A STUDY OF THE NITRIFYING ORGANISMS AS ISOLATED FROM BRITISH COLUMBIA SOILS.

by

Frederick P. Sparks

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PREFACE.

The bacteria responsible for the production of nitrates in the soil perform a service of inestimable value to mankind. Such being the case, a study of the factors controlling their growth and activity, is of great importance.

To date, so far as the writer is aware, no attempt has been made to study the nitrifying bacteria as found in Canadian soils. Since a review of the literature indicates that either distinct species, or definite differences within the same species are to be found in the different countries of the World, the writer undertook a comparative study of the organisms as isolated from the soils of British Columbia.

Simultaneously with the isolation processes, observations were made, as to some factors governing the growth and oxidative powers of the organisms.

It is with great pleasure that the writer expresses his indebtedness to Dr. H.W. Hill and to Mr. D.G. Laird, for their interest, advice, and criticism, throughout the progress of this work.

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A STUDY OF THE NITRIFYING ORGANISMS AS ISOLATED FROM BRITISH COLUMBIA SOILS.

INTRODUCTION.

Nitrogen is an essential plant food element which is taken up by the plant, largely, in the form of nitrates. Nitrification, or the conversion of ammonia to nitrates, is, in the soil, a biologic process brought about by a certain group of organisms which may be designated as "Nitrifiers". These forms are distinctive, in that, unlike most bacteria, they grow and secure their necessary supply of energy in the entire absence of organic materials. This would suggest that they are elementary forms of life. Further, their growth and activity in laboratory cultures, at least, are believed to be inhibited by such materials, yet in a soil with a high humus content they appear to be extremely active. Practically all the nitrates in the soil are produced by these organisms, in spite of the fact, that there never appears to be more than a few thousand per gram of soil at most, while hundreds of thousands of other species, whose functions are not definitely understood, are present.

The nitrifiers were first isolated from European soils and later from soils of South America, Africa, and the United States. Differences in morphology are apparent from data published in these countries. This, together with the fact, that cultures isolated from Canadian soils have never been studied, makes the nitrifiers an intensely interesting and inviting field for investigation.

HISTORICAL

Prior to 1830 very little was definitely known regarding the nutrition and physiology of plants. The men who were working on these subjects, had been trained in other branches of science, and each, therefore, approached the work from the angle of his previous training. It was recognized that plants receive most of their food from the soil, and humus, a prominent constituent, was considered to be the important plant food. The early workers held that all the complex organic materials were broken down chemically to humic acids and utilized as such by the plants. Thus, if humus was used as a source of food, it should gradually disappear, as the growing season progressed. That such was not the case was demonstrated by Leibig, a German chemist, (7). This research worker, who is known as the father of Agricultural Chemistry, pointed out the fact that there was not enough humus in the soil, to support the plant life living on it.

About this time, ammonia and nitrates were discovered in the soil and Leibig, in 1842, stated that carbon-dioxide, ammonia, and water were all-sufficient for normal plant growth. De Saussure (7), on the other hand, stated that ammonia and nitrates were not the sources of the plants' nitrogen supply, but that these acted only as solvents for the humus which was the real source of the nitrogenous food.

Boussingault (7), a follower of Leibig carried out experiments on plants during the years 1851-1855 and proved that plants could not take nitrogen from the air as had at one time been held, but would thrive well, if supplied with nitrogen in the form of nitrates. He also

proved that plants would grow normally, in a soil free from organic materials, if the ash constituents and nitrates were added.

Thus, the period 1840-1860, gave us the statement that ammonia and nitrates, particularly the latter, are the sources of plant nitrogen.

The following question then presented itself:- "Granted that plants do use ammonia and nitrates, from what are they derived, and how?" Various purely chemical theories were advanced in an effort to explain the presence of these substances in the soil. Pasteur (12), was the first to suggest that the oxidation of ammonia to nitrates might be caused by living organisms. To Schloesing and Müntz (15), however, belong the credit of proving that nitrification is a biologic process. While endeavouring to solve the problem of sewage disposal, for the city of Paris, these two men in 1877 found, that the organic nitrogen contained in the effluent was converted to nitrate nitrogen, provided the sewage was permitted to stand in settling tanks for a time. They also found, that when the sewage was treated with chloroform or other antiseptics, the process was stopped, but was again renewed on the addition of a little fresh sewage. It was observed that similar changes taking place in the soil, could be stopped, through the application of heat at 100° C., and that the transformation of ammonium compounds to nitrates was renewed, by the addition of a little fresh soil. Thus, the establishment of nitrification as a biological process, became an accepted fact.

This conclusion was confirmed by Warington (16), an English chemist, who proved that nitrification of a biological nature would proceed in ammoniacal liquid solutions inoculated with soil. He found that by raising the temperature to 30°C., nitrification was increased, and that "nitrite" production was predominant, whereas, if the depth of the solution was reduced, "nitrate" production acquired the ascendency. Later he established the fact, that there was a purely nitrous producing organism, as well as a nitric producing organism.

From this time on, many men attempted to isolate the organisms responsible for nitrification, and Warington, though he worked hard and did observe the organism without recognizing it, was doomed to disappointment. To Winogradsky belongs the credit for isolating the nitrifying bacteria. In 1891 he published his descriptions of these organisms in the Annals de l'Institut Pasteur, (19), (20).

Winogradsky, respecting the ability of his predecessors and appreciating the failure which attended their efforts in an attempt to isolate the nitrifiers, suggested that these bacteria must be distinct, in one or more respects. He conceived the idea from some previous work that possibly the nitrifiers received their carbon supply from inorganic sources rather than from organic matter, and thus ultimately succeeded in isolating them, through the use of inorganic carbon, selective culture media, and enrichment culture methods. As these organisms refused to grow on nutrient gelatin, he picked off magnesium carbonate particles which remained apparently sterile on the gelatin, and put them back into inorganic nutrient solutions. In this way he succeeded in obtaining

pure cultures.

The introduction of silica jelly for plating by Kuhne, greatly simplified the study of these bacteria, and a little later Beijerinck demonstrated that they would grow quite satisfactorily on washed agar plates containing inorganic salts.

Winogradsky gave a morphological and a partial physiological description of the nitrifiers, as found in European soils. He also examined and described forms found in the soils of Brazil, Ecuador and Australia. Strangely enough, he did not examine North American soils. Among other things, he definitely stressed the importance of furnishing a plentiful supply of oxygen. In order to secure good aeration, he used flasks with very large bottoms (300 sq.cm.) and in these, placed very thin layers of the medium (19), (20).

Jordan and Richards (10), in 1890, were the first men to attempt to isolate the nitrifiers from American soils. These men were working on sewage disposal, and, apparently isolated the nitrifiers, but did not separate the "nitrite" and "nitrate" producing forms, since in their flasks, the ammonium salts were converted directly to nitrates.

Following this work, few, if any, publications appeared, describing the nitrifiers of America, till those of Bonazzi 1915-1919, (2) (3), Gibbs 1919, (8), and Fred and Davenport 1921, (5); but as these are comparatively recent works, their findings will be compared with the results of the present study.

SCOPE OF WORK PLANNED.

In this study, the writer undertook to isolate the nitrifying

bacteria from the soils of British Columbia, and to compare them with those as isolated from the soils of other parts of the World. The work includes specifically, a study firstly, of morphological characters, and secondly, of some of the factors which influence the intensity of nitrification.

CRITERION OF PURITY.

A criterion of purity must needs be set in a work of this kind. Theoretically, a "pure culture", is one which arises from a single cell, but in practice, it is seldom attained. The common method is to take a small amount from a colony and use it as the inoculum. This, however, is not sufficient in the case of the nitrifying organisms, in the light of later work.

Winogradsky found that the nitrifiers of different lands possessed the common character of not growing in bouillon. So in spite of the differences in morphology, he made this fact the basis of his criterion of purity (21). He states as follows:-

"Introduce a loopful of a nitrified culture in ordinary bouillon and keep at 30° C., during ten days; at the end of this time, the bouillon must not show turbidity. The purity of the nitrifying organisms is then proven".

That this is a rather indefinite criterion of purity, is shown by the following statement from Jordan and Richards (10), who discussed the purity of some of their cultures as follows:-

"We are not even prepared to say that there may not have been a mixture of two or more species in our flasks, agreeing closely in morphological characters, and in giving no growth on gelatin, but differing

in important physiological respects".

Omelianski (11), states that, "after renewed ammonia additions it is necessary to control the purity by microscopical examinations, as well as inoculations in bouillon."

Since that time, Wimmer (18), found, that a nitrifying culture which proved pure on a bouillon of one reaction, proved to be impure when tested on bouillon of a more alkaline reaction. Such being the case, Bonazzi (3), adopted the following points as a basis for his criterion of the purity of a culture:-

(1) "The culture must be in full nitrification before any attempt to determine the purity by the bouillon method is made.

(2) The bouillon used in testing for purity should be of an alkaline reaction; better results would be obtained if the testing were made in bouillon of different reactions.

(3) The time limit necessary for absolute reliability for the growth in bouillon should be fixed at ten or more days.

(4) The inoculum used in the testing for purity by the bouillon method should be as large as permissible by ordinary technic.

(5) Microscopical examination must reveal a picture uniform within the limits of individual morphological variation in the species, and within the different phases of the

same organisms, provided such phases are established as correct."

These rules were adopted by the author, and no culture was considered pure,which did not measure up to these requirements.

PROCEDURE

In the isolation of the nitrifying organisms selective culture media were used. The type of medium depended upon the form of organism which was being isolated.

Liquid Media

For the "nitrite" and "nitrate" producers, the following media (6) were used respectively:-

1.	Ammonium sulphate $(N H_4)_2 S O_4$	1.0 gm.
	Dibasic Potass. phosphate $(K_2 H P O_4)$	1.0 gm.
	Magnesium sulphate $(Mg S O_4 + 7 H_2 O)$	0.5 gm.
	Sodium chloride (Na Cl)	2.0 gm.
	Ferrous sulphate $(Fe S O_4 + 7 H_2 O)$	0.4 gm.
	Magnesium carbonate $(Mg C O_3$ in excess about	5.0 gm.
	Distilled water	1000 cc.
2.	Sodium nitrite $(Na N O_2)$	1.0 gm.
	Dibasic potass. phosphate $(K_2 H P O_4)$	0.5 gm.
	Magnesium sulphate $(Mg S O_4 + 7 H_2 O)$	0.3 gm.
	Sodium chloride (Na Cl)	0.5 gm.
	Ferrous sulphate $(Fe S O_4 + 7 H_2 O)$	0.4 gm.
	Sodium carbonate $(Na_2 C O_3)$ anhydrous	0.3 gm.
	Distilled water	1000 cc.

In order to prevent the loss of ammonia or nitrite as the case may be, 10% solutions of these salts were made up separately, sterilized, and added in correct amounts to their respective media by means of sterile pipettes.

One gram of soil was placed in a 20 cc portion of each of the above culture media in large flasks, (125 cc, 300 cc, 500 cc, and 1 L flasks tried out). Active cultures were built up by renewing the ammonium, or nitrite salts, as they became exhausted. After every 4 or 5 renewals of the nitrogenous salts subinoculations were made into fresh culture media. Following a number of such subinoculations plates were poured using a variety of solid media.

Isolated colonies were picked, under the low power of the microscope, by means of a very fine needle or capillary glass tubes, and inoculated back into 20 cc portions of sterile media. These, upon becoming active, were tested according to the criterion of purity as outlined; and, if necessary, were replated.

Solid Media:

Washed agar used for the first isolations was made up in the following manner (6).

Bacto Agar was dissolved in distilled water, poured into flasks and allowed to harden in thin layers. These layers were then covered with distilled water which was changed every few days. After three weeks of washing in this manner all the soluble organic material had been removed as indicated by the clear wash water.

The agar was then ready for use, and as needed, was tubed in 15 cc portions of a 2.5% solution and sterilized. The nutrient salts were sterilized separately and added at the time of plating.

Several other types of solid media were tested during the isolations; these included:

1. Silicic acid jelly undialyzed.

   This was made according to a modification of the method of Stevens and Temple as outlined by Fred (6).

2. Silicic acid jelly partially dialyzed.

   The silicic acid was prepared in a similar manner to No. 1, but was dialyzed to reduce the concentration of the sodium chloride (6).

3. Silicic acid jelly dialyzed.

   This jelly was prepared and dialyzed in a manner similar to that used by Winogradsky in his original work (19) as outlined by Waksman (24). Certain definite changes, however, were made with respect dialyzation and these will be discussed under a subsequent heading.

4. Magnesium carbonate - gypsum black.

   These plates were made in the usual manner as suggested by Waksman (24).

5. Direct isolation on silicate jelly.

   In this medium the silicate jelly was made up according to Winogradsky (4) but, only, ammonium sulphate was added as a source of energy.

Tests used to detect the growth of the nitrifiers.

All tests for the growth of the nitrifiers are, of necessity, chemical in nature, since the only way to demonstrate their activity, is to show the rapidity of oxidation of the respective nitrogenous compounds. All these tests are qualitative in nature and were made on a spot-plate.

The tests as outlined by Fred (6) are as follows:

1. For "Ammonia"; Nessler's Reagent.

   This solution contains potassium iodide, mercuric chloride, and potassium hydroxide and on coming in contact with ammonia gives a deep golden yellow color.

2. For "Nitrites"; Trommsdorf's Reagent.

   This reagent contains zinc chloride, starch and zinc iodide and when added to a nitrite salt with a little dilute sulphuric acid (1:3) produces a deep violet color.

3. For "Nitrates"; Diphenylamine Reagent.

   This reagent consists of a solution of diphenylamine in sulphuric and hydrochloric acids. This solution, on coming in contact with a nitrate in the presence of concentrated sulphuric acid gives a deep blue color. This test is of no value in the presence of nitrites or any other strong oxidizing agent.

   By means of the methods as outlined above several strong oxidizing cultures of each of the forms were isolated.

CRUDE CULTURES IN LIQUID MEDIA.

Considerable time was necessary to build up an enrichment culture of sufficient strength and purity to be of use in the preparation of plates for isolation purposes. This is well demonstrated in Table I which shows the time required by one culture to oxidize the original nitrite salts and also, the time elapsing between renewals of the salt in this and subsequent generations. Four cultures in addition to the one cited in Table I were started and carried along. These required approximately the same time as the one reported.

Table I - Time required to build up a strong culture of a nitrate producer from the original soil inoculation.

Number of generations and renewals.	Time for Oxidation days
Soil in Flask	18
Sub Culture F 1	8
1st Renewal	4
2nd Renewal	4
3rd Renewal	3
F 2	7
1st Renewal	4
2nd Renewal	3
2rd Renewal	3
F 3	7
1st Renewal	3
2nd Renewal	2
3rd Renewal	2
F 4	6

The five cultures in the F_4 generation were used for plating on washed agar containing the necessary nutrient salts. These plates were allowed to grow for 25 days at the end of which time colonies were visible and the nitrite reaction absent. Twenty colonies were picked from the plates showing most active nitrification and streaked on washed agar slants** and only after 18 days did four of them show oxidation. These were Nos. 7, 8, 16 and 19. The small amount of inoculum probably explains the slow initial growth, and the fact that some of the slants did not show oxidation was that colonies of bacteria other than nitrifiers were picked. Flasks of nitrite culture solution were inoculated from the above nutrient slants and the records of their oxidation powers and growth will be found in the section on Associative Action.

The original cultures were maintained and nitrite salt added as required without keeping accurate records. It became apparent that some of the cultures ceased to oxidize and hence the necessity for further study with them.

Subinoculations (F_5 generation) were made immediately and the data concerning these as well as the original cultures are presented in Table II.

** Wherever washed agar slants are referred to it is to be understood that they contain the inorganic nutrient salts.

TABLE II

THE INFLUENCE OF CONTINUED ADDITIONS OF NITRITE ON OXIDATION.

Renewals of Na NO_2 F_4 gener.	Time for oxidation - days. 1	2	3	4 *	5 *
Renewal	11	11	11	No	ox.
"	6	6	10	"	"
"	5	5	6	"	"
"	6	6	7	"	"
"	7	7	8	"	"
"	8	8	17	"	"
"	9	9	7	"	"
"	7	7	10	"	"
"	3	10	10	"	"
"	7	10	4	"	"
"	10	9	5	"	"
"	5	6	6	‡Discard	
"	2	5	10+		
"	2	7	‡Dis.		
"	3	3			
"	4				
"	3	‡Dis.			
"	4				
"	5				
"	*Dis.				

Renewals of Na NO_2 F_5 gener.	Time for Oxidation days 1A	2A	" 3A	4A	5A	" 6A
sub.culture	11	11	11	17	11	11
1st	6	6	6	5	6	6
2nd	5	5	5	13	5	5
3rd	6	6	6	17	6	6
4th	7	7	7	7	7	7
5th	3	3	3	3	3	3
6th	5	5	5	7	5	5
7th	11	11	11	10	11	11
8th	7	7	7	14	7	7
9th	10	10	3	4	3	3
10th	10	10	7	3	7	7
11th	6	6	10	5	10	10
12th	3	5	10	3	4	6
13th	3	6	3	3	4	5
14th	4	9	2	3	2	4
15th	4	4	8	2	2	13
16th	5	10	6	3	1	9
17th	5	12+	4	6	2	12+
18th	4		3	5	3	
19th	3		3		4	
20th	3		3		3	
21st	6		4		3	
22nd	5		6		3	
23rd			5		4	
24th					3	
25th					6+	

‡ Dis. = Discarded.

* No ox. = No Oxidation.

+ Nitrite still present when last record taken.

" Duplicate cultures.

A review of the table indicates that cultures 4 and 5 of the F_4 generation had ceased to oxidize the sodium nitrite but upon subinoculating into fresh media the nitrite was oxidized quite readily. The cultures continued active for a considerable period as the data indicates. Renewals of nitrite have been added until as indicated in the table cultures 2A, 5A and 6A have ceased to oxidize. There is no doubt but that if subinoculations were made from these cultures oxidation would again become active. This table very definitely demonstrates that these organisms though paralyzed by the products of their metabolism are not killed.

Gibbs (8) reports a similar condition in some of his cultures which showed a total of .502 and 527 mgms. of nitrate nitrogen per 100 cc of solution when the organisms ceased to oxidize.

CRUDE CULTURES ON SOLID MEDIA

The growth of the "nitrifiers" on solid media is scanty at best. The washed agar gave no trouble, as both types of organisms grew well on this medium. Washed agar, however, permitted the growth of a greater number of contaminants than did the silicate jelly.

Of the silicic acid jellies No. 3 proved to be the most satisfactory. Difficulty was experienced in getting the sodium silicate to harden in the case of Nos. 1 and 2. Even where the plates did jell and the excess moisture was eliminated the writer failed in getting satisfactory growth even with a heavy inoculation; the cause for this may have been that the concentration of soluble salts in this medium plasmolyzed the bacterial cells. Gibbs (8) reports a similar trouble when using plates made up according to Stephen and Temple.

As mentioned above, No. 3 silicate jelly was made up successfully, and employed in isolating the "nitrate" producing organism. In the preparation of this jelly, instead of using parchment as a dialyzer, the writer used Cellophane sack as made up and described by Wilson (17).

The gypsum blocks did not give any signs of growth, either, because the organisms failed to develop, or, because the colonies, very small in any case, sank into the medium.

The direct isolation method from soil particles did not give satisfactory results, in that, no flasks were successfully started from these plates, although the plates themselves, showed that the ammonia was being oxidized to nitrites. Therefore, this method, in spite of the above, cannot be said to be a failure, as it was not tested sufficiently to warrant any statement as to its value.

MORPHOLOGY

The Nitrite Producing Organisms.

These forms grow on the bottom of the flasks around the magnesium sulphate particles. No motility of any kind has been found either in the hanging drops or in liquid solutions going opalescent. The organisms appear in two forms:-

1. A large coccoid form about 1 ± u in diameter. This form stains a deep purple by Winogradsky's malahite green method. The large coccoid form while resembling that described by Bonazzi (3) also resembles nitrosomonas europaea Winogradsky as described by Bergy, one difference noted is that no flagella have as yet been demonstrated. However, these forms need to be investigated very carefully before they are placed in any one species. (These organisms appeared singly, in pairs, and in short chains).

2. A small coccus form appeared in a large mass as if the individuals were surrounded by a gelatinous substance. These appear similar to the B. forms of Bonazzi being about 0.5u.

Colonies appear only after ten to twelve days as minute rounded forms, at first colorless, later turning a brown color.

The Nitrate Producing Organisms.

Form - Small rods, oval (1 - 1.7 u) x 0.5u occurring singly and in pairs.

Stain irregularly with carbol fuchsin.

Motility - non-motile.

Nitrite agar streak - After 7 days.

A very thin, whitish growth, not spreading beyond the shape of the needle is apparent to the naked eye, this being thickest at the butt of the slant. On examination with a 100 x objective the streak is seen to be composed of small, glistening, granular colonies, light brown in color, similar to those found on plates.

Colonies on nitrite agar plates - 26 days.

Growth - Very slow, colonies small.

Form - Rounded, or irregular.

Surface - Smooth.

Elevation - Convex.

Edge - Entire.

Internal structure - Coarsely granular with a nucleus of darker, thicker granules, at centre of colony.

Colonies on silicate jelly.

These colonies appeared very similar to those described on nitrite agar. They were small, however, and appeared in greater numbers on the needle stroke.

In Liquid culture media.

The nitrate producers may be found in the clear liquid but are usually found in gelatinous flakes which appear at the bottom of the flask after two or three additions of sodium nitrites. The organisms when stained with carbol fuchsin appear as very small oval dots. In one preparation small flagella like structures appeared. This same structure has been previously reported by Fred and Davenport (6) who have published micro-

photographs to illustrate the point.

DISCUSSION OF CONTAMINANTS.

As was mentioned in the preceding pages, some contaminants appeared in these nitrifying flasks, which were hard to eliminate. These correspond to some noted by Gibbs. The chief one is a very small bacillus, which forms colonies on washed agar, similar to those of the nitrifiers. The morphology as worked out in part is as follows:-

Form - Small rods 1.2 - .9u x .5u occurring singly and in pairs.

Gram Stain - Gram negative.

Capsules - a thin capsule.

Motility - Actively motile.

Nut. Agar Slant. - Thin, whitish, raised, glistening growth, with a slight odor and very viscid.

Nut. Agar Plates - Colonies are small, round, colorless and convex.

Bouillon - Turbid, slight surface growth after 48 hours.

Milk - Unchanged.

This small bacterium cannot be distinguished microscopically from the nitrifiers.

Another bacterial contaminant has been noted; one which gives a white pigment on nutrient agar, with a very thick, glistening, moist growth after 48 hours. This organism has not been worked out, save that it appears on agar as chains of short rods, and in broth as long rods.

An actinomycete appeared as a contaminant on the nitrate flasks.

It always appears as floating, white, specks on the surface of the medium. Beijerinck in 1914 reported a number of forms, belonging to the family actinomycetes, which contaminated his flasks and proved very troublesome. One characteristic of this actinomycete is worth noting, which is, that this form, while developing colonies on nitrite washed agar, either does not grow, or changes its morphology when placed on nutrient agar. More work is necessary, however, before any of these contaminants can be definitely classified.

ASSOCIATIVE ACTION

An interesting point noted, when studying the activities of the nitrifying cultures, was that impure or mixed cultures of nitrate producing organisms oxidized the nitrates more rapidly than the pure cultures.

Four strong nitrate producing cultures were isolated, from nitrite agar plates, No. 7, No.8, No. 16, and No. 19. No. 19 was the only culture which was pure according to our criterion of purity. No. 7 was highly contaminated, as shown, by an apparent growth on nutrient agar at the end of 24 hours, whereas, No. 16 took 48 hours before any growth was apparent. The activity of these cultures varied conversely with their purity, No. 7 being the most active, No. 16 intermediate, and No. 19, the pure culture, the slowest. This variance of activity was carried throughout the subinoculations unchanged, as illustrated by the time taken for the second subinoculations of these three cultnres to oxidize the nitrites for the first time. The subinoculations as shown by a table at the end of this chapter were sown at the same time, and tested at the same time, and kept in the same incubator, also an equal amount of incoulum was placed in each flask. These flasks were the 125 cc. size and contained 20 cc. of sterile media. No. 7, required four days to nitrify, whereas, No. 16, required 9 days, and No. 19, nineteen days.

In an article which came to hand since the preceding conclusion has been arrived at, Beijerinck is reported to have emphasized the fact, that pure cultures do not grow as rapidly as mixed cultures.

The following table gives a comparison of the rate of oxidation by cultures No. 7, No. 8, No. 16 and No. 19.

TABLE III.

COMPARISON OF RATES OF OXIDATION BY PURE AND IMPURE CULTURES.

Date of Inoc.	Dates of Tests										
	Feb.11	Feb.16	Feb.20	Feb.22	Feb.24	Feb.27	Feb.29	Mar.2	Mar.5	Mar.8	Mar.10
No. 7 (21/1/28)	-	+	-	-	-	-	+	-	-	+	-
No. 8 (21/1/28)	-	+	+	+	-	-	+	-	-	-	+
No.16 (21/1/28)	-	+	-	+	-	sl +	+	-	+	-	+
No.19 (21/1/28)	-	+	-	+	+	-	+	-	-	+	-
Sub culture No. 7 (20/2/28)			+	+	-	-	-	-	-	-	-
No.16 (20/2/28)			+	+	+	-	+	-	-	+	-
No.19 (20/2/28)			+	+	+	+	+	+	+	-	+
No. 7 (27/2/28)						+	+	+	-	-	-
No.16 (27/2/28)						+	+	+	-	+	-
No.19 (27/2/28)						+	+	+	+	-	+

TABLE III (Cont'd)

Date of Inoc.	Dates of Tests									
	Mar.12	Mar.14	Mar.16	Mar.19	Mar.21	Mar.24	Mar.26	Mar.28	Mar.30	Apr.2
No. 7 (1/21/28)	+	-	+	-	+	-	Discarded March 24/28.			
No. 8 (1/21/28)	-	+	-	+	-	+				
No.16 (1/21/28)	+	-	+	+	-	+				
No.19 (1/21/28)	+	-	+	+	-	+				
No. 7 (2/20/28)	-	+	+	-	+	-	-	+	-	-
No.16 (2/20/28)	sl ±	+	-	-	+	-	+	-	+	-
No.19 (2/20/28)	sl ±	+	-	-	+	-	+	-	+	-
No. 7 (2/27/28)	-	+	-	-	+	-	+	-	+	-
No.16 (2/27/28)	+	+	+	+	+	+	+	+	+	+
No.19 (2/27/28)	+	+	+	+	+	+	+	+	-	-
No. 7 (3/14/28)			+	-	-	-	-	-	-	-
No. 8 (3/14/28)			+	+	-	-	-	-	-	-
No.16 (3/14/28)			+	+	-	-	+	-	sl ±	-
No.19 (3/14/28)			+	+	-	-	-	-	+	-
No.5-7 (3/26/28)									+	-
No.3-8 (3/26/28)									+	-
No.4-16(3/26/28)									+	+
No.4-19(3/26/28)									+	+

When a ± (plus minus) sign appears there was a very slight reaction showing the presence of nitrite; the nitrite, however, was renewed in these flasks.

TABLE III (Cont'd)

Date of Inoc.	Dates of Tests										
	Apr.4	Apr.7	Apr.9	Apr.12	Apr.14	Apr.17	Apr.20	Apr.23	Apr.26	Apr.28	May 2
No.2-7 (20/2/28)	+	-	+	-							
" 2-16(20/2/28)	+	-	+	-		Discarded April 14th					
" 2-19(20/2/28)	+	-	+	-							
" 3-7 (27/2/28)	+	-	+	-							
" 3-16(27/2/28)	+	+	+	+		Discarded April 14th					
" 3-19(27/2/28)	+	-	+	-							
" 4-7 (14/3/28)	-	-	-	-	-	+	-	-	-	+	-
" 2-8 (14/3/28)	-	-	-	-	-	-	-	-	-	+	-
" 3-16(14/3/28)	-	-	+	-	+	-	+	-	+	-	+
" 3-19(14/3/28)	+	-	+	-	+	-	+	-	+	-	+
" 5-7 (26/3/28)	-	-	-	-	-	-	-	-	-	+	-
" 3-8 (26/3/28)	-	-	-	-	-	-	-	-	-	+	-
" 4-16(26/3/28)	-	-	+	-	+	-	-	-	+	-	+
" 4-19(26/3/28)	-	-	+	-	+	-	+	-	+	-	-
" 6-7 (20/4/28)								+	-	-	-
" 4-8 (20/4/28)								+	-	-	-
" 5-16(20/4/28)								+	+	-	-
" 5-19(20/4/28)								+	-	+	-

Wherever a - (minus) appears in this table it indicates the absence of nitrites and that a portion of nitrite was added to renew that salt in the culture flask.

A + (plus) indicates the presence of the nitrite salt in a culture flask.

AERATION AND THE NITRIFIERS.

In order to secure the maximum amount of aeration, a klinostat was built as described by Bonazzi (2) in his experiments on intensive nitrification. This instrument carried the flasks at an angle of 5° and completed one revolution in six minutes, thus a thin layer of liquid was being exposed to the air on one-half of the flask, and the liquid was continually changing by the slow rotary movement.

To further facilitate aeration, large flasks were employed (1 litre and 500 cc.), which would increase the surface exposed. Only 20 cc. of culture media were placed in each flask which gave a layer of 3 mm. for the former and 7 mm. for the latter, when the flask rested on an even surface.

Small 125 cc. flasks were first used in this study, with 20 cc. of liquid, but these gave a layer about 1.5 cm. deep and a very small surface exposure.

The influence of these large flasks is apparent, when it is realized that no small flasks, however active the cultures, converted the nitrites to nitrates in 24 hours, whereas three flasks of the 1 lt. and 500 cc. sizes did do this, and did it regularly. The influence of aeration or nitrification in crude cultures is well illustrated in Table IV.

Flask 4 B I, contained a number of pieces of pumice stone which projected above the surface of the media cultures. 4 B II and 4 B III, did not contain any pumice. Cultures 4 B I and 4 B II, were placed on the klinostat, and 4 B III, was placed in the incubator on an even surface and not disturbed except at the time of testing.

TABLE IV.

COMPARISON OF THREE DEGREES OF AERATION ON NITRIFICATION.

(Inoculated - April 9th, 1928).

	4B I.	4B II.	4B III.
April 9th	+	+	+
April 13th	+	+	-
April 14th	sl.	sl.	sl.
	+	+	+
April 15th	sl.		
	+	-	-
April 16th			sl.
	-	-	+
April 17th	-	-	-
April 18th	sl.	sl.	
	+	+	+
April 19th	-	-	-
April 20th		sl.	sl.
	-	+	+
April 21st	-	-	-
April 22nd		sl.	sl.
	-	+	+
April 23rd	-	-	-
April 25th	-	-	-

It will be noted that while 4B I, was slow in getting started, once it did get started, it oxidized the nitrates in less than 24 hours, until April 23rd, when the writer stopped checking the record. The slow period might be explained by the fact, that the organisms took time to spread themselves over the pumice. This result compares favorably, with that recorded by Bonazzi, where he used lumps of earth and large thin areas of liquid. The quick nitrification by 4B III, may be explained on the temperature basis, as discussed in another section.

It is evident from a study of 4B I and 4B II, that if 4B III, had been left at the same temperature without being disturbed, it would have been much less active than 4B II.

TEMPERATURE RELATIONS.

The range in temperature to which these organisms are exposed under natural conditions are great. As would be expected, the organism can live at low temperatures better than at high temperatures. The lowest temperatures have been recorded by Müntz ( ), 1890 who found these organisms living, but inactive, in materials which had been ice-clad for years. He reports at the same time, that the nitrifiers were found in great numbers on rocky cliffs where there was nothing but rock for them to live on, and concludes that they must be agencies in the decomposition of the rock materials.

The organisms begin active oxidation under laboratory conditions at a temperature at least as low as 18° C., as proved by one crude culture of the nitrate producing organism, which stood on a bench in a large room, the temperature of which varied from 15° - 18° C. When this culture was removed to a warmer room, 19°- 25° C., the oxidation became more intense, and when this flask was placed on the Klinostat, this culture oxidized .2 mls. 10% Na NO_2 solution in 24 hours for 5 successive days.

To test the temperature relations two duplicate flasks were inoculated with equal amounts of inoculum, one 4B II, being placed on the klinostat at 17°- 25°C. and the other, 4B III, in the incubator at

28°- 30°C. The influence of these different temperatures on oxidation is indicated in Table V.

TABLE V. TEMPERATURE AS AFFECTING NITRIFICATION.

Date of Inoculation, April 9th, 1928.

	4B II. NO_2	4B III.
April 13th	+	-
April 14th	sl. +	sl. +
April 15th	-	-
April 16th	-	sl. +
April 17th	-	-
April 18th	sl. +	sl. +
April 19th	-	-

From April 20th to date, these flasks have been equal, both converting the nitrite in the same time. A study of the Table indicates that there is little difference between the two cultures. The one, 4B II, was well aerated and the other 4B III, maintained at a higher temperature. The influence of aeration has been demonstrated, therefore temperature must also be equally important.

The thermal death point of these organisms has been set at 50°- 55°C., by Bonazzi (2), for nitrosomonas and at 56°- 58°C., for the nitrobacter by Gibbs. No thermal death point experiments have been conducted by the writer, so no comparisons can be given.

TEMPERATURE AND AERATION.

When the rates of nitrification as observed in this Laboratory, are compared with those reported by Bonazzi (2), and others, it is noted that the cultures from other laboratories were much more efficient oxidizers of nitrogen. The importance of aeration and temperature has been emphasized in the preceding pages, but in the work conducted at this laboratory, the writer was unable to achieve maximum aeration and optimum temperature at the same time. Conditions of maximum aeration were attained when the cultures were placed on the klinostat, but as incubator facilities did not permit the placing of the klinostat under controlled conditions, the cultures placed on this apparatus were not at their optimum temperature. The temperature on the klinostat varied from 19°- 25°C. while the optimum temperature for these organisms is 28°- 30°C.

From the data submitted, it appears logical to expect that had the writer's cultures been maintained at optimum temperature and aeration, much greater activity would have been secured.

CONCLUSION

The work reported in this thesis is more of a preliminary study than a finished piece of research. In it the writer has demonstrated that the nitrifiers are present in B.C. soils, that they are similar in the more important characters to some of those isolated by Winogradsky, and, that, at the same time, they show some apparently minor differences from those described by workers in Europe and the United States. This is to be expected since there is so much contradictory evidence on the subject. The presence of a number of contaminants was very definitely demonstrated, and the difficulty in eliminating those brought about additional complications. All these facts, combined with the inherent nature of the organisms make them a subject for study which demands careful observation, unlimited patience and dogged perserverance.

It is sincerely hoped that this work will serve as a basis for a more extended study of these elementary forms of life which at the same time perform such an important function to the nitrogen cycle.

SUMMARY.

1. Nitrobacter was isolated in pure culture from the soils of British Columbia.
2. Nitrite producing organisms were isolated which resemble those described in Bergey's Manual of Determinative Bacteriology (27), but more work is necessary before these can be assigned to any particular species.
3. The nitrite producers oxidized ammonium carbonate to nitrite.
4. The nitrobacter oxidized nitrite to nitrate.
5. These cultures when inoculated into beef peptone broth and nutrient agar failed to show any visible growth.
6. Washed agar and silicate jelly containing the correct nutrient salts proved to be quite satisfactory solid media, providing the latter was dialyzed.
7. Normal growth on solid media was very scant as compared with that produced by the commonly studied bacteria. The growth appears to the naked eye as very small beads. Under the microscope these beads are seen to be separate colonies.
8. Aeration has a marked effect on nitrification. Cultures under intensive aeration on the klinostat have oxidized .2 ml. of 10% Sodium nitrite solution to nitrates in 18-24 hours.
9. The temperature best suited to the needs of these organisms varies between 28-30^{o}C. They will grow and function at 15-18^{o}C.

10. Mixed cultures oxidize nitrogen more rapidly than pure cultures, suggesting an associative action between the nitrifying and the contaminating forms.

11. A number of contaminants were found to be associated with the nitrifiers. They were very difficult to eliminate, but eventually pure cultures of these were obtained.

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