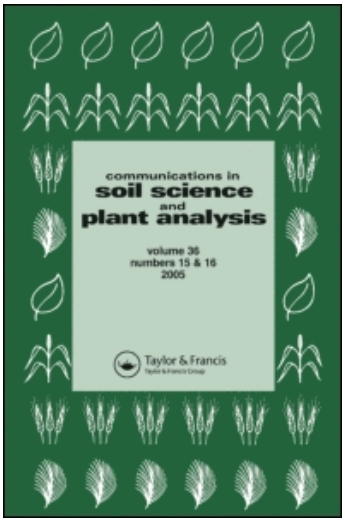

Communications in Soil Science and Plant Analysis
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597241

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Online publication date: 24 September 2010

To cite this Article Yu, Kewei , Seo, Dong-Cheol and DeLaune, Ronald D.(2010) 'Incomplete Acetylene Inhibition of Nitrous Oxide Reduction in Potential Denitrification Assay as Revealed by using ^{15}N-Nitrate Tracer', Communications in Soil Science and Plant Analysis, 41: 18, 2201 — 2210
To link to this Article: DOI: 10.1080/00103624.2010.504800
URL: http://dx.doi.org/10.1080/00103624.2010.504800

Communications in Soil Science and Plant Analysis, 41:2201–2210, 2010

ISSN: 0010-3624 print / 1532-2416 online
DOI: 10.1080/00103624.2010.504800

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Incomplete Acetylene Inhibition of Nitrous Oxide Reduction in Potential Denitrification Assay as Revealed by using ^{15}N-Nitrate Tracer

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One lake sediment and three soils for rice production were used to test the effectiveness of inhibiting of nitrous oxide (N_2O) reduction to dinitrogen gas (N_2) by acetylene (C_2H_2) using ^{15}N tracer. Regardless of the sources of the samples, results show that in presence of C_2H_2, significant isotopic enrichment of ^{15}N of N_2 was found at end of a typical denitrification assay. The $\delta^{15}N$ of N_2 value increased from 0‰ to 7.8–19.3‰ and 7.5–10.6‰ for the treatment with addition of 0.05 and 0.2 mg ^{15}N nitrate, respectively. Such ^{15}N enrichment can be interpreted as N_2 formation accounting for 15.3% and 2.5% of the total added N in these two treatments, respectively. Nitrous oxide accumulation in presence of C_2H_2 could not account for the total added N. The result indicates incomplete inhibition of N_2O reduction to N_2 by C_2H_2 in denitrification when N_2O reduction enzyme is developed.

Keywords Acetylene, denitrification, ^{15}N nitrate, N_2O reduction, stable isotope, wetland

Introduction

Denitrification, mainly a microbial process involving the reduction of nitrogen (N) oxides to N gases, is a major mechanism by which part of the N in the biosphere returns to the atmosphere. Denitrification is an essential process that removes excessive N in different ecosystems to maintain the system's function and sustainability. Different approaches to quantify denitrification have been developed, and all existing methods have some difficulties and limitations under various circumstances, which have been recently reviewed (Groffman et al. 2006).

It is very difficult to directly quantify the dominant end product, dinitrogen gas (N_2), of denitrification because of its high ambient concentration in the atmosphere. An indirect approach is to quantify an intermediate product of denitrification, nitrous oxide (N_2O), by using the acetylene (C_2H_2) blockage technique. Under anaerobic conditions, 10% C_2H_2 in gas-phase volume can inhibit N_2O reduction to N_2, making N_2O an end product of denitrification for easy detection. This technique, since its early development (Balderston,

Received 9 February 2009; accepted 12 January 2010.
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Sherr, and Payne 1976; Yoshinari and Knowles 1976), has become the most widely used method to determine denitrification rates. A complete inhibition of N_2O reduction to N_2 by C_2H_2 is essential for the method to be valid. In a potential denitrification assay (PDA) using dry soils with addition of a high concentration of nitrate, this technique is likely to be more effective. A comparison experiment using a dry grassland soil has shown no significant difference ($P > 0.05$) in total flux of N gases by using the ^{15}N-tracer technique versus the C_2H_2 blockage technique (Malone, Stevens, and Laughlin 1998). This is because nitrate is more favorable than N_2O for obtaining electrons from organic matter and because the N_2O reduction enzyme level in dry soils is generally low but can be synthesized under anaerobic conditions (Dendooven and Anderson 1995). For the same reasons, the C_2H_2 blockage technique may not always completely inhibit N_2O reduction for wet soils and sediments, where N_2O reduction enzyme will likely be developed, with low concentration of nitrate (Knowles 1990). Bernot et al. (2003) have suggested combining the treatment of C_2H_2 and antibiotic (to inhibit new enzyme synthesis) for estimating denitrification rate.

The objective of this study was to test the validity of the C_2H_2 inhibition technique in flooded soils and sediment using the ^{15}N-tracer technique. One freshwater lake sediment and three anaerobically pre-incubated rice soils were used for PDA with 10% C_2H_2. Nitrous oxide reduction enzyme is assumed to be present in the lake sediment and can be fully synthesized in the three rice soils during the pre-incubation. In the presence of C_2H_2 and labeled ^{15}N nitrate, no labeled N_2 should be formed if N_2O reduction is completely inhibited by C_2H_2.

Materials and Methods

Samples and Major Characteristics

The surface lake sediment (0–15 cm) sample was collected from Lake Cataouatche (29° 51.35′ N, 90° 14.40′ W), Louisiana, USA. The lake receives discharges of freshwater and nutrients from the Mississippi River, as part of the Davis Pond Mississippi River Freshwater Diversion Facility. More information on site description and studies of denitrification conducted at this location has been documented in recent publications (Yu, DeLaune, and Boeckx 2006; Seo, Yu, and DeLaune 2008). The three surface rice soils (0–20 cm) were collected from different rice-cultivating states in the United States: Arkansas (35° 04′ N, 092° 51′ W), Mississippi (31° 19′ N, 089° 06′ W), and Texas (29° 59′ N, 096° 44′ W). The rice soils were air dried, sieved (1-mm sieves), thoroughly mixed, and stored at room temperature (20 °C) before the experiment. Major characteristics for the sediment and three rice soils were analyzed and summarized in Table 1.

Potential Denitrification Assay Incubation Procedure

Fresh lake sediment was used for PDA incubation by weighting 30 g sediment (wet weight with water content 80.6%) into a 155-mL glass vial with 30 mL deionized (DI) water. The sediment contained trace and variable amounts of nitrate, which were not determined for this study. The three rice soils were first incubated in three separate soil microcosms (each 2200 mL in volume) with 300 g dry soil and 1500 mL DI water, until soil redox potential (Eh) reached approximately −150 mV in about a month. There are two purposes for this pre-incubation: (1) to allow soil denitrifying enzymes, especially N_2O reduction enzyme, to be fully developed under anaerobic conditions and (2) to remove any original soil nitrate

Table 1
Selected physical and chemical characteristics of the lake sediment and three rice soils

Sample	Original pH	pH before PDA	Eh (mV)	OM[a] (mg kg^{-1})	Total N (mg kg^{-1})	NH_4^+-N (mg kg^{-1})	Fe (mg kg^{-1})	Mn (mg kg^{-1})	S (mg kg^{-1})	Sand (g kg^{-1})	Silt (g kg^{-1})	Clay (g kg^{-1})
LA sediment	5.8	5.8 ± 0.1	263 ± 6[b]	56.7	4.8	ND[c]	39	0.7	18	177	278	545
AK rice	6.0	6.2 ± 0.0	−164 ± 16	14.6	0.7	6.2	134	105	13	150	41	809
MS rice	7.7	6.2 ± 0.0	−142 ± 25	25.3	1.0	4.9	71	9	12	582	126	292
TX rice	5.1	6.0 ± 0.0	−172 ± 7	25.4	1.1	4.8	115	35	38	641	75	284

[a]OM: organic matter.
[b]Data represent mean ± SD ($n = 2$). Eh was measured before PDA incubation.
[c]ND: not determined.
Note. Original soil nitrate and nitrite content was not included, because the soils were pre-incubated before the PDA incubation.

to minimize isotope dilution of added ^{15}N nitrate. After the pre-incubation, 50 mL homogenous soil slurry were withdrawn from each microcosm and transferred into a 155-mL glass vial. The rice soil slurry maintained the same soil–water ratio as in the microcosm. All experiments were conducted at room temperature (20 °C) with two replicates.

To provide an N source for denitrification in such almost nitrate-free sediment and soil slurries, 0.05 and 0.2 mg N were amended by adding 1 and 4 mL, respectively, of 3.33 mM labeled potassium nitrate ($K^{15}NO_3$) with 99.3 atom% ^{15}N ($^{15}N/^{14}N = 142$) into the soil/sediment slurries. These amendments were equivalent to 8.6 and 34.4 mg N kg^{-1} for the lake sediment slurry and 5 and 20 mg N kg^{-1} for the three rice soil slurries, respectively. This range of N concentration is commonly seen for denitrification studies, and the two levels of nitrate addition can be used to study the effect of nitrate on efficiency of C_2H_2 inhibition. After nitrate amendment, the vials were flushed with pure N_2 for a minute to remove the air in headspace of the vials, and then immediately sealed with a rubber stopper. Pure C_2H_2 was injected, replacing 10 mL of the headspace volume of each vial to inhibit reduction of N_2O to N_2 in the denitrification process. The vials were incubated for 3 days on an automatic shaker. Gas samples (3 mL each time) were collected, after vigorously shaking the bottles for gas equilibrium, from headspace of the vials once a day during the incubation for analyzing N_2O concentration. After taking the last gas samples for N_2O analysis, a 12-mL gas sample was withdrawn and immediately transferred into a 12-mL pre-evacuated Labco Exetainer Vial (Labco Limited, U.K.) for ^{15}N of N_2 isotope analysis. Afterward, slurries in incubation vials were extracted for 1 h with 1 mL of 5 M potassium chloride (KCl) solution. The soil/sediment slurries were filtered (0.45 μm) and later analyzed for nitrate, nitrite, and ammonium concentrations in the solution.

Sample Analysis

Total organic matter (OM) was measured colorimetrically after oxidizing with potassium dichromate ($K_2Cr_2O_7$) and concentrated sulfuric acid. Total iron (Fe), manganese (Mn), and sulfur (S) concentrations in the samples were analyzed by inductively coupled plasma (ICP) after digestion (Yu and Patrick 2003, 2004). Nitrate, nitrite, and ammonium concentrations were analyzed using a Lachat auto-analyzer (Hach Company, Loveland, Col., USA) with reliable detection limit of 0.01 mg N L^{-1}. Particle-size distribution of the sediment was obtained by a hydrometer method (Patrick 1958). Redox potential was measured using two replicate platinum (Pt) electrodes with a calomel reference electrode. A portable pH meter was used for pH measurement. A subsample of the fresh sediment was dried at 105 °C to a constant weight for determining moisture content. All data are presented based on dry weight of the samples.

Nitrous oxide concentration was analyzed using a Tremetrics 9001 (Tremetrics, Lawrenceville, Ga.) gas chromatograph (GC) with an electron capture detector (ECD), and calibrated with a certified N_2O standard (Scott Specialty Gases, Inc., Plumsteadville, Penn.). Gas samples were injected through a 2.0-mL sample loop connected to a Valco valve. The system was equipped with a back-flush mechanism operated by a 10-port Valco valve to prevent moisture in the samples from entering into the detector. Operating temperatures were 50 °C for the oven and 310 °C for the detector. All gas analyses were subject to conventional quality control with a standard spike in every three samples. An isotope signature of N_2 in the gas samples was analyzed by the stable isotope laboratory at the University of California, Davis, using a Europa Hydra Model 20/20 (Europa, Crewe, UK) continuous flow isotope ratio mass spectrometer (IRMS). Atmospheric N_2 ($\delta^{15}N = 0‰$) was used as a working standard.

Calculation and Statistical Analysis

Nitrous oxide flux rates were calculated by N_2O concentration increase in the incubation vials. The amount of N_2O dissolved in the liquid phase was calculated by using the molar fraction solubility of 5.07×10^{-4} (Lide 1991). Nitrogen gas flux rates were calculated from the enrichment of samples' $^{15}N/^{14}N$ ratios, which can be expressed by the δ value of the samples:

$$\delta^{15}\text{ N of } N_2\,(\%) = \left[\frac{^{15}\text{N}/^{14}\text{N (sample)}}{^{15}\text{N}/^{14}\text{N (atmosphere)}} - 1\right] \times 1000$$

where $^{15}N/^{14}N$ (atmosphere) = 0.00367 (an international standard with 0.366 atom% ^{15}N). The ratio of two enriched N_2 species formed in denitrification, $^{14}N^{15}N$ and $^{15}N^{15}N$, reflects the ratio of $^{15}N/^{14}N$ in the added labeled $K^{15}NO_3$ (the slurries were nitrate-free before addition), provided that N_2 is formed by random pairing of N atoms. Given the reduction of N_2O, the only source of $^{15}N^{15}N$, quantity of N_2 evolved can be calculated from the samples' $^{15}N/^{14}N$ ratio contributed by two N_2 sources: (1) N_2 production from denitrification with the same $^{15}N/^{14}N$ ratio as in the added labeled $K^{15}NO_3$ and (2) N_2 in the headspace of the incubation bottles with the same $^{15}N/^{14}N$ ratio as in the atmosphere. The isotope mixing model from these two sources is

$$\frac{\text{V (1)}}{\text{V (2)}} = \frac{\delta^{15}\text{N}\left(\text{K}^{15}\text{NO}_3\right) - \delta^{15}\text{N (sample)}}{\delta^{15}\text{N (sample)} - \delta^{15}\text{N (atmosphere)}}$$

where V (1) and V (2) represented the volume of N_2 from these two sources, respectively. Redox potential was calibrated to the standard H_2 electrode by adding 247 mV (the correction factor for calomel reference electrode at 20 °C) to the observed instrument reading. Statistical analysis was conducted using SAS (version 8 for Windows, SAS Institute Inc., Cary, N.C., USA). The significance level was chosen as $\alpha = 0.05$.

Results and Discussion

Lake Cataouatche is shallow, with water depths of only about 2 m. The redox potential of the lake sediment was 263 ± 6 mV before the PDA incubation. The moderately reducing condition of the lake sediment was optimum to sustain denitrification activity (Ciarlo et al. 2007; Yu and Patrick 2003, 2004). The strongly reducing condition (Eh about −150 mV) that developed during the pre-incubation of the three rice soils was sufficient to remove soil original nitrate (Yu et al. 2007). However, Eh was subject to change when nitrate was amended during the PDA incubation because of the redox buffering effect of nitrate. (Eh was not monitored during the PDA incubation.) Significant changes in pH were found in the Mississippi and Texas soil microcosm during the pre-incubation. All soil/sediment slurry pH was close to 6.0 before the PDA incubation (Table 2).

Following the 3-day PDA incubation, only a trace amount of nitrate remained in the system, indicating rapid nitrate removal by denitrification activity. Meanwhile, in the presence of C_2H_2, a significant enrichment of isotope ^{15}N of N_2 was found in the incubation vials. The $\delta^{15}N$ of N_2 value increased from 0‰ to 7.8–19.3‰ for the treatment with 0.05 mg N-labeled nitrate and to 7.5–10.6‰ for the treatment with 0.2 mg N-labeled

Table 2
Delta value of N_2 end product and remaining $NO_3^- + NO_2^-$ concentration

Sample	Treatment	$\delta^{15}N$ of N_2 (‰)	$NO_3^- + NO_2^-$ (mg N kg^{-1})	NH_4^+ (mg N kg^{-1})
LA sediment	$-C_2H_2$ + 0.05 mg N	56.3 ± 1.7	0.03 ± 0.01	20.41 ± 0.08
	$-C_2H_2$ + 0.2 mg N	238.0 ± 45.0	0.03 ± 0.01	20.35 ± 1.91
	$+C_2H_2$ + 0.05 mg N	10.5 ± 2.7	0.02 ± 0.00	22.14 ± 0.48
	$+C_2H_2$ + 0.2 mg N	10.6 ± 0.4	0.03 ± 0.00	24.22 ± 4.13
AK rice	$+C_2H_2$ + 0.05 mg N	19.3 ± 1.7	0.09 ± 0.03	46.39 ± 8.24
	$+C_2H_2$ + 0.2 mg N	7.5 ± 2.7	0.14 ± 0.05	61.74 ± 14.46
MS rice	$+C_2H_2$ + 0.05 mg N	17.0 ± 2.6	0.03 ± 0.00	22.99 ± 5.44
	$+C_2H_2$ + 0.2 mg N	9.5 ± 0.1	0.07 ± 0.06	25.40 ± 0.40
TX rice	$+C_2H_2$ + 0.05 mg N	7.8 ± 3.4	0.06 ± 0.03	26.19 ± 3.59
	$+C_2H_2$ + 0.2 mg N	8.1 ± 3.5	0.08 ± 0.01	33.90 ± 7.11

Notes. Data represent mean ± SD ($n = 2$). Delta values of N_2 as well as $NO_3^- + NO_2^-$ and NH_4^+ concentrations were determined at the end of PDA incubation. The two levels of nitrate amendments were equivalent to 8.6 and 34.4 mg N kg^{-1} for the lake sediment slurry and 5 and 20 mg N kg^{-1} for the three soil slurries, respectively.

nitrate (Table 2). The results provide direct evidence of N_2 formation in the presence of C_2H_2, confirming an incomplete inhibition of N_2O reduction to N_2 in this experimental condition. More N_2 gas was produced in the low-nitrate-addition treatment in the Arkansas and Mississippi soil slurries, probably a result of less competition of nitrate with N_2O for electrons (Knowles 1990). However, no difference in N_2 formation for the two levels of nitrate addition was found in the lake sediment and Texas soil slurries for unknown reasons. If denitrification was the only mechanism involved in nitrate removal and $^{15}N^{15}N$ formed was only from incomplete inhibition of $^{15}N^{15}NO$ reduction, on average each 1‰ increase of $\delta^{15}N$ of N_2 values could be interpreted as 0.0663 mg N kg^{-1} in the incubation vials. Therefore, the average N_2 formation at the end of the incubation accounted for 15.3% of the total added N in the 0.05 mg N treatment and 2.5% in the 0.2 mg N treatment, respectively.

The dynamics of N_2O concentration during the incubation, monitored daily, is presented in Figures 1 and 2. The lake sediment showed strong N_2O reduction activity in treatment without C_2H_2, and over time there was no detectable N_2O at end of the PDA incubation (Figure 1). Without C_2H_2, it is assumed that all the N_2O produced from denitrification was reduced to N_2 in the lake sediment, resulting in a large increase in $\delta^{15}N$ of N_2 value (Table 2). Comparison of isotopic analysis from the treatments with and without

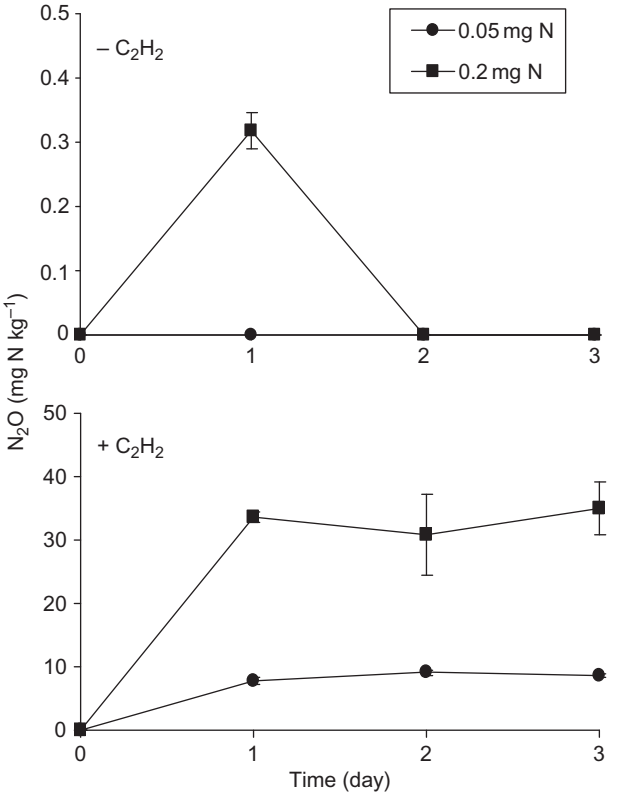

Figure 1. Nitrous oxide emissions in incubation with lake sediment. Data are presented by means with standard deviation in error bars. Addition of 0.05 and 0.2 mg N represents 8.6 and 34.4 mg N kg^{-1}, respectively, in the slurry.

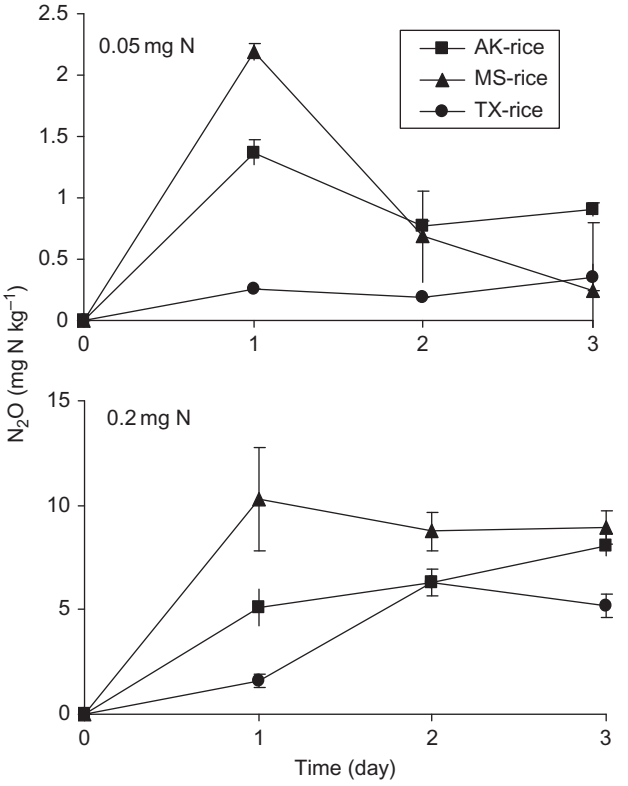

Figure 2. Nitrous oxide emissions in incubation with three rice soils. Data are presented by means with standard deviation in error bars. Addition of 0.05 and 0.2 mg N represents 5 and 20 mg N kg^{-1}, respectively, in the slurry.

C_2H_2 can be used as an indirect approach for interpreting the $\delta^{15}N$ of N_2 results with similar conclusions. In the presence of C_2H_2, N_2O accumulation could not recover the quantity of added N in all slurries (more obviously in the three rice soils), and N_2O concentration tended to decline after reaching a maximum, especially in the treatments with low level of N addition (Figure 2). Recent denitrification studies have shown that nitrate reduction rate is much greater than measured denitrification rate using C_2H_2 blockage technique (Laverman et al. 2007; Yu et al. 2008). Incomplete inhibition of N_2O reduction is partially responsible for this observation, with the N_2O reduction reaction likely more significant when nitrate concentration is low. However, in the lake sediment slurries, N_2 formation without C_2H_2 addition accounted for only half of the added N (Table 2), suggesting that other reasons are involved. Nitric oxide (NO) is also an intermediate product of denitrification (Ryden 1981; Ye, Averill, and Tiedje 1994). Dynamics of NO concentration during the PDA incubation was not monitored, but NO gas plays a role in the N balance involving denitrification. In addition, dissimilatory nitrate reduction to ammonium has been reported as a mechanism of nitrate consumption under anaerobic condition (Kaspar, Tiedje, and Firestone 1981), which would also contribute to nitrate removal during the PDA incubation. A large amount of ammonium accumulated in the three rice soil slurries during the pre-incubation (Tables 1 and 2). Greater concentrations of ammonium were found in the greater nitrate addition treatment, indicating a likely source from dissimilatory nitrate

reduction. Although formation of ammonium through the ammonification process could not be excluded, the contribution was likely insignificant in the anaerobic conditions of this study. The isotopic signature of ammonium was not analyzed in this study. If some of the labeled nitrate was converted to ammonium, there would be chances for $^{15}N^{15}N$ formation through nitrate and nitrite reaction with ammonium (anaerobic ammonium oxidation – anammox). However, formation of $^{14}N^{15}N$ is expected in this reaction (Jetten et al. 2003; Penton, Devol, and Tiedje 2006).

This study presents clear evidence of the incomplete inhibition of N_2O reduction to N_2 by C_2H_2 in the PDA. Nitrous oxide reduction enzyme, fully developed in flooded soils and sediments, as was the case in this study, may limit the validity of the C_2H_2 blockage technique. The cause of incomplete inhibition of N_2O reduction to N_2 deserves future investigation. Lesser concentrations of nitrate in the incubation could be a cause for many cases (Knowles 1990). However, lesser concentrations of nitrate alone could not explain the results of the similar $\delta^{15}N$ of N_2 values observed under the two levels of nitrate addition in the lake sediment and Texas rice soil slurries (Table 2). Reaction of C_2H_2 and NO in the gas phase has been reported to decrease C_2H_2 partial pressure during denitrification incubation (Bollmann and Conrad 1997; McKenney, Drury, and Wang 1997), which ultimately lessens the inhibition of N_2O reduction to N_2. The acetylene blockage technique has been the simplest and most commonly applied technique to quantify denitrification. Nitrogen gas formation in the presence of C_2H_2 seriously limits some applications of the C_2H_2-based techniques in denitrification studies, because the denitrification rate will be underestimated if N_2O reduction to N_2 is not completely inhibited.

Acknowledgment

This work was supported in part by the Louisiana Department of Natural Resources and by a Korea Research Foundation grant funded by the Korean government (MOEHRD) (KRF-2006-214-F00003).

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