Production and Loss of Nitric Oxide from Denitrification in Anaerobic Brookston Clay

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Nitric oxide, nitrous oxide, and nitrite ion production was measured in a Brookston clay column undergoing anaerobic denitrification. A flow system method was used whereby argon carrier gas continuously stripped soil gases from the column, allowing steady-state rates to be obtained. Over several days the temporal change in rates of these gases and NO2− followed a pattern of increase and decay which may be expected of a reaction proceeding by several consecutive steps. The method permits observation of the relatively large net production rate of NO, which is normally not observed in static systems based on head space analysis of gaseous denitrification products. In the first several hours after the onset of anoxic conditions, the net rate of NO production, fNO, increased sharply to a maximum (~1 x 10−16 mol of N/g of soil per min), paralleling the rapid increase in NO2− level, and then followed a more gradual decline extending over approximately 45 h. A similar but less pronounced pattern was observed for N2O, with net rates of production being considerably less than for NO. The ratio [NO-N]/[N2O-N] decreased with time from ~2.5 at 6 h to ~2.0 at 45 h. Estimated rates of N2 production appeared to be initially high, decreased rapidly within a few hours, and then gradually increased with time after the establishment of anaerobic conditions.

In anaerobic soils, NO is produced both by chemodenitrification (6, 27, 30) and by biological processes by a variety of denitrifying bacteria (6, 22, 30, 33). The first step in the reduction of NO3− produces NO2− and is common to denitrifying, respiring, or fermentative organisms (22). Nitrite can also be formed by nitrification of ammonium (30, 33), and the NO2− can lead to both N2O (11) and NO (27). In aerobic soil, NO2− is also readily oxidized to NO3−. Depletion of O2 results in rapid reduction of NO3− and an increase in NO2− concentration. Nitrite decomposition by chemical or biological denitrification processes then produce NO, N2O, and N2 in anaerobic soil (6, 19, 33). The measured amounts of NO are usually very small, however, for several reasons. First, in aerobic soil both NO2− and NO are oxidized by O2, the latter in a termolecular reaction to form NO2, which in turn reacts with soil water to form NO2− and HNO2 (1, 3). Thus, moist soil can be an effective sink for NO plus NO2 (19, 21). Second, in anoxic soils NO can also be enzymatically reduced to N2O and N2 (6). Therefore, it is not surprising that relatively few measurements of NO production from soils have been made (9).

We have recently described a steady-state gas flow system or gas-stripping method (18), similar in many respects to that used by Gersberg et al. (10), Tiedje et al. (29), and Wickramasinghe et al. (32). The system has considerable advantages over static methods for measurement of gaseous production rates. In particular, continuous stripping prevents accumulation of evolved gases and minimizes their subsequent transformation, a complication inherent in static methods (15). The flow technique allows rate measurements to be made over relatively short time intervals and thus under relatively constant substrate conditions. Because chemical and biological removal of NO is considerably reduced in our system, net NO production rates can be readily measured in the same way as was demonstrated previously for N2O (18).

In this paper we present some measurements of NO and N2O production rates from an anaerobic Brookston clay soil column. The results may have implications concerning the mechanisms of denitrification processes, and particularly the role of NO in the sequence.

MATERIALS AND METHODS

A Perkin-Elmer model 3920B gas chromatograph (GC) equipped with a 63Ni electron capture detector and a Porapak Q stainless-steel column (3 m by 3 mm) was used for N2O, C2H4, and C2H2 analyses. Operating conditions for N2O were as described previously (18).
Nitric oxide levels were determined by using a Columbia Scientific Industries NO/NO₂/NO₃ analyzer, model 1600. Analysis is based on the rapid chemiluminescent reaction of NO with O₂. Continuous monitoring over a range of 0.002 to 50 ppm (vol/vol) of NO can be achieved. Our measurements normally ranged from ~0.01 to 3 ppm. Use of the GC for NO determination according to Kaspar and Tiedje (13) was ruled out, since many of our measurements were below or near the GC detection limit for NO.

Nitrite analyses were carried out according to the procedure recently developed by Cox (4) whereby NO₂⁻ was reduced to NO by I⁻ in weakly acidic medium, and the NO was then determined with the NO/NO₂/NO₃ analyzer.

Nitrate was determined by specific ion electrode (Orion Ionalyzer model 401). The pH was determined by using filtered soil water extracts (10 g of soil to 100 ml of water). Soil moisture was determined gravimetrically.

The apparatus (Fig. 1) was a modified version of the one described previously (18). Soil samples, each about 100 g, were contained in five thermostatted Pyrex tubes (2.5 cm [inside diameter] by 40 cm long). Each tube was connected via a needle valve (Nupro Fine Metering) to a copper pipe manifold which provided a constant supply of argon carrier gas (Liquid Carbonic, 99.995%). Flow rates of argon to each column were controlled by adjustment of the needle valve and measured at the outlet of each column by means of a calibrated rotameter flowmeter (Gilmont, size 1). Direct connections were made to both the GC and the NO/NO₂/NO₃ analyzer as shown in Fig. 1.

During operation, the NO/NO₂/NO₃ analyzer required a steady flow of sample gas or zero gas at constant pressure. To achieve this, the sample tube from the soil column was connected to the analyzer inlet via a “tee” in flask A which was continuously flushed with zero gas, nitrogen (Liquid Carbonic, 99.995%, NO < 0.005 ppm) and maintained at a pressure only slightly above atmospheric. With this arrangement, flow rates through the soil columns could be varied over a very wide range (0 to 500 cm³/min) without any significant variation in flow to the analyzer. In practice, a constant flow of ~300 cm³/min to the analyzer was maintained and, when necessary, appropriate dilution factors for determination of concentration of NO in the column gas effluent could be precisely (~1.9%) (see below) determined from the ratio of the measured flow rates at R₁ (which measures carrier plus effluent gas flow through the column) and R₂ (which measures total gas flow, including zero gas).

Nitric oxide calibrations were made by using commercial analyzed gas mixtures, ~10 ppm of NO in N₂ (Scott Specialty Gases). Flow rates of the calibration standards were chosen to span the range of NO levels normally measured (~0.01 to 3 ppm). Calibration procedures and standards for N₂O were described previously (17).

Samples of Brookston clay soil (Orthic Humic Gleysol) were obtained from the surface (~10 cm) and stored at 4°C in plastic bags without drying. Before use, moist soil was screened through a 5-mm sieve. About 600 g of sieved soil was spread on a plastic sheet and evenly sprayed with about 25 ml of 0.03 M NH₄NO₃ solution. After thorough mixing, approximately 100-g weighed portions of the soil were loosely but uniformly packed into each of the five flow tubes.

To establish anaerobic conditions and start an experiment, the soil column was quickly flushed with argon at a relatively high flow rate. After several minutes, the flow was reduced to a somewhat lower constant level. At carrier flow rates greater than 60 cm³/min, steady-state conditions were established within about 10 to 20 min.

Net production rates of NO and N₂O were calculated by multiplication of the fraction of these gases in the effluent flow by the flow rate through the soil column measured at that time, corrected for any dilution at the tee. The pH, water, NO₂⁻, and NO₃⁻ levels at a particular time were obtained by analysis of each replicate soil column in turn over a 48-h period.

To determine NO₂⁻ levels, 20-g soil samples were stirred vigorously for several minutes with 100 ml of water, vacuum filtered through a Whatman no. 3 filter paper, and then vacuum filtered through a 0.22-μm membrane filter (Millipore type GS) for removal of soil bacteria. The samples were stored in the dark at 4°C until the analysis could be completed (2 days maximum). Experiments showed that NO₂⁻ was stable under these conditions for periods of at least up to 4 days. The solution was then acidified to 2.3 M acetic acid, placed in reaction vessel D at 20°C, and purged with a constant flow (~340 cm³/min) of argon while connected to the NO/NO₂/NO₃ analyzer. Addition of O₂-free I₂ (1 ml, 0.2 M NaI) to the reaction vessel immediately released NO which was swept as a “plug” into the chemiluminescent analyzer. The peak
observed by the analyzer was recorded and automatically integrated. Typically N\textsubscript{02}- levels over the range of \(3 \times 10^{-8}\) to \(35 \times 10^{-8}\) g of NO\textsubscript{2}N\textsubscript{2}O\textsubscript{3} of soil (30 to 350 ppb, wt/wt) were obtained. Calibrations were made by using carefully prepared standard NaNO\textsubscript{2} solutions. Cox (4) reported no apparent interference by up to \(10^4\) M excess NO\textsubscript{3}. In our studies, NO\textsubscript{3} was never in excess of NO\textsubscript{2}- by more than \(7 \times 10^{-8}\) M.

The following sterilization procedure was used to approximately estimate the relative importance of chemical production of NO and N\textsubscript{2}O from Brookston clay soil treated with NH\textsubscript{4}NO\textsubscript{3} and NaNO\textsubscript{2}. Columns, each containing \(100\) g of soil which had been sprayed with approximately \(10\) ml of \(2.9 \times 10^{-2}\) M NH\textsubscript{4}NO\textsubscript{3} or \(1.6 \times 10^{-3}\) M NaNO\textsubscript{2} solution, were autoclaved at 123°C and a pressure of 18 lb/in\(^2\) for 15 min. These were then studied anaerobically at 20°C along with nonsterile but otherwise identical control soil columns. Since rapid depletion of NO\textsubscript{3} occurred at the high autoclave temperature, an additional soil column with the NO\textsubscript{3}- solution added after the sterilization procedure was studied.

Estimations of random errors including column-to-column variability were made for each of the experimental measurements as follows. Standard deviations \((\sigma)\) were determined for a number of replicate measurements of water, pH, NO\textsubscript{3}, and NO\textsubscript{2}, lying within the range normally encountered. Error limits of \(\pm 2\sigma\) (95% confidence level) were then assigned to individual measurements as a percentage of the particular measurement. These were \(\pm 2.1, \pm 0.5, \pm 7.3\), and \(\pm 8.3\)%, respectively.

In the case of N\textsubscript{2}O, random errors in the measurements are relatively small, estimated to be \(\pm 1\) or less (17). For NO, similar estimates apply to reproducibility of successive measurements of air, soil column gas, or standard mixtures. In both cases, however, rather substantial systematic errors may arise, partly because of the error limits in the calibration gases. These were assigned by the manufacturers as \(\pm 2\%\) for NO and \(\pm 10\%\) for N\textsubscript{2}O.

To determine error limits on net rates of production of NO and N\textsubscript{2}O and of f\textsubscript{NO} and f\textsubscript{N\textsubscript{2}O}, respectively, the propagation-of-errors equation was applied following Cvetanovic et al. (5):

\[
\sigma_Q = \left[ \sum_i \left( \frac{\partial F}{\partial M_i} \sigma_i \right)^2 \right]^{1/2}
\]

(1)

where \(Q\) is the quantity being determined in the experiment from measurements \(M_i\), \(Q = F(M_i)\), \(\sigma_i\) is the standard deviation of the uncorrelated separate measurements \(M_i\).

Since f\textsubscript{NO} = \(f_c\text{[NO]}/m\), where \(f_c\) is the measured total flow rate of column effluent gas, [NO] is determined from the instrument reading (in parts per million), and \(m\) is the mass of soil in the column, it was necessary to obtain error limits on \(f_c\), NO, and \(m\). The rotameters were carefully calibrated against bubble flowmeters, and their precision was found to be within the manufacturer's specifications (~1%). In practice, values of \(f_c\) were taken from plots of scale reading versus calibrated flow rate. The error limits, calculated as the standard error of the estimate (20), amounted to \(\pm 1.9\%\). The error in [NO] was taken as \(\pm 2\%\) (above), and the error in \(m\) was only 0.01%. Substitution of these values into the propagation-of-errors equation leads to an error \(2\sigma\) of \(\pm 5.4\%\) in f\textsubscript{NO}.

Similarly, using \(\pm 10\%\) for [N\textsubscript{2}O] in f\textsubscript{N\textsubscript{2}O} = \(f_c\text{[N\textsubscript{2}O]}/m\), we calculate an error \(2\sigma\) of \(\pm 6.0\%\) in f\textsubscript{N\textsubscript{2}O}.

The quantity \((d\text{[NO\textsubscript{3}]/dt})\) is calculated from the slope of the [NO\textsubscript{3}]-versus-time plot (see Fig. 4) for which the least-squares regression equation is \([\text{NO\textsubscript{3}]} = -2.55 \times 10^{-11}t + 2.76 \times 10^{-8}\), where the concentration is expressed in units of moles of NO\textsubscript{3}N per gram of soil, and time, \(t\), is in minutes. The correlation coefficient \(r\) is 0.988, and the coefficient of determination \(r^2\) is 0.976. The error limits \(2\sigma\) in the slope (16) are \(\pm 16\%\). The combined effect of these errors on f\textsubscript{N\textsubscript{2}O} is calculated by using equation 1 to be \(\pm 26\%\).

**RESULTS**

Some effort was made to ensure positive identification of NO in soil gas effluent and to determine whether other gases would seriously interfere with analyses, since unsaturated hydrocarbons such as C\textsubscript{2}H\textsubscript{4} and C\textsubscript{2}H\textsubscript{2} could interfere with NO determinations as a result of possible reaction of these gases with O\textsubscript{3} in the analyzer reactor. To check this possibility, carefully metered flows of C\textsubscript{2}H\textsubscript{2} and C\textsubscript{2}H\textsubscript{4}, with and without NO, were added to the NO/NO\textsubscript{2}/O\textsubscript{3} analyzer. The results indicated a very low sensitivity for either C\textsubscript{2}H\textsubscript{2} or C\textsubscript{2}H\textsubscript{4}. The lower detectable limit was ~600 to 800 ppm, whereas under identical conditions the lower limit for NO was 0.005 ppm. To be certain that NO was indeed being detected and measured, retention times of NO relative to N\textsubscript{2}, O\textsubscript{2}, N\textsubscript{2}O, C\textsubscript{2}H\textsubscript{4}, and C\textsubscript{2}H\textsubscript{2} were determined on a Porapak Q column, using the electron capture detector GC. Injection of a relatively large sample of soil gas effluent to a Porapak Q column attached to the inlet of the chemiluminescent analyzer confirmed the identity of NO and showed that unsaturated hydrocarbon levels were completely negligible.

Figure 2 shows a typical recorder trace of the NO production obtained from a Brookston clay soil column at 20°C. Anaerobic conditions, constant temperature, and flow rate were established at time zero. The NO yield increased to a maximum level within about 4 h and then exhibited a very gradual decrease of ~6 \(\times 10^{-5}\) ppm/min over the next 14 h. Similar behavior was observed for N\textsubscript{2}O (see Fig. 4 and reference 18). Over very short time intervals (minutes) the rate could be considered nearly constant, implying that substrate and enzyme concentrations also remained nearly constant.

Both NO and N\textsubscript{2}O are subject to further reduction in the denitrification process, with N\textsubscript{2} being the ultimate product. In this flow system, the production of N\textsubscript{2} (and N\textsubscript{2}O from NO) is mediated by the removal of reactant gases, NO and N\textsubscript{2}O, by carrier. The rates of subsequent reduction reactions of NO and N\textsubscript{2}O will be
achieved primarily by use of direct on-line sampling at a fixed pressure. Previously (18), injection of sample by means of a gas-tight syringe was used. Total flow rates of about 300 to 500 cm³/min were usually used to ensure that steady-state conditions were obtained.

Although during short time intervals net production rates of NO and N₂O were nearly constant, over a much longer time period after the maximum at ~4 h, net rates of both gases gradually declined in response to the slowly changing substrate or enzyme concentrations. Figure 4 shows the concentrations of these gases in the effluent versus time along with NO₃⁻ and NO₂⁻ in the soil from a series of columns at 20°C. The pH and water remained constant at 7.26 ± 0.02 and 14.6 ± 0.2%, respectively. The NO₃⁻ concentration, which was moderately high, decreased slowly at a rate of only 2.54 × 10⁻¹⁰ mol of N/g of soil per min (0.213 ppm/h). The most striking feature of Fig. 4, however, is the variation in NO concentration, which approximately paralleled the change in NO₃⁻ concentration. A similar although perhaps less obvious change was observed for N₂O. After the onset of anoxic conditions, there was a rapid increase in NO₂⁻ level, 5.23 × 10⁻⁹ to 3.69 × 10⁻⁸ mol of NO₂⁻/g of soil in the first 645 min, followed by a more gradual decrease of 1.42 × 10⁻¹⁰ mol of N/g of soil per min. Similar behavior was observed for NO, which increased in the gas effluent from ~0 to 1.40 × 10⁻¹³ mol of NO-N/cm³ per g of soil over the first 400 min, followed by a decrease of 3.60 × 10⁻¹⁷ mol of N/cm³ per g of soil per min. It should also be noted

![Figure 2](http://aem.asm.org/Downloaded from http://aem.asm.org/)

**FIG. 2.** Typical recorder trace of NO measurements obtained for a Brookston clay soil column at 20°C. Instantaneous rates can be calculated at any time.

decreased, the extent of which will depend on carrier gas flow rate. It is expected, therefore, that NO and N₂O concentrations measured in the outlet stream of the soil column will be a function of total flow rate (carrier plus soil gases). This effect was noted previously for N₂O (18). Figure 3 shows NO and N₂O results obtained for Brookston clay soil at 20°C. The net production rates of both gases increased to a plateau as the total flow rate was increased by changing the carrier gas flow rate. Constant rates were obtained for both gases at all total flow rates between ~120 and ~550 cm³/min. This range of flows was considerably greater than was used in our earlier study (18) because of improved analytical sensitivity which was

![Figure 3](http://aem.asm.org/)

**FIG. 3.** Net NO and N₂O production rates as a function of total flow rate. The curves show the trend toward constant rates at high carrier flows. The data shown were obtained by using a Brookston clay soil column at 20°C. Similar results were obtained at other temperatures below 25°C. Reference 18 provides more data for N₂O at the lower total flow rates.

![Figure 4](http://aem.asm.org/)

**FIG. 4.** Variation of concentrations of NO₃⁻, NO₂⁻, NO, and N₂O with time for a Brookston clay soil column at 20°C. The total flow rate remained constant at 522 cm³/min. The concentrations are expressed as N-atoms per gram of soil for NO₃⁻ and NO₂⁻ and as N-atoms per gram of soil per cubic centimeter of gas effluent for NO and N₂O.
that NO concentrations in the column effluent were considerably greater than N₂O levels. The ratio [NO-N]/[N₂O-N] varied from ~2.6 at 400 min to ~2.0 at 2,800 min.

Figure 5 shows the variation in net production rates of NO, N₂O, and estimated N₂. The changes in rates corresponded to the changes in concentration shown in Fig. 4 as expected, since the carrier gas flow rates remained constant at 522 cm³/min over the period. The calculation of the rate of N₂ production was based on the assumption that all losses of NO₃⁻ ultimately result in gaseous NO, N₂O, or N₂, following the simplified mechanism described in the discussion below (see equation 5). It was also assumed that assimilatory removal of NO₃⁻ was negligible, as observed for a variety of soils by Reddy et al. (24). The rate of disappearance of NO₃⁻ was taken as the slope of the concentration-time curve in Fig. 4. Whereas net NO and N₂O rates increased to a maximum and then slowly decreased, the calculated net N₂ rate appeared to follow a reverse trend, first decreasing rapidly to a minimum and then gradually increasing (Fig. 5).

Figure 6 represents results from an early experiment showing the variation in rates of NO and N₂O with temperature. This experiment was done by using a similar Brookston clay column, but in this case injection of sample by syringe was used for the N₂O measurements. The trends clearly showed the rise and fall in rates typical of biological enzyme processes (23).

Table 1 summarizes a few experiments in which NO and N₂O production from sterile soils was measured after addition of NH₄NO₃ and NaNO₂. Results of control experiments are shown for comparison. Inspection of data presented in rows 1 and 2 shows that sterile soil treated with NH₄NO₃ was completely inactive with respect to NO or N₂O production (fNO < 1.0 × 10⁻¹² mol of N/min per g of soil; fN₂O < 5.0 × 10⁻¹⁴ mol of N/min per g of soil) in spite of the relatively high concentration of NO₃⁻. In contrast, the control soil showed considerable activity. The data in rows 3, 4, and 5 show the effects of addition of NaNO₂. The net rate of NO production from the sterile soil was ~45% of the rate from the control for roughly equal NO₂⁻ levels. With much lower NO₂⁻ concentrations (row 4), chemical production of NO was insignificant. In both cases, fN₂O was less than 5.0 × 10⁻¹⁴ mol of N/min per g of soil.

**DISCUSSION**

Figure 4 shows the rapid increase in [NO₂⁻] followed by its gradual decline about 4 h after anaerobic conditions are established. Similar behavior is exhibited by NO and N₂O, although the initial increase in N₂O is less pronounced. This behavior is typical of a reaction occurring via consecutive steps. The relative concentrations of the intermediates and the time scale observed depend on the magnitudes of the various rate constants in the sequence. Use of the flow system decreases the rates of disappearance of the gaseous intermediates within the soil system by simply lowering concentrations, assuming that the gas removal rates are not zero order. Thus, the transitory behavior of each intermediate is more obvious.

Our data are not sufficient to resolve more detailed aspects of the mechanism, for example, whether NO is a free gaseous intermediate or in equilibrium with another enzyme-bound precursor. Questions such as these have been discussed by others (6, 7, 12, 28, 33), particularly

**FIG. 5.** Observed net production rates of NO and N₂O and estimated production rates of N₂ as a function of time. The rates were calculated from the data for NO, N₂O, and NO₃⁻ concentration changes shown in Fig. 4.

**FIG. 6.** Effect of temperature on the net rates of production of NO and N₂O for Brookston clay soil.
for specific denitrifying organisms. The flow method, however, facilitates the determination of reactive gaseous intermediates and, more importantly, permits the relative magnitudes of their rates in relation to the overall process to be readily determined.

The results can conveniently be discussed in terms of the simplified overall mechanism suggested by Payne (22):

\[
\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2
\]

The kinetic reaction orders with respect to each intermediate are not known, but for simplicity are all assumed to be unity with the exception of \(\text{NO}_3^-\) removal, which is taken to be zero order (Fig. 4).

In the scheme, all four steps are enzymatic processes. A fifth reaction,

\[
\text{NO}_2^- \rightarrow \text{NO}
\]

accounts for nonenzymatic chemical decomposition of \(\text{NO}_2^-\).

Table 1 provides evidence to suggest that chemodenitrification production of \(\text{NO}\) may be relatively significant in the soil. Under mildly acidic conditions, self-decomposition of nitrous acid may constitute a major mechanism of nitrite loss (30). The decomposition of \(\text{HNO}_2\) is pH dependent and is most significant at \(\text{pH} < 5\). Although the measured \(\text{pH}\) in the soil studied here was greater than 5, there could have been local sites within the soil where the \(\text{pH}\) was lower (27). The fraction of \(\text{NO}\) produced by reaction 5 may be as large as \(\sim 45\%\) (Table 1). Figure 6 shows that biological production of \(\text{NO}\) or at least biological production of its precursor, presumably \(\text{NO}_2^-\), is important. This is consistent with the correlation previously noted between \(\text{NO}\) and \(\text{NO}_2^-\) concentration and the relatively high chemical production of \(\text{NO}\) in sterile soil to which \(\text{NO}_2^-\) has been added.

Some reasons why \(\text{NO}\) levels in aerobic and anaerobic soils are low and thus why measurements of rates of production and removal have often eluded experimentalists have been mentioned in the introduction. In this work, it was possible to measure net production rates of \(\text{NO}\) because subsequent removal of \(\text{NO}\) by further reaction was minimized by continuous stripping from the soil. In earlier work (18), using this technique, we measured net rate and temperature dependence of \(\text{N}_2\text{O}\) production in the same type of soil. Since \(\text{N}_2\text{O}\) is a less reactive substance than \(\text{NO}\), the process or processes govern- ing removal of \(\text{N}_2\text{O}\) are not as rapid, and therefore higher levels of \(\text{N}_2\text{O}\) can build up in the soil atmosphere in natural field situations. Thus, measurements of rates of diffusion of \(\text{N}_2\text{O}\) even from aerobic soils have been made by many groups (e.g., see references 2, 17, 25, and 26 and references therein). On the other hand, there appears to be only one report (9) of direct measurements of \(\text{NO}\) flux from soils in natural field conditions where the ability to measure concentrations of \(\sim 2\) to 16 ppb (vol/vol) was required. In contrast, determination of \(\text{N}_2\text{O}\) flux in the field requires only a capability of measuring \(\text{N}_2\text{O}\) at approximately 300 ppb (ambient level). The \(\text{NO}\) fluxes measured by Galbally and Roy (9) were in the range \(0.06 \times 10^{-11}\) to \(0.73 \times 10^{-11}\) kg of \(\text{N/m}^2\) per s, which can be compared with rates of \(\text{N}_2\text{O}\) evolution of \(\sim 0.05\) to \(500 \times 10^{-11}\) kg of \(\text{N/m}^2\) per s measured over Brookston clay in natural field situations (17).

The surprisingly high net production rates of \(\text{NO}\) obtained in this study (Fig. 5) are themselves evidence of rapid removal rates by subsequent reactions if the \(\text{NO}\) had not been stripped out of the soil. Thus, the \(\text{NO}\) measurements, in particular, illustrate the advantage of this flow

### Table 1. Net rates of production of \(\text{NO}\) and \(\text{N}_2\text{O}\) from sterile and nonsterile Brookston clay soil (column temperature, 20°C)

<table>
<thead>
<tr>
<th>Soil treatment</th>
<th>(\text{NO}_3^-) (mol of (\text{N} \times 10^{-3}/\text{g of soil}))</th>
<th>(\text{NO}_2^-) (mol of (\text{N} \times 10^{-3}/\text{g of soil}))</th>
<th>Time (min)</th>
<th>(f_{\text{N}_2\text{O}}) (mol of (\text{N} \times 10^{-11}/\text{g of soil per min}))</th>
<th>(f_{\text{NO}}) (mol of (\text{N} \times 10^{-11}/\text{g of soil per min}))</th>
<th>pH</th>
<th>Water (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH(_4)NO(_3) Control</td>
<td>3.89</td>
<td>1.74</td>
<td>140</td>
<td>4.00</td>
<td>7.75</td>
<td>7.0</td>
<td>19.0</td>
</tr>
<tr>
<td>Sterile</td>
<td>3.57</td>
<td>0.161</td>
<td>140</td>
<td>&lt;0.005</td>
<td>&lt;0.10</td>
<td>7.0</td>
<td>17.1</td>
</tr>
<tr>
<td>NaNO(_2) Control</td>
<td>1.32</td>
<td>13.1</td>
<td>140</td>
<td>5.35</td>
<td>15.4</td>
<td>6.5</td>
<td>20.2</td>
</tr>
<tr>
<td>Sterile</td>
<td>1.32</td>
<td>0.246</td>
<td>140</td>
<td>&lt;0.005</td>
<td>&lt;0.10</td>
<td>6.6</td>
<td>17.8</td>
</tr>
<tr>
<td>A</td>
<td>1.29</td>
<td>11.4</td>
<td>157</td>
<td>&lt;0.005</td>
<td>7.01</td>
<td>6.1</td>
<td>20.8</td>
</tr>
</tbody>
</table>

* These are the times after onset of anoxic conditions at which samples were taken for analysis.

* Soil was sprayed with NaNO\(_2\) solution before (A) and after (B) sterilization.
method for kinetic studies of gaseous intermediates in soils and other media.

Reference to the time scale in Fig. 2 and 4 shows that, except for the initial ~20-min period, related to the onset of anaerobic conditions in the soil column, the concentrations of substrates and intermediates change relatively slowly. As a result, at constant carrier flow rate, steady-state conditions can be assumed provided the temperature remains constant and the rate of change of bacterial population, pH, moisture, and concentrations of available carbon, nitrate, and enzyme are negligibly low relative to the time required to complete a measurement. Since measurements can be made continuously or in minutes, the assumption would seem to be justified. This is even the case for NO, where the variation in rate is relatively significant over the time intervals of several hours (Fig. 5).

In the steady state, the rates of production of NO and N2O are balanced by their removal by carrier and by any further reaction within the soil column. Figure 3 shows that the steady-state condition corresponding to the plateau regions is obtained over a relatively wide range of total flow rates. An explanation of the dependence of net production rate of N2O on total flow rates was presented previously (18). The rate of production of N2O, expressed as \( k_3[NO] \), is balanced by removal by carrier, \( f_{N_2O} \), and by reaction to form N2:

\[ k_3[NO] = f_{N_2O} + k_4[N_2O] \tag{2} \]

It is assumed that concentrations of gases obtained are proportional to their concentrations in aqueous solution within the soil and at the enzyme site. The plateau region at high total flow rates corresponds to the condition where formation of N2 from N2O is reduced to a minimum level, which is obtained when a relatively low concentration of N2O reactant remains in the soil.

For NO, a similar argument can be applied:

\[ (k_2 + k_3)[NO_2] = f_{NO} + k_3[NO] \tag{3} \]

where the production of NO is balanced by removal by carrier gas and in this case by reaction to form N2O (\( k_3 \) is the chemical reduction rate constant). Since N2 is the terminal product, it is removed only by carrier gas:

\[ k_4[N_2O] = f_{N_2} \tag{4} \]

The terms \( f_{NO} \), \( f_{N_2O} \), and \( f_{N_2} \) correspond to the net rate of production of each gas. Net rates of NO production were always greater than net rates of N2O production (Fig. 5). This same pattern was observed in similar experiments (not shown) for periods of up to 12 days (20°C, continuous flow). Also in the steady-state approximation,

\[ \frac{d[NO_2]}{dt} = k_1 - (k_2 + k_3)[NO_2] = 0 \tag{5} \]

where nitrate reduction is assumed to be zero order, i.e., \( d[NO_2]/dt = -k_1 \).

Combination of equations 2 to 5 leads to the following relationship:

\[ \frac{d[NO]}{dt} = f_{NO} + f_{N_2O} + f_{N_2} \tag{6} \]

Equation 6 provides the basis for an estimate of \( f_{N_2} \), since all other terms are known (Fig. 4 and 5). The results (Fig. 5) show that the calculated rates of N2 production apparently decline rapidly during the initial period (0 to 300 min), whereas net rates of NO and N2O increase. This behavior is then followed by a slow increase in the net N2 production rate as NO and N2O rates gradually decline. Although our results for N2 may not be conclusive, they would appear to support the observations of Firestone and Tiedje (8), who obtained a somewhat similar pattern of net N2 and N2O production rates by using a static system. Over an extended period of time the ratio N2O/N2 is therefore predicted to decrease with time, in agreement with experimental observations of Letey et al. (14, 15) and Firestone and Tiedje (8). Thus, our results may support the proposals expressed by these authors; i.e., the pattern of increase and decline of N2O with a gradually increasing proportion of N2 results from staggered synthesis of denitrification enzymes under anaerobic conditions (8). Specifically, the enzyme dissipimilatory nitrate reductase develops rapidly, but the enzyme dissipimilatory nitrous oxide reductase develops more slowly after the onset of anoxic conditions (14, 15). Other explanations have been considered (15). Wickramasinghe et al. (31, 32) attribute some nitrate losses to immobilization in the soil.

The continuous-flow method we describe provides a major advantage over static techniques based on head space analysis of gaseous products of denitrification. The flowing inert carrier gas lowers the concentrations of gaseous intermediates within the soil matrix and thereby reduces the rates of subsequent and back-reactions, best illustrated by the results obtained for NO. Very low concentrations would exist over a static soil column because of the pronounced reactivity of NO. The flow system described here overcomes this disadvantage and permits measurement of NO by the chemiluminescent analyzer, which we found to be ideal for the purpose in terms of both sensitivity and specificity. Thus, the intermediate steps in the denitrification sequence can be more readily identified and characterized. Over several days the change
in rates of these gases and NO$_3^-$ followed a pattern of increase and decay which might be expected of a reaction proceeding by several consecutive steps.

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LITERATURE CITED


