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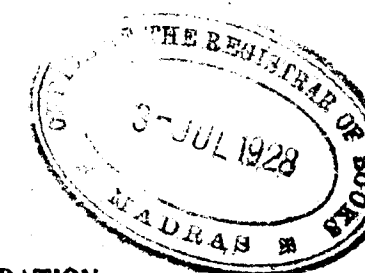
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JOURNAL

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STUDIES IN INTENSIVE BACTERIAL OXIDATION.
THE OXIDATION OF AMMONIA TO NITRIC ACID, PARTS I—IV.

BY

Gilbert J. Fowler, Y. N. Kotwal, Roland V. Norris,
S. Ranganathan and M. B. Roy.

MASTER, F.R.S., CHAIRMAN OF EDITORIAL BOARD.

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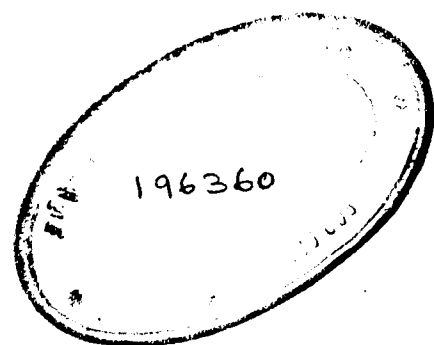
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PART I.—INTRODUCTION.

By Gilbert J. Fowler.

The practice of nitre-farming is of very ancient origin probably having been first brought to a state of proficiency in China and thence imported to Europe.

The suggestion by Pasteur that the development of nitrates from organic matter was due to micro-organisms was, however, not made till about 1878. The correctness of this suggestion was confirmed experimentally by Sclösing and Muntz, who found that nitrification in the soil was inhibited by the presence of antiseptics.

The conditions for successful nitrification have been studied by numerous investigators, but the first experiments on modern lines, directed to obtain large yields of nitrate capable of commercial application, appear to be due to Muntz and Lainé (*Ann. de la Science Agronomique*, 1926). They state that the nitrifying organisms operating at a temperature of 30° were active up to a concentration of 22 per cent. calcium nitrate, although these will not survive in a concentration of more than one per cent. of ammonium salts.

Muntz and Lainé considered peat to be the best medium on which to carry out the nitrification process and they claim to be able to nitrify per day and per cubic metre 1000 litres of solution containing 7.5 gms. ammonium sulphate per litre. They finally obtained a solution containing 5 or 6 per cent. of calcium nitrate, and state that it would be possible, according to their experiments, to obtain about 90 tons of calcium nitrate per hectare of peat-filter per day.

These experiments were taken up and developed with great care by E. Boullanger (*Ann. de l'Inst. Pasteur*, 1921. 35, 575 and 1922, 36, 395). Boullanger's experiments were carried out both in the laboratory and on a semi-technical scale using other media besides peat, the most satisfactory of which was found to be natural pozzuolana in granules the size of cherry-stones.

Boullanger's conclusions are of great interest and value, and the more important of them may be noticed here. It is necessary to begin the process of intensive nitrification very gradually as the nitrifying organism is paralysed by excess of ammonia. Nitrates also have an inhibitory action at the beginning of the process and therefore

ammonium sulphate was used as raw material at the outset, and ammonium nitrate only later. This salt was obtained either by direct solution in water or by double decomposition of ammonium carbonate with calcium nitrate formed by the process. It was found best to use bicarbonate or sesquicarbonate of ammonia rather than sulphate, and so avoid formation of insoluble calcium sulphate.

The strongest solution obtainable was found to be 138.2 gms. of calcium nitrate per litre and this was slightly inhibitive. A regular output was obtained with 120 gms. or 12 per cent. calcium nitrate.

A very careful and special technique, for details of which the original paper must be consulted, was necessary to obtain these results, the underlying principle being to maintain the necessary minimum of ammoniacal nitrogen while allowing a gradually increasing concentration of nitric nitrogen. In order to obtain this concentration it was necessary to dissolve each fresh addition of ammonium nitrate in a portion of the liquor from the previous operation, fresh water being added equivalent to the amount of nitrate solution withdrawn for recovery of the dissolved nitrate.

All the conclusions reached in the laboratory were verified on the semi-industrial scale. If the proper conditions are fulfilled, liquors can be obtained containing 11 to 13 per cent. calcium nitrate by the use of solutions containing 15 to 18 gms. nitric nitrogen and 1.5 ammoniacal nitrogen per litre. Under these conditions about 50 gms. of nitric nitrogen per day, corresponding to 286 gms. ammonium nitrate or 293 gms. calcium nitrate, can be obtained per cubic metre of medium. A nitrifier of one hectare with a working depth of 1.8 metres or 18,000 cubic metres would give per 24 hours rather more than 5 tons of ammonium or calcium nitrate. This is about one-eighteenth of the figure given by Muntz and Lainé whose results could not be maintained in continuous practice.

Boullanger gives a detailed financial estimate of the cost of nitrate production in this way and arrives at a price of 2,200 francs per ton of ammonium nitrate, the ordinary price per ton of ammonium nitrate at that date (1922) being 1,600 francs. Except as a war emergency, therefore, the process could not be considered economical.

Joshi (*Agric. J. of India*) has made a number of interesting experiments at Pusa on the use of an intensive nitrifying bed as a means of preventing nitrogen losses from cattle urine. Joshi's results are somewhat better than Boullanger's, probably owing to the nitrogen in the urine being in a form more readily attacked by micro-organisms than the simple ammoniacal solution used by Boullanger. He was

able to oxidise a solution containing 1.5 gms. ammoniacal nitrogen per litre at the rate of 100 litres daily per cubic metre of medium, or 0.15 gm. ammoniacal nitrogen daily per litre of space, which is 33 per cent. higher than the figure given by Boullanger. In the hot weather Joshi reached the figure of 0.2 gm. ammoniacal nitrogen oxidised per litre of space.

The experiments which are described in the following pages were undertaken with the object of determining how far intensive nitrification could be carried, using the principle of the activated sludge process, i.e., by moving the organisms through the solution to be nitrified instead of passing the solution over a fixed surface on which the organisms were developed. The experiments were started in 1920 before the publication of the results of Boullanger or of Joshi and although a number of interesting results were obtained in the early stages it was not until a careful study of Boullanger's paper had shown some of the necessary conditions for success that results of technical importance were reached.

Under the best conditions it has been possible slightly to exceed the highest rate of nitrification reached by Joshi. He was able to oxidise 0.2 gm. of ammoniacal nitrogen in one day per one litre space, while in his last experiment Kotwal attained a rate of 0.25 gm. ammoniacal nitrogen per litre per day.

The subsequent experiments of Norris and Ranganathan, however, seem to indicate that this high rate is not maintained as the concentration of nitrate in the solution increases.

Both Roy and Ranganathan noticed the beneficial action of traces of iron salts, an observation of interest in view of recent work by Warburg and others on the mechanism of biological oxidations. Ranganathan's results with animal charcoal lend further support to these views.

The outstanding result of all the experimental work is to show the necessity in the first place for very carefully cultivating organisms of special activity, and also for the maintenance of certain limiting conditions of concentration if the most satisfactory results are to be obtained. This is of great importance in connection with the practical technique and economical working of activated sludge plants employed for the purification of sewage, especially where highly concentrated liquors have to be used. The possibility is also indicated of employing activated sludge tanks for producing nitrates from various nitrogenous raw materials as a preliminary to their application to the purposes of agriculture.

PART II.—THE EFFECT OF VARIOUS SUBSTANCES ON THE RATE OF NITRIFICATION.

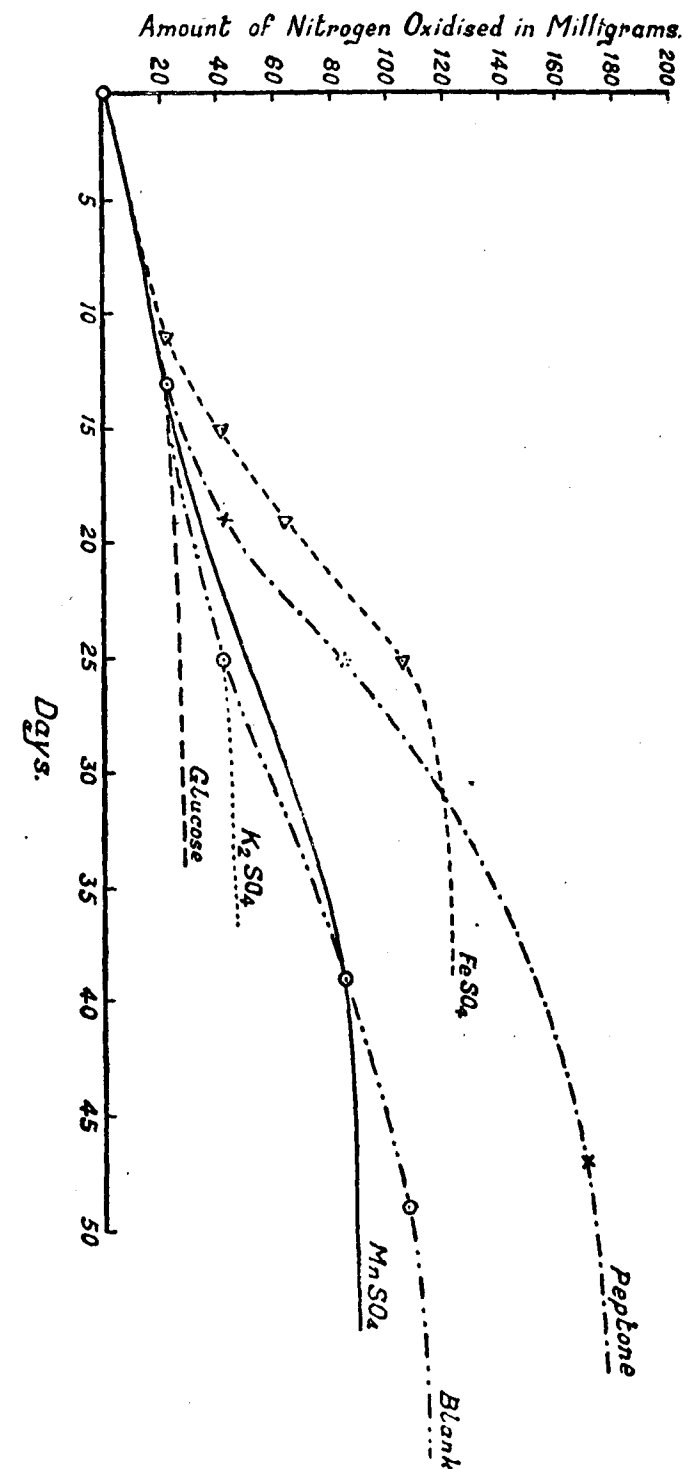
By M. B. Roy.

When nitrification takes place under natural conditions in the soil or during the various processes of sewage purification, numerous mineral salts and other substances may be present which are liable to affect the activity of the nitrifying organisms. The experiments described in this section were undertaken in order to obtain some quantitative knowledge of the effect of such substances on the nitrification process.

For this purpose 0.25 per cent. solutions were made of the following substances:—Manganous, ferrous, calcium, magnesium and potassium sulphates; chlorides of calcium and sodium; calcium superphosphate, basic slag, sodium phosphate, glucose, peptone and caramel.

Five c.c. of each of these solutions was added to 100 c.c. of a suspension of activated sludge in water (20 per cent. by volume of sludge). The mixtures were contained in a series of test-tubes of about 100 c.c. capacity. To each of these mixtures was added one or two c.c. at a time of a 10 per cent. solution of ammonium sulphate, giving a concentration of ammoniacal nitrogen available for nitrification of from 20 to 40 parts per 100,000. Precipitated calcium carbonate was added as a base and the liquid tested for acidity from time to time. The test-tubes were connected in series and a current of air, freed from any traces of ammonia by passage through sulphuric acid, was passed through the series so that any variation in the air-supply affected each test-tube equally.

One c.c. from each test-tube was taken out at intervals and tested qualitatively for ammonia and nitrites, and in some cases for nitrates, a fresh dose of sulphate of ammonia solution being added as soon as the previous dose had been oxidised. The more important results are summarised in the attached graph. It is clear from the form of the curves that some time is required, viz., about 12 days, before nitrification becomes really active and there is a point beyond which, under the conditions of these experiments, no further oxidation takes place. Both peptone and iron salts considerably increase the activity of the nitrification process as compared with the blank. Manganese accelerates the process up to a point and afterwards exercises a



depressing effect. Potassium salts exercise a depressing effect, while glucose may be almost said to inhibit nitrification, owing, doubtless, to its affording favourable conditions for the activity of denitrifying organisms.

It is reasonable to conclude that the stimulating effect of peptone is due to its furnishing necessary food for the organisms and also the possibility of its decomposition products being capable of conversion into nitrates.

The action of iron salts is doubtless catalytic, and has been confirmed in subsequent experiments by Norris and Ranganathan.

In the case of the other substances it may be stated generally that they show little influence on the rate of nitrification as compared with the blank, and consequently experiments with them were not continued.

The experiments with calcium sulphate were extended to a concentration of 14 per cent. in the actual experimental tube without any appreciable effect on the rate of nitrification. This is of interest in connection with the use of ammonium sulphate as a fertiliser on limed soils.

Another interesting isolated result discovered is the possibility of oxidising dilute solutions of ammonium hydrate in presence of calcium carbonate. This indicates that the ammoniacal liquor of gas works, if sufficiently diluted, could be oxidised directly to nitrate in presence of calcium carbonate without the necessity for the intermediate formation of ammonium sulphate.

In view of the statement by Muntz and Lainé that the nitrifying organism will tolerate concentration of nitrate up to 20 per cent. this amount of calcium nitrate was added to the nitrifying solution, with the result, however, of immediately inhibiting the process. It is clear from the work of Boullanger, referred to in the introduction that such concentration of nitrate is higher than the organisms will tolerate, unless very carefully acclimatised.

PART III.—THE NITRIFICATION OF AMMONIA IN STRONG SOLUTIONS.

By Y. N. Kotwal.

As explained in the introduction the object of these experiments was to ascertain whether by an application of the activated sludge principle, i.e., the circulation of appropriate bacteria in presence of air through a solution of the substances to be nitrified, solutions of nitrate could be produced sufficiently concentrated to make the recovery of the dissolved nitrates by evaporation economically possible.

Muntz and Lainé having stated that nitrifying organisms would tolerate 20 per cent. of nitrate, a preliminary experiment was made by bubbling air through a 15 per cent. solution of calcium nitrate containing an efficient activated sludge in suspension, and adding gradually a 4.5 per cent. solution of ammonium hydrate. No evidence of nitrification either by loss of ammonia or by development of nitrite was obtained. This result confirmed the observations of Roy (Part II), but is a little difficult to understand in view of the fact that under natural conditions nitrification appears to take place in slightly moist soils with the production of actual crystals of nitrate. Some condition present in nature evidently remains to be investigated.

Shortly after this failure the papers of Boullanger came to hand and were studied, and on proceeding further on the lines therein described, positive results were obtained. The general method of experiment was as follows:—500 c.c. of activated sludge in good 'condition' was mixed with 1500 c.c. of effluent in a tall narrow graduated cylinder of 2000 c.c. capacity. Air was drawn through by means of a filter pump and the solution to be nitrified slowly added from a tap funnel.

The effluent from the activated sludge installation originally contained ammoniacal and nitrous nitrogen but only traces of nitric nitrogen. In order to eliminate as far as possible all but the bacteria of nitrification, aeration was continued for 5 days, testing every day for ammonia and nitrites. At the end of 4 days nitric nitrogen only was present in solution to the extent of 2.6 parts per 100,000, equivalent to 0.015 per cent. calcium nitrate.

At this date, August 20th, a solution of ammonium sulphate was prepared containing 2.5 gms. per litre equivalent to 53 parts ammoniacal nitrogen per 100,000 which would give 318 parts per 100,000 of

calcium nitrate on oxidation. To this solution precipitated calcium carbonate was added in the proportion of 5 gms. to each 2.5 gms. of ammonium sulphate. Before use this mixture was thoroughly shaken, and 120 c.c. placed in a dropping funnel. The suspended calcium carbonate was allowed to settle in the funnel and dropped into the aeration cylinder all at once, the remaining clear solution being slowly but continuously added during 3 to 5 hours, aeration continuing without intermission both day and night. The added ammoniacal nitrogen dissolved in 120 c.c. solution amounted to 0.06 gm., equivalent to 0.378 gm. calcium nitrate. Fresh additions were not made until ammoniacal nitrogen had completely disappeared. During the first days this generally occupied about 48 hours so that fresh additions were made on alternate days only. The procedure was to stop aeration for 1 hour daily in the morning between 9 and 10 a.m. to allow complete settlement. The supernatant liquor was tested for ammoniacal nitrogen by Nessler solution and for nitrite nitrogen by the Griess-Ilosvay reagent; in the event of complete oxidation, a volume of supernatant liquor was removed equal to the volume of ammonium sulphate solution to be added. Nitrous nitrogen was still present at the end of 48 hours, but this was disregarded at first. By the 31st September both the ammoniacal and nitrous nitrogen present in the 120 c.c. of solution added were completely nitrified.

From September 1st to 15th both days inclusive the daily volume of ammonium sulphate added was gradually increased until at the end of this period it amounted to 400 c.c. per day. No increment was made until the rate of nitrification had maintained itself unimpaired for 3 or 4 days, an additional 50 c.c. of solution being then added. Under these conditions the oxidation proceeded very smoothly. It was noticed however, as a general observation that if the increment exceeded the established limit of oxidation at the concentration reached, nitrites as well as ammonia were found in solution in quantity. It was further observed that even if the normal daily dose was added irregularly the oxidation was not completed within 24 hours. Thus if 400 c.c. were normally oxidised during 3 to 5 hours the process was checked if 100 c.c. was added all at once.

From 16th to 27th September both days inclusive the solution of ammonium sulphate added was doubled in strength, an equivalent increase in the calcium carbonate being made simultaneously. The solution thus contained 100 parts of ammoniacal nitrogen per 100,000. This strong solution was added gradually, 100 c.c. only being used on the first day and the additions gradually increased to 250 c.c. per day by the last two days of the period. At this stage 265 mgms. of ammoniacal nitrogen were being oxidised in a volume of two litres per 24 hours.

On the 28th September a further increase of concentration was made in the solution, viz., from 5 gms. to 7.5 gms. of ammonium sulphate per litre with an accompanying 11.5 gms. calcium carbonate. This solution contained 157 parts ammoniacal nitrogen per 100,000. By the 8th October 150 c.c. of this, i.e., 237 gms. of ammoniacal nitrogen were being oxidised per day.

The important observation was made that these higher concentrations of ammonium sulphate could be oxidised quite smoothly provided that when a change was made from a weaker to a stronger solution, the actual quantity of nitrogen added was slightly lower than the quantity which was previously being oxidised, the full additional quantity not being added till 2 or 3 days had elapsed, otherwise, although oxidation did not actually cease, the amount of nitrogen oxidised in 24 hours diminished, nitrites being sometimes present for 48 hours. At this stage it was decided to add ammoniacal nitrogen in the form of ammonium nitrate rather than in the form of ammonium sulphate.

The advantage of this procedure, as pointed out by Boullanger, is to eliminate the formation of calcium sulphate which, in his case, tended to block up the filters and, in the present case, to deposit as heavy mud which was liable to impede aeration. Moreover by using ammonium nitrate the rate of concentration of nitrogen in the solution was hastened and the limit, which it was desired to determine, more quickly reached. A solution of ammonium nitrate was prepared containing 8.266 gms. per litre or 150 parts of ammoniacal nitrogen and 150 parts of nitric nitrogen per 100,000; 10.33 gms. calcium carbonate were added to a litre of this solution.

The addition of ammonium nitrate to the oxidising medium was begun on the 9th October at which time 237 mgms. of ammoniacal nitrogen were being oxidised in 2 litres of medium. The ammonium nitrate solution was added cautiously and it was not until the 11th day, i.e., 21st October that 100 c.c. of the solution was oxidised in 24 hours. Thus although at the beginning of this period 237 mgms. of ammoniacal nitrogen as ammonium sulphate were being oxidised in 24 hours, only 150 mgms. of ammoniacal nitrogen in the form of ammonium nitrate could be oxidised in 24 hours after 10 days acclimatisation. After 10 days further work an increase of 25 per cent. on this figure was possible, but it was not until the 15th December that 200 c.c. of ammonium nitrate, i.e., 300 mgms. of ammoniacal nitrogen together with 300 mgms. of nitric nitrogen were being easily oxidised per 24 hours.

Up till then as much liquid had been withdrawn from the cylinder as had been added. In order to obtain a real increase in the concentration of the calcium nitrate it was clearly necessary that the ammonium nitrate should be actually dissolved in a portion of the supernatant liquid from the cylinder and this solution returned to the vessel. This was accordingly done, 2.86 gms. ammonium nitrate or 500 mgms. ammoniacal nitrogen being dissolved in 100 c. c. of the supernatant liquor which was then slowly returned to the cylinder through the tap funnel. The equivalent amount of calcium carbonate was added to the cylinder separately.

The additional quantity of ammoniacal nitrogen could not, however, be at once oxidised and it took nine days for every trace of ammonium nitrate to disappear. The rate of oxidation gradually increased till January 14 when, through an unfortunate accident owing to a loss of water pressure during the night, aeration practically ceased, resulting in the production of a large quantity of ammonia and of nitrite. This led to a loss in activity from which the solution did not recover, and it appeared that it would be necessary to rebuild a special activated sludge. It had, however, been demonstrated that 300 mgms. of nitrogen could be readily oxidised daily in a volume of 2 litres, and a rate of 500 mgms. of ammoniacal nitrogen oxidised per 2 litres had just been attained when the unfortunate set-back occurred. The concentration of calcium nitrate at that date was 2.5 per cent.

Shortly after these difficulties were encountered the writer left to take up an appointment and further study of the subject passed to other investigators whose results are recorded in Part IV. The results described in the present paper are summarised in the tabular statement attached. It should be noted that no appreciable loss of ammonia from the solution could be found when the air drawn through the cylinders was passed through decinormal sulphuric acid and the acid tested for the presence of ammonia at the end of a month.

TABULAR STATEMENT OF RESULTS OBTAINED, AUGUST—JANUARY.

Date.	1923.										1924.	
	Aug. 8	Aug. 27.	Sept. 1.	Sept. 7.	Sept. 11.	Sept. 22.	Sept. 26.	Oct. 1.	Oct. 16.	Oct. 23.	Nov. 1.	Jan. 14.
Nature of Am. salt	...	...	...	...	...	...	...	...	...	...	...	...
Percentage of Am. salt	0.25	0.25	0.25	0.25	0.25	0.5	0.5	0.75	0.826	0.826	0.826	0.826
Volume in c.c. added	120	120	120	200	300	250	250	150	100	100	125	...
Am. Nitrogen added in mgms.	63	63	63	106	159	265	265	240	151	151	189	...
Percentage of Calcium nitrate.	0.015	0.06	0.12	0.24	0.30	0.35	0.62	0.72	1.17	1.8	1.95	2.5

Note.—From December 22 to January 14 the percentage of calcium nitrate in solution gradually rose to 2.5 as no liquor was taken out but the solid ammonium nitrate was dissolved in a portion of the supernatant liquor which was slowly returned to the main volume.

PART. IV.—FACTORS INFLUENCING THE RATE OF OXIDATION.

By S. Ranganathan and Roland V. Norris.

In the foregoing Part Kotwal has described certain experiments on intensive nitrification as carried out in suspensions of 'activated sludge.' These experiments had as their object (1) to study the rate of nitrification producible in such circumstances and (2) to ascertain the concentration of nitrate attainable by these methods. The work was interrupted before the second of these factors could be investigated and this aspect of the case has, therefore, been taken up by the present authors, and extended to a study of certain other factors bearing on the process of nitrification.

The method employed by Kotwal consisted essentially in aerating suspensions of activated sludge in large cylinders, ammonium salts being added from time to time in suitable proportions.

In the beginning of the present investigation the same procedure was followed and the work begun with two nitrifying solutions A and B handed over by Kotwal and which already contained nitrate equivalent to 2.17 and 1.08 per cent. respectively of calcium nitrate.

The first object being to ascertain the concentration of nitrate attainable, ammonium nitrate was used as the source of ammonia instead of ammonium sulphate. By this means, assuring equal rates of nitrification, the accumulation of nitric nitrogen would be twice as rapid as when ammonium sulphate was the source of ammonia.

The ammonium nitrate was dissolved in water, a suitable amount of calcium carbonate (usually equivalent to the amount of acid to be produced) added and the suspension mixed with 50 c.c. of supernatant liquid taken from the aeration cylinder. The mixed solution thus prepared was then slowly added through a dropping funnel to the aeration vessel, the liquor in the latter being aerated for not less than twelve hours a day by means of compressed air. Further additions of ammoniacal solution were made only when the previous amount added had been fully oxidised. Moreover the volume added at any one time was kept constant until evidence was forthcoming that nitrification was taking place with increasing rapidity when the dose was augmented. In practice it was found that as a rule not less than three days should elapse before such an increase was made. The

concentration of the ammoniacal solution was also increased from time to time. The solution initially used contained 17.14 gms. ammonium nitrate per litre equivalent to 0.3 gm. of ammoniacal nitrogen per 100 c.c.

Periodical determinations of the nitrate-content of the reaction mixtures were made by means of the modified Devarda method (*J. Ind. Eng. Chem.*, 1920, **12**, 352). A correction was made for loss of ammonia due to the aeration, such ammonia, if any, being caught in suitable traps.

When the experiment was begun on March 27th the two aeration mixtures A and B were nitrifying respectively 150 mgs. and 90 mgs. of ammoniacal nitrogen in 24 hours. The rate in A containing the higher nitrate-content, however, soon began to fall off while that in B remained unimpaired with the result that after one month the amount of nitrogen oxidised was only 1.65 gms. as against 2.66 gms. in B. The concentration of nitrate was at the end of the period equivalent to 3.02 per cent. calcium nitrate in A and 1.98 in B. The concentration of the ammoniacal solution was increased at this stage to 28.57 gms. ammonium nitrate per litre or 0.5 gm. ammoniacal nitrogen per 100 c.c. Nitrification proceeded normally for another two weeks when the rate began to slacken in both cylinders. The decrease became steadily more marked, and in June the two solutions could oxidise only 25–30 mgs. ammoniacal nitrogen per day. At the beginning of July the concentration of calcium nitrate was 3.40 per cent. and 2.70 in A and B respectively, while the two solutions which previously had been able to nitrify 50 c.c. of the ammoniacal solution in 24 hours could only with difficulty oxidise 10 c.c. of the same solution in this space of time; after a further two months only 5 c.c. were oxidised daily. The concentration of nitrate was then 4.79 per cent. in A and 4.55 in B.

Shortly before this stage trouble was caused by the development of green algae in the nitrifying vessels. Attempts were made to check this in the first place by adding copper sulphate (1 in 50,000). Control experiments, however, indicated that even in this high dilution nitrification was checked and this method was not further used. It was ultimately found that the simple device of excluding light by covering the cylinders with black paper was quite efficacious in preventing such growths.

Apart from this difficulty, however, nitrification continued only for a few weeks more, and in the middle of December the process came to a standstill when the nitrate concentration in the two flasks was 5.34 per cent. and 5.56 respectively. This concentration seems in fact to be the highest that can be attained under these conditions of working, using activated sludge alone without adding other salts.

Attention has already been drawn to the fact that the rate of nitrification steadily decreased as the concentration of nitrate in the reaction mixture increased, solution A, e.g., nitrifying 150 mgs. ammoniacal nitrogen in 24 hours when the nitrate concentration was 2.17 per cent. and only 5 mgs. when there was 5.26 per cent. calcium nitrate present. The phenomenon is clearly seen in the following table:—

TABLE I.

Influence of Nitrate-concentration on Rate of Nitrification.

Period of observation	Experiment A		Experiment B	
	Average speed of nitrification	Percentage calcium nitrate	Average speed of nitrification	Percentage calcium nitrate
March ...	150	2.17	90	1.06
April ...	45	2.69	77.7	1.61
May ...	30	3.08	44.3	2.46
June ...	25	3.40	28	2.73
July ...	24	4.08	25	3.19
August ...	17	4.46	25	3.66
September ...	21.8	4.61	24	4.37
October ...	16.8	5.08	24	4.93
November ...	8.3	5.34	24	5.4

It is noteworthy that the fall in activity is much more marked in A, which initially was nitrifying the more vigorously of the two.

Influence of Salts on Nitrification.

Though nitrification had for all practical purposes ceased in the above experiments this was not due to the death of the nitrifying organisms, but was apparently caused either by a paralysing effect of the high concentration of nitrate, or by the lack of some essential nutrient material in the mixture.

Other experiments having indicated the beneficial action of iron salts and phosphates in moderate concentration, an experiment was carried out to ascertain whether nitrification could be again set up in one of the mixtures used above by adding such salts.

Two one-litre aeration cylinders were therefore prepared and equal quantities of the solution from the previous experiments (i.e., containing already 5.5 per cent. calcium nitrate) placed in each. To them was added in small quantities at a time one of the following solutions:—

- (1) 1,000 c. c. water containing 28.57 gms. ammonium nitrate, 37.71 gms. calcium carbonate and 1 per cent. ferrous sulphate.
- (2) As above, but containing 1 per cent. potassium phosphate instead of ferrous sulphate.

In both cases nitrification was resumed with fair vigour, but the rate again fell after about two weeks and then continued very slowly for the next three months, about 2 c. c. of the solution being oxidised per 24 hours. The final concentrations of nitrate obtained were 7.55 and 7.44 per cent. respectively and in no experiment have we been able to exceed this concentration.

It has been suggested that ammonium nitrate is less favourable for nitrification than ammonium sulphate, the nitrate ion exercising a paralysing action on the multiplication of the organisms. An experiment similar to that just described was therefore carried out using a nutrient solution containing ammonium sulphate. To obviate the disadvantages of using calcium carbonate as the neutralising agent, namely, deposition of calcium sulphate, a solution of ammonium carbonate containing ammoniacal nitrogen equal in amount to that in the ammonium sulphate solution was added between two additions of the sulphate solution. No improvement was, however, effected by this procedure and it was not found possible to obtain more than 7.5 per cent. of calcium nitrate.

It is of interest to note that in the above experiments it was never possible to obtain a quantitative oxidation of ammoniacal nitrogen to nitric nitrogen, the highest figure obtained being a 96 per cent. conversion.

Nutrition-requirements of the Nitrifying Organisms.

A mixed culture of the nitrite and nitrate-producing organisms was isolated by frequent sub-culturing of an actively nitrifying solution into Winogradsky's medium. After six sub-cultures the growth was inoculated on the surface of sterile gypsum blocks placed in the above medium and a mixed culture of nitromonas and nitrosomonas obtained.

This mixed culture was utilised to inoculate fresh suspensions of activated sludge in subsequent experiments in order that nitrification might be more rapidly induced.

Influence of added phosphates.—Two 2-litre cylinders each containing 500 c.c. of well aerated sludge (settlement in 15 minutes) and 1,500 c.c. tap-water were arranged for aeration as in the previous experiments. To the one was added in suitable quantities from time to time a solution containing in 1,000 c.c. of distilled water the indicated grams of the following substances:—Ammonium nitrate, 28.57, calcium carbonate, 37.71, sodium potassium tartrate, 5.00, potassium phosphate, 2.50, and magnesium sulphate, 1.00. To the second cylinder was added the same solution without the phosphate.

From the beginning of the experiment, the phosphate solution nitrified more than double the amount of ammoniacal nitrogen, as compared with the control. The experiment continued for about three months, the results being recorded in the following table:—

TABLE II.

Influence of Phosphate on Rate of Nitrification.

Period	Phosphate cylinder		Control	
	c. c. of amm. solution oxidised	Percentage of calcium nitrate	c. c. of amm. solution oxidised	Percentage calcium nitrate
1st month	150	...	75	...
2nd "	135	0.499	47	0.393
3rd "	118	0.813	54	0.512
Total	403	0.813	176	0.512

It is quite evident from the above figures that the phosphate exercised a very beneficial influence. This may be brought about by either (1) direct stimulation of the nitrification process, or (2) increase in the number and vigour of the nitrifying organisms. While the latter factor cannot be excluded there appears to be a good deal of evidence to suggest that the phosphate has a direct accelerating influence on the process of nitrification though the manner in which this is exercised is not clear. The point is being further examined.

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Influence of Other Salts.

The influence of various salts on the rate of nitrification of soils has been investigated with varying results by a number of different workers. Of such salts, calcium and manganous sulphates have seemed the most promising and an experiment was therefore carried out to ascertain whether similar results would be obtained in culture solutions by using these salts.

Four 1-litre aeration cylinders were therefore set up each containing 250 c. c. of well aerated sludge and 750 c. c. tap-water. To one cylinder, serving as a control, was added at intervals a suitable amount of nutrient ammonium nitrate solution while to the other three cylinders was added a similar solution containing 1 per cent. of calcium, ferrous or manganous sulphate. As before, additions of the ammoniacal solution were only made when the previous dose had been nitrified. All four solutions were aerated for the same period each day and the nitrate nitrogen estimated at frequent intervals.

During the first week the rate of nitrification in the control was slightly slower than in the other three cylinders. This, however, was only a temporary effect and after a further short period the solution containing ferrous sulphate was the only one which was ahead of the control, the other salts exhibiting a retarding influence. The concentration of ferrous sulphate required to produce the above acceleration was small and higher amounts ultimately checked the rate of nitrification. The critical strength appeared to be about 0.07 per cent. ferrous sulphate, higher concentrations being toxic. The above results are illustrated by the figures given in Table III, showing the nitrification brought about at two stages of the experiment.

TABLE III.

Salt added	Early stage			Later stage		
	Percentage salt in re-action mixture	Percentage calcium nitrate	Relative ratio	Percentage salt in re-action mixture	Percentage calcium nitrate	Relative ratio
Control	...	0.377	100	...	1.115	100
CaSO ₄	0.035	0.254	67.4	0.048	0.828	74.3
FeSO ₄	0.074	0.48	127.3	0.088	1.058	94.7
MnSO ₄	0.051	0.353	93.5	0.055	0.926	83

It is obvious that in a culture solution the organisms are highly sensitive to the action of such salts. In soil experiments much higher

concentrations have been successfully employed. In such cases, however, absorption of the salts undoubtedly takes place and the actual concentration of the salt in the soil solution at any particular moment would probably be quite low. Further experiments with lower concentrations of ferrous sulphate are therefore indicated.

Influence of Surface.

The rapid rate at which oxidation is brought about by activated sludge is due to two causes. In the first place the surface at which oxidation can take place is very large owing to the finely divided state of the sludge; and secondly the oxidisable material, probably absorbed on the sludge particles, is brought into immediate contact with relatively large quantities of oxygen by the aeration employed.

It was not therefore surprising to find that the rate of nitrification in an activated sludge suspension could be increased by adding substances such as silt or charcoal which increased the area of the oxidation surface. Preliminary experiments with silt, animal charcoal and ordinary charcoal indicated that animal charcoal assisted the process to the greatest extent. The influence of this substance and of the aeration is shown in the following experiment:—Six aeration cylinders were set up in the usual way, four containing 500 c.c. of sludge and 1,500 c.c. of distilled water and two 2,000 c.c. of distilled water. Animal charcoal in the proportion of 1 gm. charcoal per 100 c.c. of solution was added to four of the cylinders as shown in the schedule below. A nutrient solution of ammonium nitrate was added in the usual way for nitrification. Aeration was given to three only of the flasks as indicated in Table IV which summarises the results obtained.

TABLE IV.

Composition of mixture	Treatment	A		B	
		Percentage calcium nitrate	Relative efficiency	Percentage calcium nitrate	Relative efficiency
1. Sludge alone (control)	Aerated	0.523	100	0.815	100
2. Sludge alone	Not aerated	0.038	7.3	0.044	5.4
3. Sludge and animal charcoal	Aerated	0.818	156.4	1.112	136.5
4. Sludge and animal charcoal	Not aerated	0.038	7.28	0.044	5.4
5. Animal charcoal alone	Aerated	0.412	78.8	0.668	82
6. Animal charcoal alone	Not aerated	0.104	19.9	0.168	22.8

The importance of the aeration is strikingly demonstrated by the figures given above, as was anticipated, and the effect of adding animal charcoal is highly interesting. In the first place it is clear that animal charcoal alone was able to bring about the oxidation, for even

in the non-aerated flask there was a definite production of nitrate. It is of interest, moreover, to note that in the absence of aeration, animal charcoal alone gave better results than animal charcoal with sludge. This was doubtless due to the fact that the limited amount of oxygen available under such conditions was utilised by the organisms in the sludge and hence was not available for the oxidation of ammonia. It may be mentioned that in all the flasks which were not aerated nitrites were present. No trace of these was found where aeration was given.

These experiments bear out the results of Warburg and others in so far as they demonstrate the power of animal charcoal to bring about biological oxidations. Working with cystine, Warburg showed that animal charcoal alone could oxidise this substance to ammonia and sulphuric acid. He did not however obtain any further oxidation of the ammonia to nitrite or nitrate such as has been observed in the present case.

Optimum Reaction for Nitrification.

It is generally known that nitrification proceeds more rapidly in alkaline than in acid media. To order to ascertain the extent to which changes in reaction would influence nitrification under the experimental conditions employed in the present investigation the following experiment was carried out. A series of nine flasks was prepared, each containing 5 c.c. of a dilute solution of 2 per cent. ammonium carbonate. Each flask was inoculated with 1 c.c. of an actively nitrifying culture and the volume made up to 100 c.c. The pH of the mixtures was approximately 8.0. In eight of the flasks the reaction was now adjusted in order to obtain pH values of 7.6, 7.2, 6.8, 6.4, 6.0, 5.8, 5.6 and 5.4.

The flasks were frequently shaken, samples being removed every six hours and tested for ammonia and nitrites; the absence of both indicates complete nitrification. The results obtained are given in Table V.

TABLE V.

Flask No.	pH	Time required for complete nitrification
1	8.0	28 hrs.
2	7.6	18 "
3	7.2	18 "
4	6.8	48 "
5	6.4	60 "
6	6.0	72 "
7	5.8	120 "
8	5.6	Not complete in 10 days
9	5.4	

It is clear that nitrification proceeded well when the pH was between 8.0 and 6.0. In more acid reactions the rate diminishes rapidly and nitrification was completely checked when the acidity was greater than 5.8. The optimum reaction is between 7 and 8, that is to say just on the alkaline side of the neutral point.

Requirement of Calcium Carbonate for Efficient Nitrification.

It has been the contention of some investigators, notably Fischer (*Landw. Versuchs Stat.*, 1909, **70**, 335-342) that thrice or even more times the theoretical amount of calcium carbonate is necessary for efficient nitrification. To find out whether this was really the case, the following experiments were conducted. Ten 125 c.c. conical flasks each containing 25 c.c. of a dilute ammonium nitrate solution (8 grams in 500 c.c.) were inoculated with 1 c.c. each of an actively nitrifying culture. Flasks I and II received no calcium carbonate; II and VII received 0.5 gm. each, the amount theoretically necessary to neutralise the acid resulting from the nitrification of the 25 c.c. of the above dilute ammoniacal solution. To flasks III and VIII was added exactly twice the theoretical amount, namely, 1 gram; to IV and IX twice, and to V and X four times (i.e., 2.0 gms.) the theoretical amount of calcium carbonate. The volume in each flask was made up to 100 c.c. The flasks were then divided into two series, the first series being analysed after one week for ammoniacal and nitric nitrogen while the second series was examined after two weeks.

The figures obtained however indicated that no advantage at all resulted by increasing the amount of calcium carbonate above that theoretically required to neutralise the acid produced in the oxidation; on the other hand high amounts tended to bring about a loss of nitrogen and were therefore detrimental.

SUMMARY AND CONCLUSIONS.

1. The speed of nitrification decreased with increased concentration of nitrate.
2. With dilute solutions of ammonium salts and with calcium carbonate in quantity necessary to neutralise the resulting acid, but employing no nutrient salts, a concentration of 5.56 per cent. of calcium nitrate was obtained.
3. The maximum concentration of calcium nitrate obtained in this laboratory in culture solutions was 7.5 per cent.
4. Only about 96 per cent. of the nitrogen added was recovered as nitric nitrogen.

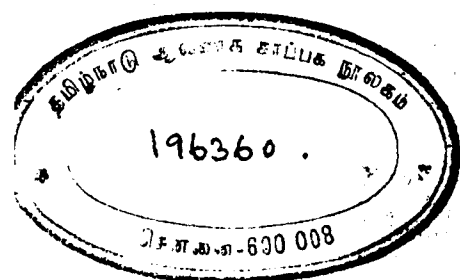
5. The influence of potassium phosphate, with ferrous, calcium and manganous sulphates has been studied ; as also the effect of surface and aeration on the process of nitrification.

6. The theoretical quantity of calcium carbonate required to neutralise the acid produced is quite sufficient for efficient nitrification.

7. The nitrifying organisms thrive in media of pH ranging from 5.8 to 8.0, but function best when the pH lies between 7.0 and 8.0.

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