Distinguishing between Nitrification and Denitrification as Sources of Gaseous Nitrogen Production in Soil

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The source of N₂O produced in soil is often uncertain because denitrification and nitrification can occur simultaneously in the same soil aggregate. A technique which exploits the differential sensitivity of these processes to C₂H₂ inhibition is proposed for distinguishing among gaseous N losses from soils. Denitrification N₂O was estimated from 24-h laboratory incubations in which nitrification was inhibited by 10-Pa C₂H₂. Nitrification N₂O was estimated from the difference between N₂O production under no C₂H₂ and that determined for denitrification. Denitrification N₂O was estimated from the difference between N₂O production under 10-kPa C₂H₂ and that under 10 Pa. Laboratory estimates of N₂O production were significantly correlated with in situ N₂O diffusion measurements made during a 10-month period in two forested watersheds. Nitrous oxide production from nitrification was most important on well-drained sites of a disturbed watershed where ambient NO₃⁻ was high. In contrast, denitrification N₂O was most important on poorly drained sites near the stream of the same watershed. Distinction between N₂O production from nitrification and denitrification was corroborated by correlations between nitrification N₂O and water-filled pore space and between nitrification N₂O and ambient NO₃⁻. This technique permits qualitative study of environmental parameters that regulate gaseous N losses via denitrification and nitrification.

Nitrous oxide can be produced by both nitrifying and denitrifying bacteria. Development of aerobic and anaerobic microsites within close proximity, indeed in the same soil aggregate, permits both nitrification and denitrification to occur simultaneously (12). Given this possibility, the source of observed N₂O emitted from soils is often uncertain (1, 3, 7, 8, 13).

Acetylene has been widely used to inhibit nitrous oxide reductase in denitrification studies, and it is also a potent inhibitor of nitrification (21). The concentrations of C₂H₂ required to inhibit these two processes differ by 1 to 3 orders of magnitude. Partial pressures of 0.1 to 5.0 kPa of C₂H₂ (depending upon NO₃⁻ availability) were needed to completely inhibit N₂O reductase (18). In contrast, nitrification was completely inhibited by 10 Pa of C₂H₂, with partial inhibition observed at 0.1 Pa of C₂H₂ (2). Klemmedtsson et al. (L. K. Klemmedtsson, P. Berg, and B. H. Svensson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, N39, p. 184) have suggested that this differential sensitivity to C₂H₂ concentration could be utilized to distinguish between the processes of N₂O production in soil. A procedure is proposed here which exploits this differential sensitivity. Forest soils which were known to support both nitrifying and denitrifying activity were used to examine the utility of the proposed procedure.

MATERIALS AND METHODS

Study sites. Two north-facing watersheds (WSs) at the U.S. Department of Agriculture Forest Service Coweeta Hydrologic Laboratory were studied: (i) WS18, a 12.6-ha reference WS supporting an uneven-aged, aggrading, mixed-hardwood forest that had not undergone major disturbance since the chestnut blight in the late 1920s; and (ii) WS6, an 8.9-ha disturbed WS that was clear cut, limed, fertilized, and planted in fescue grass (Festuca arundinacea Schreb.) in the late 1950s (10), was treated with herbicide in 1966 and 1967 (6), and then was allowed to regenerate naturally. Black locust (Robinia pseudo-acacia L., Fabaceae) was the dominant woody species during early stages of succession on WS6 but is now declining due to natural succession processes, including a recent infestation by the locust stem borer (Megacyllene robiniae Forester).

Within these WSs, five study areas were identified: (i) a strip along an intermittent stream on the reference WS; (ii) a well-drained toeslope position on the reference WS supporting an oak-hickory-hemlock stand; (iii) a strip along a permanent stream on the disturbed WS; (iv) a well-drained toeslope position on the disturbed WS supporting a mixed locust-tulip poplar-maple stand; and (v) a well-drained midslope position on the disturbed WS supporting a stand dominated by black locust. No equivalent of the locust-dominated area existed on the reference WS. The stream areas were 10 by 20 m, and the upslope areas were 20 x 20 m.

Within each of these study areas, three soil sampling sites and adjacent frames for in situ measurement of N₂O diffusion were established. The classification of these soils and selected properties are given in Table 1. Although only one soil series has been identified for each area, spatial heterogeneity with respect to ground cover and soil moisture content was obvious within each area. Therefore, the three sampling sites within each area were purposely chosen to sample a broad range of variability rather than to attempt random replication of an area inappropriately assumed to be homogeneous. The sampling sites were not considered true replicates in statistical analyses.
TABLE 1. Selected characteristics of soils

<table>
<thead>
<tr>
<th>WS</th>
<th>Area</th>
<th>Series (classification)</th>
<th>Site</th>
<th>PH</th>
<th>BS (%)</th>
<th>Tot-C (%)</th>
<th>Tot-N (%)</th>
<th>NO₃⁻ (mg/kg)</th>
<th>NH₄⁺ (mg/kg)</th>
<th>WFPS (%)</th>
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<td>Midslope, locust</td>
<td>Tusquitee loam (umbric dystrochrepts)</td>
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<td>WS6</td>
<td>Toeslope, hardwood</td>
<td>Tusquitee loam (umbric dystrochrepts)</td>
<td>D</td>
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<td>81</td>
<td>3.0</td>
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*Top 6 cm of mineral soil. Abbreviations: BS, base saturation; Tot-C, total carbon; Tot-N, total nitrogen; NO₃⁻, ambient nitrate; NH₄⁺, ambient ammonium.

Soils were sampled and in situ N₂O diffusion was measured simultaneously on a bimonthly basis from September 1984 through July 1985. A composite sample of 12 mineral soil cores (2.5 cm in diameter, 6 cm deep) was collected from each site. Pebbles and root fragments were removed by hand when these composite samples were subsampled.

In situ nitrous oxide diffusion measurements. Square aluminum frames were constructed with a strip of rubber garage door insulation attached to the bottom edge (Fig. 1). These frames were placed on top of the forest floor, and soil was packed around the outside of the frames, burying the rubber flap, providing a good seal, and leaving the forest floor within undisturbed. The frames were left in place throughout the study period. Covers were made for each frame from aluminum materials and styrofoam insulation (Fig. 1). A curved 7-cm, 3-mm diameter stainless steel tube fitted through the cover top served as a vent for equalizing pressure. A similar straight stainless steel tube, connected to perforated Tygon tubing below and stoppered Tygon tubing above, served as a gas sampling port.

Preliminary studies indicated that short sampling periods recommended in agricultural studies (14) were inadequate for detectable enrichment of headspace N₂O at many sites. Rates of N₂O enrichment were generally linear throughout 2 or 4 h for even the most active site (Fig. 2). Covers were routinely placed over frames during late morning, and duplicate gas samples were removed with a syringe at hourly or half-hourly intervals throughout 2 to 3 h, depending on the rates previously observed at each site. The air temperatures within closed covers never varied more than 2°C from ambient air. Gas samples were transferred to evacuated Hungate tubes for later analysis by gas chromatography (see below). Rates of N₂O diffusion were determined by linear regression.

Effects of acetylene in laboratory incubations. To test the assumptions that 10 kPa of C₂H₂ effectively inhibits N₂O reduction and that 10 Pa of C₂H₂ inhibits only nitrification and not N₂O reduction, the effect of C₂H₂ on depletion of added N₂O was observed in samples of one of the Spivey soils (site I, Table 1). This sample was known to produce N₂ as a denitrification endproduct. Sixteen subsamples, each 20 g of field-moist soil, were placed in 125-ml serum bottles. The headspace gas was replaced with argon, and the bottles were preincubated in the dark for 24 h at 20°C to reduce ambient NO₃⁻ levels and thus provide a rigorous test of C₂H₂ inhibition. Four of the samples were then extracted with 100 ml of 2 M KCl for later NO₃⁻ determination.
Headspace gas in the remaining 12 bottles was reequilibrated with room air for 5 min, the stoppers were replaced on the bottles, and sufficient N\textsubscript{2}O was injected into each bottle to yield headspace partial pressures of about 1 Pa. Acetylene, generated from CaC\textsubscript{2} and H\textsubscript{2}O, was injected into the bottles so that four had partial pressures of 10 kPa, four had 10 Pa, and four received no C\textsubscript{2}H\textsubscript{2}. These bottles were incubated as before. Headspace gas was sampled with a syringe at regular intervals for 24 h and analyzed for N\textsubscript{2}O by gas chromatography. A Varian 3700 gas chromatograph was operated with an electron capture detector at 330°C, a Porapak-Q analytical column and a Porapak-R precolumn at 65°C, and a carrier gas (95% argon, 5% methane) flow rate of 30 to 40 cm\textsuperscript{3} min\textsuperscript{-1}. A 10-port valve permitted the heavier C\textsubscript{2}H\textsubscript{2} and water vapor to be back-flushed from the precolumn, whereas N\textsubscript{2}O and lighter gases were separated on the analytical column. The effects of acetylene treatment on N\textsubscript{2}O depletion rates were tested by analysis of variance. Logarithmic transformations of the data were necessary to equalize variances across treatment means.

**Laboratory incubations.** At each sampling date (except March 1985), 15 composite soil samples, one from each sampling site, were brought to the laboratory on ice and stored in plastic bags at 4°C. This storage period never exceeded 1 week. Nine 20-g subsamples of field-moist soil from each sample bag were placed in 125-ml serum bottles, and stoppers were placed in the bottles. Acetylene was injected into these bottles so that three had headspace partial pressures of 10 kPa, three had 10 Pa, and three received no C\textsubscript{2}H\textsubscript{2}. The bottles were incubated in the dark for 24 h at temperatures recorded in the field with soil thermometers at 6-cm depth. Headspace gas was then sampled by syringe and analyzed for N\textsubscript{2}O by gas chromatography as described above.

Six plausible combinations of hypothetical results exist for these incubations (Fig. 3). Production of N\textsubscript{2}O under the 10-Pa treatment was assumed to estimate denitrification N\textsubscript{2}O, since all autotrophic nitrification should be inhibited by the presence of C\textsubscript{2}H\textsubscript{2}. The difference between N\textsubscript{2}O production under no C\textsubscript{2}H\textsubscript{2} and that under 10 Pa was assumed to estimate nitrification N\textsubscript{2}O, since any decrease in N\textsubscript{2}O production when 10 Pa C\textsubscript{2}H\textsubscript{2} was added should have been due to inhibition of nitrification. Production of N\textsubscript{2} by denitrification was estimated by the difference between N\textsubscript{2}O production under 10 kPa of C\textsubscript{2}H\textsubscript{2} and that under 10 Pa.

The Q statistic (5) was calculated for each of the 249 treatment means of this study to screen for extraneous values that would skew the mean. In 17 cases, one of the observations was identified as extraneous and was not included in the calculation of that mean. Estimates of nitrification N\textsubscript{2}O, denitrification N\textsubscript{2}O, and denitrification N\textsubscript{2} were obtained by subtraction of treatment means for each composite soil sample as described above. These estimates and the in situ N\textsubscript{2}O diffusion rates were log transformed because, (i) as in other denitrification studies (16), the data more closely fit log-normal distributions; and (ii) within the measured range of parameters such as temperature and moisture content, gaseous N production increased exponentially as these parameters increased arithmetically, and thus logarithmic transformations facilitated linear regression analyses.

Check for nitrate limitation during incubations. When nitrification is inhibited by C\textsubscript{2}H\textsubscript{2} and when denitrification rates are high, NO\textsubscript{3}\textsuperscript{-} could become depleted during the incubation, thus causing an error in denitrification estimates (17). One of the Spivey soil samples (site I, Table 1) was known to exhibit high denitrification rates and low ambient NO\textsubscript{3}\textsuperscript{-} levels. For samples from this site, each of the two levels of C\textsubscript{2}H\textsubscript{2} was also applied to three subsamples which had been amended with 0.1 ml of 36 mM KNO\textsubscript{3}, providing 1
mg of NO\textsubscript{3} nitrogen per kg of dry soil. The effects of C\textsubscript{2}H\textsubscript{2} treatment, NO\textsubscript{3}\textsuperscript{-} amendment, and their interactions were determined by analysis of variance. Spot checks were made on other soil samples, but C2H2-induced NO\textsubscript{3}\textsuperscript{-} limitation was important for only one Spivey site.

**Time course profiles of laboratory incubations.** Time course analyses could not be conducted for each of the several hundred incubations in this study. To determine whether rates of N\textsubscript{2}O production were generally linear throughout these incubations, the incubation procedures described above were repeated on Spivey, Tusquitee, and Haywood soil samples, with N\textsubscript{2}O concentrations determined at regular intervals over a 50-h period. These analyses were conducted in May 1985, when conditions were generally favorable for microbial activity.

**Other soil parameters.** Ambient NO\textsubscript{3}\textsuperscript{-} was determined by extraction for 1 h in 2 M KCl (5:1 [wt/wt] ratio of solution to moist soil) and colorimetric analysis of filtered extracts with a Technicon Autoanalyzer. The water-filled pore space (WFPS) was determined from gravimetric moisture content, gravimetric bulk density estimates (mean of four cores [7.6-cm diameter by 6 cm deep] at each site) and an assumed particle density of 2.65 (20). On two occasions, the WFPS exceeded 100% because of standing-water.

**RESULTS**

**Laboratory incubations with acetylene: test of assumptions.** Anaerobic preincubation reduced ambient NO\textsubscript{3}\textsuperscript{-} levels in the Spivey soil sample from 932 (±67, 95\% confidence interval) to 67 (±11) µg of NO\textsubscript{3}\textsuperscript{-} nitrogen per kg of dry soil, thus providing a rigorous test of the 10-kPa C\textsubscript{2}H\textsubscript{2} block of N\textsubscript{2}O reduction at low NO\textsubscript{3}\textsuperscript{-} concentration during the following aerobic incubation. Nitrous oxide concentration increased during incubation under 10-kPa C\textsubscript{2}H\textsubscript{2}, indicating that the remaining NO\textsubscript{3}\textsuperscript{-} was reduced to N\textsubscript{2}O (62 ± 25 µg of N per kg of dry soil) and that none of this N\textsubscript{2}O or the added N\textsubscript{2}O was reduced to N\textsubscript{2} (Fig. 4). The 10-kPa C\textsubscript{2}H\textsubscript{2} block appeared to be effective.

The 10-Pa C\textsubscript{2}H\textsubscript{2} treatment may have caused a slight inhibition of N\textsubscript{2}O reduction relative to the rate of N\textsubscript{2}O depletion in the samples not receiving C\textsubscript{2}H\textsubscript{2} (Fig. 4), but analysis of variance revealed that the rates of N\textsubscript{2}O depletion for these two levels of C\textsubscript{2}H\textsubscript{2} were not significantly different (α = 0.05).
exhibited N₂ production (Fig. 5c). This same soil exhibited significant increases in N₂O production under all C₂H₂ treatments when NO₃⁻ was added before incubation. Therefore, partial inhibition of N₂O production during incubation of this soil under 10 Pa of C₂H₂ could be due to either inhibition of nitrification N₂O or inhibition of NO₃⁻ production and subsequent NO₃⁻ limitation for denitrification.

**Time course analysis of laboratory incubations.** Rates of N₂O accumulation within incubation bottles were generally linear during the first 24 h for most of the nonsaturated soil samples (Fig. 6a). Incubation beyond 24 h sometimes led to nonlinear rates (Fig. 6a, 10 kPa). The saturated Spivey soil sample discussed above exhibited nonlinear rates under all C₂H₂ treatments (Fig. 6b), probably because of NO₃⁻ depletion during the incubation.

**Comparison of in situ and laboratory results.** Laboratory nitrification N₂O and denitrification N₂O were significantly correlated (a = 0.05) with in situ N₂O diffusion determinations (r = 0.51 and 0.29, respectively). When included in the multiple regression model log IS-N₂O = β₀ + β₁ log N-N₂O + β₂ log D-N₂O + e, where IS-N₂O is in situ N₂O; N-N₂O is nitrification N₂O (laboratory), and D-N₂O is denitrification N₂O (laboratory), both independent variables were significant (a = 0.01; R² = 0.40). Laboratory estimates of N₂O and N₂ from denitrification were also significantly correlated (r = 0.80).

**Gaseous N estimates versus soil parameters.** The four estimates of gaseous N appear distinct with respect to their correlations with soil parameters. For example, WFPS was poorly correlated with in situ N₂O (Fig. 7a) and nitrification N₂O (Fig. 7b), but was significantly correlated with denitrification N₂O (Fig. 7c) and denitrification N₂ (Fig. 7d). In contrast, nitrification N₂O exhibited the strongest correlation with ambient NO₃⁻ (R² = 0.58; P < 0.01). Denitrification N₂O also exhibited a correlation with ambient NO₃⁻ for those samples with low denitrifying activity (R² = 0.41; P < 0.01), but there were notable outliers which are discussed in the accompanying paper (4).

**DISCUSSION**

The use of C₂H₂ to estimate denitrification N₂ production has been problematic for soils with either very high or very low ratios of available carbon to NO₃⁻. Nitrous oxide reduction was not completely inhibited by 10 kPa of C₂H₂ for samples of sewage sludge where available C was extremely high and NO₃⁻ was extremely low (11). In contrast, C-limited soils which had been amended with NO₃⁻ appeared to metabolize C₂H₂ as an energy source, especially in soil samples which had previously been exposed to C₂H₂ (9). For short-term incubations of soils of intermediate available C/NO₃⁻ ratios, the assumption that 10 kPa of C₂H₂ completely inhibits N₂O reduction and does not become significantly metabolized is probably valid (18, 19). In the present study, N₂O reductase inhibition under 10 kPa of C₂H₂ appeared complete even when ambient NO₃⁻ levels were low.

The lower threshold of C₂H₂ concentration where inhibition occurs is also somewhat controversial. Partial inhibition of N₂O reduction has been observed at C₂H₂ partial pressures as low as 1 Pa (22). In the present study, no significant difference in the rate of N₂O depletion was observed between treatments of no C₂H₂ and 10 Pa of C₂H₂ (Fig. 4). Since the effect of low C₂H₂ concentration may vary with soil types and denitrifier populations, the assumption that N₂O reduction is unaffected by 10 Pa of C₂H₂ may need verification for specific soils of interest.

Laboratory incubations under three levels of C₂H₂ permitted the qualitative distinction of the source of N₂O production. Both the nitrification N₂O and denitrification N₂O estimates were correlated with in situ N₂O diffusion measurements, and their contributions to a multiple regression were nearly additive, suggesting that both processes contributed to N₂O production in situ. The laboratory incubations provided information to assess which process was most important at each site and sampling time. For example, samples exhibiting the highest denitrifying activity were all from the riparian zone sampling area of the disturbed WS (Fig. 7c and d), whereas high rates of nitrification N₂O occurred at midslope positions of the same WS (Fig. 7b). A quantitative ratio of nitrification N₂O to denitrification N₂O would not be appropriate, since this ratio could vary between laboratory conditions and field conditions. Similarly, a quantitative ratio of N₂O to N₂ produced by denitrification would be unwarranted, because samples which have sufficient reducing conditions to produce N₂ would probably exhibit nonlinear rates of N₂ and N₂O production (Fig. 6b). The distinction between nitrification N₂O and denitrification
tion N\textsubscript{2}O by this method is corroborated by the observation of expected relationships between these estimates and soil parameters. Denitrification N\textsubscript{2}O production often exceeded 1 \textmu g of N\textsubscript{2}O nitrogen per kg of dry soil when the WFPS was above 70\% (Fig. 7c), which agrees well with results of Linn and Doran (13). Sufficient reducing conditions for appreciable N\textsubscript{2} production occurred only in samples with WFPS above 80\% (Fig. 7d). The highest values for nitrification N\textsubscript{2}O were observed near 50 to 60\% WFPS (Fig. 7b). The relationship between WFPS and nitrification N\textsubscript{2}O is more thoroughly discussed in the accompanying paper (4). Nitrification N\textsubscript{2}O was strongly correlated with the other end product of nitrification, ambient NO\textsubscript{x}.

This technique could be modified for studies of intact soil cores. However, a gas circulating system would be necessary to maintain appropriate \textsubscript{C}2\textsubscript{H}2 concentrations throughout the core (15). Such designs increase the difficulty in obtaining adequate replication for statistical determinations of \textsubscript{C}2\textsubscript{H}2 effects when variation among cores is high. Alternatively, a single core could be incubated repeatedly under successively higher \textsubscript{C}2\textsubscript{H}2 concentrations. However, preliminary work on soil cores in our laboratory indicated that denitrification rates increased during successive incubations at one \textsubscript{C}2\textsubscript{H}2 level, probably because incubation conditions favored denitrifying enzyme synthesis. Therefore, the effects of cumulative incubation time would probably confound successive \textsubscript{C}2\textsubscript{H}2 treatment effects. In the present study, we employed composite soil samples to improve homogeneity across \textsubscript{C}2\textsubscript{H}2 treatments for a given soil sample and to allow simultaneous processing of a large number of samples.

When the source of N\textsubscript{2}O is uncertain, study of the factors affecting N\textsubscript{2}O production is complicated. Qualitative distinction between nitrification and denitrification as sources of N\textsubscript{2}O production permits investigation of the environmental parameters regulating gaseous N losses via each of these two pathways. This study is the subject of the accompanying paper (4).

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LITERATURE CITED