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cropping did not indicate a loss of N, but the $N^{15}$ data presented above do suggest that there was some loss of fertilizer N.

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# Sequence of Products Formed During Denitrification in Some Diverse Western Soils[1]

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## ABSTRACT

The sequential products of anaerobic denitrification were determined on seven Western soils (four alkaline, two acid, and one neutral) by soil and gas analysis. The soils with 1% alfalfa and added $KNO_3$ were incubated at moistures slightly greater than field capacity and with an atmosphere of He. The soils and gases were periodically analyzed and balance sheets prepared. The sequence of $NO_3^- \rightarrow NO_2^- \rightarrow N_2O \rightarrow N_2$ operated in all soils. The $N_2O$ and $N_2$ were determined on a gas chromatograph. The rates of nitrogen interchanges and maximal amounts of nitrite, $N_2O$, and $N_2$ were determined. From this data it was postulated that the rate-limiting process for denitrification in acid soils is the reduction of nitrate, while in alkaline soils it is the reduction of nitrite. The reduction of $N_2O$ was rapid in all instances. The total time for complete reduction of 300 ppm. N as nitrate to $N_2$ at 30°C. varied only from 28 to 96 hours for the 7 soils. Lowering the temperature to 25° or 20°C. influenced all rates for 3 alkaline soils and resulted in a twofold increase in denitrification time for the 10°C. drop. Decreases in initial concentration of added $KNO_3$ to 150, 75, and 37.5 ppm. did not change the overall rate of denitrification. There was, however, a marked reduction in the maximal amounts of $N_2O$ found in the gaseous atmosphere when the initial $KNO_3$ concentration was reduced.


IT HAS BEEN SUGGESTED for many years that denitrification is a source of loss of soil N (1). Until recently, however, evidence supporting this idea consisted primarily of failure to account for all the soil nitrogen in lysimeter studies. The quantitative identifications of nitrogen gas ($N_2$) with the mass spectrometer (4, 5, 6, 7, 8, 11, 12, 13, 14, 17) and of nitrous oxide ($N_2O$) with the mass spectrograph and infrared spectrophotometer (2, 8, 14, 16) have led to differences of opinion among investigators concerning the production and fate of various N compounds and oxides during denitrification.

Kluyver and Verhoeven (12) proposed a scheme for the reduction of nitrate in which the reductive process involves two electron transfer steps. In this scheme, the gases that can be identified are $N_2O$ and $N_2$. Arnold (2) postulated that the earth is the source of atmospheric $N_2O$ and that the $N_2O$ is produced through a chemical reaction involving nitrous acid and hydroxylamine. Schwartzbeck et al. (14) concluded that $N_2O$ was produced during denitrification when ammonium and nitrate were both present, while only $N_2$ was produced when nitrate alone was present. Wijler and Delwiche (17) stated that $N_2O$ is the major gas evolved during denitrification in acid soils but that $N_2$ production predominates in alkaline soils. McGarity et al. (13) agreed with Hauck and Melsted (8) that $N_2$ is the gas evolved under anaerobic conditions while small quantities of both $N_2$ and $N_2O$ will be evolved in the presence of any free oxygen. Cady and Bartholomew (6, 7), using $N^{15}$, presented a sequence of gaseous production during anaerobic denitrification in an acid soil. In their studies, the length of time for complete reduction of nitrate to $N_2$ varied from 7 to 25 days, depending upon the moisture content of the soil. Nitric oxide (NO), as well as $N_2O$ and $N_2$, were quantitatively measured.

The study reported here involved use of a gas chromatograph to study nitrogen losses in Western soils. The simultaneous analysis of the soils for the inorganic N components facilitated preparation of a complete N balance sheet for the soils and conditions involved. The sequential appearance and fate of the various N componets in the system are delineated.

## METHODS AND PROCEDURES

### Soils

Some physical and chemical characteristics of the seven Western soils used in this study are given in table 1. All were obtained from the surface 6 inches and were air-dried, screened through a 2-mm. sieve, and stored dry until used.

In all cases, 2 g. of ground alfalfa was mixed dry with 200 g. of the air-dried soil. Sufficient water and $KNO_3$ solution were added to the soil to bring the moisture content up to field capacity and the N level up to that desired. The N level

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was 300 ppm. in all cases except one. This exception was made in order to check the influence of nitrate concentration on the magnitude and the fate of the various nitrogenous components. In this case, therefore, N levels of 37.5, 75, and 150 ppm. were established. Sufficient quantities of all soils were prepared so that 4 replicates of treated and untreated (no N but including alfalfa) soils could be harvested periodically in order to analyze for the inorganic soil N. Nitrates and nitrites were determined on a $Ca(OH)_2$ extraction of the soils. Nitrates were determined on a portion of the extract by the phenoldisulfonic acid method (9) after destroying the nitrites with sulfamate. The nitrites were determined by the method of Shinn (15) which employs sulfanilamide and a coupling reagent.

### Incubation Flasks and Gas Sampling

Following the mixing of the soil plus alfalfa with the necessary water and $KNO_3$ solution, the soils were placed into 500-ml. suction flasks which served as the incubating flasks. A piece of latex rubber tubing was fastened over the side-arm tubulation to facilitate exchanging the gaseous atmosphere inside the flask. Ten milliliters of 4*N* KOH, to trap the $CO_2$ produced, was put into a small test tube which was placed inside the flask. An additional 10 ml. of water was added to bring the soil in each flask to the moisture content to the level indicated in table 1 (5% above field capacity).

The stopper for each flask was drilled with a ½-inch hole and fitted with a silicon disc (chromatograph inlet seal) that would reseal after repeated puncturing with a hypodermic needle. The disc was held in place by two one-holed number zero stoppers, one on each side of the disc, that were cemented in place. The atmosphere within each flask was exchanged for one containing 100% He by introducing the gas mixture into the flask through a needle inserted through the silicon disc and allowing the gas to flow out through the side-arm tubulation. For analysis, samples of the gaseous atmosphere were taken periodically through the silicon disc with a 1-ml. hypodermic syringe fitted with a sheathed 28-gauge needle and were injected directly into the chromatograph.

The GC-2 gas chromatograph was equipped with dual columns made of ¼-inch aluminum. One column was 6 feet in length and filled with molecular sieve for the identification of $N_2$. The second column, for separation and identification of $N_2O$, was 14 inches in length and filled with charcoal. The carrier gas was He. The columns were maintained at 40°C., while the carrier gas was passed through the columns at 40 pounds per square inch. This gave a flow rate of approximately 100 ml. per minute. Standard curves relating known quantities of $N_2$ or $N_2O$ with peak heights were obtained. The peak heights were used since they correlated highly with the area under the curve and were easier of the two values to obtain. Corrections were made for differences in flask volumes and internal flask pressure. The amounts of N reported, either gaseous or in the soil, are those determined after the amount determined in the control (no added nitrogen) has been subtracted.

**Table 1—Some physical and chemical characteristics of the seven experimental soils used in the anaerobic denitrification study.**

| Soil | Location | pH (paste) | Organic matter | Moisture characteristics | | Moisture content used in present study |
|---|---|---|---|---|---|---|
| | | | | 1/3 bar | saturation | |
| | | | | Percent | | |
| Millville loam | N. Utah | 7.8 | 1.9 | 18 | 31 | 23 |
| Yolo loam | N. California | 6.8 | 1.4 | 20 | 40 | 25 |
| Manhattan silt loam | S. Montana | 7.8 | 2.3 | 25 | 48 | 30 |
| Reeves loam | S.E. New Mexico | 7.8 | 1.1 | 18 | 33 | 23 |
| Gila loam | C. Arizona | 7.9 | 1.4 | 19 | 35 | 24 |
| Walla Walla silt loam | S.E. Washington. | 6.1 | 2.8 | 25 | 41 | 30 |
| Wysaro clay | S. Wyoming | 6.1 | 2.6 | 25 | 50 | 30 |

## RESULTS AND DISCUSSION

The sequence of the different nitrogenous components determined appeared to be:

$$NO_3^- \rightarrow NO_2^- \rightarrow N_2O \rightarrow N_2$$

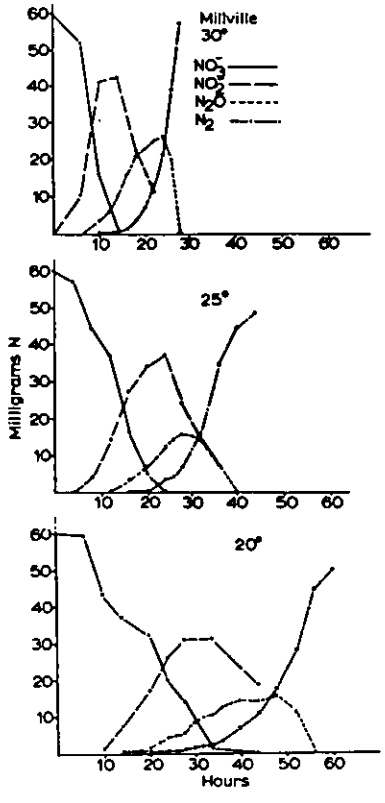


Figure 1—Sequence and magnitude of N products formed and utilized during anaerobic denitrification of Millville loam (pH 7.8) at 30°, 25°, and 20°C.

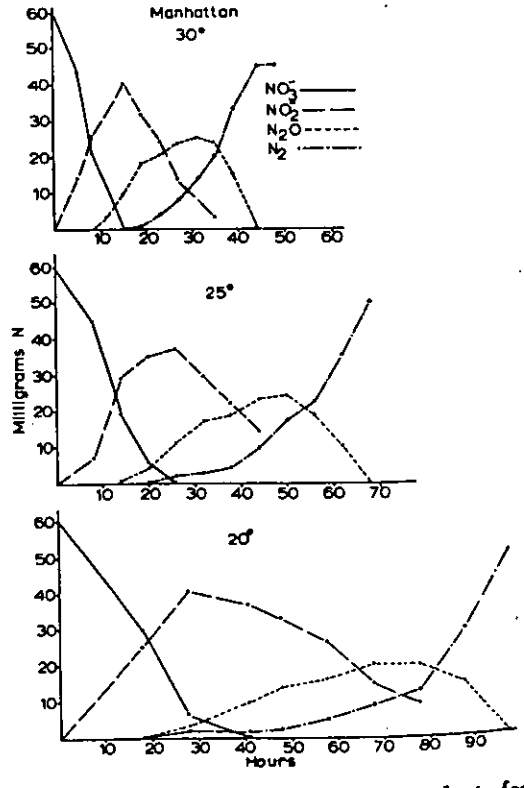


Figure 2—Sequence and magnitude of N products formed and utilized during anaerobic denitrification of Manhattan silt loam (pH 7.8) at 30°, 25°, and 20°C.

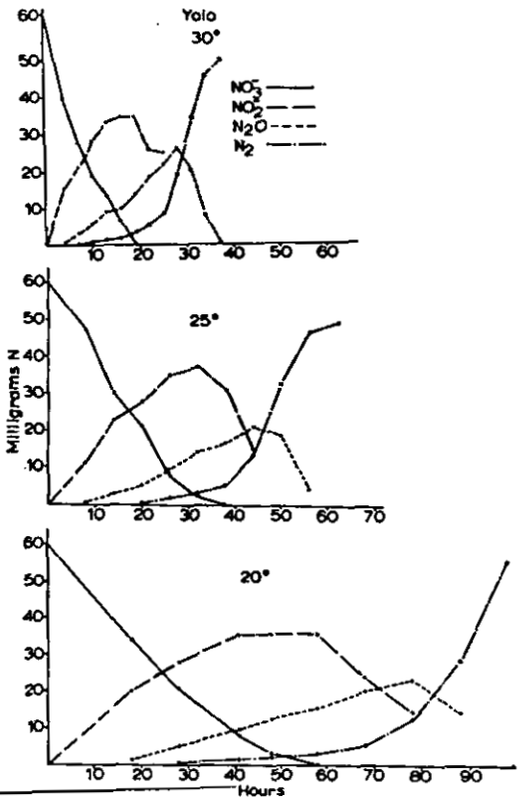


Figure 3—Sequence and magnitude of N products formed and utilized during anaerobic denitrification of Yolo loam (pH 6.8) at 30°, 25°, and 20°C.

This sequence, which has been suggested by others (6), apparently operated in all the soils under the various conditions imposed (figures 1 to 5). Most recent investigators, however, have based their conclusions only on studies of the gases evolved into the atmosphere. It is obvious that magnitude and rate of production and utilization of the various products will vary with the soil and the conditions imposed. In this experiment, however, definite patterns could be discerned. The reduction of N from nitrate through the various products to $N_2$ was rapid and varied only over a range of 28 to 96 hours for all the soils when kept at 30°C. These rates are several times more rapid than most reported in the literature (4, 5, 6, 7, 8, 13, 14, 16, 17). Some studies, however, give comparable rates. Jones (11) showed reduction of 200 ppm. nitrate-nitrogen to $N_2$ in 3 days under vacuum for an acid soil (pH 5.8). Bremner and Shaw (3) reported complete disappearance of nitrate within 2 days, the shortest time they investigated, for an alkaline, waterlogged soil. Some of the slower dentitrification rates reported might be explained on the basis of low pH (6, 8), the lack of readily oxidizable material (8, 14), or because the soils were not fully anaerobic (4, 5, 8, 14).

The maximum amounts of the added N that were detected as nitrite or $N_2O$ (table 2), along with the rates of change of the various N components, give clues as to the rate-limiting process in dentitrification. In alkaline soils, the reduction of nitrate was rapid but there was a lag in reduction of nitrite to $N_2O$. This lag gave rise to high levels of nitrite. In the acid soils, the low level of bacteria (10) may have caused a concomitant low rate of nitrate reduction. The high levels of nitrite encountered in the

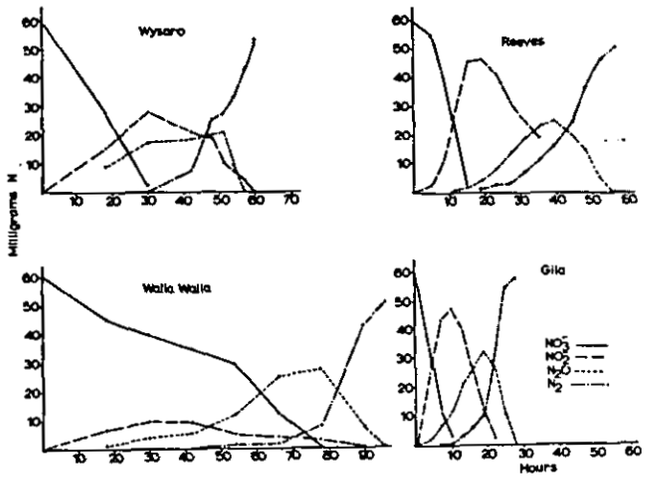


Figure 4—Sequence and magnitude of N products formed and utilized during anaerobic denitrification of Reeves loam (pH 7.8), Gila loam (pH 7.9), Wysaro clay (pH 6.1), and Walla Walla silt loam (pH 6.1) at 30°C.

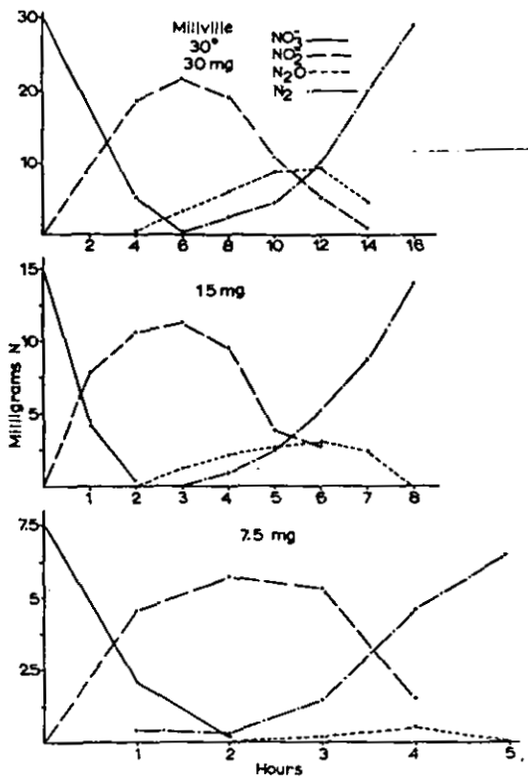


Figure 5—Effect of nitrate concentration on the sequence and magnitude of N products formed and utilized during anaerobic denitrification of Millville loam at 30°C.

alkaline soils may poison the system until some adaptation is made. The maximum amount of N as $N_2O$ detected in the gaseous atmospheres above the alkaline soils was 50 to 65% of the maximum nitrite detected. Only in the acid Walla Walla soil did the amount of $N_2O$ exceed the maximum quantity of nitrite recovered. In all instances, the $N_2O$ was rapidly reduced to $N_2$. The complete and rapid utilization of $N_2O$ noted in this experiment is in accordance with the results of Cady and Bartholomew (6) but differs from the work of Schwartzbeck et al. (14)

**Table 2—The magnitude of the nitrogen products formed during anaerobic denitrification in seven Western soils.**

| Soil | Temperature | N as $KNO_3$ | $NO_2^-$-N | | $N_2O$ | | $N_2$ | |
|---|---|---|---|---|---|---|---|---|
| | °C. | mg. | mg. | % | mg. | % | mg. | % |
| Gila | 30 | 60 | 47 | 78 | 32 | 53 | 57 | 95 |
| Reeves | 30 | 60 | 47 | 78 | 25 | 42 | 51 | 85 |
| Wysaro | 30 | 60 | 29 | 45 | 21 | 35 | 54 | 90 |
| Walla Walla | 30 | 60 | 9 | 15 | 27 | 45 | 51 | 85 |
| Millville | 30 | 60 | 42 | 71 | 27 | 45 | 57 | 95 |
| Millville | 25 | 60 | 37 | 62 | 16 | 27 | 49 | 82 |
| Millville | 20 | 60 | 32 | 53 | 16 | 27 | 50 | 83 |
| Manhattan | 30 | 60 | 41 | 68 | 26 | 43 | 45 | 75 |
| Manhattan | 25 | 60 | 38 | 63 | 25 | 42 | 51 | 85 |
| Manhattan | 20 | 60 | 41 | 68 | 21 | 35 | 51 | 85 |
| Yolo | 30 | 60 | 35 | 58 | 27 | 45 | 50 | 83 |
| Yolo | 25 | 60 | 38 | 63 | 21 | 35 | 49 | 82 |
| Yolo | 20 | 60 | 37 | 62 | 23 | 38 | 56 | 93 |
| Millville | 30 | 30 | 22 | 73 | 8.5 | 28 | 28 | 93 |
| Millville | 30 | 15 | 12 | 80 | 3.0 | 20 | 14 | 93 |
| Millville | 30 | 7.5 | 6 | 80 | 0.5 | 7 | 7 | 93 |

and Wijler and Delwiche (17), who implied that $N_2O$ does not function as an electron acceptor in all soils.

Based on this experiment, it appears that $N_2O$ is a major factor in denitrification in alkaline soils as well as in acid soils (6, 8, 17). The speed at which $N_2O$ can be reduced may explain why this gas was not identified when the interval of sampling was several days (13). The speed of reduction also could explain why liming an acid soil would lead to the conclusion that $N_2$ is the major gas produced during denitrification of an alkaline (limed) soil while $N_2O$ is the major gas produced in the unlimed soil (8).

Lowering the temperature from 30°C. to 25° or 20°C. slowed down the rate of N interchanges in the 3 alkaline soils studied (figures 1 to 3). There was a twofold increase in time for complete reduction to $N_2$ for the 10°C. drop. A greater "tailing off" effect of nitrite reduction was noted as the temperature decreased. The decrease in the amounts of $N_2O$ that could be detected in the gaseous atmosphere as the temperatures were reduced (table 2) would indicate that the reduction of $N_2O$ to $N_2$ was less temperature dependent than was the reduction of nitrate or nitrite.

The influence of initial nitrate concentration on the sequence of nitrogen transformations and on the magnitude and rate of production of N compounds is shown in figure 5 for Millville loam. The similarity of the curves for nitrate, nitrite, and $N_2$, for the three levels of nitrate added, indicate that the initial nitrate concentration did not influence the overall rate of denitrification (17). As pointed out by Wijler and Delwiche (17), marked reduction occurred in the amount of $N_2O$ that could be detected in the gaseous atmosphere as the initial nitrate concentration was lowered (table 2). This would suggest that $N_2O$ was utilized more rapidly than nitrite.

It should not be assumed that the differences in nitrification rates are due only to differences in soil pH, even though artificially altering the soil pH has brought marked changes in nitrification rate (4, 6, 8, 17). The soils used in the present study can be grouped according to pH into those of pH 6.1 (Walla Walla silt loam and Wysaro clay), of pH 6.8 (Yolo loam), and of pH 7.8 (Millville loam, Manhattan silt loam, Reeves loam, and Gila loam). The differences among denitrification rates were as great within a pH group as they were between pH groups. Since all soils were treated the same with respect to temperature, organic matter, and moisture level, the differences noted must have been due to other factors.

The possibility that $N_2O$ and $N_2$ might arise by other pathways than that given by the proposed sequence should not be overlooked. Nor is it implied that the components of the suggested sequence are the only intermediates in the reduction of nitrate to $N_2$.

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