allow the coarse particles to settle (15 seconds) and withdraw 1 ml aseptically with a sterile pipette and discharge it into a dilution bottle containing 99 ml of sterile tap water. This second suspension will contain 0.001 grams of soil, comprising a dilution of 1:1,000 or  $10^{-3}$ . Complete the dilution and plate inoculation steps according to the plan in the dilution plating diagram.

3. To each petri dish add about 15 ml of the appropriate agar medium, melted previously in a bath of boiling water and cooled to 42-45°C. (At this temperature the tube will feel uncomfortably warm when held against the cheek as a rough temperature test, but it can be easily tolerated and will not burn the skin.) Quickly mix medium and inoculum by rotating the dishes with a broad swirling movement of the hand while the plate is in contact with the table top, and while the medium is still liquid. Allow the agar to solidify after mixing.

4. Pour a tube of medium into an uninoculated plate as a check on the sterility of the medium and the technique. Provide one such control plate for each dilution plated.

5. Incubate the plates in the inverted position upside down for 7-10 days at 25-30°C.

6. Examine the plates carefully after incubation. Select plates with 30-300 well distributed colonies for counting. Do not include colonies of filamentous fungi in the count, but do include both surface and sub-surface colonies of bacteria that are so small as to be barely visible.

7. The numbers per gram of oven dry soil are calculated by multiplying the average count per plate by the dilution factor. For example, if plates at the 1/100,000 dilution averaged 150 colonies per plate, and the moisture content of the soil was 12 percent:

$$\begin{aligned} \text{Bacteria/g dry soil} &= \frac{\text{average count} \times \text{dilution}}{\text{dry weight of 1 gram moist soil}} \\ &= \frac{150 \times 10^5}{1.00 - 0.12} \\ &= 17,045,454 \text{ or roughly } 17 \times 10^6 \end{aligned}$$

Record the number of colonies that appeared on all plates that were in the correct range for counting. Compare the number of bacteria per gram of each soil as determined with nutrient agar and with soil extract agar. Compare numbers of bacteria per gram of each soil with numbers of actinomycetes as estimated by plating with caseinate agar.

8. Note the range of size, shape, color, and appearance of bacteria as a function of soil and of plating medium. Observe the difference between bacteria and actinomycetes and note how selective the caseinate medium is in favoring actinomycetes almost exclusively. What are the main features of actinomycetes colonies?

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#### METHOD 9

##### SOIL FUNGI BY DILUTION PLATING

Filamentous fungi of a great many different kinds are to be found as a normal part of the soil microflora. The contribution of fungi to the total microbial activity is probably substantial in most soils, and in certain special environments such as occur in many forest soils, fungi may dominate the microflora. Fungi are usually highly oxidative in their metabolism, highly efficient in cell synthesis, and may immobilize substantial quantities of the nitrogen available in a soil environment.

Numbers of fungi counted by plating methods vary from a few thousand to around 1 million per gram of soil. The conventional plate dilution method favors the development of fast growing, heavily sporulating forms and hence exaggerates the importance of these forms at the expense of slower growing, nonsporulating fungi. Direct examination of soil suggests that most fungi produce relatively few spores in the soil. Some groups of fungi, such as the Basidiomycetes, are almost completely overlooked by dilution plating procedures, due to the nutritional conditions imposed by the media used. Additional difficulties are presented in the interpretation of the counts of fungi obtained in dilution plates, since it is not possible to know if the colonies represent the spores from one parent culture in the soil, or from many. Since bacteria usually are more numerous than fungi, plates meant for the fungi would be overgrown with bacteria unless special selective media are used. In the medium used in this method the dye, rose bengal, and the antibiotic, streptomycin, inhibit bacteria and provide a selective medium for the fungi. Other combinations of dyes and antibiotics may also be suitable.

#### MATERIALS REQUIRED

- Soil samples with moisture content determined.
- Balance with sensitivity to  $\pm 0.05$  mg.
- Sterile water blanks: 95 ml, 99 ml, 9 ml, and 49 ml, one of each for each soil plated.
- Pipettes, 1 ml pipettes (5 for each soil plated).
- Rose bengal - streptomycin agar medium: 100 ml/flask, 3 flasks per soil:  
Glucose, 10 g; peptone, 5 g;  $\text{KH}_2\text{PO}_4$ , 1 g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g; agar, 15 g; rose bengal, 0.033 g; tap water, 1,000 ml. Streptomycin added to melted medium after it has cooled to  $50^\circ\text{C}$ , as filter-sterilized solution to give final concentration of 30 micrograms per ml. Hold complete medium at  $45^\circ\text{C}$ .

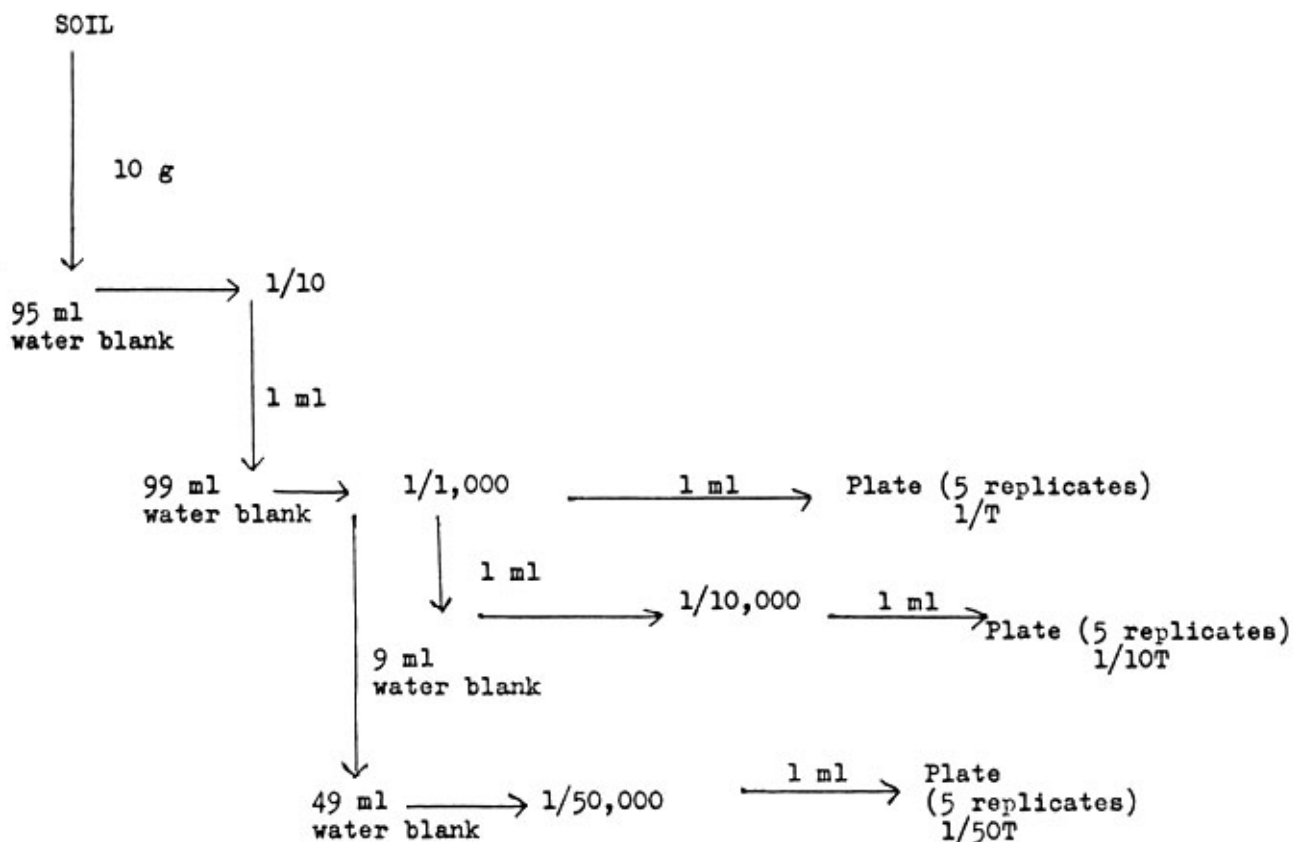
Petri dishes, sterile.

Transparent pressure - sensitive tape.

Lactophenol-cotton blue mounting medium: Add phenol, 20 g; lactic acid, 20 g; and glycerol, 40 g; to 20 ml water. Dissolve with mild heating and add 0.05 g cotton blue.

### PROCEDURE

1. Make dilutions of soil according to the shaking and handling procedure outlined in Method 8. Use the following diagram to guide the preparation of dilutions and the inoculation of plates for the estimation and study of soil fungi:



2. Take a flask of the rose bengal - streptomycin agar medium from the 45°C bath (rose bengal medium was previously autoclaved in the flask and then cooled to 50 degrees, at which point the sterile streptomycin was added and the complete medium was maintained at 45°C). Allow the medium to cool to about 42°C and then quickly pour about 15 ml into each inoculated plate until the flask is emptied. Before continuing with a second flask, mix the medium and inoculum in the plates that have been poured before the agar solidifies. After all plates (including uninoculated controls) have been poured and have solidified incubate at about 28°C for one week. Unused portions of agar that solidify before pouring cannot be melted for later use unless more streptomycin is added to replace that destroyed as the medium is heated to boiling.

3. After a week of incubation observe the plates and enumerate the colonies of fungi present. Observe the diversity of forms that occur and pay particular attention to the efficiency of the medium in restricting colony size of fungi and stopping growth of bacteria and actinomycetes.

4. Choose three well isolated colonies that differ in macroscopic appearance and describe the gross features of each. Examine each colony with the low power objective of the microscope to view the aerial mycelium.

5. Prepare pressure tape mounts on slides for detailed microscopic study. The transparent tape is placed in contact with the aerial mycelium which adheres virtually intact to the adhesive on the tape, and the whole preparation is transferred to a glass slide. Carry out the following procedure:

- (a) Deposit a small drop of mounting medium on the center of a clean slide.
- (b) Cut a piece of the special tape about 1 inch long from the stock roll. Avoid contaminating the adhesive surface with the fingers or dust, by grasping the loose end of the tape with forceps as it is removed from the roll. A dissecting needle will aid in freeing the tape from the forceps.
- (c) The adhesive side of the tape is applied to an area of the fungous colony where sporulation is in evidence, but near the advancing edge of the colony so that reproductive structures are not fully mature. Take care to avoid excessive pressure on the tape as this will result in too dense a mass of fungal tissue.
- (d) Remove the tape from contact with the colony and apply it adhesive side down to the drop of mounting medium on the glass slide. Rub the tape gently with a smooth flat instrument to get rid of air bubbles. If the tape is sufficiently long and the drop of medium not too large, the ends of the tape strip will be free of mounting fluid and will adhere to the side.

6. Examine the slide preparations with the microscope. Prepare adequate sketches to describe the appearance of each colony studied. Attempt to identify the fungi with the aid of identification manuals.

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#### METHOD 10

##### EXAMINATION OF THE RHIZOSPHERE

Micro-organisms are more numerous and more active on the surfaces of roots and in soil immediately adjacent to roots, than elsewhere in the soil. That portion of the soil environment wherein microbial development is influenced by the living root is known as the rhizosphere. The rhizosphere not only has a greater density of population as



compared to soil not affected by the plant root, but the composition of the population differs as well. This selective environment imposed by the plant root is generally considered to be the result of root excretions and sloughed off root tissues providing energy and nutrients for the micro-organisms. The micro-organisms that develop in the rhizosphere must, in turn, have significant effects on the growth of the green plant, but few specific mechanisms of interaction have been studied.

#### MATERIALS REQUIRED

Seedlings with intact root system surrounded by soil.  
Surface soil from same field but not containing roots.  
Knife or large spatula.  
Sterile wide mouth flask containing 95 ml sterile water and sterile glass beads.  
Weighing dishes.  
Sterile pipettes, 1.0 ml or 1.1 ml, and 10 ml.  
Sterile petri plates.  
Sterile water blanks.  
Soil extract agar (Method 8).  
Balance.

#### PROCEDURE

1. A block of soil encompassing the complete root system of a seedling is cut out and placed in a clean container.
2. Gently crush the block of soil to recover the root system in such a way that some soil still adheres to the roots even after gentle shaking. Weigh the flask with glass beads to get a rough tare weight. Cut off the top of the plant at the crown and deposit the roots into the sterile flask with water and glass beads. If the root system is sparse and the amount of soil attached to the roots seems substantially less than about 10 grams as estimated by rough weighing, introduce additional root systems.
3. Shake the flask containing the roots vigorously until soil is washed from the roots. Remove the plant roots and check the weight of the flask plus rhizosphere soil to insure that about 10 grams of soil is present. Suspend the soil uniformly, and quickly remove a 10 ml aliquot to a tared weighing dish which is then dried and weighed to determine the amount of dry rhizosphere soil obtained.
4. Withdraw 1 ml of rhizosphere soil suspension and transfer to 99 ml water blank. Assume the suspension in the flask to be about 1/10, so that this dilution would result in approximately 1/1,000 dilution. Prepare subsequent dilutions, following the procedures of Method 8 so that the dilution of approximately 1/1,000,000 and 1/10,000,000 may be plated. The exact amount of dry rhizosphere soil in each dilution can be calculated when moisture content is determined. Pour 5 replicate plates at each of these two dilutions, using soil extract agar.
5. Weigh out 10 grams of control soil which does not contain plant roots and determine the moisture content. Weigh out a second 10 gram portion of control soil, place in a 95 ml water blank, shake well and from this prepare dilutions of 1/100,000 and 1/1,000,000. Plate the 1/100,000 and 1/1,000,000 dilutions with 5 replicate plates each, using soil extract agar.
6. Incubate all plates for 7-10 days and make colony counts. Record counts on each plate. Determine average counts, and calculate the total number of bacteria per gram of dry rhizosphere soil and control nonrhizosphere surface soil. Compare rhizosphere and nonrhizosphere plates as to diversity of bacteria, based on pigmentation and colony type.

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Section 4

ACTIVITIES OF SOIL MICRO-ORGANISMS

## METHOD 11

### CARBON DIOXIDE EVOLUTION AND PLANT RESIDUE DECOMPOSITION

The quantity and the rate of carbon dioxide evolution from soil has proved to be one of the best methods for measuring the activity of the soil microflora as a whole. The method has found its widest application in the study of organic matter decomposition in soil. It is especially useful in comparative studies, in which the response of a given soil to various treatments is followed, or in which a given treatment is compared for several soils.

Carbon dioxide is a principal product of the metabolism of all the life of the soil. Heterotrophic micro-organisms grow on organic materials in the soil. A part of the carbon that they assimilate from the organic matter is metabolized into their own microbial tissue; a larger and relatively constant, proportion of the carbon assimilated is necessarily oxidized during cell respiration, and released as carbon dioxide. All factors that affect microbial development will, of course, influence carbon dioxide evolution. Thus the amount and the rate of carbon dioxide is not only the generalized expression of the metabolic activities of the many different kinds of micro-organisms, but the generalized expression of the interactions of soil environmental factors as well.

This method is planned to compare changes that may result in the release of carbon dioxide from a soil, when the level of microbial activity is altered by the addition of various plant residues to the soil. More elaborate techniques and equipment are available for more accurate determinations of carbon dioxide evolution; such techniques use a CO<sub>2</sub> free air stream which passes over the soil and into absorption vessels where any respiratory CO<sub>2</sub> picked up from the soil is precipitated as the carbonate.

#### MATERIALS REQUIRED

Soil wetted to the moisture equivalent.  
Legume meal (legume plant material, dried and ground).  
Cereal meal (cereal grain straw, dried and ground).  
Ammonium phosphate, dibasic, powder.  
Flasks; 500 ml wide mouth, Erlenmeyer or extraction.  
Crucibles, or plastic beakers, to fit through neck of flask, and with 25 ml capacity.  
Oxalic acid, N/6 and barium hydroxide N/6 solutions. Weigh out oxalic acid so as to provide several liters of N/6 acid. Make up about a 0.2N solution of barium hydroxide and allow it to stand for several days to allow dissolved carbon dioxide to precipitate as barium carbonate. The normality of the solution will change during this time. Finally withdraw an aliquot and titrate it against N/6 oxalic acid with phenolphthalein as indicator. Calculate the amount of CO<sub>2</sub> - free water that must be added to the barium hydroxide in order to adjust the stock solution to N/6.  
Phenolphthalein indicator.  
Burettes.  
Balance.

#### PROCEDURE

1. Assemble 10 incubation flasks in accordance with Fig. 3.

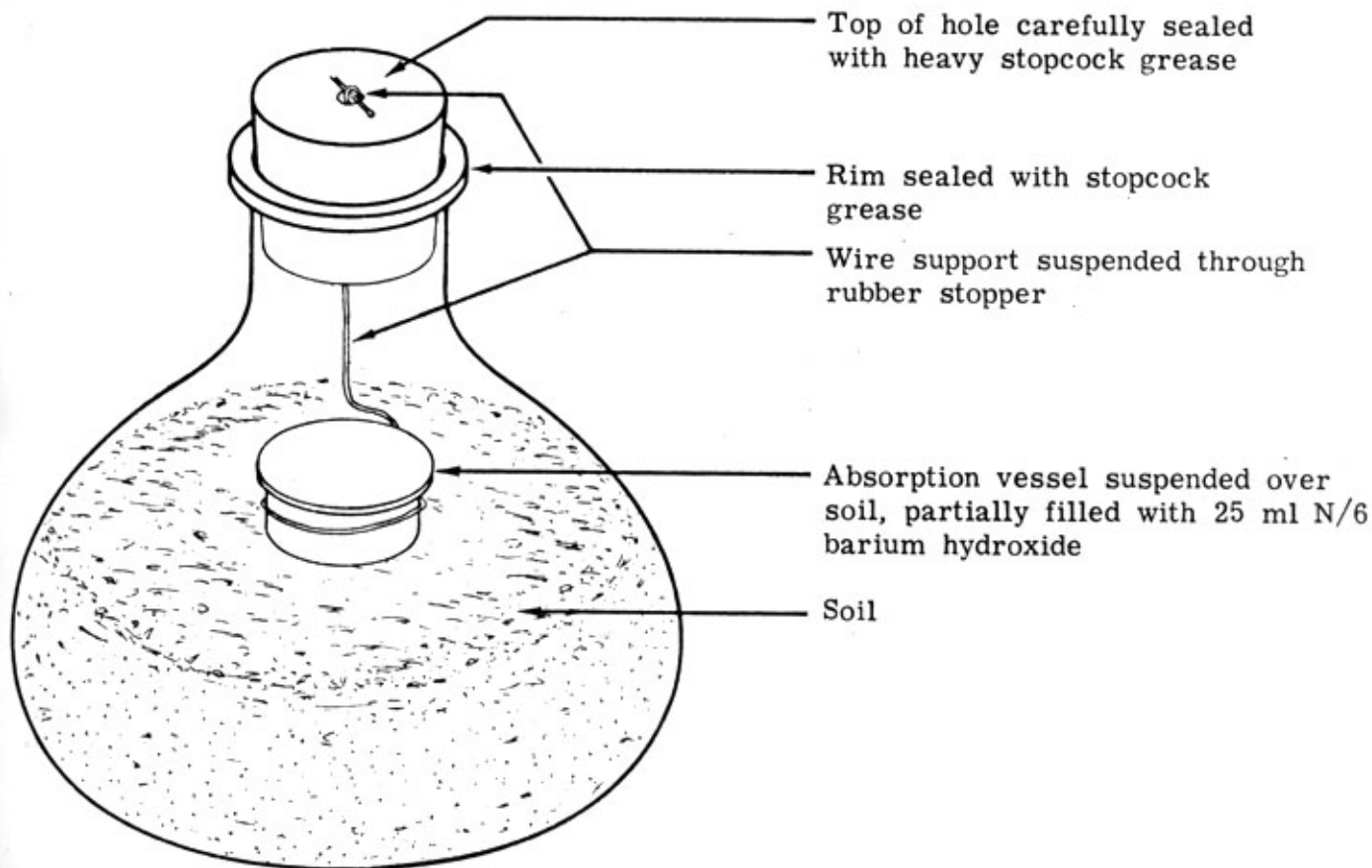


Fig. 3

2. Weigh out 100 grams of soil moistened to the moisture equivalent and transfer to a wide mouth, 500 ml flask. Prepare a total of 8 flasks in this matter. Treat as follows:

- 2 flasks - no treatment (soil control)
- 2 flasks - add 0.5 grams legume meal/flask, mix well
- 2 flasks - add 0.5 grams cereal meal/flask, mix well
- 2 flasks - add 0.5 grams cereal meal and 50 mg dibasic ammonium phosphate/flask, mix well

Weigh and record the weights of soil plus flasks (unstoppered) as a check against drying of the soil during subsequent incubation.

3. Arrange 2 empty flasks as a fifth set, to comprise an atmosphere control.

4. Place 25 ml of N/6 barium hydroxide in the plastic absorption beaker or crucible, place the beaker in the wire collar suspended from the rubber stopper and position it in the flask so that the absorption vessel is suspended just over the soil. The stopper must be fitted tightly to the flask to avoid gas leakage. Insure a tight seal by moistening the lower rim of the stopper before inserting, and then sealing all around the lip of the flask with vaseline or stopcock grease.

5. Assemble all remaining flasks similarly and incubate the 8 soil units and the 2 empty control units together at 28-30°C.

The procedure described in this method is a crude but relatively simple technique to study the diverse cellulolytic microflora found in soil. Even a crude estimate of the abundance of cellulolytic micro-organisms will underline their widespread occurrence in soils. The method uses the most probable number technique, applied earlier (Method 4) for the enumeration of soil algae. The most probable number method is modified only slightly, but in an essential detail, for the estimation of cellulose decomposers. That essential detail involves the use of appropriate media selective for cellulolytic micro-organisms. Cellulose in the form of filter paper strips will provide the necessary selectivity as either the main source of carbon, or the only source of carbon.

#### MATERIALS REQUIRED

Organic soil or forest soil.

Medium (a) - Cellulose as sole carbon source - 50 tubes, 9 ml/tube:

$K_2HPO_4$ , 1.0 g;  $NaNO_3$ , 0.5 g;  $MgSO_4 \cdot 7H_2O$ , 0.3 g; KCL, 0.3 g;

$FeSO_4 \cdot 7H_2O$ , 0.01 g; distilled water, 1,000 ml.

Medium (b) - Cellulose as main carbon source, yeast extract supplement.

50 tubes, 9 ml/tube: Medium (a) plus 1.0 g yeast extract.

99 ml, 95 ml, 90 ml sterile water blanks.

10 ml pipettes, 1.0 ml pipettes, sterile.

100 sterile filter paper strips - 1.0 cm x 10 cm.

Sterile forceps.

#### PROCEDURE

1. Weigh out 10 grams of soil and suspend in a 95 ml sterile water blank to obtain a 1/10 dilution. Plan a dilution procedure analogous to that diagrammed in Method 8, but use only 90 ml and 99 ml water blanks and provide dilutions of 1/10,000, 1/100,000, 1/1,000,000, and 1/10,000,000.

2. Inoculate 10 replicate tubes of medium (a) and 10 replicate tubes of medium (b) each with 1.0 ml of the 1/10,000 dilution. Be sure to shake the dilution frequently during the inoculation process. Repeat for each of the remaining dilutions, so that finally 40 tubes of each medium will be inoculated. The remaining 10 tubes of each medium are retained as uninoculated controls.

3. Place one sterile filter paper strip in each tube by means of a sterile forceps. A portion of the paper strip must extend above the liquid surface.

4. Incubate all tubes at room temperature for 2 to 3 weeks. Without opening the tubes, examine them frequently during incubation to observe macroscopically the development of discoloration on the paper strips.

5. After 14-21 days make final observations. Note carefully the number of paper strips in each dilution series that are positive for growth. Look for evidence of growth on the filter paper strips with the low power objective under the microscope or with a hand lens, paying particular attention to that portion of the filter strip at the liquid-air interface. Determine the most probable number of cellulose decomposing micro-organisms per gram of soil, using the most probable number table of Method 8. Compare the number of cellulolytic micro-organisms as estimated in the medium in which cellulose was the sole source of carbon (medium a) with that as estimated in the medium in which the cellulose was supplemented with yeast extract (medium b). Account for the differences noted.

6. Record the appearance of areas of deterioration on filter paper strips representative of each medium. Make wet mounts in lactophenol cotton blue of fibers taken from both control strips and from zones of degradation on inoculated strips, and examine microscopically.



The procedure described in this method is a crude but relatively simple technique to study the diverse cellulolytic microflora found in soil. Even a crude estimate of the abundance of cellulolytic micro-organisms will underline their widespread occurrence in soils. The method uses the most probable number technique, applied earlier (Method 4) for the enumeration of soil algae. The most probable number method is modified only slightly, but in an essential detail, for the estimation of cellulose decomposers. That essential detail involves the use of appropriate media selective for cellulolytic micro-organisms. Cellulose in the form of filter paper strips will provide the necessary selectivity as either the main source of carbon, or the only source of carbon.

#### MATERIALS REQUIRED

Organic soil or forest soil.

Medium (a) - Cellulose as sole carbon source - 50 tubes, 9 ml/tube:

$K_2HPO_4$ , 1.0 g;  $NaNO_3$ , 0.5 g;  $MgSO_4 \cdot 7H_2O$ , 0.3 g; KCL, 0.3 g;

$FeSO_4 \cdot 7H_2O$ , 0.01 g; distilled water, 1,000 ml.

Medium (b) - Cellulose as main carbon source, yeast extract supplement.

50 tubes, 9 ml/tube: Medium (a) plus 1.0 g yeast extract.

99 ml, 95 ml, 90 ml sterile water blanks.

10 ml pipettes, 1.0 ml pipettes, sterile.

100 sterile filter paper strips - 1.0 cm x 10 cm.

Sterile forceps.

#### PROCEDURE

1. Weigh out 10 grams of soil and suspend in a 95 ml sterile water blank to obtain a 1/10 dilution. Plan a dilution procedure analogous to that diagrammed in Method 8, but use only 90 ml and 99 ml water blanks and provide dilutions of 1/10,000, 1/100,000, 1/1,000,000, and 1/10,000,000.

2. Inoculate 10 replicate tubes of medium (a) and 10 replicate tubes of medium (b) each with 1.0 ml of the 1/10,000 dilution. Be sure to shake the dilution frequently during the inoculation process. Repeat for each of the remaining dilutions, so that finally 40 tubes of each medium will be inoculated. The remaining 10 tubes of each medium are retained as uninoculated controls.

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4. Incubate all tubes at room temperature for 2 to 3 weeks. Without opening the tubes, examine them frequently during incubation to observe macroscopically the development of discoloration on the paper strips.

5. After 14-21 days make final observations. Note carefully the number of paper strips in each dilution series that are positive for growth. Look for evidence of growth on the filter paper strips with the low power objective under the microscope or with a hand lens, paying particular attention to that portion of the filter strip at the liquid-air interface. Determine the most probable number of cellulose decomposing micro-organisms per gram of soil, using the most probable number table of Method 8. Compare the number of cellulolytic micro-organisms as estimated in the medium in which cellulose was the sole source of carbon (medium a) with that as estimated in the medium in which the cellulose was supplemented with yeast extract (medium b). Account for the differences noted.

6. Record the appearance of areas of deterioration on filter paper strips representative of each medium. Make wet mounts in lactophenol cotton blue of fibers taken from both control strips and from zones of degradation on inoculated strips, and examine microscopically.

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### METHOD 13

#### CELLULOSE DECOMPOSITION IN SOIL

Micro-organisms capable of decomposing cellulose in soil are both diverse and widespread but soils differ nevertheless in cellulolytic activity. Cellulose decomposition goes on most rapidly in soils that are well aerated, high in organic matter and inorganic nitrogen, about neutral in pH. At the same optimal conditions of moisture and temperature, acid soils, very heavy soils, or low organic matter soils still decompose cellulose, but at a slower rate. Differences among soils as to the activities of their cellulose decomposing microfloras may be demonstrated in soil burial tests. In this test, cellulose in the form of cotton fabric is buried in a soil, and is incubated under favorable moisture and temperature conditions. The strip is recovered from the soil, and the extent of degradation may be estimated by loss of weight or, if appropriate equipment is available, by loss in tensile strength. The soil burial test is a convenient means of demonstrating that soils differ in cellulose decomposing ability. If it is desirable to observe the microscopic appearance of the micro-organisms that colonize cellulose, the method may be modified to include the burial of cellophane strips (Tribe, 1957). The transparent cellophane strips may be examined microscopically after recovery from soil, and some of the cellulolytic micro-organisms may be seen quite easily.

#### MATERIALS REQUIRED

Soils that differ in properties or that differ with respect to cropping history or treatment; provide about a kilogram of sieved soil that has been collected recently and has not dried. Rectangular pans approximately 5" wide by 4" deep by 10" long, glass or plastic preferred. Other size pans may be substituted easily.

Cellulose strips (cotton "duck", "canvas" or "sailcloth" weighing 8 to 10 ounces per square yard), cut about 1" x 7". Provide 6 such strips for each soil to be tested. Strips should be numbered separately, dried at 70°C for several hours, and cooled in a desiccator.

Pan with distilled water to soak strips.

Pan with sodium nitrate, 100 mg/ml, to soak strips.

#### PROCEDURE

1. Select 6 cotton strips and weigh each on an analytical balance as soon as it is taken from the desiccator. Record the number and weight of each strip.

2. Place 3 of the strips to soak in distilled water, and the remaining 3 to soak in sodium nitrate solution.

3. Weigh about a kilogram of moist soil and put approximately half of the soil into the rectangular burial pan, so that a layer of soil at least 2 inches deep is provided. Smooth the surface of the soil, compact it lightly, and place the distilled water-soaked strips on the soil surface. Cover the strips with the remaining soil to a depth of 1/2"-2". Add sufficient water to the surface of the soil to approximate the moisture equivalent. Prepare a second burial pan in which the strips have been soaked in sodium nitrate solution.

4. Repeat the preceding steps for each soil sample provided. Incubate all soil burial systems for about 10 days in a high moisture chamber or in a high humidity incubator at 25-30°C, at 95-100 percent relative humidity.

5. At the end of the incubation period, remove the strips very carefully from the soil and wash each strip gently. Place the washed strips on a paper towel and dry in the oven at 70°C for 24 hours. Weigh the dried strips and calculate loss in weight for each strip during incubation. Carefully examine each strip for evidence of cellulose decomposition as indicated by pigmentation, holes in the fabric, or thin areas in the fabric. Make sketches of typical areas of deterioration.

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#### METHOD 14

#### SOLUBILIZATION OF PHOSPHATE BY SOIL MICRO-ORGANISMS

Large numbers of phosphate dissolving micro-organisms are present in soil, especially in the vicinity of plant roots. Many different kinds of soil micro-organisms bring more phosphate into solution than is required for their own nutrition. This phosphate-dissolving action, although widespread, is not specific. Inorganic phosphates are dissolved by various by-products of microbial growth, such as carbon dioxide, chelation substances, and especially organic acids. Although micro-organisms that appear capable of placing the most phosphate in solution occur in greatest abundance in the root zone, it is not yet established that phosphate utilization by the plant is benefitted. Competition from rhizosphere micro-organisms may prevent the plant root from assimilating the phosphate solubilized in the root zone (Katznelson, 1965). Considerable attention has been given to some large scale field experiments in which soil was inoculated with a bacterium thought to be especially active in dissolving phosphate - Bacillus megaterium var. phosphaticum. The variable results obtained in these experiments was reviewed by Mishustin and Naumova (1962).

This method merely illustrates that many of the micro-organisms isolated from soil are capable of placing inorganic phosphate into solution. Inorganic phosphate is suspended in an agar medium. Micro-organisms that develop on this medium and dissolve the phosphates during growth, are indicated by a zone of clearing produced around the colony. There is no assurance that the ability to dissolve phosphate in such a culture system means that the same organism is equally active in dissolving phosphate in the soil or in the rhizosphere.

#### MATERIALS REQUIRED

- Sections of fresh plant roots, or fresh, sieved mineral soil.
- Phosphate precipitated medium (Gerretsen, 1948): Carrot infusion, 1,000 ml (steam 1 kg finely chopped carrots in 1 liter of tap water in the autoclave for 2 hours, filter); asparagine, 1 g; glucose, 1.0 g; agar, 20 g. Sterilize.

Sterilize a 10% solution of  $K_2HPO_4$  (solution A) and sterilize separately a 10% solution of  $CaCl_2$  (solution B). Add 0.5 ml solution A and 1.0 ml solution B quickly and aseptically to the molten basal agar medium at 50°C. A homogeneous precipitate of  $CaHPO_4$  forms. Use 15 ml portions immediately in petri plates.

Sterile petri dishes.

Sterile pipettes, 1 ml.

Glucose - peptone - yeast extract slants. Glucose, 1.0 g; yeast extract, 0.5 g; peptone, 0.5 g; agar, 15 g; tap water, 1,000 ml.

#### PROCEDURE

1. Pour plates of the phosphate-precipitated agar and allow plates to dry overnight or for several hours to rid the agar surface of excess moisture.
2. Imbed small sections of freshly harvested rootlets into the agar of several plates, and distribute soil crumbs about the surface of several additional plates.
3. Incubate for 10-14 days at about 25°C and look for the development of colonies characterized by surrounding zones of clearing.
4. Isolate and obtain pure cultures of phosphate-dissolving micro-organisms by streaking out growth from colonies surrounded by clear zones. Streak on plates of the same phosphate-precipitated agar.
5. Examine the growth on streak plates and transfer pure cultures that exhibit phosphate-dissolving activity to slants of glucose - peptone - of each isolate.

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#### METHOD 15

#### SULFATE REDUCING MICRO-ORGANISMS IN SOIL

Microbial reduction of sulfate is brought about exclusively by the activities of certain specific bacteria, referred to as the sulfate-reducing bacteria. These bacteria are widespread in nature; they occur in soil, in water, in sediments, and in sewage. In soil the sulfate reducing bacteria are most active where the water table is high and anaerobic conditions prevail as in paddy soils, bog soils, and heavy clay soils. The specificity of the sulfate reducing bacteria results from their use of sulfate as the final, and generally indispensable, hydrogen acceptor, reducing the sulfate to sulfide. Thus under anaerobic conditions in soil sulfate levels may fall and limit the availability of sulfate to higher plants, especially at soil pH levels above 5.5. Hydrogen sulfide formed by sulfate-reducers may react with iron to form ferrous sulfides to account for the corrosion of iron pipes buried in wet soils. A pipe with walls one-half cm thick may be completely corroded and destroyed in seven or eight years.



The procedure outlined in this method is designed to demonstrate the occurrence of sulfate reducing bacteria in soil, and to estimate their abundance. The technique is adequate to reflect rough differences in numbers of sulfate reducers in different soils. It is useful to demonstrate as well that these bacteria occur in normal arable soils, that they are most active in waterlogged soils, and that sulfides accumulate during their growth. For best results soils from arable fields should be compared with anaerobic soils from bogs or paddy fields. Other comparisons might involve differences due to pH and soil texture.

#### MATERIALS REQUIRED

Aerobic field soil and soil from waterlogged soil or bog.  
Tubes, glass, 1 x 15 cm, sterile and plugged with cotton.  
Medium for sulfate-reducers (Postgate 1963):  $\text{KH}_2\text{PO}_4$ , 0.5 g;  
 $\text{NH}_4\text{Cl}$ , 1.0 g;  $\text{Na}_2\text{SO}_4$ , 1.0 g;  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ , 1.0 g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2.0 g;  
sodium lactate, 3.5 g; yeast extract, 1.0 g; ascorbic acid, 0.1 g;  
thioglycolic acid, 0.1 g;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g; agar, 15 g; distilled  
water, 1,000 ml. Adjust to pH 7.6 with NaOH. Place in 125 ml or 250 ml  
Erlenmeyer flasks, stopper with cotton and autoclave for 15 minutes at  
121°C. (15 lbs pressure). Hold molten agar in water bath at 42-44°C  
until used.  
Sterile water blanks, 95 ml, 99 ml, 9 ml.  
Pipettes, 1 ml, sterile.  
Agar, 1.5 percent.

#### PROCEDURE

1. Prepare 1/10, 1/100, and 1/1,000 dilutions of each soil, and inoculate 1.0 ml of each dilution, in triplicate, into the sterile cotton-plugged, 1 x 15 cm tubes.
2. Add approximately 10 ml of the molten Postgate agar to each inoculated tube and mix the inoculum thoroughly into the agar medium. Allow the inoculated agar medium to solidify and then seal the top with a 1.5 cm layer of agar to prevent access of air to the inoculated portion. Replace the cotton stopper and incubate tubes for 7-14 days at 25-28°C.
3. Identify the sulfate-reducing bacteria by the appearance of black colonies dispersed in the medium. The color is due to the release of hydrogen sulfide which reacts with iron to form the black iron sulfide. Hydrogen sulfide results from the reduction of sulfates by the specific bacteria. Count the number of black colonies and calculate numbers of sulfate-reducing bacteria per gram of dry soil.

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## METHOD 16

### SULFUR OXIDATION IN SOIL

Oxidation of elemental sulfur by biological mechanisms takes place readily in most soil environments. The element, sulfur, may be added to soils in agricultural practices or it may be formed chemically through the ready oxidation of sulfides. Soils are treated with elemental sulfur to relieve deficiencies of that element, or to increase acidity as in the control of potato scab disease. Plant remains sometimes introduce elemental sulfur insofar as they may carry residues of some common fungicidal sulfur sprays. Elemental sulfur and reduced forms of sulfur must be oxidized in the soil to sulfate prior to use as a nutrient by plants.

Activation of elemental sulfur is generally attributed to certain autotrophic bacteria of the genus Thiobacillus, notably T. thiooxidans. The fact that various heterotrophic soil organisms may also oxidize elemental sulfur to sulfate is well established, but the microbiology of the transformation has received little attention. It is likely that heterotrophic forms account for most of the disappearance of sulfur in soils not regularly treated with sulfur, so long as decomposable organic matter is present.

The method outlines below required close attention to the analytical techniques involved in the quantitative estimation of the sulfate sulfur content of the soils studied. Sulfur in a finely divided elemental form is added to the soils and the effects of additions of the specific sulfur oxidizing bacterium, Thiobacillus thiooxidans, and readily decomposable organic matter are observed. If cultures of T. thiooxidans are not available, effects of organic matter alone, or the effects of factors such as temperature, moisture content, or crop history may be studied. In any case, the index of sulfur oxidation is the appearance of sulfate, and successful use of the method depends on mastery of sulfate analyses.

#### MATERIALS REQUIRED

Soils, to provide comparison of an acid soil with a nearly neutral soil.  
Elemental sulfur, precipitated.  
Legume meal.  
Culture of Thiobacillus thiooxidans.  
Erlenmeyer flasks, 300 ml, and 125 ml.  
Balance.  
pH meter.  
Extracting solution - dissolve 39 g. of anhydrous ammonium acetate in 1 liter of 0.25 N acetic acid.  
Sulfate standard solution (100 ppm  $\text{SO}_4^{--}\text{S}$ ) - dissolve 0.7703 g. of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  in 1 liter of extracting solution.  
Activated charcoal (Norit A).  
Barium chloride crystals - ground to pass 20 mesh, but not 60 mesh screen.  
Acacia stabilizer solution, 0.5% - dissolve gum arabic powder in distilled water.  
Filter paper, Whatman 42, volumetric flasks, 50 ml.  
Filter funnels.  
Pipettes, volumetric 5 ml and 10 ml.  
Colorimeter, Klett-Summerson Photoelectric Colorimeter or equivalent.

#### PROCEDURE

1. Place 100 gram samples of soil into each of 4 Erlenmeyer flasks and treat as follows:

1. Untreated control nothing added
2. Add 0.5 g elemental S
3. Add 0.5 g elemental S and 0.5 g legume meal
4. Add 0.5 g elemental S and 1.0 ml T. thiooxidans both cultures

Mix in all treatments and adjust to a favorable moisture content.

2. After 7 days and again after 14 days remove a 10 g sample for sulfate determination, a 10 g sample for pH determination, a 10 g sample for moisture determination.

3. Determine moisture content by drying at 105°C for 24 hours, and determine pH on the soil paste prepared by adding water to a 10 gram sample of soil.

4. Prepare a standard curve for sulfate determination: Dilute the standard solution of  $MgSO_4$  containing 100 ppm  $SO_4^{--}S$  with extracting solution so as to contain 0, 2, 5, 10, 15, 20, 25, and 40 ppm  $SO_4^{--}S$ . Use 50 ml volumetric flasks for these working solutions. Pipette 10 ml of each working solution into a 50 ml Erlenmeyer flask and add 1 ml of acacia gum solution. After mixing, add 0.5 g.,  $BaCl_2$  crystals, allow to stand 1 minute, then swirl until the  $BaCl_2$  crystals are dissolved and make to volume. Transfer to a colorimeter tube and read the turbidity at once on a Klett-Summerson Colorimeter using the 420 (blue) filter. Plot colorimeter scale units against sulfate-sulfur concentration for standard curve.

5. Extract one 10 g sample of soil for sulfate determination: Add 0.2 grams of charcoal and 25 ml of extracting solution to the 10 g of soil in a 125 ml Erlenmeyer flask. Shake well over a 5 minute period. Allow to settle for 10-15 minutes and filter the supernatant fluid through Whatman 42 paper. Return the filtrate through the filter paper if turbidity is evident. To 10 ml of filtrate in a 50 ml volumetric flask add 1 ml of gum arabic stabilizer and 0.5 g barium chloride crystals. Allow to stand 1 minute, then swirl until  $BaCl_2$  crystals are dissolved while adding water to make to 50 ml volume. Transfer to a colorimeter tube and read the turbidity at once on the colorimeter using the 420 millimicron range (blue) filter.

Refer values obtained to the standard curve. Filtrates too high in sulfate should be diluted quantitatively with extracting solution to fall within the range of the standard curve.

6. Organize all data. Record sulfate sulfur as milligrams per gram of dry soil. Include data on pH, report all turbidity measurements, and plot the standard curve.

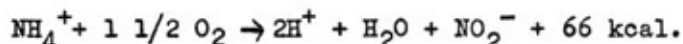
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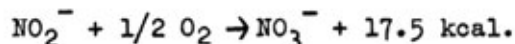
## METHOD 17

### NITRIFICATION IN ENRICHMENT CULTURE

The activities of the autotrophic nitrifying bacteria in soils may be demonstrated by introducing a small amount of soil into an inorganic medium containing ammonium or nitrite nitrogen. The ammonium ion serves as the specific nitrogen and energy source for bacteria mainly of the genera Nitrosomonas and Nitrosococcus. It is oxidized to nitrite in the reaction:



Energy released in this reaction is used to reduce soluble carbon dioxide, carbonates, or bicarbonates, to cellular carbon. A few other specific autotrophic bacteria, notably members of the genus Nitrobacter, oxidize nitrite to nitrate:



The autotrophic nitrifying bacteria are generally considered to be the sole biological agents responsible for the formation of nitrate in soils, sewage, and aquatic environments. The importance attributed to these bacteria stems from the fact that nitrate serves as the principal source of nitrogen for higher plants. Nitrifying bacteria are notoriously difficult to isolate and this has been a contributing factor to a lack of precise information concerning their existence in soil.

Nitrification is not restricted to the autotrophic nitrifying bacteria however, since certain heterotrophic micro-organisms are known to form nitrate or nitrite (Schmidt 1954). The most active of the heterotrophic nitrifiers is the soil fungus Aspergillus flavus, but it is not known if this or any other heterotroph actually participates in nitrification in soil environments. Nitrification is illustrated in this method merely by the inoculation of soil into an inorganic solution. The method is satisfactory as a demonstration that soil contains organisms that will oxidize ammonium or nitrite. The method further serves as an enrichment procedure if the nitrifying bacteria are to be studied in more detail. Rates of nitrification in such solution cultures however, are a poor index of the nitrifying capacity of the soil itself.

### MATERIALS REQUIRED

Soils, moist and recently collected.

Basal nitrification medium:  $\text{KH}_2\text{PO}_4$ , 0.5 g;  $\text{K}_2\text{HPO}_4$ , 1.5 g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g;  $\text{CaCl}_2$ , 0.25 g;  $\text{Fe}_2(\text{SO}_4)_3$ , 0.01 g. Distilled water, 1,000 ml. Distribute 50 ml quantities into 250 ml Erlenmeyer flasks, plug with cotton, autoclave 10 minutes at 121°C.

Ammonium sulfate solution - 5 mg/ml, sterile.

Sodium nitrite solution - 2.5 mg/ml, sterile.

Pipettes, sterile, 1 ml.

Ammonium nitrogen test reagent (see Appendix).

Nitrite test reagent (see Appendix).

Nitrate test reagent (see Appendix).

Spot plates.

### PROCEDURE

1. Obtain 4 flasks of basal medium for each soil to be included. Complete the basal medium as follows:

- (A) Add 1 ml of ammonium sulfate solution aseptically to each of 2 flasks containing 50 ml of basal medium. This medium will test for the presence of ammonium oxidizing micro-organisms.

- (B) Add 1 ml of sodium nitrite solution aseptically to each of 2 flasks containing 50 ml of basal medium. This medium is used to test for nitrite oxidizing forms.

2. Inoculate 1 flask of medium A and 1 flask of medium B with about 0.2 g of fresh, moist soil. Retain the other flasks as uninoculated controls.

3. Incubate at room temperature. Twice weekly over a period of 3-4 weeks, withdraw samples for qualitative spot tests for ammonium, nitrite, and nitrate. Indicate the intensity of each test on a scale ranging from + (weak) to ++++ (strong). Prepare a complete record of the progress of nitrification as reflected in the qualitative spot test data.

4. If all ammonium nitrogen disappears from the inoculated flasks of medium A, transfer 1 ml to a new flask of medium A and observe the rate of ammonium disappearance a second time with this enriched inoculum. Do the same if all nitrite in medium B is oxidized. Ammonium will be oxidized to nitrite, but the nitrite will not accumulate in medium A since it will in turn be oxidized to nitrate, but low concentrations of nitrite should be detected until all the ammonium is gone. In medium B the nitrite will be converted to nitrate and this medium reflects the activity of nitrite oxidizers only. Note carefully that the qualitative test for nitrate is valid only after all nitrite has disappeared.

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#### METHOD 18

#### AMMONIFICATION AND NITRIFICATION IN SOILS

Under normal soil conditions with the supply of readily available energy to micro-organisms very limited, there is only a low level of ammonia formation. The ammonium nitrogen forms slowly but steadily as the organic nitrogen complexes of the soil organic matter and the nitrogenous constituents of dead microbial tissue are decomposed. This process, referred to as ammonification, goes on in poor soils and in good soils under a wide range of conditions and is participated in by a wide variety of soil micro-organisms. When fresh residues are added to soil ammonia is evolved in much larger amounts than in unamended soil. Evolution of ammonia may take place very early in the decomposition of those residues high in nitrogen, or somewhat later in the case of low nitrogen residues.

The ammonia released in soils by microbial activity is retained by the colloidal surfaces of soil particles. Under rather poor soil conditions the ammonia formed will constitute the end product of nitrogen mineralization. Under the soil conditions associated with good fertility however, ammonia will be oxidized almost as rapidly as it is formed, due to the activity of nitrifying organisms (Method 17). Ammonia is oxidized to nitrite and the nitrite is further oxidized to nitrate. The greater reaction rate of the oxidation of nitrite to nitrate prevents the accumulation of the nitrite ion in soil.



Follow the course of ammonification and nitrification reactions in different soils in the laboratory. The method provides for the qualitative examination of the changes in ammonium and nitrate concentrations with time in normal soils, and in soils amended with a residue high in nitrogen.

#### MATERIALS REQUIRED

Soils.

Legume meal.

Flasks to hold 50 grams of soil each.

Sodium chloride, 1N.

Buchner funnels.

Whatman 42 filter paper.

Suction flasks marked at 250 ml volume level.

Small test tubes.

Nessler's reagent (ammonium test reagent, Appendix).

Diphenylamine reagent (nitrate test reagent, Appendix).

#### PROCEDURE

1. Weigh out and distribute 6 replicate 50 gram portions of each soil into flasks. Treat as follows:

- (a) 3 flasks - untreated soil
- (b) 3 flasks - add 0.5 grams of legume meal, mix well.

2. Bring all soils to favorable moisture level by the addition of tap water. Cover the containers and record the weight of each so that any moisture losses during incubation can be restored as necessary. Incubate at 25-30°C.

3. After 4 days incubation analyze 1 untreated soil and 1 amended (legume meal) soil, qualitatively for ammonium nitrogen and for nitrate. Since the ammonium is held in the soil on the colloidal complex, an exchange extraction procedure is required to release the ammonium. Prepare the extract and test for both ammonium nitrogen and nitrate in the extract as follows.

4. Pour 100 ml of 1N solution chloride into the flask containing soil. Stopper and shake intermittently for 30 minutes. Set up a Buchner filter fitted with a moist, retentive filter paper circle, and connected to a suction flask for collecting the filtrate. Mark the side of the suction flask to indicate a volume of 250 ml. Wash the soil onto the filter and collect filtrate while leaching the soil with additional sodium chloride. Continue the leaching under gentle suction until 250 ml of extract are collected.

5. Test the undiluted extract for ammonium by adding 0.5 ml of Nessler's reagent to 5 ml of extract in a small test tube. If orange color is intense or if a precipitate forms, dilute the extract quantitatively with distilled water (1:2, 1:4, etc.) until a light orange color is obtained. Be sure and record the dilution observed and the color. Similarly test for nitrate by adding 0.5 ml of diphenylamine reagent to 5 ml of extract. Again dilute as necessary to obtain a light blue color.

6. Repeat the analyses after 7 days with a second set of soil samples and again after 14 days with the final set. Test at exactly the same dilutions as used in the 4 day analyses. Tabulate data for the various incubation periods.



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## METHOD 19

### DENITRIFICATION IN SOIL

Nitrate is subject to ready reduction by many micro-organisms in soils and culture media. Most of the reductions of nitrate merely result in the circulation of nitrogen atoms in reduced forms in the soil. Some reductive changes may be of considerable importance however, in that they tend to deplete the supply of available nitrogen.

It is essential to distinguish among the types of nitrate reductions that may occur. Nitrates are reduced in the course of nitrogen assimilations by a great many micro-organisms and higher plants. The nitrate ion serves in nitrogen assimilation as a source of nitrogen in the synthesis of cell material, and intermediate compounds are not detectable. Nitrates are reduced also in reactions generally referred to as nitrate reductions. In these transformations the nitrate acts as the principal hydrogen acceptor in the absence of molecular oxygen; nitrate is most commonly reduced to nitrite but also is reduced to ammonia by some bacteria. Many anaerobic and facultative bacteria use nitrate in this manner as a hydrogen acceptor. In the third type of reaction nitrate also serves as the acceptor of hydrogen derived from the oxidation of various organic compounds (hydrogen donors); however, the nitrate is reduced to nitrogen gas or to gaseous oxides of nitrogen. Such reductions are referred to as denitrification, and are carried out by a few facultative anaerobic bacteria. Since gaseous nitrogen is readily lost, denitrification reactions may constitute a serious loss of available nitrogen under some conditions. Denitrification reactions are favored by circumstances that include the presence of nitrate together with anaerobic conditions, alkaline reaction (pH 7.0-8.-2), and readily available energy supply (hydrogen donator).

The method described here merely shows the disappearance of nitrate from soil as influenced by anaerobic conditions (waterlogging) and presence of decomposable organic matter (glucose). All data are based on nitrate determinations. Much more elaborate analyses would be required to prove that the nitrate disappeared because of denitrification. However, as this experiment is set up, any marked losses of nitrate that are observed are almost certainly due to denitrification. This is because ammonium nitrogen is added together with nitrate, and the greater part of the nitrogen locked up in cell synthesis will be ammonium nitrogen. Marked losses of added nitrate then, will not be due to nitrogen assimilations, but will be directly related to denitrification.

### MATERIALS REQUIRED

- Soil of known low moisture content, pH 7-8.
- Erlenmeyer flasks, 250 ml, large mouth, or drinking glass tumblers.
- Balance.

Erlenmeyer flasks, 1 liter, and 50 ml.

Copper sulfate extractant, 0.02N, 500 ml for each soil sample to be extracted.

Calcium sulfate, powder, to flocculate soil colloids during extraction.

Filter funnel and Whatman 42 filter paper.

Volumetric pipette; or graduated cylinder, 50 ml.

Kjeldahl flask, 1 liter, and condenser to fit.

Magnesium oxide, powder.

Distilled water, ammonia free.

Boric acid - indicator - Dissolve 20 g of pure  $H_3BO_3$  in about 700 ml of hot water, cool, transfer to a 1 liter volumetric flask containing 200 ml of ethanol and 20 ml of mixed indicator prepared by dissolving 0.330 g of bromocresol green and 0.165 g of methyl red in 500 ml of ethanol. After mixing the contents of the flask, add approximately 0.05N NaOH cautiously until a color change from pink to pale green is just detectable when 1 ml of the solution is treated with 1 ml of water, then dilute the solution to volume with water and mix it thoroughly (Bremner, 1965).

Nessler's reagent for colorimetric ammonia test (Appendix).

Devarda alloy.

Standard sulfuric acid, 0.005N.

Microburette, 10 ml.

## PROCEDURE

1. Weigh 100 gram portions of soil and place in 250 ml Erlenmeyer flasks or in glass tumblers (drinking glasses). Prepare 4 replicates. Treat as follows:

- a) 2 sets: soil plus 0.25 g  $NH_4NO_3$
- b) 2 sets: soil plus 0.5 g glucose plus 0.25 g  $NH_4NO_3$

2. Mix soil and amendments thoroughly before wetting the soil. Add water to 1 soil of set a, and 1 soil of set b to approximately one half the water holding capacity. These will represent aerobic soil systems. Add water to the 2 remaining soils to 100 percent water holding capacity (saturated). These high moisture soils will represent anaerobic (waterlogged) conditions. Weigh each soil and container to establish weight at start of incubation.

3. Incubate all soils at 25-30°C for a period of 14 days. During incubation check the weight of each soil frequently and add water as indicated to restore moisture losses.

4. Analyze for nitrate nitrogen after 14 days. The procedure involves (a) extraction of nitrate, (b) distillation and discard of any ammonia present in the extract, (c) reduction of nitrite and nitrate still present in the extract, with Devarda alloy, and (d) distillation and determination of the ammonia formed from the reduction of nitrite and nitrate. Since the amount of nitrite present will probably be negligible, the amount of ammonium nitrogen finally measured, will represent almost solely, nitrate extracted from the soil.

Proceed as follows: Transfer all soil from the incubation container to a 1 liter flask or bottle by washing with a portion of 500 ml of 0.02N copper sulfate. When all soil is transferred add the remainder of the 500 ml of copper sulfate and about 0.5 g of calcium sulfate. Stopper well and shake vigorously for 10 minutes, and decant the supernatant through a relatively fine filter paper (Whatman 42 or equivalent), collecting about 100 ml of clear filtrate. If first portion of filtrate is cloudy, return it to the filter. Place a 50 ml aliquot of the filtrate into a Kjeldahl flask (1 liter), add 100 ml of ammonia-free distilled water, and 0.5 grams of magnesium oxide. Attach the Kjeldahl flask to a condenser tube and heat to mild boiling, testing the condensate as it distills over for ammonium, using Nessler's reagent.

When the Nessler's test is very faint on the distillate as it comes off, stop the heating and discard any distillate collected. Allow the Kjeldahl flask to cool. Add 5 ml of boric acid indicator solution to a 50 ml Erlenmeyer flask marked so as to indicate a volume of 30 ml. Place the flask under the condenser so that the end of the condenser delivery tube is just below the surface of the boric acid. Add additional ammonia-free distilled water if necessary, to the Kjeldahl flask. Place 0.3 g of Devarda alloy into the extract, connect the condenser quickly and heat to gentle boiling. Collect the distillate until the 30 ml mark is reached on the receiving flask, then withdraw the receiving flask and stop the heating. Determine the ammonium nitrogen in the distillate by titration with 0.005N H<sub>2</sub>SO<sub>4</sub>, from a microburette. The end point is reached with a color change from green to a permanent, faint pink.

Calculate the amount of ammonium present in the distillate (nitrate plus nitrite present in the 50 ml aliquot of copper sulfate extract). One ml of 0.005N H<sub>2</sub>SO<sub>4</sub> is equivalent of 70 micrograms of ammonium N or, in this case, assume 70 micrograms of nitrate nitrogen.

5. Collect data on all soils and record results of nitrate nitrogen analyses. Tabulate data and interpret.

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#### METHOD 20

##### ORGANIC RESIDUES AND SOIL AGGREGATION

Arrangement of individual soil particles into stable crumbs or granules is a feature of the most productive agricultural soils; soil microbes contribute to the formation of this stable crumb structure. The relative contribution of soil micro-organisms to the formation of soil structure cannot be evaluated, for the process is not well understood. Physical-chemical reactions, plant development, and the activities of the microfauna and macrofauna also contribute to the evolution of stable structural aggregates in soil.

The impact of soil micro-organisms appears to be important during the early stages of structure formation with the cementation of newly formed aggregates into crumbs (Jack, 1963). Cementation and glueing substances include "humic" by-products of microbial action on soil organic matter, and polysaccharide and other capsular substances associated with bacterial development; temporary binding effects result from fungal hyphae extension (McCalla, 1945; Martin, et al. 1955; Iimura and Egawa, 1956). Addition of decomposable organic matter to soil results in a marked increase in water stable aggregates, at least during the period of virorous microbial activity (Iimura and Egawa, 1956).

Effects of intensive microbial activity on the water stability of soil extracts are examined in this method. Soil is incubated both with and without an added source of energy. The marked increase in microbial activity in response to available organic matter will influence the water stability of aggregates present in soil. In some soils the aggregates may become more water stable due to slimes and gums formed during intense microbial activity; in other soils the aggregates may already be highly water stable, and intense microbial activity may either have no effect or may even reduce the water stability to some extent. The method should be applied to several soils to note various effects, but the method should not be considered as a technique to measure soil structure. It is meant only to demonstrate effects of microbial activity on soil aggregates.

#### MATERIALS REQUIRED

Soil samples.  
Sucrose.  
Legume meal.  
Ammonium chloride, powder or crystalline.  
Moist chamber for incubation.  
Sieve assembly: screen numbers 5(5 mm), and 3(3 mm).  
Analytical balance.  
100 ml beakers, dry and tared.  
1-liter flasks.  
1-liter graduated cylinder.  
Volumetric pipette, 50 ml or 25 ml.  
Machine shakes if available.

#### PROCEDURE

1. Prepare 500 gram samples of soil as follows:
  - a) Untreated
  - b) Treat with 0.5 percent sucrose and 0.1 percent ammonium chloride
  - c) Treat with 0.5 percent ground legume meal

Add water to about 60% moisture holding capacity and incubate in a moist chamber for 2 weeks.

2. Separate an aggregate fraction. After incubation spread the soils on paper and allow to air dry. Transfer the dry soil to a sieve assembly composed of a top sieve with 5 mm screen, second sieve with 3 mm screen, and unscreened bottom pan. Sieve the soil and collect the portion that passes through screen 5(5 mm), and is retained on screen 3. This is the 3-5 mm aggregate fraction. Weigh out 10 g of 3-5 mm aggregates from each treatment.

3. Test the water stability of the 3-5 mm aggregates. Add 10 grams of aggregates to 500 ml of water in a 1 liter flask or bottle. Stopper tightly and shake vigorously. If by hand, shake intermittently for 25 minutes. If by reciprocal shaking machine, shake for 10 minutes. Transfer the shaken suspension to a 1 liter graduated cylinder and add water to the 1 liter mark. Up-end the cylinder 3-4 times to suspend soil, place at rest and after exactly 3 minutes of settling, withdraw a 50 ml aliquot using a 50 ml volumetric pipette (or 2 samples with a 25 ml pipette) inserted into the suspension column to a depth of 10 cm. Discharge the 50 ml aliquot into a previously dried and tared beaker. Repeat to obtain a total of 3 replicate aliquots from each cylinder but be sure to resuspend the soil by up-ending prior to each sampling. Dry for 24 hours at 105°C and determine weight of soil.

4. Record data and compare soil treatments. Note that the method measures water stability indirectly. If the aggregates are fully water stable during the shaking procedure, they will not break or slake but will settle out completely during the 3

minute settling period. If the aggregates are not fully water stable there will be some disruption and slaking with the release of smaller aggregates, and particles of sand, silt, and clay. Some of the silt and clay particles will remain in suspension and will be collected in the aliquot after 3 minutes. The weight of suspended material will provide an index of the stability of the soil aggregates when shaken in water, as in this experiment.

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Section 5

NITROGEN FIXATION IN SOILS

## METHOD 21

### ISOLATION OF RHIZOBIUM ROOT NODULE BACTERIA

The most important system by which molecular nitrogen is fixed biologically results from a symbiotic association between a soil bacterium and a legume plant. Practical agriculture since ancient times has made use of the benefits of this symbiotic association. Members of the genus of soil bacteria, Rhizobium, infect legume seedlings through root hairs and stimulate the formation of tumor-like nodules on the roots. The nodules, comprised of both plant and microbial tissue, are capable of using atmospheric nitrogen. Nitrogen fixed by the symbiotic structure is stored by the legume in the form of protein and other constituents of the protoplasm. The rhizobia are aerobic, non-sporulating rods that develop well in carbohydrate media, but are unable to use free nitrogen when cultured independently of the plant host. Within the nodule the bacteria assume various growth forms and usually appear in microscopic examination of crushed nodule material as highly vacuolated, pleomorphic cells.

The objective of this method is to provide a procedure for the isolation of Rhizobium species from nodules found on the roots of legume plants. It is important to know if legumes grown from uninoculated seed under conditions of local agriculture do become nodulated. If nodules are not present the effect of seed inoculation with appropriate strains of Rhizobium should be studied in the field or in the greenhouse according to Method 23. If nodules are present on plants grown from uninoculated seed, it is necessary to determine the effectiveness of these nodules formed by "wild" strains of Rhizobium in the local soils. The first step in this procedure is the isolation of the "wild" Rhizobium strains (this Method).

Isolations may lead to recovery of several strains of Rhizobium of varying effectiveness, even from the same plant, and even the inadvertent presence of non-nodulating bacteria that resemble Rhizobium. Thus it is necessary to test all isolates that look like Rhizobium, first for ability to incite nodule formation, and then to test further those that can nodulate, for their effectiveness in fixing atmospheric nitrogen.

Isolation is accomplished by the procedure listed in this Method. Testing of a relatively large number of such isolates simply to tell whether each will, or will not, nodulate the legume of interest follows in the next procedure (Method 22). Finally in Method 23, the techniques for comparing the effectiveness of the more promising isolates is outlined.

#### MATERIALS REQUIRED

Root systems of nodule-bearing legume plants, freshly collected.  
Sterile water.  
Sterile Petri dishes.  
Ethanol, 70 percent.  
Sterile forceps.  
Scalpel or razor blade.  
Inoculating loop.  
Mannitol-yeast extract-congo red agar: Mannitol, 10 g; yeast extract, 0.1 g;  
 $K_2HPO_4$ , 1.0 g;  $MgSO_4 \cdot 7H_2O$ , 0.2 g; NaCl, 0.1 g; Congo red dye, 2.5 ml of  
a 1% solution; agar, 15 g; tap water, 1,000 ml.

#### PROCEDURE

1. Wash the soil from the roots and nodules of the legume plant chosen for study. Note the appearance, distribution, and abundance of nodules.

2. Carefully cut, do not tear, a nodule from the root so that a small portion of the root remains attached. If possible, select a plump firm nodule preferably pinkish in color. Wash under running water to insure the removal of all soil particles.

3. Put the nodule into a petri dish containing 0.1 percent HgCl<sub>2</sub> and leave it immersed for 5 minutes.

4. Transfer the nodule to a sterile petri dish containing sterile water. Use sterile forceps to make the transfer. Wash the nodule in the sterile water.

5. Transfer the nodule to another petri dish containing 70 percent ethanol for 3 minutes. Remove to another petri dish of sterile water and rinse thoroughly, then transfer to a second plate of sterile water for final rinsing.

6. Add 1.0 ml of sterile water to each of six sterile petri dishes. Remove the nodule from the rinse water to petri dish No. 1 and crush it with flamed forceps. Mix the nodule tissue with the water.

7. Transfer two loopfuls of the suspension in the No. 1 petri dish to petri dish No. 2 and mix it with the sterile water previously added. Repeat this loop dilution progressively for plates Nos. 3, 4, 5, and 6.

8. Add 15 ml of mannitol-yeast extract-congo red agar (melted in a boiling water bath and held at 42°C for pouring) to plates Nos. 2 to 6 inclusive. Mix the agar and dilutions thoroughly by swirling, and incubate at 28°C for 7 days.

9. At the end of one week representative colonies of Rhizobium should have developed and isolations can be made for further use or storage. Select the mucoid, or flat, watery colonies that are most abundant in the loop dilution plates. Choose an isolated colony among the dominant, Rhizobium - like colonies derived from a single nodule, and transfer aseptically to several slants of the same medium. Label, incubate, and store for further testing.

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#### METHOD 22

##### PRELIMINARY TESTING OF RHIZOBIUM ISOLATES FOR ABILITY TO NODULATE

Bacteria often are isolated from the nodules of legume plants, in order to obtain strains of Rhizobium that are suitable for seed inoculation. The isolation procedure may lead to a relatively large number of isolates derived from different nodules on the

same plant species, or from the same plant species in different locations, or from different species of legume. It is important to know which isolates of Rhizobium form the most efficient nodules on a given legume, but it is first necessary to screen the isolates for ability to form nodules at all. It may not be assumed that all isolates obtained by following the procedure of Method 21 will form effective nodules; some may not form any nodules, and some may form nodules that fix little or no nitrogen gas.

It is the purpose of this method to provide procedures to test a large number of isolates in a preliminary fashion. Those isolates that are contaminants, those Rhizobium isolates that fail to nodulate, and those Rhizobium isolates that form typically ineffective appearing nodules, are detected and discarded. The technique of surface sterilization of legume seeds (step 3) must be mastered before this Method is started.

#### MATERIALS REQUIRED

Test tubes: approximately 2.5 cm x 15 cm for small seed legumes;  
approximately 4 x 20 cm for large seed legumes.

Vermiculite.

Nitrogen-free nutrient solution, sterile. (Bryant's modification of Crone's salt solution - Allen, 1959) Stock salt mixture: Potassium chloride, 100 g; calcium sulfate, 2.5 g; magnesium sulfate, 2.5 g; tricalcium phosphate, 2.5 g; ferric phosphate, 2.5 g; mix all salts and grind to a fine powder. Add 1.5 gram of this stock salt mixture per 1,000 ml water and autoclave for 40 minutes at 15 pounds pressure (121°C).

Legume seeds of appropriate species, based on isolates to be tested.

H<sub>2</sub>O<sub>2</sub>, 30 percent. Fresh solution, refrigerate when stored.

Petri dishes and sterile water.

Sterile blotting paper in Petri dishes.

Sterility test media (see step 3).

Suspensions of bacterial isolates to be tested. Grow isolates on mannitol-yeast extract agar (Medium 21 with Congo red solution omitted) on large test tube slants or on bottle slants until good growth is obvious on the surface. Wash with 5-10 ml of sterile nutrient solution (above) to obtain a dense suspension of each isolate.

Sterile forceps.

Sterile pipettes, 10 ml.

#### PROCEDURE

1. Fill test tubes half way to the top with air dry vermiculite. Use the same weight of vermiculite in each tube. Plug with cotton and autoclave for 20 minutes at 15 pounds pressure (121°C).

2. Moisten the sterilized vermiculite by adding aseptically an appropriate volume of sterile nitrogen-free nutrient solution. The vermiculite should be thoroughly moistened, but must not be waterlogged. Determine the amount of solution by trial and error, to arrive at a volume suitable for routine use.

3. Surface sterilize the legume seed to eliminate any rhizobia that may be present. Immerse seed in fresh 30 percent hydrogen peroxide for 20 to 30 minutes. Rinse several times with sterile distilled water. Spread the seeds on sterile blotting paper in Petri dishes. Large seeds should be inoculated and planted promptly after surface sterilization. A check on the effectiveness of the surface sterilization should be a part of each experiment. Therefore, if small seed legumes are studied, transfer 10-20 surface sterilized seeds aseptically to the surface of a Petri plate containing mannitol-yeast extract agar (Method 21 with congo red solution omitted) and incubate for 2-3 days. For large seeded legumes, set up about 10-12 test tubes containing sterile mannitol-yeast extract broth (Method 21 with congo red solution and agar omitted);

introduce one surface sterilized seed aseptically into each tube of broth and incubate. If contamination is obvious in most test seeds (colonies around seeds on the agar, or turbidity in the tubes) the entire experiment must be repeated and material already set up in the following steps must be discarded.

4. Inoculate the appropriate surface sterilized test seeds with a test strain of Rhizobium. Be sure that the isolate of Rhizobium to be tested is matched to an appropriate legume seed. It is essential, therefore, to inoculate a given isolate into seeds of either the same species of legume from which the isolate was obtained originally, or on seeds of legumes in that same inoculation group. Place 5 surface sterilized seeds in a sterile Petri dish and add a dense suspension of the isolate to be tested, in sufficient volume to submerge the seeds when the plate is tilted. Allow seeds to soak in the inoculum for 10 minutes.

5. Transfer inoculated seeds to the vermiculite growth tubes. Use a sterile forceps and drop 1 seed per tube onto the surface of the sterile moistened vermiculite. Replace cotton stopper.

6. Cover seeds with sterile moistened vermiculite. It is convenient to prepare extra tubes of sterile moistened vermiculite (steps 1 and 2) and to use these to add enough vermiculite to cover the seed completely to a depth of 1 cm. Simply pour sufficient vermiculite from the extra tubes into the tubes with seeds.

7. With cotton stoppers in place incubate all tubes exposed to adequate light, and at temperature between 60-80°C. Temperatures must not exceed 80°C. Retain the cotton plug until the plant grows up to the base of the plug and starts to push against it. Then discard plug and leave the growth tube open. Incubate the growth tubes for a total of 4-6 weeks. Check the moisture level after 2 weeks and add sterile water (not nutrient solution) if necessary. If 2 additional waterings are required, make the second one with sterile nutrient solution. Under most conditions it will not be necessary to add any additional moisture during the entire growth period.

8. After 4-6 weeks growth, recover as many of the 5 original replicates as had germinated and developed into growing plants. Record the number of plants harvested in each series, and examine each for the presence of nodules. Note carefully those nodules that are pink colored and located near the larger roots. Such nodules are presumed to be effective nodules. Record also the presence and distribution of smaller, whitish colored nodules frequently found on fine lateral roots. Such nodules are presumed to be ineffective nodules. The legume-Rhizobium associations that consistently yield the most abundant effective nodules are of greatest interest, and these should be tested further to evaluate the effectiveness of the association more quantitatively in Method 23.

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## METHOD 23

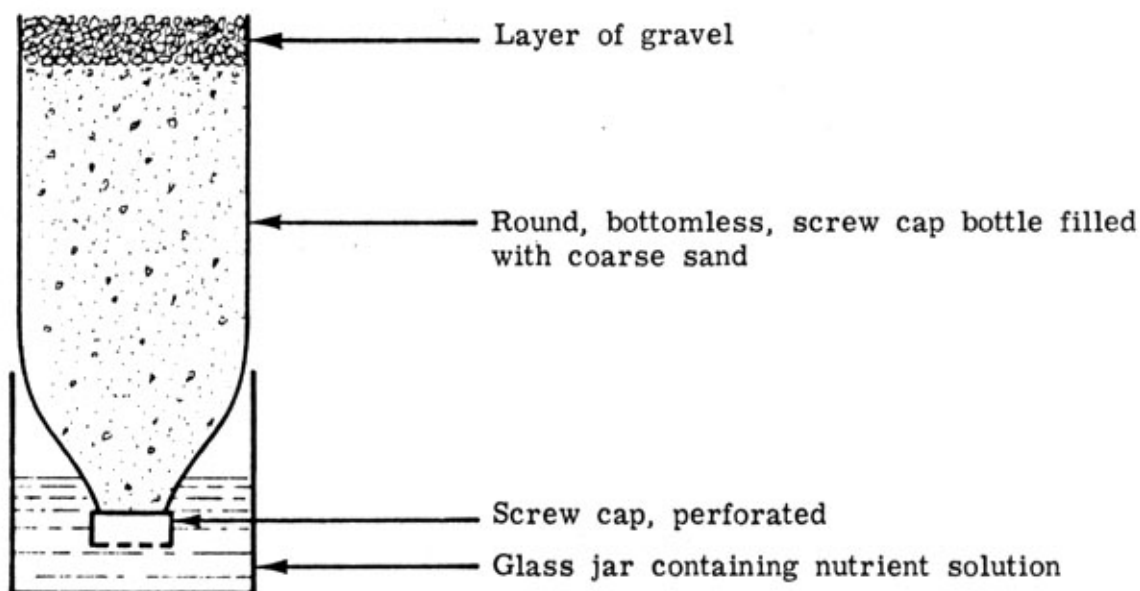
### TESTING RHIZOBIUM ISOLATES FOR NITROGEN-FIXING ABILITIES

Different strains of Rhizobium vary considerably with respect to the efficiency with which the nodules that they form are able to use atmospheric nitrogen. The nitrogen-fixing ability of any given isolate of Rhizobium can be determined only by plant tests in a properly constructed greenhouse or plant-growth chamber. This Method describes procedures for a more detailed evaluation of the efficiencies of those isolates that appeared most interesting in the preliminary tests.

Isolates are tested for efficiency by growing the inoculated legume in sterilized sand treated with nitrogen-free plant nutrient solution. Under these conditions plant growth is dependent on the nitrogen fixed by the symbiotic relationship. The more efficient the strain of Rhizobium, the better the appearance of the plants, and the higher the plants in total nitrogen and in dry matter produced. Tests designed to study efficiency should provide good growing conditions for the legume host in clean, dust-free surroundings. Strict attention to sanitation must be observed throughout, to insure that the growth response of the host is solely a function of the applied Rhizobium, and is not due to contaminating bacteria.

#### MATERIALS REQUIRED

Self irrigating assembly for growing legumes.<sup>1/</sup> (See Fig. 4 and step 1)  
Provide 1 uninoculated unit and 5 replicates for each Rhizobium isolate.  
Clean washed sand, coarse, about 80% total weight retained on a 50 mesh screen, and about 20% of this should be larger than 30 mesh.



Modified Leonard type self-irrigating assembly for growing legumes (courtesy Dr. L. W. Erdman, formerly Research Microbiologist, Soil and Water Conservation Division, United States Department of Agriculture, Beltsville, Maryland U.S.A.)

Fig. 4

<sup>1/</sup> A simpler culture method employs crockery pots filled with moist sand. The pots are wrapped in paper and steam sterilized for one hour on three successive days. The legume seed is planted aseptically in the sand, the inoculant is added and the plant nutrient solution is added as needed.



Bryant - Crone nitrogen-free nutrient solution (Method 22).  
Sterile forceps.  
Sterile Petri dishes.  
Hydrogen peroxide, 30 percent.  
Seeds of the legume under test.  
Rhizobium isolate, 50 ml heavy suspension (Method 22).

#### PROCEDURE

1. The unit is made up of a bottomless (approximately one liter), round, screw cap bottle filled to within 7-8 cm of the top with dry washed sand and inverted into a jar of slightly larger diameter. The top of the bottle should extend to about 2 cm from the bottom of the jar to insure continuous nutrient supply. The larger jar serves as a reservoir for the nitrogen-free nutrient solution and as a support for the inverted bottle used for growing the test seedling. Avoid sand that is too fine, as it may become waterlogged. Check pH of sand-nutrient solution system and adjust pH of the nutrient solution so that the final pH of the rooting system is about 6.5. The bottom of the bottle is removed first by making a circular scratch with a file just above the bottom of the bottle. The bottle is then placed in a revolving holder and a fine acetylene flame is played on the revolving bottle along the scratched line. The bottom will drop off in about 30 seconds. The cut edge can be smoothed with emery paper.
2. The self-irrigating assembly is filled with sand and the open top is covered with heavy wrapping paper or aluminum foil. Next, sterilize the apparatus in the autoclave at 15 lbs pressure (1 kg/cm<sup>2</sup>) for two hours. The units are then placed on greenhouse benches about eight inches apart. If possible, a separate isolated section of the greenhouse should be used which is kept scrupulously clean and free from dust and air currents.
3. Immerse the legume seeds in 30% hydrogen-peroxide for 30 minutes to free the seed coats of bacteria. Then rinse the seeds several times in petri dishes containing sterile water. Aseptic conditions must be carefully observed.
4. Transfer the seeds (15 for small seeded legumes, 5 for the larger species) to the culture assembly, distributing them evenly over the surface of the sterile sand.
5. Inoculate each jar with 10 ml of a heavy suspension of the inoculant being tested, using five replicates for each inoculant (Method 22).
6. Cover the seeds lightly with sterile sand followed by a thin layer of fine gravel. Replace the paper cover until germination takes place. In the greenhouse, it is important that chance contamination, particularly of Rhizobium bacteria, is eliminated. This involves a strict control of insects and dust, as well as isolation from ordinary greenhouse operations. One or two uninoculated controls must be included in each test.
7. Permit the plants to grow 45 days keeping careful notes of the progress of growth and the degree of green color which develops. In particular, signs of nitrogen deficiency should be noted. Add additional nutrient solutions as necessary.
8. At the end of the growth period, it is often possible to locate the best strains of rhizobia by appearance, but this should be verified by removing the plants, cut at the sand surface level, and determining the total plant dry matter for each pot. The tests can be further verified by:
  - (a) determining the total nitrogen content of the plant material;
  - (b) removing the root systems carefully from each pot, and determining its mass and the number of nodules which developed.

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## METHOD 24

### NITROGEN FIXATION BY NON-SYMBIOTIC SOIL MICRO-ORGANISMS

The most widely studied of the free living, or non-symbiotic, biological agents of nitrogen fixation is undoubtedly the Gram negative soil bacterium, Azotobacter. These bacteria fix substantial amounts of atmospheric nitrogen into cell protein when grown in pure culture under conditions of good aeration, adequate energy supply, pH above 6, and absence of combined nitrogen. Since conditions for nitrogen fixation appear to be quite specific, the practical importance of Azotobacter in adding nitrogen to field soils has been questioned. Large scale inoculations of soil with Azotobacter have been carried out, but the practicality of such inoculation has not been established. The review by Jensen (1954) should be consulted for various aspects of the biology of this interesting genus.

Nitrogen fixation by Azotobacter in aerobic circumstances, and by Clostridium in anaerobic, has been known since about 1900. In recent years  $N_2^{15}$  isotope techniques have shown that 5 or 6 other genera of soil bacteria fix atmospheric nitrogen (Virtanen and Miettinen, 1963). The list of soil bacteria which fix at least some nitrogen may become quite large. The method described below is meant to demonstrate the presence of free living nitrogen fixing micro-organisms in soil. A portion of each soil is first enriched in nitrogen fixers by incubating with a readily available energy compound added to the soil. The enriched soil is used to inoculate a culture medium containing no added nitrogen, and is compared to an inoculum of non-enriched soil. After growth the total amount of nitrogen added to the nitrogen-free medium will be measured by Kjeldahl analysis. The test conditions, especially those of the soil enrichment, will favor development of Azotobacter species.

### MATERIALS REQUIRED

- Soil samples, recently collected and not air dried.
- Corn starch, powdered.
- Nitrogen-free basal salts solution:  $K_2HPO_4$ , 1.0 g;  $MgSO_4 \cdot 7H_2O$ , 0.5 g;  $CaCO_3$ , 2.0 g;  $FeCl_3 \cdot 6H_2O$ , 0.1 g;  $Na_2MoO_4 \cdot 2H_2O$ , 0.010 g; distilled water, 1,000 ml. Place 50 ml portions into 500 ml Erlenmeyer flasks, stopper with cotton plugs and autoclave for 15 minutes at 121°C (15 lbs pressure).
- Sucrose, 20 percent solution, sterile.
- Pipettes, 5 ml, sterile.

### PROCEDURE

1. Add about 4 percent corn starch to a portion of each soil to be studied. Mix well, adjust to about 60 percent water holding capacity and incubate uncovered in a moist atmosphere for 5-7 days. Prepare a second portion of each soil, without added corn starch, moisten and incubate similarly.

2. Inoculate each of 4 flasks of nitrogen-free, basal salts solution with 0.2 g (weighed out) of soil as follows:

- (a) 2 flasks - inoculate with enriched (corn starch treated) soil
- (b) 2 flasks - inoculate with normal (incubated, no corn starch) soil.

Complete 1 flask of (a) and 1 flask of (b) by adding 5 ml of 20% sterile sucrose solution. The 2 remaining flasks are to be incubated without sucrose in the medium. There are two reasons for omitting the sucrose energy source: first, the two soil-minus-sucrose systems serve as controls, to indicate the small amount of nitrogen present in the basal medium and in the soil inoculum; secondly, controls are analyzed much more conveniently if carbohydrate is absent, since foaming and frothing can be avoided during Kjeldahl digestion.

3. Incubate flasks at 28-30°C. Note that the use of 50 ml of solution in a relatively large (500 ml) flask provides a shallow layer with large surface area to maintain aerobic conditions during incubation.

4. At the end of 7-10 days incubation determine total nitrogen by the procedure "Total nitrogen of culture suspensions by Macro-Kjeldahl" (see Appendix).

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#### METHOD 25

#### THE NITROGEN-FIXING GENERA, AZOTOBACTER AND BEIJERINGKIA IN SOIL

The genus Azotobacter comprises a group of free-living, non-symbiotic, nitrogen fixing bacteria native to soil. Azotobacter, principally the species A. chroococcum, occurs commonly but not uniformly, in soils of near neutral pH all over the world. Isolation and counting techniques are based on the use of media made selective by the omission of nitrogen in a combined form. Since these bacteria do not require organic substances other than some suitable source of carbon, the medium can be quite simple. It must, however, be well buffered, well supplied with phosphate and potassium salts, and must provide trace amounts of molybdenum.

Most of the bacteria in the azotobacter family fix nitrogen at pH 6-8, but an acid tolerant form was reported by Starkey and De in 1939. This bacterium is now known as Beijerinckia indicum, and the genus is thought to occur mainly in tropical soils, especially in acid lateritic soils (Becking 1961). The genus Azotobacter apparently occurs more commonly than the genus Beijerinckia in soils of temperate regions, and the reverse seems to be the case in tropical soils. However most soils probably contain both genera. Isolation and enumeration of these two nitrogen-fixing genera of the azotobacter family may be accomplished with nitrogen-free media with pH adjusted to favor either Azotobacter or Beijerinckia.

Meiklejohn (1965) has suggested a procedure which avoids the use of both acid and alkaline media to count and isolate Azotobacter and Beijerinckia. A single medium, that of Brown, Burlingham, and Jackson (1962), with sucrose as the carbon source, is used to count both genera. The method outlined in this experiment is based on Meiklejohn's suggestion.

#### MATERIALS REQUIRED

- Soils, freshly collected and maintained at field moisture, sieved if necessary.
- Petri plates, sterile. 6 for each soil.
- Dilution bottles, 90 ml and 20 ml.
- Pipettes sterile, 1 ml and 5 ml.
- Nitrogen-free sucrose agar. (a) sucrose, 5.0 g;  $MgSO_4 \cdot 7H_2O$ , 0.2 g;  $FeSO_4 \cdot 7H_2O$ , 0.4 g;  $NaMoO_4$ , 0.005 g;  $CaCl_2$ (anhy.), 0.15 g; agar, 15.0 g; distilled water, 1,000 ml. Autoclave together at 15 lb pressure for 15 minutes.
- (b) Autoclave separately a solution of  $K_2HPO_4$  containing 100 mg/ml and add 10 ml of this to part (a) when both have cooled to about 50°C. Hold the completed medium at a temperature of 42-45°C until plates are to be poured.
- Inoculating wires.
- Microscope.
- Microscope slides.
- India ink, dense suspension in distilled water. Concentrate the diluted ink by heating to evaporate excess water if necessary.

#### PROCEDURE

1. Pour 6 plates of the nitrogen-free sucrose agar for each soil to be studied, allow plates to harden well and dry overnight in a low moisture incubator at 30-35°C.
2. Use freshly collected soil and prepare a 1/10 dilution by shaking 10 g of soil in 90 ml of sterile water vigorously for 10 minutes. Transfer 5 ml of the 1/10 dilution to 20 ml of sterile water to obtain a 1/50 dilution.
3. Make triplicate 1 ml inoculations from the 1/10 and 1/50 dilutions onto the dry surface of the sucrose agar plates. Tilt each plate so as to distribute the inoculum evenly over the surface, place in a plastic bag or wrap in foil to avoid further drying and incubate at 30°C.
4. After 3 days incubation count the flat, soft, milky, mucoid colonies that develop. These are Azotobacter colonies. Use an inoculation wire and isolate from several different appearing colonies of Azotobacter by streaking out each selection on a fresh plate of nitrogen-free sucrose agar. Hard and tough bacteria-like colonies are probably Streptomyces and should be ignored since they do not fix nitrogen. Do not discard the soil dilution plates and, after making Azotobacter isolations, mark the location of all colonies present at 3 days, and continue incubation.



5. After 14 days count the Beijerinckia colonies that have developed. These will be white, raised colonies, sometimes wrinkled, with a tough elastic consistency. Streak several different colonies of Beijerinckia out on fresh plates to obtain isolates.

6. Compare the numbers of the two genera found in each soil. Transfer from isolated colonies on the streak plates onto slants of nitrogen-free sucrose agar in attempts to get pure cultures.

7. Make microscopic examinations of either streak plate isolates or of material taken from isolated colonies on the soil dilution plates. A wet mount, negative stain procedure is convenient for microscopic examination. To make such a preparation place several loopfuls of a dense suspension of india ink in water on a microscope slide, and add a small amount of the bacterial growth. Mix, cover with a coverslip, and examine with high dry and oil immersion objectives. The background will appear dark, due to carbon particles in suspension. Individual cells of bacteria will be seen surrounded by a clear light zone. Capsule materials around the bacteria are so dense that the colloidal ink particles are held away from the cell. Describe the appearance of the cell types seen. Did the cultures examined microscopically appear to be pure culture?

8. If desired, isolates of Beijerinckia or of Azotobacter could be inoculated into the nitrogen-free, sucrose solution medium and tested quantitatively for nitrogen-fixing ability (Method 24).

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Section 6

APPENDIX

## NITROGEN SPOT TEST REAGENTS

### NESSLER'S REAGENT FOR AMMONIUM

Dissolve 34.9 g potassium iodide (KI) and 45.5 g of mercuric chloride ( $\text{HgCl}_2$ ) in a minimum of ammonia-free water (Solution A).

Dissolve 112 g of potassium hydroxide (KOH) in 120 ml of ammonia-free water (Solution B).

Add solution A to solution B and dilute to 1,000 ml with ammonia-free water.

Store in a tightly stoppered brown glass bottle in a dark place.

To test for ammonia, add 2 drops of Nessler's reagent to 3 drops of the solution to be tested in a spot plate.

An orange-brown color indicates the presence of ammonia.

### GRIESS REAGENTS FOR NITRITE AND NITRATE

Dissolve 5 g of sulfanilic acid in 500 ml of 30 percent (by volume) acetic acid. Heat gently. (Solution A).

Add 1.5 g of alpha-naphthylamine to 350 ml of water and boil for two minutes. After filtering, add 150 ml of glacial acetic acid (Solution B).

Free zinc dust from traces of nitrites and nitrates by boiling for one hour in 0.1 N acetic acid. Filter the dust under vacuum, wash with distilled water and dry thoroughly at  $105^\circ\text{C}$ .

To test for nitrite, place 0.5 ml of the solution to be tested in a spot plate and add three drops each of solutions A and B. A red color indicates the presence of nitrite.

Nitrate in the absence of nitrite is detected by reducing the nitrate to nitrite with zinc dust. Place 0.5 ml of the solution in a spot plate, add 3 drops each of solutions A and B. No color should develop. Next add 1-5 mg of the prepared zinc dust. The development of a red color is evidence of the presence of nitrate. The color may fade as the test is not as sensitive as for nitrites.

To detect nitrate in the presence of nitrite the latter is first destroyed. Add 3 ml of solution A and 5 ml of the solution under test to a glass test tube, and boil gently over a flame for 3 minutes. Withdraw one ml of this solution and place it on a spot plate. Add 3 drops each of solutions A and B. If no color develops, the destruction of nitrite is complete. Then test for nitrate as described above.

### DIPHENYLAMINE REAGENT FOR NITRATE

Dissolve 50 mg of diphenylamine in 25 ml of conc. sulfuric acid ( $\text{H}_2\text{SO}_4$ ). Store away from light in glass stoppered dropping bottles. Prepare a fresh solution every 2 weeks.

In a spot plate add 3 drops of the solution under test to 5 drops of the reagent. A blue color indicates the presence of nitrate.

### Caution

The reagent is corrosive and is not suitable for solutions containing nitrites.

## SOIL MOISTURE DETERMINATION

The numbers of micro-organisms are usually expressed on the basis of oven-dry soil. This applies also to other determinations which are associated with microbiological activities. The usual procedure is to dry the soil at 105°C until the weight becomes constant. This requires at least five hours, but ordinarily the sample is left in the oven overnight.

### MATERIALS REQUIRED

Tared glass or aluminum containers provided with a cover.  
A desiccator.

### PROCEDURE

Weigh 5 to 10 g of the sieved thoroughly mixed sample into duplicate previously weighed and dried containers (weighing to one hundredth of a gram gives sufficient accuracy).

Place in a 105°C oven until constant weight is attained. This usually requires at least five hours, but preferably overnight.

Remove the containers from the oven, and immediately transfer to a desiccator.

When cool, weigh and calculate the moisture content according to the following formula:

$$\text{Moisture percent} = \frac{A - B}{B - C} \times 100$$

Where A = weight of the dry container and soil before drying  
B = weight of the container and soil after drying  
C = weight of the empty container

## DETERMINATION OF MOISTURE-HOLDING CAPACITY OF SOILS

Moisture control is very important to the microbiological studies of soils. Too much moisture leads to anaerobic conditions and a reduction of the numbers of aerobic micro-organisms in the soil. On the other hand, if a soil becomes too dry, the numbers of micro-organisms and the biological process are depressed. Thus, in microbiological experimental work it is necessary to standardize the moisture of the soil under study and hold it at a point relevant to the subject being studied. To do this, a reference point is necessary and the one usually chosen is the Moisture-Holding Capacity or Field Capacity of the soil. This point is determined experimentally and for ordinary aerobic studies the moisture content is adjusted somewhere between 50 to 70 percent of the moisture-holding capacity. The variation is necessary to take into consideration the different textures and organic matter contents of soils.

### MATERIALS REQUIRED

A perforated bottomed crucible (Gooch) or other similar container.  
Filter paper.  
A tight vessel to provide a water saturated atmosphere, e.g. a large desiccator where the drying agent is replaced with water.

## PROCEDURE

Line the bottom of a Gooch crucible (or similar container) with filter paper cut to fit the perforated bottom snugly, and record the weight. Moisten the filter paper and then record the weight of the crucible and moistened filter paper.

Fill the crucible to within 1-1/2 cm of the top with soil.

Weigh the cups containing soil and moistened filter paper. Wet from the bottom by immersion in water to about half the height of the crucible. Allow to stand until the moisture content of the soil has reached equilibrium throughout.

The samples of different soils or the samples of different horizons of the same soil may require different lengths of time for complete saturation. Usually overnight or 24 hours will be sufficient to saturate soil completely with water. Occasionally heavy clay or organic soils require 3 days for saturation.

Transfer the crucible to a water-saturated atmosphere (for 24 hours) taking care to remove the excess water adhering to the outside of the container.

To obtain a water-saturated atmosphere, simply pour some distilled water into a container with a glass plate cover large enough to accommodate the crucible of wet soil. The crucible should not be in contact with water and should be supported above the water on a glass dish or glass rods. A large desiccator can be converted for this purpose by removing the desiccant and replacing it with water.

Record weight of crucible containing saturated soil and filter paper.

Dry in the oven at 105°C to constant weight. This may require 24 hours. Record weight of dry crucible containing soil and filter paper.

Calculate the Moisture-Holding Capacity (M.H.C.) expressed in percent as follows:

$$\text{M.H.C.(\%)} = \frac{\text{Weight of saturated soil} - \text{Weight of oven dry soil}}{\text{Weight of oven dry soil}} \times 100$$

## DETERMINATION OF TOTAL NITROGEN IN SOIL

Because of the complexity and variety of organic nitrogen forms present in the soil, the determination of the total nitrogen content of soil is not simple. The original, Kjeldahl method has been modified many times. At the present time, many laboratories use a micro-Kjeldahl method for the total nitrogen determination.

The following procedure is a semi-micro adaptation of the Ranker method for total nitrogen, using the Kemmerer-Hallett distillation unit. This method includes nitrate, inasmuch as the salicylic acid retains the nitrate until it is reduced by the sodium thiosulfate. The method is inclusive for amino, amide, ammonia, nitrate and other N compounds if all precautions are followed. Moisture should be avoided because in its presence the salicylic acid is not quantitatively nitrated.

## MATERIALS REQUIRED

Digestion unit. This is not mandatory but is preferable as these units include ventilation systems for removal of fumes.

Distillation unit.

Boric acid dispensing unit.

Titration unit, 10 ml micro-burette preferably with reservoir.

30 ml Kjeldahl flasks.

Salicylic acid-sulfuric acid mixture: Dissolve one g salicylic acid in each 30 ml conc.  $H_2SO_4$ . Make up two liters and store in special acid bottle equipped with dispensing burette. This burette is protected with drying tubes containing drierite so that the conc.  $H_2SO_4$  will not take up water.

Sodium thiosulfate crystals. (Be sure to use coarse crystals several millimeters in size; with finely ground thiosulfate the reduction process is so rapid and violent as to cause excessive foaming and extrusion of the contents through the neck of the flask.)

Forty percent NaOH solution. (Make this solution with container in the sink so that if the container breaks due to the intense heat, the NaOH can be flushed down the drain.)

Two percent boric acid solution. (This need not be accurately weighed.)

Approximately 0.05 N  $H_2SO_4$  (Standardize accurately).

Phenolphthalein indicator (0.5 g phenolphthalein in 100 ml 50 percent ethanol).

Brom-cresol green methyl red indicator. Prepare a 0.1 percent brom-cresol green aqueous solution adding 2 ml of 0.1 N NaOH per 0.1 g of indicator. Also prepare a 0.1 percent methyl red solution in 95 percent ethanol adding 3 ml of 0.1 N NaOH per 0.1 g of methyl red indicator. Mix 75 ml of the brom-cresol green solution, 25 ml of the methyl red solution, and 100 ml of 95 percent ethanol.

## PROCEDURE

### A) Digestion:

Weigh accurately 200 to 250 mg of oven-dry finely ground (to pass 40 mesh screen) soil material in a tared weighing pan (aluminum dish). Transfer with camel hair brush to a cigarette paper. Fold carefully and place in Kjeldahl flask.

Add 4 ml of the salicylic-sulfuric acid reagent, stopper tightly, and let stand for at least an hour - or overnight.

Add about 0.5 g of sodium thiosulfate and heat on digestion rack with very low flame for five minutes (dense white fumes should come off).

Rotate flask occasionally during the heating. Allow to cool and add about 0.8 g of the sodium sulfate-copper sulfate-selenium mixture.

Digest with a low flame at first, increasing the flame as danger of frothing decreases. Digest for about 15 minutes after the solution "clears". Be sure that all material washes down from the neck of the flask. Total digestion should take about 40 minutes.

Allow to cool and add about 15 ml of water just before solution solidifies.

Stopper the flasks if they are to stand. Distillation may be started at once or the flasks may stand for as long as a day or two if stoppered. Make sure that there are no fumes from  $NH_4OH$  in the hood during the time Kjeldahls are being run or the acid will absorb the ammonia and erroneously high values will be obtained.

### B) Distillation:

Make sure steam-generating flask is originally about two-thirds full of water. Do not let water level go much below one quarter.

Heat steam-generating flask to boiling with drain tube open. Introduce digested sample from Kjeldahl flask into middle flask, i.e. between generator and condenser. Rinse Kjeldahl flask with several washings of water, adding these to flask.



Add 20 ml of 40 percent NaOH, several drops of phenolphthalein, and thoroughly rinse-in base and indicator with water.

Close stopcock, place 50 ml beaker containing 10 ml 2 percent boric acid and five drops of brom-cresol green-methyl red indicator under condenser outlet with tip beneath surface of solution. Close drain tube from steam-generating flask.

Distill until 50 ml beaker is about two-thirds full. Prevent strong drafts which might cool steam-generator and cause boric acid solution to be sucked over into middle flask.

At end of distillation period, lower the contents of the beaker below tip of condenser for a minute or two, rinse off tip of condenser with water and remove beaker and contents.

Remove flame from steam-generating flask and contents of middle flask will siphon into top compartment of steam-generator. Empty by opening drain tube. Apparatus is now ready for next sample.

C) Titration:

The boric acid solution with the indicator will have changed to a clear green with the first drop of distillate. Titrate with the 0.05 N  $H_2SO_4$ . The end point is a light pink. Just before reading the end point, the indicator is neutral or grey in color.

D) Blank:

A blank should be run with each lot of samples. Add cigarette paper to a Kjeldahl flask and proceed as with a sample. Titration for the blank should be about 0.1 ml or less of acid.

Calculation:

Subtract "blank" titration from all other titrations. Then one ml of 0.05 N  $H_2SO_4$  equals 0.7 mg of nitrogen. Report as % N to two decimal places.

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TOTAL NITROGEN OF CULTURE SUSPENSIONS BY MACRO-KJELDAHL

Transfer 50 ml of culture suspension to an 800 ml, Kjeldahl flask. Add approximately 5 grams (1 tsp.) of a mixture of 10 parts anhydrous sodium sulfate and 1 part copper sulfate and then add 25 ml of concentrated sulfuric acid. Place a few glass beads in the flask and mix ingredients by swirling the flask.

Place the digestion mixture over low heat on the digestion apparatus. Watch closely those samples high in sugar during the carbonization of the sample in the early stage of digestion so as to avoid loss of the sample through frothing. When the danger of frothing is past, digest with higher temperature and continue the heating until the solution is clear green in appearance (about 2 hours).

While the digestion flask is cooling, set up the receiving flasks on the distilling apparatus. Use a 500 ml Erlenmeyer flask as the receiving vessel, and approximately 40 ml of saturated boric acid indicator solution (Method 19). With the receiving flasks in place, the tube from the condenser must extend below the surface of the boric acid solution.

When the digest has cooled, add 200 ml of distilled water, and cool a second time.  
CAUTION HERE! When the diluted digestion mixture is thoroughly cool, add carefully 100 ml of 40% NaOH solution down the side of the flask. Layer the NaOH on the bottom of the flask and do not agitate flask. If contents of flask are mixed at this point the reaction is violent and dangerous. Add additional beads, boiling chips or powdered Zn to reduce bumping. Attach the flask to the distilling apparatus without agitation.

With the flask in place on the distillation rack, mix the layers in the flask by gentle rotation at first, followed by increasingly greater agitation until contents are thoroughly mixed so as to avoid explosion on subsequent heating. Start the burner immediately with low heat and commence heating the mixture as soon as agitation is complete.

Collect approximately 150 ml of distillate in the boric acid. During the distillation the  $\text{NH}_3$  is volatilized in the alkaline digestion flask and passes through the condenser into the receiving solution.

When distillation is complete first withdraw the receiving flask and then turn off the burner.

Titrate with N/14 sulfuric acid to a change from green to a permanent, faint pink endpoint.  $\text{NH}_3\text{-N}$  in the sample is calculated:

$$\text{mgm NH}_3\text{-N} = (\text{ml of N/14 acid}) (\text{acid factor}), \text{ where}$$

$$\text{acid factor of N} = (\text{normality of standard acid}) (\text{equivalent weight of N})$$