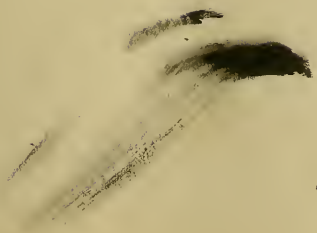

SOIL BIOLOGY

LABORATORY MANUAL

BY

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FIRST EDITION

NEW YORK
JOHN WILEY & SONS, INC.
LONDON: CHAPMAN & HALL, LIMITED
1917

Stanhope Press
F. H. GILSON COMPANY
BOSTON, U.S.A.

PREFACE.

Soil biology treats of the microörganisms which inhabit soils in their relation to soil fertility, crop production, and permanent agriculture. It includes the rapidly developing fields of soil bacteriology, soil protozoölogy, soil mycology, and others which may later merit study. The purpose of this manual is to present the important principles of soil biology, particularly as they point to the intelligent control of the essential elements of plant food. The principles are incorporated in practices which acquaint the student with the various forms of life in the soil and their activity. Special attention is given to all biochemical reactions influencing soil conditions. The sequence of arrangement of the practices is not fixed but that given has been found best in this laboratory. The choice of materials which are tested is based upon farm practice. Students are encouraged to undertake these studies on their own soils.

The laboratory course is a part of a five-hour course consisting of two lectures, one quiz, and three laboratory periods per week.

Soil fertility and bacteriology are prerequisites while organic chemistry and plant physiology are desirable for the course as outlined.

The questions, problems, and references accompanying the practices have been found by experience to be valuable supplements in fixing the principles and applying the information obtained.

Emphasis is laid upon quantitative results and the measure applied consists of biochemical and chemical methods. The results thus obtained are interpreted as far as possible in terms of soil fertility and crop yields.

In Part II are included bacteriological, chemical, mechanical, and pot-culture methods as applied or developed in this laboratory. In the last section will be found Suggestions for Instructors and Students Preparing to Teach. An attempt has been made to satisfy the demand to have this information ever at hand and in a classified form.

This little work would not be complete without an acknowledgment to Professor C. G. Hopkins for suggestions and encouragement in its development and to Mr. Warren R. Schoonover, assistant in soil biology, whose able assistance and careful observations have proved invaluable; also to certain former graduate students for testing new methods.

A. L. WHITING.

URBANA, ILLINOIS,
February, 1917.

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PART II

PART I.

LABORATORY PRACTICES.

SOIL BIOLOGY

PRACTICE 1.

EXAMINATION OF MICROÖRGANISMS IN SOILS AND MANURES.

Many kinds of microörganisms inhabit soils and manures. Their presence in soils is essential to agriculture in general since the soil is the basis of all agriculture. It is also true that life would cease on the earth were it not for the activity of these organisms.

Study the typical forms as they occur in their natural media as outlined below:

(*a*) Prepare an infusion of each of the following samples:

1. Fresh horse manure.
2. Sandy loam.
3. Rich loam.

Place 50 grams in 100 cc. of sterile water contained in a 200 cc. sterile Erlenmeyer flask. Shake vigorously for 5 minutes and then allow it to stand until after the following procedure has been carefully carried out.

(*b*) 1. Place the microscope on the table in a position which permits of comfortable use.
2. Bring the draw tube to standard length.
3. Remove the eyepiece and arrange the plane mirror, using Abbé condenser, so that the field of light is clear and free from obstructions such as window bars, trees, etc.
4. The illumination should be central. (Transmitted axial light.)
5. Examine with medium power a specimen of algæ, diatom, a sand or soil grain, cotton fiber or an air-bubble.

6. Change the illumination to oblique (transmitted oblique light) by placing the finger below and half over the light opening of the iris diaphragm of the condenser. Note the advantage of oblique light for surface and morphological studies.
7. Repeat numbers 5 and 6, varying the opening of the iris diaphragm as follows: Wide open, open $\frac{1}{2}$, and $\frac{2}{3}$ the diameter of the rear lens opening of the objective. This is determined by removing eyepiece and looking into the tube.
8. Focus oil immersion objective and lower it with coarse adjustment until it comes into contact with the oil. Determine this by watching carefully with the head to one side, complete focusing with fine adjustment. Always focus upward when looking into the instrument.

Examine each in the following manner: By means of a sterile glass rod remove two drops of the liquid. Place it upon a clean slide. Examine with low, medium, and oil immersion objectives, using a cover slip. In a similar manner, make stained preparations on the slides, using carbol-fuchsin, methylene blue, gentian violet, and iodine, and examine with oil immersion objective. Do not use cover slips for the stains on the slides. Note the different forms present and which one predominates. Examine for cells of higher plants, fungous growths (mycelial and sporal), algæ, diatoms, and protozoa (amœbæ, ciliates, flagellates).

Material	Different forms found	Predominant class of bacteria		
		Bacillus	Coccus	Spirillum

References.

1. U. S. Dept. Agr., O. E. S., Bul. 194, 6, 7, and 13.
2. Read the booklet accompanying your microscope.
3. Elementary Chemical Microscopy, Chamot (1915), 1–53, 102–158.
4. Bacteriological methods, pages 91–93, this manual.

Questions.

1. Which of the microörganisms found are vegetable and which animal?
2. Discuss the distribution of bacteria in soils.
3. How do the microörganisms obtain their food in a soil?
4. Name the sources of the twelve essential elements for soil microörganisms.
5. Which class of the microörganisms is the most important and why?

PRACTICE 2.

OCCURRENCE OF BACTERIA AT DIFFERENT DEPTHS IN SOILS.

QUANTITATIVE BACTERIOLOGICAL EXAMINATION OF SOILS.

Fresh samples of the surface soil ($6\frac{2}{3}$ inches) and the subsoil (at 35–40 inches) are collected in the manner prescribed on page 121.

Place 100 grams of the soil in a sterile 400 cc. shaker bottle and add 200 cc. of sterile water. Submit the mixture to five minutes shaking in the mechanical shaker or by hand. This soil infusion is used for inoculating purposes. Allow it to settle 15 minutes to facilitate measuring. By means of sterile pipettes make the following dilutions:

2 cc. of the infusion in 98 cc. of sterile water (*A*) 1–100.
10 cc. of (*A*) into 90 cc. of sterile water (*B*) 1–1000.
10 cc. of (*B*) into 90 cc. of sterile water (*C*) 1–10,000.
10 cc. of (*C*) into 90 cc. of sterile water (*D*) 1–100,000.
10 cc. of (*D*) into 90 cc. of sterile water (*E*) 1–1,000,000.
10 cc. of (*E*) into 90 cc. of sterile water (*F*)* 1–10,000,000.
20 cc. of (*F*) into 80 cc. of sterile water (*G*)* 1–50,000,000.

Boiling flasks containing the correct amounts of sterile water will be found on the supply shelf. Letter the flasks as above. Place 1 cc. of dilutions (*D*), (*E*), (*F*), and (*G*), with sodium asparaginate or synthetic agar. Place the 1 cc. in the sterile Petri dish and pour the agar quickly, tilting the dish to effect uniform seeding. Reserve dilutions (*C*) and (*D*) for further use in practices 3 and 4. When cool, place in the Petri dish containers and invert

* (*F*) and (*G*) not necessary if a poor soil.

the container. Place in room-temperature incubator. Count plates after 3–4 days. After counting, replace plates in incubator and allow the colonies to develop for a week or 10 days. Make further observations on the growth, using the Society of American Bacteriologists' Chart for descriptive notes. Consult laboratory charts for identifying colony characteristics.

Each student should enter his results together with the results of another student using the same soil on the data sheet below.

Date	Soil surface	Medium	Incubation		Dilution	Number per 1 cc. of dilution	Av. per gram of soil	No. per acre	Notes
			Temp.	Time					
					Average				
	Sub-soil								
					Average				

References.

1. Agricultural Bacteriology, Percival (1910), 118–124.
2. Iowa Exp. Sta., Research Bul. (1912), 8.
3. U. S. Dept. Agr., O. E. S., Bul. 194, 8–13.

Problems.

1. Calculate in pounds per acre the dry weight of the bacteria found in this soil.

500 million dry bacteria weigh 0.2 milligram, living 1 milligram.

2. Calculate in cubic feet the volume occupied by the living bacteria in this soil.

100 million occupy 0.2 cubic millimeter.

3. How many pounds of nitrogen and phosphorus are contained in the bacterial bodies of an acre, as based upon the dry weight figures obtained under number one?

Analysis of Bacteria.

(Dry Basis.)

Nitrogen 2.3 per cent. Phosphorus 1.2 per cent.

Questions.

1. Do numbers of bacteria in a soil indicate efficiency as to biochemical reactions?
2. Explain the differences found between the surface and subsoil?
3. How does the number of bacteria compare with the number of soil particles in a gram of a silt loam? Explain the reason for this difference.

PRACTICE 3.

OCCURRENCE OF NON-VEGETATIVE FORMS OF BACTERIA AND FUNGI IN SOILS.

The number of non-vegetative forms is determined in samples of the surface soil of the same type as that used in the previous practices. Add to each tube of melted agar (synthetic or sodium asparaginate) and melted fungi gelatin 1 cc. of dilution (*d*). Heat duplicate tubes of each medium at the following temperatures, 70°, 85°, and 100° C. for ten minutes. Pour plates at 40–42° C. and, when cool, place in Petri dish container, invert and place them in the room-temperature incubator. Examine at the end of two days. Count in the manner already described after 3–4 days. Return the plates to the incubator and allow the colonies to further develop for 2 weeks or longer. Note pigment formation and colony characteristics. Observe which form disappears at the various temperatures. Calculate the percentage of the total number that are in the non-vegetative stage.

Date	Soil	Medium	Incubation		Dilution	Heated to temp.	Number per 1 cc. of dilution	Av. per gram of soil	No. per acre	Notes
			Temp.	Time						

Question.

1. How do you explain the presence of the non-vegetative forms in normal soils?

PRACTICE 4.

OCCURRENCE OF FUNGI AT DIFFERENT DEPTHS AND IN DIFFERENT SOILS.

Prepare 1–10,000 dilutions of the surface soils and 1–1000 dilutions of the subsoils used in practice 2. Plate in duplicate 1 cc. of each dilution employing the fungi gelatin. Incubate at room temperature for 3–4 days or until the colonies are easily recognized as fungi. Make the counts at this time, using the binocular or hand lens. Study the microscopic appearance of the colony. Draw the typical colonies under the binocular. Allow the fruiting bodies to mature. Examine the mycelia and fruiting bodies carefully. Which are septate or non-septate? Are the fruiting bodies perfect or imperfect? The $\frac{1}{6}$ objective is used for the fruiting bodies.

Date	Soil	Medium	Incubation		Dilution	Depth	No. per cc.	Av. per gram	No. per acre	Notes
			Temp.	Time						

References.

1. Household Bacteriology, Buchanan (1913), 50–84, 487–523.
2. Cornell Agr. Expt. Sta., Bul. 315 (1912), 415–419, 437–501.

Questions.

1. What kinds of fungi inhabit soils?
2. Which of these kinds predominate?
3. Of the factors necessary for growth which are most important for soil fungi?
4. In the struggle for food how are soil fungi at an advantage?

PRACTICE 5.

AMMONIFICATION IN SOILS.*

INFLUENCE OF MOISTURE CONTENT ON THE PROCESS.

Ammonification, which is the production of ammonia from organic compounds by microörganisms, is greatly influenced by the moisture content of a soil. There is an optimum moisture content for all bacterial activities in soils and it is determined as outlined in this exercise with the exception that periodic determinations are usually made while here one suffices to show the influence the moisture factor exerts on this process.

On the bulletin board will be posted the soil type, the organic matter (kind and amount) and the amount of sterile water to add to each treatment in addition to that indicated below.

Weigh ten 100 gram portions of the air-dry sieved soil into the jelly glasses. Add the organic matter with a sterile spatula and thoroughly mix. Add the sterile water as indicated below with a sterile pipette slowly and evenly throughout the entire mass. Leave the surface level.

1 and 2	6 cc.
3 and 4	12 cc.
5 and 6	18 cc.
7 and 8	24 cc.
9 and 10	30 cc.

* Organisms concerned in the liberation of ammonia from organic compounds are not isolated in this course as they have been studied in the general course in bacteriology. They are furnished to those students who wish to further study them from pure cultures.

Place the tin covers on the glasses and set them in the room-temperature incubator. After 14 days remove and transfer contents to a Kjeldahl flask. Determine ammonia by direct distillation with magnesium oxide as outlined on page 112.

Group..... Soil Type..... .

Blank on Method.....mgs. 1 cc. NH_4OH.....mgs. N.

Date	Sample number	Period of incubation, days	Treatment	HCl ⇌ NH_4OH		Titrated back, NH_4OH	Equivalent in sample, NH_4OH	Mgs. N	Notes

References.

1. Consult the data sheets on the reference shelf.

Problems.

1. Prepare a graph on cross-section paper, the abscissas representing percentages of moisture, the ordinates percentages of nitrogen recovered of the initial applied.

Nitrogen content of the materials used will be found on the bulletin board.

2. Show by chemical equations the reactions which yield ammonia from protein, protein derivatives, amides, and amino acids.

3. Tabulate in your laboratory manual the names of ten typical ammonifiers.

Questions.

1. Ammonification is the result of cell activity; what are the active agents which enable the cell to assimilate and digest organic material?

2. How does moisture influence these agents?

3. What farm crops are able to utilize ammonia directly?

4. How does ground limestone and rock phosphate influence ammonia production?

5. What is the normal ammonia content of the corn-belt soil?

6. What influence does the mechanical composition of a soil exert on ammonification?

7. How does chemical composition influence ammonification?

8. What effects do the biological factors have on ammonification?

PRACTICE 6.

AMMONIFICATION OF UREA AND ISOLATION OF UREA ORGANISMS.

Place 20 cc. of urea solution in each of six 200 cc. Erlenmeyer flasks, plug and sterilize in the autoclave at 10 pounds pressure for 10 minutes.

Inoculate as follows:

1 and 2, nothing (sterile).
3 and 4, 1 gram of fresh soil.
5 and 6, 1 gram of fresh horse-manure.

Place in the incubator at room temperature and after 48 hours remove 5 cc. of each treatment with a sterile pipette, filter and titrate against standard acids. Use weak acid for No. 1 and 2, strong acid for the others. Make a second titration in a similar manner 24 hours later. Calculate the per cent of urea changed at each period.

Isolation of Urea Organisms. — Pour plates of each of the soil and manure treatments by transferring a loopful to 10 cc. of sterile water. From these dilutions, inoculate tubes of sterile, liquefied, and cooled (40° C.) urea agar. Plate as usual. Incubate 2–4 days at 20° C. and transfer after 4 days to other plates and further transfer until pure cultures are obtained.

Stain the organisms and examine in a hanging drop. Describe them carefully as to size, shape, motility, and rate of ammonia production. Inoculate tubes containing 10 cc. of sterile urea solution with a loopful from typical colonies. Note turbidity. Titrate to obtain ammonia production at end of 2 days. Determine whether the

ammonia is produced under aërobic or anaërobic conditions by heating 10 cc. of urea solution to expel dissolved gases; cool and inoculate, immediately covering the surface with 1 inch of heavy oil and plugging the test tube tightly with cotton.

Group..... Soil Type.....
Blank on Method.....mgs. N. 1 cc. NH_4OH.....mgs. N

Date	Sample number	Period of incubation, days	Treatment	HCl ⇌ NH_4OH		Titrated back, NH_4OH	Equivalent in sample, NH_4OH	Mgs. N	Notes

References.

1. Handbuch der technischen Mykologie, Lafar (1904–6), **3,** 71–85.
2. Centbl. f. Bakt. 2 Abt. (1913–14), **39,** 209–358, plates after 358.

Problem.

1. Calculate how many pounds of nitrogen, as nitrate, are possible from the urea occurring in the urine of one cow, for a year, if 20 pounds of urine are voided per day.

Questions.

1. Write the chemical reactions showing the ammonification of urea and the calories yielded.
2. Are the common ammonifiers able to decompose urea?
3. Of what special importance are the urea organisms?

PRACTICE 7.

NITRITATION.

OXIDATION OF AMMONIA TO NITRITE BY NITROSOMONAS.

Influence Exerted by Carbonates, Ignited Soil, Soluble Organic Matter, and Aëration.

(*This experiment also illustrates denitrification.*)

Place 25 cc. of the salt solution for nitrite formation (page 95) in each of 12 one-liter Erlenmeyer flasks (ratio of depth to diameter 1 : 20–22).

Make the following additions:

1 and 2, nothing.
3 and 4, 1 gram ground limestone or dolomite.
5 and 6, 1 gram magnesium carbonate.
7 and 8, 50 grams ignited soil.
9 and 10, 50 grams ignited soil + 1 gram limestone or dolomite.
11 and 12, 0.5 gram dextrose + 1 gram limestone or dolomite.

Some students will perform this practice using 100 cc. flasks (ratio, depth to diameter, 1 : 3–4), plugging tightly with cotton. Plug the flasks loosely and sterilize in the autoclave at 12 pounds pressure for 15 minutes. When cool, add with a sterile graduated pipette the required amount of a standard solution of ammonium sulfate, carbonate, or nitrate, to give 10 milligrams of nitrogen per flask.

Inoculate each with 1 gram of fresh soil obtained preferably from some continuous soil or crop experiment. Place flasks in the 30° or the room-temperature incubator

as indicated by instructor. At the end of 2, 3, and 4 weeks test all for nitrite by the method outlined on page 116. Show each test to the instructor. The quantitative determination will be made upon advice of the instructor.

Determine the nitrite in 1–10 inclusive by the permanganate method (see page 113).

Group..... Soil Type.....
Blank on Method...mgs. N. 1 cc. N/10 $KMnO_4$.....mgs. N.

No.	Treatment	cc. N/10 $KMnO_4$ taken	cc. N/10 NaS_2O_3 titrated	Equivalent N/10 $KMnO_4$	Mgs. N	Per cent of initial nitrogen changed	Qualitative tests							

References.

1. U. S. Dept. Agr., O. E. S., Bul. 194, 57–67.
2. Agricultural Bacteriology, Percival (1910), 134–168.
3. N. J. Agr. Exp. Sta. Ann. Rept. (1908), **29**, 117–119.

Questions.

1. Write the reaction for this oxidation, using ammonium sulfate, carbonate, and nitrate in the presence of calcium carbonate.
2. Do nitrites exist in normal soils?
3. Are nitrites assimilable by plants?

4. What three factors are most detrimental to the growth of this organism under field conditions?

5. Give the notable exception exhibited by this organism in its nutrition.

6. What is the chief source of this element?

7. Name 10 elements which will suffice to neutralize the acid formed.

8. What substances retard and check nitritation and in what concentrations?

9. What substances accelerate nitritation?

10. Discuss the importance of nitrite production from the standpoint of the liberation of insoluble minerals.

PRACTICE 8.

NITRATATION.

OXIDATION OF NITRITE TO NITRATE BY NITROBACTER.

Influence Exerted by Carbonates, Ignited Soil, Soluble Organic Matter, and Aëration.

(*This experiment further illustrates denitrification.*)

Place 25 cc. of the salt solution for nitrate formation (page 95) in each of 12 one-liter flasks. Make the following additions:

1 and 2, nothing.
3 and 4, 1 gram ground limestone or dolomite.
5 and 6, 1 gram magnesium carbonate.
7 and 8, 0.025 gram sodium carbonate.
9 and 10, 50 grams ignited soil.
11 and 12, 0.5 gram dextrose + 1 gram ground limestone or dolomite.

As in the previous practice, this practice will also be conducted with 100 cc. flasks plugged tightly with cotton. Plug flasks loosely and sterilize in the autoclave at 12 pounds pressure for 15 minutes. When cool add to each the required amount of standard sodium nitrite solution which gives 10 milligrams of nitrogen per flask.

Inoculate each flask with 1 gram of soil as in the previous practice. Place flasks in the 30° or room-temperature incubator as indicated by instructor. At the end of one week test qualitatively for nitrate by the method given on page 117. At the end of 2 weeks test for nitrites by the method used in the previous practice and then determine nitrate nitrogen in all but 11 and 12 by the method given on page 113.

Group..... Soil Type.....
Blank on Method.....Mgs. 1 cc. NH_4OH.....Mgs.

No.	Treatment	HCl	NH_4OH	Titrated back, NH_4OH	Equivalent in sample, NH_4OH	Mgs. N	Per cent of initial N changed	Qualitative tests				

References.

1. Centbl. f. Bakt. 2 Abt. (1899), **5,** 329, 377, 429.
2. Centbl. f. Bakt. 2 Abt. (1910), **27,** 169.
3. Science (1912), **35,** 996.
4. Expt. Sta. Record (1908–9), **20,** 518, 519–520.
5. Ohio Expt. Sta. Bul. Tech. Series (1915), **7.**
6. Ann. Inst. Pasteur (1904), **18,** 181–196.

Problem.

1. Calculate the pounds of carbon dioxid reduced when 35 pounds of nitrogen are oxidized.

Questions

1. What is the function of the base in this reaction?
2. Explain the effect of sodium carbonate.
3. How is the effect represented by the action of sodium carbonate obviated under field conditions?

4. What is the cause of the action produced by the ignited soil?
5. Name ten nitrites which are changed to nitrates.
6. What substances retard or check nitratation, and in what concentrations?
7. What substances accelerate nitratation?
8. Write the reaction for this transformation.
9. Discuss the value of nitrates compared with nitrites, ammonia, and soluble organic nitrogen in soils.

PRACTICE 9.

ISOLATION AND STUDY IN PURE CULTURE OF NITROSOMONAS AND NITROBACTER.

In the isolation of the nitrite and nitrate organisms advantage is taken of the already vigorous growths existing in treatments 9 and 10 of practices 7 and 8. When the qualitative tests have given a strong reaction (consult instructor for advice at this time), follow the procedure outline below.

Isolation of Nitrosomonas. — *Procedure:*

1. Place 1 cc. of the solution (numbers 7 and 8 in practice 7) in a sterile Petri dish.

2. Add 10 cc. of sterile silica jelly (Solution I) in the Petri dish and thoroughly mix.

3. Add 1 cc. of sterile solution of sodium carbonate and ammonium sulfate (Solution II), and immediately tilt to mix the contents of the dish as the jelly solidifies rapidly.

Caution should be exercised in the manipulation described under 3 as rough plates resulting from uneven mixing of the carbonate solution with the jelly make it difficult to see the colonies. The plates should be left level until solid. An excess of moisture is undesirable and will not occur if the above procedure is followed. After the medium has become solid, label the plates and place them in Petri dish containers and incubate at 30° C. Allow the colonies to develop until the centers become yellow or orange, when the microscopical study should begin. Test colonies with nitrite and nitrate reagents before transferring. Transfer from typical colonies to magnesium ammonium phosphate agar. When typical colonies are well developed (4–6 days), transfer to the

sterile magnesium plaster of Paris blocks, which are half submerged in a solution for nitrite formation, in Petri dishes. Inoculate silica jelly and agar slants. Study the colony characteristic on the various media. Stain the organism with carbol-fuschin, gentian violet, and methylene blue. Study the size and shape, and compare with nitrate organism. Prepare a permanent slide. Inoculate a small sterile flask containing ignited soil and solution for nitrite formation and incubate at 30° C. Test for nitrite production at the end of 5 days. This method together with the microscopic study will determine if your culture is pure.

Isolation of Nitrobacter. — Proceed as in the isolation of *Nitrosomonas*, using instead inoculating solution from 9 or 10, practice 8, and 1 cc. of a solution of sodium carbonate and sodium nitrite (Solution III). Omit the use of magnesium ammonium phosphate agar, otherwise make similar studies with this organism and inoculate a small flask after studying the organism in pure culture.

References.

1. Exp. Sta. Rec. (1890), **2,** 751–757 (Winogradsky).
2. Jour. Chem. Soc. (London) (1878), **33,** 44 (1898), **59,** 484 (Warington).
3. Jour. Chem. Soc. (London) (1891), **60,** 352 (Franklands).

Questions.

1. From the qualitative tests which organism is the more rapid grower and what are the comparative rates of oxidation?
2. Give the group number for both these organisms (Soc. Am. Bact. Chart).

PRACTICE 10.

PRODUCTION OF INORGANIC NITROGEN IN SOILS.

COMPARATIVE AMMONIFICATION AND NITRATATION OF CROP RESIDUES, GREEN MANURES, AND FARM MANURES IN TYPICAL SOILS.

Influence Exerted by Carbonates, Soil Type, Moisture, and the Condition of the Organic Substances.

This experiment is designed to show the weekly ammonia and nitrate production from organic materials, such as are used in agricultural practice and under as nearly similar conditions as possible. Such materials as clover, sweet clover, soybeans, cowpea, and alfalfa hays, corn stalks, wheat and oat straw, and farm manures are applied in both the green and dry condition according to common usage.

It would be advisable to use fresh soil for this practice if convenient. The materials applied in the dry condition should all pass a 10-mesh sieve. Green materials may be applied much coarser.

This practice is conducted in groups. Students are permitted to use soils from their own farms or ones in which they are particularly interested. Each group of students conducts four sets of treatment on a given soil.

Weigh out fifty-six 100 gram portions of the soil to be studied. Place in the jelly glasses and make the following applications:

1–14, nothing.
15–28, 1 gram of carbonate (limestone or dolomite).
29–42, organic matter.
43–56, 1 gram carbonate + organic matter.

The students are permitted to choose the kind of organic matter they desire to test and the amounts to be added are posted.

The sterile water to be added will be found on the bulletin board and is the optimum for the various treatments.

Mix thoroughly and place glasses in the room-temperature incubator. Each week 1–3 cc. of sterile water is added to each glass to compensate for loss due to evaporation.

Ammonia and nitrate determinations are made on duplicates of all treatments at the beginning of the experiment, and every 7 days, for 7–10 weeks.*

The sample is divided into two equal parts by weight. On one-half determine the ammonia by direct distillation or by aëration. Dry the other half at 108° C. for 6–8 hours in the electric oven, then add 300 cc. of dilute hydrochloric acid (5 cc. per liter). Shake vigorously several times. Allow the solution to settle a few minutes when 200 cc. is removed by suction. Proceed as indicated for the determination of nitrites and nitrates, page 113.

If the total nitrogen content is not known it will be necessary to determine it on the soil used. The total nitrogen content of the typical soil types and the organic materials will be posted.

* It is sometimes convenient to make the intervals 9 and 11 days to conform to the laboratory periods.

Other materials such as raw rock phosphate are used in this experiment. The number of jelly glasses may be increased to extend through the growing period of a crop.

AMMONIA DETERMINATIONS.

Date	Sample No.	Treat-ment	HCl	≎ NH_4OH	Titrated back, NH_4OH	Equiva-lent in sample, NH_4OH	Mgs. N	P.P.M.	Notes

AMMONIA DETERMINATIONS.

Date	Sample No.	Treatment	HCl	≎ NH_4OH	Titrated back, NH_4OH	Equivalent in sample, NH_4OH	Mgs. N	P.P.M.	Notes

NITRATE DETERMINATIONS.

Date	Sample number	Treatment	Weight of soil taken	CC. solution taken	HCl	NH_4OH	Titrated back, NH_4OH	Equivalent in sample, NH_4OH	Mgs. N	P.P.M.	Notes

NITRATE DETERMINATIONS.

Date	Sample number	Treatment	Weight of soil taken	CC. solution taken	HCl	NH$_4$OH	Titrated back, NH$_4$OH	Equivalent in sample, NH$_4$OH	Mgs. N	P.P.M.	Notes

SUMMARY OF THE NITROGEN DETERMINATIONS.

Group..... Soil Type.....
Group..... Soil Type.....

Treatment	Nitrogen as	Parts per million of nitrogen in water-free soil									Pounds per acre
		Date of determinations									
		Beginning								Av.	
	Nitrate Ammonia										
	Nitrate Ammonia										
	Nitrate Ammonia										
	Nitrate Ammonia										
	Nitrate Ammonia										
	Nitrate Ammonia										
	Nitrate Ammonia										
	Nitrate Ammonia										

References.

1. Soil Fertility and Permanent Agriculture, Hopkins, 194–198.
2. Soil Conditions and Plant Growth, Russell (1913), 78–89.
3. Centbl. f. Bakt. 2 Abt. (1908), **25,** 64.
4. Centbl. f. Bakt. 2 Abt. (1910), **27,** 169–186. Exp. Sta. Recd. (1910), **23,** 721.
5. Hawaii Agr. Exp. Sta. Bul. 39 (1915), 24–25.
6. Jour. Indus. & Eng. Chem. (1915), **7,** 521.
7. N. J. Rept. of Soil Chemist and Bacteriologist (1914), 217, 220.

Problems.

1. Plot the ammonia and nitrate data for all treatments on cross-section paper, the abscissas representing the time and the ordinates the milligrams of nitrogen as ammonia and nitrate. Use red for nitrate, blue for ammonia and solid, broken, single, and double dotted lines for the treatments.

2. Explain the fluctuations of both the ammonia and nitrate curves.

3. Calculate the yield of oats, wheat, and corn possible from the nitrate and ammonia found.

4. Correlate the ammonia and nitrate production on the soil used with crop yields.

Questions.

1. Under field conditions is there need of an application of nitrate nitrogen?

2. Does nitratation go on in acid soils?

3. Do non-nitrifiable soils exist?

4. How should a non-nitrifiable soil be treated?

5. How does the crop influence nitrate and ammonia formation?

6. What effect does cultivation have on nitratation?

7. Are nitrifying organisms active in the fall and winter months?

8. What means may be used to check the loss of ammonia and nitrate from soils?

9. Is the loss of ammonia from the brown silt loam appreciable?

10. Why does organic matter not inhibit the growth and activity of these organisms in soils?

11. What factors are most important in nitrate production in field soils?

12. What factors are most important in ammonia production in field soils?

13. What is the average annual loss of nitrogen per acre?

14. Is there any nitrification below the surface soil?

15. How does the nitrate content of a soil vary from the surface soil to a depth of 5 feet?

PRACTICE 11.

CARBON DIOXID PRODUCTION.

Carbon dioxid is produced by respiration of the microorganisms in soils. It is evolved from soil into the air in large amounts. A large amount of carbon dioxid bathes the soil and liberates insoluble elements by the production of acid and salts. The importance of the carbon cycle is understood.

The rate and amount of carbon dioxid evolved depends upon many factors, chiefly the kind, amount, and stage of decomposition of organic matter present in the soil. A determination of nitrogen as ammonia and nitrate makes possible a calculation of the carbon nitrogen ratio of decomposition.

Place six 100-gram portions of the soil to be tested in a beaker and add the organic materials. Thoroughly mix and place in 500 cc. Erlenmeyer flasks equipped with glass tubes on the end of which are rubber tubings which may be opened for the admission of air and a pair of Wortmann valves (Greiner and Friedricks, Cat. No. 3459), one above the other. Into both valves place 10 cc. of standard potassium hydroxid. The carbon dioxid from the soil is collected in the lower valve, while the upper valve serves as a trap collecting carbon dioxid from the air.

Arrange as follows:

1 and 2, soil alone.
3 and 4, soil + 2 grams dextrose.
5 and 6, soil + 2 grams organic matter.

Place the stopper containing the glass tube and valves in the flask and allow the flasks to remain at room temperature in a reasonably shaded place (dark not necessary).

Every two days remove the valves and titrate the contents of the lower one as indicated on page 12. At the end determine the ammonia on 50 grams and the nitrate on 50 grams of treatment numbers 5 and 6.

Group..... Soil Type.....

10 cc. KOH.....cc....Acid 1 cc. KOH.....mgs. CO_2

Number	Treatment	Milligrams carbon dioxid — Days	P.P.M. total CO_2	P.P.M. NH_3	P.P.M. NO_3	CO_2/NO_3 ratio

References.

1. Soil Fertility and Permanent Agriculture, Hopkins, 33.
2. Soil Conditions and Plant Growth, Russell (1913), 78.
3. Ill. Agr. Exp. Sta. Bul. 145, 105–111, 121.
4. U. S. Dept. Agr., O. E. S. Bul. 194, 55–56.
5. Iowa Exp. Sta. Research Bul. 3, 135–154.
6. Jour. Agr. Sci. (1915), **7**, 44.
7. Centbl. f. Bakt. 2 Abt. (1910), **28**, 45.

Problems.

1. Calculate how long the carbon supply of the atmosphere over an acre would be sufficient for 100 bushel crops of corn if micro-organisms failed to maintain the supply.

2. From the figures obtained in this practice, what can be deduced as to the stage of decomposition of the soil and the organic matter used? Indicate this by carbon, nitrogen ratios.

Questions.

1. How does carbon dioxid originate from soil organic matter?

2. Which classes of organisms are most active in carbon dioxid production and under what conditions?

3. What per cent of carbon dioxid is found in a normal soil atmosphere?

4. What factors are important in the fluctuations occurring in the carbon dioxid content of the soil atmosphere?

5. In what way does carbon dioxid prove injurious?

6. What beneficial action does it produce?

7. How would you overcome the injury arising from planting immediately after plowing under a green crop?

PRACTICE 12.

CELLULOSE DECOMPOSITION.

AËROBIC DECOMPOSITION BY FUNGI AND BACTERIA.

Contrary to earlier conceptions, the decomposition of cellulose and fibrous residues takes place very actively under aërobic conditions. Fungi play a very important rôle in this decomposition.

Place 2 grams of filter paper, cut into squares of about $\frac{1}{8}$ of an inch, and 2 grams of corn stover or straw in jelly glasses with 100 grams of soil. Add sterile water to make the optimum moisture content. Place all in the 30° C. incubator for 10–14 days, observing the fungous growth at frequent intervals.

1 and 2, paper 2 grams.
3 and 4, straw or stover 2 grams.
5 and 6, soil, only.

Plate from 1, 3, and 5 on cellulose and starch agar. Incubate at 30° C. for 3 weeks, then transfer to cellulose and starch agar. At this time expose plates of cellulose and agar to the laboratory air for 5 minutes. Study the dissolving of the cellulose in the zone around the colony. Stain the organisms with carbol-fuchsin. Describe the organism and draw colonies and individuals. Allow 2, 4, and 6 to remain 8–10 weeks, when an examination of the residual cellulose or stover should be made under the microscope.

References.

1. Centbl. f. Bakt. 2 Abt. (1904), **11,** 689–695; (1908), **21,** 700; (1909), **23,** 300–304; (1910), **26,** 222–227; (1910), **27,** 1–7, 449–451, 633.

2. Centbl. f. Bakt. 2 Abt. (1912), **34,** 63, 485–494.
3. U. S. Dept. Agr. Bur. Pl. I. Bul. 266 (1913).
4. Soil Science, 1916, **1,** 437–487.

Questions.

1. What kinds of fungi decompose cellulose?
2. What kinds of bacteria decompose cellulose?
3. Is there a selective action exhibited by these organisms?
4. What products are formed in the aërobic decomposition of cellulose?
5. What is the enzyme concerned?
6. Will the cellulose of a green crop be more easily attacked than that of a dry drop?
7. Name the important processes for which cellulose serves as a source of energy.

PRACTICE 13.

ANAËROBIC CELLULOSE DECOMPOSITION.

In each of five 200 cc. Erlenmeyer flasks, place 150 cc. of solution for anaërobic cellulose decomposition. Weigh accurately 3 sets, of 3 each of $5\frac{1}{2}$ cc. diameter filter paper and place a set in each of three flasks, making sure that the papers are entirely submerged. In the other two flasks, place 3 grams of wheat or oat straw. Plug and sterilize at 12 pounds pressure for 10 minutes.

Inoculate as follows:

1, 2, and 3 with 5 cc. of a filtered infusion of well-rotted horse-manure.

4 and 5 with 5 cc. of a filtered infusion from surface soil.

Place in the room-temperature incubator. After decomposition has started, as judged by the frayed appearance of the edge of the paper, transfer, with a platinum needle, a small portion to a tube to be used in isolating the organism.

Allow these flasks to remain several weeks or until such a time when the papers are apparently decomposed. Note the odor of hydrogen sulfide. Test with lead acetate paper. When decomposition has progressed sufficiently, filter the contents of the flasks on a weighed filter paper, wash with water, dry, and weigh. Report the loss of carbon. Isolate the organisms under anaërobic conditions and test their ability to reduce sulfur compounds and to produce hydrogen sulfide from inorganic and organic sources.

	Paper			Straw		
	1	2	3	4	5	
Initial weight.....						
Final weight.....						
Loss.....						
Weight after drying.....						
Weight of filter paper used as filter.....						
Final weight.....						

References.

1. Microbiology, Marshall (1912), 246–249.

2. Vorlesungen über landwirtschaftliche Bakteriologie, Löhnis (1913), 171–177.

3. Centbl. f. Bakt. 2 Abt. (1902), **8,** 192–201, 225–231, 257–263, 289–294, 321–326, 385–391 (Omelianski); (1904), **11,** 369–377; (1904), **12,** 33–43 (1906), **15,** 673–687.

4. Centbl. f. Bakt. 2 Abt. (1908), **20,** 682; (1912), **34,** 485–494.

Problems.

1. Calculate how many pounds of straw per acre will be decomposed under anaërobic conditions in 6 months at the rate found in this experiment.

Questions.

1. Which decomposes more rapidly the straw or pure cellulose?

2. What are the chief products formed?

3. What beneficial purposes may cellulose serve under anaërobic conditions?

4. Why is an excess of straw or coarse manure often very injurious to soil?

5. For what 3 processes does cellulose serve as a source of energy under anaërobic conditions?

PRACTICE 14.

SYMBIOTIC NITROGEN FIXATION.

INOCULATION OF LEGUME SEEDS.

Growth of Nodules and Determination of Nitrogen Fixed.

(*B. radicicola is to be isolated from nodules obtained in this practice.*)

This practice may be omitted if the students have already had the work given in soil fertility or its equivalent.

Place 12 pounds of washed sand in each of 6 one-gallon earthen jars and in two others place 10 pounds of brown silt loam. The jars may be rinsed with 1–300 mercuric chloride solution and then with sterile water if recently used for similar work. Select 5 of the larger legume seeds (cowpeas) and 15 of the smaller (clover) and sterilize by the method outlined on page 127. Arrange the jars as below, and place them in the greenhouse.

1 and 2, inoculate with soil.
3 and 4, inoculated by the glue method.
5 and 6, uninoculated.
7 and 8, inoculated (plant in soil).

Plant the seeds in the usual manner and water with sterile water. Apply sterile plant food solution every ten days, omitting the nitrogen. After about 10–15 days, depending upon the legume, wash out the plants from numbers 1, 3, and 5, and examine the roots carefully. Study the nodules. Make a drawing showing the manner of attachment, shape, and size, and carefully describe the

appearance as to color and surface. Make cross and longitudinal sections and describe the internal appearance. Compare your description with that of other groups and report the differences found between the various legumes grown. After 20–30 days analyze 3 plants, either green or dry, of each of the inoculated and uninoculated, for total nitrogen.

Remove small nodules from some of the plants washed out in 1 or 3 and proceed with practice 15.

Sample No.	Treatment	HCl ⇌ NH_4OH		Titrated back, NH_4OH	Equivalent in sample, NH_4OH	Mgs. N	Gain from nodules

References.

1. Ill. Agr. Exp. Sta. Cir. 86, Bul. 94.

2. Ill. Agr. Exp. Sta. Bul. 179, 471–482, 493–499, 503–522.

3. Consult article dealing with Legume Inoculation (Reference Shelf).

4. Vorlesungen über landwirtschaftliche Bakteriologie, Löhnis (1913), 361–378.

Questions.

1. Explain why the soil and glue methods are superior to the chemical cultures at the present time.

2. Why is the glue method an ideal method?

3. Explain the method of collecting, storing, and inoculating with soil.

4. Name the legume groups as arranged for cross inoculating under field conditions.

5. What percentage of the total nitrogen is derived from the air by inoculated legumes, on normal soils?

6. Discuss the nitrogen enrichment of a sandy soil, a silt soil, and a heavy clay soil by this symbiosis.

7. Calculate the gain in nitrogen due to inoculation on the basis of standard crops of cowpeas and soy beans.

8. Through what organs do legumes obtain the atmospheric nitrogen?

9. What American scientists first noted and carefully studied nitrogen fixation?

PRACTICE 15.

ISOLATION OF B. RADICICOLA FROM LEGUME NODULES.

If the previous practice was not conducted, proceed as follows: Plant the various legume seeds in both sand and soil in the one-gallon earthen jars, in the ordinary way, inoculating either by the glue method or by an infusion from the nodule.

When the plants in the sand are well started, remove a small nodule with sterile forceps and place in a 1–500 mercuric chloride solution for 3 minutes, remove and rinse in sterile water, pass into three test tubes of sterile water and finally crush in 1 cc. sterile water with a sterile glass rod or sterile forceps. Pour 5 loopfuls in a test tube of saccharose-ash-agar, agitate thoroughly and remove 5 loopfuls to a second tube of the agar. Pour these plates in the usual way and incubate at 30° C. It is essential to transfer several times on the saccharose-ash-agar before making the detailed study. After 10–12 days examine with a hand lens and describe the colony characteristics. Stain the organisms with aniline gentian violet and carbol-fuchsin. Examine in hanging drop. Describe the organisms as to size, shape, motility, and reaction to stain. Make a permanent slide for your own collection. Examine slides of other groups, and make notes on the organisms from the different legumes.

Remove an old nodule from a legume plant growing in the soil and examine the organisms in the hanging drop.

Draw the bacteroids as you see them. Stain the bacteroids with carbol-fuchsin, heating to steaming twice with an interval of two minutes between. Examine some of

the bacteroids from the liquid-culture media which will be furnished.

References.

1. Ill. Agr. Exp. Sta. Bul. 179, 482–488.
2. Centbl. f. Bakt. 2 Abt. (1909), **23,** 59–91.
3. Vir. Agr. Exp. Sta. Ann. Rpt. (1909–10), 123–142, 145–174.
4. Centbl. f. Bakt. 2 Abt. (1907), **19,** 264–272, 426–441.

Questions.

1. Are these bacteroids an involution form?
2. How do they differ from bacilli?
3. In what form does the organism exist in the soil?
4. How long are the organisms viable under field conditions?
5. Does freezing of the soil kill the organisms?
6. What effect does drying a soil have on these organisms?
7. Will these bacteria live in an acid soil?
8. Are these organisms able to fix nitrogen without the legume plant?
9. Discuss the importance of this symbiosis as related to a permanent agriculture.

PRACTICE 16.

NON-SYMBIOTIC NITROGEN FIXATION.

AËROBIC NITROGEN FIXATION IN SOILS.

Influence of Carbonates on the Process.

Fifty grams of soil (see posted sheet for soil type assigned to your group) are placed in each of ten jelly glasses. Treat as follows:

1 and 2, nothing.
3 and 4, 0.3 gm. mannite.
5 and 6, 0.3 gm. mannite and 1 gm. $CaCO_3$.
7 and 8, 15 gms. fresh clover or rye tops.
9 and 10, rich algæ slime, place in the direct light.

The amount of water to be added will be found on the posted sheet. Place in the room-temperature incubator for four weeks, adding 3 cc. sterile water each week and stirring the soil in all but 9 and 10 after adding it. At the end of this time dry the soil and grind it to pass 100-mesh sieve and determine the total nitrogen on 10-gram samples of each treatment. The total nitrogen content of the original soil before incubation together with the nitrogen content of the clover, algæ, carbonate, etc., will be posted.

Group..... Soil Type.....
Blank on Method.....mgs. N. 1 cc. NH_4OH.....mgs. N.

Date	Sample number	Treatment	HCl	NH_4OH	Titrated back, NH_4OH	Equivalent in sample, NH_4OH	Mgs. N	P.P.M.	Notes

References.

1. Centbl. f. Bakt. 2 Abt. (1901), **7,** 561–582. Centbl. f. Bakt. 2 Abt. (1902), **9,** 3–43.

2. N. J. Exp. Sta. Ann. Rpt. (1903), **24,** 217–286; (1904), **25,** 237–268; (1906), **27,** 178–187; (1907), **28,** 141–169; (1908), **29,** 137–147.

3. Wis. Agr. Exp. Sta. Research Bul. 12 (1910).

4. Col. Agr. Exp. Sta. Bul. 155.

5. Soil Conditions and Plant Growth, Russell (1913), 93–95.

6. Ill. Agr. Exp. Sta. Bul. 179, 494.

Problems.

1. How many pounds of wheat straw, corn stover, and fresh clover tops must be oxidized to yield a fixation of nitrogen sufficient to supply a 50 bushel wheat crop and 100 bushel corn crop?

2. Calculate the fixation of nitrogen per acre, subtracting the results obtained in numbers 1 and 2 and the nitrogen content of the added materials from the various treatments.

3. Explain the practical difficulties arising in determining the activity of *Azotobacter* in normal soil, such as a brown silt loam.

4. Explain the difference between *Azotobacter* and *B. radicicola* in relation to organic carbon and the carbon cycle.

Questions.

1. Name the common *Azotobacter* organisms.
2. Why is isolation from a soil easier in the fall or winter?
3. What influence does the reaction of the soil have on the growth of *Azotobacter?*
4. Name the chief sources of organic carbon for nitrogen fixation by *Azotobacter.*
5. What part may algæ play in assisting *Azotobacter* fix nitrogen?
6. In what kind of soils does the greatest fixation occur?
7. What organisms usually are found associated with *Azotobacter?*

PRACTICE 17.

NITROGEN FIXATION IN SOLUTION BY SOIL BACTERIA.

Place 25 cc. of solution for aërobic non-symbiotic nitrogen fixation in each of ten 450 cc. Erlenmeyer flasks. Add 20 grams of ignited soil, plug with cotton and sterilize at 12 pounds pressure for 15 minutes. Inoculate as below with one gram of soil.

1 and 2, brown silt loam.
3 and 4, gray silt loam.
5 and 6, brown sandy loam.
7 and 8, yellow silt loam.
9 and 10, nothing.

Incubate at 30° C. for 25 days and do not disturb the scum any more than possible when transferring. Note any peculiar odors during incubation. Analyze the contents of each flask for total nitrogen. Calculate the fixation.

Group.....

Blank on Method.....mgs. N. 1 cc. NH_4OH.....mgs. N.

Date	Sample number	Treatment	HCl ≈	NH_4OH	Titrated back, NH_4OH	Equivalent in sample, NH_4OH	Mgs. N	P.P.M.	Notes

Reference.

1. U. S. Dept. Agr., O. E. S. Bul. 194, 8–15.

Questions.

1. What arguments have been advanced against this method of experimentation?

2. What advantage has been claimed for this method?

3. Give the general results of the workers who have shown this method to be less related to soil fertility than the methods which involve the soil instead of the solution.

PRACTICE 18.

ISOLATION OF AZOTOBACTER FROM SOIL.

This practice is dependent upon practice 17. When a good scum has formed (7–10 days) transfer 3–5 loopfuls to a 100 cc. Erlenmeyer flask containing 20 grams ignited soil and sufficient solution for aërobic non-symbiotic nitrogen fixation, to half cover the soil slope. Likewise, transfer 2 or 3 times. After the second transfer pour plates of mannite agar, using 1 cc. of the solution for the first tube and 3 loopfuls from this for the second tube. Repeated transfers on the mannite agar are decidedly advantageous in studying the organism. Study the colony characteristics and stain the organism with carbol-fuchsin, gentian violet, methylene blue, and iodine. In using the iodine on the earlier preparations note any clostridia forms. Make slants on this mannite agar. Study the multiplication of the organism and the zooglea. Measure the organisms. Classify the organism isolated. Describe carefully the pigment formation at the various stages. Grow the organism on the magnesium plaster of Paris block. Make permanent slides and drawings of the organism.

References.

1. Handbuch der technischen Mykologie, Lafar (1904–6), **3,** 8, plates after 8.
2. Vorlesungen über landwirtschaftliche Bakteriologie, Löhnis (1913), plates after 170.
3. Centbl. f. Bakt. 2 Abt. (1913), **38,** 14–25, plates after 24.
4. Jour. Ag. Res. (1915), **4,** 225–239, plates after 239.
5. N. J. Agr. Exp. Sta. Ann. Rpt. (1908), 137.
6. Jour. Agr. Sci. (1907), **2,** 35–51.
7. Jour. Agr. Res. (1916), **6,** 675–702.

Questions.

1. What advantage is derived from repeated transfers?
2. Of what is the zoöglea composed?
3. What characteristic reaction does the brown pigment give and what erroneous conclusions might be drawn therefrom?
4. How may the media on which growth occurs be responsible for differences in chemical analyses of bacteria?
5. If combined nitrogen is present will it prevent the growth of *Azotobacter?*

PRACTICE 19.

NON-SYMBIOTIC ANAËROBIC NITROGEN FIXATION AND ISOLATION OF B. CLOSTRIDIUM PASTEURIANUM.

The fixation of nitrogen by this organism may in part counterbalance the activity of the denitrifying organisms which thrive under similar conditions.

In each of 10 reduction test tubes, place 100 cc. of the solution for anaërobic nitrogen fixation. Plug and sterilize at 10 pounds pressure in the autoclave for 10 minutes. Obtain samples of soil at $6\frac{2}{3}$, 20, and 40 inches and air dry in the dark. Heat the sample for 15 minutes at 75° C.

1 and 2, 1 gram surface soil at $6\frac{2}{3}$ inches.
3 and 4, 1 gram soil collected at 20 inches.
5 and 6, 1 gram soil collected at 40 inches.
7 and 8, surface soil saturated with the solution.
9 and 10, solution sterile for transfers.

In 7 and 8 place enough surface soil to fill the 1 inch of the top, add solution to completely saturate the soil. Tubes 9 and 10 are to be used for transfers.

Pour oil on the surface of 1–8 inclusive (about $\frac{1}{2}$ inch of paraffin oil), plug tightly, and incubate at 30° C. Transfer from 3 and 4 to 9 after examining to see how growth has developed. Make transfers until cultures are pure. Study the organism by the usual methods. Stain with iodine solution. Note carefully a characteristic odor. Determine total nitrogen on 1–6 and consult bulletin board for nitrogen control. Calculate the fixation.

Group..... 1 cc. NH_4OH.....mgs. N.

Date	Sample number	Treatment	HCl	$\approx$	NH_4OH	Titrated back, NH_4OH	Equivalent in sample, NH_4OH	Mgs. N	P.P.M.	Notes

References.

1. Compt. Rend. Acad. Sci. (Paris) (1893), **116,** 1385.
2. Exp. Sta. Rec. (1906–7), **18,** 429.
3. Exp. Sta. Rec. (1893–94), **5,** 1010.
4. Centbl. f. Bakt. 2 Abt. (1902), **9,** 43–54, 107–112.

Questions.

1. How important is this fixation under normal conditions?
2. What factor may be responsible for considerable fixation by these organisms?
3. Compare the carbon required by clostridia and *Azotobacter* forms.
4. Are endospores formed by these organisms?
5. What organic acids do they produce?
6. What sources of carbon will suffice for their growth?

PRACTICE 20.

DENITRIFICATION AND FORMATION OF CALCIUM CARBONATE.

Denitrification, the reverse of nitrification, includes the evolution of nitrogen gas, ammonia, oxides of nitrogen and various other volatile nitrogen compounds from nitrates, nitrites, ammonium compounds, and nitrogenous organic compounds. It should be considered as important in soil studies only when conditions are brought about which result in the nitrogen being driven into the air. Practices 7 and 8 represent the denitrification of nitrites and ammonium compounds. In its stricter sense, it means the reduction of nitrates with the loss of nitrogen as the gas.

Into eight 100 cc. Erlenmeyer flasks, place the following solutions and materials:

1 and 2, 90 cc. denitrification solution + 0.5 gram dextrose.

3 and 4, 90 cc. $Ca(NO_3)_2$ denitrification solution + 0.5 dextrose free from carbonate.

5 and 6, 90 cc. base solution.

7 and 8, 90 cc. base solution.

Plug and sterilize at 10 pounds for 10 minutes. Inoculate when cool as follows:

1 and 2, 1 cc. fresh soil infusion (surface).

3 and 4, 1 cc. fresh soil infusion (surface).

5 and 6, 3 grams fresh horse manure.

7 and 8, 5 grams rich brown silt loam.

Pour paraffin oil over the surface and incubate at 30° C. for 10 days or longer if necessary. Test for nitrate, nitrite, and ammonia in 1–2 and observe the precipitation

of calcium carbonate in 3 and 4. Filter, dry, and test for carbonate by treating with acid. Allow numbers 5, 6, 7, and 8 to remain several weeks, after which time determine the loss of nitrogen by analyzing the contents of each tube for total nitrogen. The analyses of samples of the manure and soil used should also be made at the beginning.

Date	Sample number	Treatment	$HCl = NH_4OH$		Titrated back, NH_4OH	Equivalent in sample, NH_4OH	Mgs. N	Loss

QUALITATIVE TESTS.

	1	2	3	4	5
Nitrate					
Nitrite					
Ammonia					

References.

1. Soil Conditions and Plant Growth, Russell (1913), 98–99.
2. U. S. Dept. Agr., O. E. S. Bul. 194, 68–71.
3. Jour. Agr. Sci. (1911–12), **4**, 145–149.
4. Science (1913), N. S., **37**, 552.
5. Science (1915), N. S., **41**, 624.

Questions.

1. Is denitrification of importance in normal soils?
2. In what kind of soils is it of importance?
3. What common substances hasten denitrification?

4. Write a typical reaction showing denitrification.
5. Write the reaction occurring in flasks 3 and 4.
6. Explain the theory of the precipitation of calcium carbonate or ground limestone and the formation of dolomite.
7. Explain the physiological significance of denitrification.
8. What other reductions are closely related to denitrification?

PRACTICE 21.

SULFOFICATION AND DESULFOFICATION IN SOILS.

The oxidation and reduction of sulfur, sulfide, sulfite, thiosulfate, and organic sulfur is a function of certain higher soil bacteria. The sulfur cycle like that of nitrogen is extremely difficult to control as sulfur is only returned to the soil by rain. No organisms are known to fix sulfur from the air as occurs with carbon dioxid or nitrogen.

Place 100 grams of soil in each of ten 400 cc. shaker bottles. Treat as follows:

1 and 2, nothing.

3 and 4, 5 cc. of sodium sulfide solution = 0.2 gram Na_2S.

5 and 6, 0.2 gram free sulfur.

7 and 8, 5 cc. of calcium sulfate = 0.1 gram $CaSO_4$ (saturated).

9 and 10, 2 grams clover tops.

Mix the treatment thoroughly and add water to the optimum for all but 7 and 8, which are saturated. Plug the bottles and incubate at room temperature or at 30° C. for 14 days. At the end of that time add 300 cc. of acidified water (HCl 5 cc. per liter), shake for 5 hours and let stand over night, remove 200 cc., and determine the sulfates as indicated on page 117.

Group..... Soil Type.....
Standard Reading.....mm. Date.....

	Treatment	Total volume	Volume taken	Reading, mm.	Mgs. S	P.P.M. S oxidized or reduced	

References.

1. Handbuch der technischen Mykologie, Lafar (1904–6), **3**, 214–244.

2. Vorlesungen über landwirtschaftliche Bakteriologie, Löhnis (1913), 192–197.

3. Wis. Agr. Exp. Sta. Research Bul. 14.

4. Ken. Agr. Exp. Sta. Bul. 174 and 188.

5. Soil Science (1916), **1**, 533–539.

Questions.

1. In what form is most of the sulfur in soils and what form do plants prefer?

2. In what form does most of the sulfur pass into the air from bacterial decomposition and combustion?

3. How are the losses of sulfur compensated?

4. What bacteria effect the oxidation and reduction of sulfur and its compounds?

5. On what compounds is their action most necessary for the continuance of the sulfur cycle?

6. Write the reactions involved in the oxidation of the sulfide, free sulfur and sulfite to sulfate, the reduction of sulfate to sulfide.

7. Write a reaction showing the reduction of sulfur by a cellulose decomposer; the reduction of a sulfide with the formation of calcium carbonate.

8. Write the reaction showing the oxidation of tetrathionate.

PRACTICE 22.

FUNGI IN SOILS.

RELATION TO SOIL NITROGEN.

Factors Influencing Fungous Growths in Soils.

Weigh out fourteen 50-gram portions of the soil to be tested and place in the jelly glasses.

Treat as follows:

1 and 2, nothing.

3 and 4, 10 milligrams of nitrogen as nitrate.

5 and 6, 3 grams organic matter.

7 and 8, 3 grams organic matter (moisture 80 per cent of saturation).

9 and 10, 3 grams organic matter + 10 milligrams of nitrogen as nitrate.

11 and 12, 5 cc. sulfuric acid.

13 and 14, 5 cc. sulfuric acid + 3 grams organic matter.

The amount of water to be added will be posted on the bulletin board. Mix thoroughly and place in room-temperature incubator. Examine closely every 2 days for growth and finally examine a portion of the soil from numbers 1 and 5 with the binocular microscope for mycelial threads.

At the end of 14 days analyze numbers 3 and 9 for nitrate, at 30 days analyze 4 and 10 for nitrate.

Make observations on the growth in all the treatments after one week and then discard all except those on which the nitrates are to be determined.

Date	Soil No.	Treatment	Incubation		Growth	Mgs. N as nitrate found	Original N as nitrate	Gain or loss of N as nitrate	Notes
			Temp.	Time					

References.

1. Household Bacteriology, Buchanan (1913), 50–84, 487–523.
2. N. J. Agr. Exp. Sta. Bul. 270.
3. Centbl. f. Bakt. 2 Abt. (1914), **40,** 555–647.
4. Science (1914), **39,** 35–37.

Questions.

1. What kinds of fungi inhabit soils?
2. Which of these kinds predominate?
3. Of the factors necessary for growth which are most important for soil fungi?
4. In the struggle for food how are soil fungi at an advantage?
5. Name 10 forms of nitrogen which fungi are able to assimilate.
6. Are fungi able to fix atmospheric nitrogen?
7. Did the fungi feed on organic matter or nitrates in this experiment?
8. Are fungi able to ammonify?

PRACTICE 23.

PROTOZOA IN SOILS.

ISOLATION AND STUDY OF AMŒBÆ, CILIATES, AND FLAGELLATES FROM SOIL.

Determination of Active Protozoa in Soils.

The three classes amœbæ, ciliates, and flagellates are commonly found in normal soils. At times they are more easily found owing to increases in moisture content, but they exist in an active state in all normal soils yet examined.

Prepare a 1.5 per cent solution of blood meal in tap water, filter and add a crystal of di-potassium phosphate, and place 10 cc. in each of six 100 cc. Erlenmeyer flasks. Plug and sterilize in the autoclave at 12 pounds pressure for 15 minutes. Treat as follows:

1, nothing. Inoculate, 5 grams surface soil (normal).
2, 1 gram ground limestone, inoculate, 5 grams surface soil (normal).
3, 1 gram ground limestone, inoculate, 5 grams subsoil (normal).
4, 1 gram ground limestone, inoculate, 5 grams surface soil (poor).
5, Make slightly acid with hydrochloric acid, 5 grams surface soil.
6, 1 cc. rich algal slime, 5 grams surface soil.

Examine each treatment at the end of two days for ciliates and flagellates and at intervals of two days for three to four laboratory periods. Note shape of these organisms, size, means and kind of motility, feeding habits and source of food. Stain some organisms from No. 2 with aqueous methylene blue and gentian violet (equal parts). Kill and stain with picrosulphuric acid. Carefully examine for cysts. With the experience now

acquired a direct examination for active protozoa in field soils may be made as follows:

Place 100 grams of soil in a 400 cc. shaker bottle, add 200 cc. of sterile water and 1 gram of calcium carbonate, shake and let settle a few minutes. Remove several drops from the surface with a glass rod and place them in a hollow cell or on an ordinary slide. Examine with the binocular, using the high power lens, or with the regular microscope, using the low power lens. Make several examinations. Note the forms which are motile. Study the plates in Dolfein, Minchin, and Bul. 2 Geological and Natural History Survey, Conn.

The large forms observed are not known to possess an encyst stage. It should also be stated that authorities differ as to the time required for excystment.

Those students desiring to make permanent slides are referred to advanced practice Active Protozoa in Soils.

Sample number	Treatment	Amœbæ		Ciliates		Flagellates	
		Large	Small	Large	Small	Large	Small

References.

1. An Introduction to the Study of Protozoa, Minchin (1912).
2. Conn. Geo. and Nat. His. Survey (1904–5), **1,** Bul. 2.
3. Lehrbuch der Protozoenkunde, Dolfein (1911).
4. Jour. Agr. Sci. (1909), **3,** 111–144.
5. Jour. Agr. Sci. (1915), **7,** 49–74, 106–119, plates after 118.
6. Centbl. f. Bakt. 2 Abt. (1914), **39,** 596; **41,** 625–630.
7. Jour. Agr. Res. (1915), **4,** 511–559; **5,** 137–139; **5,** 477–487.
8. Protozoa in Illinois Soils, Reference Shelf.
9. Soil Science (1916), **1,** 135–153.
10. Jour. Bact. (1916), **1,** 423–433.

Questions.

1. Give the distinguishing characteristics of the three classes of protozoa.
2. What constitutes the food of each class?
3. Describe the process of encystment.
4. What is enflagellation and exflagellation and how may they lead to erroneous conclusions?
5. Give the names of typical members of each class.
6. What factor is most important for most of the protozoa in soils to lead an active existence?
7. How do you explain the fact that the protozoa may be active in a soil with a low moisture content?

PRACTICE 24.

ALGÆ IN SOILS.

RELATION OF ALGÆ TO SOIL NITROGEN.

Comparative Numbers of Algæ in Different Soils. Factors Influencing the Growth of Algæ in Soils.

Place 50 grams of clean white sand in each of ten 100 cc. Erlenmeyer flasks. Add 20 cc. of algæ solution to each, plug with cotton, and sterilize for 30 minutes at 12 pounds pressure in the autoclave. Slant the flasks so that a sand slope is formed.

Prepare an infusion of the soils to be studied. In each of ten flasks place 10 cc. of the infusion = 5 grams of soil, and treat as follows:

1 and 2, place in incubator (dark).
3 and 4, place in window sill (direct sunlight).
5 and 6, 0.5 gram sodium carbonate (direct sunlight).
7 and 8, 2 cc. $N/7$ hydrochloric acid (direct sunlight).
9 and 10, 6 cc. $N/7$ hydrochloric acid (direct sunlight).

When a green color is observed in those on the window sill make a microscopic examination of all the flasks. Note the forms and their appearance. Stain with iodine solution and explain the results. After several weeks, make stains with picronigrosin. Test all the flasks for nitrate. Filter the solution before testing it.

Prepare the following dilutions of the soil infusion 1–1000, 1–5000, 1–10,000, 1–20,000. In flask 11 place 1 cc. of infusion 1–1000, in No. 12, 1–5000, in flask 13, 1–10,000, in flask 14, 1–20,000. Shake the flasks and slant to form a sand slope. Place in direct sunlight.

Report the number of algæ per gram of the soil tested. Counting is accomplished by observation of the green color and represents the minimum number.

Date	Soil	No.	Treatment	Iodine test	Nitrate test	No. per gram	No. per acre	Notes

References.

1. Chem. Absts. (1915), **9**, 2923.
2. Tufts College Studies Scientific Series **2**, No. 3, The Green Algæ of North America, Collins, also **3**, No. 2.
3. Morphologie und Biologie der Algen, Oltmann. I Speciellie Tiel.
4. Col. Exp. Sta. Bul. 184.
5. Minnesota Algæ I (Tilden).

Problems.

1. How many pounds of algæ would it require to furnish the energy for a fixation of 15 pounds of atmospheric nitrogen?
2. Calculate the nitrogen as nitrate that would be conserved per acre with 13½ inches of drainage from the results obtained in your experiment.

Questions.

1. In what kinds of soils do algæ grow best?
2. What recently isolated constituent of algæ accounts for the symbiosis with *Azotobacter?*

3. Explain the symbiosis in view of the fact that algæ require inorganic nitrogen and *Azotobacter* cannot nitrify.
4. In what region of the soil do the algæ grow?
5. What factor is most essential for their existence?
6. Explain the symbiosis between algæ and paramœcium.

PRACTICE 25.

A STUDY OF ENZYMES.

Enzymes are a product of living cells but after formed may act independently of the cell. Their exact composition and their relation to the reactions they produce is yet undecided. They are likened to catalyzers, magnetism, and colloids and perform like all three. Two kinds are recognized, the endo-enzyme and the ecto-enzyme. Oxidations, reductions, hydrolytic, synthetic, and intramolecular processes are brought about by these agents. Thus it will be seen that the ecto-enzyme makes food soluble without, for assimilation by the cells and the endo-enzymes are responsible for digestion and fermentation within.

The importance of enzyme action is better realized when it is stated that all our soil microörganisms perform their functions through these agents.

Isolation of Proteolytic Enzyme (Proteases). Make 2 stab cultures of nutrient gelatin with *B. subtilis*, 2 with *B. liquefaciens*, 2 with *B. coli* and 2 of *Aspergillus niger*.

Incubate at room temperature until the gelatin is liquefied by *B. subtilis*. Add 1 cc. of toluene to each tube, shake, grind in an agate mortar with a small amount of fine sand. Filter through paper, infusorial earth, or by using a Berkefeld apparatus.

Inoculate tubes of solid gelatin and milk with 5 cc. of the filtered product. Place a small amount of barium sulphate in the gelatin tubes and mark on the outside with a red pencil the height of the gelatin. This serves to show the rate and point of liquefaction. Examine each day and note the action which occurs.

Add to a 5 per cent solution of guiacin in alcohol 5 cc. of the filtered product and note the results. If a color does not develop at once add hydrogen peroxide and observe carefully the formation of gas or a color.

Isolation of Urease. — (1) Inoculate 10 cc. of sterile urea solution with the culture obtained in practice 6. Incubate 24 hours at room temperature. Add 1 cc. of toluene to the culture and grind the solution in a mortar, using sand, filter and inoculate another tube of sterile solution with a portion of the filtered product. Add toluene to the tube and after 24 hours titrate the ammonia produced. (2) Place 0.9 gram of powdered soy bean seeds in 10 cc. of water and allow the mixture to stand over night. Separate the aqueous portion by centrifugation and mix the aqueous extract with an equal volume of glycerol. Test the soy bean urease preparation on urea solution. Tabulate your observations on the action of the different organisms on the gelatin and milk and the results of the chemical tests.

References.

1. General Chemistry of Enzymes, Euler (Pope), (1912).
2. The Nature of Enzyme Action, Bayliss (1914), 1–6, 10–13, 22, 139–43, 146.
3. An Introduction to the Chemistry of Plant Products, Haas and Hill (1913), 334–372.
4. Bacteriological and Enzyme Chemistry, Fowler (1911), 256–272.

Questions.

1. What function does the toluene perform?
2. Explain the production of color with and without the addition of hydrogen peroxide.
3. The gas formed proves the presence of what enzyme?
4. Classify the enzymes as endo- or ecto-enzymes.
5. What important action does urease produce?
6. How does the energy liberated by the two classes of enzymes compare?
7. Classify the enzymes studied according to the chemical action produced.

CLASS PRACTICE 1.

GROWTH AND STUDY OF IRON BACTERIA OF SOILS.

In the soil all the mineral elements are subject to direct or indirect bacterial action. Iron and manganese are typical of the elements which are attacked by special forms of bacteria. In studies of drinking water the action of these bacteria has presented important economic problems. These organisms occur in soils and, when suitable conditions for their multiplication are brought about, they act on the iron and manganese compounds and oxidize them, thereby obtaining energy.

In a liter Erlenmeyer flask place 500 cc. of solution for iron bacteria. Titrate 20 cc. of the solution for ferrous iron. Introduce an iron wire bent in the form of a crook at the end immersed and extending to the top of the flask bent at right angles to hold it in place. Plug and sterilize at 10 pounds for 15 minutes.

Inoculate with 100 grams of fresh surface soil. Place the flask on the window sill and observe carefully the yellowish-red slime which forms on the wire and in the liquid. Remove some of the growth and wash the iron deposits from the cells with 1 per cent hydrochloric acid. Stain with carbol-fuchsin and methylene blue. Describe the organisms and draw typical cells. Remove 20 cc. portions and determine the ferrous iron. The difference between the original and the final titrations represents the iron oxidized to ferric.

Original titration	cc. *N* 10 $KMnO_4$
Final titration	cc. *N* 10 $KMnO_4$
	cc. *N* 10 $KMnO_4$ mgs. Fe

References.

1. Centbl. f. Bakt. 2 Abt. (1904), **11,** 215–219, 277–287.
2. Centbl. f. Bakt. 2 Abt. (1908), **20,** 97–99.
3. Proc. Soc. Am. Bact. Pubs. in Science (1916).

Questions.

1. What influence on solubility of iron and manganese do these bacteria exert?
2. Are these simple forms of bacteria?
3. Why is the hydrochloric acid necessary?

CLASS PRACTICE 2.

DENITRIFICATION IN SOLUTION BY SOIL BACTERIA.

This practice is suggested where laboratory facilities and time are limiting factors. It serves to illustrate denitrification, gas production, desulfofication, and ability of soil organisms to reduce organic compounds.

In a large flask or bottle with a long neck ($5\frac{1}{2}$ liter boiling flask) fitted with a one-hole rubber stopper, insert a glass tube which should extend to within $\frac{1}{4}$ inch of the bottom and project 2 or more feet above the stopper. Bend the end of the tube so it will deliver into a graduated glass cylinder. Place 100 grams of surface soil in the flask, and fill with solution for denitrification (class practice) so that the solution will be visible just above the stopper.

Put the apparatus in some convenient place, covering all but the neck with a paper or cloth to shut out the light.

The students should mark the height of the liquid on the tube at the beginning and every two days and record it on a card attached to the apparatus in inches. The liquid displaced by the gas should be recorded. This liquid may be tested for nitrates, sulfates, and sugar from time to time. An explanation of the process should be given by the instructor.

ADVANCED PRACTICE 1.

ACTIVE PROTOZOA IN SOILS.

The method of Martin and Lewin with careful manipulation gives excellent results.

Place 20 grams of soil in a 100 cc. evaporating dish and add through a funnel at the bottom of the soil mass enough of the picric acid fixative or the corrosive fixative to completely cover the soil, shake the dish immediately. Mark a cover slip to indicate the film side and float it on the surface to obtain a film. Fix, stain, and prepare the film for mounting by the method found on page 106. Mount in balsam. By means of the camera lucida draw the different forms observed. Stain some films with aqueous solution of methylene blue and gentian violet.

Number of Protozoa in Soils. — The number of protozoa per gram of soil is at present best obtained by use of the (Blutkorperzahl apparat) blood-counting apparatus. Prepare an infusion of the soil to be examined, remove 10 cc., and thoroughly shake. Make a dilution of 1–100 or 1–10 with mixing pipettes. Discard 3 drops of the dilution and then place a drop in the counting chamber. Exercise care not to have the liquid run into the moat. Count the protozoa in the drop and repeat on 3 more drops taking the average number of the four determinations and multiply by the dilution. Report the number per gram of soil taken.

Separation of Protozoa. — Filter 10 cc. portions of stock solutions of soil protozoa through single and quadruple filters (Schleicher and Schull's 589), which have been sterilized by alcohol. Count the protozoa in the filtrate by the method used above, and at the same time count the

forms in the stock culture. After making a separation of this kind, prepare concentrated solutions by incubation and inoculate into sterile, unsterile, moist and dry soils and study the nitrate formation.

Reference.

1. Jour. Agr. Research (1915), **5**, 137–139.

ADVANCED PRACTICE 2.

DECOMPOSITION OF CYANAMID.

Calcium cyanamid is a product of the electric furnace. It is made at a high temperature (1300° C.) from calcium carbide and nitrogen gas obtained from the air according to the reaction:

$$CaC_2 + N_2 \rightleftarrows CaNCN + C.$$

Microörganisms decompose it into nitrate as shown by the reactions below.

1. $\begin{cases} CaNCN + CO_2 + H_2O = CaCO_3 + H_2NCN. \\ CNCaN + 2\,H_2O = CNNH_2 + Ca(OH)_2. \end{cases}$
2. $H_2NCN + H_2O = CO(NH_2)_2.$
3. $CO(NH_2)_2 + 2\,H_2O = (NH_4)_2CO_3.$
4. $(NH_4)_2CO_3 + 6\,O + CaCO_3 = Ca(NO_2)_2 + 2\,CO_2 + 4\,H_2O.$
5. $Ca(NO_2)_2 + O_2 = Ca(NO_3)_2.$

The manufacture of this product has been developed to a point where it is cheaper than nitrate of soda and is destined to replace nitrate of soda in foreign countries.

As seen from above it presents a most interesting chemical phenomena, a diamide (urea) resulting from bacterial action on calcium, carbon, and nitrogen.

Place 100 grams of soil in each of 10 jelly glasses. Arrange as follows:

1 and 2, nothing.
3 and 4, nothing.
5 and 6, 0.1 gram $CaCN_2$.
7 and 8, 0.1 gram $CaCN_2$.
9 and 10, 0.2 gram $CaCN_2$.

Add moisture to the optimum as usual.

Determine ammonia on 1, 2, 4. and 5 after 2 weeks. After 4 weeks determine the nitrate in 3, 4, 6, 7, 9, and 10.

Sample number	Treatment	HCl ⇌ NH_4OH		Titrated back, NH_4OH	Equivalent in sample, NH_4OH	Mgs. N as NH_2	Mgs. N as NO_3
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							

Into four 500 cc. Erlenmeyer flasks place 50 cc. of the following solution:

Water	1000 cc.
Dipotassium phosphate	1.0 gram
Asparagin	0.1 gram
Dextrose	0.1 gram
Calcium cyanamid	2.0 grams

Inoculate with 10 grams of surface soil and incubate for 4 weeks.

Prepare cyanamid gelatin by adding 10 per cent of gelatin, and sodium hydroxid to make faintly alkaline, to this solution. After 2 weeks transfer several loopfuls from the flasks inoculated with soil to the other two and after 2 weeks plate out from these solutions to the cyanamid gelatin.

Study the colony characteristics and inoculate urea agar and urea solution with typical colonies.

References.

1. Centbl. f. Bakt. 2 Abt. (1905), **14,** 87, 389.
2. Centbl. f. Bakt. 2 Abt. (1909), **22,** 254–281.
3. Centbl. f. Bakt. 2 Abt. (1910), **26,** 633, 643.
4. U. S. Dept. Com. & Labor, Bur. Mfgs. Special Agent Series No. 52 (1912), 116–168.
5. Dictionary of Applied Chemistry, Thorpe (1912), **3,** 698–712.
6. Ill. Agr. Expt. Sta. Bul. 179, 494, footnote.

Question.

1. Discuss the importance of the cyanamid industry as related to general agriculture.

ADVANCED PRACTICE 3.

PROTEIN FORMATION IN SOILS.

This practice serves to demonstrate that certain bacteria use nitrate nitrogen to make protein instead of evolving the nitrogen gas into the air. It tends to show that these organisms with normal moisture content may act as conservers of nitrogen instead of denitrifiers.

Place 100 grams of soil in each of ten 500 cc. Erlenmeyer flasks. Sterilize six of them at 15 pounds pressure for 2 hours.

Inoculate and treat as follows:

No. 1. Normal soil, nothing, 18 per cent water.
No. 2. Normal soil, 0.5 gram dextrose, 18 per cent water.
No. 3. Normal soil, 0.5 gram dextrose, 18 per cent water + 10 mgs. N as nitrate.
No. 4. Normal soil, 0.5 gram dextrose, 18 per cent water, + 50 mgs. N as nitrate.
No. 5. Sterilized soil, 0.5 gram dextrose, 18 per cent water, 10 mgs. N as nitrate.
No. 6. Sterilized soil, 0.5 gram dextrose, 24 per cent water, 10 mgs. N as nitrate.
No. 7. Sterilized soil, 0.5 gram dextrose, 30 per cent water, 10 mgs. N as nitrate.
No. 8. Sterilized soil, 3 grams dextrose, 18 per cent water, 50 mgs. N as nitrate.
No. 9. Sterilized soil, 0.5 gram dextrose, 24 per cent water, 50 mgs. N as nitrate.
No. 10. Sterilized soil, 0.5 gram dextrose, 18 per cent water, 100 mgs. N as nitrate.

The sterilized treatments are inoculated with cultures of *B. fluorescens liquefaciens* and *B. pyocyaneus*. Total nitrogen determination should be made on the soil and added materials in the beginning.

The sugar and nitrate are added, together with the inoculation, in the sterile water applied.

Incubate at room temperature for 3–4 weeks, after which time, dry and grind the soil and determine total nitrogen in duplicate in samples of ten grams. Determine the nitrate nitrogen in samples of 50 grams.

Number	Treatment	Total nitrogen			Nitrate nitrogen			Protein N, mgs. formed
		Found	Mgs. calculated	Gain + or loss −	Found	Mgs. calculated	Gain + or loss −	

References.

1. Centbl. f. Bakt. 2 Abt. (1910), **26**, 335–345.
2. Soil Conditions and Plant Growth, Russell (1913), 99.

Question.

1. Explain the results obtained in terms of the conservation of nitrogen.

ADVANCED PRACTICE 4.

FLAGELLA STAINING OF B. RADICICOLA, B. NITROSOMONAS, B. DENITRIFICANS, B. SUBTILIS, B. TYPHOSUS.

Some of the common and more easily stained organisms are used for comparison with the more difficult, such as *B. radicicola*. This is a difficult procedure and requires careful technique. The organisms to be stained should have been transferred every two days for several times and before staining examined in the hanging drop. It is best to have transfers of each organism as some are better than others. The cover slips must be absolutely clean (test with water). After removing from the alcohol, if suspicious, pass through the flame three times allowing time between each to avoid cracking. Place 1 loopful of water on the cover slip. Spread by pulling and do it gently. Care at this point is highly essential. The end of the platinum wire may be used for this purpose. First fix with gentle heat and then apply the mordant (Loeffler's Flagella Stain) which should be filtered on to the cover slip until completely flooded and heat until steam arises (use care not to overheat). Hold the cover slip with cornet forceps and keep the finger extended over it when passing through the flame. Heat $\frac{1}{2}$–1 minute. Wash in water and blot with filter paper. Stain with the carbol-fuchsin, taking care to completely flood the cover slip so that no material will be precipitated out during the 3 minutes heating. Wash and examine. Mount in balsam if a satisfactory stain is obtained. Keep accurate notes on the procedure.

ADVANCED PRACTICE 5.

CROSS INOCULATION OF LEGUMES.

Some valuable cross inoculations have already been made by this station. There are no doubt many other similar cross inoculations which may be made. The following method will serve to enable advanced students to test some of these possibilities. Sterilize clean white sand in 600 cc. beakers. Add 5 cc. sterile plant food solution (nitrogen omitted) in 140 cc. of sterile water. Sterilize the legume seeds in 1–500 mercuric chloride solution and with a sterile pipette add the legume bacteria in the following experiment:

1– 5, 1st legume, inoculated with bacteria common to it.
6–10, 2nd legume, inoculated with bacteria of legume No. 1.
11–15, 2nd legume, inoculated with bacteria common to it.
16–20, 1st legume, inoculated with bacteria of legume No. 2.
21–25, 1st legume, uninoculated (sterile).
26–30, 2nd legume, uninoculated (sterile).

Failure to prove out organisms in some such manner at the conclusion of experiments may lead to erroneous work.

There are many wild legumes which should be tested on the cultivated. Wild vetches, beans, peas, lespedeza, desmodium (ticks), beggarweed, coffeeweed and many more and their relation to beans (common), soybeans, and many others are typical cases which are not yet solved.

If organisms are not available, gather soil about the wild legumes and grow the nodules with which to start. Some of the crosses made by earlier workers should be repeated as only a few are known to be reliable. The plants are allowed to grow in the greenhouse. If a suitable undis-

turbed portion of a greenhouse is available, it is not necessary to keep the plants under sterile conditions. Insects and people should be excluded. Separating the treatments is a further precaution.

References.

1. Ill. Agr. Exp. Sta. Bul. 179, 497–498, 475.
2. Manual of Botany, Gray, 7th Edition (1908), 499–531.
3. Illustrated Flora of the Northern States and Canada, Britton and Brown (1913), II Amaranth-Logania, 330–425.

Questions.

1. How closely related were the orders crossed?
2. Discuss the possibility of these organisms growing on plants not legumes.

ADVANCED PRACTICE 6.

SOLVENT ACTION OF SOIL BACTERIA ON MINERALS.

SOLUBLE PHOSPHORUS AND CALCIUM PRODUCED BY NITROSOMONAS.

The formation of nitrites requires a base and the calcium of insoluble phosphates is a suitable one. This results in a liberation of soluble phosphorus.

Place 50 cc. of base solution for nitrite formation in each of eight 1 liter Erlenmeyer flasks, plug loosely and sterilize in the autoclave at 10 pounds pressure for 15 minutes. Add 20 milligrams of nitrogen as ammonium sulfate and 100 milligrams of the raw rock phosphate to each flask. The phosphate should have been dried and analyzed for phosphorus and calcium before using.

Arrange as follows:

1 and 2, sterile rock phosphate.*
3 and 4, inoculated rock phosphate.
5 and 6, inoculated rock phosphate.
7 and 8, inoculated rock phosphate.

Inoculate each with 5 cc. of a fresh infusion of soil and then again sterilize flasks 1 and 2. Place in 30° incubator and at the end of 40 days proceed as follows: Filter the contents of the duplicate flasks 3 and 4 through an S. and S. 589 and make up to 200 cc. Analyze two 50 cc. for nitrogen as nitrite and nitrate and two 25 cc. portions of phosphorus and calcium. Use the Devarda method for the nitrogen and the volumetric methods for phosphorus and

* Pure tricalcium phosphate and various kinds of insoluble natural phosphates are used in this experiment.

calcium. After 50 days analyze flasks 5 and 6 and after 60 days flasks 1, 2, 7, and 8. Calculate the ratio of nitrogen oxidized to phosphorus and calcium made soluble.

Number	Nitrogen oxidized	Phosphorus soluble	Calcium soluble	Ratios		
				N : P	N : Ca	Ca : P

References.

1. Ill. Agr. Exp. Sta. Bul. 190.
2. Centbl. f. Bakt. 2 Abt. (1904), 11, 724.
3. Soil Science (1916), **1**, 533–539.

Problems.

1. Write the chemical reaction to which the results conform.
2. Calculate the possible amounts of phosphorus available for standard crops of corn, wheat, oats from the reaction occurring.

Questions.

1. What organisms exert a solvent action on minerals?
2. Of these which are the most important?

3. Upon what will the availability of the raw rock phosphate depend?

4. Explain why the nitrite organisms are effective in this connection and the nitrate not.

5. Discuss the importance of the plant in an equilibrium reaction of this kind.

PART II.

METHODS IN SOIL BIOLOGY.

BACTERIOLOGICAL METHODS.

FOOD OF SOIL MICROÖRGANISMS.

Soil microörganisms consisting of bacteria, fungi, algæ, protozoa, and others require certain elements for growth and for energy. Ten elements are essential for vegetable microörganisms while animal microörganisms require the same elements with the addition of sodium and chlorine.

The twelve essential elements are carbon, hydrogen, oxygen, phosphorus, potassium, nitrogen, sulfur, calcium, iron, magnesium, sodium, and chlorine. Manganese is associated with many microörganisms, but is not known to be an essential element. Silicon is thought by some to be necessary for diatoms.

Vegetable microörganisms require the same ten essential elements as green plants, and animal microörganisms require the same twelve as the higher animals. In this respect there is a great similarity, but when the ability of certain microörganisms to utilize these elements from diverse sources is considered there appears exceptional differences.

Noteworthy among them may be mentioned the following: First, the utilization of free nitrogen by nitrogen fixers (*B. radicicola, B. clostridium* and *Azotobacter*) as a source of food; second, the acceptance of either inorganic or organic carbon (carbon dioxide, bicarbonate or sugar) as a source of food by nitrifying and sulfur organisms (*Nitrosomonas, Nitrobacter,* and sulfur bacteria); third, the appropriation of mineral compounds for the production of energy (ammonia, nitrite, hyposulfite, sulfur, iron, manganese, and even elementary carbon are used by various bacteria); fourth, the utilization of organic nitrog-

enous compounds (urea and amino acids) as a source of both energy and food by urea and other bacteria.

Other rare exceptions to the general sources of the essential elements are, methane which serves as food for growth and energy, and hydrogen gas which serves as food for growth only, for a few bacteria.

It is well to recall that of the non-nitrogenous compounds, alcohols (simple and complex), carbohydrates (sugar, starch, and cellulose), organic acids, fats, and as recently shown even benzene, carbolic acid, paraffin, and formaldehyde are decomposed by bacteria and used as food for growth and energy. Among the nitrogen compounds bacteria find a source of food for growth and for energy and here they exhibit great variations compared with plants and animals. Bacteria utilize free nitrogen, nitrate, nitrite, ammonia, urea, amino acids, primary and secondary derivatives of protein (proteans and peptone), and protein. It is claimed there is no organic compound which cannot be decomposed by some bacteria.

Plants and animals would soon become extinct if forced to depend upon the nitrogen of the atmosphere. Legumes, by symbiosis with *B. radicicola*, gain nitrogen coming from the air, but without this association they cannot use atmospheric nitrogen. Plants depend upon inorganic substances for their elements. Nitrates, ammonia, and possibly some nitrogenous organic compounds constitute their nitrogen sources of food. Animals require proteins, peptones, and some may assimilate amino acids.

Bacteria, fungi, and yeasts possessing no photo-synthetic process as green plants do, obtain their energy chiefly by oxidation or intramolecular changes, or a breaking down of compounds (destructive metabolism). In doing this, they oxidize enormous amounts of material for energy compared with small amounts taken for cell growth. Plants reverse this process, requiring large amounts of food for growth and obtain their energy from the radiant

energy of the sun at the same time building up compounds (constructive metabolism).

The food for energy is an important practical consideration in dealing with microörganisms especially such as *Azotobacter* or *B. radicicola*. It is valuable to recognize the source of energy of our soil organisms and supply the form necessary for the desirable organisms. This phase of nutrition has not as yet received much investigation.

An understanding of the sources and the changes through which the essential elements for microörganisms pass is necessary, before their rôle in permanent agriculture can be appreciated.

To this end, it has become necessary to develop many kinds of media which serves to enable the soil biologist to acquaint himself with the characteristics of these organisms.

PREPARATION OF CULTURE MEDIA.

In considering culture media, it must be stated that logically and ultimately there is only one kind of culture media and that is the soil. For soil biological studies to have their proper relation to soil fertility and to aid in developing better systems of agriculture, they must be conducted ultimately with the natural medium of the soil. The more scientific research advances along these lines, the more convincing the above statement becomes.

It should now be said, however, that the soil biologist has need of other culture media than soils. One must admit that most of our important discoveries would have been impossible had soil been employed solely as the culture medium. A simpler medium, than a complex mixture such as a soil, is necessary in order to establish the biochemical reactions brought about and to study the characteristics of the organisms.

These being established, application to the soil then is of greatest importance. This is not always easily done and

often requires a great amount of difficult research before it is possible to prove that a similar reaction occurs in soil and before its relative importance is understood. We therefore apply food for microörganisms to liquid and solid materials of various kinds, such as water, agar, and gelatin, and as a result we have the so-called liquid and solid media in or on which the organisms grow.

For convenience in use the following tabular method of presenting the formulæ of the various culture media has been adopted.

FORMULÆ OF SOLUTIONS (LIQUID MEDIA).

(Grams per liter.)

Constituents.	Nitritation.	Nitratation.	Soil extract.	Denitrification.	Urea.	Starch solution.	Anaërobic cellulose decomposition.	Protozoa.
Water (cc.)	1000 cc.	1000 cc.	3000 cc.	1000 cc.	1000 cc.*	800 cc.	500 cc.	1000 cc.*
Soil (grams)	.....	.....	1500 g.	.....	.....	boiling water	.....	.....
Asparagin	.....	.....	Extract 2 hours	.....	.....	.....	.....	.....
Beef extract	.....	.....	in the auto-	.....	1	.....	.....	.....
Blood meal	.....	.....	clave at 20	.....	.....	.....	.....	15
Cellulose solution	.....	.....	pounds pres-	.....	.....	.....	500 cc.†	.....
Iron wire No.	.....	.....	sure. Filter	.....	.....	.....	.....	.....
Peptone	.....	.....	and use	.....	.....	.....	.....	.....
Urea	.....	.....	directly	.....	5	.....	.....	.....
Starch (potato)	.....	.....	.....	.....	.....	Add 10 in cold	.....	.....
Wood ashes (hard)	.....	.....	.....	.....	.....	water, reduce	.....	.....
Dextrose	.....	.....	.....	.....	.....	volume to	.....	.....
Saccharose	.....	.....	.....	4	.....	500 cc. by	.....	.....
Maltose	.....	.....	.....	.....	.....	boiling	.....	.....
Mannite	.....	.....	.....	.....	.....	.....	.....	.....
Di-potassium phosphate	1	1	.....	2	1 cryst.	.....	0.5	3 cryst.
Mono potassium phosphate	.....	.....	.....	.....	.....	.....	.....	.....
Ammonium sulfate	1	.....	.....	.....	.....	.....	0.1	.....
Magnesium sulfate	0.2	0.2	.....	2	.....	.....	0.5	.....
Manganese sulfate	.....	.....	.....	.....	.....	.....	.....	.....
Sodium nitrate	.....	.....	.....	.....	.....	.....	.....	.....
Calcium nitrate	.....	.....	.....	2	.....	.....	.....	.....
Sodium nitrite	.....	1	.....	.....	.....	.....	.....	.....
Calcium chloride	.....	.....	.....	0.2	.....	.....	.....	.....
Ferric chloride	1 drop ‡	1 drop	.....	1 drop	.....	.....	1 drop	.....
Sodium chloride	1	1	.....	.....	2	.....	0.5	.....
Calcium carbonate	.....	.....	.....	.....	.....	.....	0.1	10
Sodium carbonate	.....	.....	.....	4.25	.....	.....	.....	.....
Ferrous ammonium citrate	.....	.....	.....	.....	.....	.....	.....	.....
Citric acid	.....	.....	.....	5	.....	.....	.....	.....
Hydrochloric acid	.....	.....	.....	.....	.....	.....	.....	.....
Potassium hydroxid	.....	.....	.....	.....	.....	.....	.....	.....

* Tap water. † Under special medium. ‡ Drops refer to 10 per cent solutions.

FORMULÆ OF SOLUTIONS (LIQUID MEDIA). — *Continued.*

(Grams per liter.)

Constituents.	Algæ.	Wood ash.	Non-symbiotic aërobic nitrogen fixation.	Sulfate reduction.	Bouillon.	Symbiotic nitrogen fixation, B. radicicola.	Iron oxidation.	Non-symbiotic anaërobic nitrogen fixation.
Water (cc.)	1000 cc.	1000 cc.	1000 cc.	1000 c .*	1000 cc.	1000 cc.	1000 cc.*	1000 cc.
Soil (grams)	.....	.....	.....	.....	.....	.....	.....	.....
Asparagin	.....	.....	.....	1	.....	.....	.....	.....
Beef extract	.....	.....	.....	.....	5	.....	.....	.....
Blood meal	.....	.....	.....	.....	.....	.....	.....	.....
Cellulose solution	.....	.....	.....	.....	.....	.....	.....	.....
Iron wire No.	.....	.....	.....	.....	.....	.....	12 in. long	.....
Peptone	.....	.....	.....	.....	10	.....	.....	.....
Urea	.....	.....	.....	.....	Boil and filter	.....	.....	.....
Starch (potato)	.....	.....	.....	.....	.....	.....	.....	.....
Wood ashes (hard)	.....	15	.....	.....	.....	.....	.....	.....
Dextrose	.....	Boil and filter,	.....	.....	.....	.....	.....	20
Saccharose	.....	then add	.....	.....	.....	.....	.....	.....
Maltose	.....	.....	.....	.....	.....	15	.....	.....
Mannite	.....	.....	10	.....	.....	.....	.....	.....
Di-potassium phosphate	.....	.....	1	1	.....	1	1 cryst.	1
Mono-potassium phosphate	1	3	.....	.....	.....	.....	.....	.....
Ammonium sulfate	.....	which gives	.....	.....	.....	.....	.....	.....
Magnesium sulfate	0.2	correct reaction	0.2	1	.....	0.2	.....	1
Manganese sulfate	.....	.....	.....	.....	.....	.....	.....	0.01
Sodium nitrate	.....	.....	.....	.....	.....	3 drops	.....	.....
Calcium nitrate	1	.....	.....	.....	.....	.....	.....	.....
Sodium nitrite	.....	.....	.....	.....	.....	.....	.....	.....
Calcium chloride	.....	.....	.....	.....	.....	3 drops	3 drops	.....
Ferric chloride	5 drops	.....	1 drop	.....	.....	3 drops	.....	1 drop
Sodium chloride	1	.....	1	1	.....	3 drops	.....	1
Calcium carbonate	.....	.....	10†	.....	.....	.....	.....	10
Sodium carbonate	.....	.....	.....	.....	.....	.....	.....	.....
Ferrous ammonium citrate	.....	.....	.....	.....	.....	.....	.....	.....
Citric acid	.....	.....	.....	.....	.....	.....	0.5	.....
Hydrochloric acid	Use to	.....	.....	.....	.....	.....	.....	.....
Potassium hydroxid	make acid	.....	.....	.....	.....	Use to make neutral	.....	.....

* Tap water. † Special cases.

FORMULÆ OF SOLID MEDIA (AGAR).

(Grams per liter.)

Constituents.	Sodium asparaginate.	Synthetic.	Soil extract.	Fungi gelatin.	Bouillon.*	Bouillon.	Starch.
Water	1000 cc.	1000 cc.	.....	1000 cc.	.....	.....	500 cc.
Soil extract	.....	.....	1000 cc.	.....	.....	.....	.....
Agar	12	15	12	20	.....	15	10
Bouillon	.....	.....	.....	.....	1000 cc.	1000 cc.	.....
Cellulose solution	.....	.....	.....	.....	.....	.....	.....
Gelatin	.....	.....	.....	300 g.	150 g.	.....	.....
Peptone	.....	0.5	.....	.....	.....	.....	.....
Urea solution	.....	.....	.....	.....	.....	.....	500 cc.
Starch solution	.....	.....	.....	.....	.....	.....	.....
Wood-ash solution	.....	.....	.....	.....	.....	.....	.....
Dextrose	1	10	.....	.....	.....	.....	.....
Saccharose	.....	.....	.....	.....	.....	.....	.....
Maltose	.....	.....	.....	.....	.....	.....	.....
Mannite	.....	.....	.....	.....	.....	.....	.....
Mannite solution	.....	.....	.....	.....	.....	.....	0.5
Di-potassium phosphate	.....	0.5	2	.....	.....	.....	.....
Mono-potassium phosphate	.....	.....	.....	2	.....	.....	.....
Di-ammonium phosphate	1.75	.....	.....	.....	.....	.....	.....
Mono-ammonium phosphate	or 1.50	.....	.....	.....	.....	.....	.....
Magnesium ammonium phosphate	.....	.....	.....	.....	.....	.....	1
Ammonium sulfate	.....	.....	.....	.....	.....	.....	0.5
Magnesium sulfate	0.2	0.2	.....	0.2	.....	.....	.....
Potassium sulfate	.....	.....	.....	.....	.....	.....	.....
Sodium nitrate	.....	.....	.....	2	.....	.....	.....
Ammonium nitrate	.....	.....	.....	.....	.....	.....	.....
Sodium nitrite	.....	.....	.....	.....	.....	.....	.....
Calcium chloride	0.1	.....	.....	.....	.....	.....	.....
Ferric chloride	3 drops	.....	.....	.....	.....	.....	0.5
Sodium chloride	.....	.....	.....	0.2	.....	.....	.....
Potassium chloride	0.1	.....	.....	.....	.....	.....	1
Calcium carbonate	.....	.....	.....	.....	.....	.....	.....
Sodium asparaginate	1	.....	.....	.....	.....	.....	.....

* Tap water.

FORMULÆ OF SOLID CULTURE MEDIA (AGAR). — *Continued.*

(Grams per liter.)

Constituents.	Cellulose.	Magnesium ammonium phosphate.	Wood ash, saccharose B. radicicola.	Mannite Azotobacter.	Dextrose.	Nitrite.	Urea.
Water	500 cc.	1000 cc.	.....	.....	1000 cc.*	1000 cc.*	.....
Soil extract	.....	.....	.....	.....	.....	.....	.....
Agar	10	15†	10	15	15	15†	15
Bouillon	.....	.....	.....	.....	.....	.....	.....
Cellulose solution	500 cc.‡	.....	.....	.....	.....	.....	.....
Gelatin	.....	.....	.....	.....	.....	.....	.....
Peptone	.....	.....	.....	.....	.....	.....	.....
Urea solution	.....	.....	.....	.....	.....	.....	1000 cc.
Starch solution	.....	.....	.....	.....	.....	.....	.....
Wood-ash solution	.....	.....	1000 cc.	.....	.....	.....	.....
Dextrose	.....	.....	.....	.....	10	.....	.....
Saccharose	.....	.....	10	.....	.....	.....	.....
Maltose	.....	.....	10	.....	.....	.....	.....
Mannite	.....	.....	.....	5	.....	.....	.....
Mannite solution	.....	.....	.....	1000 cc.	.....	.....	.....
Di-potassium phosphate	0.5	.....	.....	.....	.....	0.5	.....
Mono-potassium phosphate	.....	.....	.....	.....	.....	.....	.....
Di-ammonium phosphate	.....	.....	.....	.....	.....	.....	.....
Mono-ammonium phosphate	.....	.....	.....	.....	.....	.....	.....
Magnesium ammonium phosphate	.....	2	.....	.....	.....	.....	.....
Ammonium sulfate	1	.....	.....	.....	.....	.....	.....
Magnesium sulfate	0.5	.....	.....	.....	.....	.....	.....
Potassium sulfate	.....	0.5	.....	.....	.....	.....	.....
Sodium nitrate	.....	.....	.....	.....	.....	.....	.....
Ammonium nitrate	.....	.....	.....	.....	.....	.....	.....
Sodium nitrite	.....	.....	.....	.....	.....	2	.....
Calcium chloride	.....	3 drops	.....	.....	.....	.....	.....
Ferric chloride	.....	3 drops	.....	.....	.....	.....	.....
Sodium chloride	0.5	.....	.....	.....	.....	.....	.....
Potassium chloride	.....	.....	.....	.....	.....	.....	.....
Calcium carbonate	1	.....	.....	.....	.....	.....	.....
Sodium asparaginate	.....	.....	.....	.....	.....	.....	.....

* Tap water. † Purified. ‡ Under special media.

SPECIAL MEDIA.

Silica Jelly. — This solid medium possesses the advantage of being exceptionally selective for the growth of the nitrite and nitrate organisms. Its successful preparation and use require careful observance of the following details.

Procedure. — Standardize a sufficient amount of a solution of sodium silicate to last several years. This is easily accomplished by adding hydrochloric acid, filter, wash, dry, and weigh as silicic anhydride (SiO_2). Stock solutions containing 4–5 per cent of silicic anhydride are prepared from this solution as desired.

Prepare a 4–5 per cent solution of silicic anhydride in water and a standard solution of hydrochloric acid of equivalent strength, using methyl orange as the indicator. This (4–5 per cent silicic anhydride) stock solution of sodium silicate will keep indefinitely. When it is desired to prepare the jelly for plating proceed as follows:

Solution I.

106 cc. standard hydrochloric acid.
Add the following salts to the acid.
Pour the sodium silicate solution in the acid.
100 cc. sodium silicate solution (4–5 per cent silicic anhydride).
200 milligrams di-potassium phosphate.
100 milligrams calcium chloride.
40 milligrams magnesium sulfate.
1 drop ferric chloride.

Add methyl orange as indicator and sterilize in the autoclave at 12 pounds pressure for 15 minutes. Dilute two 10 cc. portions to 100 cc. and titrate against solution II, III, or IV (sodium carbonate solution 7.5 grams per liter), to determine the amount necessary to solidify the jelly. One cc. only should be added and if more is required, add more carbonate to the solution.

Solution I should not stand more than 5–6 days before using. It is best used immediately.

Solution II.....	For nitrite organisms
Sodium carbonate.....	7.5 grams per liter
Ammonium sulfate....	10 grams per liter
Solution III.....	For nitrate organisms
Sodium carbonate.....	7.5 grams per liter
Sodium nitrite.....	10 grams per liter
Solution IV.....	For nitrite and nitrate organisms
Sodium carbonate.....	7.5 grams per liter
Ammonium sulfate....	5 grams per liter
Sodium nitrite.....	5 grams per liter

Solutions II, III, IV, when it is necessary to sterilize them, should not be autoclaved, but the dry salts should be placed in sterile water in sterile flasks.

Pour plates as below:

I. Place 1 cc. of the solution to be plated in the sterile Petri dish.
II. Add 10 cc. of Solution I and thoroughly mix.
III. Add 1 cc. of Solution II, III, or IV as desired and tilt to mix.

The jelly solidifies rapidly, changing in color from red to pale yellow, and care must be exercised to keep the plates level. Place in the tin boxes and incubate at 28° and 22° C.

Transferring the organisms from the silica jelly to ammonium, magnesium, phosphate agar, and nitrite agar has been found very advantageous, as they grow more rapidly on these media than on the silica jelly, but for first plating the silica jelly is unexcelled.

Magnesium Plaster of Paris Blocks. — These blocks are sometimes used for the growth of nitrite, nitrate, and nitrogen fixing organisms.

Procedure. — Thoroughly mix 1 gram of magnesium carbonate with 200 grams of plaster of Paris and slowly add water until the mass becomes pasty. By means of a spatula spread the mixture on a piece of glass and mark into the desired shape (squares are very convenient for use in Petri dishes). When dry they may be pried loose and stored indefinitely.

When desired, place in Petri dishes or other receptacles and pour in enough of the nitrite, nitrate, or nitrogen fixation solution to half submerge the block. Sterilize in the autoclave at 10 pounds pressure for 10 minutes. Inoculate with 1 cc. of infusion or 3 loopfuls of medium.

Cellulose Solution (Scales Method). — Dilute 100 cc. of concentrated sulfuric acid with 60 cc. of distilled water in a 2 liter Erlenmeyer flask. Keep the acid at 60° C. Moisten 5 grains of white ribbon S. & S. filter paper with water and add to the acid. Agitate until the paper is dissolved and quickly fill the flask with cold tap water. Do not take over 1 minute for this operation. Filter the precipitate and wash free of acid and then make up to 500 cc. with water. To prepare a solution for anaërobic studies or the agar consult the table of formulæ for solid and liquid media.

Reaction of Culture Media. — When it is desired to bring the medium to a definite degree of acidity or alkalinity, proceed as follows:

Place 5 cc. of the solution in an evaporating dish or casserole. Add 45 cc. of water and boil 1 minute. Add 1 cc. of phenolphthalein, and then run in from a burette $N/20$ NaOH or HCl until a faint but stable pink or no color with HCl remains after 1 minute of boiling. Make several titrations and average the results. The formula,

$$\frac{\text{cc. } N/20 \text{ NaOH or HCl used}}{20} : 5 :: x : 1000 \text{ cc.},$$

makes the calculation of the number of cc. of normal

HCl or NaOH necessary to be added to neutralize one liter a simple matter.

x is the number of cc. of normal alkali or acid required to exactly neutralize one liter of medium. The amount of alkali or acid actually added to any medium to give a definite degree of alkalinity or acidity is easily calculated as below.

One cc. of normal alkali or acid per liter is designated as $+1°$ for the acid and $-1°$ for the alkali on Fuller's scale. Neutral solutions are indicated as 0 on this scale.

Example. — Prepare a medium $+8°$ on Fuller's scale and one $-8°$.

When the initial titration was made with NaOH then
$x - 8$ = number cc. normal alkali to add to give $+8°$.
$x + 8$ = number cc. normal alkali to add to give $-8°$.
When the initial titration was made with HCl, then
$x + 8$ = number cc. normal acid to add to give $+8°$.
$x - 8$ = number cc. normal acid to add to give $-8°$.

The erroneous use of per cent of acidity or alkalinity in this connection is omitted here. If the student meets this method in the literature, he should consider only degrees + or − as they are cc. of normal acids or alkalies per liter and from them correct calculations may be made. Per cent cannot be relied upon as indicative of normality or hydrogen ion concentration.

The student and instructors as well should bear in mind that true acidity means hydrogen ion concentration. In adjusting the reactions of solutions where accuracy is desired, they had best be titrated cold or after sterilizing as some of the constituents of certain solutions react and hydrogen is lost.

Many of the culture media outlined in this manual require no adjustment of the reaction. In most experiments for soil bacteria when it becomes necessary to adjust the medium, $+8°$ is commonly used.

STAINING AND PREPARATION OF STAINS.

Simple Staining.

In general, the simple stains are most satisfactory in soil biological studies. For simple staining the procedure below should be followed:

1. By means of clean cornet forceps pass the clean slide through the flame three times to remove the alcohol.

2. By means of a sterile platinum loop place a small drop of sterile water in the center of the slide; this serves to give a better spreading of organisms and to indicate to the student if his slide is clean. With a sterile loop place the bacterial substance in the water and spread.

3. Air dry and fix by passing through the flame 3 times, film side up. With the forefinger at the tip of the forceps and describing a circle about 1 foot in diameter one round per second there is no danger of overheating.

4. Flood with the stain desired. Heating is permissible if carefully done and sometimes is a great advantage. Allow Loeffler's methylene blue and gentian violet to act 3 minutes, carbol-fuchsin 30 seconds, iodine 5 minutes, Lugol's iodine 3 minutes, picro-sulfuric acid nigrosin 24 hours, Bismarck brown 3 minutes.

5. Wash with distilled water, taking care not to injure the film.

6. Dry and examine with the oil immersion lens.

7. For permanent mounts the stain should be made on the cover slip and mounted in Canada balsam properly labeled, with date, name of student, and organism.

Special Staining.

Without the aid of special stains the presence and study of flagella, capsules, spores, and other characteristics would be unknown.

Many of these methods require extreme care and patience to obtain successful results.

Flagella Stain.

The staining of flagella is perhaps the most difficult method encountered by the soil biological student. At times it is easy to stain flagella and at other times difficult. The cultures should be repeated transfers of young and moist colonies from agar, not over 48 hours old and should be examined in the hanging drop first to see if they are motile. Proceed as in simple staining until after spreading the bacteria, when they should be allowed to diffuse one-half hour; then spread with platinum needle and fix by gentle heating. Apply the mordant by filtering it upon the cover glass, warm the cover glass, allow mordant to act 4–6 minutes, wash with water and apply stain immediately, allow warm stain to act for 3 minutes. Wash with water, dry, and examine.

Loeffler's Flagella Stain.

1. Mordant (warm, allow to act 4–6 minutes).
   Solution of tannin (20 per cent in water), 10 cc.
   Saturated cold aqueous solution of ferrous sulfate.
   Remove iron oxide by filtering. Use pure crystals, 5 cc.
2. Stain carbol-fuchsin (warm, allow to act 3 minutes).

Spore Stain.

(*Hansen's Spore Stain.*)

It is sometimes desirable to stain for spores (endospores) and for this purpose, make a simple stain first which will partially demonstrate the presence of spores. Fix as usual and wash with chloroform to remove fat. Wash with water and dry. Fix again and then drop on carbol-fuchsin and heat 5 minutes over a flame, renewing the stain as it boils away. Nearly decolorize in dilute acetic acid (5 per cent). Wash and counterstain with dilute aqueous methylene blue or Loeffler's methylene blue. Wash, air dry, and examine.

Capsule Stain.

In selecting bacteria for capsule staining a viscid growth on agar or a slimy appearance on the surface of a solution suggest the presence of capsulated forms. The capsules do not take up the simple stains sufficiently strong owing to their mucilaginous nature. The capsules surround the cell wall and when several capsules are united the mass is the so-called zoöglea.

Hiss Capsule Stain.

Spread film, dry, and fix; then stain with a 5 per cent solution of gentian violet and steam slightly, wash at once with 20 per cent copper sulfate solution, dry between filters, and examine.

Nodule Tissue Stain.

(Flemming's Triple Stain.)

This method of stain is especially adapted to differentiating tissues in the nodule (Microtome sections).

The section is first placed in:

(1)	Safranin 0 (sat. al. solution)	50 cc.
	Distilled water	50 cc.
	Aniline water	5 cc.

After washing in water, it then goes into (2):

(2) Saturated aqueous solution of gentian violet.

It is then washed in water and passed into (3):

(3) Aqueous solution orange G. or other suitable stain, strong or weak (about one-half saturated).

Protozoa Fixative, Stain, and Mount.

(Martin and Lewin Method.)

Place soil in some receptacle which will give a large ratio of depth to diameter and add through a funnel to the bottom of the soil layer enough of picric fixative to cover the soil and shake disk immediately.

Picric Fixative.

Picric acid, 1 per cent	100 cc.
Alcohol, 70 per cent	100 cc.

Float cover slips marked to indicate film side on the surface to obtain film. Stain and mount by the method below: Films may be obtained by the corrosive fixative.

Mercuric chloride (sat. solution)	100 cc.
Alcohol, 70 per cent	100 cc.

Treat picric films as indicated below and corrosive films likewise, omitting No. 1:

1.	Corrosive solution	2 minutes
2.	Alcohol, 70 per cent + iodine in potassium iodide	5 minutes
3.	Water, distilled.	
4.	Hæmalum *a*: hæmatoxylin	1 gram
	Hæmalum *a*: water, distilled	1000 cc.
	Hæmalum *a*: sodium iodate	0.2 gram
	Hæmalum *a*: alum	50 grams, 5 minutes
5.	Wash with *b*: alum	1–2 grams, 3 times
	Wash with *b*: water	100 grams
6.	Tap water (till blue).	
7.	Alcohol, 70 per cent	5 minutes
8.	Eosin in absolute alcohol	3–5 minutes
9.	Absolute alcohol I	1 minute
10.	Absolute alcohol II	1 minute
11.	Xylol I	2 minutes
12.	Xylol II	1 minute

Mount in balsam and preserve to be used to demonstrate the presence of active protozoa in normal field soils.

Protozoa Stain.

The use of certain stains serves to emphasize some of the functions within the cell and to differentiate the structures.

Neutral red (1–800 physiological salt solution or tap water) distinguishes nucleus, nutrition vacuoles (alkaline

yellowish red), acid fermentation granules, and fatty granules.

Neutral violet colors the metachromosomes. Bismarck brown (1–20,000–30,000) colors the nutrition vacuoles. Congo red proves acid in the nutrition vacuoles. Litmus and alizarin sulfate also prove this. Vesuvin stains bacteria in the nutrition vacuoles of protozoa a very brown color.

Methylene blue and gentian violet in equal parts in aqueous solution are useful for distinguishing protozoa.

Algæ Fixative and Stain.

(*Picro-nigrosin.*)

This stain has the advantage of fixing the organism and staining at the same time. It is useful in collecting specimens on that account. It should be allowed to act 24 hours. Combine in equal parts the aqueous solutions of picric acid and nigrosin.

FORMULÆ OF STAINS.

It is of advantage to have certain stock solutions of the stains and of other materials which do not deteriorate and which are used in large amounts.

Formulæ of Stock Solutions of Simple Stains.

Methylene blue	7 grams
Alcohol, 95 per cent	100 cc.
Gentian violet	8 grams
Alcohol, 95 per cent	100 cc.
Fuchsin	4 grams
Alcohol, 95 per cent	100 cc.
Aniline	10 cc.
Distilled water	500 cc.

The stock solutions are best made by the application of heat. The aniline should be shaken for 10 minutes and filtered through filter paper (this solution will not keep over a week).

Simple Stains.

The simple stains are prepared as follows: It is always best to filter the stock solution.

1. Methylene blue:
   - Saturated alcoholic solution methylene blue ..... 30 cc.
   - Potassium hydroxide, 0.01 per cent solution 100 cc.
2. Carbol-fuchsin:
   - Saturated alcoholic solution fuchsin ..... 5 cc.
   - Carbolic acid, 5 per cent solution ..... 45 cc.
3. Aniline gentian violet:
   - Aniline solution ..... 50 cc.
   - Saturated solution gentian violet ..... 8 cc.
4. Lugol's iodine solution:
   - Iodine ..... 1 gram
   - Potassium iodide ..... 2 grams
   - Water, distilled ..... 300 cc.
5. Iodine solution:
   - Iodine ..... 3 grams
   - Alcohol, 70 per cent ..... 100 cc.
6. Picro-sulfuric acid:
   - Picric acid ..... 2.5 grams
   - Sulfuric acid, conc. ..... 5 cc.
   - Water, distilled ..... 1000 cc.
7. Picric acid, alcoholic:
   - Picric acid ..... 10.0 grams
   - Water ..... 1000 cc.
   - Alcohol, 70 per cent ..... 1000 cc.
8. Nigrosin, alcoholic:
   - Nigrosin ..... 1 gram
   - Alcohol ..... 196 cc.
   - Water ..... 4 cc.
9. Nigrosin, aqueous:
   - Nigrosin ..... 2 grams
   - Water, distilled ..... 1000 cc.
10. Bismarck brown:
    - Bismarck brown ..... 2 grams
    - Alcohol, 70 per cent ..... 100 cc.
11. Fuchsin, aqueous:
    - Fuchsin ..... 1 gram
    - Water ..... 100 cc.

Formulæ of Stock Solutions of Disinfectants.

1. Carbolic acid	50 grams
Water, distilled	1000 cc.
2. Carbolic acid	20 grams
Alcohol	100 cc.
3. KOH (sticks)	1.25 grams
Water, distilled	1000 cc.
4. Corrosive sublimate, $HgCl_2$ sat. in water	1000 cc.
Alcohol, 70 per cent	1000 cc.
5. Corrosive sublimate, $HgCl_2$	1 gram
Water	300 cc.
6. Corrosive sublimate, $HgCl_2$	1 gram
Water	500 cc.
7. Corrosive sublimate, $HgCl_2$	1 gram
Water	1000 cc.
8. Formalin alcohol:	
Commercial formalin (40 per cent formaldehyde)	2 cc.
Alcohol, 70 per cent	100 cc.
9. Formalin aqueous:	
Formalin	2 cc.
Water, distilled	98 cc.
10. Formalin strong for preserving specimens:	
Formalin	4 cc.
Water, distilled	100 cc.

Special Stains.

1. Orange G.:	
Orange G	1 gram
Water, distilled	100 cc.
2. Safranin:	
Safranin	1 gram
Alcohol, 95 per cent	50 cc.
Water, distilled	50 cc.
3. Hæmatoxylin:	
Saturated solution ammonia alum	100 cc.
Add drop by drop solution of hæmatoxylin 6 cc. alcohol	1 gram
Expose to air one week. Filter and add 25 cc. glycerin and 25 cc. methyl alcohol. Let age 2 months before using.	

4. Carbol-methylene blue:
   - Methylene blue ..... 1.5 grams
   - Absolute alcohol ..... 10 cc.
   - Titrate in an evaporating dish and add gradually carbolic acid, 5 per cent aqueous solution ..... 100 cc.
5. Eosin aqueous:
   - Excellent for cell contents and cellulose walls.
   - Eosin ..... 1 gram
   - Water ..... 100 cc.
6. Hæmalum:
   - Hæmatoxylin ..... 1 gram
   - Alcohol, 95 per cent, hot ..... 50 cc.
   - Then add to:
   - Alum ..... 50 grams
   - Water, distilled ..... 1000 cc.
   - Cool, let settle, filter, and preserve from mold by use of a crystal of thymol.

FIXATIVES.

1. Chromic acid, 1 per cent:
   - Chromic acid ..... 1 gram
   - Water ..... 100 cc.
2. Osmic acid, 2 per cent:
   - Osmic acid ..... 2 cc.
   - Water, distilled ..... 100 cc.
3. Picric acid:
   - Picric acid ..... 1 gram
   - Water ..... 100 cc.
   - Alcohol, 70 per cent ..... 100 cc.
4. Corrosive fixative:
   - Corrosive sublimate, sat. solution ..... 100 cc.
   - Alcohol, 70 per cent ..... 100 cc.

For other information on stains, fixatives, and special methods consult texts on bacteriology, Plant Anatomy, by Stevens, Methods in Plant Histology, by Chamberlain, Behrens Tabellen, bei W. Behrens, and botanical literature.

CHEMICAL METHODS.

The chemical methods described in this manual have been selected from the various possibilities in the different fields of chemistry, after thorough and painstaking research conducted under the conditions required in soil biological studies.

QUANTITATIVE DETERMINATION OF NITROGEN.

In all these methods determinations should be made in duplicate and blank determinations run on all reagents. All analyses are reported in milligrams per 100 grams of water-free soil, or air-dry soil, and as pounds per acre.

Total Nitrogen in Soil. — The Kjeldahl method modified to include nitrates is the most reliable method for the determination of total nitrogen in soils.

Procedure. — Place 10 grams of soil (5 grams if an alkali or marine soil, 2 grams if a peat) in a 500 cc. Kjeldahl flask, add 20 or 30 cc. sulfuric acid (according to organic matter content) containing 1 gram of salicylic acid, mix thoroughly and add 5 grams of sodium thiosulfate, heat slowly at first; after 10 minutes boiling add 1 drop of metallic mercury (0.6 gram), continue digestion until the contents are grayish in color (about 2 hours), add potassium permanganate to a permanent pink color, transfer to a liter Kjeldahl flask (glass), using 250 cc. nitrogen-free distilled water. Place the receiving flask containing the standard acid in position and turn on the steam and air of the pipe distillation apparatus. Add the required alkali (60 cc.) containing the potassium sulfide to the Kjeldahl flask, connect with apparatus and distill at least 40 minutes, obtaining about 200 cc. of distillate. Titrate the distillate against ammonium hydroxid with sodium alizarin sulfonate or cochineal, as indicator.

Total Organic Nitrogen in Soil. — Employ the method previously described, omitting the salicylic acid and sodium thiosulfate. This method is very reliable where nitrates are not a factor in soil studies.

Total Nitrogen in Microörganisms, Plants, and Other Organic Materials. — The following method developed in this laboratory is a modification of the Kjeldahl-Gunning Arnold method and has given excellent success. It is rapid, accurate, and convenient. This method has been used constantly for determining nitrogen in green and dry plants and under these conditions it is unexcelled.

Procedure. — Place the sample (0.2–1 gram) in the 500 cc. Kjeldahl, add 20 cc. sulfuric acid, about 6–8 grams of potassium bisulfate (fused) and mercury as in Kjeldahl method. Digest $1\frac{1}{2}$–2 hours. Proceed to distill as in the Kjeldahl method, using either pipe or tank distillation apparatus. The acid and alkali should be about $N/20$ for accuracy. Sulfuric acid and sodium hydroxid are used when the amounts of nitrogen are small. If nitrates are a factor, apply the salicylic acid and sodium thiosulfate modification and delay the addition of the mercury until reduction has proceeded 10–15 minutes. Titrate as usual.

Ammonia Nitrogen. — The ammonia in soils, especially in ammonification studies where large amounts are present, is most easily and satisfactorily determined by direct distillation with magnesium oxide. A slight hydrolysis occurs, but aside from this the method is very reliable in the hands of students with the pipe distillation apparatus used in this laboratory which has entirely eliminated the usual troubles.

Procedure. — Place 100 grams (more may be used) of soil, either dry or moist, in a liter Kjeldahl flask, add 250 cc. water and 6–8 grams magnesium oxide. Place receiving flasks in position, turn on steam and air and connect

Kjeldahl flasks with apparatus, light burners, and distill 45 minutes. Titrate as in total nitrogen method.

Ammonia Nitrogen by Aëration. — The aëration method for the determination of ammonia is accurate and applicable to small amounts. The method herein described is a modification of the Folin method applied to soil and was developed in this laboratory.

Procedure. — Place 50 grams of fresh soil (dry may be used) in a 500 cc. Kjeldahl flask, add 100 cc. water and 5 grams of heated magnesium oxide, connect with a 400 cc. shaker bottle containing standard sulfuric acid ($N/20$). The apparatus is set up in battery of as many as 20 or more, and connected with a vacuum pump run by a motor. The air is washed with sodium hydroxid and sulfuric acid and drawn through the apparatus for 17–19 hours, or conveniently over night. The titration is made against weak alkali ($N/30$), using rosolic acid indicator where small amounts of ammonia are present.

Nitrite Nitrogen. — The determination of nitrite nitrogen in solutions is easily accomplished by the following method in which ammonia and nitrate do not interfere.

Procedure. — Filter the soil or the medium containing the nitrite into a 250 cc. Jena beaker and wash residue 2–3 times with distilled water, add 50 cc. of dilute sulfuric acid (4 cc.–4000 water) very slowly, keep cool, and then add an excess of $N/10$ potassium permanganate, let stand 5 minutes, and then add 5 cc. of 10 per cent potassium iodide solution and titrate the free iodine with $N/10$ sodium thiosulfate. (Hot starch solution may be used but is not necessary.) 1 cc. $N/10$ permanganate $\simeq$ 0.7005 milligrams of nitrogen.

Nitrate Nitrogen. — The determination of nitrates alone is made by the Devarda method or the aluminum reduction method. Ammonia and nitrites must be expelled.

Procedure. — (1) The filtered solution or acid extract of a soil is first made alkaline with nitrogen-free potassium

hydroxide and boiled until the ammonia is expelled, which requires about 20–30 minutes, depending upon the solution and the amount of ammonia present. Acidify with strong acetic acid, adding it frequently during evaporation. Evaporate to dryness on steam bath and take up with 5 cc. acid and again run to dryness. The procedure in both methods is identical to this point.

(2) In the Devarda method transfer to a 500 cc. Kjeldahl flask using 200 cc. water and add 0.5 gram Devarda metal and 4 cc. potassium hydroxid (300 grams per liter), distill one hour using heat, collecting in standard hydrochloric acid. Titrate as usual.

(3) In the aluminum reduction method after running to dryness, the salts are transferred to a reduction tube, 50 cc. of water added, and 1–4 cc. nitrogen-free potassium hydroxid, a strip of aluminum, and reduction allowed to proceed over night. With solutions high in organic matter reduction is slow, and the solutions should be tested for absence of nitrites or nitrates with diphenylamine sulfuric acid or a little Devarda metal should be added when distillation is carried out. Distill 40 minutes and titrate as usual. Both these methods are accurate and convenient. The Devarda method is more rapid. The aluminum method is convenient where large numbers of analyses are to be made, and especially where only a limited amount of apparatus is available during a laboratory period.

Nitrite and Nitrate Nitrogen. — The determination of both nitrites and nitrates is made by either the Devarda or aluminum reduction methods.

Procedure. — The solution should be made alkaline and the ammonia expelled; and then follows the procedure as outlined in the method for the determination of nitrate nitrogen under 2 or 3.

Inorganic Nitrogen. — The determination of total mineral nitrogen is made by direct reduction and distillation with Devarda metal or by reducing in the cold

and then distilling with the aluminum, taking care in the latter method to make the water in the trap acid, as ammonia may be given off if present in large amounts.

Qualitative Tests for Nitrogen.

Organic Nitrogen. — Ignite a small portion of the substance on platinum foil and test the residue for inorganic salts. Test some of the original substance for nitrate.

Prepare a stock solution by placing a piece of clean metallic sodium (sodium is kept in kerosene; remove and clean so no vapors of the oil will be mistaken for the sodium vapors) the size of a pea in a small 2-inch test tube. Place test tube in an iron clamp attached to an iron stand, add a little material and heat until the vapors of sodium form a layer $\frac{1}{2}$ inch high. Place 3 drops of the liquid, or if a solid an equivalent amount, letting it fall at intervals of one to two seconds upon the sodium vapors, taking care not to let the substance touch the side walls. Quickly add a second piece of sodium and ignite strongly. By means of a pair of forceps carefully lower the hot tube into 10 cc. of distilled water in a beaker. Warm, filter, and use this stock solution for the tests of nitrogen and sulfur. It may also be used to test for chlorine, bromine, and iodine, if desired. If ammonia is a factor, add strong alkali and note the odor of ammonia. Organic ammonium salts give the nitrogen test and odor with alkali.

Procedure. — Boil a few cc. of the *alkaline* stock solution for two minutes with five drops $FeSO_4$ solution and one drop of $FeCl_3$ solution. Cool, acidify carefully with HCl. If the precipitate does not disappear, leaving a blue or bluish green precipitate or a clear yellow solution, warm gently. Cool, and filter through a clean white filter paper and wash. A blue precipitate of Prussian blue shows the presence of nitrogen. Often, if iodine is present, a blue coloration may appear at this point. To distinguish from

the nitrogen test, wash the filter with alcohol to dissolve out the iodine.

Test for Organic Nitrogen and Sulfur when Present Together. — Acidify one cc. of the stock solution with HNO_3 and add a drop of ferric chloride. A deep red coloration is due to the formation of ferric sulfocyanide. Always carry on tests for organic nitrogen and organic sulfur together with this test.

Ammonia. — Place a few drops of the solution to be tested in a test tube or a Nessler tube, add 10 cc. or 50 cc. of water to mark on tube, and then add 0.1 cc. Nessler reagent. Yellowish coloration indicates ammonia. Test is accurate to 0.00025 of a part per million. Large amounts of ammonia are best shown by the ammonium chloride test. Whenever possible distill a portion of the solution to be tested.

Nitrites. — The value of this qualitative test depends upon its use in the presence of nitrates and ammonia. The Greiss method is well suited to demonstrate the presence of nitrite. It is a delicate test and cannot be relied upon to indicate quantity unless performed as below.

Place 0.2 cc. of the solution to be tested in 10 cc. of water, another 0.2 cc. in 20 cc. of water in test tubes, add 0.5 cc. sulfanilic acid, and then 0.5 cc. of alpha naphthylamine acetate freshly made up. Note the number of minutes required at room temperature (22°–24° C.) for the color to appear as faint, medium, and strong. Compare with the table found below:

				Time, min.	Color
1	mg. of N as nitrite	from 50 cc.	solution in 10 cc.	$1\frac{3}{4}$–2	faint
1	"	"	" 10 cc.	5	medium
1	"	"	" 20 cc.	5	faint
3	"	"	" 10 cc.	1	medium
3	"	"	" 10 cc.	3	strong
3	"	"	" 20 cc.	3	faint
3	"	"	" 20 cc.	5	medium
3	"	"	" 20 cc.	12	strong
5	"	"	" 10 cc.	$\frac{1}{2}$–1	strong
10	"	"	" 10 cc.	.....	strong

A standard solution will be available for comparison. Always run blanks on water and reagents.

Nitrates. — The most useful test for nitrates where nitrites are present is the paratoluidine sulfate reagent. Nitrites do not interfere permanently and 100 parts per million of nitrogen as nitrite may be present and not cause error on the nitrate determination. There must be at least 80 parts per million of nitrogen as nitrate to give a reliable test. This test serves to show nitrates in sufficient amounts to be relied upon for making transfers and to determine when quantitative analysis should be attempted.

Place 1 cc. of solution to be tested in a test tube, add an equal volume of concentrated sulfuric acid without mixing the liquids; then add 4 drops of the reagent paratoluidine sulfate solution. The test should stand 3–5 minutes. A red ring at the point of contact indicates the presence of nitrates. Nitrites give a yellowish brown coloration. There is present at least 4 milligrams in 50 cc. of solution when a good red ring develops after 3 minutes. Brucine sulfuric acid is a delicate and excellent reagent for nitrates, giving a red coloration which later becomes yellowish red. Diphenylamine sulfuric acid gives an excellent blue coloration with the nitrate, while the nitrite color is brownish.

Quantitative Determination of Sulfur.

Total Sulfur in Soil and Organic Materials. — The total sulfur content of a soil or crops is determined by the bomb combustion method.

Procedure. — The materials are first finely ground. Place 5 grams of soil, or 1 gram organic material, in the bomb cup and add 12 grams sodium peroxide for the soil, 3 grams for the organic matter, 1 gram magnesium, powder, thoroughly mix using caution not to scatter the sodium peroxide. Place the cover on the bomb cup and make tight with the lock nut. Heat until bottom of bomb is red. Cool in water. Transfer fusion to beaker using warm

water, acidify with concentrated hydrochloric acid (34 cc. for 12 grams peroxide), adding 10 cc. in excess of neutrality. Evaporate to dryness on steam bath; take up with 1–1 HCl and again evaporate to dryness a second time. Take up with hot water and filter hot. The filtrate and washings are heated to boiling and 15 cc. of a ten per cent solution of barium chloride added with constant stirring. Place on steam bath and keep warm six hours. Filter and wash free of chlorides, dry and burn to constant weight, adding sulfuric acid after the first weighing and again burn to expel excess acid. Weigh as barium sulfate. 7.04 mgs. of $BaSO_4 \Rightarrow$ 1 mg. S.

Another more convenient and a rapid method is that of the sulfur photometer. With the sulfur in solution ready for precipitation proceed as below.

Make slightly acid with hydrochloric and make up to 250 cc. in a graduated flask. Mix and take out 10 cc. to which 90 cc. of water is added. Place the 100 cc. in an Erlenmeyer flask, add 0.3–0.5 gram of barium oxalate powder (equal parts barium chloride and oxalic acid), cork immediately, and shake occasionally for 20 minutes. Adjust the graduated tube in the water in the crystallizing dish so the rounded end is under water ($\frac{1}{2}$ inch of water). Use the electric light. Darken the room with the black shades. Place some of the solution in the separatory funnel, admit solution in tube until the last tip of the cone of the light just disappears. Remove tube, read in millimeters the depth of liquid. Repeat reading three times, using same solution. Refer to the chart and report as milligrams of solution per 100 grams of soil and as pounds per acre. This method is accurate to 0.2 or 1 per cent and gives excellent results for sulfur in soils. Read the standard solution and report it with the results obtained.

Sulfates in Soils. — Place 100 grams of soil in a 400 cc. shaker bottle, add 200 cc. of water acidified with hydrochloric, 5 cc. per 1000, and shake for 7 hours in the

mechanical shaker, filter and make up to 250 cc. and proceed as in the total sulfur determination, using sulfur photometer.

Qualitative Test for Sulfur.

Organic Sulfur. — To 1 cc. of the stock solution prepared for organic nitrogen test, add acetic acid and then lead acetate. A black precipitate indicates sulfur.

Inorganic Sulfate. — Add barium chloride to the solution. A white finely divided crystalline precipitate indicates sulfate.

Hydrogen Sulfide. — For the gas use moistened lead acetate paper; add lead acetate in acetic acid solution.

Determination of Phosphorus, Carbon, Dry Matter, Acidity, and Magnesium.

Total phosphorus, carbon (total organic and inorganic), and magnesium in soils. Organic materials and raw rock phosphates.

The methods given in "Soil Fertility Laboratory Manual," by Hopkins and Pettit, are used for these determinations.

Determination of Calcium.

Proceed as in the manual referred to above with the exception of titrating the oxalate against $N/10$ potassium permanganate instead of weighing as CaO. 1 cc. $N/10$ $KMnO_4$ = 2 mgs. Ca.

Determination of Iron.

Total Iron. — The total iron is determined by reducing all the iron present to the ferrous iron and then oxidizing to ferric iron by $N/10$ permanganate.

Procedure. — Place 20 cc. of the solution to be analyzed in a 250 cc. beaker, add 50 cc. water and 15 cc. concentrated sulfuric acid. Add zinc dust and heat if neces-

sary. Test a drop for complete reduction by placing it on the porcelain plate in contact with ammonium thiocyanate. Red coloration indicates ferric iron. If no ferric iron is present cool and titrate with $N/10$ potassium permanganate. 1 cc. $N/10$ $KMnO_4$ = 0.0056 Fe, = 0.0072 FeO, = 0.0080 Fe_2O_3.

Carbon Dioxid. — The carbon dioxid evolved during decomposition of organic residues is determined by collecting it in standard potassium hydroxid solution contained in fermentation valves. Remove the valve, wash the contents with 200 cc. hot water into an Erlenmeyer flask, add 3–5 cc. of a ten per cent solution of neutral barium chloride, shake, let stand a few minutes and titrate the excess potassium hydroxid with standard acid preferably of equivalent strength. 1 cc. $N/2$ KOH = 11 mgs. CO_2.

MECHANICAL METHODS.

Collecting Soil Samples for Biochemical Analysis. — The collection of soil samples for biochemical studies should be carried out in the same manner as sampling for soil analysis which consists of taking 16–20 borings per tenth acre plot, by means of the soil auger or soil tube. This ensures a representative sample. Duplicate samples are taken to a depth of $6\frac{2}{3}$ inches, 20 inches, and 40 inches as desired. The borings are mixed and the required amount placed in a properly labeled Mason fruit jar. This makes the work comparable and meets the practical requirements.

Collecting Soil Samples for Bacteriological Analysis. — A different problem is presented in sampling a given area for bacteriological analysis than that of sampling for soil analysis. The irregular results obtained from samples which include the surface two inches have caused them to be discarded in taking the sample. It has also been found that the soil auger is responsible for contamination, especially when the subsurface and subsoil are sampled.

The sampling is accomplished by removing the surface two inches with a sterile spatula and then with a sterile spatula drawing the sample. Place it on a sterile oilcloth or in a sterile soil pan, mix, and place the composite sample in a sterile container. This does not disturb the soil materially, and suffices for surface samples. A special soil tube which is constructed of brass, pointed at one end, and which is plugged with cotton at the other end, has recently been recommended. Such a tube made longer than the one proposed by Noyes (Jour. Am. Soc. Agron. (1915) **5,** 239) should prove valuable for obtaining samples

deeper than $6\frac{2}{3}$ inches. For studying the bacterial flora at greater depths than the surface $6\frac{2}{3}$ inches the pit method has been employed.

It consists in digging a pit to the required depth to which it is desired to obtain the last sample. The sides of the pit are cut down with a spade as sharply as the soil will permit, and then they are sterilized either by direct flaming or scraped with a sterile spatula. The samples are then removed from the sides at right angles to the vertical axis. This may be done with a sterile soil tube or a sterile spatula. The sample is treated as in the other method outlined from this point.

The container used may be either a sterile cotton-plugged bottle or a sterile fruit jar. The spatulas and other apparatus are sterilized by flaming in the field. (An alcohol lamp is used.) A sterile agate pan is a suitable substitute for the oilcloth. The samples should not be taken when the wind is blowing over a light breeze unless the soil is moist on the surface. Early in the morning, 6–9 o'clock, is usually the best time for taking samples.

The error due to contamination of the sample in the field is very slight if the above conditions are fulfilled. The soil samples are used in the desired experiments immediately after collection.

Preparation of the Soil Samples. — Soil samples for biochemical analysis of field experiments are not dried for the determination of ammonia. For the moisture and nitrate determinations the samples are dried in an electric oven at 108° C. for 8 hours. Samples collected for bacteriological analysis are immediately used for inoculation or incubation.

Samples intended for laboratory practices (to be used from December to April) are air-dried; sieve to remove stones and large roots and place in barrels or soil bins, *for use later*. Some soils are pulverized, but in no case are they ground as in soil analysis. Dry soil is satisfactory

for demonstrating the principles involved in most soil biological studies. A supply of fresh, moist soil is always maintained in the greenhouse which serves any time as a source of fresh inoculating material.

Sampling of Crops. — The use of common farm materials instead of artificial materials, such as casein, blood meal, etc., is taken as the standard in these studies. Samples of hays, straws, stover, and such like should be collected in the field so that their history may be accurately known. Decomposition is related to the stage of development of crops and it is, therefore, of advantage to know the condition of sample. Samples should be air-dried and ground if used in beaker experiments. Grinding to pass a 2-mm. or 10-mesh sieve is sufficient. A card catalogue of all soil samples, limestones, phosphates, and crop samples is a great convenience. The analysis is entered on the card together with the other necessary data. Farm and green manures are collected and used whenever possible.

Shaking. — A mechanical shaker is convenient for use in preparing soil infusions and soil extracts.

Preparing a Soil Infusion. — The bacteria are satisfactorily removed from the soil particles by shaking the soil with water. Place 100 grams of soil in a 400 cc. sterile shaker bottle and add 200 cc. sterile distilled water. Place a clean rubber in the bottle and shake for 5 minutes. Allow solution to settle 15 minutes and then use as desired by means of sterile pipettes.

Ignition of Soil. — Ignited soil is used in culture solutions, especially for nitrite and nitrate bacteria. Place the soil in an iron or nickel crucible and burn at a red heat until all the organic matter is completely oxidized. Large quantities may be burned in a kiln and stored for use.

Centrifuge. — A rapid separation of bacteria and soil can be made by use of the centrifuge. It is also valuable in obtaining extracts containing enzymes and the like.

Filtration. — Soil solutions are filtered for chemical determinations and for making soil extract media. A battery of suction filters is of great advantage in obtaining clear soil extracts. A rapid filtration can be made through glass wool or absorbent cotton. Most soil biological media are filtered through cotton. The Berkefeld and Pasteur-Chamberland filters are indispensable for obtaining bacteria free filtrates.

Cleaning Glassware. — It is absolutely necessary that all glassware shall be perfectly clean. Acids, alkalies, and organic matter do not permit equal distribution of the solutions to be examined.

The test tubes, Petri dishes, and flasks are cleaned in the following way: Boil in soap and water for 10–15 minutes or immerse in the following hot cleaning solution and leave over night:

Potassium bichromate	60 parts
Concentrated sulfuric acid	460 parts
Water	300 parts

(Add acid slowly with constant stirring.)

Wash with water, rinse with distilled water, and invert to dry. Test tubes may be easily dried in the hot-air oven.

The tumblers are washed in the ordinary way with tap water, rinsed with distilled water, and inverted to dry.

It is well to plug the test tubes and flasks with cotton when clean and dry. (See the instructor about rolling plugs.)

For most glassware which is used for chemical work simple washing in tap water with a cleanser, then with distilled water and finally rinsing with distilled water is sufficient. Dipping in weak hydrochloric acid and then rinsing in distilled water is efficient for a great deal of the glassware employed in the determination of nitrogen.

Cleaning cover-slips and slides requires especial attention since the success of flagella staining and obtaining

good permanent preparations depends to a great extent upon clean slips and slides. The following method has given best results in this laboratory. Wash in distilled water, boil 10 minutes in strong nitric acid, remove and wash in distilled water (do not handle the slips or slides with your hands or dirty forceps), and then wipe dry. Place in absolute alcohol in containers used only for this purpose. Test the cover-slips and slides with distilled water to see if they are clean by drawing the film over the surface at will.

If alkali is used caution must be exercised as hot alkali or strong alkali dissolves the glass, producing an etched appearance.

Autoclave. — The autoclave represents sterilization by moist heat under pressure. It is by far the most satisfactory means of sterilization for most media, solutions, and materials that will stand heating under pressure.

The autoclaves 4B and 2B are connected with high-pressure steam. To operate, proceed as follows: Open cocks under doors and lower cock under autoclave. Turn on slowly the high pressure steam cock (upper cock under autoclave), one-fourth turn at first, increasing gradually later.

The table below is a guide to the use of the autoclaves.

Material	Pressure, pounds	Time.
Water	12	10 min.
Solutions	12	15 min.
Solid media	15	15 min.
Sand and solution	15	30 min.
Urea, dextrose	10	15 min.
Sand	15	2–4 hours
Soil	15	6–8 hours

In sterilizing soil, wait until all the air is expelled from it before closing the autoclave. For such masses use the maximum thermometers in the interior of the mass during sterilization.

TABLE OF AUTOCLAVE — TEMPERATURES AND PRESSURES.

Steam pressure, pounds	Temperature.	
	C.	F.
0	100	212
5	109	228
10	115.5	240
12	118.0	244
15	121.5	250
20	126.5	260
25	131	268
30	134.5	274

Hot-air Oven. — The hot-air sterilizer has four compartments. The burners are in the lower compartments. To operate turn on the gas by pulling the levers at either end. Light burners and close doors. When the thermometer in the door registers 350° F. (20–25 minutes), pull the levers at each end shut. Usually the oven is loaded with all the glassware to be needed for a long time or with duplicate sets and allowed to run 5–8 hours.

Sterilization of Glassware. — Invert the clean Petri dishes and place them in the round seamless tin boxes* assigned for this purpose. Place the cover on the box and sterilize by heating in the hot-air oven at least 1 hour at 350° F. The boxes are inverted before opening to prevent possible contamination of the Petri dishes.

Pipettes are handled as follows: Plug the mouth end with cotton and then place them in the horizontal copper boxes used in sterilizing them. Heat in the hot-air oven as above. It is not always necessary to plug the pipettes if commonplace caution is exercised in their use after sterilizing.

Tumblers, cylinders, and other glassware which cannot

* A 24-ounce round seamless tin box used in the manner above has been found to give excellent satisfaction. The boxes should be heated several hours before using the first time, owing to their being lacquered.

be safely subjected to dry heat or steam sterilization can be effectively sterilized by letting them stand in (1–300) solution mercuric chloride for 20 minutes and rinsing with sterile water.

Glass bottles, lipless Jena glass beakers, or tile pots can be used in place of tumblers and permit of sterilization in the autoclave.

Sterilization of Seeds. — It has been found extremely difficult to completely sterilize seeds for use in experiments where sterile conditions are required in the beginning or throughout. A satisfactory method is wanting for all kinds of seeds. However, in a great deal of the work, complete sterilization is not necessary, especially is this true of legume studies. To recognize the possibilities and eliminate the necessity of complete sterilization will often spell success in these experiments.

A solution of mercuric chloride (1–500) for 10 minutes gives an efficient sterilization against legume, non-symbiotic nitrogen fixers, nitrite, nitrate, ammonifiers, and many other organisms but does not insure killing mold spores.

Solutions of mercuric chloride, hydrogen peroxide, copper sulphate, bromine, silver nitrate, and sulfuric acid sometimes give rather unsatisfactory results owing to the persistence of air bubbles on and inside the seeds.

Hutchinson and Miller used a solution of mercuric chloride in a vacuum apparatus. This obviated the trouble from air bubbles.

Treatment with a 5 per cent solution of chloride of lime for three hours has given good results at this laboratory.

Harrison and Barlow suggest a method which is suited to special investigations. Pods are picked from plants while they are yet green. They are then washed in 1–1000 mercuric chloride for one hour and dried in folds of sterile cotton. The pods are then burned by holding in a flame with sterile forceps, after which they are opened and the seeds placed in folds of sterile cotton. When dry they

are removed to plugged sterile test tubes by means of sterile forceps.

Large seeds with tough seed-coats are taken in forceps, dipped in alcohol (95 per cent) and passed through a low flame. Cowpeas and soybeans have been successfully treated in this way in this laboratory.

Sterilization of Nodules. — The nodules are effectively sterilized by treatment with 1–500 mercuric chloride for 3 minutes, washing in sterile water, and then crushing in another portion of sterile water in a sterile container with sterile glass rod.

Sterilization of Parts of Plants. — It is sometimes necessary to sterilize the stem or leaf of a plant for inoculation purposes. It has been found that 1–1000 mercuric chloride rubbed into the leaf or stem will not only give very satisfactory sterilization but seems to penetrate the tissue and tends to keep the cells sterile. In inoculating experiments this fact should be considered as it may destroy the inoculation. For further methods consult Irwin Smith's "Bacteria in Relation to Plant Diseases, I."

Sterilization of Soil. — It is often necessary to sterilize a soil for experimental purposes. This subject has been studied by many workers and the present methods are included below.

It is important for the student of soil biology to note the changes which have been found to occur in the sterilization of a soil, as they account for the irregular behavior observed in many experiments. It will be recalled that sterile conditions have been stated as being very unsatisfactory for plant growth. Some of the reasons are to be found in the following paragraphs.

The three common methods are:

1. Moist heat (autoclave).
2. Dry heat (hot-air oven).
3. Volatile antiseptics.

Moist Heat. — The autoclave is the only satisfactory means of attaining complete sterilization aside from fire. The changes which occur increase with a rise in temperature.

The chemical changes brought about by steam under pressure in the soil are many and complex, but only a few are of importance. These are the nitrates, nitrites, ammonia, and precipitation reactions, such as formation of insoluble calcium, phosphorus, and iron compounds.

Lyon and Bizzell concluded that 30 pounds pressure for two or four hours reduced the soil nitrates and nitrites to ammonia. They also found a great increase in the ammonia content after sterilization. This ammonia originates chiefly from organic matter. Schreiner and Lathrop found a notable increase in many forms of organic nitrogen, an increase in water soluble constituent and in acidity upon treating at 30 pounds pressure for three hours. Thus it is seen that steam sterilization greatly increases the soluble matter of a soil and changes the organic matter more than either of the other methods.

The biological effects are evidenced by complete death of all forms of life in the soil and the deleterious influence exerted on plant growth for 2–3 months after treatment. Some investigators have noted very great beneficial results to plant growth after sterilization by this method, planting, however, after the soil has been well aërated. Sometimes this increase amounts to 4–10 times the crop obtained on the unsterilized soil.

The physical characters suffer in a similar way as in the dry heat method which is described below.

In order to determine correctly the temperature of the interior of the mass of soil a maximum thermometer should be inserted into the soil.

Dry Heat. — The use of dry heat for soil sterilization changes the chemical, biological, and physical properties of the soil.

The chemical studies have shown that the nitrogen undergoes changes. The total nitrogen may or may not remain constant according to its state of decomposition or according to the type of soil. Some soils yield ammonia and volatile organic nitrogenous decomposition products when heated much above 110° C.

The soluble nitrogen is greatly increased by the heating due to changes brought about in the insoluble forms, and the amount made soluble in this way is related to the moisture content of the soil.

Heating at 200° C. changes the organic matter of soils. At this temperature the soluble material is increased from 6 to 10 times.

Heating at 100° C. and 250° C. increases the solubility of all the mineral constituents except sodium in both water and fifth normal nitric acid as solvents. At 100° C., there is an increase in water-soluble calcium, magnesium, phosphorus, sulfur, and bicarbonates. Potassium, silicon, and aluminum increased in half the soils tested while iron decreased in most instances.

Heating at 250° C. or ignition produced similar results. At 150° C. nitrates decomposed while at 200–250° C., practically total destruction of nitrates took place. Ammonia was produced in large amounts at 200° C. At 200° C., 25 per cent of the total nitrogen was lost.

The life of the soil is rendered almost extinct for the time being when a soil is heated at 95° C., or above. This temperature kills the vegetative stages of most of the plant life and the active stages of animal life. The spores are not destroyed completely until ignition is reached. The fauna and flora of any soil may be changed at any temperature above 45–50° C. Russell and Hutchinson found from two to four times the crop on a soil heated at 95° C. as on an unheated soil. Many results indicate that plants grow better on heated than unheated soils.

Physically the differences appear to be in the increased

capillary and absorptive power of the soil as determined by Richter in 1896, when he heated a soil to 100° C. for six hours on three consecutive days.

Volatile Antiseptics. — This method is being studied with great interest at the present time in many laboratories. At best, it is only partial sterilization acting very similar to dry heat at 98° C.

Carbon bisulphide and toluene are used most commonly. Chloroform, ether, xylol, and others may be employed for partial sterilization. Four per cent of toluene is effective.

This method of treatment kills the living organisms but does not injure the spores or the encyst forms of life. The life of the soil greatly increases after this treatment and plant growth is more vigorous than on untreated soils. The increased chemical results noted are due to biological changes. Ammonia and nitrate production are greatly increased at first. Unless kept under sterile conditions, these differences gradually subside. Air-drying a soil rapidly or soils which have been dried a long time give similar results when the soil is again placed under normal conditions. This is due to a suppression of the fauna and flora during drying. The rate of multiplication of the bacteria under such conditions makes them the predominant form o life and the consequent yield of ammonia and nitrate is high at first, but later, when the soil remains under normal conditions, the numbers and activities fall to the same level as fresh or untreated soil.

Sterilization of Sand. — No permanent changes are caused by sterilizing sand. It should be well aërated after sterilization before being used as a medium for plants. The ignited soil is unchanged by sterilization.

POT-CULTURE METHODS.

The value of data obtained in pot-culture experiments is recognized by the soil biologist. Under the controlled conditions afforded in the well-equipped greenhouse many facts, unsolvable under field conditions, are established.

The following paragraphs are included to assist the student in considering the essentials for successful pot-culture experiments.

Containers. — The one-gallon earthen jar, one-gallon battery jar and the four-gallon earthen jars are satisfactory containers. A galvanized pot of the Wagner type is excellent as the water may be added beneath the surface. Evidence of zinc poisoning has been obtained in experiments of long duration with zinc pots. For accurate studies glass jars are preferable although the cost and breakage are high. The one-gallon earthen jar has a capacity of 10 pounds of soil and 12 pounds of sand. Drainage is provided by placing a glass tube in the bottom.

Sand Medium. — Crystal white sand is an excellent medium for studying the elements of plant food. It should be washed free of salts with hydrochloric acid and with distilled water until free of acid. When great accuracy is required other methods such as ignition and reduction are necessary.

Soil Medium. — Soil should be used as the medium ultimately and whenever it will not vitiate the results. The previous history is desirable. It should be sieved and mixed carefully before being placed in the container. Clay soils are more difficult to experiment with owing to their preventing drainage and root development. Carbonates will greatly decrease this trouble.

Moisture. — The optimum moisture content should be established and maintained throughout accurate experiments. This in many cases is determined by an empirical method. Weighing the jars at 4–7 day intervals and

adding water in equal amounts during these periods to all treatments has proved very satisfactory.

Plant Food. — The plant food elements are conveniently applied in solutions as given in "Soil Fertility Laboratory Manual," Hopkins and Pettit. Calcium carbonate and dolomite are applied as the dry salt, 10 grams per gallon jar.

Inoculation of Legume Seeds. — Excellent results are obtained when inoculating sand and soil cultures by the following method. Wash the nodules to be used for inoculation with mercuric chloride 1–300 for 10–12 minutes; then wash in sterile water and finally crush in another portion of sterile water. This avoids the transference of an unnecessary number of organisms not concerned in the legume experiment. Place a few cubic centimeters (5 cc. per seed if large or with small seeds 10–20 cc. per jar) of this infusion on each seed before covering with sand or soil. This method is the simplest and best for pot-culture experiments. A few nodules are sufficient to make several liters. It is not necessary to sterilize with the mercuric chloride solution in all cases. This method of inoculation has never failed to give excellent results at this station. Pure cultures from the laboratory or a soil infusion may be used with good results. Where added soil will not affect the experiment, it may be employed for inoculation. The glue method may also be used.

Planting. — A careful selection of seeds to be planted is important. Irregular seeds and those with ruptured coats should be discarded. In many experiments the seeds should be accurately weighed in order to have a check on their chemical composition. A high per cent of germination is desirable. Usually it is advisable to plant a few more seeds than necessary for the final stand so that they may be thinned to a definite number per jar which number depends upon the size of the jar and the kind of plant.

Crops. — Plants differ in their adaptability to grow under greenhouse conditions and only experience can make fine distinctions as to the best choice of crop. As a rule, annuals are more uniform in their growth than biennials or perennials; this is especially true of the legumes. Cowpeas, soybeans, and the cereal crops are well suited to these methods.

In the selection of the crop, the temperature should be considered as well as the light values. The fall is a poor time for the growing of most crops as the light is not intense enough. Warm-weather crops will stand the temperature of the greenhouse even in summer. Sudden changes in temperature should be avoided as they are disastrous.

Plant diseases and insects are a serious menace to greenhouse experiments and a careful operator should be employed to regulate the temperature and fumigate the house properly. Distilled water sprayed on the plants checks the ravages of red spiders. Sulphur may be found useful as a temporary remedy for mildews. An interesting case of contamination came under the writer's observation in which red spiders carried the cowpea organisms from one jar to another until all became inoculated.

Growth of Plants under Sterile Conditions. — This is not a desirable method of experimentation owing to the abnormal conditions which it involves. It is, however, sometimes necessary to grow plants under sterile conditions in order to establish the influence of a definite factor or to determine a specific reaction. Bell jars, Woulfe bottles, beakers, or battery jars covered with cotton will be found of service in this connection. A side tube jar is often valuable for such studies. Erlenmeyer flasks and test tubes can be used by plugging with cotton. Various devices have been tried with partial success.

Records. — Accurate records must be kept of all the

facts of importance, such as the history of the soil, plant food applied, moisture content, kind of seed, number of seeds planted and the number allowed to remain, irregularities in growth, injuries due to insects, or from other causes, and of greatest importance, the weights or yields.

Photographs should be taken as their worth may prove inestimable. Measurements of growth are often valuable data. The harvested materials are usually air-dried or oven-dried and placed in properly labeled receptacles for storage until ready for analysis.

SUGGESTIONS FOR INSTRUCTORS AND STUDENTS PREPARING TO TEACH

ACID, ALKALI, AND OTHER STANDARD SOLUTIONS.

The number of standard solutions required in a course of this kind is necessarily greater than in courses dealing with but a single field of chemistry or biology.

Experience has demonstrated the value of the solutions herein found:

	Solution.	*Use.*
1.	$N/20$ NaOH.....	Titrating media and for use in titrating small amounts of nitrogen.
2.	$N/20$ HCl.....	Titrating media. Not used very much as most solutions are at the correct reaction.
3.	$N/1$ NaOH.....	Correcting reaction.
4.	$N/1$ HCl.....	Correcting reaction.
5.	N/KNO_3.....	Acidity determinations.
6.	$N/7$ HCl.....	Nitrogen determination when amount is sufficient to give more than a difference of 2 cc. in titration.
7.	$N/7$ NH_4OH.....	Used as above.
8.	$N/20$ H_2SO_4.....	Titrating small amounts of nitrogen.
9.	$N/1483$ KOH.....	Det. P. 1 cc. = 0.2 mg. phosphorus.
10.	$N/1483$ HNO_3.....	1 cc. = 0.2 mg. phosphorus.
11.	$N/10$ $KMnO_4$.....	1 cc. = 2 mg. Ca, calcium nitrite. 1 cc. = 0.7005 mg. nitrite, iron determination.
12.	$N/10$ $Na_2S_2O_3$.....	Nitrite and iron determinations.
13.	6 per cent $FeCl_3$....	Phosphorus determination.
14.	10 per cent $FeCl_3$...	Culture media.
15.	10 per cent NaCl...	Culture media.
16.	10 per cent $CaCl_2$..	Culture media.
17.	10 per cent KI.....	Nitrite determination.

18. Reduced KOH (300 grams per liter) solution.....	Nitrite and nitrate determinations.
19. Ammonium molybdate solution....	Phosphorus determination.
20. Saturated ammonium oxalate solution.....	Calcium determination.
21. Nessler solution....	Ammonia determination.
22. NaOH (2 pounds per liter (26.6 grams K_2S).....	Nitrogen determinations.

INDICATORS.

The following indicators have been selected from a large number tested as being most reliable.

Formulæ of Indicator.	*Alkali and Acid Color and Use.*
1. Sodium alizarin sulfonate, 1 gram.....	Alkalies red — acids yellow.
Alcohol, 60 per cent, 100 cc. ..	In all nitrogen determinations where ammonia is distilled. (See 6. Rosolic acid.)
Best indicator where H_2S is not present. Volatile organic bases do not affect this indicator as much as the others.	
2. Cochineal, 3 grams.....	Alkalies violet — acid yellowish-red.
Macerate in the solution below:	
Water, 200 cc.....	In nitrogen determinations where sulfides or sulfur distill over in amounts sufficient to obscure the end point of number 1.
Alcohol, 50 cc.	
This indicator is only slightly affected by H_2S or CO_2.	
3. Lacmoid, 3 grams.....	Alkalies blue —acids red.
Water, 700 cc.....	Alternative for Number 1.
Alcohol, 300 cc.	
This indicator is affected by H_2S.	

4. Methyl orange, 1 gram.....	Alkalies yellow — acids red.
Water, 1000 cc.....	Strong acids.
Not affected by H_2S or CO_2...	Sodium silicate, hydrochloric acid, sodium carbonate.
5. Phenolphthalein, 1 gram.....	Alkali red — acid colorless.
Alcohol, 50 per cent, 100 cc..	Phosphorus, media, acidity.
Very sensitive to CO_2.....	Valuable for weak alkalies, carbonates, alkali earths.
Useless for NH_4S.....	Bicarbonates neutral to this indicator, organic acids.
6. Rosolic acid (commercial), 1 gram.....	Alkali rose red — acid yellow.
Alcohol, 60 per cent, 100 cc...	Ammonia titration when aluminum or copper are used for reduction. Use sodium hydroxid and sulfuric acid as large amounts of ammonium salts interfere.
Useless for acetic acid. Not affected by ammonia but by ammonium salts.	

COLORIMETRIC REAGENTS.

Soil solutions and other turbid solutions which retain their color upon filtering through the ordinary filters may be decolorized by use of aluminum cream (alkali-free). Two to three cc. per liter usually suffices; repeat if necessary. It is best, however, to avoid as much as possible the use of such materials on account of occlusion.

Formulæ of Reagents.	*Use and Color.*
1. Diphenylamine sulfuric acid....	Nitrite — brownish blue.
Diphenylamine, 1 gram.....	Nitrates — blue.
Sulfuric acid (conc.), 100 cc....	Rings on a palate.
Small amount of ferric salts do not interfere.....	Delicate test.
2. Brucine sulfuric acid.....	Red ring shows nitrates.
Brucine, 1 gram.....	Delicate test.
Sulfuric acid (conc.), 100 cc.	
3. Paratoluidine sulfate.....	Red ring with nitrates.
Paratoluidine, 0.5 gram.....	Not delicate.
Sulfuric acid (conc.), 100 cc. Very well adapted to indicating nitrates when more than 2–3 mgs. of nitrogen present in 25 cc. of solution.	

4. Alpha naphthylamine acetate and sulfuric acid.....
   (*a*) Alpha naphthylamine, 1 gram.
   Acetic acid, sp. gr. 1.04, 200 cc.
   (*b*) Sulfanilic acid, 1 gram.
   Acetic acid, sp. gr. 1.04, 250 cc.
   Use equal amounts of each when making a test. The alpha-naphthylamine should be made up fresh each 2–3 days.
   Nitrites. Very delicate. See qualitative test for nitrites, page 116.

A list of chemicals and apparatus have been included as a guide and represent the materials which have been found essential with the present state of development of the course. For special work a larger number of sugars, organic acids, organic salts, stains, and special chemicals than found in the list included are necessary. A greater variety of chemicals and apparatus are necessary in a course of this kind owing to the varied nature of the experiments.

CHEMICALS USED BY STUDENTS IN SOIL BIOLOGY.

Acid, acetic.
" carbolic.
" chromic.
" citric.
" hydrochloric.
" molybdic.
" nitric.
" oxalic.
" osmic.
" picric.
" salicylic.
" sulfanilic.
" sulfuric.
Agar agar shredded.
" " powdered.
Alcohol, ethyl.
Alpha naphthylamine.
Aluminum metal strips.
" metal powder (coarse).
" metal powder (fine).
" and potassium sulfate.
Ammonium carbonate.
" hydrate, sp. gr. 0.90.
" nitrate.
" oxalate.

Ammonium diphosphate.
" monophosphate.
" sulfate.
" thiocyanate.
Anilin oil.
Asparagin.
Balsam, Canada.
Barium chloride.
Barium oxalate powder.
Barium hydroxid.
Blood meal.
Beef extract.
Brucine.
Calcium carbonate (ground limestone).
" acetate.
" chloride.
" nitrate.
" oxide.
" triphosphate.
" diphosphate.
" monophosphate.
" sulfate.
Calcium sulfate (gypsum).
Calcium sulfate (plaster of Paris).
Carbon bisulfide.
Casein.
Chloroform.
Cleanser.
Collodion.
Copper sulfate.
Dextrin.
Dextrose.
Diphenylamine.
Dolomite.
Ether.
Feldspar.
Ferric ammonium citrate.
" wire.
" chloride.
Ferrous sulfate.
Formaldehyde.
Gelatin.
Glass wool.
Glycerin.
Glue (furniture).
Hydrogen peroxide.
Iodine.
Kaolin.
Kieselguhr.
Lacmoid.
Lead acetate.
" oxide.
Lysol.
Magnesium ammonium phosphate.
" carbonate.
" chloride.
" metal
" oxide.
" sulfate.
Maltose.
Manganese sulfate.
Mannite.
Mercury metal.
Mercuric chloride.
Metal, Devarda's alloy.
Oil, immersion cedar.
Oil, cedarwood.
Oil, cylinder, heavy, light.
Oil, cloves.
Oil, paraffin.
Paratoluidine.
Paraffin, M. P., 52° C.
Paraffin, M. P., 40–42° C.
Paraffin, M. P., 50–53° C.
Peptone, Witte's.
Potassium bichromate.
Potassium bisulfate (fused).
Potassium carbonate.
Potassium chloride.
Potassium hydroxid.
Potassium iodide.
Potassium nitrate.
Potassium permanganate.

Potassium phosphate, monobasic.
Potassium phosphate, dibasic.
Potassium sulfate.
Potassium sulfide.
Rock phosphate.
Saccharose.
Silver nitrate.
Silver nitrite.
Sodium asparaginate.
" carbonate.
" chloride.
" hydroxid.
" (metal).
" nitrate.
" nitrite.
" peroxide.
" silicate.
" sulfate.
" sulfide.
Sodium thiosulfate.
Stains, Bismarck brown.
Eosin.
Fuchsin.
Gentian violet.
Hæmatein.
Hæmatoxylin.
Methyl blue.
Methylene blue.
Nigrosin
Orange G.
Safranin O.
Versuvin.
Indicators:
Cochineal.
Congo red.
Litmus.
Methyl orange.
Phenolphthalein.
Sodium alizarin sulfonate.
Starch, potato.
" corn.
Toluene.
Urea.
Vaseline.
Xylol (xylene).
Zinc, granulated.
" dust.
Wood ashes.

APPARATUS.

Asbestos.
Balance, triple beam.
" metric solution scale.
" weighing scoops.
Beakers, various sizes.
Bottles, various sizes.
Boxes, copper for pipettes.
" seamless tin for Petri dishes.
" slide.
Brushes, test tube, flasks.
Bulbs, distilling, Hopkins.
Burettes, various sizes.
Burners, Bunsen, pilot, and micro.
Casserole, 210 cc.
Cell, blood counting chamber.
Clamps, test tube, burette.
Corks.
Cotton.
Crucibles, porcelain, Gooch, iron.
Cylinders, graduated, various sizes.
Dishes, evaporating.
" Petri, dia. larger dish 100 mm.
" depth of lower dish 16 mm.
Files.
Filter cases for 6 sizes.

Flasks, Erlenmeyer, boiling, filter.
" Kjeldahl (500, 1000 cc.).
" volumetric and Kolle.
" various sizes.
Forceps, cornet, dissecting.
Funnels, glass, Buchner, copper.
Glasses, jelly, with tin covers.
Glass rods, tubing.
Jars, anatomical, staining, specimen.
Lamp, alcohol.
Lenses, magnifying, reading glass.
Lens paper.
Microscopic slides 3×1, concave 3×1.
" cover glasses, round, square, several thicknesses.
Motors, porcelain, agate.
Paper, litmus.
" filter, 6 sizes.
Pencils, red, blue for glass.
Pipettes, 1, 2, 5, 10, 25, 50, 100 cc.
" graduated.
" stopcock, 10 cc.
Plates, porcelain, 12 cavities.
Plates, Lafars counting.
Platinum foil, wire 0.41 mm. diameter.
Pump filtèr.
Rings, iron.
Rubber stoppers, policemen, tubing.
Section lifters.
Shears, steel.
Sieves, brass nested, set of 5.
Spatulas, 3 sizes.
Spoons, bone.
Stopcocks, Geissler.
Supports, iron, funnel test tube.
Test tubes, 150 mm. long outside.
Thermometers, several kinds.
Towels, barber.
Tongs.
Triangles.
Tripods.
Tubes, fermentation (Smith's).
fermentation safety valve in top.
Watch glass.
Wire baskets.
Wire gauze.

SPECIAL APPARATUS.

Aëration apparatus, battery of 16
Autoclave, 2B, 4B (large).
Auger, soil, 1, $1\frac{1}{4}$ inch.
Balance, analytical, No. 10 and weights.
Balance, analytical, No. 16 and weights.
Baths, steam.
Centrifuge and accessories (electric).
Colorimeter, Nessler tube, double mirrors.
Crocks, earthen, for waste material.
Digestion racks.
Distillation apparatus, battery of 20.
Filters, Berkefeld.
" Pasteur Chamberland.
Incubators, 37°, 20°, room temperature.

Jars, dialyzers.
" sample.
Microscopes, regular and binocular, and accessories.
Microtome.
Oven, electric, drying and incubator (small).
" gas, hot-air sterilizer (large).
Pans, soil.
Plate, electric, hot.
Photometer sulfur.
Pump, vacuum for aëration apparatus.
Shaking machine.
Stools, laboratory.
Trays, laboratory, various sizes.
Tables, laboratory.
Tube, soil, King.