Production of Nitric Oxide in Loam Under Aerobic and Anaerobic Conditions

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Measurements of gas flow through soil columns of loam from Kjettslinge, Uppland, Sweden, gave average NO production rates of 0.06 ± 0.01 ng of NO N g of soil⁻¹ min⁻¹ in aerobic conditions and 3.7 ± 0.6 ng of NO N g of soil⁻¹ min⁻¹ in anaerobic conditions at 25°C. Approximately 30% of the NO₃⁻ loss in anaerobic conditions was as NO. In aerobic conditions an equilibrium concentration for NO was found. Above this concentration there was uptake of NO. Autoclaved samples indicated that less than 10% of the NO production was abiological, and there was no abiological NO uptake. The NO production reached anaerobic rates at soil O₂ levels between 0.5 and 0.05%.

Nitric oxide, NO, can be produced biologically during the microbial processes of nitrification and denitrification, chemically in soil, and during combustion. It participates in the chemistry of the lower atmosphere. Furthermore, the transport of NO from the sites of soil microbial activity to the atmosphere constitutes one of the pathways of plant nutrient loss (8, 13, 13a, 15, 23, 24).

In spite of the considerable interest in these processes (21), little understanding has been achieved until recently because of the lack of adequate instrumentation to make the necessary measurements. This situation has been remedied, and there are now observations of nitric oxide fluxes from soils (8, 13, 13a), studies of nitric oxide production by pure cultures of nitrifying (14) and denitrifying (7) bacteria, and from soil columns in the laboratory under anaerobic conditions (17).

A full understanding of nitric oxide evolution from soils will require knowledge of the following: (i) to what extent possible biological and abiological processes are involved as sources or sinks for NO in soils, (ii) whether biological processes are involved and whether NH₄⁺ oxidation or NO₃⁻ reduction is the main contributor, and (iii) how these processes influence NO production and consumption.

There are three processes that are likely to cause the production of NO in soils: biological nitrification and denitrification and chemical denitrification.

Ritchie and Nicholas (22) showed that NO release is an alternative terminal pathway in the nitrifying process by Nitrosomonas sp., and they proposed NO as an intermediate before NO₂⁻ production, but after the branch leading to N₂O production. They suggested that this same bacterium, when reducing NO₂⁻, produces NO first as an intermediate and subsequently N₂O. There has been some debate about whether NO is an obligatory intermediate (presumably enzyme bound) in the nitrifying process, because so far there has been no demonstration of an enzyme-catalyzed conversion of NO to NO₂⁻ (11, 12).

One laboratory study has shown increased NO released relative to NO₂⁻ production for a soil Nitrosomonas sp. at reduced O₂ levels, 0.5 versus 21% (14). No other evidence is available about environmental factors influencing NO release by nitrifying bacteria.

More detailed information exists about NO production in denitrifying processes. However, some points of conflict remain as to whether either free (gaseous) NO or enzyme-bound NO is an obligatory intermediate (7, 10). It appears that in some bacteria NO is either a free intermediate or is in rapid equilibrium with an intermediate between NO₂⁻ and N₂O in the reduction process. The sequence of denitrification of nitrate NO₃⁻ is usually written as follows (21): NO₃⁻ → NO₂⁻ → NO → N₂O → N₂. Various possible biochemical pathways have been proposed to explain this sequence (2) and provide a basis for designing further experiments. It has been shown that the reduction of gaseous NO is energy yielding for the denitrifying bacterium Paracoccus denitrificans (9). This and other work (7) provide direct evidence of the uptake of NO by denitrifying bacteria. No equivalent evidence has been produced for nitrifying bacteria.

A strong temperature dependence of NO release from denitrification in soil was observed by McKeeney et al. (17). The influence of other environmental factors has not been investigated.

Chemical denitrification involves the release of NO due to the self-decomposition of NO₂⁻ in soils or by the reaction of NO₂⁻ with soil organic matter and minerals (1, 4). There have been many laboratory studies of this process (5, 18–20, 25, 26), but there is no general agreement on the mechanisms or environmental factors influencing the rate of NO production.

MATERIALS AND METHODS

In this paper we report on NO production in laboratory experiments with soil columns run at different environmental conditions with a system similar to that of McKeeney et al. (17). The soil, a loam topsoil from an agricultural field, Kjettslinge, Uppland, Sweden, was crushed, sieved (2 mm), and air dried to 4.5 g of water per 100 g of dry soil. The soil was stored at 4°C. The soil properties were pH 6.3, 2.2% organic carbon, 0.23% total N (E. Steen, P.-E. Jansson, and J.Persson, Acta Agric. Scand., in press). The soil moisture was made up to ~30 g of H₂O per 100 g of dry soil for all but one of the laboratory measurements.

The experimental layout used is shown in Fig. 1. A 250-g
A sample of moist soil was held in a Plexiglas tube (5.0-cm internal diameter), occupying 12 to 15 cm of the tube, giving a bulk density of ~1 g cm⁻³ or a porosity of around 50%. The soil was held in place by membrane filters (in holders) at each end. These filters were Millipore type Zefluor, 2-μm pore size. There was a continuous gas flow through the soil columns.

The gases used were industrial grade nitrogen and air from AGA, Sweden. The concentrations of gases measured in both nitrogen and air cylinders were as follows: NO, ~1 ppbv (10⁻⁹ m³/m³); N₂O, <0.5 ppmv (10⁻⁶ m³/m³). A molecular sieve (1.0 nm) gas scrubber was installed after the cylinder regulator to remove any organic contaminants.

Experiments conducted removing and replacing the sieve showed no change in the NO evolution from the soil. Gas flow regulation and measurement were achieved with thermal conductivity mass flow meters-regulators, integrating gas meters, and soap bubble meters. The gas flows obtained from the mass flow meter readings were within ±3% of the values derived from the integrating gas meters and soap bubble meters.

The gas was humidified by bubbling through humidifiers (containing ~250 ml of water) before entering the soil column. The relative humidity was probably >99%, but precise measurements could not be made at this high relative humidity. The soil columns showed negligible (<1-g) weight change during a sequence of 3 days of gas flow. All gas lines after the soil tubes were made of Teflon and were covered on the outside with heating wires to prevent condensation. Tube couplings were made of polypropylene. A Teflon 2-μm, 47-mm-diameter particle filter was in the line before the analyzer. There was no detectable uptake of NO in tubing, filters, and empty soil tubes. The nitric oxide analyzer was a Thermo Electron series 14 modified by the procedures of Delany et al. (6). The detection of NO is based on the chemiluminescence of NO₂, which is produced during the reaction between NO and O₃. Excess O₃ is added to the sample containing NO, which is then passed through a chamber with infrared-reflective walls. Interfering signals from ozonolysis of other materials are prevented by a filter, which blocks radiation below 600 nm. Nitrogen dioxide (NO₂) was measured after conversion to NO. Two types of converters were used, a thermal converter with metallic molybdenum and a chemical converter with ferrous sulfate (FeSO₄).

The sensitivity of the experiment was ±0.2 ppbv. Calibration was made with 1.02 ± 0.05 and 50.5 ± 1.0 ppmv NO in N₂ gas standards obtained from Alfax, Malmö and AGA Special Gas, Lidingö, Sweden, respectively. Nitrous oxide gas samples were collected in Venoject (Meditratt, Stockholm) evacuated tubes for blood sampling and analyzed by gas chromatography with an electron capture detector. Soil samples were taken before and after each experiment, frozen, and subsequently analyzed for NO₃ and NH₄⁺ by the method of Berg et al. (3).

Three soil columns were run simultaneously in each experiment and generally sampled sequentially on a 30-min cycle. The soil temperature was 24 to 25°C.

The net production or uptake of NO by the soil column, \( f_{NO} \), was calculated from the relationship \( f_{NO} = \nu ([NO₃]₀ - [NO₃]) \), where \( \nu \) is the gas flow through the column, the brackets denote concentration, and the subscripts denote the position along the column (e and o denote exit and entrance, respectively). Appropriate corrections were made when the column effluent was diluted before the NO measurement. The experimental limit of detection of \( f_{NO} \) was 0.001 × 10⁻⁹ g of NO g of soil⁻¹ min⁻¹. The accuracy of the flux measurements was approximately ±10%, allowing for errors in both the NO concentration measurements and the flow rate measurements.

RESULTS AND DISCUSSION

Sixteen soil tubes were run under aerobic (air or air plus N₂) to decrease oxygen concentrations) or anaerobic (N₂) conditions (or both) to evaluate NO fluxes. Table 1 gives details of the experimental arrangements used, the total production of NO, and the concentrations of NH₄⁺ and NO₃⁻ before and after the flux measurements.

Five tests were made to clarify the processes taking place in the experimental arrangement.

(i) Controls were run to ensure that the compound measured was NO and not any unsaturated hydrocarbon, e.g., C₂H₄. A combination of tests with oxidation of the column effluent by O₃, where NO is converted to NO₂, and thermal catalytic conversion of NO₂ to NO suggested that it was improbable that the signal originated from any compound except NO. Quantitative recovery of the original signal was obtained by this oxidation/reduction procedure. The sensitivity of the detector to C₂H₂ was observed to be ~10⁻⁹ of its sensitivity to NO.

(ii) The relative importance of biological and abiological NO production was investigated by comparing the NO production in control tubes with autoclaved tubes (treated for 20 min at 2 to 3 bar water absolute pressure).

An increase of the production in the autoclaved columns after 36 to 48 h indicated a reestablishment of the microflora. The production of NO during the first 24 h after autoclaving was smaller by a factor of 10 to 10³ compared with the production occurring in the control samples. Hence, to a first approximation, abiological NO production can be neglected in this system, provided, of course, that any source of abiological NO production, e.g., NO₃⁻, was not destroyed by the autoclaving. These experiments do not rule out the possibility that chemical decomposition of biologically generated NO₃⁻ is an important mechanism for NO production in soils. The results of McKenney et al. (17) indicated that decomposition of NO₃⁻ might account for 45% of the NO produced. However, we interpret these experiments as indicating that under their control conditions...
TABLE 1. Total production of NO and soil concentrations of \( \text{NH}_4^+ \) and \( \text{NO}_3^- \) before and after flux measurements

<table>
<thead>
<tr>
<th>Column no.</th>
<th>Time used (h)</th>
<th>Gas</th>
<th>( \mu \text{g of NO N} \text{g of soil}^{-1} )</th>
<th>( \mu \text{g of N g of soil}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NO evolved</td>
<td>( \text{NH}_4^+ ) before</td>
<td>( \text{NH}_4^+ ) after</td>
</tr>
<tr>
<td>1</td>
<td>190</td>
<td>Air-( \text{N}_2 )</td>
<td>4.4</td>
<td>NM</td>
</tr>
<tr>
<td>2</td>
<td>62</td>
<td>Air</td>
<td>0.21</td>
<td>9.4</td>
</tr>
<tr>
<td>3</td>
<td>73</td>
<td>Air</td>
<td>0.11</td>
<td>10.0</td>
</tr>
<tr>
<td>4</td>
<td>62</td>
<td>Air</td>
<td>0.21</td>
<td>5.4</td>
</tr>
<tr>
<td>5</td>
<td>48</td>
<td>( \text{N}_2 )</td>
<td>4.5</td>
<td>6.2</td>
</tr>
<tr>
<td>6</td>
<td>48</td>
<td>( \text{N}_2 )</td>
<td>4.6</td>
<td>6.5</td>
</tr>
<tr>
<td>7</td>
<td>48</td>
<td>( \text{N}_2 )</td>
<td>4.8</td>
<td>7.0</td>
</tr>
<tr>
<td>8</td>
<td>25</td>
<td>Air</td>
<td>0.13</td>
<td>12.6</td>
</tr>
<tr>
<td>9</td>
<td>48</td>
<td>Air-( \text{N}_2 )</td>
<td>2.6</td>
<td>17.0</td>
</tr>
<tr>
<td>10</td>
<td>68</td>
<td>Air</td>
<td>0.31</td>
<td>12.2</td>
</tr>
<tr>
<td>11</td>
<td>60</td>
<td>Air-( \text{N}_2 )</td>
<td>0.02</td>
<td>15.5</td>
</tr>
<tr>
<td>12</td>
<td>36</td>
<td>Air-( \text{N}_2 )</td>
<td>0.01</td>
<td>10.7</td>
</tr>
<tr>
<td>13</td>
<td>48</td>
<td>Air-( \text{N}_2 )</td>
<td>0.01</td>
<td>12.2</td>
</tr>
<tr>
<td>14</td>
<td>43</td>
<td>( \text{N}_2 )</td>
<td>6.5</td>
<td>3.9</td>
</tr>
<tr>
<td>15</td>
<td>43</td>
<td>Air-( \text{N}_2 )</td>
<td>6.1</td>
<td>3.6</td>
</tr>
<tr>
<td>16</td>
<td>43</td>
<td>Air</td>
<td>0.19</td>
<td>3.4</td>
</tr>
</tbody>
</table>

\( ^a \) Varied oxygen concentrations (from 0 to 21\% in \( \text{N}_2 \)).
\( ^b \) NM, Not measured.
\( ^c \) This column was later used in an experiment with acetylene. The concentrations of \( \text{NH}_4^+ \) and \( \text{NO}_3^- \) after the experiment with this column are not representative of an aerobic soil column.
\( ^d \) Autoclaved soil.

(no \( \text{NO}_3^- \) addition) only 14\% of the NO produced could have been of biological origin.

(iii) The uptake of NO by nonbiological processes in the soil was measured on the autoclaved soil columns. The NO uptake by these sterile columns was in three cases indistinguishable from the noise level of the measurements (Table 2).

(iv) When a soil column was biologically active, an increase of [NO] in the incoming gas decreased the NO release from the soil. A situation was reached, at some particular [NO] in the incoming gas where NO release from the soil ceased, but no uptake occurred. This concentration can be called the compensation point (the concentration at which no net uptake or release of NO was occurring). At higher incoming [NO], uptake occurred in the soil column. The compensation points were approximately 250 ppbv for column 4 and 400 ppbv for columns 9 and 10 (Fig. 2).

The uptake (calculated as the difference between incoming NO concentration and the concentration at the compensation point divided by the incoming NO concentration) was a factor of 5 to 10 larger than that in the sterile columns; the exact size of this ratio cannot be determined as the uptake in sterile conditions sometimes was less than the resolution of the measurements. It appears that biological uptake was the major process responsible for the loss of NO in these soil columns.

(v) Measurements on columns 14 (anaerobic) and 16 (aerobic) showed that NO\(_2\) production was less than 2\% of the NO production.

These investigations indicated that the gas being evolved was NO and that abiological processes only to a small extent may contribute to the NO production and have a negligible influence on the NO uptake within these columns. The latter point is quite startling in view of the common opinion that physical and chemical processes within the soil efficiently remove NO (1, 4). We suggest that biological processes are the major sink for NO in the soil.

Measurements of NO production made on the soil col-
TABLE 3. Initial productions of NO from freshly made soil columns

<table>
<thead>
<tr>
<th>No. of columns</th>
<th>Gas</th>
<th>NO production (10⁻⁹ g of NO N g of soil⁻¹ min⁻¹)</th>
<th>Special treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>Air</td>
<td>0.057 ± 0.011</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Air</td>
<td>0.020</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Air</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>N₂</td>
<td>3.7 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>N₂</td>
<td>0.003</td>
<td></td>
</tr>
</tbody>
</table>

* Columns contained 250 g of moist soil (30 g of water per 100 g of soil), and gas flow rates were ca. 0.5 liters min⁻¹.

umns during the first 24 h after preparation are presented in Table 3. A standard deviation of ±20% was obtained for multiple flux measurements made on similarly prepared columns. All observations were in a range of ±30%, which provides a measure of the inherent variability of these flux measurements. A remarkable difference in the NO fluxes between aerobic and anaerobic columns was observed. In N₂, the fluxes were 65 times those in air. One soil column (no. 3) was treated with additional water (total of 35 g of water per 100 g of dry soil; Table 3). In aerobic conditions this column showed a factor of 3 lower NO production than the nine columns treated with 30 g of water per 100 g of dry soil (Table 3). As this change in NO production is significant, further experiments are warranted.

To our knowledge, measurements of the production of NO from soil columns during aerobic (nitrifying) conditions have not previously been reported. McKenney et al. (17) made measurements under denitrifying conditions, and their results are compared with ours in Table 4. The production observed in the present investigation at the same flow rate was about a factor 4 larger than that observed by McKenney et al. (17) (see further discussion below). The temperature difference (25 and 20°C) is not believed to cause such a large difference, which may more likely be due to differences in energy supply for the bacteria involved or their abundance (or both).

McKenney et al. (17) stress that the NO and N₂O productions are independent of flow rate in their observations. The establishment of this condition is justified in an earlier paper (16) on the grounds that when this condition is reached the concentration in the soil atmosphere is probably so low that consumption reactions are negligible compared with production. Then the flux from the column approximates the true net production rate.

The NO fluxes from columns during aerobic conditions were independent of flow rate, whereas during anaerobic conditions, the flux increased with increasing flow rate (Fig. 3 and 4). This finding accentuates the difference in NO production between aerobic and anaerobic conditions. The lines connect values from the same columns. Symbols: ⊙, column 1, 10 to 12 January; +, column 1, 5 January; ○, column 2; □, column 3; △, column 4; ×, column 8; ●, column 9; ○, column 10; ◦, column 14; ☐, column 15.

Complete sequences of three soil column measurements are shown in Fig. 5. During most of the time columns 14 and 15 were run under anaerobic conditions, whereas column 16 was run under aerobic conditions. One particular feature of the behavior of column 14 is that the NO production between 30 and 31 March. Similar features were observed with columns 5, 6, and 7, which were also run under anaerobic conditions. The subsequent soil analyses of columns 14 and 15 (Table 1) showed very low NO₃⁻ concentrations and probably these decreases in the production of NO are the result of exhaustion of soil NO₃⁻. The integrated NO loss up to the time of this exhaustion accounted for approximately 30% of the NO₃⁻ N initially present within

TABLE 4. Comparison of the NO production measurements of McKenney et al. (17) with those presented here for denitrifying conditions

<table>
<thead>
<tr>
<th>Source of data</th>
<th>Column mass (g)</th>
<th>Water content (% of dry soil)</th>
<th>pH</th>
<th>Temp (°C)</th>
<th>NO₃⁻ (µg of N g of soil⁻¹)</th>
<th>Flow rate (liters min⁻¹)</th>
<th>NO production (ng of N g of soil⁻¹ min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>McKenney et al. (17)</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Fig. 5</td>
<td>100</td>
<td>14.6</td>
<td>7.3</td>
<td>20</td>
<td>38</td>
<td>0.5</td>
<td>1.2</td>
</tr>
<tr>
<td>Table 1</td>
<td>100</td>
<td>19.0</td>
<td>7.0</td>
<td>20</td>
<td>55</td>
<td>0.5</td>
<td>0.91</td>
</tr>
<tr>
<td>This work</td>
<td>250</td>
<td>30</td>
<td>6.3</td>
<td>25</td>
<td>18</td>
<td>0.5</td>
<td>3.7b</td>
</tr>
</tbody>
</table>

* Average of six columns.
* The true net production rate was at least 14 ng of NO N g of soil⁻¹ min⁻¹; see the text.
the soil; the rest was probably lost as N₂O or N₂ (or both). McKenney et al. (17) made more detailed measurements of the NO₃⁻ loss and found that the NO flux could account for 40% of the NO₃⁻ loss at around 10 h after the onset of anaerobic conditions. This comparatively great loss of N as NO in denitrifying conditions shows clearly the profound difference between these flow column measurements and the field situation. In field measurements of NO emission from fertilized soil at Kjettslinge the loss of N as NO may be estimated to be only about 0.2% of the applied NO₃⁻ N (13a).

Different amounts of NH₄⁺ were present in some of the soil columns (3.4 ± 17 μg of N g of soil⁻¹; Table 1). This varied NH₄⁺ content had no apparent effect on the NO production rate in aerobic conditions. Only about 3% of the total NH₄⁺ present was lost as NO during aerobic conditions (Table 1).

Generally for the soil columns run in aerobic or mixed aerobic-anaerobic conditions (Table 1) an increase in the NO₃⁻ concentration in the column was obtained. The loss of N as NO was generally much smaller than the increase in NO₃⁻ during aerobic conditions. Assuming denitrification and NO₃⁻ immobilization to be small during aerobic conditions, this suggests that even in flow columns, where the NO loss is maximized, the NO loss during aerobic conditions was only a small fraction of the ammonium oxidized.

This difference in behavior between denitrifying and nitrifying bacteria in regulating NO loss may be the result of evolutionary optimization of NO usage, because diffusive losses of NO from the soil atmosphere would be small in an anaerobic (wet, isolated) environment, but could be large in an aerobic (dry, aerated) environment. In the field situation in soils with an underlying anaerobic zone and an overlying aerobic zone there may be cycling of NO within the soil with net NO emission from the anaerobic zone and uptake in the aerobic zone. Consequently the NO release from the soil surface to the atmosphere would be small compared with the NO production rate in the anaerobic zone.

The effect of O₂ on the production of NO, which is evident from the results shown in Table 3 and Fig. 5, is shown explicitly in Fig. 6. The lines were drawn as a visual aid connecting observations on one soil column. It should be noted that column 9 had been run during aerobic conditions (for 48 h) before these measurements were made and that the highest production of NO observed for 0%–100% N₂ when column 9 was started was 3 × 10⁻⁷ g of NO N g of soil⁻¹ min⁻¹. The data in Fig. 6 indicate that at some O₂ level between 0.5 and 0.05% O₂ in the soil atmosphere, NO production occurs at a rate comparable with the rate during anaerobic conditions. At these low O₂ concentrations aerobic microorganisms probably consume the O₂ rapidly enough so that anaerobic conditions prevail at many microsites within the soil.
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LITERATURE CITED