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

ERIC A. DAVIDSON,[1]‡* WAYNE T. SWANK,[2] AND THOMAS O. PERRY[1]

Department of Forestry, North Carolina State University, Raleigh, North Carolina 27695-8002,[1] and U.S. Department of Agriculture Forest Service Southeastern Experiment Station, Coweeta Hydrologic Laboratory, Otto, North Carolina 28763[2]

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The source of N_2O 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 C_2H_2 inhibition is proposed for distinguishing among gaseous N losses from soils. Denitrification N_2O was estimated from 24-h laboratory incubations in which nitrification was inhibited by 10-Pa C_2H_2. Nitrification N_2O was estimated from the difference between N_2O production under no C_2H_2 and that determined for denitrification. Denitrification N_2 was estimated from the difference between N_2O production under 10-kPa C_2H_2 and that under 10 Pa. Laboratory estimates of N_2O production were significantly correlated with in situ N_2O 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 NO_3^- was high. In contrast, denitrification N_2O was most important on poorly drained sites near the stream of the same watershed. Distinction between N_2O production from nitrification and denitrification was corroborated by correlations between denitrification N_2O and water-filled pore space and between nitrification N_2O and ambient NO_3^-. 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 N_2O 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 C_2H_2 required to inhibit these two processes differ by 1 to 3 orders of magnitude. Partial pressures of 0.1 to 5.0 kPa of C_2H_2 (depending upon NO_3^- availability) were needed to completely inhibit N_2O reductase (18). In contrast, nitrification was completely inhibited by 10 Pa of C_2H_2, with partial inhibition observed at 0.1 Pa of C_2H_2 (2). Klemedtsson et al. (L. K. Klemedtsson, P. Berg, and B. H. Svensson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, N39, p. 184) have suggested that this differential sensitivity to C_2H_2 concentration could be utilized to distinguish between the processes of N_2O 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 successional 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 × 20 m.

Within each of these study areas, three soil sampling sites and adjacent frames for in situ measurement of N_2O 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.

* Corresponding author.

† Paper no. 10425 of the journal series of the North Carolina Agricultural Research Service, Raleigh, NC 27695-7601.

‡ Present address: Department of Plant and Soil Biology, University of California, Berkeley, CA 94720.

TABLE 1. Selected characteristics of soils[a]

WS	Area	Series (classification)	Site	pH	BS (%)	Tot-C (%)	Tot-N (%)	NO_3^-[b] (mg/kg)	NH_4^+[b] (mg/kg)	WFPS[b] (%)
WS6	Midslope, locust	Tusquitee loam (umbric dystrochrepts)	A	5.6	75	3.9	0.11	5.5	3.0	41
			B	5.6	78	4.2	0.12	7.5	2.0	46
			C	5.9	85	4.1	0:13	5.7	2.6	47
WS6	Toeslope, hardwood	Tusquitee loam (umbric dystrochrepts)	D	5.8	81	3.0	0.10	1.6	2.4	43
			E	5.9	88	4.7	0.13	5.8	4.1	53
			F	5.5	80	3.5	0.12	1.6	4.5	40
WS6	Stream	Spivey stony loam (typic haplumbrepts)	G	5.9	87	11.6	0.28	9.9	4.1	65
			H	5.9	83	6.1	0.16	8.0	1.5	84
			I	5.2	58	4.4	0.12	1.0	3.9	106
WS18	Toeslope, hardwood	Trimont loam (typic hapludults)	J	4.6	27	7.7	0.14	0.1	4.0	45
			K	4.6	33	4.6	0.13	0.1	4.5	52
			L	4.7	21	4.5	0.12	0.1	3.4	45
WS18	Stream	Haywood loam (cumulic haplumbrepts)	M	5.1	48	7.3	0.17	0.1	3.9	38
			N	5.1	48	4.9	0.15	0.1	3.1	35
			O	4.8	36	5.6	0.15	0.1	3.9	47

[a] Top 6 cm of mineral soil. Abbreviations: BS, base saturation; Tot-C, total carbon; Tot-N, total nitrogen; NO_3^-, ambient nitrate; NH_4^+, ambient ammonium.
[b] Mean of values from six sampling dates.

Soils were sampled and in situ N_2O 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 N_2O at many sites. Rates of N_2O 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 N_2O diffusion were determined by linear regression.

Effects of acetylene in laboratory incubations. To test the assumptions that 10 kPa of C_2H_2 effectively inhibits N_2O reduction and that 10 Pa of C_2H_2 inhibits only nitrification and not N_2O reduction, the effect of C_2H_2 on depletion of added N_2O was observed in samples of one of the Spivey soils (site I, Table 1). This sample was known to produce N_2 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 NO_3^- levels and thus provide a rigorous test of C_2H_2 inhibition. Four of the samples were then extracted with 100 ml of 2 M KCl for later NO_3^- determination.

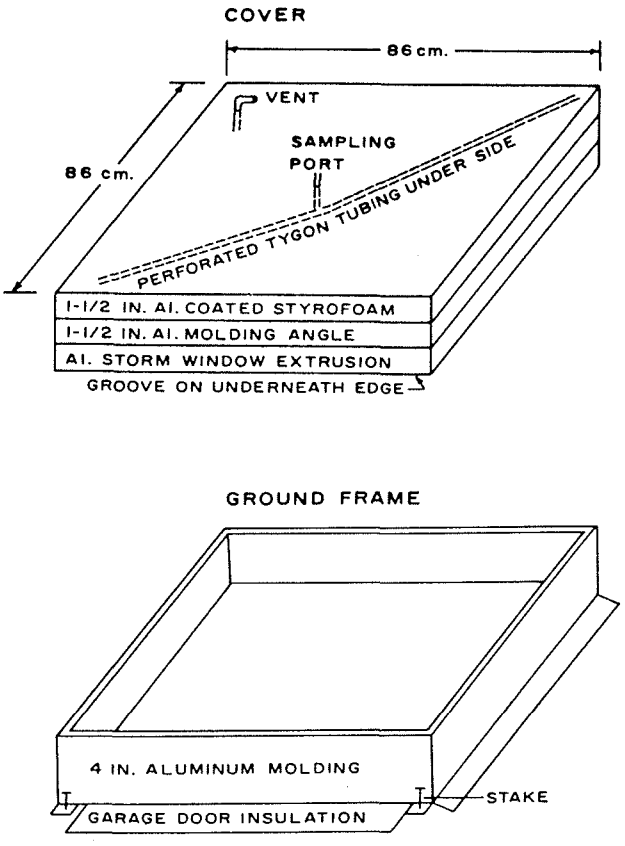

FIG. 1. Diagram of frame and cover for in situ N_2O diffusion measurement.

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_2O was injected into each bottle to yield headspace partial pressures of about 1 Pa. Acetylene, generated from CaC_2 and H_2O, was injected into the bottles so that four had partial pressures of 10 kPa, four had 10 Pa, and four received no C_2H_2. These bottles were incubated as before. Headspace gas was sampled with a syringe at regular intervals for 24 h and analyzed for N_2O 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^3 min^{-1}. A 10-port valve permitted the heavier C_2H_2 and water vapor to be back-flushed from the precolumn, whereas N_2O and lighter gases were separated on the analytical column. The effects of acetylene treatment on N_2O 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_2H_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_2O by gas chromatography as described above.

Six plausible combinations of hypothetical results exist for these incubations (Fig. 3). Production of N_2O under the 10-Pa treatment was assumed to estimate denitrification N_2O, since all autotrophic nitrification should be inhibited by the presence of C_2H_2. The difference between N_2O production under no C_2H_2 and that under 10 Pa was assumed to estimate nitrification N_2O, since any decrease in N_2O production when 10-Pa C_2H_2 was added should have been due to inhibition of nitrification. Production of N_2 by denitrification was estimated by the difference between N_2O production under 10 kPa of C_2H_2 and that under 10 Pa.

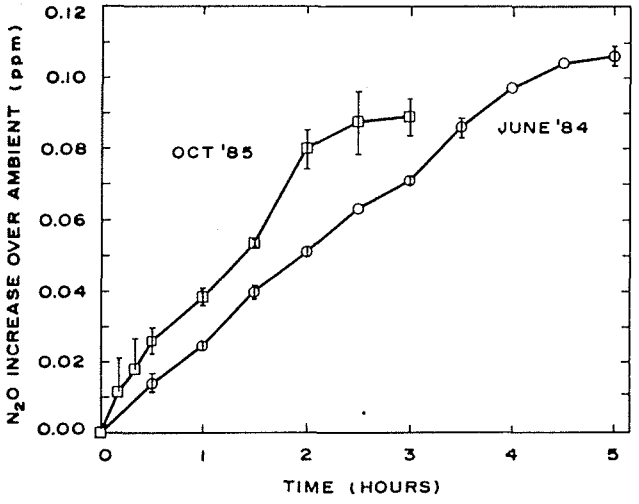

FIG. 2. Profiles of N_2O accumulation within soil covers at a midslope position on the disturbed WS: means and standard deviations (bars) for duplicate or triplicate gas analyses.

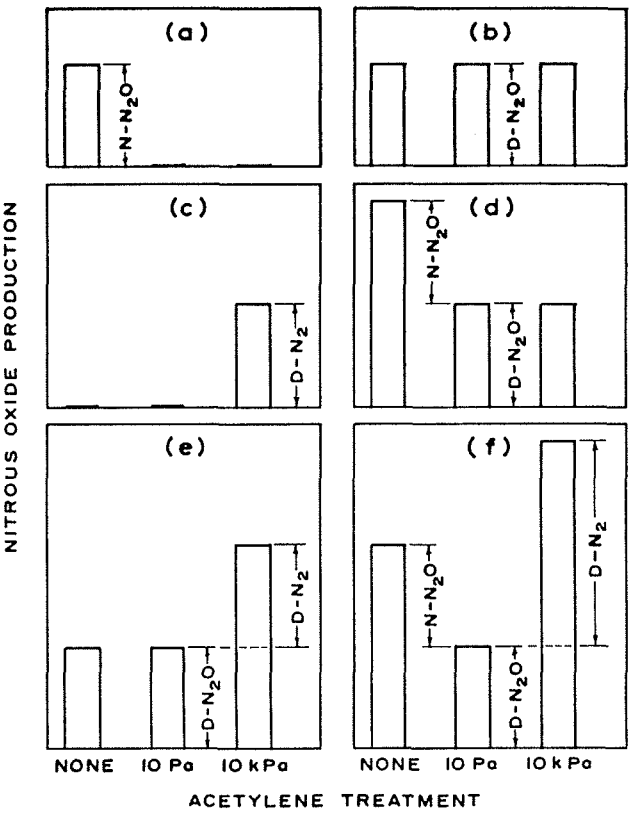

FIG. 3. Theoretically possible results of C_2H_2 treatment on N_2O production: (a) nitrification N_2O ($N\text{-}N_2O$) only, (b) denitrification N_2O ($D\text{-}N_2O$) only, (c) denitrification N_2 ($D\text{-}N_2$) only, (d) N_2O only from both nitrification and denitrification; (e) denitrification only with both N_2O and N_2 produced; and (f) all three sources of gaseous N.

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_2O, denitrification N_2O, and denitrification N_2 were obtained by subtraction of treatment means for each composite soil sample as described above. These estimates and the in situ N_2O difussion 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_2H_2 and when denitrification rates are high, NO_3^- 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_3^- levels. For samples from this site, each of the two levels of C_2H_2 was also applied to three subsamples which had been amended with 0.1 ml of 36 mM KNO_3, providing 1

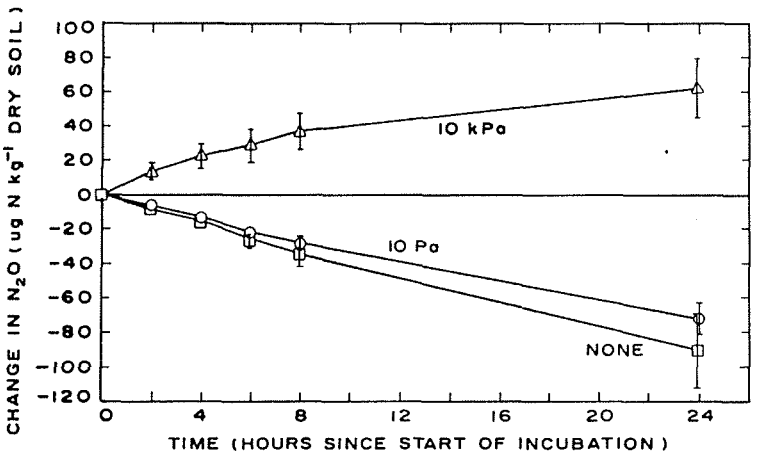

FIG. 4. Test of assumptions of C_2H_2 effects: means and standard deviations (bars) for four incubations per C_2H_2 treatment.

mg of NO_3^- nitrogen per kg of dry soil. The effects of C_2H_2 treatment, NO_3^- amendment, and their interactions were determined by analysis of variance. Spot checks were made on other soil samples, but C_2H_2-induced NO_3^- 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_2O production were generally linear throughout these incubations, the incubation procedures described above were repeated on Spivey, Tusquitee, and Haywood soil samples, with N_2O 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_3^- 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_3^- levels in the Spivey soil sample from 932 (±67, 95% confidence interval) to 67 (±11) μg of NO_3^- nitrogen per kg of dry soil, thus providing a rigorous test of the 10-kPa C_2H_2 block of N_2O reduction at low NO_3^- concentration during the following aerobic incubation. Nitrous oxide concentration increased during incubation under 10-kPa C_2H_2, indicating that the remaining NO_3^- was reduced to N_2O (62 ± 25 μg of N per kg of dry soil) and that none of this N_2O or the added N_2O was reduced to N_2 (Fig. 4). The 10-kPa C_2H_2 block appeared to be effective.

The 10-Pa C_2H_2 treatment may have caused a slight inhibition of N_2O reduction relative to the rate of N_2O depletion in the samples not receiving C_2H_2 (Fig. 4), but analysis of variance revealed that the rates of N_2O depletion for these two levels of C_2H_2 were not significantly different ($\alpha = 0.05$).

Estimation of activity in laboratory incubations. Of the six hypothetical combinations of results given in Fig. 3, three were observed in this study (Fig. 5). Most of the samples in which N_2O production occurred exhibited both nitrifying and denitrifying activity (Fig. 5b). Soil samples from only one site (Spivey soil under saturated conditions) consistently

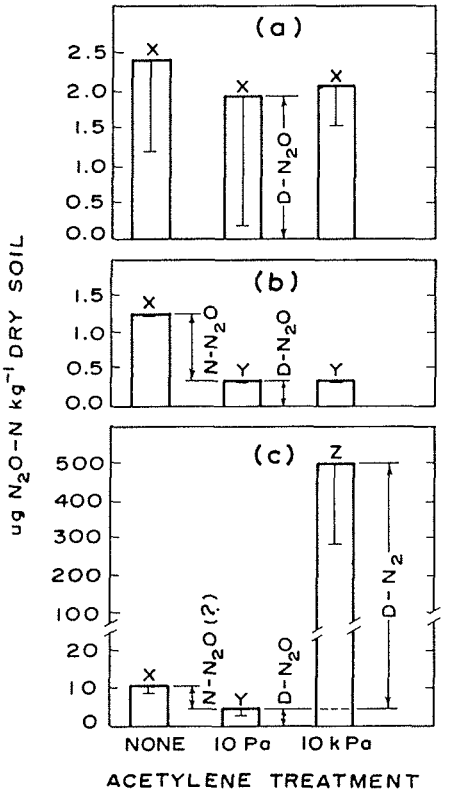

FIG. 5. C_2H_2 treatment effects on N_2O production: means and standard deviations (bars) for triplicate 24-h incubations. Means labeled by the same letter (X, Y, or Z) are not significantly different by least significant difference ($\alpha = 0.05$). Soil samples collected from the disturbed WS in May 1985 were (a) Spivey from a stream area, (b) Tusquitee from a midslope area; and (c) saturated Spivey from a stream area.

exhibited N_2 production (Fig. 5c). This same soil exhibited significant increases in N_2O production under all C_2H_2 treatments when NO_3^- was added before incubation. Therefore, partial inhibition of N_2O production during incubation of this soil under 10 Pa of C_2H_2 could be due to either inhibition of nitrification N_2O or inhibition of NO_3^- production and subsequent NO_3^- limitation for denitrification.

Time course analysis of laboratory incubations. Rates of N_2O 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 C_2H_2 treatments (Fig. 6b), probably because of NO_3^- depletion during the incubation.

Comparison of in situ and laboratory results. Laboratory nitrification N_2O and denitrification N_2O were significantly correlated ($\alpha = 0.05$) with in situ N_2O diffusion determinations ($r = 0.51$ and 0.29, respectively). When included in the multiple regression model $\log IS\text{-}N_2O = \beta_0 + \beta_1 \log N\text{-}N_2O + \beta_2 \log D\text{-}N_2O + \varepsilon$, where $IS\text{-}N_2O$ is in situ N_2O; $N\text{-}N_2O$ is nitrification N_2O (laboratory), and $D\text{-}N_2O$ is denitrification N_2O (laboratory), both independent variables were significant ($\alpha = 0.01$; $R^2 = 0.40$). Laboratory estimates of N_2O and N_2 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 N_2O (Fig. 7a) and nitrification N_2O (Fig. 7b), but was significantly correlated with denitrification N_2O (Fig. 7c) and denitrification N_2 (Fig. 7d). In contrast, nitrification N_2O exhibited the strongest correlation with ambient NO_3^- ($R^2 = 0.58$; $P < 0.01$). Denitrification N_2O also exhibited a correlation with ambient NO_3^- for those samples with low denitrifying activity ($R^2 = 0.41$; $P < 0.01$), but there were notable outliers which are discussed in the accompanying paper (4).

DISCUSSION

The use of C_2H_2 to estimate denitrification N_2 production has been problematic for soils with either very high or very low ratios of available carbon to NO_3^-. Nitrous oxide reduction was not completely inhibited by 10 kPa of C_2H_2 for samples of sewage sludge where available C was extremely high and NO_3^- was extremely low (11). In contrast, C-limited soils which had been amended with NO_3^- appeared to metabolize C_2H_2 as an energy source, especially in soil samples which had previously been exposed to C_2H_2 (9). For short-term incubations of soils of intermediate available C/NO_3^- ratios, the assumption that 10 kPa of C_2H_2 completely inhibits N_2O reduction and does not become significantly metabolized is probably valid (18, 19). In the present study, N_2O reductase inhibition under 10 kPa of C_2H_2 appeared complete even when ambient NO_3^- levels were low.

The lower threshold of C_2H_2 concentration where inhibition occurs is also somewhat controversial. Partial inhibition of N_2O reduction has been observed at C_2H_2 partial pressures as low as 1 Pa (22). In the present study, no significant difference in the rate of N_2O depletion was observed between treatments of no C_2H_2 and 10 Pa of C_2H_2 (Fig. 4). Since the effect of low C_2H_2 concentration may vary with soil types and denitrifier populations, the assumption that N_2O reduction is unaffected by 10 Pa of C_2H_2 may need verification for specific soils of interest.

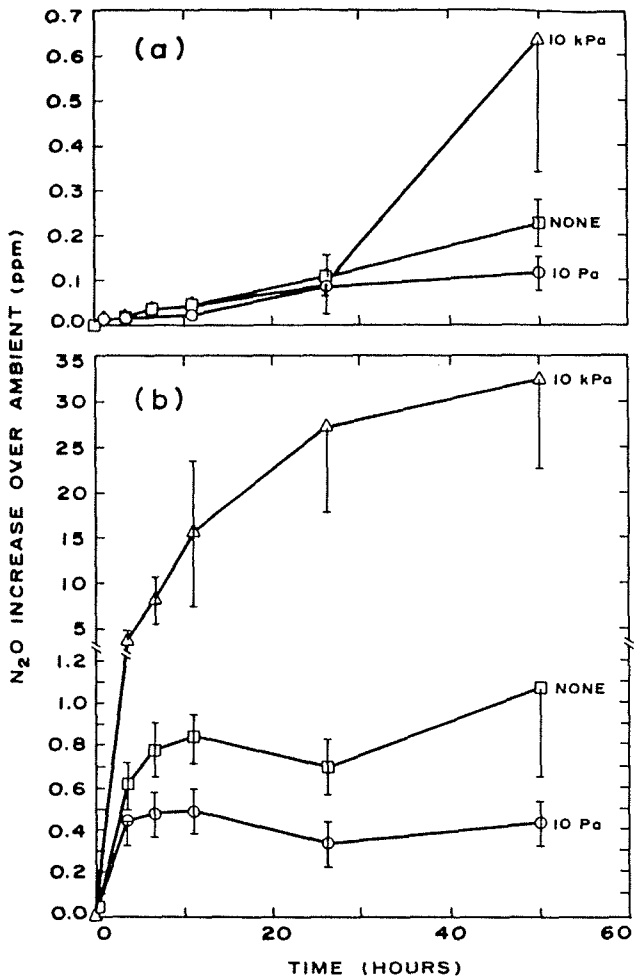

FIG. 6. Time-course profiles of N_2O production during laboratory incubations under three levels of C_2H_2: means and standard deviations (bars) of triplicate incubations per treatment-soil combination. Spivey soil samples from the disturbed WS stream area were collected (a) not saturated and (b) saturated.

Laboratory incubations under three levels of C_2H_2 permitted qualitative distinction of the source of N_2O production. Both the nitrification N_2O and denitrification N_2O estimates were correlated with in situ N_2O diffusion measurements, and their contributions to a multiple regression were nearly additive, suggesting that both processes contributed to N_2O 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 N_2O occurred at midslope positions of the same WS (Fig. 7b).

A quantitative ratio of nitrification N_2O to denitrification N_2O would not be appropriate, since this ratio could vary between laboratory conditions and field conditions. Similarly, a quantitative ratio of N_2O to N_2 produced by denitrification would be unwarranted, because samples which have sufficient reducing conditions to produce N_2 would probably exhibit nonlinear rates of N_2 and N_2O production (Fig. 6b).

The distinction between nitrification N_2O and denitrifica-

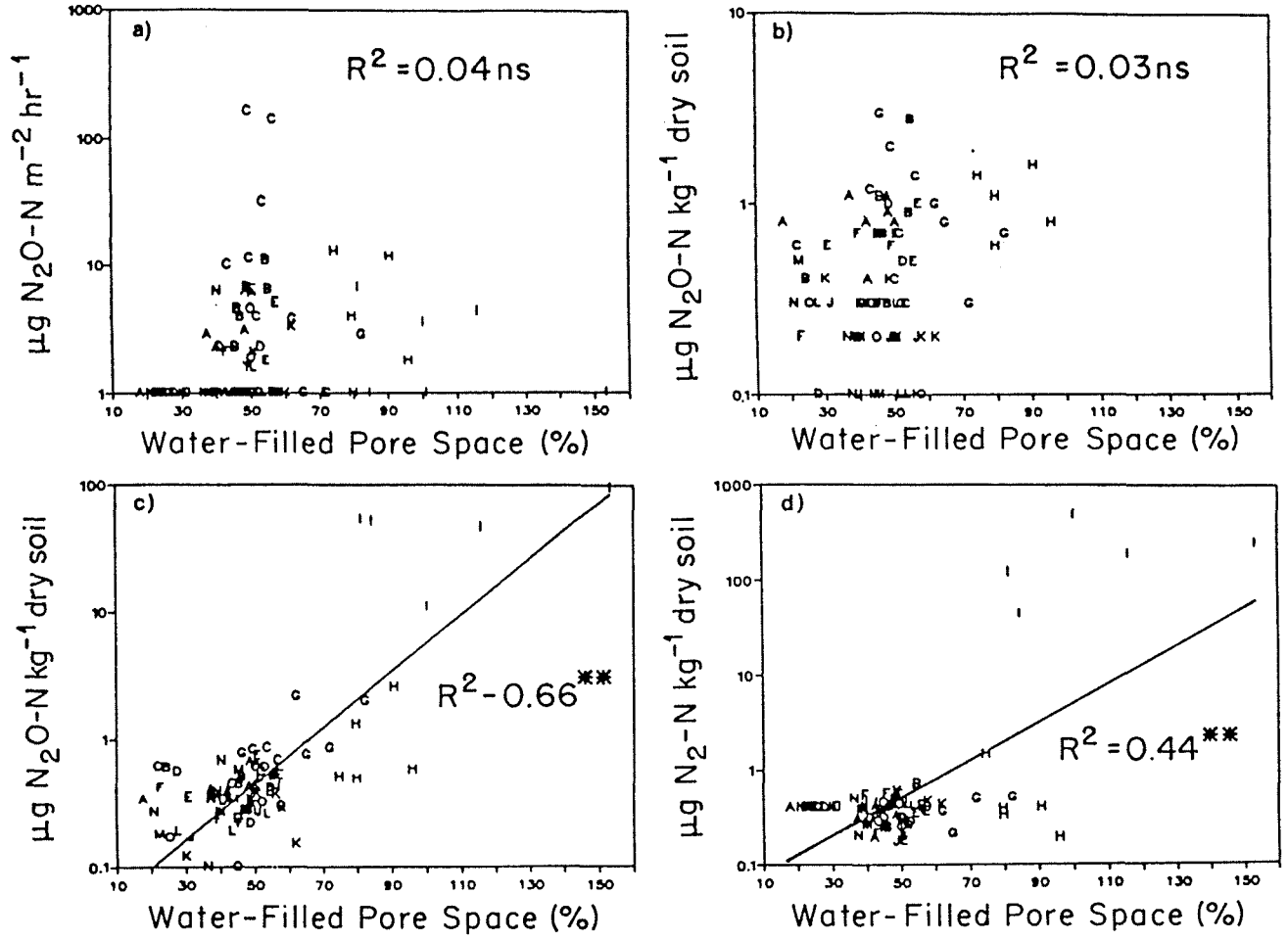

FIG. 7. Gaseous N production versus WFPS. Measurements taken from September 1984 through July 1985 from a midslope locust area of disturbed WS (sampling sites A, B, and C); a toeslope mixed-hardwood area of disturbed WS (D, E, and F); stream area of disturbed WS (G, H, and I); toeslope area of reference WS (J, K, and L); and stream area of reference WS (M, N, and O). (a) In situ N_2O versus WFPS; (b) nitrification N_2O versus WFPS; (c) denitrification N_2O versus WFPS; (d) denitrification N_2 versus WFPS.

tion N_2O by this method is corroborated by the observation of expected relationships between these estimates and soil parameters. Denitrification N_2O production often exceeded 1 μg of N_2O 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_2 production occurred only in samples with WFPS above 80% (Fig. 7d). The highest values for nitrification N_2O were observed near 50 to 60% WFPS (Fig. 7b). The relationship between WFPS and nitrification N_2O is more thoroughly discussed in the accompanying paper (4). Nitrification N_2O was strongly correlated with the other end product of nitrification, ambient NO_3^-.

This technique could be modified for studies of intact soil cores. However, a gas circulating system would be necessary to maintain appropriate C_2H_2 concentrations throughout the core (15). Such designs increase the difficulty in obtaining adequate replication for statistical determinations of C_2H_2 effects when variation among cores is high. Alternatively, a single core could be incubated repeatedly under successively higher C_2H_2 concentrations. However, preliminary work on soil cores in our laboratory indicated that denitrification rates increased during successive incubations at one C_2H_2 level, probably because incubation conditions favored denitrifying enzyme synthesis. Therefore, the effects of cumulative incubation time would probably confound successive C_2H_2 treatment effects. In the present study, we employed composite soil samples to improve homogeneity across C_2H_2 treatments for a given soil sample and to allow simultaneous processing of a large number of samples.

When the source of N_2O is uncertain, study of the factors affecting N_2O production is complicated. Qualitative distinction between nitrification and denitrification as sources of N_2O 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).

ACKNOWLEDGMENTS

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