Bacteriological studies of nitrification

A Thesis

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Clifford L Mc Arthur, B. S.

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Bacteriological studies of nitrification.

During the past twenty years the science of the bacteriology of the soil has been slowly but steadily progressing, and in fact the greater part of this progress has been within the last eight or ten years. It has been studied by various investigators and only in the last few years have their results been uniform. Text books in bacteriology written ten years ago spoke of soil bacteriology as merely a problem of sanitation, the object being to prevent the soil from becoming contaminated with pathogenic organisms and thus polluting the water supply.

It has been known for years that nitrogen is one of the essential elements of plant food, although but little attention was directed along this line until a scarcity of nitrogen in various soils became noticeable. Various theories were advanced as to the probable source of the nitrogen which the different plants used, but all of these early theories were of a chemical, instead of a bacteriological, nature.

One of the first theories advanced was that plants take their nitrogen supply directly from the air since the air is four-fifths nitrogen. The "Ammonia Theory" was also introduced about this time by Liebig in which he maintained that plants absorbed their nitrogen from the air in the form of ammonia. It was soon discovered however that plants do not take their their nitrogen from the air, except perhaps a small amount, and that the ammonia of the air was not sufficient to supply the amount needed by the plants, hence various new theories were

brought forth. In the meantime it was discovered that plants take their nitrogen through their roots and that it must necessarily come from the soil in the form of nitrates. But the principle question was" Where did the soil obtain its nitrogen?" for, although the plants used large amounts of nitrogen and equally as much was washed from the soil by rain, still the supply was not exhausted. It was also known that soils that were left uncultivated gradually gained in nitrogen.

Pasteur (1) as early as 1862 suggested that nitrification in the soil was due to bacterial action but there was no proof of this until fifteen years later , when Schloesing and Muntz(2) proved by carefully planned experiments that the formation of nitrates in soils was due to bacterial action. By chemical analysis they were able to show that tubes containing sand and lime and a small amount of sewage, gained somewhat in their nitrate content when kept at warm temperatures, while they decreased proportionately in ammonia. When chloroform was added no nitrates were formed but when this was removed and a small quantity of soil added nitrates were formed again. It was also discovered that heating the soil up to a point higher than bacteria could live, stopped nitrification. This showed that there was some relationship between the formation of nitrates and the development of bacteria, and that the soil seemed to contain some living element suitable to the formation of nitrates. It also showed that the nitrates were probably formed from some form of organic nitrogen, although practically nothing more was accomplished along the line of nitrification until fifteen years later when Winogradskys(3) experiments

placed the problem of nitrification and especially the formation of nitrates from organic nitrogen on its present basis.

In the meantime various investigators had been experimenting with plants and the probable source of nitrogen used by the plants and had discovered the ability of the legumes to fix free nitrogen when aided by bacteria. Hellriegel and Wilfort (4) had been doing considerable work along this line, but it was still a problem as to how fields which did not contain legumes , nor had not been cultivated could could still show a marked increase in nitrogen. The problem was still unsolved but from that time foreward various discoveries were made and theories advanced.

The discovery of the Clostridium Pastorianium in 1895 by Winogradsky (5) and the discovery by Berjerinck (6) of the Azotobacter were the next important discoveries in soil bacteriology and in nitrogen fixation, as these two groups of bacteria fix nitrogen from atmospheric nitrogen. However it was in 1899 and 1900 that Winogradsky conducted a series of exepriments which really opened the way for modern soil bacteriology. It was in these experiments that he first isolated and studied the bacteria which cause nitrification in the soil. Since the isolation of these bacteria by Winogradsky(3) work along the same line has been conducted by Beijerinck (7) and Omeliansky(8). The work with these bacteria caused these investigators to conclude that there were at least three different organisms necessary to the formation of nitrates from organic nitrogen. The bacteria that cause the first step are the putrefactive organisms forming ammonia. The second step is

how these proteids may be reduced to the simple form of nitrates

Part of the proteid thus built up into the bodies of plants and animals remains there until the plant or animal dies and at death is still a proteid and as complex as ever. In this form it may reach the soil when the animal or plant dies or it may be used as food, and pass through the body of some other animal, but much of it will finally reach the soil while still in the form of proteids.

A portion of the proteid is used up in the animal body to furnish energy. When it is thus used its complex chemical molecule is broken up and it is reduced to much simpler compounds, all of which assumes the form of urea. Though urea is a nitrogenous compound far simpler than proteid still it is entirely too complex to be used as a plant food. Urea or some closely allied compound is the form in which nearly all of the nitrogenous material resulting from proteid metabolism in the animal body , is finally excreted. Urea thus represents one stage in the destruction of proteid compounds and to this stage the proteids are finally brought as a result of the life processes of animals. The amount of nitrogen excreted in this manner is enormous, it being estimated that 38,000 tons of urea are excreted daily by the human race besides the still greater amount excreted by other animals.

Thus the nitrogen of the nitrate that was formerly taken up and metabolized by the plant has reached two quite different conditions, part of it is still in the highly complex form of proteid, eithr in the dead body of the animal or plant, while a second portion has been partly broken down in its passage

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through the animal body and has reached the condition of urea or some allied body. But in neither condition is it within reach of another generation of green plants. It must be still further broken down before it is available for plant food.

The destruction and breaking down of these nitrogenous compounds such as urea, proteids and other similar bodies is brought about by several different agencies after they are no longer connected with the living plant or animal. The chief one of these agencies is microorganisms. A small amount of the proteid appears to broken down in plant tissue without the aid of bacteria, another portion is broken down by yeasts, molds and fungi, while decomposition of the remainder is due chiefely to a class of bacteria called "decomposition bacteria".

Complete decomposition is brought about by certain microorganisms working in the presence of oxygen. Their end products are quite simple and are varied as are also the bacteria which produce this decomposition. These bacteria are so numerous, that they are sure to attact any nitrogenous organic matter, which has become lifeless, and soon break up and decompose it.

The chemical nature of this decomposition is very complicated and highly varied. The products consist of both gases and solids, and of the gases ammonia is the one in which we are most interested.

After passing through several intermediate stages the nitrogen of the decaying mass assumes, in large part, the condition of ammonia. One of the first and easiest substances to undergo

this ammoniacal fermentation is urea. There are several species of bacteria which cause this ammoniacal. Under proper conditions as much as 97 percent of the nitrogen in urea is converted into ammonia in the space of four days, providing the conditions are favorable. Although urea shows the ammoniacal fermentation most readily, still other nitrogenous bodies, like proteids, may give rise to ammonia which is indeed one of the common end-products of proteid decomposition . The chemical changes that occur in proteid decomposition are complex and not wholly understood but it seems that they are first converted into peptone-like bodies and these are further converted into ammonia. The occurence of such a decomposition is no new discovery for it has long been known that this was natures method of dealing with the remains of past animals and plants but it was not recognized until recent years that this decomposition was also one step in natures method of preparing this nitrogenous matter once more for plant food.

As has been previously stated plants receive practically all of their nitrogen from the soil in the form of nitrates and are able to absorb but a very small amount of their nitrogen from the air, consequently if ammonia formed by the decomposition of nitrogenous substances, as previously discussed, is to be used as plant food it must be changed from ammonia to nitrates.

The term nitrification is used in the sense of the formation of nitrates from some form of nitrogen. It is a process of vigorous oxidation and is now considered as taking

place in three different stages. First the breaking down of organic nitrogen into ammonia, second the formation of nitrites from the ammonia, and third the formation of the nitrates from the nitrites. Each step is supposed to be the result of bacterial action. The breaking down of organic nitrogen into ammonia is the result of the activities of one or more common decomposition bacteria, among which are, B.Subtilis, B.Vulgaris, B.Fluorescens, and B.Mycoides.

According to the investigations of winogradsky(3) and various other investigators the formation of nitrates from the ammonia is divided into two steps, the change being due to a specific group of organisms in each case. In the formation of the nitrite from the ammonia there are two closely related bacteria which have been named the Nitrosomonas and Nitrosococcus while in the formation of nitrate from nitrite the organism concerned has been named the Nitrobacter.

In ordinary soil these two kinds of nitrifiers work together. So closely connected is their action that it is difficult to find any traces of nitrites in the soil since they are converted into nitrates as rapidly as they are formed. But although occuring simultaneously the two steps in the nitrification appear to be distinct and produced by distinct organisms.

It has been claimed recently that there are other classes of soil organisms which combine the two steps in one, converting ammonia and even organic matter directly into nitrates. If this is true they represent distinct classes of nitrifiers,

but the observations have not yet been sufficiently verified.

The importance of the phenomenon of nitrification makes it very desirable to understand thoroughly the conditions under which it may best occur and the means for stimulating or hindering it. Up to the present time the results obtained by various investigators show that the conditions regulating the life of these organisms,are, in some respects peculiar. In respect to food these nitrifying bacteria behave the very opposite to ordinary bacteria, for ordinary bacteria will not grow upon anything except organic media, while withthese nitrifying bacteria the presence of organic matter in their solutions or media is directly injurious. These bacteria will grow readily in mineral solutions, but if a small quantity of organic matter is added the growth stops immediately. Thus in laboratory solutions a very small amount of organic matter acts like an antiseptic. In the soil, however, the nitrifiers behave somewhat differently, and are not checked in their growth by such small quantities of organic matter as serves to check them in laboratory solutions. This injurious action of organic matter is a curious phenomenon for the more valuable the material for ordinary bacteria the greater is its injury to these bacteria. Since these bacteria are injured by organic matter it follows that nitrification cannot be expected to take place in such highly concentrated decomposing masses, but as these organic substances are decomposed and reduced, nitrification can begin. Proper testing has also shown that nitrification takes place more vigorously in soil than in solutions used in the laboratory, and investigators have also shown that the organic matter in the soil does not prevent nitrification like it does

in solutions.

Thus we see that although the plant may absorb a very limited amount of its nitrogen from the air and may secure a still greater amount by the fixation process, still the great problem of soil bacteriology is the utilization of organic waste matter and the formation of nitrates from it, for plant food as noted in the previous discussion. In this thesis only the one phase will be studied, namely the subject of nitrification from organic nitrogen.

THE PURPOSE OF THIS THESIS.

The purpose of this thesis is to study the occurence, the activity, and if possible some manner of determining the number of nitrifying bacteria in the soil.

GENERAL METHODS FOLLOWED.

The organisms were studied almost entirely by means of solutions and these solutions were prepared by dissolving certain salts in distilled water. The formula of each will be given with the experiment. The solutions were divided into 100 CC portions, placed in flasks plugged with cotten and sterilized under steam pressure at 15 lbs. for 15 min. Where agar was used it was prepared in various ways explanation of which will be given with the experiment. Most of the experiments were carried on at 28 C. although some of them were kept at 37.5 C. The glassware was all carefully sterilized in the dry oven at 150 C. for 2Hrs.

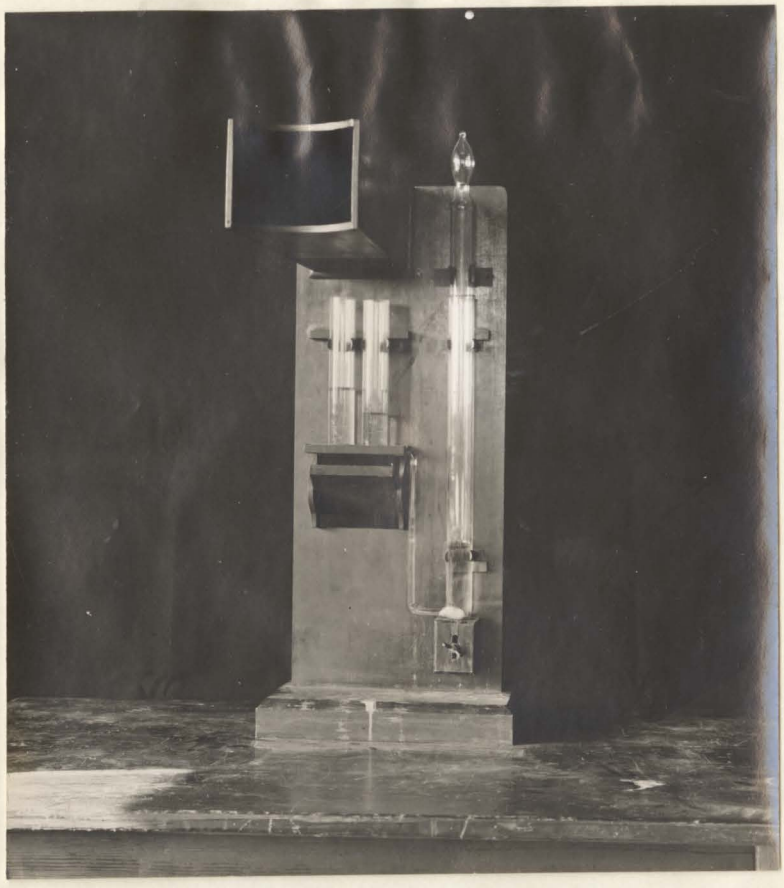

Plate I. Colorimeter used in making the tests for nitrites and nitrates.

The test used for nitrites was Greiss (9) colorimetric test as modified by Ilosvay (10) and the test used for nitrates was Sprengelâ (11) as modified by Gill (12). Both of these tests are quantitative. Soil samples were taken with a soil sampler which had been sterilized each time before using. The samples were collected in paper sacks which had been sterilized in the dry oven.

In order to study these bacteria it was first necessary to determine whether or not they were present in the soil and this was accomplished by inoculating solutions with samples of the soil.

Exp. I, Part 1.

This experiment was carried on in order to determine whether or not nitrite forming bacteria were present in the different soils, and if they were present to determine their power to form nitrites from ammonia in solution. The solution was prepared as follows.

$(NH_4)_2SO_4$	1.0 Gr.
KH_2PO_4	1.0 "
$MgSO_4$	0.5 "
$Fe_2(SO_4)_3$	0.4 "
NaCl	2.0 "
H_2O Distilled	1000 C.C.

This solution was divided into 100 C.C. portions placed in soxhlet flasks, and 1 gr. of $CaCO_3$ added to each flask after which they were plugged with cotton and sterilized in

the autoclav at 120 C. for 15 minutes.

The soils used in this experiment were taken from four different sources namely, Orchard, Garden, Cornfield, and Compost heap. Five grams of each sample were placed in a 50 C.C. water blank and from this 1 C.C. was used to inoculate each solution. The solutions were run in duplicate, one being left blank and another inoculated and sterilized so as to determine the amount of nitrite in the 1 C.C. used for inoculation. All ten of the flasks were then incubated for ten days at 28 C. after which they were tested for nitrites with the following results.

Flask No.	Source	Mg. of nitrite formed in the flasks.
1	Compost heap	.067
2	" "	3.200
3	Garden	.085
4	" "	.105
5	Cornfield	.075
6	" "	.060
7	Orchard	.025
8	"	.050
9	Sterilized	.026
10	Blank	.000

From the results it was quite evident that there were nitrite organisms in all of the samples, for the blank showed no formation at all and the sample that was sterilized showed much less than the others. All of the samples, except one,

from twice to twelve times as much nitrite as the sterilized sample, thus indicating that there was some agent present which was very active in the formation of nitrites. The soil from the compost heap gave the best results.

Exp. I, Part 2.

This experiment was conducted in the same manner as Part 1. except that it was test for the formation of nitrates from nitrites. The solution used for this experiment was prepared as follows.

$NaNO_3$	1.0 Gr.
Na_2CO_3	1.0 "
KH_2PO_4	0.5 "
Na Cl	0.5 "
$Fe_2(SO_4)_3$	0.4 "
$MgSO_4$	0.3 "
H_2O Distilled	1000 C.C.

This solution was divided into 100 C.C. portions, placed in flasks and sterilized after which it was inoculated and incubated at 28 C. the same as part 1. At the end of 14 days it was tested for nitrates with the following results.

Flask No	Source	Mg. of nitrite formed in the flasks.
1	Compost heap	102.4 Mg.
2	" "	128.0 "
3	Garden	120.0 "
4	"	121.6 "
5	Cornfield	1.4 "
6	"	22.8 "
7	Orchard	4.4 "
8	"	4.1 "
9	Sterilized	2.4 "
10	Blank	2.1 "

There was nitrate formation in all of the flasks although the duplicates did not check with any degree of accuracy, still the amount of nitrate formed in all of the flasks compared to the amount formed in the blanks, shows that nitrate formation had taken place quite vigorously.

The sample from the compost heap shows the greatest formation but the samples from the garden and cornfield both showed a considerable amount of nitrate formation. The sample from the Orchard didnot show as vigorous nitrification as the others, thus indicating that cultivation and the effect of manure have something to do with the distribution of the nitrate organisms.

After having found from the two preceding experiments that the nitrite and nitrate organisms were present in the different soils, it was decided to detremine if possible whether or not

they were present in the solutions in sufficient numbers to be transferred from one solution to another and still continue the nitrification process. In order to do this fresh solutions were prepared, the same as in the preceding experiments, and inoculated from each of the preceding experiments, showing the highest amount of nitrite and nitrate formation with.1C.C., .5 C.C., and 1 C.C. of the two solutions.

Exp. I, Part 3.

The ammonia solutions being prepared and sterilized were inoculated as follows. From flask No.2 of Exp.1 Part 1 , three flasks numbered 1, 2, and 3 were inoculated with .1 C.C., .5 C.C., and 1 C.C. of the above mentioned flask. From flask No. 4 of exp.1 Part 1 three flasks numbered 4, 5, and 6 were inoculated using .1 C.C., .5 C.C., and 1 C.C. respectively of the solution. Flask No. 7 was inoculated with 1 C.C. of the solution from flask No. 2 of Exp.1 Part 1. and flask No. 8 was inoculated with 1 C.C. of the solution from flask No. 4 Exp. 1 Part 1. Flasks No. 7 and 8 were sterilized. Flasks No. 9 and 10 were left blank. All were incubated at 28 C for 10 days, at the end of which period they were tested for nitrates with the following results.

Flask No.	Source Exp. I Part 1.	Amt. Added	Mg of nitrite formed in the flasks.
1	Flask No. 2	1 C.C.	.000 Mg.
2	" " "	.5 C.C.	.360 "
3	" " "	.1 C.C.	.860 "
4	Flask No. 4	1 C.C.	.000 "
5	" " "	.5 C.C.	.000 "
6	" " "	.1 C.C.	Trace "
7	Flask No. 2	1 C.C. Steril	.000 "
8	" " 4	1C.C. "	.000 "
9	Blank		.000 "
10	"		.000 "

Flasks No.2 and 3 which were inoculated with .5 and .1 C.C. of the solution from flask No. 2 Exp. I Part 1. showed considerable nitrite formation, and No. 6 showed a trace, while none of the other flasks showed any formation at all. The experiment proves that the nitrite forming organisms were present in great enough numbers so that could easily be transferred in dilutions.

Exp. 1,Part 4.

This experiment was conducted in exactly the same manner as the preceding one , except that it was with nitrate forming bacteria and the flasks used to inoculate from were flasks No. 2 and 4 of experiment 1 part 2. 5hey were all incubated at 28 C for 14 days after which they were tested for nitrates with the following results.

Flask No.	Source Exp.I Pt.2.	Amt: Added	Mg of nitrate formed in the flasks.
1	Flask No. 2	1CC	91.2 Mg
2	" " "	.5CC	112.0 "
3	" " "	.1CC	72.0 "
4	Flask No. 4	1.CC	91.2 "
5	" " "	.5CC	91.2 "
6	" " "	.1CC	97.4 "
7	Flask No. 2	Sterilized	1.6 "
8	Flask No. 4	"	1.5 "
9	Blank		.9 "
10	Blank		1.9 "

All of these flasks showed a vigorous nitrification and proved that the organisms were present in large numbers in the original solution.

From the experiments conducted thus far it had been proven quite conclusively that the nitrifying organisms were present in all of the soils that had been tested. The sample taken from the compost heap showed the most vigorous nitrification ,with the sample from the garden slightly less. All of the samples were taken at a depth of six inches, and it might be well to state, that the one taken from the compost heap was soil over which a compost heap had stood for several winters. Since it was plainly evident that these organisms were present in the soil one of the first questions arising is " at what depth are they present in the greatest numbers and and what are some of the factors that favor the growth of these organisms? In

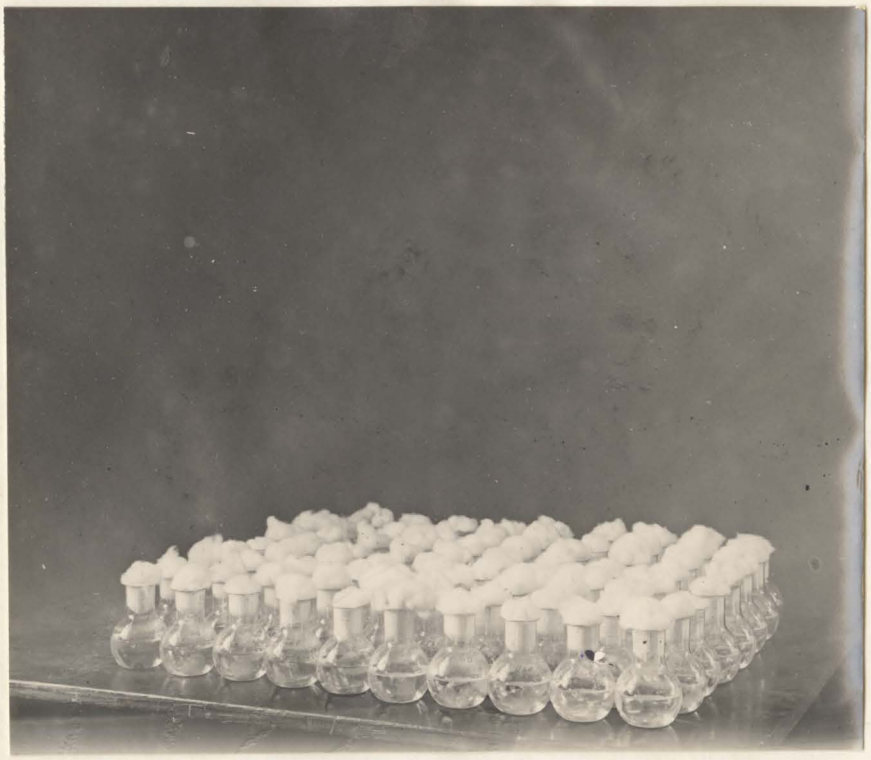

Plate II. Series of flasks inoculated with soil for the formation of nitrites and nitrates.

other words in what kind of soils do they grow thebest? In order to determine these facts the following experiments were conducted.

Exp. II, Part 1.

The object of this experiment wasto determine at what depth the greatest number of these organisms are present.

The soil samples were taken at the following depths from the soil where the compost heap had stood., 6 in., 10 in., 12 in. , 15 in., 18 in., and 24 in. Five grams from each of these depths were placed in 50 CC water blanks and 1 CC ofthis dilution used for inoculations. In this part of the experiment the test was for nitrites. Each sample was run in duplicate. Two were inoculated and sterilized and two were left blank. They were all incubated at 28 C and at the end of ten days tested for nitrites with the following results.

Flask No.	Depth	Mg of nitrite formed in the flasks.
1	6 in	.1125
2	" "	.1000
3	10 "	.0800
4	" "	.0725
5	12 "	.0500
6	" "	.0750
7	15 "	.0250
8	" "	.0300
9	18 "	.0200
10	" "	.0275
11	24 "	.0275
12	" "	.0175
13	6 " Sterilized	.0300
14	" " "	.0250
15	Blank	.0225
16	"	.0275

Exp. II, Part 2.

This experiment is the same as Exp. II Part 1. except that the test is for nitrate forming bacteria. The soil used was the same as that used in pt.1. The samples were incubated at 28 C. At the end of 14 days they were tested for nitrates with the following results.

Flask No.	Depth		Mg of nitrate formed in the flask.
1	6 in		121. 60
2	" "		92. 40
3	10 "		68. 80
4	" "		112. 00
5	12 "		49. 60
6	" "		43. 20
7	15 "		28. 00
8	" "		16. 00
9	18 "		12. 00
10	" "		7.20
11	24 "		.34
12	" "		.46
13	6 "	Sterilized	.26
14	" "	"	.34
15	Blank		.44
16	"		.28

Exp. II, Part 3.

In this experiment soil was taken from a garden and treated in every way like parts 1 and 2 except they were run for nitrites. The results were as follows.

Flask No.	Depth		Mg of nitrite formed in the flask.
1	6 in		.1575
2	" "		.1225
3	10 "		.0800
4	" "		.0950
5	12 "		.0675
6	" "		.0725
7	15 "		.0325
8	" "		.0425
9	18 "		.0400
10	" "		.0300
11	24 "		.0200
12	" "		.0275
13	6 "	Sterilized	.0250
14	" "	"	.0250
15	Blank		.0275
16	"		.0225

Exp. II, Part 4.

In this part soil was taken from a garden and tested for nitrates in the same manner as part 2 of this experiment. The results were as follows.

Flask No.	Depth		Mg of nitrate formed in the flasks.
1	6 in		75.20
2	" "		113.60
3	10 "		102.40
4	" "		24.00
5	12 "		68.80
6	" "		40.00
7	15 "		15.00
8	" "		8.80
9	18 "		10.80
10	" "		4.80
11	24 "		2.80
12	" "		.30
13	6 "	Sterilized	.22
14	" "	"	.28
15	Blank		.32
16	"		.20

From the results of these experiments using garden soil and compost soil, it is evident that the majority of the nitrifying bacteria are in the first six inches of soil and that there are parctically none below the first foot. This is probably due first, to the fact that there is very little decaying organic matter for them to work on below six or eight inches, and second to the fact that they receive but a very small amount of air below the surface soil or that part which is stirred by farming.

Exp. III

The object of this experiment is to determine the relative number and activity of the nitrifying organisms in some extreme cases. It was generally understood that adding fertilizer to the soil in the form of barnyard manure or the cropping of a field to alfalfa greatly increases its nitrogen content. Of course this is largely due to nitrogen fixation but it was also thought that possibly the nitrifying bacteria might be more vigorous and be present in greater numbers than if the field had not been cropped to alfalfa or manured. The experiments were conducted as follows.

Exp. 3 ,Part 1.

The soils for this experiment were taken from four different plats. Plat 1 had been manured heavily for six consecutive years, and had been cropped in either wheat or oats each year. Plat 2 had been in alfalfa for twelve consecutivre years, and had not been plowed or stirred in any way. Plat 3 was ordinary soil that had not been manured but had been in some crop each year for several years. Plat 4 was unbroken prairie that had never been plowed or tilled in any way. All of these samples were taken at a depth of six inches. The dilution used was the same as that used in the preceding work, namely, 5 Gr. in a 50 CC water blank and 1 CC of this used for the inoculation. The samples were run in duplicate. This part of the experiment

was for nitrites and was incubated at 28 C. At the end of ten days it was tested for nitrites with the following results.

Flask No.	Plat	Mg of nitrite formed in the flasks.
1	1	.148
2	"	.161
3	2	.096
4	"	.120
5	3	.064
6	"	.044
7	4	.040
8	"	.032
9	1 Sterilized	.036
10	" "	.020
11	Blank	.036
12	"	.028

Exp. III, Part 2.

This was an exact duplicate of the preceding experiment except that it was for nitrates instead of nitrites. It was incubated at 28 C. for 14 days after which it was tested for nitrates with the following results.

Flask No.	Plat	Mg of nitrates formed in the flasks.
1	1	91.00
2	"	74.00
3	2	55.00
4	"	61.00
5	3	20.00
6	"	7.00
7	4	3.80
8	"	2.80
9	1 Sterilized	.40
10	" "	.44
11	Blank	.30
12	"	.36

The results of this experiment show that the most active nitrite and nitrate organisms are in the plat which has been manured heavily each year, thus showing that the addition of decaying organic matter tends to increase their numbers and vitality. Plat 2 was almost equal in nitrifying power to plat 1 but not as strong as that plat, while plats 3 and 4 both showed some formation but not nearly as much as the first two. Thus the experiment tends to show that the nitrite and nitrate organisms are more vigorous and present in greater numbers in soil well supplied with organic matter, than in soils not so well supplied.

Exp. IV.

The next experiment was to determine the value of different solutions for growing nitrite and nitrate organisms

in the laboratory. All of the solutions were prepared by dissolving different salts in distilled water. These solutions were varied somewhat in order to determine which were the most favorable for the organisms to work in. Blanks were used with each sample and the soils used were from the same respective samples. The solutions for nitrites will be dealt with first.

Exp. IV, Part 1.

In this work there were three different solutions used which will be numbered 1, 2, and 3 and will each be dealt with by number. The ingredients of solution 1 are as follows.

$(NH_4)_2SO_4$	1.0 Gr.
$Mg\ CO_3$	5.0 "
KH_2PO_4	1.0 "
Na Cl	2.0 "
H_2O Distilled	1000 CC

Solution No.2 was prepared as follows.

$(NH_4)_2SO_4$	1.0 Gr.
KH_2PO_4	1.0 "
$Mg\ SO_4$	.5 "
$Fe_2(SO_4)_3$	.4 "
Na Cl	2.0 "
H_2O Distilled	1000 CC

Solution No 2 was divided into 100 CC portions placed in flasks and 1 Gr. of $Ca\ CO_3$ added to each

Solution No 3 was prepared as follows. It was an exact duplicate of No2 except that 1/2 Gr. of $Mg\ CO_3$ was added to

each flask instead of 1 Gr. of Ca CO_3. All of the solutions were divided into 100 CC portions placed in flasks and sterilized, after which they were inoculated with the same soil, the dilution being the same as that used in the previous samples. At the end of ten days they were tested for nitrites with the following results.

Flask No.	Solution tested		Mg of nitrite formed in the flasks.
1	No. 1		.196
2	" "		.216
3	" "		.184
4	" 2		.132
5	" "		.164
6	" "		.084
7	" 3		.096
8	" "		.068
9	" "		.076
10	" 1	Sterilized	.044
11	" 2	"	.054
12	" 3	"	.048
13	Blank		.040
14	"		.044
15	"		.048

Exp. IV, Part 2.

This experiment was for nitrates in different solutions Two different solutions being used. Solution No, 1 was prepared as follows.

$NaNO_3$	1.0 Gr.
KH_2PO_4	.5 "
Na Cl	.5 "
$Fe_2(SO_4)_3$	.4 "
$MgCO_3$	5.0 "
$CaCO_3$	1.0 "

Solution No. 2 contained the following.

$(NH_4)_2SO_4$	1.0 Gr.
KH_2PO_4	1.0 "
$MgCO_3$	5.0 "

These solutions were divided into 100 CC portions and inoculated with 1 CC of the same sample as the preceding experiment. It was incubated at 28 C and at the end of 14 days it was tested for nitrates with the following results.

Flask No.	Solution used	Source	Mg of nitrate formed in the flasks.
1	No. 1	Compost heap	32.40
2	" "	"	29. 20
3	" "	"	31.20
4	" 2	"	5.40
5	" "	"	6.00
6	" "	"	2.80
7	" 1	Sterilized	3.00
8	" 2	"	2.20
9	Blank		1.80
10	"		2.00

The results show that solution No. 1 in testing both for nitrites and nitrates gave the best results. These two were the solutions that had been used in practically all of the work thus far.

Exp. V.

It was decided to determine the length of time best adapted to nitrification, also the percentages of moisture that were best adapted to the growth of the nitrifying bacteria. In order to do this samples were first taken from moisture experiments which were being conducted in the greenhouse. The soil used for this experiment was sand to which two percent of finely pulverized manure was added in order to add a nitrifiable substance and also the nitrifying organisms. These samples were placed in tumblers and each seried was kept at a different percentage of moisture by weighing each day and adding the proper amount of distilled water. The percentages of moisture varied from .9 % in series I to 12 % in series 8.

In order to determine whether or not the nitrifiers were present in the sand mixture a sample of it was taken and solutions inoculated as follows.

Exp. V, Part 1.

This was the test for nitrites and the dilutions used were the same as those used in previous inoculations. Three flasks were inoculated and allowed to incubate. A fourth flask was inoculated and sterilized , while the fifth was left blank. At the end of ten days they were tested for

nitrites with the following results.

Flask No.	Source	Mg of nitrite formed in the flask.
1	Sand Mixture	.100
2	"	.025
3	"	.017
4	"	.022
5	"	.000

Exp. V, Part 2.

This experiment was conducetd in exactly the same manner as part 1 except that it was for nitrates instead of nitrites. At the end of 14 days theyawere tested for nitrates with the following results.

Flask No.	Source	Mg of nitrate formed in the flask.
1	Sand Mixture	43.20
2	"	72.00
3	"	.72
4	" Sterilized	.62
5	Blank	.80

From both of the preceding tables it was plainly evident that both the nitrite and nitrate forming bacteria were present in sufficient numbers to justify the continuance of Exp. parts 3, 4, 5, and 6. These experiments were conducted as follows. At the end of each two weeks samples were taken from series 1, 3, 6, and 8, and a series of three flasks flasks inoculated from each sample for nitrites and a series of three for nitrates. At the end of 5, 10, and 15 days a flask was

taken for analysis the last one being used at the end of 15 days. The results were as follows .

Exp. V, Part 3.

No Days	Flask No.	Source Sand Mixture	Mg of nitrite formed in the flasks.
5	1	Moisture .9 %	.024
10	2	" " "	.096
15	3	" " "	.096
5	1	Moisture 3.5 %	.076
10	2	" " "	.136
15	3	" " "	.052
5	1	Moisture 9.%	.024
10	2	" " "	.084
15	3	" " "	.088
5	1	Moisture 12 %	.040
10	2	" " "	.060
15	3	" " "	.064
5	1	Blankure	.000
10	2	"	.040
15	3	"	.044

Exp. V, Part 4.

This was an exact duplicate of part 3 except that it was for nitrates. The same samples were used for inoculation as in part 3. The results were as follpws .

No days	Flask No.	Source Sand Mixture	Mg of nitrate formed in the flasks.
5	1	Moisture .9 %	10.00
10	2	" " "	20.00
15	3	" " "	21.00
5	1	Moisture 3.5 %	14.80
10	2	" " "	16.40
15	3	" " "	18.40
5	1	Moisture 9.0 %	10.00
10	2	" " "	122.00
15	3	" " "	36.00
5	1	Moisture 12.00%	8.00
10	2	" " "	42.00
15	3	" " "	60.00
5	1	Blank	8.40
10	2	"	2.60
15	3	"	8.00

At the end of two weeks the experiment was repeated with new samples, the samples having been in the greenhouse 4 weeks at this time. These experiments were conducted in exactly the same manner as the two preceding ones.

Exp. V, Part 5.

This table shows the results for the nitrites of the 4 weeks old sand mixtures incubated at 28 C.

No Days	Flask No.	Source Sand Mixture	Mg of nitrite formed in the flasks.
5	1	Moisture .9 %	.032
10	2	" " "	.080
15	3	" " "	.160
5	1	Moisture 3.5 %	.040
10	2	" " "	.044
15	3	" " "	.128
5	1	Moisture 9.0 %	.032
10	2	" " "	.068
15	3	" " "	.100
5	1	Moisture 12.0 %	.032
10	2	" " "	.048
15	3	" " "	.120
5	1	Blank	.032
10	2	"	.052
15	3	"	.060

Exp. V, Part 6.

This table is the same as Part 5 except that it shows the results of the nitrates at the different lengths of time

No. Days	Flask No.	Source Sand Mixture	Mg of nitrate formed in the flasks.
5	1	Moisture .9 %	12.00
10	2	" " "	12.80
15	3	" " "	14.00
5	1	Moisture 3.5 "	12.40
10	2	" " "	11.40
15	3	" " "	12.00
5	1	Moisture 9.0 "	8.00
10	2	" " "	14.00
15	3	" " "	12.00
5	1	Moisture 12.0 "	64.00
10	2	" " "	88.00
15	3	" " "	102.00
5	1	Blank	5.60
10	2	"	6.40
15	3	"	6.80

From the results of the preceding experiment it was evident that there was practically no difference in the activity of the organisms where they had been kept in the different tumblers and then transfered to the solutions in the laboratory. This showed that they lived in the .9 percent of moisture from two to five weeks and were just as active if not more so than those that had been in the 12 percent of moisture. As to the length of time require for

nitrification to take place in the flasks it was found that the samples which were run five days usually showed some nitrite and nitrate formation but it was not as large an amount as at the end of ten days, however those allowed to run fifteen days usually showed more than either of the other two, thus indicating that the longer the flasks were allowed to incubate the more nitrite and nitrate they showed and that the best length of time for allowing the flask experiments to incubate was from ten to fifteen days, preferably fifteen and never less than ten.

Exp. VI.

Thus far none of the work had dealt with higher dilutions All of the dilutions used before were 1-50 and it was decided to conduct an experiment to determine whether or not it was possible to find the number of nitrite and nitrate bacteria in soil by using high dilutions. This was to be determined by inoculating flasks from these dilutions and allowing them to incubate for the proper length of time after which they were tested to determine whether or not nitrification had taken place. The soil used was taken from some experiments which were in progress in the greenhouse. The soil was in flasks which were kept at different percents of moisture. The dilutions used were as follows. 1-100,000 , 1-1,000,000 ,1-10,000,000, 1-20,000,000 , 1-50,000,000 . Thus each series contained five flasks besides the necessary blanks.

Exp. VI, Part 1.

This part of the experiment was for determining the number of nitrite bacteria and was conducted as follows. The solutions were inoculated with the dilutions previously mentioned, incubated at 28 C. and at the end of 14 days tested for nitrites with the following results. The results are given in Mg. of nitrite in each flask which contained 100 CC of the solution.

Series	Dilutions				
	1-100000	1-1000000	1-10000000	1-20000000	1-50000000
1	.076	.052	.072	.062	.058
2	.044	.020	.048	.052	.052
3	.048	.040	.020	.640	.068
4	.060	.060	.048	.068	.076
5	.072	.060	.092	.052	.048
6	.072	.076	.076	.048	.020
7	.076	.076	.068	.020	.064
Blanks	.072	.020	.092	.068	.088

At the end of two weeks this experiment was repeated with the following results.

Series	Dilutions				
	1-100000	1-1000000	1-10000000	1-20000000	1-50000000
1	.040	.020	.030	.032	.032
2	.032	.028	.040	.032	.036
3	.048	.044	.024	.028	.028
4	.032	.036	.044	.036	.048
5	.056	.036	.036	.048	.044
6	.044	.029	.036	.032	.032
7	.032	.028	.020	.020	.036
Blanks	.024	.032	.040	.024	.040

Exp. VI, Part 2.

This experiment was conducted in the same manner as Part 1 except that it was for determining the number of nitrate bacteria. The results given are Mg of nitrate in each flask containing 100 CC of the solution.

Series	Dilutions				
	1-100000	1-1000000	1-10000000	1-20000000	1-50000000
1	9.60	8.40	7.20	9.20	8.20
2	5.80	6.60	8.00	4.60	7.20
3	9.20	18.20	7.00	8.60	7.80
4	7.80	8.80	7.80	11.00	8.00
5	9.40	9.00	8.60	10.20	8.20
6	11.20	4.60	9.00	8.40	8.00
7	10.00	9.80	9.00	11.00	9.00
Blank	12.40	11.60	9.00	9.80	11.00

At the end of two weeks the experiment was repeated with the following results.

Series	Dilutions				
	1-100000	1-1000000	1-10000000	1-20000000	1-50000000
1	6.40	6.00	9.80	9.40	7.40
2	7.20	6.00	9.40	6.40	8.60
3	12.40	12.00	7.40	8.40	12.20
4	10.00	12.00	6.00	9.40	14.00
5	7.80	8.40	10.40	12.00	12.20
6	7.40	7.00	7.20	9.00	8.40
7	10.00	6.40	9.80	8.00	10.80
Blank	11.00	10.20	7.40	8.20	5.40

Fromthe results of the preceding experiments it is quit evident that the number of nitrite and nitrate bacteria is less than 100000 per gram or the organisms are less active than those in the other experiments. Probably if the experiment had been conducted with smaller dilutions the relative number of nitrifiers per gram might have been found, but as it was the highest dilutions did not show any more nitrification than the blanks, and in some cases even less. In all of the experiments conducted thus far the best results were obtained from dilutions of 1-50 from fresh samples of soil.

Exp. VII.

From the results obtained thus far it was quite evident that the nitrite and nitrate organisms were present in practically all soils which ware available for tillage and that they can be studied to quite an extent, in the solutions. But as none of them had been isolated or studied on solid media it was decided to devote the remainder of this thesis to work along the line of isolation and their growth upon solid media.

The most of the work with solid media was with modified agar, which will be explained under the experiments. In this experiment 15 grams of agar was placed in 1 liter of distilled water and melted. It was then divided into two portions 500 CC being placed in each of two liter flasks. After solidifying, the flasks were filled with distilled water and placed in the incubator. They were allowed to wash for 21

days the water being changed every other day. At the end of the 21 days the agar was remelted and different salts dissolved in it according to the work that it was to be used for. The experiment was divided into several parts each of which will be given below.

Exp. VII, Part 1.

The above treated agar was melted and the following salts added for nitrite bacteria.

$(NH_4)_2SO_2$	1.0 Gr.
Mg CO_3	5.0 "
KH_2PO_4	1.0 "
Na Cl	2.0 "

The agar was not allowed to solidify until all of the chemicals had dissolved, that would dissolve. Part of the chemicals did not dissolve entirely but settled to the bottom so that the clear agar could be tubed and sterilized without any difficulty. The agar solidified nicely. In order to determine whether or not the soil germs would grow upon this agar, it was inoculated from a flask which had shown nitrite formation. Several dilutions were made and the results were as follows.

Dilutions	No. of bacteria per CC
1/100	1,440,000
1/1000	4,800,000
1/10000	32,000,000

a. At the end of a week the experiment was repeated with the following results.

Dilutions	No.of bacteria per.CC.
1/100	920,000
1/1000	2,560,000
1/10000	8,000,000

Exp. VII, Part 2.

In this part of the experiment the above treated agar was melted and the following salts added for the nitrite bacteria

$(NH_4)_2SO_4$	1.0 Gr.
KH_2PO_4	1.0 "
$Mg\ SO_4$	.5 "
$Fe_2(SO_4)_3$	.4 "
Na Cl	2.0 "

The agar solidified nicely after the salts were added but as soon as it was heated it became watery and would not again solidify. At first it was thought that there had not been sufficient agar used, but the experiment was repeated using different amounts of agar from 10 grams to 20 grams per liter, but in no case would the agar solidify as long as these salts were added. It was finally concluded that it was due to some chemical change and this media was discarded.

Exp. VII, Part 3.

In this part of the experiment the agar was prepared for nitrate bacteria instead of nitrite. The agar was washed the same as in the preceding experiments and the following salts

were added to it.

$NaNO_2$	1.0 Gr.
KH_2PO_4	.5 "
Na Cl	.5 "
$Fe_2(SO_4)_3$	.4 "
$MgCO_3$	5.0 "
$CaCO_3$	1.0 "

The agar was tubed and sterilized and solidified nicely on cooling. In order to detremine whether or not the soil organisms would grow upon this agar it was inoculated with solution from a flask that had shown nitrate formation. Three dilutions were made . It was incubated a week after which the colonies were as follows.

Dilutions	No. of bacteria per. CC.
1/100	2,000,000
1/1000	18,000,000
1/10000	128,000,000

At the end of a week the experiment was repeated with the following results.

Dilutions	No.of bacteria per CC.
1/100	400,000
1/1000	1,600,000
1/10000	20,000,000

From the results of Exp. VII it was plainly evident that there were a large number of bacteria in the flasks which had shown nitrification, an that these bacteria would grow well upon a medium which was inorganic or as near inorganic as it was

possible to make it. There were a number of different kind of bacteria on the plates and some of these gave a good growth when isolated on lactose agar, this showing that some bacteria grow well on either organic or inorganic media. The principle difficulty was the determination of the organisms which were the the nitrite and nitrate formers. Since there were several kinds on each plate. Various methods were employed for isolating the nitrite forming bacteria. Each of the methods will be given in following experiments.

Exp. VII.

In this experiment the agar was not melted but was washed in the shredded form. The water was changed every other day, and at the end of 21 days the agar was placed in one liter of distilled water and melted. The salts were added the same as in Exp. VII but it did not seem to give as good results, in that, it was more brittle than that which had been melted before it was washed. It was difficult to melt and did not pour well. It also seemed to become dry much quicker than that in the preceding experiment. None of the agar prepared in this experiment was used for growing the nitrifying bacteria.

It might be well to say a word, however, in regard to all the agar prepared in the above experiments. It was rather difficult to prepare. It did not always give the same results but this was soon overcome after becoming more familiar with the work. Practically all of this agar became dry much sooner than ordinary agar. It was easily setrilized and did not show any growth if left unsterilized for several weeks.

ISOLATION OF THE NITRIFYING BACTERIA.

As previously stated various methods were employed for isolating the nitrifying bacteria. The principle object being to determine which were the nitrifying organisms and then become familiar with the colonies so that they could be distinguished from other bacteria when making counts from the plates.

The first method used for isolating the organisms was by isolating from the colonies in the plates and transplanting to agar slants which were of the same medium as the plates. It was intended to inoculate flasks from the growth on the agar slants and then test at the end of fifteen days in order to determine the amount of nitrite and nitrate formed. The organisms did not grow well on the slants although they gave good sized colonies in the plates. Some of them gave a good growth on lactose agar, thus proving according to the observations of Winogradsky (3) that they were not nitrifying organisms. Others however did not give any growth on any media except in colonies in the plates so that some of them were in all probability the nitrifying bacteria. The method did not prove very successful for isolating the organisms as there were too many different organisms to contend with.

The second method used in attempting to isolate these organisms was with tumblers of soil. The soil was was placed in the tumblers and covered with a petri dish after which they were heated in a dry oven at 110 C for several hours. After being sterilized in this manner they were watered with a sterile

solution, this solution being the same as that used in the flasks in Exp. I Part 1 and 2. This was to adda a nitrifiable substance to the flasks. After this the flasks were watered each day with a solution which was distilled water containing a colony from the plates. The tumblers were kept at about 15 percent of moisture by weight. The were watered several days in succession with the suspension until they were thoroughly inoculated with the bacteria, after which distilled water was used for keeping up the moisture content. Several different germs were tried in this manner. The principle difficulty encountered was that the soil in the tumblers became musty if kept covered with the petri dishes, and dried out to fast if the petri dishes were removed, as they were kept at 28 C. in the incubator. Another difficulty was with the soil used. The soil was a fine black loam and it was very difficult to get the moisture evenly distributed to all parts. It also began to bake as soon as it began to dry. If the soil had been at least half sand it could have been worked to a much better advantage. Few of the tumblers showed any increase in nitrites or nitrates, although one did show a marked increase in nitrite Since it was difficult to conduct the experiment and also required considerable time, from four to eight weeks, it was not repeated although it certainly offers some possibilities if worked out more completely.

Another method used to isolate the germs was with silicate jelly. This was prepared according to the method given by Smith (13) except that parchment was used for dialyzing instead of the collodion sacks. The silicate jelly is easily prepared except for the one difficulty, namely, that it solidifies as

soon as it becomes neutral or slightly acid, and must be left rather strongly alkaline in order to pour it into test tubes and petri dishes before it solidifies. Several tubes and plates were inoculated with cultures from the different plates but no growth could be detected, which might have been due to the strong alkalinity of the silicate jelly. Another noticeable feature in the silicate jelly is the tendency to dry and crack when left in the incubator a few days. It is also difficult to remove from the glassware and in general it does not offer near the possibilities that modified agar does.

The last and most successful method used was conducted as follows. Two series of flasks were inoculated with dilutions from the soil, one series for nitrites and one series for nitrates. At the end of ten and fifteen days they were tested. The flasks which showed a marked increase in nitrites and nitrates were then used for inoculating a new series, 1 CC being used for the inoculation . These flasks were incubated at 28 C, and then tested twice the same as the first series and those showing a marked increase were used to inoculate a new series. Stains were made from all the flasks in each series as soon as the flask showed nitrification. In the stain from the first series there was a mixed culture, although there was one particular organism in each which seemed to be present in greater numbers than any of the others. The stains from the second series gave these same organisms in almost pure

Plate III. Showing colonies on modified agar which had been inoculated from a nitrite solution.

Plate IV. Showing colonies on modified agar which was inoculated from a nitrate solution

culture while inethethird series these organisms were present in practically a pure culture. It was decided that these were the bacteria which caused nitrification, as the nitrification was increasing and there were practically no other bacteria present.

After thus isolating the bacteria in solutions the next step was to grow them on the washed agar. The agar used in this work had been melted and then washed for two months the water being changed twice a week, so that it contained but very little if any organic matter. The agar was divided and half of it prepared for nitrite and half for nitrate organisms. The same salts were added in both cases as had been used in exp.VII, parts 1 and3. Plates were poured using dilutions of 100, 1000 and 10000, from the flasks which had shown a pure culture. These plates were incubated at 28 C. for several days and then examined. A slight growth was beginning to make its appearance. on the nitrite plates but there was practically no growth on the nitrate plates. At the end of two weeks they were examined again and by this time the growth in the nitrite plates had reached the size of a pinhead and some growth was evident in the nitrate plates although the colonies were very thin, but were larger than the nitrite colonies and remained practically the same when allowed to grow for several weeks. Stains were made from the different colonies to determine whether or not the colonies contained the same germ as that which was in the solution in pure culture. The stains showed that the colonies were the same organisms that had been found in the solutions.

The nitrite colonies were smaller than the nitrate colonies but were more definite and present in greater numbers. The nitrate colonies were very slow in making their appearance and the growth was always light and indefinite, although they were plain enough to be counted. The nitrite organism was a small bacillus while the nitrate organism was a bacillus but very short and plump.

This method seemed to give the best results in isolating the nitrifiers. The principle difficulty was in getting a good active organism in the first series of flasks. If it was not active it was rather difficult to transfer it from flask to flask and get any growth. The success of the experiment depends upon the number and activity of the organisms in the first sample. In making the transfers the other organisms seemed to gradually disappear until there is practically a pure culture in the last dilutions.

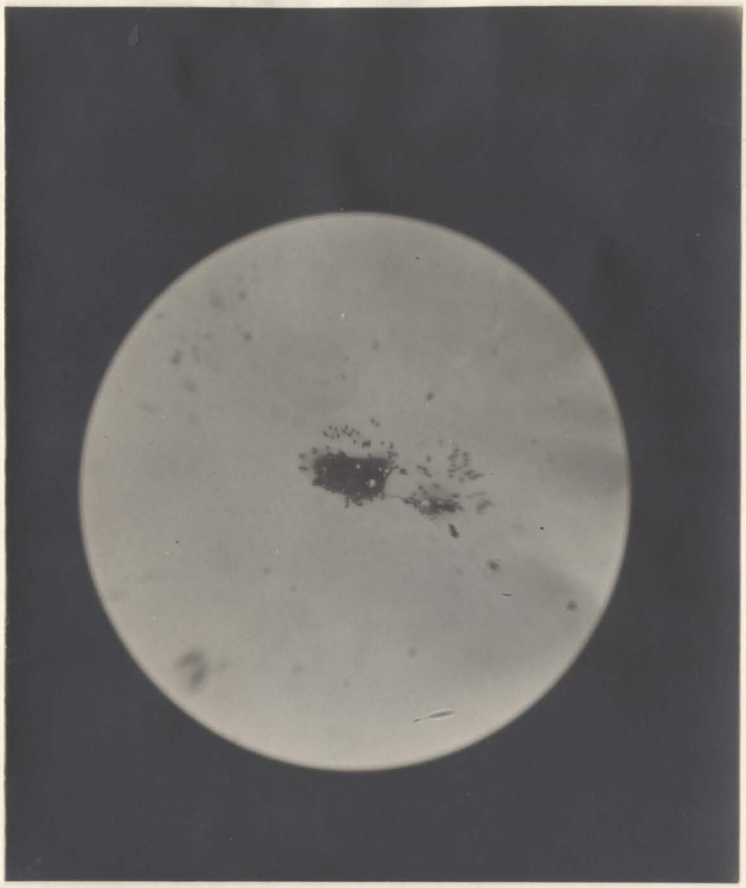

Plate V. Photomicrograph of an organism isolated from a nitrite solution. Stained with carbol fuchsin X 1000

Plate VI. Photomicrograph of an organism isolated from a nitrate solution. Stained with carbol fuchsin. X 1000

Summary.

1. The nitrifying organisms were present in all of the soils that were tested.

2. They were more numerous and more active in cultivated soils, or soils that had been manured.

3. They were more active in the early fall and spring than during the winter.

4. Some of the organisms from the same soil were much more active than others.

5. They can be studied in solutions and can be grown on modified agar with difficulty.

6. The principle difficulty in working with these organisms is in determining which are nitrifying and which are not, in that several kinds of non-nitrifying bacteria were found growing in the modified agar plates.

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