I. HYDROXYLAMINE AS AN INTERMEDIATE IN NITRATE REDUCTION BY BACTERIA

II. NITROGEN TRANSFORMATION STUDIES IN MIXED SOIL CULTURES USING N^{15}

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I. HYDROXYLAMINE AS AN INTERMEDIATE IN NITRATE REDUCTION BY BACTERIA

LITERATURE REVIEW

Most earlier investigators accepted the hypothesis that ammonia was the central intermediate compound in the amino acid synthesis believing that nitrate as well as molecular nitrogen was reduced to ammonia. However, the validity of this hypothesis was doubted as early as 1884, when Meyer and Schulze (1) proposed hydroxylamine as the likely key intermediate. They suggested that this compound could arise from ammonia and molecular nitrogen by oxidation or from nitrate by reduction. Blom (2) and then Endres (3) showed the presence of a compound with an =NOH group linked to carbon to be normally present in cultures of Azotobacter using nitrate or molecular nitrogen. By hydrolyzing such compounds with sulfuric acid and then oxidizing them with iodine, nitrite was produced. Consequently, the intermediate must contain either the oxime group (=C=NOH), or the hydroxamic acid group (O=C-NOH). The findings of Virtanen and Laine (4,5) showed that the compound in the medium that was oxidized to nitrite arose either from hydroxylamine or oximino-nitrogen.

Virtanen and Csáky (6), examining the formation of the bound =NOH group in Torula yeast growing on nitrate, found that a considerable amount of oximino-nitrogen was formed within the first 10 minutes but disappeared rapidly thereafter. Because the amount of oximino-nitrogen decreased during the course of the experiment, the authors concluded that the cells utilized this form of nitrogen. They also pointed out that these findings do not give conclusive evidence of the significance of oximino-nitrogen nor explain its mode of formation, because oximino-

nitrogen is also produced from ammonium-nitrogen by certain aerobes. In both cases oximino-nitrogen might have been formed only after ammonia formation. If this is the course of the reaction, the oxime formation noted in nitrate reduction would not be adequate proof of the formation of hydroxylamine as an intermediate. Oximino-nitrogen formation from ammonium-nitrogen was noted by Lees (7). His experiments with washed suspensions of Nitrosomonas have shown that while hydroxylamine is toxic to nitrification in excess of a few micrograms of N per ml., yet at concentrations below about 1.5 micrograms of N per ml., the rate of formation of nitrite from hydroxylamine is at least as great or greater than its rate of formation from ammonia at the same concentration of nitrogen. It might be argued that the hydroxylamine was first dismuted to ammonia and nitrous oxide or nitrogen and actually the ammonia arising from the dismutation was nitrified, but the maximum amount of ammonia that could be formed in this way would contain half the nitrogen concentration of the original hydroxylamine and ammonia at this concentration was nitrified far more slowly. At a pH lower than 8.4, hydroxylamine was found to be nitrified considerably more rapidly than ammonia. From these results, it seems probable that hydroxylamine is an intermediate in the nitrification of ammonia, and therefore could presumably act as an intermediate in the reductive process.

Rautanen (8) obtained positive tests of the presence of oximino-nitrogen in green plants assimilating nitrate. This observation agrees with those of Burström (9) who stated that the reduction in green plants proceeds only as far as hydroxylamine. This then reacts with carbonyl compounds with subsequent amino acid and protein synthesis.

Virtanen and Järvinen (10) also examined the formation of oximino-nitrogen by Azotobacter vinelandii in nutrient solutions containing either ammonium or nitrate salts as the sole source of nitrogen, and in nitrogen-free solutions where active nitrogen fixation was occurring. The rate of formation of the bound hydroxylamine was found to be much more rapid when nitrate or molecular nitrogen served as the nitrogen source than when ammonium-nitrogen was the source, even though the assimilation of ammonium-nitrogen was as rapid as, or more rapid than the other nitrogen forms. They concluded that during nitrogen fixation and nitrate reduction, hydroxylamine was formed before the complete reduction of nitrogen to ammonia. The results also indicated that ammonium or amino-nitrogen was oxidized to some extent to yield hydroxylamine.

On the other hand, those opposing the hydroxylamine hypothesis have demonstrated the toxicity of hydroxylamine and oximes to microorganisms (11,12) and have presented evidence indicating that they are not available even in non-toxic concentrations (13,14). Burke and Horner (13) early demonstrated, by using a micro respiration method, that hydroxylamine in non-toxic concentrations of 1 to 3 micrograms per ml. was not metabolized by Azotobacter.

Novak and Wilson (15), in a series of carefully controlled trials, showed that the oximino-nitrogen of the oximes of pyruvic, oxalacetic and α-ketoglutaric acids was not available to Azotobacter vinelandii. Similarly, Segal and Wilson (14) have demonstrated that hydroxylamine, in extremely low non-toxic concentrations, was not utilized as a source of nitrogen by Azotobacter. Burris and Wilson (16) have concluded from their studies with aerated cultures of Azotobacter, that ammonia is the first stable intermediate formed in nitrogen fixation.

EXPERIMENTAL

Organisms— The organisms used in these studies were as follows: Pseudomonas fluorescens, strain from the stock of the Department of Agricultural Bacteriology, University of Wisconsin; Pseudomonas fluorescens, NRRL-B-6 of the Northern Regional Research Laboratory; Bacillus subtilis, strain from the Department of Bacteriology, Oklahoma Agricultural and Mechanical College; Pseudomonas denitrificans, isolate from soil from the Division of Plant Nutrition, University of California.

Culture Conditions— All of the organisms were trained to grow on simple media containing: Inorganic nitrogen salts as the source of nitrogen; glucose, potassium gluconate, or potassium citrate as the carbon source; Henderson's and Snell's salts C as a source of mineral salts; and biotin. The medium was buffered with monobasic potassium acid phosphate or monobasic potassium acid citrate, and adjusted to pH 6.8. Adaptation of the organisms to the different nitrogen and carbon sources was accomplished by long serial subculture on nutrient media in the presence of varying concentrations of the desired compound. Transfers were made every 24 or 48 hours depending on the rate of growth.

Culture Methods— Two types of culture methods were used in these studies, small still cultures and larger cultures with mechanical aeration.

The shallow-layer, still cultures were used to compare the toxicity and utilization of the nitrogen compounds studied. Nineteen ml. portions of nitrogen free media (normal medium containing all of the required constituents except a nitrogen source) were placed in 125 ml. erlenmeyer flasks, and the stoppered flasks were autoclaved. Each flask then received an aseptic addition of 1 ml. of a solution of the nitrogen

source which had been previously made neutral in pH and sterilized. Each flask was then inoculated with 0.2 ml. of a homogeneous cell suspension of a 24 or 48 hour subculture of the organism under study. Incubation was conducted at constant temperature (25° C. for Ps. fluorescens and 38° C. for the other organisms) until heavy growth had occurred on the control inorganic nitrogen salts. The cells were killed and separated from the medium as described below under sampling procedures. The washed cells were then analyzed for cellular nitrogen. The cell free filtrate and washings were made to 100 ml., and portions of this were used to determine the kinds and amounts of nitrogenous compounds remaining in the medium.

Studies with larger aerated cultures were performed using one liter of media in two liter filter flasks fitted with fritted glass dispersion tubes. A stream of compressed air was bubbled through water (in order to minimize evaporation of the medium), filtered through a cotton filter and passed into the medium through the fritted disc. The passage of air was so adjusted to agitate the medium and maintain a dispersion of minute air bubbles throughout. The medium was inoculated with 10 ml. of a 24 hour cell suspension of the organism, which had been previously subcultured on the same medium. Upon depletion of the original nitrogen form, as determined by testing an aliquot by the brucine sulfate method (17) for nitrate or by aeration and testing for ammonia by nesslerization, an initial sample was removed. The nitrogen compound under investigation was then added, and the culture was again sampled. Sampling was thereafter repeated periodically for 6 or 8 hours.

Sampling Procedures— Samples were obtained by withdrawing 25 ml.

of the culture and pipetting into 2 ml. of boiling 0.8% H_2SO_4. The samples were then brought to a boil, stoppered and stored under refrigeration until analyzed. The bacterial cells were removed from the sample media either by filtration or centrifugation. When the cells were light and did not clump readily, it was found that filtration of the sample gave more precise results. The cells were washed on the filter paper and the paper containing the cells was placed in a Kjeldahl flask. The contents was made basic with NaOH and boiled 5 minutes to remove occluded ammonia. The filtrate of the sample along with the washings was made up to 100 ml. It was then sterilized or frozen and reserved for analysis.

Analytical Procedures— Cellular nitrogen was determined by a semi-micro modification of the Kjeldahl procedure. Total medium nitrogen (to include nitrate-nitrogen) was determined by a semi-micro modification of the iron-sulfuric acid reduction procedure as developed by Pucher et al.(18). When oximino-nitrogen was present, this method recovered only about 90% of the nitrogen. Therefore, a modification was introduced that resulted in higher recovery of oximino-nitrogen. This procedure was as follows: About 25 ml. of the solution containing the oxime (1 to 5 mg. N) were placed in a semi-micro Kjeldahl flask. Eight ml. of concentrated H_2SO_4 containing 50 gm. of salicylic acid per liter was added, and this followed by the addition of 0.5 gm. of zinc dust. After completion of the reaction, the flask was heated on a digestion rack until no more white fumes escaped. After cooling, 4 ml. of H_2SO_4 and a small amount of selenium-copper sulfate catalyst were added and digestion continued. This procedure, while not ideal, was found serviceable.

Recovery experiments showed a range of 96 to 100 %, 94 to 98 %, and 92 to 97 % of the nitrogen of the oximes of α-ketoglutaric, oxalacetic and pyruvic, respectively.

Analysis for ammonia was made on suitable aliquots of the supernatant by the aeration method of Pucher et al.(19). The distillates from the aeration apparatus were diluted to known volumes, and ammonia determined by nesslerization.

Nitrate analysis was made on aliquots of the supernatant by a modification of the brucine sulfate method developed by Gad (20). This is a rapid and convenient method, which has been checked and found to give results which agree within 5 % of those obtained by the Devarda's alloy reduction of nitrate to ammonia.

Free hydroxylamine and the hydroxylamine liberated by acid hydrolysis of oximes was determined by Endres' modification (21) of Blom's method (22) as presented by Csáky (23). Hydroxylamine is oxidized to nitrite with iodine-acetate solution. With sulfanilic acid, the nitrite forms a diazo compound that is then coupled with α-naphthylamine to form a red dye that is estimated colorimetrically. Since hydroxylamine rapidly decomposes in aqueous solution, particularly if basic conditions exist, all samples and sample filtrates were made acidic (pH 4) and either frozen or refrigerated until analyzed.

Oximino-nitrogen of the cell free supernatant was determined by acid hydrolysis of the oxime by the method of Csáky (23). The oxime was boiled with 3 N H_2SO_4 containing some sulfanilic acid for 6 hours in a boiling water bath. Nitrous and hyponitrous acids are totally destroyed by this treatment, while hydroxylamine is stable against boiling with

H_2SO_4 if it is alone in solution, whereas it is partially destroyed in the presence of one of these oxidized forms. Oximino-nitrogen determined by this method thus tends to be low. Oxime determinations were performed as shortly after sampling as possible due to the instability of oximino compounds. Usually only an hour or two elapsed between sampling and analysis.

Preparation of Oximes— Three equivalents of the various keto acids were used for each equivalent of hydroxylamine in order to reduce the concentration of free hydroxylamine in the oxime preparations. The acid was dissolved in a minimal amount of 50 % alcohol and water and this solution was neutalized with NaOH. The weighed amount of hydroxylamine hydrochloride was added to the solution, and a slight excess of NaOH was added. The resulting solution was refluxed 10 minutes in a boiling water bath. The refluxed solution was made acidic, quantitatively transferred to a volumetric flask and made up to volume. The flask was then set in a refrigerator for a day or two until analysis for free hydroxylamine showed a non-toxic concentration.

Attempts to Demonstrate the Presence of Hydroxylamine and Oximes— Investigations by Dishburger and MacVicar (24) on the assimilation of ammonium and nitrate nitrogen by Ps. fluorescens and Ps. denitrificans suggested the presence in the medium of a form of nitrogen, which appeared to be neither nitrate, nitrite nor ammonia and which was utilizable by the cells for synthesis of cellular constituents. A high concentration of isotopic nitrogen was found to remain in the medium at the termination of the sampling period in a culture of one of these organisms supplied with labelled nitrate. Hall and MacVicar (25) observed that in aerated cultures of B. subtilis to which $KN^{15}O_3$ and $(NH_4)_2SO_4$ were added simultaneously, the nitrate was reduced extremely rapidly to a soluble form of nitrogen with a high N^{15} content that was then utilized for cellular synthesis. All analytical methods indicated that this compound with the exceptionally high concentration of N^{15} was ammonia. Inspection of the data suggest that nitrate was first converted to some other soluble nitrogen compound which was then further reduced to ammonia. Characterization of this unidentified form of nitrogen would possibly assist in the elucidation of the pathway of nitrate reduction.

The theory that hydroxylamine is an intermediate in nitrate reduction is supported by the detection of oximino-nitrogen in the cultures of certain aerobes utilizing nitrate-nitrogen. In previous studies with Ps. fluorescens and B. subtilis, it was impossible to detect the presence of any free hydroxylamine or oximino-nitrogen in the culture media, despite the sensitivity of the method (0.05 ug. N per ml.). These experiments, however, had not been designed to test the possible role of hydroxylamine, and the results were, therefore, subject to some question.

Accordingly, experiments were so designed to determine whether it was possible to detect hydroxylamine or oximino compounds under conditions designed to permit their identification. Oximino-nitrogen has been detected in the culture medium of Azotobacter on nitrate and ammonia nutrition, but its quantitative estimation was said to be indefinite because the free carbohydrate present in the medium decomposed giving a color which interfered with the estimation of the red dye (10). In these studies, this difficulty was avoided by adapting the organisms to citrate as the source of carbon. No interfering colors developed upon treatment with acid.

One liter of a medium containing 1 gm. of $NaNO_3$, 31.2 gm. of potassium citrate, and the required salts was prepared and sterilized. The medium was inoculated with 10 ml. of a 48 hour subculture of B. subtilis, and incubated at 38° C. with vigorous aeration. Upon depletion of the nitrate-nitrogen, 2.5 mg. NO_3-N and 2.5 mg. NH_4-N per ml. were added. A sample was immediately withdrawn; followed by sampling at regular intervals up to 8 hours. No free or bound hydroxylamine could be detected in any of the sample filtrates through 8 hours of growth, even though the samples were analyzed within 1 hour after sampling and so treated that very little decomposition could have occurred during this time. The same experiment was repeated, with the only differences being that the medium was inoculated with a 24 hour culture of Ps. fluorescens and incubation was at 25°. Likewise, no hydroxylamine could be detected in any of the samples through 8 hours of reduction of nitrate.

A liter of media was prepared and inoculated with B. subtilis as described in the preceeding experiment. After depletion of the original

nitrate, 2.5 mg. NO_3-N and 2.5 mg. NH_4-N per 100 ml. were added. The cells were harvested from the medium by filtration after 90 minutes of growth. The greatest reduction of nitrate occurs between the first and second hour. The filtrate was made 3 N with respect to H_2SO_4. A 600 ml. portion of the filtrate was reduced to 60 ml. by vacuum distillation. Any hydroxylamine in the medium would be stable under these conditions, and any oxime present would be hydrolyzed to yield hydroxylamine. High concentration of the medium caused slight coloration, which interfered somewhat with the estimation of hydroxylamine. When hydroxylamine determination was performed on 5 ml. of the concentrated medium, a faint red color developed, which corresponded to about 0.01 µg. N per ml. as compared to a standard. Since the medium was concentrated about 10 times, this represents about one millimicrogram of N per ml. of medium derived from either free or bound hydroxylamine.

Failure to detect hydroxylamine in anything but the minutest traces doesnot preclude its functioning as an intermediate in the reduction path from nitrate to ammonia. All the evidence in these studies, however, fail to provide any support for the concept that it, rather than ammonia, is the key compound in the reduction of nitrate. It does not seem probable that hydroxylamine is the intermediate of unknown character observed to accumulate in the reduction of nitrate to ammonia by B. subtilis. There was indication that some other compound in the medium was converted to ammonia, but no detectable accumulation of hydroxylamine could be found.

Attempts to Demonstrate Utilization of Hydroxylamine and Oximes--Those opposing the hypothesis that hydroxylamine is an important intermediate in nitrogen fixation and reduction have drawn support from evidence based on demonstrations that hydroxylamine is either toxic to or is unavailable to micro-organisms. Such toxicity studies and studies of the utilization of hydroxylamine have been confined primarily to strains of Azotobacter and Nitrosomas. It was therefore deemed of interest to determine whether or not utilization of hydroxylamine could be demonstrated using the organisms that had been used in these laboratories in previous studies on the mechanism of nitrate reduction, namely Ps. denitrificans, Ps. fluorescens, and B. subtilis.

Three series of flasks, each containing 19 ml. of a nitrogen free medium, were used for each organism. One ml. of solution containing 10 to 200 mg. NH_4-N was added to each flask in the first series; each flask in the second series received 0.5 ml. of the same solutions plus 0.5 ml. of hydroxylamine solution of equal nitrogen concentration. The only nitrogen in the third series was that supplied by hydroxylamine from the 1 ml. addition to each flask in concentrations of 10 to 200 mg. N. The inoculated flasks were incubated at 38° and the time of appearance of growth in the clear medium was noted. In the media containing only hydroxylamine, no growth appeared before 48 hours.

The toxicity of free hydroxylamine to the different organisms was determined by comparison of the rate of growth in the series containing ammonia with the series containing both ammonium and hydroxylamine nitrogen. After 24 hours of incubation, the cells in both series were killed. They were removed from the medium and total cellular nitrogen

was determined on them. At a concentration of 0.5 µg. N. per ml., hydroxylamine slightly retarded the growth of Ps. fluorescens and B. subtilis, and complete inhibition occurred in the presence of about 0.8 ug. NH_2OH-N per ml. Ps. denitrificans was found to be more sensitive with a concentration of 0.5 µg. NH_2OH-N per ml. producing more than 50% inhibition.

Growth appeared in the media containing only hydroxylamine nitrogen in levels up to 1 and 2 µg. N per ml. between 24 and 48 hours. Also some growth in all of the flasks containing higher concentrations of hydroxylamine had occurred by 48 or 72 hours. Analysis of uninoculated flasks of the same hydroxylamine concentration showed that by the time growth was appearing the concentration of hydroxylamine had been reduced to less than 1 µg. N per ml. due to its instability in the media under these conditions (pH 6.8 to 7.0). This decomposition yields ammonia which the cells then utilize for growth.

Since the non-toxic level of hydroxylamine is so low and it is so unstable in culture media, actual utilization of this form of nitrogen by these organisms could mot be conclusively demonstrated by our present analytical techniques. Therefore, the demonstration of the utilization or the non-utilization of hydroxylamine bound in the form of oxime was attempted.

The studies of the toxicity and the utilization of oximino nitrogen were performed with the same three organisms on shallow media in still cultures using three α-keto oximes prepared from the acids of pyruvic, oxalacetic and α-ketoglutaric. An experiment with each organism consists of three series of flasks prepared as has been previously

described. Each flask received a 1 ml. addition of solution of the nitrogen source, which contained 0.20 to 4.00 mg. of nitrogen. The flasks were inoculated with 0.2 ml. of a homogeneous cell suspension of the organism and were then incubated at constant temperature for a fixed period. The cells were killed and total cellular nitrogen was determined. Total medium nitrogen and oximino nitrogen were determined on aliquots of the cell free filtrate.

The results are tabulated in Tables 1, 2, and 3. No growth appeared in any of the flasks that contained oximino nitrogen as the sole source of nitrogen. After 4 days of incubation, the medium was filtered and total nitrogen determined on the material remaining on the filter paper. In no case was the amount of cellular nitrogen more than 0.05 mg., which corresponds closely to the amount of cellular nitrogen in the inoculum. Recovery of the medium nitrogen amounted to at least 90% of the added oximino nitrogen in all cases.

That growth did not take place due to the unavailability of this form of nitrogen to the organisms, and not to the toxicity of the preparations can be seen by a comparison of the rate of growth on the inorganic nitrogen with growth on the toxicity controls (Tables 1, 2, 3,). When heavy growth appeared in the inorganic nitrogen series, the cells in that series and in the toxicity controls were killed and filtered from the medium. Total medium, oximino and cellular nitrogen were determined. The α-ketoglutaric oxime preparation was found the least toxic, being only slightly inhibitory at the highest concentration used (200 ug. N per ml.) to any of the three organisms. The oxalacetic was only slightly toxic at concentrations of 100 ug. N per ml. and did

TABLE 1. UTILIZATION OF OXIMINO NITROGEN BY *PS. FLUORESCENS*, NRRL-B-6

20 ml. still cultures incubated at 38° C.

	NH_4-N added mg.	Cell N found mg.	Oximino-N added mg.	Oximino-N recovered mg.	Oximino-N recovered %	Total Medium-N mg.	Medium-N recovered %	Free NH_2OH µg N/ml.
				INORGANIC-N CONTROL [1]				
	0.20	0.24	none					none
	1.00	0.89						
	2.00	1.46						
	4.00	1.59						
				TOXICITY CONTROLS [1]				
Pyruvic Oxime	1.00	0.55	1.00	0.93	93			0.08
	2.00	0.77	2.00	1.89	94			0.16
	4.00	0.05	4.00	3.69	92			0.32
Oxalacetic Oxime	1.00	0.81	1.00	0.93	93			0.10
	2.00	1.15	2.00	1.86	92			0.20
	4.00	0.62	4.00	3.70	92			0.40
Ketoglutaric Oxime	1.00	0.79	1.00	0.94	94			0.06
	2.00	1.31	2.00	1.82	91			0.12
	4.00	1.33	4.00	3.68	92			0.32
				OXIMINO-N UTILIZATION [2]				
Pyruvic Oxime	none	0.03	0.20	0.16	80	0.19	95	0.04
		0.04	1.00	0.91	91	0.95	95	0.08
		0.04	2.00	1.79	91	1.86	93	0.16
		0.05	4.00	3.68	92	3.72	93	0.32
Oxalacetic Oxime	none	0.05	0.20	0.18	90	0.18	90	0.05
		0.04	1.00	0.92	92	0.94	94	0.10
		0.03	2.00	1.84	92	1.87	94	0.20
Ketoglutaric Oxime	none	0.03	0.20	0.18	90	0.19	95	0.03
		0.04	1.00	0.94	94	0.96	96	0.06
		0.04	2.00	1.81	91	1.86	93	0.12
		0.02	4.00	3.66	91	3.73	93	0.24

[1] Cells killed following 48 hours incubation
[2] Cells killed following 96 hours incubation

TABLE 2. UTILIZATION OF OXIMINO-NITROGEN BY *B. SUBTILIS*
20 ml. still cultures incubated at 38° C.

	NH_4-N added mg.	Cell.N found mg.	Oximino-N added mg.	Oximino-N recovered mg.	Oximino-N recovered %	Total Medium-N mg.	Medium-N recovered %	Free NH_2OH µg N/ml.
				INORGANIC-N CONTROL [1]				
	0.20	0.23	none					none
	1.00	0.70						
	2.00	1.26						
	4.00	1.86						
				TOXICITY CONTROLS [1]				
Pyruvic Oxime	1.00	0.42	1.00	0.93	93			0.08
	2.00	0.31	2.00	1.83	92			0.16
	4.00	0.09	4.00	3.70	92			0.32
Oxalacetic	1.00	0.42	1.00	0.92	92			0.10
	2.00	0.85	2.00	1.84	92			0.20
	4.00	0.59	4.00	3.66	91			0.40
Ketoglutaric	1.00	0.67	1.00	0.93	93			0.06
	2.00	1.11	2.00	1.86	93			0.12
	4.00	0.90	4.00	3.64	91			0.24
				OXIMINO UTILIZATION[2]				
Pyruvic Oxime	none	0.04	0.20	0.18	90	0.19	95	0.04
		0.02	1.00	0.91	91	0.95	95	0.08
		0.04	2.00	1.80	90	1.88	94	0.16
		0.04	4.00	3.70	92	3.78	95	0.32
Oxalacetic	none	0.02	0.20	0.18	90	0.19	95	0.05
		0.05	1.00	0.92	92	0.97	97	0.10
		0.01	2.00	1.82	91	1.91	96	0.20
		0.04	4.00	3.65	91	3.73	92	0.40
Ketoglutaric	none	0.01	0.20	0.19	95	0.18	90	0.03
		0.04	1.00	0.93	93	0.96	96	0.06
		0.04	2.00	1.82	90	1.85	92	0.12
		0.02	4.00	3.60	90	3.68	92	0.24

[1] Cells killed following 51 hr. incubation
[2] Cells killed following 96 hr. incubation

TABLE 3. UTILIZATION OF OXIMINO NITROGEN BY PS. DENITRIFICANS

20 ml. still cultures incubated at 38° C.

	NH_4-N added mg.	Cell.N found mg.	Oximino-N added mg.	Oximino-N recovered mg.	Oximino-N recovered %	Total Medium-N mg.	Medium-N recovered %	Free NH_2OH-N µg.N/ml.
				INORGANIC-N CONTROL[1]				
	0.20	0.23	none					none
	1.00	0.92						
	2.00	1.56						
	4.00	1.62						
				TOXICITY CONTROLS[1]				
Pyruvic Oxime	1.00	0.77	1.00	0.90	90			0.08
	2.00	1.11	2.00	1.78	91			0.16
	4.00	0.21	4.00	3.67	92			0.32
Oxalacetic Oxime	1.00	0.87	1.00	0.92	92			0.10
	2.00	1.19	2.00	1.79	91			0.20
	4.00	1.02	4.00	3.69	92			0.30
Ketoglutaric Oxime	1.00	0.88	1.00	0.90	90			0.06
	2.00	1.23	2.00	1.84	93			0.12
	4.00	1.28	4.00	3.66	92			0.24
				OXIMINO-N UTILIZATION[2]				
Pyruvic Oxime	none	0.05	0.20	0.17	85	0.18	90	0.04
		0.04	1.00	0.90	90	0.92	92	0.08
		0.04	2.00	1.81	92	1.87	93	0.16
		0.02	4.00	3.65	91	3.84	95	0.32
Oxalacetic Oxime	none	0.04	0.20	0.18	90	0.19	91	0.05
		0.00	1.00	0.91	91	0.93	93	0.10
		0.04	2.00	1.83	92	1.88	94	0.20
		0.05	4.00	3.61	90	3.53	89	0.40
Ketoglutaric Oxime	none	0.02	0.20	0.19	95	0.18	90	0.03
		0.07	1.00	0.92	92	0.94	94	0.06
		0.04	2.00	1.76	89	1.86	93	0.12
		0.03	4.00	3.64	91	3.69	92	0.24

1 Cells killed following 50 hours incubation
2 Cells killed following 98 hours incubation

not totally inhibit growth until present in concentrations exceeding 200 ug. N per ml. The pyruvic oxime preparation was quite toxic to the organisms tested in excess of 50 µg. N. per ml. The highest concentration of free hydroxylamine in any of these cultures was 0.4 µg. N per ml. which is definitely below the toxic level to these organisms.

An attempt was made to see if B. subtilis or Ps. fluorescens, NRRL-B-6 could be adapted to the utilization of oximino nitrogen. Strains of both organisms previously adapted to nitrate or ammonia nutrition were further subcultured on media containing glucose and ammonia or nitrate nitrogen in the presence of 50 µg. of oximino nitrogen per ml. The subculturing was continued every 48 hours for more than two months, but when these organisms were used to inoculate media containing oximino nitrogen as the only source of nitrogen, no growth occurred.

Studies on the utilization of keto oximino nitrogen by large aerated cultures of actively growing cells of Ps. fluorescens, Ps. fluorescens NRRL-B-6, and B. subtilis were performed so that more conclusive nitrogen balance information could be obtained.

One liter of medium containing 16.5 mg. of NH_4-N per 100 ml., 20 gm. of glucose, and the required salts were prepared and sterilized. The medium was inoculated with 10 ml. of a 24 hour subculture of Ps. fluorescens NRRL-B-6, and incubated at 25° with rapid aeration. At the time of depletion of the original ammonia (31 hr.), the medium was sampled and 25 ml. of an α-ketoglutaric oxime solution was added to the culture to make a concentration of 5.0 mg. oximino N per 100 ml. Samples were withdrawn at the definite intervals to 8 hours after the

oxime addition. The cells were killed and separated by filtration. After washing and removal of occluded ammonia, cellular nitrogen was determined. The medium and cell washings were made up to 100 ml., and aliquots were used for the determination of total medium, oximino and hydroxylamine nitrogen. Table 4 shows that there was no increase in cellular nitrogen and hence no evidence of growth after the addition of the oximino nitrogen. Both cellular and total medium nitrogen remained constant throughout the 8 hour sampling period, and, as far as can be determined from the recovery of oximino nitrogen ranging from 90 to 93 %, the oxime concentration remained unchanged throughout. That the cells could still use ammonium or nitrate nitrogen for cellular synthesis was demonstrated by the addition of one of these forms of nitrogen at the end of the sampling period (when the oxime concentration was unchanged) with the subsequent detection of an increase in cellular nitrogen within one hour.

The results in Table 4 are typical of those obtained in other experiments with the above mentioned organisms when non-toxic levels of any of the three keto oximes were added to actively growing cells.

TABLE 4. UTILIZATION OF α-KETOGLUTARIC OXIME NITROGEN BY B. SUBTILIS

1 liter aerated culture incubated at 38° C.

Time minutes	Cellular-N mg./ 100 ml.	Total Medium-N mg./ 100 ml.	Oximino-N mg./ 100 ml.	Oximino-N % recovered	Hydroxylamine ug. N/ ml.
-2	13.61	2.57 (residual)	none		
0	13.57	7.38	4.61	92	0.12
15	13.62	7.11			
30	13.58	7.17	4.58	92	0.10
45	13.55	7.10			
60	13.60	7.19	4.67	93	0.10
80	13.49	7.20	4.62	92	0.09
100	13.53				
120	13.66	7.18	4.50	90	0.08
150	13.61	7.23			
180	13.55	7.12	4.63	92	
230	13.47				
270	13.53	7.09	4.47	90	0.09
300	13.61	7.19			
360	13.59	7.21	4.56	92	0.09
480	13.49	7.11	4.49	90	0.08
490 [1]					
510	13.78				
540	13.93		4.54	91	0.08

[1] Ammonium sulfate was added to medium to give it a concentration of 5 mg. NH_4-N per 100 ml.

[2] An increase of about 0.3 mg. of cellular-N per 100 ml. occurred, showing that the presence of the oxime was non-toxic to the utilization of NH_4-N

DICUSSION

The hydroxylamine hypothesis is supported by the direct evidence of the detection of hydroxylamine and oximes from nitrate reduction or nitrogen fixation by Azotobacter, Nitrosomonas, yeast cells and green plants. In the experiments presented here on the reduction of nitrate by Pseudomonas fluorescens and Bacillus subtilis, hydroxylamine or oxime could be detected only in extremely small amounts in the media. Those opposing the hydroxylamine hypothesis, have demonstrated that hydroxylamine in non-toxic concentraticns is not used by micro-organisms (13,14) and that oximino nitrogen is unavailable to Azotobacter (15). Any hydroxylamine, if present in the bacterial system, in such low, non-toxic concentrations would most probably combine with carbonyl compounds present to form oximino compounds. The carbonyl compounds present in the largest amounts usually are the α-keto acids formed from carbohydrate metabolism. The more important keto acids are pyruvic, oxalacetic and α-ketoglutaric. Oximino nitrogen of the α-keto oximes of these acids was demonstrated to be an unavailable form of nitrogen for growth of Ps. fluorescens, Ps. denitrificans, and B. subtilis. The possibility still remains that difficulties of permeability may account for this non-utilization, but this seems unlikely as the detection of oximes excreted into medium by many aerobes has been reported and the free keto acids have likewise been found to be produced by many bacteria. The choice of the NRRL strain of Ps. fluorescens was, in fact, largely because of its tendency to form large amounts of α-ketoglutaric acid (26).

This lack of utilization prevents the use of specific critical tests that previously had been used to establish ammonia as an intermediate

in nitrate reduction by these organisms. In some respects, the negative results presented here are more definitive than if they showed that either free or bound hydroxylamine was utilized, for if growth had occurred it would have been difficult to prove that it was from the nitrogen of hydroxylamine and not from its decomposition product--ammonia.

Until the utilization of hydroxylamine or oximino nitrogen is demonstrated so that critical tests can be applied or until the lack of utilization of oximino nitrogen can be proven to be due to permeability difficulties, the hydroxylamine hypothesis finds no support in the results of these experiments designed to test its role as an intermediate. This, coupled with the previous work from these laboratories, supports ammonia as the key intermediate in nitrate reduction.

SUMMARY

1. No hydroxylamine or other oximes could be detected in the media of cultures of Pseudomonas fluorescens or Bacillus subtilis actively reducing nitrate. Even concentration of the media only gave a faint, rather indefinite indication of the presence of hydroxylamine, corresponding to about one microgram of hydroxylamine nitrogen per liter of media.

2. Attempts to demonstrate the utilization of free hydroxylamine by Ps. fluorescens, Ps. denitrificans, and B. subtilis were inconclusive. Growth would not take place unless the concentration of free hydroxylamine was less than one microgram per milliliter. The toxicity of hydroxylamine for each organism on ammonia nutrition was determined and made use of in the studies with the oxime preparations. Combination of hydroxylamine with α-keto acids rendered the compound non-toxic at levels from 50 to 200 times the toxicity of free hydroxylamine .

3. Attempt to adapt Ps. fluorescens and B. subtilis to the utilization of the nitrogen of oximes of α-keto acids failed.

4. Experiments with both still cultures and aerated cultures of rapidly growing cells of the three organisms mentioned above showed that the oximino nitrogen of the α-keto oximes of pyruvic, oxalacetic, and α-keto-glutaric acids was unavailable to those orgamisms as a source of nitrogen.

II. NITROGEN TRANSFORMATION STUDIES IN MIXED SOIL CULTURES USING N^{15}

LITERATURE REVIEW

Oklahoma soils have been gradually depleted of their natural fertility in past years, and now if their utilization, with any degree of productivity, is to be continued, agronomic practices most conductive to the creation and maintenance of soil fertility must be employed. Increasing the fertility of these depleted soils has necessitated the use of increasing amounts of nitrogenous fertilizers, which has in turn, made the questions of the most efficient forms and methods of addition of fertilizer nitrogen of utmost importance. Nitrate, particularly as ammonium nitrate, is making up an increasing fraction of fertilizer used, both in terms of quantity and in dollars spent. Losses of nitrogen due to denitrification represent, therefore, an increasing loss. Another problem of importance in the wheat producing areas of Oklahoma and the Southwest is the best method of disposal of wheat straw. It is quite possible that during the decomposition of straw, some of the nitrogen is lost by conversion to gaseous nitrogen by soil microorganisms. More nitrogen is lost in the production of synthetic manure from straw to which nitrates are added as compared with the addition of ammonium salts. It has also been observed that nitrates formed in the soil disappear when leached into the subsoil.

These problems have not received extensive study because of lack of suitable methods whereby nitrogen transformations of this kind could be accurately measured. Such techniques are now available by use of the stable isotope of nitrogen, N^{15}.

The bacterial reduction of nitrates to nitrogen gas is a process

that has been known for nearly a century. That denitrification in soil could be brought about by many soil bacteria was established by early workers. Denitrification was found to take place when organic complexes were available as sources of energy (27, 28); in wet soils even when no additional carbon had been added (29); even during the cold seasons (30); and that it is favored by the addition of large quantities of manure (31) or other difficulty decomposable organic materials (32). Later workers found that soils lost nitrogen through denitrification only when considerable amounts of organic matter were added along with nitrate. Voorhees (33) reported that little denitrification takes place in normal soils. Waksman (34) reached a similar conclusion-- that denitrification is of no economical importance in well aerated, not too moist soils, in the presence of moderate amounts of organic matter or nitrate. This viewpoint seemed to have been generally accepted until quite recently, but certain evidence suggests that this may not be entirely true. Corbet and Wooldridge (35) reported that in soils there is a biological mechanism that is able to effect the loss of nitrogen in the presence of carbonaceous material together with nitrate or nitrite. Meiklejohn (36) described two species of Pseudomonas capable of denitrification in aerated cultures as well as under anaerobic conditions. Nitrate reduction by Pseudomonas aeruginosum and Achromobacter siccum was found by Korsakova (37) not to be affected by aeration or the partial pressure of oxygen, but was dependent upon the amount of organic matter present. Broadbent (38), after studying nitrogen losses in incubated sandy loam soils in an apparatus designed to vary the rate of aeration of the soil, likewise, concluded that low oxygen concentration in the

soil atmosphere is not a necessary condition for the denitrification process, and that denitrification is affected more by the quantities of nitrate and oxidizable carbon in the soil than by partial pressure of oxygen. In similar experiments using isotopic nitrogen, Broadbent and Stojanovic (39) have presented evidence that denitrification is inversely related to partial pressure of oxygen, but is of apprecible magnitude even under fully aerobic conditions. The maximum recovery of the added nitrate nitrogen under fully aerobic incubation conditions was 75 %. Their data suggests that denitrification is not restricted to soils receiving recent additions of organic material. Isotope data showed that a negligible amount of the added nitrate was reduced to ammonia, but ammonification of organic nitrogen compounds did occur, except when glucose was added as a source of energy material. Their findings emphasize the advantages of ammonia fertilization from the standpoint of its retention in the soil.

Jansson and Clark (40) have investigated the nitrogen changes occurring during the decomposition of oat straw and alfalfa hay in the presence of added organic nitrogen under different conditions of incubation. Their experiments demonstrated that in the presence of abundant nitrate, the denitrification process is self-inhibitory in acid substrates, because of the toxicity of the nitrous acid developed to the bacteria. It was found that mature oat straw constituted a less suitable substrate for denitrification than did alfalfa hay. Under standard conditions of incubation, and with the moisture content at 2/3 saturation, there was no denitrification in oat straw treated cultures. However, if the water concentration was increased to saturation, or a more finely ground plant

material was employed, or if sugar or peptone was added to the substrate, denitrification occurred. The oxygen supply was thus influenced by these modifications in incubation, and the authors suggest that the " biological oxygen demand" in such instances should not be overlooked.

Despite its great importance in the field of agriculture, the process of nitrification has received relatively little atttention and that primarily with pure cultures of nitrifying organisms. Practically no work has been done with mixed soil cultures. The early work in this area was comprised of attempts to isolate the responsible organisms. The fact that the presence of organic matter in the medium tended to inhibit the growth of nitrifying organisms led early workers to assume that organic matter per se was deleterious to the growth of these organisms. Later this assumption became the subject of much discussion but comparatively little experiment. The nature of the effect of organic matter on nitrifiers is still undecided.

An experiment was designed, therefore, to study the changes in the nitrogen fractions of three soils of widely differing types to which were added two forms of labelled nitrogen, nitrate and ammonia, each with and without added organic matter. A fifth culture contained plant residues, the nitrogen of which previously had been labelled with N^{15}.

EXPERIMENTAL

Soils— Three Oklahoma soils of widely differing type and structure were selected for comparison. They were: Stidham loamy fine sand; Renfro clay loam; and Vanoss fine sandy loam.

Treatments— Each of these soils, containing their original bacterial population, were divided into five 1000 gm. portions and given the following treatments: (1) $KN^{15}O_3$, 60 p.p.m. N; (2) as 1 plus 20000 p.p.m. cellulose; (3) $(N^{15}H_4)_2SO_4$, 60 p.p.m. N; (4) as 3 plus 20000 p.p.m. cellulose; and (5) plant residue labelled with N^{15}, 4153 p.p.m. supplying 60 p.p.m. N. The nitrogen was added in solution and the soils were thoroughly mixed. This addition of nitrogen as either nitrate or ammonia corresponded to an addition of 120 pounds of nitrogen per acre. The amount of cellulose added was approximately equivalent to the amount of wheat straw that would have to be added to yield this amount of nitrogen (60 p.p.m.).

Incubation and Sampling— After treatment, the soils were incubated for a period of seven months under conditions simulating those of normal aerobic soils— maintained at near optimum moisture levels, in secluded light, and at 20 to 25° C. The soil cultures were sampled weekly for 4 weeks and at essentially monthly intervals thereafter. All soil samples were rapidly dried by exposure to a current of air in an oven at 65° C.

Analytical Procedures— Total nitrogen determinations were made on 5 gm. of the air dried soil by the customary semi-micro Kjeldahl procedure.

Ammonia and nitrate nitrogen were determined by a semi-micro aeration method adapted from Pucher et al. (19). Ten grams of homogeneous

soil were placed in one tube of an aeration train. Twenty ml. of distilled water were added and the pH brought to 9 by the addition of 3 ml. of $NaOH-Na_3PO_4$ buffer. The alkaline mixture was then rapidly aerated into 10 ml. of N/56 H_2SO_4 for 4 hours. The liberated ammonia was determined by nesslerization. To the same mixture, now free of ammonia, was added 1 gm of Devarda's alloy. The mixture was rendered more strongly alkaline by adding 2 ml. of 40% NaOH, and the mixture first gently and then rapidly aerated to carry over the ammonia, produced by reduction, into an acid trap.

The Kjeldahl titration mixtures from the total nitrogen analyses and aliquots of the ammonium solutions from the determinations of ammonia and nitrate were prepared for conversion as suggested by Schoenheimer (41). The conversion apparatus was similar to that described by him. Analyses for N^{15} were made on a Westinghouse mass spectrometer under the supervision of Mr. Glenn Hallmark of the Department of Electrical Engineering, A. and M. College of Texas.

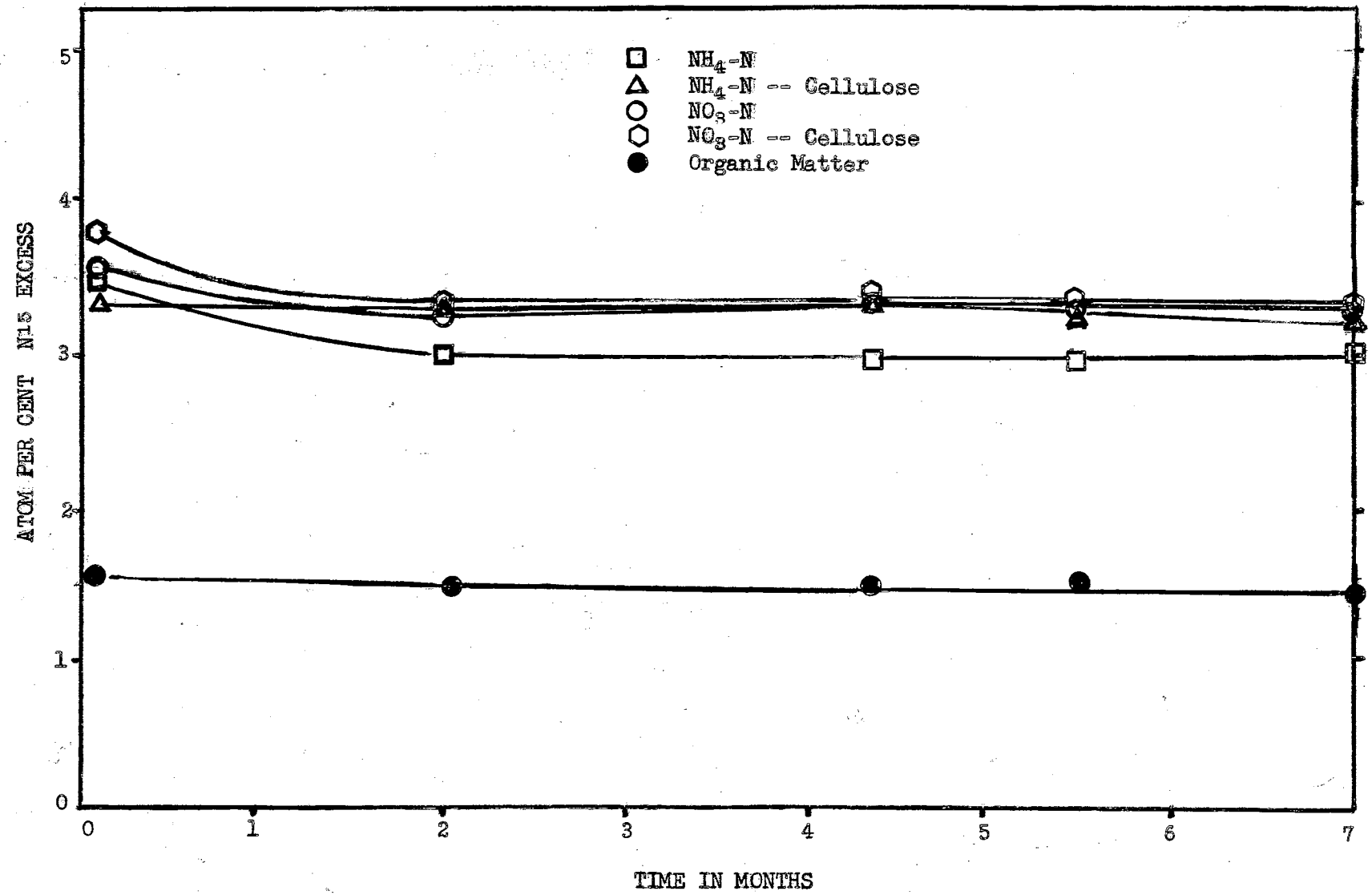
RESULTS AND DISCUSSION

Changes in Total Nitrogen of the Soil Cultures— The total nitrogen contents and isotope ratios of all the soils are presented in Appendix Table 1. The total nitrogen of all the soils remained quite constant throughout the 7 month incubation period. The largest variation at the seventh month from the initial value was about 5 %. This is close to the precision of the Kjeldahl procedure used. It is therefore difficult to deduce from these results whether absolute changes in the total nitrogen content took place. In general, however, there was no substantial evidence to suggest that denitrification had occurred to any appreciable extent when the total soil nitrogen is considered. This is in contrast to previous observations by Bowers and MacVicar (42) with Chickasha silt loam in which some losses of nitrogen appeared to occur during a similar incubation.

The isotope ratios of the soils showed a gradual decrease in N^{15} content. The largest amounts of decrease were about 0.4 to 0.6 atom % N^{15} excess, which is an apprecible loss corresponding to about 10 to 15 % of the N^{15} present. A general trend in the total nitrogen of all the soils and their various treatments can be discerned from study of the isotope data presented in Appendix Table 1 and as represented by a typical soil in Figure 1. The greatest loss of N^{15} occurred within the first two months and thereafter the amount remained quite constant throughout the rest of the incubation.

In soils to which ammonium nitrogen had been added, analysis for N^{15} showed that there were measureable losses at the end of two months. These losses varied from 11 to 13 % of the added fertilizer nitrogen in those

ISOTOPE RATIOS OF THE TOTAL NITROGEN OF RENFRO SOIL



soils to which no organic matter in the form of cellulose had been added. Losses in the soils containing cellulose additions were less, being from 4 to 6 % of the nitrogen added. After this initial decrease, the N^{15} level remained fairly constant throughout the remainder of the period. The greater sensitivity of the N^{15} measurements as compared to the analysis of total nitrogen permited the detection of these losses which are relatively small in comparison to the total nitrogen of the soil, but represent substantial losses of added nitrogen. If such losses were continued over long periods of time, as would be the case under natural conditions, the total effect might be substantial.

The smaller losses in the presence of organic matter is presumed to be due to the stimulatory effect of a source of readily-available material for bacterial growth of the microbial population of the soil. In the presence of cellulose, the available nitrogen is taken up by the bacteria and incorporated into cellular material. This reduces the "pool" of nitrogen available for transformations which might ultimately result in denitrification. These results are in agreement with those of Bowers (42) who found smaller losses in a virgin soil, high in organic matter, than in the corresponding cultivated soil.

A similar, though smaller, loss of N^{15} was noted in corresponding soils treated with nitrate nitrogen. In this situation, also, losses occurred in N^{15} during the first few months of incubation. The magnitude of the losses, however, seemed less related to the presence or absence of added organic matter; in fact, the higher losses occurred in those cultures to which cellulose had been added. This might be rationalized on the basis that nitrate nitrogen is available both as a source of nitrogen

and as an acceptor of hydrogen arising from the dehydrogenations of metabolizable compounds. Thus, if there is a deficit in hydrogen acceptors, denitrification might be promoted in the presence of nitrate by an excessive amount of available organic matter. This would not occur if the available nitrogen were in a reduced form such as ammonia.

Analysis for total nitrogen of soils treated with plant residues labelled with N^{15} failed to reveal any significant changes. Isotope data, however, showed that N^{15} decreased throughout the incubation period. The amount of losses ranged from about 11 to 14 %. These data are in general agreement with the observations of Jansson and Clark (40) who showed that loss of nitrogen occurred when alfalfa forage was incorporated into soil and allowed to decompose.

Apparently equilibration between the available nitrogen of the soil and the added fertilizer nitrogen as ammonium and nitrate nitrogen takes place relatively rapidly, since after two months there was little further loss of N^{15}. Changes with respect to nitrogen transformations in the presence of added plant residues occurred more slowly. The data indicates that the major losses in nitrogen occurred after the fourth month. As will be seen in Figure 4, it was only during this time that nitrate was present in significant quantity in the culture.

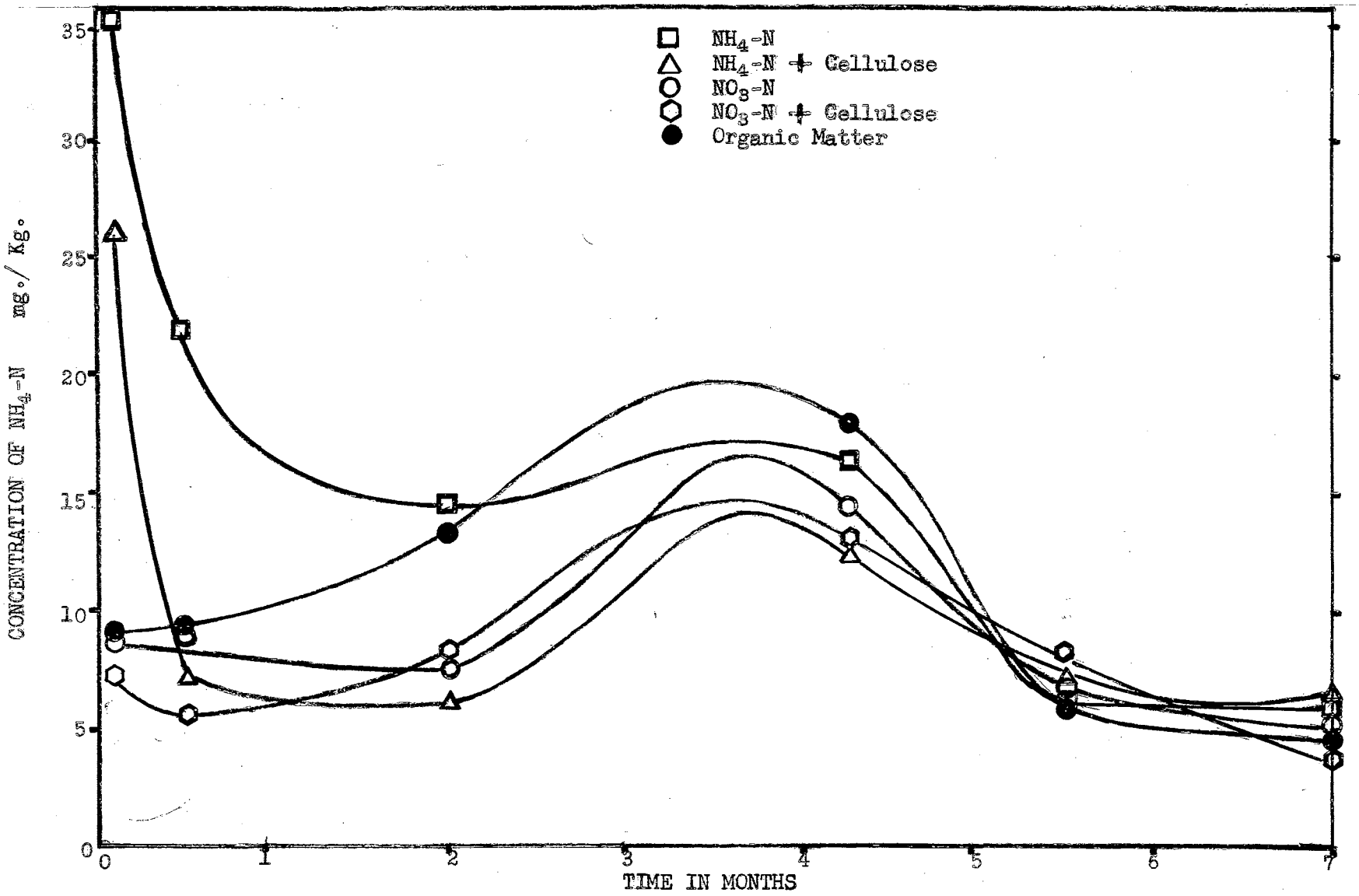
Changes in the Ammonium Nitrogen Fractions of the Soil Cultures--
The ammonium nitrogen fraction of the three soils was determined, and the results are presented in Appendix Table 2. The results were all found to be qualitatively very similar, and, therefore, the medium soil, Renfro clay loam, was chosen for further isotope analyses (Tables 1, 2).

The ammonia content of the cultures treated with only ammonium nitrogen rapidly decreased during the first and second months of incubation. A slight increase occurred from the second month with the maximum amount of ammonia accumulating at about 4 months. Thereafter a decrease again took place with the result that by 6 or 7 months the ammonium nitrogen concentration had reached a relatively low level of about 10 p.p.m. N.

The soil cultures to which cellulose had been added along with the ammonium nitrogen showed an even more rapid deminution of ammonia. By two weeks, the ammonia concentration was already below 10 p.p.m. N. The ammonia concentration was found to increase slightly from the second to the fourth month, and then to decrease again to a level below 10 p.p.m. N. The added carbon source stimulated rapid cell growth, and hence the free ammonium nitrogen was assimilated more rapidly and was tied up in the form of nitrogenous cellular components.

The ammonium nitrogen concentration of the soils treated with nitrate or both nitrate and cellulose was low initially (7 or 8 p.p.m. N). The addition of cellulose apparently had little effect on the concentration of ammonia in these cultures, as the ammonia concentrations of the cultures containing added cellulose were only slightly less than those cultures having no additional source of organic matter. There was a slightly greater increase of ammonium nitrogen in the cultures containing add-

FIGURE 2. AMMONIUM NITROGEN FRACTION OF RENFRO SOIL



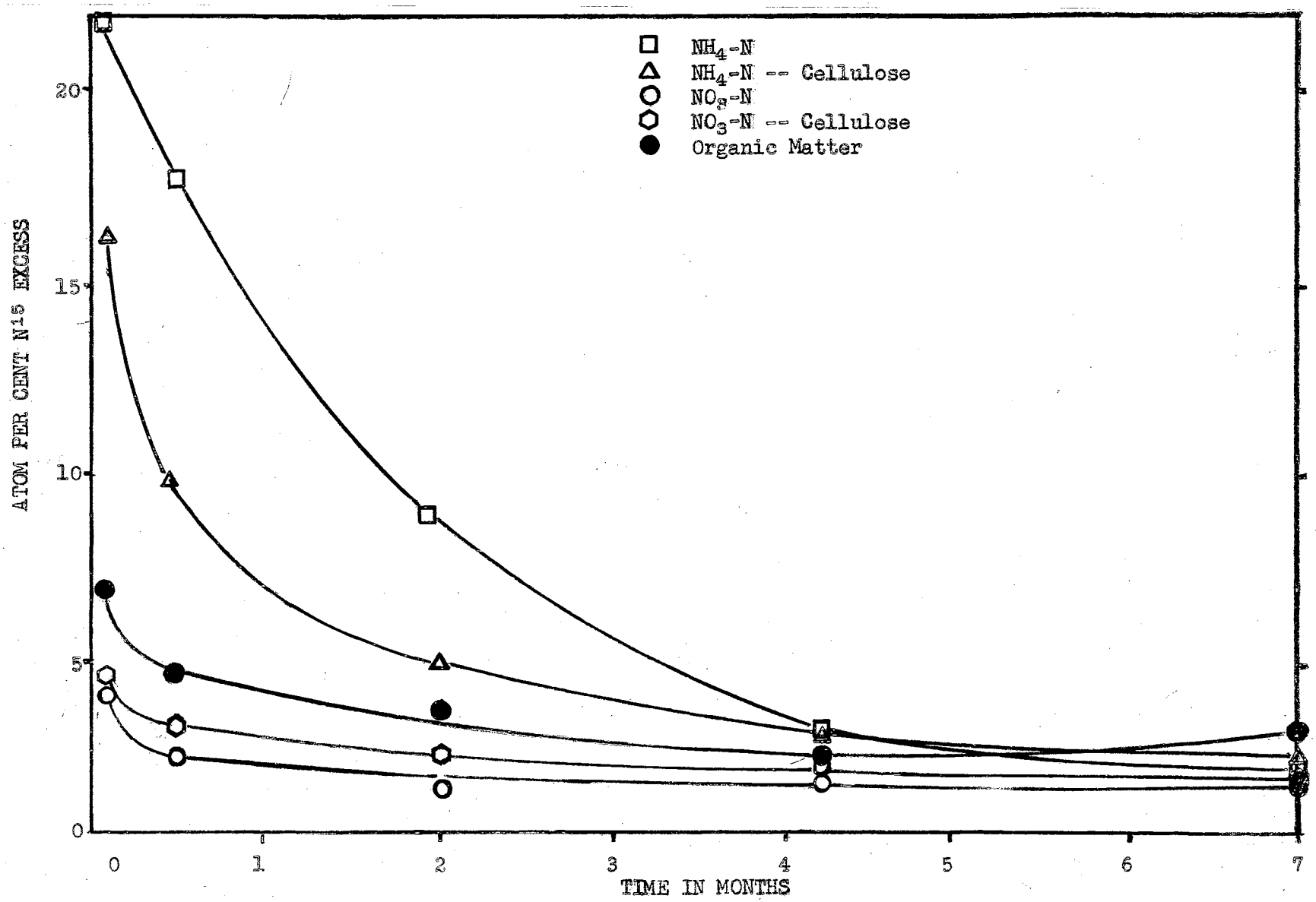
ed nitrate from the second to the fourth month. The peak in the concentration occurred about 4 months (10 to 25 p.p.m. N) and was followed by a rapid decrease so that by 7 months the concentration of ammonia was about 5 p.p.m. N.

The ammonia content of all the soils treated with plant residues was low (about 8 p.p.m. N) at the beginning of incubation and followed very closely the curves of the nitrate-treated cultures.

By 6 or 7 months the ammonium nitrogen content of all the soils, regardless of treatment, was below 10 p.p.m. N. The reason for the increase in ammonium nitrogen, reaching a peak about 4 months, is not clear but this corresponds to the time of year when the cultures were exposed to increasing temperatures of incubation due to higher temperatures in the room in which they were kept.

The rapid decrease of the ammonium nitrogen of the culture treated with ammonia is confirmed by a rapid decrease in the N^{15} content of this fraction (Appendix Table 2 and Figure 3). This decrease is due to the entry of the added nitrogen, which had a high N^{15} excess, into the "metabolic nitrogen pool" with subsequent dilution. The decrease in the N^{15} ratios of the culture treated with both ammonia and cellulose was much more rapid than in the culture without any additional carbon source. The N^{15} content of the ammonium nitrogen was 30 % at the initiation of incubation, but by 2 weeks it was less than 5 % and continued to decrease slightly from then on. This rapid decrease in the N^{15} content showed that not only a rapid disappearance of ammonium nitrogen had occurred, but there also was a very rapid rate of transfer of the ammonium nitrogen present with other available sources of nitrogen in the soil.

FIGURE 3. ISOTOPE RATIOS OF AMMONIUM NITROGEN FRACTION OF RENFRO SOIL



The N^{15} content of the ammonium nitrogen fraction of the soils treated with nitrate was low initially and remained low throughout the course of incubation. Cellulose added with the nitrate had little effect on the isotope ratios of the culture. The N^{15} concentration was low in the beginning and gradually decreased during the 7 month period of incubation.

The content of N^{15} of the culture treated with plant residues was 7.3 atom % excess at the start of incubation and rapidly decreased during the first 2 months and thereafter decreased gradually. This indicates that initally a substantial amount of the ammonium nitrogen in the culture was derivable from the added plant residues, probably most as amide nitrogen. In common with the free $N^{15}H_4$, however, this was rapidly incorporated into the cells of the microbiological flora, whose growth was stimulated by the presence of a readily-available source of carbon.

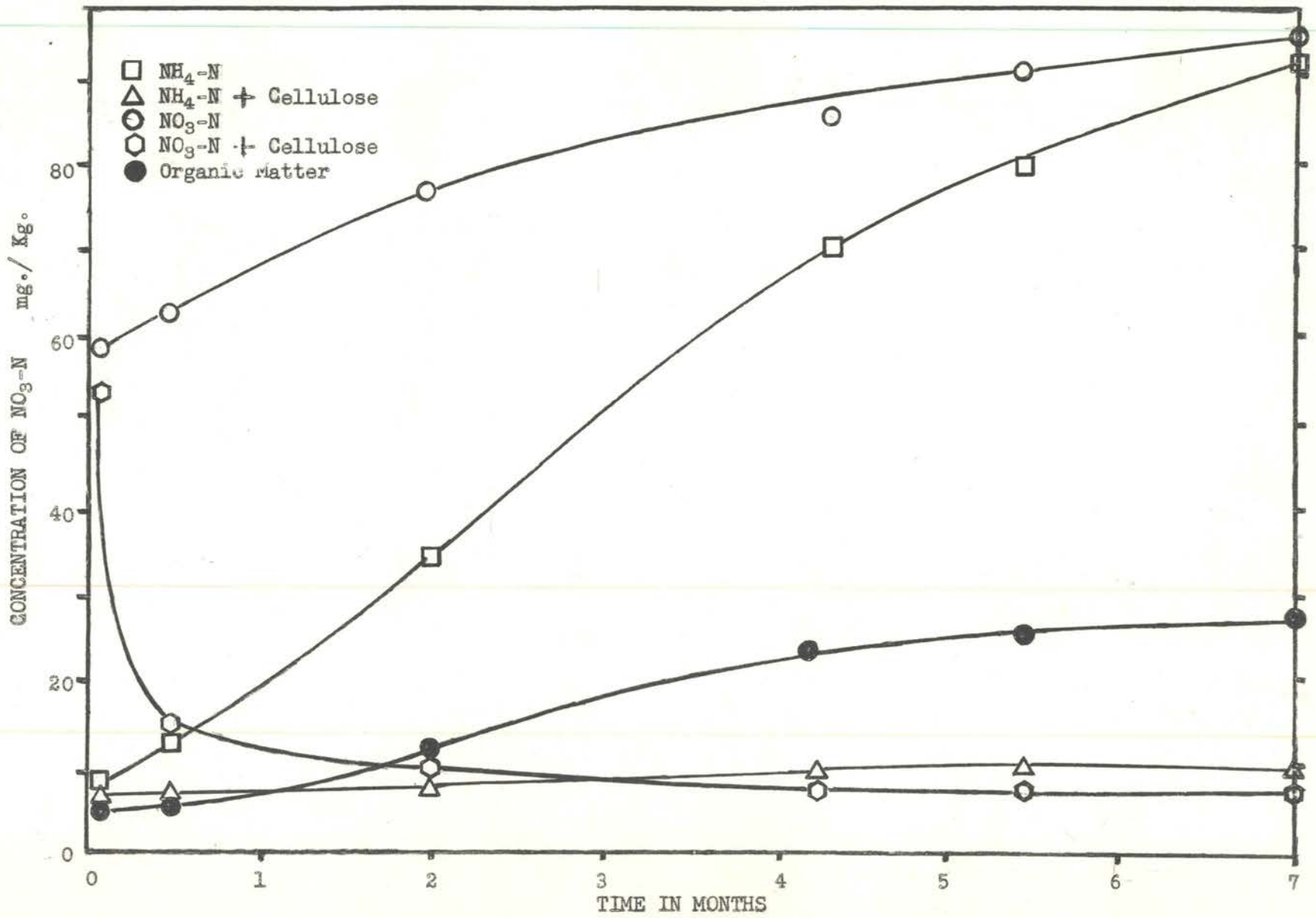
Changes in Nitrate Nitrogen Fraction of the Soil Cultures—The changes taking place in the nitrate nitrogen fraction of the soils during incubation are presented in Appendix Table 3, and the isotope ratios for Renfro soil are represented in Figure 5.

The cultures treated with nitrate showed that a steady increase in the nitrate nitrogen took place during the incubation period due to nitrification by the nitrifying organisms present in the soil. The initial concentration was 60 p.p.m. N and increased to 85-100 p.p.m. in the three soils by 7 months. This is an increase of more than 50%.

The nitrate nitrogen of the soils treated with nitrate and cellulose showed an extremely rapid decrease during the first 2 weeks of incubation. By 2 weeks the concentration of nitrate nitrogen was less than 15 p.p.m. N, for all three soils, and by 2 months the concentration had reached a low and constant level (less than 10 p.p.m. N). This is presumed to represent utilization of nitrate nitrogen for cellular synthesis by those soil organisms capable of reducing nitrate. It may also, to some degree, represent losses of nitrate nitrogen through denitrification.

The ammonia treated soil cultures showed rapid increases in their nitrate nitrogen content. The concentration of nitrate nitrogen of all the soils had reached 60 p.p.m. N or more by 3 or 4 months. That some of this increase was due to the oxidation of the original addition of 60 p.p.m. ammonium nitrogen is shown by the isotope ratio curve which shows an increase in the isotope concentration of the nitrate nitrogen fraction during the first few months. Additional evidence is that this increase corresponds to the decrease of ammonium nitrogen in the same

FIGURE 4. NITRATE NITROGEN FRACTION OF RENFRO SOIL

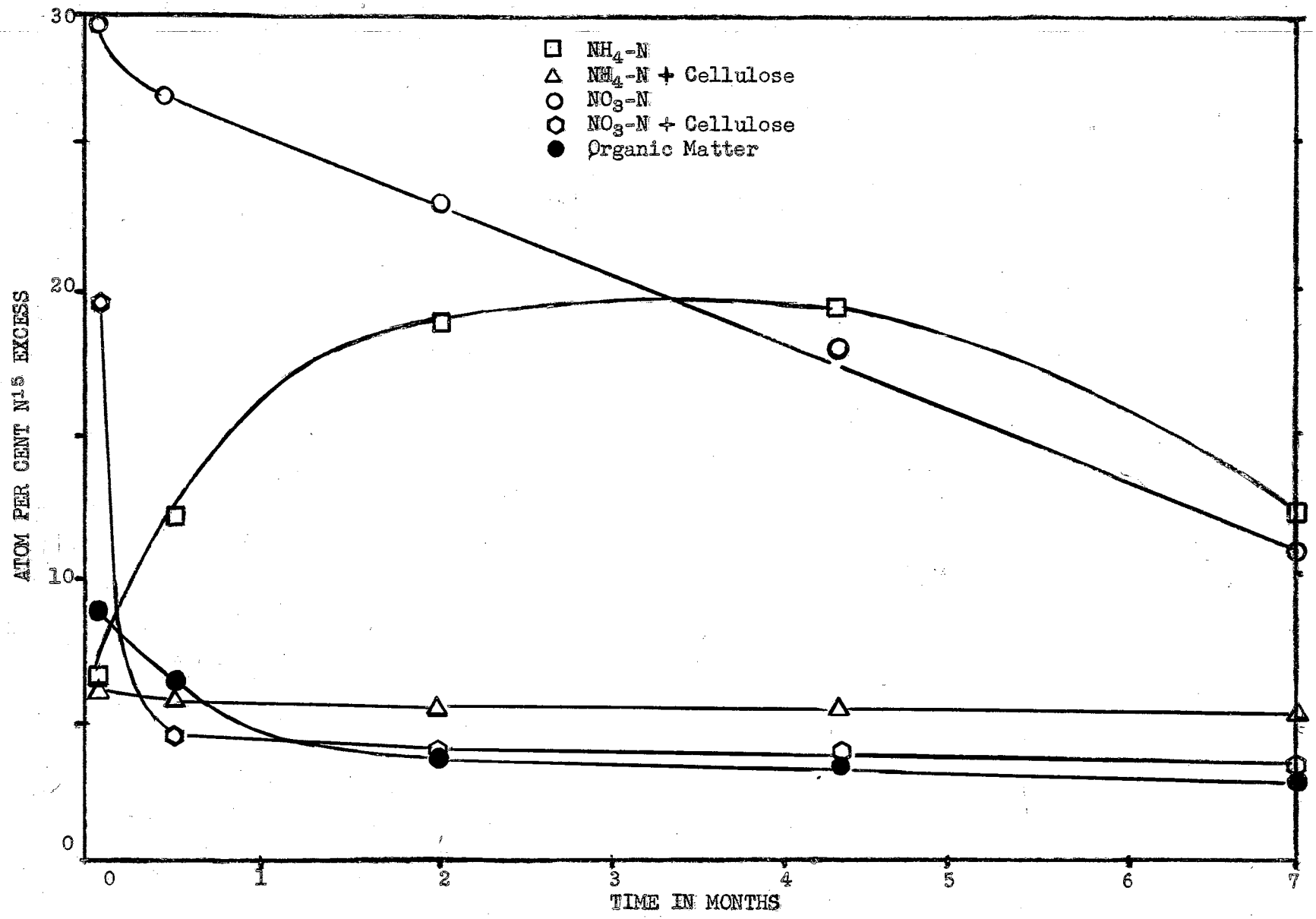


cultures. A rapid up take of N^{15} in the nitrate fraction occurred during the first 2 months corresponding to the rapid decrease of N^{15} in the ammonium nitrogen fraction taking place at the same time. After 2 months the N^{15} ratio leveled off and gradually decreased. This decrease in the N^{15} ratio is due to dilution caused by the increase in nitrate formed from other sources, primarily the "metabolic pool" nitrogen of the culture.

As was observed before, the addition of cellulose along with the added fertilizer nitrogen caused the free nitrogen form to be tied up in cellular constituents. The nitrate in the soil culture treated with both nitrate and cellulose was reduced extremely rapidly during the first month of incubation. The isotope ratio also shows this rapid decrease. The nitrate nitrogen in the soil treated with ammonia and cellulose was initially very low and remained at this low level throughout the whole incubation period as the ammonium nitrogen was rapidly reduced to cellular nitrogenous compounds and hence, no ammonia was left in the culture that could be oxidized to nitrate. The addition of added carbonaceous material when a soil is fertilized with a free form of nitrogen increases the C/N ratio and, hence, causes the free nitrogen in the soil to be incorporated into forms of nitrogen of the soil bacteria. This prevents nitrogen losses due to denitrification, but also produces conditions which may be poorly suited for crop growth.

The nitrate nitrogen of the soil treated with only the plant residues showed a rather marked increase from the second to the fourth month, apparently after the attainment of a stabilized C/N ratio, due to nitrification. While the amount of fertilizer nitrogen added as

FIGURE 5. ISOTOPE RATIOS OF NITRATE NITROGEN FRACTION OF RENFRO SOIL



plant residues was the same as that added as free nitrate or ammonia, the amount of carbon added was much less that the amount of cellulose added to corresponding cultures. It would seem that to a considerable degree, the added organic matter in the form of plant residues had been stabilized by 4 months.

SUMMARY

Three Oklahoma soil, namely, Stidham loamy fine sand, Renfro clay loam, and Vanoss fine sandy loam were used in these studies. Five treatments were set up on each soil as follows: 60 p.p.m. $(N^{15}H_4)_2SO_4$; 60 p.p.m. $(N^{15}H_4)_2SO_4$ + 20000 p.p.m. cellulose; 60 p.p.m. $KN^{15}O_3$; 60 p.p.m. $KN^{15}O_3$ + 20000 p.p.m. cellulose; and 60 p.p.m. N as N^{15} enriched plant residues. The total nitrogen, ammonium nitrogen and nitrate nitrogen fractions of each culture were analyzed periodically throughout a seven month incubation period. Also the isotope ratios of the total nitrogen of each soil were determined. The results for the three different soils were found to be qualitatively similar. Therefore, the Renfro clay loam was chosen for further isotope analysis of the ammonium and nitrate nitrogen fractions.

1. The total nitrogen of all the soils (as determined by a Kjeldahl procedure) was quite constant over the seven month incubation period. However, the isotope ratios showed that some losses had occurred, presumably due to denitrification. The greatest amount of loss occurred during the first two months in all the soils and all the treatments of each. After two months the losses of nitrogen were slight.

2. The greatest loss of nitrogen occurred in the treatments containing ammonia without added organic matter.

3. Losses of added N^{15} from fertilizer nitrogen additions were decreased in the case of $N^{15}H_4$ by adding organic matter. Addition of organic matter to soils fertilized with $N^{15}O_3$ was without beneficial effect.

4. Added ammonium nitrogen was rapidly converted to other forms. This turnover was accelerated by the addition of organic matter.

5. The nitrate concentration increased in all soil treatments, except those containing added cellulose, due to nitrification taking place more rapidly than the amount of nitrate utilized for bacterial growth. An increase of 50 per cent occurred by seven months in those soils fertilized with either ammonia or nitrate. Evidence suggests the direct conversion of added $N^{15}H_4$ to $N^{15}O_3$.

APPENDIX

TABLE 1.

TOTAL NITROGEN

Soil	Treatment No.	Time in Months 0	0.5	2.0	4.0	5.4	7.0	Change over 7 mo.	Change Over 7 mo.
		mg. N/ Kg.							%
Stidham	1	0.25	0.25	0.26	0.28	0.26	0.26	0.01	+ 5
	2	0.26	0.27	0.28	0.28	0.27	0.27	0.01	+ 5
	3	0.27	0.28	0.27	0.28	0.27	0.28	0.01	+ 5
	4	0.27	0.27	0.27	0.29	0.27	0.27	0	0
	5	0.27	0.28	0.28	0.31	0.27	0.26	0.01	- 5
Renfro	1	0.55	0.54	0.55	0.59	0.58	0.57	0.02	+ 5
	2	0.56	0.56	0.57	0.58	0.58	0.57	0.01	+ 2.5
	3	0.58	0.59	0.59	0.60	0.57	0.57	0.01	- 2.5
	4	0.58	0.56	0.58	0.60	0.57	0.57	0.01	- 2.5
	5	0.57	0.59	0.59	0.61	0.58	0.57	0	0
Vanoss	1	0.76	0.76	0.78	0.81	0.79	0.78	0.02	+ 3
	2	0.76	0.77	0.81	0.84	0.79	0.77	0.01	+ 1.5
	3	0.78	0.76	0.82	0.85	0.80	0.78	0	0
	4	0.79	0.79	0.79	0.83	0.80	0.79	0	0
	5	0.81	0.80	0.82	0.84	0.82	0.81	0	0
		Atom % N^{15} Excess							%
Renfro	1	3.44		2.94	2.97	2.99	3.02	0.42	- 12.2
	2	3.37		3.24	3.22	3.17	3.24	0.13	- 3.9
	3	3.51		3.27	3.21	3.11	3.29	0.22	- 6.3
	4	3.70		3.32	3.29	3.21	3.31	0.39	- 10.2
	5	1.61		1.48	1.50	1.53	1.48	0.13	- 8.0
Vanoss	1	2.55		2.26	2.23		2.27	0.28	- 11.4
	2	2.49		2.36	2.31		2.34	0.15	- 6.0
	3	2.63		2.47	2.48		2.49	0.14	- 5.3
	4	2.70		2.55	2.41		2.51	0.19	- 7.0
	5	1.42		1.28	1.23		1.26	0.16	- 11.2
Stidham	1	6.89		6.07	5.93		6.10	0.79	- 11.4
	2	6.39		6.11	6.05		6.00	0.39	- 6.1
	3	5.88		5.47	5.57		5.55	0.33	- 5.6
	4	6.74		6.29	5.98		6.28	0.48	- 6.8
	5	3.03		2.98	2.93		2.71	0.32	- 10.7

Treatment 1. NH_4-N
2. NH_4-N + Cellulose
3. NO_3-N
4. NO_3-N + Cellulose
5. Organic Matter

TABLE 2

AMMONIUM NITROGEN FRACTION

Soil	Treatment No.	Time in Months 0	0.5	2.0	4.2	5.4	7.0
				mg. N/ Kg.			
Stidham	1	24.7	15.4	2.81	2.63	2.95	2.87
	2	18.4	7.23	3.15	1.57	1.49	1.53
	3	7.90	4.68	2.15	6.57	2.33	2.45
	4	8.01	4.10	2.95	5.85	1.97	2.17
	5	7.23	5.20	2.75	10.8	3.35	3.70
Renfro	1	36.0	20.9	14.95	17.4	5.07	6.28
	2	26.8	6.78	6.32	12.3	5.11	6.06
	3	8.37	8.50	7.23	14.5	6.71	5.07
	4	6.55	5.34	7.35	12.5	7.20	4.27
	5	9.10	9.87	14.0	18.3	5.07	4.97
Vanoss	1	45.0	26.9	12.6	22.9	12.5	6.23
	2	38.4	14.3	7.89	22.6	9.90	6.04
	3	8.91	12.3	18.8	26.7	9.23	7.80
	4	6.56	9.30	12.3	22.7	10.5	6.05
	5	9.15	10.5	15.4	27.2	13.0	8.38
				Atom % N^{15} Excess			
Renfro	1	21.10	17.67	8.72	2.26		1.92
	2	16.32	9.96	5.10	4.84		2.23
	3	4.46	2.34	2.28	2.19		2.14
	4	4.72	2.72	2.60	2.46		2.19
	5	7.30	4.87	3.13	2.53		2.72

Treatment	1.	NH_4-N	60 p.p.m. N
	2.	NH_4-N + Cellulose	60 p.p.m. N + 20000 p.p.m. cellulose
	3.	NO_3-N	60 p.p.m. N
	4.	NO_3-N + Cellulose	60 p.p.m. N + 20000 p.p.m. cellulose
	5.	Organic Matter	60 p.p.m. N

TABLE 3.

NITRATE NITROGEN FRACTION

Soil	Treatment No.	Time in Months					
		0	0.5	2.0	4.2	5.4	7.0
		mg. N/ Kg.					
Stidham	1	8.82	34.5	60.5	73.9	78.5	82.6
	2	8.78	8.35	7.73	7.80	9.86	9.10
	3	62.5	64.8	71.0	77.9	83.5	86.5
	4	58.8	16.5	9.08	8.60	7.34	5.65
	5	8.35	8.55	9.17	47.6	48.9	54.7
Renfro	1	8.95	13.4	34.5	71.0	79.4	90.6
	2	7.55	8.25	9.75	10.4	10.9	9.88
	3	58.8	60.9	77.7	84.8	89.5	93.0
	4	53.4	15.4	10.9	7.70	7.95	7.53
	5	5.15	5.34	12.4	26.0	28.5	28.2
Vanoss	1	7.65	16.5	73.0	98.7	100.8	102.5
	2	7.10	8.71	8.85	8.10	8.35	8.20
	3	66.6	80.5	91.0	94.7	100.5	102.5
	4	63.2	13.5	10.8	6.40	6.15	4.24
	5	7.35	9.15	12.1	36.0	40.8	46.8
		Atom % N^{15} Excess					
Renfro	1	6.67	11.76	18.95	18.75		11.94
	2	6.34	6.25	6.05	5.78		5.67
	3	29.86	26.73	23.59	17.65		10.47
	4	19.87	4.13	4.08	3.67		3.06
	5	8.76	6.56	3.96	3.62		2.88

Treatment 1. NH_4-N — 60 p.p.m. N
2. NH_4-N + Cellulose — 60 p.p.m. N + 20000 p.p.m. cellulose
3. NO_3-N — 60 p.p.m. N
4. NO_3-N + Cellulose — 60 p.p.m. N + 20000 p.p.m. cellulose
5. Organic Matter — 60 p.p.m. N

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