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ON NITRIFICATION

PRELIMINARY OBSERVATIONS

By E. R. ALLEN AND A. BONAZZI

WOOSTER, OHIO, U. S. A., APRIL, 1915

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CONTENTS

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BULLETIN
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ON NITRIFICATION

I. PRELIMINARY OBSERVATIONS

I. INTRODUCTION

The effect of fertilizers and rotations on crop yields has been the subject of an immense amount of experimentation and research and much remains to be learned before satisfactory explanations can be proposed for phenomena of the complex problem of soil fertility. In recent years a very large amount of attention has been bestowed on the effect of fertilizers and soil treatments on the lower forms of plant life depending on the soil for food and habitat, and on the influence of these lower forms in turn on the higher plants.

The large number of plots at the Ohio Station furnish an unusual opportunity for the study of the action of crop rotations and soil treatments on the soil flora, and this has been selected as the main project of the laboratory of soil biology.

It is recognized at the outset, however, that this problem in its entirety is easily beyond the means for research at our disposal. Phases of the problem must be therefore taken up.

Two more or less distinct methods of attack have been employed in the investigation of the problems of soil bacteriology, the one which might be designated as the *physiological* or perhaps *biochemical* method, and the other which might be designated as the *taxonomic* or *botanical.* The underlying principle of the physiological methods is the determination of the kind and intensity of the functions of the bacteria. This is accomplished by inoculating exact portions of a given soil into nutrient solutions designed to favor a given process and determining the metabolic products after a given incubation period. Many recent investigators have proposed modifying these procedures by carrying out the tests in the "natural" soil, favoring a given process by appropriate means.

The coefficients of the different physiological activities are designated as the "nitrifying, denitrifying, ammonifying, nitrogen fixing," etc., powers. The principle of the other method of attack is the counting, isolation and classifying of the complete flora of the soil—so far as all the forms can be grown on any one medium—with an attempt to determine which forms are accidental and which are important, and to classify the important forms in easily recognizable classes. This method of attack recognizes the importance of studying not only the physiological activities of the bacteria but also the associative action between the different lower forms and between the bacteria and higher plants. The former method of attack was first suggested by Remy and later developed more fully by Lòhnis and others. The botanical method of attack has been advanced by Hugo Fischer, who contended that the Remy-Löhnis methods are unreliable and inadequate. A very concise discussion of the two methods, with excellent suggestions for future methods of attack from the taxonomic standpoint, has been given by Conn (1), who later followed out these suggestions and carried on quite extensive investigations on the flora of soils (2).

Differences of opinion exist in regard to the value of the different procedures. The criticisms of Fischer (3) have been replied to by Löhnis (4), and while the Remy-Löhnis methods have been the much more widely adopted, it by no means follows that they are superior to those conducted from the other viewpoint. Both methods undoubtedly possess merit and the ideal would be an interweaving of both methods, yet each is such an enormous problem in itself that with the facilities at our command it was necessary to choose one or the other.

It has seemed to us that the physiological method of attack is the more promising from an agricultural standpoint, since it is more important to know what the bacteria do than what they are—at least with our present meagre knowledge of such intangible phenomena as associative action of bacteria, mutual relations between higher plants and soil bacteria, etc. We must hasten to admit, however, that physiological studies which neglect morphological studies are somewhat incomplete. We are inclined to regard, however, the morphological studies as tools to be used in the solution of the greater physiological problems.

Having decided upon the physiological point of attack as preferable, it is necessary to reduce this problem further and select some group of organisms for study since the physiology of one group alone is still a very extensive problem in itself. The more

generally recognized important groups of soil bacteria are (1) the ammonifying, (2) the denitrifying, (3) the nitrifying, (4) the symbiotic nitrogen fixing, (5) the nonsymbiotic or "free living" nitrogen fixing, and (6) the cellulose decomposing bacteria. All groups are important in the nitrogen and carbon cycles of the soils and a very large literature exists on each group, from which interesting suggestions may be obtained. Just as it is impossible to decide positively in our present state of knowledge whether the morphological or the physiological methods of attack are the most important, so it is impossible in this case to decide which of the groups is the most deserving of attention, and will give the most reward as a result of research. We have chosen the nitrifying group for the reason that it may be quite accurately studied by the sensitive methods for nitric nitrogen determination, and because it is certainly an important group as evidenced by the extensive investigations that have been bestowed upon it. This group of soil bacteria we regard as of importance in the nitrogen cycle of soils, and as a valuable indicator of microbial activity in general.

It is not believed that it is desirable that all nitrogen of the soil be transformed as rapidly as possible into the nitric form, yet a large part of the soil nitrogen is transformed by these organisms and a knowledge of their activities is important. Furthermore, Gutzeit found (see page 8), for instance, that nitrifying activities were more sensitive to soil differences than were the other microbial activities studied. Moreover, Lyon and Bizzell (5) have obtained evidence indicating a possible relation existing between nitrification and higher plants. Undoubtedly then other actions of this group of bacteria besides the production of nitric nitrogen are of importance.

II. NITRIFYING POWERS OF SOILS AS AFFECTED BY SOIL TREATMENTS AND CROPPING

1. HISTORICAL

The determination of nitrifying powers of soils differently treated has been carried out by a number of investigators. In order to illustrate in a general way the nature of the results obtained by this line of research, a very brief review of some typical investigations by others is reported.

Remy (6) in one of his original papers devoted some attention to the determination of nitrifying powers of soils. By inoculating 50 cc. portions of Omelianski's nutrient solutions for nitrite and for

nitrate bacteria, and determining periodically the nitrogen transformations, differences were observed between different fields and different seasons of the year.

Löhnis (7) observed differences in nitrifying powers—as well as in other bacteriological properties,—which bore at least some relation to factors affecting crop production, e. g., method of cultivation, moisture, etc. His determinations were made by inoculating soil extract to which had been added ammonium sulphate, potassium phosphate and calcium carbonate, and determining the nitric nitrogen produced in a given time.

Gutzeit (8), following the suggestion of Wollny (9) that weeds produced deep seated effects on soils that are ill understood, studied the effects of oats and of oats and wild mustard on the bacterial activities of the soils on which they were grown. He found that the plots on which wild mustard grew possessed the following year the same decay power, the same nitrogen fixing power, but a distinctly lower nitrifying power, than the plots kept free from the above weed. These results throw new light on the action of weeds and suggest a possible explanation of the action of rotations. It is also interesting to note that Gutzeit obtained stronger nitrification in soil extract prepared according to Löhnis than he did in Omelianski's ammonium sulphate broth plus calcium carbonate, the two nutrient solutions containing essentially the same concentration of ammonium salts.

Buhlert and Fickendey (10) studied the nitrifying powers of aerated and non-aerated plots of different soils, and also other physiological activities, by inoculating quadruplicate portions of a nutrient ammonium sulphate solution with *soil suspensions* and determining the nitrate produced in 30 days. Marked differences were observed in nitrifying powers and this function stood approximately proprtional to the humus content of the soil from which the inoculum was taken. Oddly enough, they found that, with one exception, the nitrifying powers of the non-aerated soils stood above those of the aerated. The differences were small but consistent.

Stevens and Withers (11) obtained striking differences between nitrifying powers as determined in nutrient solutions inoculated with soils, and those measured in the soil itself. Their solution studies, however, are open to the objection that they overlooked the precautions Löhnis had found necessary in solution work (7), and also the fact that Gutzeit (8) had verified the statement of Löhnis that solution depth is of great importance in the growth of these bacteria, and that soil extract is preferable to Omelianski's

broth as a nutrient medium. Their conclusion that solutions as media are wholly unreliable as compared with soils as a medium for the study of soil bacteriological problems was accepted by many workers, who discarded solution methods. This trend, as well as the work of Stevens and Withers, has recently been criticised by Löhnis and Green (12), who discuss at some length the above mentioned points, contending that many of the known critical factors in solution studies on nitrification were ignored by these investigators, and they raise the point that perhaps the solutions when properly handled are as useful or more so than soil tests.

Stevens and Withers (13) also carried out studies on the ammonifying and nitrifying powers of widely separated and differently cropped North Carolina soils. Among other things they concluded that (1) the nitrifying powers, i. e., what they designated as "nitrifying energies" of North Carolina soils, are low as compared with soils studied by other investigators, and that (2) this particular bacterial potential may be increased by cropping to legumes and by the action of stable manure.

The above results are by no means all the work that has been done on the determination of nitrifying powers, but they may be taken as typical of some of the biological differences that have been revealed by this Remy or modified Remy method of attack. In all the above work observations were also recorded on other physiological groups of soil bacteria. The results on the other groups are not discussed by us for the reason that our own experimental work is confined to the nitrifying organisms. On the other hand, the investigations which we have taken up contemplate more thorough cultural and physiological studies of the causal organism than was carried out by the above workers. It is our conviction that a thorough study of one group is more contributory to progress than continued elaborate but somewhat superficial studies of all groups. It is characteristic of the extensive work on nitrification that but little has been done by way of exact studies of the organisms themselves in pure culture, and at present the organisms and their physiology are but imperfectly understood. This point is discussed more at length below.

Our preliminary experimental results on nitrifying powers which were determined in soils will now be reported.

2. EXPERIMENTAL

These investigations were begun in the summer of 1912, the object being to collect samples from representative plots at the Ohio Station to see if notable biological differences could be detected.

The experiments conducted are admittedly somewhat crude, yet they are certainly as carefully conducted as is such work generally, and the purpose in mind, that is, of hewing out in the rough, problems for future research, has been served.

Samples of slightly more than 300 grams of soil were taken to a depth of 7 inches from three borings from the plot to be studied. These were transferred to the laboratory, allowed to become air dry, 100 grams taken for "original" nitrate, while duplicate 100 gram portions were taken for the determination of nitrate producing power. These samples were moistened with 20 cc. of a 0.5% ammonium sulphate solution. This brought the soil, which is a silt loam—Wooster silt loam (14)—to approximately optimum moisture content. This amount of ammonium sulphate is equal to 212 parts nitrogen per million of soil, or 424 pounds per acre 6 inches. Stevens and Withers used an amount of ammonium sulphate equivalent to 600 parts per million, while Brown, Lipman and others used the same amount as we have in this work.

Incubation was effected in wide mouthed bottles plugged with moistened absorbent cotton. These plugs were moistened from time to time during the incubation period. The containers were kept in cupboards, the temperature of which varied from 22° to 26°. This variation in temperature is unfortunate, but, owing to incomplete laboratory facilities there was no way of avoiding it.

At the end of the incubation period the samples were transferred to aluminum pitchers, rinsing out the bottles with small amounts of a 480 cc. portion of distilled water, the remainder of which was finally added to the pitchers, the soil and water mixture was stirred well for 3 minutes, allowed to settle for a short time and then the supernatant liquid was clarified by a Berkfeld pressure filter, discarding the first 100 cc. portion of the filtrate. An aliquot of the perfectly clear filtrate was taken for nitrate determination by the phenol-sulphonic acid method. The modified reagent which has been proposed by Chamot, Pratt and Redfield (15) was used. All results are expressed as parts nitrogen per million of soil. The difference between the original and final nitric nitrogen content is recorded as the nitrifying power.

(i) Five-year Rotation: Samples were collected first from the plots of Series A of the 5-year rotation, which series was in corn in 1912. The plan of the first 18 of the 28 plots of this 5-year rotation experiment is shown in Fig. 1 [1].

[1]For a more detailed description of these and other plots of this Station, as well as the yields from the same, see Ohio Agricultural Experiment Station Circular 144 (April, 1914).

FIGURE I. Plan of fertilizing in 5-year rotation

Plots one-tenth acre—Fertilizing materials in pounds per acre

Plot No.	On corn			On oats			On wheat			
	Acid phosphate	Muriate of potash	Nitrate of soda	Acid phosphate	Muriate of potash	Nitrate of soda	Acid phosphate	Muriate of potash	Dried blood	Nitrate of soda
1										
2	80			80			160			
3		80			80			100		
4										
5			160			160			50	120
6	80		160	80		160	160		50	120
7										
8	80	80		80	80		160	160		
9		80	160		80	160		50	120	
10										
11	80	80	160	80	80	160	160	100	50	120
12	80	80	240	80	80	240	160	100	50	200
13										
14	80	80	160				160	100	50	120
15							160	100	50	120
16										
17	160	80	80	160	80	80	160	100	25	60
18	Barnyard manure, 8 tons on corn and wheat									

In addition to the above treatments the west half of all these plots has been treated with caustic lime at the rate of 1 ton per acre in 1902, the east half with a like amount of ground limestone in 1907, and the west half with 1 ton ground limestone in 1912. The lime was added to all the plots, fertilized and unfertilized alike, and was applied after the land was plowed for corn and was harrowed in[1].

Samples were collected on June 5 and June 19 from the limed and unlimed plots indicated in Table I, and were incubated 10 days. The results which represent the gain during the incubation period are expressed as parts nitric nitrogen per million of soil.

These results show that no appreciable nitrification has taken place in the ten-day period. All differences reported are probably within experimental error.

[1]Ohio Agricultural Experiment Station Bul. 279 (1914).

TABLE I. Nitrifying powers of soils from Series A of the 5-year rotation

Plot No.	June 5		June 19	
	Unlimed	Limed	Unlimed	Limed
1	*	2	11	0
2	*	10	8	0
3	5	13	11	*
5	0	1	7	*
6	6	10	22	*
7	0	6	11	*
8	*	16	7	*
9	*	11	*	*
14	4	2	*	*
18	17	6	*	6

*Less nitric nitrogen at the end than at the beginning of the incubation period.

(ii) Continuous Culture Plots. This series had been started in 1894 and the plots were, therefore, in their 19th year in 1912 when sampled. The plan and treatment of these plots is shown in Figure 2.

In addition to the above treatments, applications of lime were made as follows[1]: Wheat series—Burned lime applied at rate of one ton per acre to west half of all plots in 1899. Same treatment to east half in 1902. Oats series—Ground limestone applied to west half of all plots at rate of 2,670 pounds per acre in 1911. Corn series—Hydrated lime applied at rate of 2,200 pounds per acre to west half of all plots in 1905. Ground limestone added at rate of 4,140 pounds per acre to west half of all plots in 1911.

Samples were collected on June 11, 24, and Aug. 1, 1912, from the west half of plots 6, 7 and 8 of each series. The samples were incubated 10, 20, and 30 days, as shown in Table II.

TABLE II. Nitrifying powers of soils from continuous culture plots

Plot No.	Crop	June 11	June 24		Aug. 1
		10 da.	20 da.	30 da.	30 da.
6	Wheat	11	50	49	..
7	"	4	2	*	..
8	"	13	40	82	..
6	Oats	20	28	96	90
7	"	8	0	0	12
8	"	18	3	*	156
6	Corn	13	35	111	109
7	"	*	0	*	13
8	"	*	23	48	113

*Less nitric nitrogen at end than at the beginning of the incubation period.

The data on the 20 and 30 day incubation periods show that the samples collected June 24 from plot 8 of the oats series failed to nitrify appreciably. This seemed rather surprising since samples

[1]Unpublished results, Ohio Agricultural Experiment Station.

FIGURE II. Plan of fertilizing in continuous culture

PLOTS ONE-TENTH ACRE

	Fertilizing materials in pounds per acre
Wheat	1 None
	2 Acid phos., 160; muriate potash, 100; nitrate soda, 120; dried blood, 50*
	3 Acid phos., 60; muriate potash; 30; nitrate soda, 120; dried blood, 50*
	4 None
	5 Yard manure, 2½ tons
	6 Yard manure, 5 tons
	7 None
	8 Acid phos., 160; muriate potash, 100; nitrate of soda, 280; dried blood, 50**
	9 Acid phos., 120; muriate potash, 60; nitrate of soda, 280; dried blood, 50**
	10 None
Oats	1 None
	2 Acid phos., 160; muriate potash, 100; nitrate soda, 160
	3 Acid phos., 55; muriate potash, 50; nitrate soda, 160
	4 None
	5 Yard manure, 2½ tons
	6 Yard manure, 5 tons
	7 None
	8 Acid phos., 160; muriate potash, 100; nitrate soda, 320
	9 Acid phos., 110; muriate potash, 100; nitrate soda, 320
	10 None
Corn	1 None
	2 Acid phos., 160; muriate potash, 100; nitrate soda, 160
	3 Acid phos., 60; muriate potash, 30; nitrate soda, 160
	4 None
	5 Yard manure, 2½ tons
	6 Yard manure, 5 tons
	7 None
	8 Acid phos., 160; muriate potash, 100; nitrate soda, 320
	9 Acid phos., 120; muriate potash, 60; nitrate soda, 320
	10 None

(South)

*120 pounds nitrate soda plus 50 pounds dried blood is equivalent to 160 pounds nitrate of soda.
**280 pounds nitrate soda plus 50 pounds dried blood is equivalent to 320 pounds nitrate of soda.

of plot 8 of the other series nitrified appreciably in the same periods. Therefore samples were collected from the oats and corn series on Aug. 1 and incubated 30 days as before. The results which are included in Table II show that soils from plot 7 of the oats series do not possess the power of oxidizing appreciable amounts of ammonia to nitrate. The failure of the samples collected on June 24 to nitrify must be left unexplained for the present.

It seems from the data in Table II that the conclusions may be reasonably safely drawn that (1) very little nitrification takes place in these soils in 10 days; (2) plots 7 (untreated) of each series have a very feeble or no nitrifying power, while plot 6 (treated with yard manure) and plot 8 (treated with chemical fertilizers) possess a moderate power to oxidize ammonium salts to nitrate.

(iii) Barnyard Manure Test. Samples for laboratory studies were next taken from different plots of this experiment, the plan of which is shown in Figure 3. This experiment was begun in 1897; the cropping consists of a 3-year rotation of corn, wheat and clover.

In addition to the above treatment liming has been practiced in this experiment, the lime being applied once in a rotation. It was applied to all plots, fertilized and unfertilized alike, after they had been plowed for corn, and it was then worked into the soil during the operations of preparing the seed bed. The amounts of ground limestone which have been applied to Series A are as follows:

Year	Amount added per acre
1906	4,400 lbs.
1909	3,000 lbs.
1912	1,880 lbs.

Samples were collected from Series A, which was in corn in 1912. The date of sampling, plots studied, time incubated and results obtained are reported in Table III.

TABLE III. Nitrifying powers of soils from plots of barnyard manure experiment

Plot No.	June 11	June 24	
	Nitric nitrogen produced in		
	10 da.	20 da.	30 da.
3	16	121	169
4	1	114	179
6	3	88	196
9	*	35	87
13	13	44	171
16	6	90	139
17	*	129	194
18	*	64	127

In Table III as in Nos. I and II there is little or no nitrification in 10 days, but appreciable oxidation does take place in the 20 and 30 day periods. It should be noted that the check plots 4 and 17

FIGURE III. Arrangement of plots and plan of fertilizing in experiments with manure

PLOTS ONE-SIXTEENTH ACRE

11	Nothing	Nothing	1
12	Yard manure and gypsum	Yard manure and floats	2
13	Stall manure and gypsum	Stall manure and floats	3
14	Nothing	Nothing	4
15	Yard manure, untreated	Yard manure and acid phos.	5
16	Stall manure, untreated	Stall manure and acid phos.	6
17	Nothing	Nothing	7
18	Chemical fertilizer	Yard manure and kainit	8
19	Chemical fertilizer	Stall manure and kainit	9
20	Nothing	Nothing	10

SECTION A

11	Nothing	Nothing	1
12	Yard manure and gypsum	Yard manure and floats	2
13	Stall manure and gypsum	Stall manure and floats	3
14	Nothing	Nothing	4
15	Yard manure, untreated	Yard manure and acid phos.	5
16	Stall manure, untreated	Stall manure and acid phos.	6
17	Nothing	Nothing	7
18	Chemical fertilizer	Yard manure and kainit	8
19	Chemical fertilizer	Stall manure and kaiuit	9
20	Nothing	Nothing	10

SECTION B

11	Nothing	Nothing	1
12	Yard manure and gypsum	Yard manure and floats	2
13	Stall manure and gypsum	Stall manure and floats	3
14	Nothing	Nothing	4
15	Yard manure, untreated	Yard manure and acid phos.	5
16	Stall manure, untreated	Stall manure and acid phos.	6
17	Nothing	Nothing	7
18	Chemical fertilizer	Yard manure and kainit	8
19	Chemical fertilizer	Stall manure and kainit	9
20	Nothing	Nothing	10

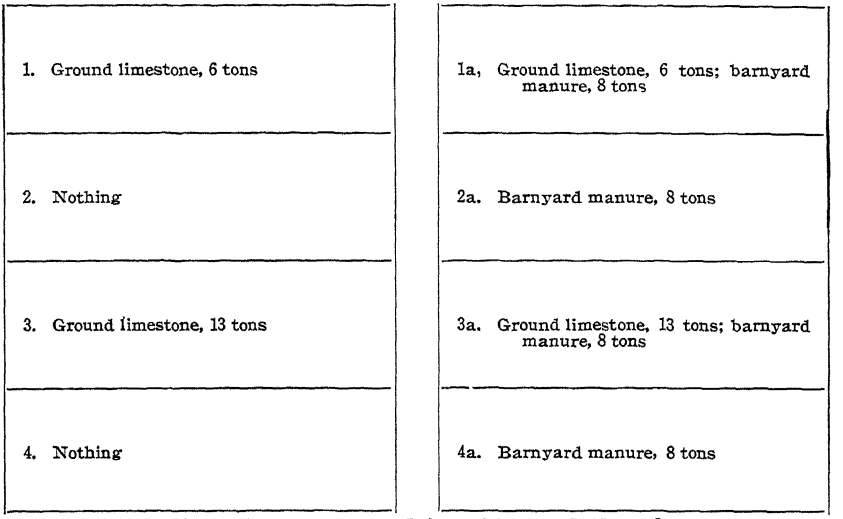
SECTION C

NORTH

are not feeble nitrificants as compared with the treated plots, as was the case in the soils from the continuous culture plots reported in Table II. Further, the general average of the nitrifying powers of the soils from the barnyard manure test is greater than that of the soils from the continuous culture plots.

(iv) Limestone Extension Experiment. The plan of this series is shown in Fig. 4.

FIGURE IV



This experiment was started in 1907 and the above treatment was applied at that time. The plots were all sown to sweet clover (Melilotus alba-Desv.) in 1912. Luxuriant growths were obtained on the limed plots, but the crop was a failure on the unlimed plots. The clover was cut on plots 1a and 3a June 13, but was left growing on plots 1 and 3 [1]. At the time of sampling, the sweet clover on plot 3 was rank and green and was about 5 feet tall. The other plots sampled contained only slight growths of weeds and grasses.

Samples were collected Aug. 1, 1912, from plots 3, 3a, 4 and 4a, and the nitrifying power determined as in the previous cases. The data are given in Table IV.

TABLE IV. Nitrifying powers of soils from plots of limestone extension experiment—Collected Aug. 1, 1912

Plot No.	Nitric nitrogen produced in		
	10 da.	20 da.	30 da.
4	...	...	23
3	6	14	23
4a	4	14	21
3a	104	208	187

[1]These variations in cropping between the two plots were a part of the experiments of the Department of Agronomy on the power of sweet clover to re-seed itself.

The data show that plots 4, 3 and 4a possess feeble nitrifying powers while plot 3a possesses a high nitrifying power, transforming considerable of the ammonium nitrogen to the nitrate form in 10 days. The failure of plot 3, which had received in 1907 13 tons of ground limestone, to nitrify strongly, was very surprising and therefore the determinations were repeated on samples collected October 9, 1912. At this time the clover was still standing but was past its stage of maximum growth.

The methods of determining the nitrifying power were varied somewhat in this case, in order that possible light might be thrown on the differences between methods. Ammonium sulphate was added in amounts equivalent to 600 parts of nitrogen per million of soil, and controls consisting of moistened soil to which no ammonium sulphate had been added were incubated with those receiving ammonium sulphate. These altered conditions would affect the absolute but not the relative results of the experiment. The nitrifying powers obtained by subtracting the amounts of nitric nitrogen produced in the controls from those produced in the soils receiving ammonium sulphate are given in Table V.

TABLE V. Nitrifying powers of soils from plots of limestone extension experiment—Collected Oct. 9, 1912

Plot No.	Nitric nitrogen produced in		
	10 da.	20 da.	30 da.
4	*	*	*
3	92	296	316
4a	*	0	*
3a	126	349	406

Thus both plots 3 and 3a show a vigorous nitrifying power, 3a averaging slightly above 3. Just why No. 3 should on Aug. 1 possess a feeble and on Oct. 9 a strong nitrifying power is unexplainable, unless it is due to the difference in the cropping on these plots (see page 16). However, such a large difference in nitrifying power as the result of differences in cropping has not, so far as we are aware, been reported as yet. These differences are striking and become especially interesting when compared with the results of McBeth and Smith (16). These investigators, in studying the nitrifying powers of highly calcareous soils of the semi-arid region (rainfall approximately 16 inches per annum), found that active nitrification took place in 10 days and this was the length of period used in all their incubation tests. The Wooster soil, which is de-

rived from non-calcareous material and is in the humid region (rainfall 36-38 inches), even after receiving moderate applications of lime failed to produce vigorous nitrification in 10 days, while this same soil, when treated with large amounts of calcium carbonate, i. e., made artificially calcareous, possessed a nitrifying power apparently comparable with that reported by McBeth and Smith from the naturally calcareous soils.

3. DISCUSSION

Interesting relations between bacterial activity and crop production are shown by comparing the nitrifying powers of the soils from the continuous culture plots with the crop yields obtained from these same areas. The nitrifying powers as obtained by the 30-day periods are compared in Table VI with the crop yields for the 5-year period, 1909-1913.

TABLE VI. Relations between crop yields and nitrifying powers of soils of continuous culture plots

Plot No.	Wheat		Oats		Corn	
	Grain bu. per acre	Nitrifying power	Grain bu. per acre	Nitrifying power	Grain bu. per acre	Nitrifying power
6	18.75	49	33.84	90	30.22	109
7	5.96	*	18.81	12	6.95	13
8	22.04	82	40.70	156	45.82	113

*Less nitric nitrogen at end than at beginning of incubation period.

The results are shown graphically in Figure 5. The crop yields are plotted as percentages of normal yields[1].

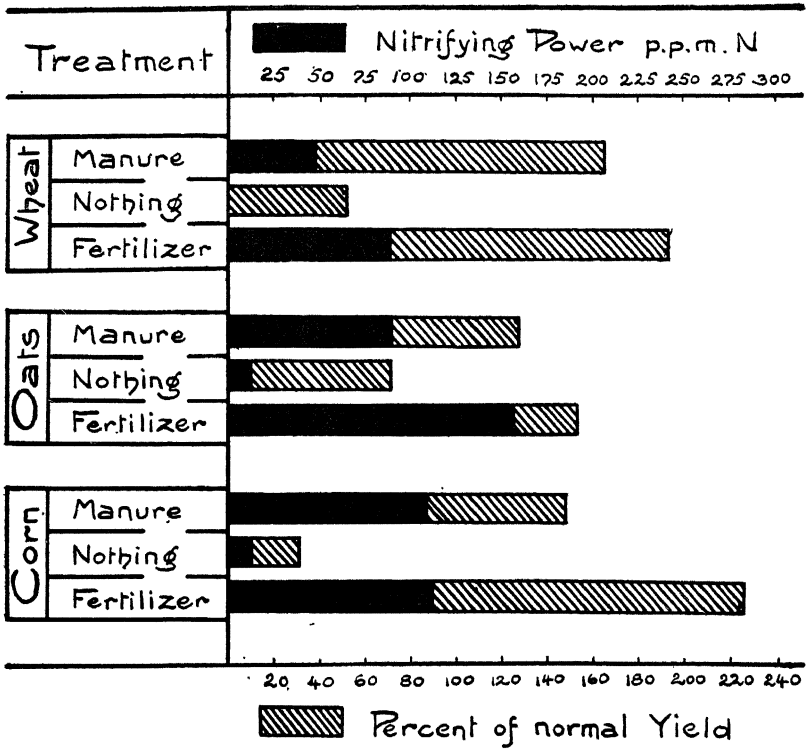
The comparison between yields and nitrifying power in the soils from the plots of the barnyard manure test are seen in Table VII and in Figure 6.

The lack of agreement between nitrifying power and crop yields is just as striking on these soils as was the agreement in the soils from the continuous culture plots. The check plots, 4 and 7, nitrified just as strongly practically as did the treated plots, while they were distinctly lower in crop producing power. Evidently, if our biological methods are to be relied upon, the factors which limit crop production in the continuous culture plots are not the same as those operating in the barnyard manure series. It is worthy of note that none of the fertilized or manured plots of the continuous culture nitrified as strongly as did the checks of the barnyard manure series.

[1]In calculating the percentages of normal yield for Figures V and VI, the 5-year average, 1908-1913, of the check plots of the 5-year rotation is taken as a normal yield.

The results obtained on the soils from the continuous culture plots are sufficiently marked to justify some discussion in regard to the causes of these differences, granting even that the data reported are meager and contain somewhat annoying sources of error (see page 10).

FIGURE V



Lack of nitrifying power may be due on the one hand to lack of organisms, or, on the other, to the presence of those conditions which prevent the growth and reproduction of the bacteria. Of course these two are mutually interdependent, and the latter operating through a period of years would produce the former.

Whatever this limiting factor or factors may be, it is, in a large part, overcome by the application of barnyard manure to plot 6 and by the application of fertilizers to plot 8. The fact that there is no considerable difference between the nitrifying powers of the corn, oats and wheat plots would indicate that these crops have not produced toxic conditions in the soil, since it is to be expected that

the different crops would act differently, or if not differently, at least in different degrees. The evidence is, however, by no means conclusive on this point. Corn does not seem to exert a beneficial effect on nitrification as might be anticipated from the work of Lyon and Bizzell (5).

TABLE VII. Relations between crop yields and nitrifying powers of soils from the barnyard manure test

Plot No.	Treatment	Yields[1] per acre			Nitrifying power
		17 yrs., 1897-1913		Hay 13 crops Lbs.	
		Corn 16 crops Bus.	Wheat 16 crops Bus.		
3	Stall manure and floats	66.38	25 85	5,054	169
4	None	32.12	10.96	2,422	179
6	Stall manure and acid phosphate	67.16	25.97	5,096	196
9	Stall manure and kainit	61.82	22.38	4,488	87
13	Stall manure and gypsum	62.38	23.50	4,116	171
16	Stall manure, untreated	60.20	21.15	4,250	139
17	None	38.10	10.96	2,891	194
18	Chemical fertilizer*	47.43	14.99	3,358	127

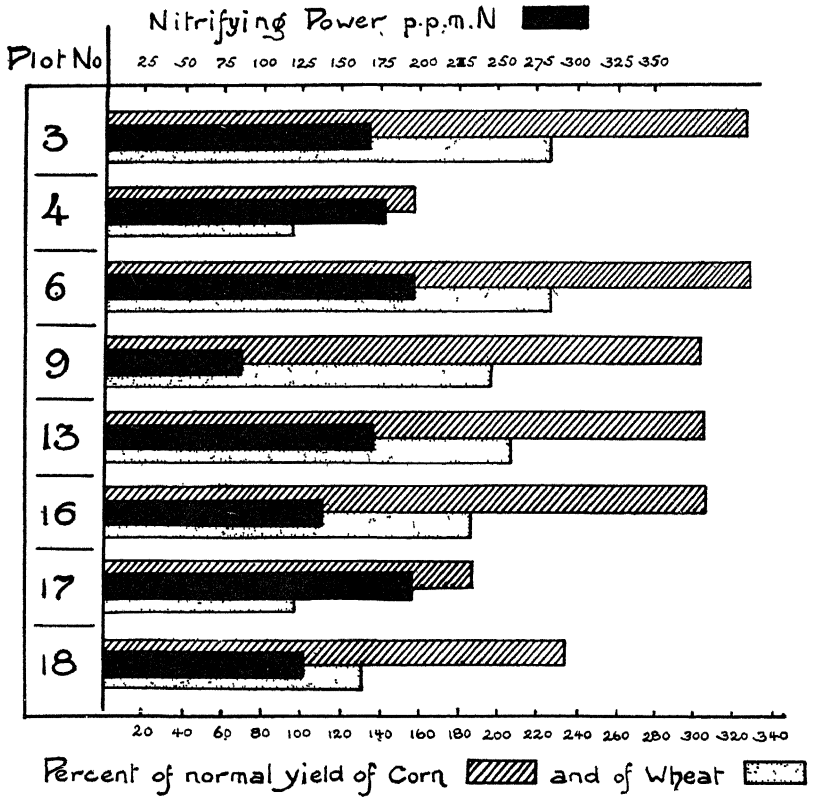
*Acid phosphate, 80 lbs.; muriate of potash, 80 lbs.; nitrate of soda, 160 lbs.
[1]Ohio Agr. Exp. Sta., Cir. 144, Table XVI, p. 88 (1914).

If, on the other hand, the differences are due to lack of one or more essential elements, this element is evidently supplied both in the manure and chemical fertilizer. Since the resultant action has been essentially the same in both cases, the theory of rare elements proposed by Kaserer (see page 29) is evidently eliminated, and the differences in bacterial activity observed are apparently related in some way to the presence of the elements nitrogen, phosphorus and potassium. But the fact that the checks of the barnyard manure series nitrified more strongly than even the fertilized plots of the continuous culture series eliminates evidently the phosphorus and potassium, since these elements were not applied to the checks of the barnyard manure series. The continuous culture checks and the checks of the barnyard manure series differ principally in the matter of nitrogen added[1]. The latter series receives the nitrogen from a legume once in three years while the former have received no extraneous nitrogen for twenty years.

[1]Examination of the data on liming shows that the barnyard manure plots have received more of this ameliorant than have the continuous culture. However, since the differences in bacteriological response, and since the corn series of this experiment was limed more heavily in 1912 than the plots of series A of the barnyard manure and yet failed to nitrify markedly in the checks, indicates that the biological difference between the plots of barnyard manure experiment and those of the continuous culture is not due to liming In view of the rapid loss of calcium carbonate from soils that has been observed by Ames and Gaither (17), it is probable that the corn plots of the continuous culture contained as much or more calcium carbonate than those of the barnyard manure series at the time these studies were made.

Since now nitrifiable material is the food for nitrifying bacteria, i. e, is the substance which they must have in order to perform their life activities—at least all researches bearing on this point indicate that these bacteria are obligate nitrifiers—it is natural to expect that the condition most likely to limit their growth is the absence of nitrifiable material.

FIGURE VI



Since, furthermore, considerable evidence has accumulated in recent years which favors the theory that the higher plants feed on ammonium compounds, and even on organic nitrogen, as well as the nitric form, it is easily conceivable that in twenty years of cropping practically all nitrifiable material has been removed and a dying of the nitrifying flora would result.

The nitrate of soda added to plots 8 of each series in continuous culture becomes, because of its transformation into other forms of nitrogen, nitrifiable material, and thus the nitrifying bacteria find food for growth and reproduction.

It seems to us that the easiest explanation of lack of nitrifying power in the check plots of the continuous culture series is that the nitrifying flora have died off because of lack of nitrifiable material as food. The fact that the fertilized and manured plots are lower than those of the barnyard manure series indicates that some other factor is operative aside from that of presence or absence of nitrifiable material. Here again, however, there is the possibility that still larger applications of nitrifiable material would have produced a normal nitrifying power.

In this connection it is interesting to note that J. G. Lipman and associates (18) have shown that the introduction of a legume catch crop into continuous cultivation produces very marked increases in crop yields. All plots in their experiment at the New Jersey Station received limestone and mineral fertilizers (1 ton per acre of ground limestone at beginning of experiment in 1908, and 2 tons per acre in fall of 1913, and an annual treatment of 400 lbs. acid phosphate and 200 lbs. muriate of potash per acre). Each of the above crops is grown with and without legume. The following data, taken from Table V of the above report, show a striking effect of legume on the sixth crop.

Wheat and rye with and without legume
(New Jersey Experiment Station)

Plot No.	Crop	Per acre		
		Bushels grain	Pounds straw	Pounds nitrogen
68	Rye, alone	8.96	1,275	12.86
69	Wheat, alone	6.25	1,025	14.38
70	Rye, followed by soybeans	17.71	3,075	29.48
71	Wheat, followed by soybeans	13.47	2,550	29.78

The following comment on the above table is made by Lipman et al.:

"From this table it will be noted that the yields were all low this year, but it is significant that the yields of grain are just about twice as great from the legume plots as they are from the plots which did not grow a legume. The yields of straw and nitrogen on the plots with the wheat and rye alone did not amount to half as much as the yields on the legume plots. It is to be noted further that the leguminous crops have a slightly increased proportion of nitrogen in rye and wheat grain, and likewise in the rye straw, but not in the wheat straw. The results for 1913 bear out the results obtained in 1911 and 1912 and make it quite clear that even a leguminous crop which has not attained full development, may add

to the soil enough humus and nitrogen to more than double the yields as compared with the plots which have grown no legume. It must be admitted, however, that the yields on all plots this year are entirely too low. The low yields on the legume plots may be due, in part at least, to the poor growth of the 1912 crop of cow peas, while the low yield on the other plots is no doubt due to the gradual depletion of nitrogen and humus of the soil." These results although extremely interesting have only been run a comparatively short time and therefore do not possess yet quite the value of the Ohio experiments.

P. E. Brown (19) reports some effects observed by him on the effects of continuous culture as compared with rotations on the bacterial activities of soils. In general, rotations were beneficial, even a two-year rotation of corn and oats producing a beneficial effect on nitrifying powers, as compared with continuous corn. Corn, oats and clover proved still better than corn and oats, and indeed better than a rotation of corn and oats followed by clover or cowpeas turned under after the oats. But what is quite significant in this connection is that continuous clover plots showed the lowest nitrifying power of the series. The results do not indicate then, as do those we have obtained on the Wooster soils, that lack of nitrifying material is the limiting factor. Since, however, the bacteriological studies on the Iowa soils were carried on in the fourth year of the plots, and since, furthermore, the soils on which the Iowa experiments were made is a naturally very fertile soil of glacial origin, possessing a high content of humus, it would not show the effects of continuous culture as readily as the Ohio and New Jersey soils, and the question of nitrogen hunger and lack of nitrifiable material could scarcely have entered at the time the experiments were conducted. The differences observed by Brown may be due to different factors than are the differences we have observed in Ohio. The three series of plots from the three Stations, however, present interesting fields of research.

4. SUMMARY

It is believed that the above reported studies on nitrifying powers of soils differently treated justify the following conclusions:

1. The nitrifying power of the naturally non-calcareous Wooster soils, even after receiving moderate applications of ground limestone, is rather feeble as compared with naturally calcareous soils.

2. Very heavy applications of ground limestone impart a high nitrifying power to Wooster soils, which is comparable with that reported from the naturally calcareous semi-arid soils.

3. The nitrifying power of a soil may or may not correlate with its crop producing power. In other words, it is conceivable that the conditions which limit the growth of higher plants in one set of plots may be different from that in another. The one factor or set of factors may limit nitrification, the other not.

4. Continuous cropping, especially without fertilization, reduces the nitrifying power of soils. Although the possibility of the production of toxic material is not eliminated, it seems that in the plots studied the deleterious effects of continuous cropping on higher plants, as well as on the bacteria, are closely connected with the nitrogen relations.

5. Such observations as have been made by Wollny, Gutzeit, P. E. Brown, Lyon and Bizzell, and those reported in this chapter, will have to be studied elaborately before satisfactory theories of crop rotations can be proposed.

III. NITRIFICATION IN SOLUTIONS AND IN POROUS MEDIA

A. SOLUTIONS

Before the process of nitrification and its relation to higher plants can be satisfactorily understood—and its relation to higher plants is as yet a matter of uncertainty—many cultural studies of the casual organisms, including detailed investigations on their physiology, must be carried out. To that end some work of this nature was taken up in this laboratory as supplementary to the above reported data.

The theory of nitrification generally held at the present time by bacteriologists is that proposed by Winogradsky in 1892 after his classical researches on the isolation and study of the nitrifying bacteria. Only ammonium salts are nitrifiable, and these in two steps by two distinct groups of bacteria, the symbiosis of which two groups is developed in nature to a marked degree. Both groups of organisms are assumed to be autotrophic, i. e., deriving their carbon from carbon dioxide, though some recent work seems to throw doubt on this point, or at least seems to be contradictory with the earlier work. At any rate, the best known method of isolating bacteria capable of oxidizing ammonium salts is by following the procedures recommended by Winogradsky. Using such methods we obtained results which indicate that the Winogradsky organisms are present in Wooster soils and therefore are probably the cause of the phenomena reported; some of the difficulties in the way of the isolation of these organisms are also shown.

1. EXPERIMENTAL

The solutions proposed by Winogradsky's colleague, Omelianski (20), were used. These had the following composition:

For nitrite bacteria

Ammonium sulphate	2.0 gm.
Sodium chloride	2.0 gm.
Potassium phosphate	1.0 gm.
Magnesium sulphate	0.5 gm.
Ferrous sulphate	0.4 gm.
Distilled water	1000 cc.

For nitrate bacteria

Sodium nitrite	1.0 gm.
Sodium carbonate	1.0 gm.
Potassium phosphate	0.5 gm.
Magnesium sulphate	0.3 gm.
Sodium chloride	0.5 gm.
Ferrous sulphate	0.4 gm.
Distilled water	1000 cc.

One hundred cc. portions of these solutions were measured into 750 cc. Erlenmeyer flasks, producing a liquid layer 1 cm. deep. After sterilization 2 grams of sterile magnesium carbonate were added to the nutrient ammonium sulphate solution and both solutions were seeded with 1 gram portions of fresh moist Woster soil taken from one of the greenhouse pots. This soil was a part of a liming experiment and had received calcium carbonate at the rate of 9,000 lbs. per acre.

(i) Cultivation of nitrite bacteria. The object aimed at is, of course, the acceleration of the nitrite producers, and the suppression of the nitrate formers, i. e., to produce nitrite, not nitrate, from ammonium salts. The symbiosis of the two forms is so well developed that their separation is a tedious task. Sub-cultures must be made at different stages of the development of the crude cultures. Inasmuch as our results may contain suggestions to others, the progressive growth of the cultures is briefly reported.

Inoculated Jan. 19. Traces of nitrite formed Jan. 31. Sub-cultures (F_1) were made on this date by transfer of 1 cc. portions of the mother culture into fresh 50 cc. nutriment ammonium sulphate solution contained in 250 cc. Erlenmeyers. Omelianski's solution minus ammonium sulphate; to each 50 cc. of this solution 0.1 cc. 2% ammonium sulphate solution was added. These were made in duplicate, the one containing $MgCO_3$, the other $CaCO_3$, and are to be designated as $F_1.MgCO_3$ and $F_1.CaCO_3$. On Feb. 28, 40 days after inoculation the mother culture (F_0) showed no ammonia and a strong nitrite reaction. Growth in the F_1 generation was slow, however, as shown in Table VIII. Attributing this to small numbers of organisms in the mother culture, at the time the sub-culture was made Feb. 14, from the F_0 generation. Only magnesium carbonate was used, and instead of the regular amount of ammonium sulphate only 0.4 gm. per liter were added. The cultures were designated as F_1—12 and and F_1—21. Results are shown in Table VIII.

TABLE VIII

Number of culture	Base	2-9		2-11	2-14	2-16	2-20	2-24		2-28		3-2	3-11
		NH_3	NO_2	NO_2	NO_2	NO_2	NO_2	NH_3	NO_2	NH_3	NO_2	NH_3	NH_3
F_1-1	$CaCO_3$	###	tr.	—	—	tr.	+	###	+	###	###	###	###
F_1-2	$MgCO_3$	###	tr.	—	—	—	+	###	—	###	##	###	tr.
F_1-12	$MgCO_3$				inoc.	—	—	—	—	tr.	##	tr.	tr.
F_1-21	$MgCO_3$				inoc.	—	—	—	+	tr.	##	tr.	0

0 _ no reaction
tr. _ traces
— _ perceptible
\+ _ distinct
_ strong
_ very strong

F_2 culture prepared as before on Mar. 2 from F_1. To 50 cc. mineral nutrient solution was added 1 cc. of a 4% solution of ammonium sulphate. No growth Mar. 11, but on April 9 all ammonia (except in checks) had disappeared and strong NO_2 reactions were obtained.

The F_3 cultures were then prepared from these F_2 cultures, using the same concentration of ammonium sulphate. In one month the ammonia had disappeared and strong nitrite, without any nitrate was manifested.

The F_4 generation was prepared from the F_3 on April 29. May 25 these showed low nitrite and no nitrate. June 12 slight amounts of nitrite and nitrate and strong ammonia were present. The cultures were then abandoned.

(ii) Cultivation of nitrate bacteria. The solutions mentioned above, which are designed to favor the growth of the nitrate producing organisms (*Bacillus nitrificans-Van Tieghem, Nitromonas-Winogradsky*) were prepared, 100 cc. portions placed in 750 cc. Erlenmeyer flasks and inoculated with 2 gram portions of the same soil that was used for the isolation of the nitrite producers. The progress of these cultures is briefly recorded.

The F_0 generation started Jan. 19. All nitrite transformed into nitrate in 23 days. F_1 generation started Jan. 31 from the F_0 (i. e., before nitrification was complete). Nitrification had proceeded markedly in 9 and completely in 11 days. The F_2 cultures were started on Feb. 9. In these and subsequent cultures up to the 5th generations, inclusive, one cc. of inoculum was used as before, and the medium was 50 cc. of nutrient nitrite solution in 250 cc. Erlenmeyer flasks. All nitrite had disappeared by Feb. 20. F_3 cultures prepared Mar. 2 showed a very strong nitrate reaction in 10 days. Owing to pressure of other laboratory work, the F_4 generation was not started till April 9, and was not tested till May 25, 46 days after inoculation, at which time all nitrite had disappeared. The F_6 generation was prepared May 25 by inoculating 50 cc. of nutrient nitrite solution, contained in 250 cc. flasks with 1 cc. of F_5 cultures. All nitrite disappeared in 9 days. The F_7 generation was then prepared in the same manner, except that they were inoculated with a spiral loop of the F_6 cultures and then one of the duplicate cultures tested daily for disappearance of

nitrite, while the other was preserved for plating. Complete oxidation took place in 9 days, which shows that the mother culture was at least fairly vigorous. Plates poured on *unwashed* nitrite agar developed only minute slow growing colonies of organisms resembling the nitromonas; the culture was, therefore, approximately pure at least; otherwise strong growths would have been developed on the unwashed agar. Pure cultures were not obtained, however. Generations F_8 and F_{18} were prepared under strictly aseptic conditions, comparing in several cases the growth when inoculated with 1 cc. with that resulting from inoculation with a spiral loop of the same culture. In each case the 1 cc. inoculum produced a culture which effected complete nitrification in 8 days, while the growth resulting from the spiral required 9 to 10 days.

The above cultures show a more rapid growth than do the nitrite producing forms. However, in view of the fact that cultures of nitrite forms had to be started again, and in view also of the fact that a relation between the condition of the mother culture and the growth of the filial culture had been indicated, new cultures of both forms were started, the method of procedure in which was to produce a much stronger growth in the mother culture before transfers were made. This point is referred to again.

(iii) Comparison of synthetic solutions with soil extracts. There is a more or less prevalent idea among investigators of soil bacteriology that soil extracts are preferable to synthetic solutions as culture media. This has been observed, for instance, by Löhnis (7) and verified by Gutzeit (8). In order now to see if these observations could be verified experiments were run in Omelianski's solution, in aqueous extract approximately according to Löhnis, and in aqueous extract of soil that had previously been ignited to destroy organic matter.

The soil used was a fertile, brown clay loam of glacial origin, containing sufficient humus to impart a dark color to it when wet. Extracts were made by boiling portions of the soil for 5 hours with 5 times their weight of distilled water under a reflux condenser. The extract from the non-ignited soil was distinctly dark straw colored, presumably as a result of soluble organic matter, while the extract from the ignited soil was reddish in color. The extracts were used in the place of water in preparing Omelianski's nitrite broth. Twenty-five cc. portions of solution were placed in 100 cc. Erlenmeyers, plugged, sterilized and then inoculated with a loopful of F_{11} culture of nitromonas (see page 26). The mineral nutrients were added thus to all flasks; the only differences in composition of the media are those derived from the soil. The soil extracts are weaker than those used by Löhnis. It is, however, almost impossible to compare extracts from different soils, except in a very approximate way.

The arrangement of the experiment was as follows:

Culture	Medium
F_{11}-0	Omeliansky's nutrient nitrite solution
F_{11}-1	Omeliansky's nutrient nitrite solution
F_{11}-2	Extract of ignited soil (25 cc.)+salts
F_{11}-3	Extract of ignited soil (25 cc.)+salts
F_{11}-4	Extract of non-ignited soil (25 cc.)+salts
F_{11}-5	Extract of non-ignited soil (25 cc.)+salts

F_{11}-O was left sterile, while all the others were inoculated. Growth was determined by testing the cultures for disappearance of nitrite. The results apear in the following table:

TABLE IX

	Aug.	September										October																					
	22	21	22	23	24	25	26	27	28	29	30	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
F_{11}-0	#	#							#							#			#		#		#	#	#	#		#	#	#				#		#	#		#	#
F_{11}-1	#	#							#							o			o		o		o	+	!	o		o		!				#		#	+		o	o
F_{11}-2	#	o							o							+			o		o		o	#	o	o		o		o				#		#	!		o	o
F_{11}-3	#	+							+							+			o		o		o	#	+	!		o		o				#		#	#		+	+
F_{11}-4	#	o							o							#			#		#		+	#	#	+		o		#				#		#	#		o	o
F_{11}-5	#	#							#							#			#		#		#	#	#	#		#		#				#		#	#		#	#

On 9-28-14 1 cc. of 1% $NaNO_2$ solution was added to each culture, and this treatment was repeated at each subsequent sampling. On 10-26-14 the cultures 3 and 5 were placed in the 37.5° incubator, while the others were left at 27°.

It is seen that the culture in the Omelianski solution grew more slowly the first week than did those in the soil extracts. After this it proceeded, oddly enough, slightly more rapidly, while the cultures in the extracts of non-ignited soils became slower in their action.

Subsequent to 10-26-14 an indication of the effect of temperature may be seen, the cultures placed at 37.5° almost failing to nitrify, while those at 27° grew rapidly as before.

DISCUSSION

The slowness with which growth proceeds in the solution cultures is typical of what may be encountered when research is taken up along these lines, and is, in our opinion, the obstruction that has prevented the proper study of the nitrifying bacteria. In view of this some discussion is merited here.

In the papers of Löhnis and associates, to which reference has already been made, it is emphasized that the conditions favoring

growth of nitrifying organisms over those suppressing it are frequently but slightly different. We observed the precautions given by Löhnis as far as was possible to do and still break down the symbiosis of the nitrite and nitrate producers. We believe, however, that many investigators have lost sight of the fact—as indeed we did in the above experiments—that decided chemical transformations in culture media correspond to extremely small bacterial growth. The chemical methods used—Nessler, Griess, etc.—are very delicate, and strong reactions indicate really only slight amounts of nitrogen. Again, the nitrifying bacteria derive their energy from the oxidation of either ammonia or nitrite, and a tremendous amount of these inorganic compounds must be transformed in order to furnish the large amount of energy required to build up protein, that is, to form bacterial cells. Thus the cultures from which sub-cultures were made in the above experiments no doubt contained very low numbers of cells, and hence the seeding into the sub-culture is very slight. The rather peculiar behavior of these bacteria in the presence of any considerable concentration, either of their food or of their by-products, must be constantly borne in mind in the growing of cultures. Observations on this point have been made by Boullanger and Massol (21). They found that nitrite production is stopped when the concentration of ammonium sulphate reaches 30 to 50 grams per liter, or when the concentration of magnesium nitrite reaches 13-15 grams per liter. Sodium and potassium nitrite exert a stronger retarding action. The nitrate producing organisms are, oddly enough, more sensitive to their food than to their by-products. Thus very low numbers introduced into a solution containing an appreciable concentration of ammonium salts or of nitrite results in feeble growth. According to this, better results should be obtained by adding to the bacterial cultures ammonia or nitrite as the original amounts have disappeared, and continuing this till very high concentrations of nitrite or of nitrate are present and appreciable bacterial growths present in the flasks, then transferring into new solutions of very low ammonia or nitrite content, and adding nitrifiable material as needed. This procedure is not uncommon in bacteriological methods, and has been used, for instance, by Wimmer (22). Cultures have already been started following these methods and very encouraging results have been obtained.

Aside from the above contributing factors there is still another possibility that must not be overlooked, which may help to explain why growth of nitrifying bacteria is slower in artificial nutrient solutions than in soil. After a number of researches Kaserer (23)

suggests that the superior properties of soil extracts over synthetic solutions, as well as the striking benefits reported by Krzmieniewski (24) from the addition of small amounts of humus and soil to Azotobacter cultures, are to be attributed to the presence in humus or soil extracts of minute traces of rare nutrients, as silico-phosphates, iron, manganese, and possibly even such elements as zinc, copper, arsenic, and titanium present in humus, soils and soil extracts, but lacking from the synthetic solutions; and that, furthermore, the autotrophic forms possess high requirements of these substances.

The results on the soil extracts and on Omelianski's solutions under identical conditions fail to indicate that extracts prepared from soils possess any superiority as a culture medium for the autotrophic form *B. nitrificans*. The solutions and containers were such that the ratio of diameter to depth was 8:1. This is less than the ratio used by Löhnis and Green, therefore these cultures cannot be said to have had optimum conditions for growth. Nevertheless, the growths from 10-5 to 10-19 must be considered as very vigorous indeed, especially those free from organic matter.

It must be pointed out, however, that the above tests were conducted with *Nitromonas* only, while it may be that different results would be obtained with *Nitrosomonas*.

The points developed by the solution studies may be summarized as follows:

1. *Micrococcus nitrificans*—(Van Tieghem) and *Bacillus nitrificans*—(Van Tieghem) are evidently the organisms producing nitrification in Wooster soils.

2. The growth of the nitrite formers is much more feeble in solution than is that of the nitrate formers. The growth of the former is so slow that isolation is a tedious process.

3. Aqueous extracts of ignited and non-ignited soils, or at least of the soil used by us, when reinforced by the regular mineral nutrients, possess no superiority over Omelianski's nutrient solution for the growth of the nitrate producer. The traces of rare nutrients, which according to Kaserer are so important for the growth of autotrophic soil bacteria, are either not of importance to Bacillus nitrificans or were not present in the soil used in this work.

B. POROUS MEDIA

1. HISTORICAL

As mentioned above Stevens and Withers contend that soil studies of nitrification are so different from those carried out in solution that the latter are unreliable, while Löhnis and Green

maintain that with proper precautions the solutions may yield valuable results. It is easily beyond the province of this paper to discuss the different results and viewpoints that have been presented on this question by the above and other authors, yet there are a few theories that have been proposed which may or may not have a bearing on the differences between nitrification in soil and in solution, which we will now very briefly mention.

Rahn (25) has studied the activities of bacteria in solution, in soil and in sand, varying the moisture contents in the porous media; ammonification of peptone by *B. mycoides* was studied for the most part. From his own work and that of others, Rahn concludes that microbial activity is very much affected when the nutrient solutions are absorbed in sand. Marked increases in activity are recorded and some evidence was obtained that the physiology was altered, since the nitrogen carbon ratios were altered. He considers that "aeration and thickness of the moisture film are the two controlling factors in quartz sand cultures. They may be considered as the two main physical factors of the soil," and that "absorption plays a minor role in the bacterial activity of quartz sand cultures."

Sohngen (26) points out that the beneficial results that have been so widely observed from the action of soil and humus are without satisfactory explanation. After discussing the work of others and obtaining beneficial actions from cellulose, blood charcoal, and inorganic colloids, he concludes that the accelerating effect of soil on microbial activity is due to its physical properties, and that these properties are those of colloids and, therefore, due to the presence of colloids in soils. Two specific properties, both absorption phenomena, are regarded as of prime importance: (1) the power of colloids to absorb the major part of mineral nutrients from solution, leaving a low concentration, then restoring this concentration as the living cells lower it—and thus that which Rahn considers unimportant; (2) the power of colloids to condense on their surface gases, such as CO_2, N, O, etc., which may be then utilized by the bacteria.

In resumé then, it may be said that Stevens and Withers obtained very wide differences in bacterial activity in soil from that in solution; that Rahn considers these differences as due to moisture and aeration; that Söhngen considers them due to the absorptive power of colloids; that Kaserer believes synthetic solutions are inferior chemically to soils and soil extracts; while Löhnis and Green consider that no necessary fundamental differences exist

between bacterial activities in soil and in liquid media. The problem is indeed a complex one and its solution one of the most urgent needs of soil bacteriology. The results reported by the different investigators have been obtained under such widely varying experimental conditions that direct comparisons are not permissible, and in order to obtain some possible indications in regard to the merits and demerits of the proposed theories—all of which are indeed lacking in the fundamental supporting facts—the following experiment was conducted.

2. EXPERIMENTAL

Quartz sand as a medium for the support of nitrification, alone and with colloidal substances, and soil untreated, ignited, and ignited plus humus were used.

This experiment essentially followed the suggestion of Löhnis and Green (12) that, "By suitable addition of glass-wool, sand, chalk, humus, etc., tests which are primarily 'in solution' may be arranged so as to separate at least partially the conglomeration of factors involved in soil tests." This suggestion is most excellent, especially in view of the above conflicting theories. The preparation of a synthetic soil involves innumerable difficulties, however, and it is interesting to note in this connection the comment of Stevens and Withers (27): "An artificial soil of high N. C. (nitrifying capacity), which might be universally standard, is desired, but our attempts to construct such an artificial medium have failed utterly."

However, it seems to us that the uncertainties involved must be attacked from the synthetic standpoint. The following experiment is crude and incomplete, but it is reported to simply record our experience in the hope that it may contain a suggestion to others. We attempted to impart to sand some of the ordinary properties of soil and to destroy in soil some of its properties which are usually considered as of importance in its property as a medium for nitrification. Igniting soils we believe simplifies the process somewhat, because the humus and other organic colloids are destroyed and some of the inorganic colloids lose their properties as such, and since it is free from organic matter it is a medium in which the competition of saprophytic forms is very much reduced.

The nutrient solution used was Winogradsky's ammonium sulphate broth, and approximately 1 gram of *calcium* carbonate was added to the 50 gm. portions of porous material. Since the different porous substances possess different water-holding capacities, different amounts of nutrient solution were added to the different media. The nutrient solution was added to the porous substances from a

burette in small portions at a time, stirring well with a spatula after each addition. The addition of solution was continued until a structure resembling that of soil at optimum water content was obtained. The amount added was then recorded. In the case of sand, and sand plus carbon black, 5 cc. of solution was added in each case. The results are therefore expressed as milligrams nitric nitrogen produced per 100 cc. of solution. Table X shows the arrangement and results of the experiment. The samples were incubated 30 days at room temperature (22-25°).

TABLE X

No.	Medium	cc. Wino's solution	Nitric nitrogen produced	
			p. p. m. of solid medium	Mgs. per 100 cc. solution
1	Sand	5.0	13.5	13.5
5	Sand + fresh Fe_2O_3 [1]	7.0	8.8	6.3
6	Sand + fresh Fe_2O_3 + humus[2]	7.2	7.6	5.2
8	Sand + fresh Al_2O_3 [3]	5.4	3.8	3.5
9	Sand + fresh Al_2O_3 + humus	7.4	21.8	14.7
20	Sand, ignited, washed	5.0	19.9	19.9
20a	Sand	5.0	8.2	8.2
21	Sand + humus	6.2	54.1	43.5
23	Sand + humus, triple amount	7.1	26.7	18.7
31	Soil	10.2	66.7	32.6
32	Soil, ignited	8.9	89.2	50.1
33	Soil, ignited + humic acid	11.4	121.1	53.0
35	Sand, 32 cc. (50 gm.)	5.0	20.0	20.0
36	Sand, 28.9 cc., carbon black, 3.2 cc.	5.0	12.8	12.8
37	Sand, 25.6 " " " 6.4 "	5.0	14.8	14.8
38	Sand, 16.0 " " " 16.0 "	5.0	14.8	14.8
39	Carbon black, 32 cc.	5.0	14.7	14.7

[1] 0.5 gm. $FeCl_3$ $6.H_2O$ was added to the 50 gm. sand contained in a large evaporating dish, dissolved in a minimum amount of water and precipitated with ammonia. It was then washed by decantation till only a slight reaction for chlorides was obtained. The mixture was then allowed to dry in the air and the Winogradsky's solution added.

[2] The humus was extracted from peat by 4% NH_4OH after having first washed the peat with acid. The "humic acid" was dialyzed in collodion bags until it was partially deflocculated, forming what would be considered in colloid chemistry a poor jelly. It was thin enough to pour, yet showed no tendency on several days standing to flocculate. Fifty cc. of such a "solution" contained 0.6796 gm. humus dried at 105°. This amount was added to 50 gram portions of sand.

[3] The Al_2O_3 was added in the same manner as was the Fe_2O_3. 9 gm. potassium alum per 50 gm. sand was used as the source of aluminum.

3. DISCUSSION

A comparison of the data expressed as milligrams per 100 cc. of solution (which is certainly the fairest way to compare the different cultures) shows among other things that sand is distinctly inferior to soil as a medium for nitrification. A comparison of the triplicates, Nos. 1, 20a, and 35, shows that the probable error of the data is high, yet the differences between any one, or the average, of the sand cultures and the cultures in the soil are certainly greater than experimental error. Additions of inorganic colloids, or of carbon black, all of which have high absorptive powers, seem not to increase the nitrifying capacity of the medium. On the other hand

sand plus humus (1.35%) surpassed ordinary soil, while with a triple amount of humus the results are lower. Soils ignited, with and without humus, are practically the same, and superior to any other medium used.

This experiment, of course, does not clear up the question as to what are the causes of higher nitrification in soil than sand. There seems to be in the data, at first glance at least, no support for Söhngen's theory of the action of colloids, yet the soil, even after ignition, may possess properties in regard to absorption of gases, and of nutrients out of solution, not possessed by the particular colloids prepared and added here.Perhaps the differences are due simply to differences in ability of the substances to absorb the toxic ammonium salts from solutions. The fact that carbon black, which possess a high absorptive capacity for ammonia, fails to aid nitrification speaks against this assumption. This point can be cleared up, it seems to us, only by comparisons of nitrifying capacity of different porous media with their power to absorb ammonia out of solution as measured by their absorption isotherms. The use of sand plus a difficultly soluble ammonium salt, as magnesium ammonium phosphate, which was used by Löhnis and Green (12) is not quite the same as a substance which absorbs the ammonium out of solution. In the former case the concentration of the solution remains constant; in the latter the concentration in the solution varies with the concentration on the solid.

The results on ignited soil are of interest and importance. It seems to us that this material gives promise of being a satisfactory medium for the study of nitrifying inoculating powers of soils. Being free from organic matter, competition of saprophytic forms is reduced, and the medium may also be sterilized for pure culture work without the entrance of the deleterious results from the breaking down of organic matter.

It seems that before more time is spent on speculations and formulations of theories in regard to the greater rapidity of bacterial activity in soil as compared with solution or with sand, that it should be first established whether or not there is a real fundamental difference. The facts are not at hand to enable us to determine this point. The wide differences reported in this paper may be due only to differences in ammonia absorption, which Löhnis and Green regard as very important.

4. SUMMARY

It seems that only the following conclusions may be drawn from the above experiment:

(1) Soil as a medium possesses the property of supporting nitrification better than sand. The cause of this is as yet uncertain. That is, the factors which limit nitrification are not yet properly understood.

(2) Ignited soil seems to be an excellent porous medium for the support of nitrification and merits further study.

IV. CONSIDERATION OF THE PRESENT METHODS OF STUDYING NITRIFICATION

1. CRITICISM OF PRESENT METHODS

In the preceding papers repeated reference has been made to errors existing in the methods of studying nitrification. Indeed the reason that there are so many incomplete experiments and so many loose ends in the experiments reported in this paper, is that the methods used (which are the customary ones) are so fraught with errors that we can never hope to solve by them the complex problems encountered. Physiological studies have therefore been largely suspended in this laboratory and efforts are being directed toward devising more accurate methods.

As mentioned by Löhnis and Green (12), there has been a marked tendency among recent workers to condemn solution methods and to adopt "soil" methods instead. The latter have been regarded as very accurate[1] and therefore satisfactory for the attack of soil biological problems. After patient endeavors with this soil culture method, as applied to nitrification studies, we cannot agree with this viewpoint, and have been forced to admit that such procedures are burdened with errors and, as mentioned above, we have abandoned further physiological research until at least the more glaring of these errors are removed. These will now be discussed.

The first method of procedure with which we must take issue is the maintenance of the moisture content of the sample, either by weighing the container and sample at stated intervals and replacing the loss, or by preventing loss by plugging the container with moistened absorbent cotton. In the former case, aside from the large amount of experimental work involved, there is a varying moisture content, the upper portion of the sample is alternately more moist and more dry than the lower portion, and with some soils the addition of water without stirring tends to produce puddling and to form a crust. The second procedure will not prevent the loss of water completely and does not permit perfect diffusion of air into the container.

[1]See, for instance, the papers of P. E. Brown.

A second source of error present in the above work is in the analytical methods employed. These are of two kinds, (1) the error in the analytical procedure itself, and (2) the error in the extraction of the nitrate from the soil or other porous medium. The phenoldisulphonic acid method considered by many as reliable is open to at least three objections,—(1) it is affected by organic matter sure to be present in greater or less degree in soil extracts; (2) it is not suitable for the determination of high amounts of nitrate, and (3) the personal equation in the manipulation of the method is large. The last two objections are faults common to colorimetric methods. Colorimetric procedures have their place in analytical operations, but their field is restricted at best. During the progress of this work numerous checks and cross checks were carried out to test the analytical methods. The results were not satisfying and indicate that the method, in our hands at least, does not possess the accuracy desired for the investigations in hand. The second error, namely, that of incomplete extraction of the nitrate, is one far too often overlooked. It has been noted by different investigators, but has never received the attention it merits. Stevens and Withers report some data which show the seriousness of this error. Amounts of nitric nitrogen from 1 to 100% of the 240 milligrams per 400 grams of soil were added to samples of three different soils. The aqueous extracts (1:3) of these soils were then analyzed for nitric nitrogen, using the phenoldisulphonic acid method for the smaller and the Tiemam-Schulze method for the larger amounts. They recovered "only about 40 percent of the nitrates when small quantities were added and more than twice this amount when larger quantities were added. These results show that figures given elsewhere in this paper are not absolute, but as the percentage recovered increases fairly regularly, the incomplete recovery does not interfere with the conclusions which are drawn" (27). The curves which are plotted from their data suggest two things: (1) the curves, although irregular, indicate by their resemblance to the adsorption isotherm type of curve that the phenomenon is one of pure physical adsorption, and (2) the irregularities in the curves indicate that the analytical procedures are not satisfactory.

Possibly the incomplete recovery of nitrates does not interfere with the conclusions Stevens and Withers have drawn, but an error in methods amounting in cases to 40% of the amount present is at best objectionable, and may mask the finer differences which are often so helpful in interpreting results from a complex phenomenon. Furthermore, as pointed out below (see page 38), the applications of nitrifiable nitrogen used by Stevens and Withers are abnormally

high. The use of lesser amounts will result in the production of smaller physiological differences, necessitating in turn more accurate analytical methods.

During the carrying out of the analytical work in our investigations some experiments were made to see if complete extraction of the nitrate were being obtained. All extractions were made 1:5 on 100-gram portions of soil. From these mixtures of 500 cc. of water and 100 grams of soil, 400 cc. of clear solution were removed, and replaced by an equal amount of distilled water. Since in the first extraction 100 cc. of water is left in contact with the soil, the nitric nitrogen obtained in the second extraction should be—if complete solution were effected—one-fifth of that obtained in the first extraction. Expressing the nitric nitrogen obtained by the first extraction as A, and that by the second as B, then in the case of complete solution, $B-\frac{A}{5}=0$, and $A+(B-\frac{A}{5})=A$.

In a large number of determinations, however, of which the following are typical, $A+(B-\frac{A}{5})$ is greater than A.

TABLE XI. The recovery of nitric nitrogen from soils with distilled water

No.	A	B	$(B-\frac{A}{5})$	$A+(B-\frac{A}{5})$	Percent in first extraction
1	150	76	46	196	76.5
2	157	72	41	198	79.2
3	187	99	62	249	75.0
4	202	98	58	260	77.6
5	194	97	58	252	76.9
6	197	93	54	251	78.4
7	199	78	38	237	83.9
8	194	89	50	244	79.5
9	124	89	64	188	65.9
10	189	83	45	234	80.7

The data indicate that either the method of extracting as ordinarily employed is very inexact or that the analytical method was markedly affected by varying amounts of organic matter and of soluble salts. The latter does not seem probable, since 5 times as much extract was analyzed in the second case as in the first. The simplest explanation of these data is that shaking a soil with 5 times its weight of water fails to extract all the nitrate.

The question of the most desirable amount of nitrifiable nitrogen must next be considered. We employed both the application of 212 parts nitrogen per million, as used by Lipman, Brown and others, and 600 parts nitrogen per million as used by Stevens and

Withers. The former would be equal to 424 pounds per acre 7 inches and the latter to 1,200, the former higher than is customary in field practice and the latter completely beyond practical operations of agriculture.

The determination of nitric nitrogen (or other metabolic products) after incubation for a certain period, is a purely arbitrary procedure and may lead to serious error. No one period of incubation can be adopted for all soils or for even different conditions of the same soil.

Granted that the above errors and weaknesses of the methods were eliminated, incubation tests in soil even then give us only part of the information desired. They give no indication of whether the differences observed are due to differences in the soil itself or in the flora. Addition of a large amount of nitrifiable material to a soil and incubation for a certain period do not furnish *natural* conditions. This particular point is discussed by Lohnis and Green and need not be dwelt upon further here.

2. MEANS OF IMPROVEMENT

The above criticisms respecting the methods of studying bacterial activity might indicate that the problem is hopelessly complicated and involved. It is, however, no more complicated than many other problems that are being attacked and the difficulties are by no means insurmountable. We have devoted considerable time to the improvement of methods during the last year and a half and while progress has been slow it has not been discouragingly slow. The methods of avoiding the above errors will now be considered.

The matter of the control of water content of soils during incubation periods was considered quite carefully. As a result a constant temperature humidor has been constructed, which is intended to maintain constant temperature and saturated atmosphere over considerable periods. While an opportunity to test the device thoroughly has not been presented, it gives promise of solving this annoying source of error.

The matter of analytical difficulties has been taken up and considerable progress made. A method has been developed which evidently can be further perfected so that the requirements of the study of nitrification can be met. The matter of extraction of the nitrate from soils is also under investigation (28).

Smaller amounts of ammonium sulphate should be used, except perhaps in special cases. The smaller amount used in this work, i. e., 100 mg. sulphate of ammonia to 100 gms. soil, is probably satis-

factory for average work. The amounts of nitric nitrogen produced from this amount of ammonium nitrogen would produce sufficient amounts of nitric nitrogen to determine with an accurate method.

Soil incubation tests should be accompanied by solution studies. The precautions to be taken in the prosecution of solution studies, as well as the need for the same, have been ably set forth by Lohnis and Green and no extended discussion is needed here. "The ideal (of difficult consummation devoutly to be wished) would be surely to build up the natural environment, a synthetic soil, step by step, at each stage gaining some insight into the separate components involved in the natural processes" (12). Such a method of attack would also shed additional light on the theories of Söhngen and of Kaserer, theories which at present lack the fundamental facts for their establishment, but which must not be overlooked in the interpretation of the results of this complex phenomenon.

The misleading conclusions, which are so likely to be drawn from single determinations of nitrite or nitrate produced, can be avoided by determining these metabolic products periodically and interpreting the results with the aid of curves as suggested by Rahn (29). This unavoidably entails a large amount of analytical work, but biochemical processes cannot be accurately interpreted otherwise.

It is believed that light can be shed on such properties of the soil as "nitrifying inoculating power," for instance, by inoculation (preferably by the infusion method as recommended by Buhlert and Fickendey (10), if their excellent results can be duplicated) into ignited and sterile soil to which calcium carbonate and moderate amounts of ammonium salts have been added. Satisfactory results could not be expected from the method of Stevens and Withers of inoculating a soil which had been autoclaved and treated with enormous amounts of ammonium sulphate.

Studies on the isolation and cultivation of the causal organisms in pure culture must be carried out. The large amount of work which has been done on nitrification without studies of the specific organisms, when the process is evidently an obligate function of two specific forms, is almost without a parallel in bacteriological science. Investigations on the physiology of the organisms are just as sorely needed as are pure culture studies, and not until they are made can such functions as "nitrifying energy, inoculating power, etc.," be properly interpreted, no matter how accurately they may be made experimentally.

The above described studies which are urgently needed are perhaps beyond the province of most laboratories, but this is all the more the reason why the attempt should be made to study one process in detail rather than superficial examinations of "nitrifying, ammonifying, nitrogen fixing," etc., properties of soils so generally studied, especially at the present time. Studies of this latter type have been useful in the past in showing that great biological differences do exist in different soils. Future studies must in addition attempt to determine the nature of these differences. Only by extensive, thorough and accurate observations of this complex phenomenon of nitrification can its role in the yet more complex phenomenon of soil fertility be determined. At present speculations in regard to relations between soil fertility and nitrifying powers, etc., are only speculations. Intense nitrification may not be a desideratum to the agriculturist, a point suggested by Winogradsky 25 years ago, but which seems to have been lost sight of by many recent workers.

V. GENERAL CONCLUSIONS

1. The different systems of cropping and fertilizing which have been carried out on the plots of the Ohio Experiment Station have produced some very wide differences in microbial activity in the soil. These differences are as marked, or in some cases more marked, than those reported by other investigators, and are worthy of careful study.

2. The organisms producing nitrification in Wooster soils are evidently *Micrococcus nitrificans*—(*Van Tieghem*) and *Bacillus nitrificans*—(*Van Tieghem*) or the *Nitrosomonas* and *Nitromonas* of Winogradsky.

3. Ignited soil is apparently a useful porous medium for the support of nitrification.

4. The methods in vogue for studying the process of nitrification—which is taken as the representative of the bacterial activities —contain many errors, which must be largely eliminated before the problem of soil bacteriological differences can be satisfactorily attacked.

5. Many of the weaknesses of the methods can be eliminated, and by the use of improved methods and more elaborate studies, designed to correctly determine the factors limiting this phase of microbial activity, useful results will be obtained.

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