NITROUS OXIDE SOURCES IN AEROBIC SOILS: NITRIFICATION, DENITRIFICATION AND OTHER BIOLOGICAL PROCESSES

G. P. ROBERTSON* and J. M. TIEDE
Departments of Crop and Soil Sciences and of Microbiology and Public Health, Michigan State University, East Lansing, MI 48824-1114, U.S.A.

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Summary—Nitrous oxide (N₂O) production in aerobic, intact soil cores collected from two forest sites was partitioned into different sources of N₂O by subjecting soils to specific inhibitors during short-term incubations. We used acetylene (10% v/v) O₂ to selectively inhibit N₂O production by nitrifiers, 100 kPa (100% v/v) O₂ to inhibit N₂O production by denitrifiers, and sterilized soil to evaluate chemical sources of N₂O. Individual soil cores were incubated in a recirculating atmosphere system under air, 100 kPa O₂, and air + 10 Pa N₂ concentrations for 3-4 h under each condition. Rates of N₂O production in air ranged from 2.8 to 8.5 ng N g⁻¹ h⁻¹ in soils from the Rose Lake site and from 0.1 to 0.94 ng N g⁻¹ h⁻¹ in soils from Warren Woods. The importance of specific N₂O sources in these soils varied among soil cores within each site. In the recently disturbed, more fertile Rose Lake site nitrifiers and denitrifiers were both important N₂O sources, but in many cores a significant portion of the N₂O flux was inhibited by neither acetylene nor O₂, implying an alternate N₂O source. Sterilized soil produced little N₂O, which suggests that the alternate source is biological. In the old-growth Warren Woods site nitrifiers were significant but relatively unimportant sources of N₂O. O₂ consistently stimulated rather than inhibited N₂O production in cores from this site, suggesting (a) that denitrifiers in this site are a sink for N₂O produced by other sources, and (b) that most of the N₂O produced in this site is from sources other than nitrification and denitrification. Biological sources of N₂O other than nitrifiers and denitrifiers were not further identified, although low soil pH values suggest that fungi may be important.

INTRODUCTION

The importance of nitrous oxide to atmospheric photochemistry has become increasingly well-defined in recent years (Crutzen, 1983; Whitten et al., 1984), and recently documented increases in atmospheric N₂O concentrations (Weiss, 1981; Rasmussen and Khalil, 1986) have stimulated substantial interest in identifying and measuring rates of N₂O flux from terrestrial sources to the atmosphere. Biological sources of N₂O appear to dominate present-day global fluxes (Soderlund and Svensson, 1976; Banin et al., 1984), but there is little agreement about which of the several soil biological processes that are capable of producing N₂O are in fact responsible for the N₂O fluxes observed in situ.

In vitro experiments using soil slurries and re-packed soil cores have demonstrated significant N₂O production by both denitrifiers (Firestone et al., 1980) and nitrifiers (Blackmer et al., 1980), although N₂O from nitrifiers may in fact be from nitrifier denitrification (Poth and Focht, 1985). Pure culture studies suggest other soil biological processes may also be important N₂O sources. These latter processes include dissimilatory nitrite and nitrate reduction to ammonium (Bleakley and Tiedje, 1982; Smith, 1982; Smith and Zimmerman, 1981), nitrate assimilation (Satoh et al., 1981), and yet to be identified processes carried out under aerobic conditions by fungi, yeasts, nitrate-assimilating bacteria and green algae (Bollag and Tung, 1972; Bleakley and Tiedje, 1982; Burth and Ottow, 1983, 1984).

Studies of agricultural soils that produce additional N₂O following NH₄⁺ but not following NO₃⁻ additions, and in which little N₂O is produced after additions of nitrate and nitrifier inhibitors (Bremner and Blackmer, 1979; Tiedje et al., 1979; Aulakh et al., 1984) suggest that nitrifiers are the principal agents of N₂O production in many agricultural soils. In soils from forested or other noncultivated sites, however, this generalization may not apply. Such soils are not heavily fertilized and pool sizes of NH₄⁺ and NO₃⁻ fluctuate very differently from pool sizes in cultivated systems. Because fluctuations of inorganic N pools can have important consequences for the strengths of both sources and sinks of N₂O in soils (Blackmer and Bremner, 1978; 1979; Firestone et al., 1979; Terry and Tate, 1980), and because the microbial community in forested versus cultivated sites is very different, N₂O dynamics in nonagricultural soils may differ substantially from those in cultivated systems. In particular, sources of N₂O in noncultivated systems may be more diverse.

Partitioning this diversity into meaningful process-level compartments has not yet been achieved. Few soils appear to contain only two or three classes of potential N₂O producers, and selective inhibitors that do not affect existing electron donor-acceptor distributions are rare. Nevertheless, two inhibitors that may only minimally affect these distributions are available: acetylene and oxygen. Acetylene severely inhibits nitrification at concentrations far below those.
Table I. Outline of experimental treatments to partition N$_2$O sources in intact soil cores. Symbols indicate whether N$_2$O production is potentially present (+) or inhibited (−).

<table>
<thead>
<tr>
<th>N$_2$O source</th>
<th>O$_2$ (100 kPa)</th>
<th>C$_3$H$_8$ (10 Pa)</th>
<th>Sterilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrifiers</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Non-denitrifying</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Denitrifiers</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Dissimilatory nitrate reduction to ammonium</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Other organisms</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chemical</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

O$_2$ has little or no effect on N$_2$O production by many of these organisms but this has not been extensively examined.

that affect N$_2$O reductase in soil (Berg et al., 1982), and oxygen in sufficient quantity inhibits respiratory denitrification (Knowles, 1982). If one can experimentally verify the effectiveness of these inhibitors in a given soil, it becomes possible to partition N$_2$O sources in otherwise undisturbed soil cores into at least four classes (Table 1): N$_2$O from nitrifiers is inhibited by 10 Pa acetylene; N$_2$O from denitrifiers, including denitrifying nitrifiers, is inhibited by elevated O$_2$; N$_2$O from chemical sources is produced in sterilized soil; and N$_2$O from other biological sources can be deduced by difference.

Our objective in the present study was to determine the relative importance of these four potential sources of N$_2$O in two temperate forest soils.

**MATERIALS AND METHODS**

**Site description and sampling strategy**

Intact soil cores (4.7 cm dia x 10 cm depth) were removed from an early successional forest at the Rose Lake Wildlife Research Area near East Lansing, Michigan, and from an old growth beech-maple forest at Warren Woods in southwest Michigan. Soils from both sites had earlier produced N$_2$O at significant rates (Robertson and Tiedje, 1984). Selected soil characteristics appear in Table 2; more detailed site and soil descriptions can be found in the description of our earlier study.

N$_2$O production in most sites studied to date demonstrates extreme spatial variability (Mosier et al., 1981; Goodroad and Keeney, 1984; Folorunso and Rolston, 1984). In order to better understand the diversity of N$_2$O sources throughout our sites, we partitioned N$_2$O sources in individual soil cores rather than in composited soil samples. This strategy reduced our ability to generalize results over the entire sites, but we believe it offers greater resolution at the process level than would a strategy that included a large number of replicated samples from a single site composite. The four cores per site that constitute the bulk of the results reported in this paper were typical of the >16 per site actually analyzed. We limit our report to these four for clarity.

**N$_2$O assays**

Short-term N$_2$O production in individual cores with and without specific inhibitors was monitored using the soil atmosphere recirculation system described by Parkin et al. (1984). In this system, four sealed, intact cores stored at 4°C since sampling 3–4 days earlier were vented, brought up to 22°C, and incubated in line with individual diaphragm pumps that continuously circulated core + headspace atmosphere from the top of each core to its base via external 0.32 mm dia stainless steel tubing. Periodically, a valve along each recirculation stream delivered 0.5 ml of core atmosphere to an injection port of a Perkin-Elmer 910 CC fitted with dual $^{60}$Ni electron capture detectors. Gas flows were optimized for separation of N$_2$O and CO$_2$, and detection of both peaks was completely independent of sample O$_2$ concentration from 0.1 to 100 kPa O$_2$ (Siever et al., 1979). Total airspace volume inside each core was measured with a pressure transducer (Parkin et al., 1984); total core plus recirculating system air space ranged from 80 to 150 ml.

We incubated soil cores on the recirculating atmosphere system under the treatments noted in Table 1 in the sequence: (1) air (21 kPa or 21% v/v O$_2$) to establish a baseline rate of N$_2$O production, (2) 100 kPa (100% v/v) O$_2$, to inhibit denitrification, (3) air to re-establish a baseline N$_2$O flux, and (4) 10 Pa (0.01% v/v) C$_3$H$_8$ to selectively inhibit nitrification. Autoclaved soil from different, otherwise untreated cores at each site was also assayed for N$_2$O production under each treatment to evaluate chemical N$_2$O production. Cores were incubated under each experimental condition for 3–4 h, with 30–45 min venting periods (100 ml min$^{-1}$ O$_2$ or air)

**Table 2. Selected soil characteristics (mean ± SE) of the two research sites (from Robertson and Tiedje (1984) except pH)**

<table>
<thead>
<tr>
<th>Site</th>
<th>Bulk density (g cm$^{-1}$)</th>
<th>Texture</th>
<th>pH</th>
<th>Organic C (%)</th>
<th>C/N</th>
<th>N$_{mn}$ (mg N m$^{-2}$ day$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NO$_3^-$</td>
</tr>
<tr>
<td>Rose Lake</td>
<td>0.88 (0.07)</td>
<td>Loamy sand</td>
<td>5.2 (0.4)</td>
<td>2.49 (0.17)</td>
<td>12.3 (1.5)</td>
<td>60.3 (15.7)</td>
</tr>
<tr>
<td>Warren Woods</td>
<td>1.14 (0.05)</td>
<td>Loamy sand</td>
<td>3.8 (0.09)</td>
<td>1.62 (0.36)</td>
<td>20.9 (6.4)</td>
<td>142.0 (28.0)</td>
</tr>
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</tr>
</tbody>
</table>
preceding each treatment to flush the previous atmosphere. Both CO$_2$ production and O$_2$ consumption were monitored along with N$_2$O production during these assays; rates of N$_2$O production were calculated from periods under each treatment when all three rates were linear. This usually occurred within the first 15 min of each treatment period. Acetylene was generated from CaC$_2$. Previous experiments with soil cores collected on the same date as our principal cores showed that rates of N$_2$O production in both the presence and absence of 10 kPa C$_2$H$_2$ were constant for at least 20 h.

To evaluate whether 10 Pa C$_2$H$_2$ effectively blocked nitrification in our soils we monitored NO$_3^-$ production in the presence and absence of 10 Pa C$_2$H$_2$ in soils from both sites. Twelve 10 g replicates of soil from each site were placed in individual 160 ml serum vials sealed and treated with air plus 10 Pa C$_2$H$_2$ or with air. Vials were held at 25°C and periodically were replenished with original atmosphere (air or air plus 10 Pa C$_2$H$_2$). On days 1, 3 and 7, three replicate vials from each site were extracted with 100 ml of 2 M KCl, and the extracts analyzed for NO$_3^-$ and NO$_2^-$ by calorimetric techniques (Technicon, 1973).

In a second experiment, to evaluate the short-term sensitivity of nitrifiers to C$_2$H$_2$, we used the chlorate inhibition assay (Belser and Mays, 1980) to examine 24 h ammonium oxidation in Rose Lake soils amended with different concentrations of C$_2$H$_2$. Warren Woods soils were also assayed but no NO$_3^-$ accumulated under control conditions. Soils (25 g) were incubated in replicate 160 ml serum vials containing 100 ml media plus NaClO$_3$; different amounts of C$_2$H$_2$ injected into the sealed headspace of each vial brought solution concentrations of C$_2$H$_2$ to 0, 0.009, 0.09 and 0.9 Pa C$_2$H$_2$. Headspace concentrations ranged to 7.8 Pa C$_2$H$_2$. Vials were held on a shaker for 24 h and each vial subsampled periodically for NO$_3^-$ determinations.

Potential effects of 10 Pa C$_2$H$_2$ on denitrification were evaluated by monitoring N$_2$O production in soil from the Rose Lake site incubated under anaerobic conditions and treated sequentially with 0, 0.01 and 10 kPa C$_2$H$_2$. Replicate serum vials (160 ml) containing 25 g soil were incubated on the recirculation system with an Ar atmosphere under each condition for ≤3 h.

To evaluate chemical sources of N$_2$O in these soils, 25 g soil samples were autoclaved in sealed serum vials three times during a 5-day period, vented to dissipate N$_2$O formed during autoclaving, and then incubated on the recirculating atmosphere system described above. N$_2$O production was monitored under air, 100 kPa O$_2$ and 10 Pa C$_2$H$_2$ atmospheres for 3 h each.

All soils assayed for N$_2$O production were subsequently analyzed for soil moisture content, pH and nitrate. Soil from cores was mixed and sieved (<4 mm). Two 10 g samples were analyzed for pH in a 2:1 H$_2$O:soil slurry.

RESULTS AND DISCUSSION

Inhibitor effectiveness and selectivity

Initial experiments were designed to test the effectiveness with which 10 Pa C$_2$H$_2$ in these soils inhibited nitrification without affecting denitrification. Figure 1 shows that nitrate production in both soils was severely curtailed by 10 Pa C$_2$H$_2$ during a 7 day incubation; that this curtailment was rapid is suggested by the results of the chlorate inhibition experiment, wherein only 0.9 Pa C$_2$H$_2$ in solution markedly depressed 24 h nitrite production in chlorate-treated Rose Lake soils (Fig. 2). The 10 Pa C$_2$H$_2$ treatment had little effect on denitrification (Fig. 3): under anaerobic conditions...
favorable to denitrifiers, N$_2$O production in Rose Lake soils was not significantly affected by 10 Pa C$_2$H$_2$ (34.2 vs 32.4 ng N g$^{-1}$ h$^{-1}$). That a 10 kPa C$_2$H$_2$ atmosphere resulted in significantly higher N$_2$O production (85.2 mg N g$^{-1}$ h$^{-1}$) shows that denitrifiers were present and active under the 0 and 10 Pa C$_2$H$_2$ treatments.

We judge the effectiveness of 100 kPa O$_2$ as a selective inhibitor of denitrification on our understandings of O$_2$ effects on denitrifiers and of O$_2$ dynamics in soil microsites. First, O$_2$ is the most effective and selective inhibitor of denitrifiers (Allison et al., 1960; Knowles, 1982; Parkin and Tiedje, 1984), for example, found that in intact soil cores only 2% of the soil's capacity to denitrify was expressed unless O$_2$ concentrations declined to <3 kPa O$_2$. Second, O$_2$ microelectrode studies have shown that soil denitrification goes on only in aerobic microsites (Sexstone et al., 1985), with the extent of the anaerobic zone around a microsite defined by macropore O$_2$ concentration, the gaseous diffusion coefficient, and O$_2$ consumption in the microsite. In our recirculating system soil macropores were brought rapidly to 100 kPa O$_2$, thereby steepening the O$_2$ gradient around soil microsites and presumably reducing zones of anaerobiosis. The rapid reduction of the zone of anaerobiosis should be especially marked in poorly structured soils such as the loamy sand soils used in this study. We cannot conclude that we have totally inhibited denitrifying sources of N$_2$O in our soils because we could not directly measure denitrification in our aerobic acetylene-free atmospheres. Nevertheless, our conclusions do not depend on complete inhibition of denitrification and it seems likely that denitrification was severely inhibited by our 100% (v/v) O$_2$ atmospheres.

Enhanced soil aeration should also affect N$_2$O production by nitrifiers (Goreau et al., 1980), especially where nitrifier-derived N$_2$O is the result of denitrification (Poth and Focht, 1985). In our experiment N$_2$O from nitrifier denitrification is classified as both nitrification and denitrification because this source should be inhibited by both 100 kPa O$_2$ and 10 Pa C$_2$H$_2$ (see Table 1).

### N$_2$O partitioning

In three of four soil cores from the Rose Lake site, 10 Pa C$_2$H$_2$ inhibited N$_2$O production 39-64% (Table 3). This implies that except in core RL4, in which acetylene did not significantly affect N$_2$O production, nitrifiers were directly responsible for a major portion of the N$_2$O flux from soils of this site. In core RL3, for example, acetylene reduced N$_2$O production from 8.5 to 3.1 ng N g$^{-1}$ h$^{-1}$.

### Table 3. Effect of 10 Pa C$_2$H$_2$ and 100% O$_2$ on rates of N$_2$O produced by intact soil cores from Rose Lake Research Area. Values in parentheses represent standard errors of the slopes; n = 10-12 sample points per 2 h incubation period

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Core</th>
<th>RL1 (ng N$_2$O-N g$^{-1}$ h$^{-1}$)</th>
<th>RL2 (ng N$_2$O-N g$^{-1}$ h$^{-1}$)</th>
<th>RL3 (ng N$_2$O-N g$^{-1}$ h$^{-1}$)</th>
<th>RL4 (ng N$_2$O-N g$^{-1}$ h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>RL1</td>
<td>8.06 (0.57)</td>
<td>2.77 (0.11)</td>
<td>8.45 (0.27)</td>
<td>4.12 (0.15)</td>
</tr>
<tr>
<td>Air + 10 Pa C$_2$H$_2$</td>
<td>RL2</td>
<td>4.91 (0.13)</td>
<td>1.51 (0.05)</td>
<td>3.07 (0.55)</td>
<td>4.00 (0.12)</td>
</tr>
<tr>
<td>100 kPa O$_2$</td>
<td>RL3</td>
<td>6.32 (1.48)</td>
<td>1.55 (0.03)</td>
<td>4.78 (0.29)</td>
<td>2.47 (0.04)</td>
</tr>
</tbody>
</table>

Denitrification also appears to be a component of the Rose Lake site's N$_2$O flux. A 100% oxygen atmosphere in these cores inhibited N$_2$O production (Table 3) by 16% in core RL4 to 46% in core RL2. Some of this lost N$_2$O may have been from denitrifying nitrifiers, but probably only a relatively small portion: in companion cores amended with 100 kPa O$_2$ to inhibit denitrification, N$_2$O production was further inhibited by 10 Pa C$_2$H$_2$ (Fig. 4). If denitrifying nitrifiers were important in these cores acetylene would have had little effect since denitrification was already inhibited by the O$_2$. Instead, acetylene depressed the N$_2$O flux under both air and oxygen to the same degree (Fig. 4, Table 3), suggesting that under both conditions denitrifying nitrifiers were unimportant relative to non-denitrifying nitrifiers.

In Warren Woods soils, 10 Pa acetylene in air also inhibited N$_2$O production, but to a lesser degree than in Rose Lake soils. N$_2$O fluxes from Warren Woods cores were about an order of magnitude lower than those from Rose Lake cores, and nitrification appeared to account for negligible portions of the flux in two cores (WW3 and WW4; Table 4) and only 11-23% in the remaining two described here. This implies that non-nitrifier denitrification and other sources of N$_2$O must account for most of the N$_2$O flux from these soils.

Surprisingly, however, results from the O$_2$-inhibition experiments suggest that denitrifiers are a net
sink rather than a net source of N₂O in these soils. In all four of our Warren Woods soils a 100% O₂ atmosphere resulted in substantially enhanced rates of N₂O production (Table 4). This increase was not due to chemical reactions at high pO₂ (Table 5), but rather appears to represent the net consumption of N₂O by denitrifiers under a normal soil atmosphere. Under the O₂ atmosphere denitrifiers were inhibited so that N₂O produced by other sources accumulated rather than was further reduced to N₂ by denitrifiers. We thus surmise that total N₂O production in these soils is actually 3-4 times greater than that observed under an ambient air atmosphere, and that consumption by denitrifiers accounts for the difference between total N₂O produced and total N₂O emitted.

Chemical production of N₂O as judged by autoclaved cores is present but it is less than 10% of the total N₂O flux (Table 5).

Figure 5 summarizes our understanding of N₂O production in these soils. Nitrification appears to account for 3-40% of the net N₂O flux from Rose Lake soils and for 0-23% of the net N₂O flux from Warren Woods soils. Some of this N₂O may be from denitrification by nitrifiers. Denitrification from all sources may account for as much as 46% of the net N₂O flux in Rose Lake soils. Denitrification in Warren Woods soils appears to be a sink rather than a source of N₂O.

In soils from both sites, organisms other than nitrifiers or denitrifiers appear to be major contributors to the net N₂O flux. Such sources appear to account for up to 80% of the N₂O flux from our Rose Lake cores and for 77-100% of the N₂O emitted by Warren Woods cores. We have not attempted to identify these sources more precisely. Boltag and Tung (1972), Bleakley and Tiedje (1982) and Burth

### Table 4. Effect of C₂H₂ and O₂ on N₂O flux in intact cores from Warren Woods. Values in parentheses represent standard errors of the slopes; n = 10-12 sample points per 2 h incubation period.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>WW1 (ng N₂O-N g⁻¹ h⁻¹)</th>
<th>WW2 (ng N₂O-N g⁻¹ h⁻¹)</th>
<th>WW3 (ng N₂O-N g⁻¹ h⁻¹)</th>
<th>WW4 (ng N₂O-N g⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>0.216 (0.012)</td>
<td>0.109 (0.011)</td>
<td>0.330 (0.013)</td>
<td>0.935 (0.063)</td>
</tr>
<tr>
<td>Air + 10 Pa C₂H₂</td>
<td>0.167 (0.002)</td>
<td>0.097 (0.002)</td>
<td>0.324 (0.004)</td>
<td>1.022 (0.017)</td>
</tr>
<tr>
<td>100 kPa O₂</td>
<td>0.871 (0.008)</td>
<td>0.447 (0.016)</td>
<td>0.880 (0.023)</td>
<td>2.574 (0.057)</td>
</tr>
</tbody>
</table>

### Table 5. N₂O production (±SE) in air, 100 kPa O₂, and 10 Pa C₂H₂ atmospheres after autoclaving soils. Values below are means and standard errors.

<table>
<thead>
<tr>
<th>Site</th>
<th>Air (ng N₂O-N g⁻¹ h⁻¹)</th>
<th>100 kPa O₂ (ng N₂O-N g⁻¹ h⁻¹)</th>
<th>10 Pa C₂H₂ (ng N₂O-N g⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rose Lake</td>
<td>0.21 (0.01)</td>
<td>0.19 (0.01)</td>
<td>0.17 (0.02)</td>
</tr>
<tr>
<td>Warren Woods</td>
<td>0.012 (0.001)</td>
<td>0.015 (0.002)</td>
<td>0.019 (0.002)</td>
</tr>
</tbody>
</table>
and Ottow (1983) reported N₂O production by fungi in pure culture. That both our Rose Lake Site and especially our Warren Woods site have low pH soils suggests that fungi may be important sources.

Overall, our results suggest that N₂O production in noncultivated soils is from a suite of organisms that encompass a greater diversity than simply nitrifiers and denitrifiers. A basic understanding of the factors underlying the global N₂O balance requires understanding this diversity and identifying the factors that regulate process-level interactions in situ.

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