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THESIS

Studies in Denitrification

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Nitrification

Soils – Bacteriology

T H E S I S

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Denitrification is, as the term implies, a reducing process in which the soil nitrogen is eliminated in one form or another by the breaking down of the nitrate or complex nitrogenous compounds, thereby becoming lost to plant growth. It is still to a great degree an open question in what form the nitrogen is lost, whether as oxides of nitrogen, free nitrogen or free ammonia or in more than one of these forms.

The denitrification phenomenon was first noticed in cultivated soil in 1862 by Goppelsröeder (Technical Mycology Lafar), and for a long time it was regarded as a purely chemical process. The first reference to the agency of bacteria in the changes produced was made in 1875 by C. Meusel (Tech. Myc.) and the earliest pure cultures of such micro-organisms were made ..

in 1882 by U. Gayon and G. Dupetit (Tech. Myc.). In 1886 W. Heraeus (Tech. Myc.) made known several species of the denitrifying class, and two years later Percy Frankland and R. Warington (Frankland's Micro-organisms in Water) found a large percentage of soil bacteria capable of reducing nitrates to nitrites although they do not state whether their cultures possessed the power of further reduction.

In later years scores of workers have entered the field, and, working upon different phases of the subject, have secured some very valuable results.

The object of the present investigation was to contribute something toward a solution of the problem by a detailed study of the denitrifying properties of three germs found commonly at a depth of two to six inches in different soils upon the college farm, viz.; B. Subtilis, B. Megaterium, and B. Mycoides.

A brief outline of the investigation pursued follows:-

1. Action upon nitrate.
2. Action upon nitrite.
3. Action upon ammonium salt.

4. Effect of the presence of organic matter.

5. Influence of abundant oxygen supply.

1. Action upon Nitrates.

As has been stated, in 1888, Frankland and Warington found a large number of soil bacteria capable of reducing nitrate to nitrite. For their work they used a mineral solution which contained besides the mineral elements necessary for plant growth, a small amount of organic matter in the form of peptone. In our studies an endeavor was made to determine quantitatively the reduction of nitrate to nitrite, and whether any further products resulted.

The culture solution used was made up as follows:-

Potassium nitrate	5. grams.
Potassium phosphate	.1 gram.
Magnesium sulphate (cryst)	.02 "
Calcium chloride (fused)	.01 "
Glucose	.3 "
Peptone (Wittes')	.25 "
Calcium Carbonate	5. "
Distilled Water	1000. c. cm.

75 c. cm. Erlenmeyer flasks, plugged and sterilized, received about 25 c. cm. each of the

culture solution and were sterilized intermittently in moist heat. The inoculations were made with .5 c. cm. of cultures of the germs in a mineral solution containing a minimum of nitrate or organic matter, some of the flasks being retained uninoculated for controls. The cultures were kept in a darkened room at an approximately average temperature of 25° C. Determinations of nitrite and free ammonia were made at the time of inoculation and at intervals thereafter. In all the work with mineral solutions bouillon tubes were inoculated with small portions from the culture and control flasks, to verify the vitality of the cultures and sterility of the controls.

TABLE II.

Nitrites expressed in m. g. per c. cm. N_2O_3

Ammonia expressed in m. g. per c. cm. NH_3

	At inoculation		At two days		At seven days		At sixty days	
	N_2O_3	NH_3	N_2O_3	NH_3	N_2O_3	NH_3	N_2O_3	NH_3
B. Subtilis	None	None	None	.0121	None	.0194	Trace	.0048
B. Megaterium	None	None	None	None	None	.0242	Trace	.0291
B. Mycoides	None	None	None	.0048	None	.0291	.011	.0194
Uninoculated Control	None	None	None	None	None	None	Trace	None

From a comparison of the tables the fact becomes evident that most of the ammonia in the nitrate cultures results from the peptone. No reaction for nitrites was obtained until the cultures were old and then only a trace. Whether this was due to spontaneous chemical changes is a question. The fact that the control, which remained sterile, showed a trace of nitrite would lead one to suspect that such was the case. The work was duplicated in order to verify the results The same methods were employed except that fewer tests were made of the results.

TABLE III.

Nitrites expressed in m. g. per c. cm. N_2O_3.

Ammonia expressed in m. g. per c. cm. NH_3.

	At inoculation		At forty-five days.	
	N_2O_3	NH_3	N_2O_3	NH_3
B. Subtilis	None	None	None	.0291
B. Megaterium	None	None	None	.0194
B. Mycoides	None	None	None	.0243
Uninoculated Control	None	None	None	None

Here again the results show that the nitrites result from the reduction of nitrate, but that at least a portion of the ammonia is the result of the decomposition of the proteid molecule. These results agree with those of Maassen (Cent. f. Bakt. 1902.II. No. 5. S. 152) who grew 109 common bacterial species in mineral solution containing peptone, and who says in stating his results;"Considerable ammonia was also produced on account of the vigorous peptonization."

2. Do the Micro-organisms Have a Reducing Action upon Nitrites?

To determine whether the micro-organisms were capable of reducing nitrites, a nutrient solution was prepared as follows:

Potassium nitrite	.3	grams
Magnesium Sulphate	.05	"
Potassium phosphate	.1	"
Glucose	.3	"
Peptone	.25	"
Calcium carbonate	5.	"
Distilled water	1000.	c. cm.

About 25 c. cm. of this solution was placed in 75 c. cm. Erlenmeyer flasks and sterilized intermittently in moist heat. They were inoculated with one-tenth c. cm. of liquefied gelatine cultures of the respective bacteria and kept in semi-darkness at 25° C.

T A B L E I V.

The amount of N_2O_3 estimated in original culture solution, equals .2329 m. g. per c. cm. Results in m. g. per c. cm. N_2O_3.

	At inoculation	At five days	At eighteen days	At forty-five days
B. Subtilis	.22	.22	.22	.22
B. Megaterium	.22	.22	.22	.22
B. Mycoides	.22	.11	.077	.077
Uninoculated Control	.22	.22	.22	.22

The only consideration was the amount of reduction of the nitrite, and in only one case, B. Mycoides, was any decrease in nitrites apparent.

3. Action of the Organisms upon Ammonia Salt.

Cultures were made in the following solution to determine, if any, the action of the germs upon ammonium sulphate.

Ammonium sulphate	1.	gram
Potassium phosphate	1.	"
Magnesium sulphate	.02	"
Calcium chloride	.01	"
Glucose	.3	"
Ferrous sulphate	.3	"
Peptone	.25	"
Calcium carbonate	5.	"
Distilled water	1000.	c. cm.

The cultures were made as before in 75 c. cm. Erlenmeyer flasks, the inoculations being made with a platinum loop from cultures of the germs in bouillon. Nessler determinations were made at intervals to discover, if any, the decrease in the amount of ammonia, standard

ammonium chloride being used for comparison. Duplicate tests were made at different periods, the results of which correspond. At no time was any change observed in the ammonia content, showing that the bacteria have not the power of reducing ammonium sulphate, and furthermore that the salt prevents the production of ammonia from the peptone present.

4. The Effect of the Presence of Organic Matter upon Denitrification.

Künneman in 1898 (Landw. Vers. Stat. 1898 I-II. S. 65-113), in experiments carried on with a micro-organism which he had isolated from horse-dung, found that the amount of denitrification decreases as the amount of organic matter increases, which observation was also made by Matz and Wagner (Landw. Vers. Stat. 1897-XLVIII, 247) who stated that as "humification" proceeds the power of destroying nitrate decreases. On the other hand, Pfeiffer (Deut. Landw. Presse. 1897-911) states that denitrification can take place only in the presence of particles of straw, faeces or vegetable tissue which act as food substance for the denitrifying organisms. Stutzer and Maul (Cent. f. Bakt. 1896- XV, S. 473-474) isolated two micro-

organisms which gave very marked nitrate reducing power when grown in a solution containing a small amount of organic matter. Maassen (loc. cit.) states that in a strictly inorganic solution he obtained very little reduction of nitrate by common bacteria. In our studies along this line five solutions were used, differing only in the nature of the material furnishing the combined nitrogen to be acted upon, the bases of the different solutions being as follows: Ammonium nitrate and Potassium nitrate, Asparagin, Potassium nitrite, and Potassium nitrate and nitrite combined in equal amounts. The other elements necessary to plant growth were added in various salts but none of the solutions, except the asparagin, contained any organic matter. The cultures were tested at the end of forty-five days for the reduction products. In only one of the solutions, Asparagin, were satisfactory results obtained. There was practically no formation of nitrite or ammonia, and upon plating in ordinary gelatine it was found that while the micro-organisms still possessed vitality, there was no vigor of growth. The cultures in Asparagin developed vigorously, and there was a very marked production of ammonia, the controls yielding no reaction for ammonia. A comparison of the results obtained in these strictly mineral solutions with those in solutions containing a small amount of organic matter, leads to the conclusion that the presence of at least a small amount of the latter is imperative in order to secure vigorous denitrification. What would be the effect of large amounts of organic matter is still an unsettled question.

5. The Effect upon Denitrification of an Abundant Supply of Air.

In a study of this question, Deherain in 1897, upon growing micro-organisms from straw and the solid excrement of animals, found that the reduction of nitrates was much more rapid in closed than in open flasks, although he does not say whether the conditions were strictly anaerobic. Maassen (loc. cit.) states, that while denitrification is not inhibited by anaerobic conditions it proceeds best in a limited supply of air. Pfeiffer in 1897 (Chem. Zeit. 21-1897) found that in a culture of B. Denitrificans the admitting of pure air did not lessen the amount of denitrification. In 1898, Ampola and Ulpiana (Tech. Myc.) isolated from soil a micro-organism which grew well both in the presence and absence of air.

In our work one purpose was to so construct a piece ofapparatus that air might be freely drawn through the culture, and yet that the danger of contaminations be eliminated. Five 75 c. cm. Erlenmeyer flasks were tightly fitted with two-hole-rubber stoppers and connected by means of U tubes, one arm of which extended to the bottom of the flask. Two each of the end flasks contained normal-fifth sulphuric acid, the middle one holding the culture solution. Thus when air was drawn through the apparatus the sulphuric acid in the first two flasks would absorb any ammonia from the air passing through, as well as any micro-organisms which might be drawn in with the current. The other two acid flasks were to catch any ammonia that might be given off from the culture and drawn over upon aeration. Aerated and non-aerated control flasks of acid were also used.

Furthermore, one series each of cultures sealed, and simply plugged with cotton wool were used, uninoculated controls being retained in each case; also cultures and controls were placed in an atmosphere entirely devoid of oxygen. The culture solution used was Frankland's denitrifying solution , which is made up as follows:-

Potassium nitrate	.168	gram
Potassium phosphate	.1	"
Magnesium sulphate	.02	"
Calcium Chloride	.01	"
Glucose	.3	"
Peptone	.25	"
Calcium carbonate	5.	grams
Distilled Water.	1000. c. cm.	

The inoculations were made from pure cultures in this solution and kept in the dark at an average temperature of 25° C. After eleven days determinations were made of nitrites and free ammonia in the cultures and controls. The acid flasks of the aeration apparatus were titrated against normal-fifth sodium hydroxide, but showed no indication of absorption of ammonia .

TABLE V.

Nitrites expressed in m. g. per c. cm. N_2O_3.

Ammonia expressed in m. g. per c. cm. NH_3.

Cultures in Absence of Oxygen	N_2O_3	NH_3
B. Subtilis	None	None
B. Megaterium	Trace	Trace
B. Mycoides	Trace	.0080
Uninoculated Control	None	None
Cultures Sealed.		
B. Subtilis	.0330	.0064
B. Megaterium	.0440	.0080
B. Mycoides	None	.0240
Uninoculated Control	None	None
Cultures Unsealed		
B. Subtilis	.0440	.0128
B. Megaterium	.0495	.0244
B. Mycoides	.0551	.0160
Uninoculated Control	None	None
Cultures Thoroughly Aerated		
B. Subtilis	.0826	.0192
B. Megaterium	.1101	.0160
B. Mycoides	.0551	.0240
Uninoculated Control	None	Trace

1 c. cm. sterile culture solution contains .1264 m. g. N_2O_3.

1 c. cm. sterile culture solution contains .0282 m. g. NH_3.

The results show that the reduction depends almost entirely upon the presence of at least a small amount of oxygen, and that, except in the case of B. Mycoides, a greater supply of air induces a greater amount of reduction.

C O N C L U S I O N S .

1. The three micro-organisms under investigation have the power of acting upon nitrates to reduce them to simpler substances, nitrites, free ammonia, and probably free nitrogen.

2. The nitrites present in a culture solution having nitrate for its basis result entirely from the nitrate.

3. A portion of the ammonia usually found in nitrate solutions containing organic matter, results from the decomposition of this latter material.

4. For a vigorous development and activity of these micro-organisms, the presence of at

least a trace of organic matter is essential.

5. Not all bacteria having the power of acting upon nitrates are capable also of exerting a reducing action upon nitrites.

6. The bacteria studied can not reduce ammonium sulphate; furthermore, the ammonium sulphate checks the production of free ammonia from the peptone in the solution.

7. These micro-organisms act more vigorously in the presence of an abundant supply of atmospheric oxygen.

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