Reports

Denitrification Studies with ^{13}N-Labeled Nitrate

Abstract. *Nitrate labeled with ^{13}N ($^{13}NO_3^-$) was produced in a cyclotron by the $^{16}O(p, \alpha)^{13}N$ reaction with protons having energies of 14.5 million electron volts. The $^{13}NO_3^-$ was used as a tracer for direct quantitative measurements of denitrification rates in soils from flooded rice fields. The ^{13}N technique provides a new tracer method for the measurement of denitrification rates in natural systems over short time intervals, without changing the concentration of NO_3^- in the system.*

Denitrification is defined as the biological reduction of NO_3^- or NO_2^- to gaseous end products, usually N_2O or N_2. This process can result in a significant net loss in the combined nitrogen available to an ecosystem unless it is balanced by nitrogen fixation. The lack of quantitative measurements of nitrogen lost through denitrification has hindered the development of accurate nitrogen budgets for both soil and aquatic ecosystems.

We report here the details of a new technique involving the use of the radioisotope ^{13}N by which denitrification rates can be measured without significantly changing the concentration of NO_3^- in the system. Some preliminary results of ^{13}N experiments on flooded rice soils are presented. The ^{13}N technique allows a measurement of the kinetics of nitrogen transformations over very short time intervals and at low substrate concentrations. As the longest-lived radioisotope of nitrogen, ^{13}N has obvious advantages if one wishes to follow the course of environmental nitrogen transformations; however, because the half-life of ^{13}N is only 10 minutes, the use of this isotope has been limited to just a few studies of biological nitrogen fixation (*1*). Two advantages of using ^{13}N rather than the stable isotope ^{15}N are the shorter incubation times necessary (10 minutes as opposed to several hours) and the increased sensitivity for detection (approximately 10^8-fold). In aquatic systems where NO_3^- concentrations may be rate-limiting, it is especially difficult to estimate denitrification rates with ^{15}N techniques, because the microgram amounts of $^{15}NO_3^-$, which must be added, significantly raise the NO_3^- concentration, thereby increasing the denitrification rate (*2*). In our experiments $^{13}NO_3^-$ additions involved trace quantities ($\sim 10^{-12}$ g).

Millicurie amounts of $^{13}NO_3^-$ were produced in the 193-cm isochronous cyclotron at the Crocker Nuclear Laboratory by the $^{16}O(p, \alpha)^{13}N$ reaction with 14.5-Mev protons (*3*). The beam of protons (1 to 2 μa) impinged on a thin-walled quartz beaker filled with approximately 55 ml of distilled water. With water as a target the radiochemical form was > 99.6 percent $^{13}NO_3^-$, and there was no measurable $^{13}NO_2^-$ or $^{13}NH_4^+$ (*4*). With an average nuclear production cross section of about 20 millibarns (*3*), an irradiation time of 20 minutes was sufficient to produce about 20 mc of ^{13}N activity. Comparable yields have been reported for water targets under similar irradiation conditions (*5*, *6*). The bombarding energy was below the threshold for the production of either ^{11}C (half-life = 20 minutes) or ^{15}O (half-life = 2 minutes), and the slope of the decay curve confirmed a 10-minute half-life for the irradiation product (*7*). During irradiation the water target and transfer tubing were flushed with helium to remove air from the system. Upon completion of the bombardment, the aqueous $^{13}NO_3^-$ was transferred by a pressure differential from the cyclotron vault, through polyethylene tubing, into a lead-lined glove box where the experiments were carried out.

We chose waterlogged rice soils for our experiments to demonstrate the applicability of the ^{13}N technique to rate measurements of natural bacterial populations. Submerged rice paddy soils are characterized by two distinct zones: an aerobic (oxidized) surface layer several millimeters thick and an underlying anaerobic (reduced) layer comprising the rest of the soil (*8*). In the aerobic layer NH_4^+ is nitrified to NO_3^-, which then enters the anaerobic zone where denitrification occurs (*9*). The rhizosphere of rice roots constitutes a second aerobic zone in the soil where NH_4^+ is nitrified to NO_3^- (*10*). Severe nitrogen loss has been shown to occur in soils subjected to periods of alternate drying (aerobic) and flooding (anaerobic) (*11*), conditions which occur annually in lowland rice soils.

Samples for ^{13}N experiments were obtained by vacuum transfer of soil from just below the soil-water interface into a helium-filled 500-ml bottle; care was taken in the transfer to prevent exposure to air because oxygen both inactivates and represses the formation of the dissimilatory nitrate reductase (*12*). The soil sample was taken immediately to Crocker Nuclear Laboratory where the bottle was fitted with a rubber stopper designed for the ^{13}N apparatus (Fig. 1). Once the aqueous $^{13}NO_3^-$ had been transferred into the incubation bottle, a continuous flow of helium (200 to 300 ml min^{-1}) main-

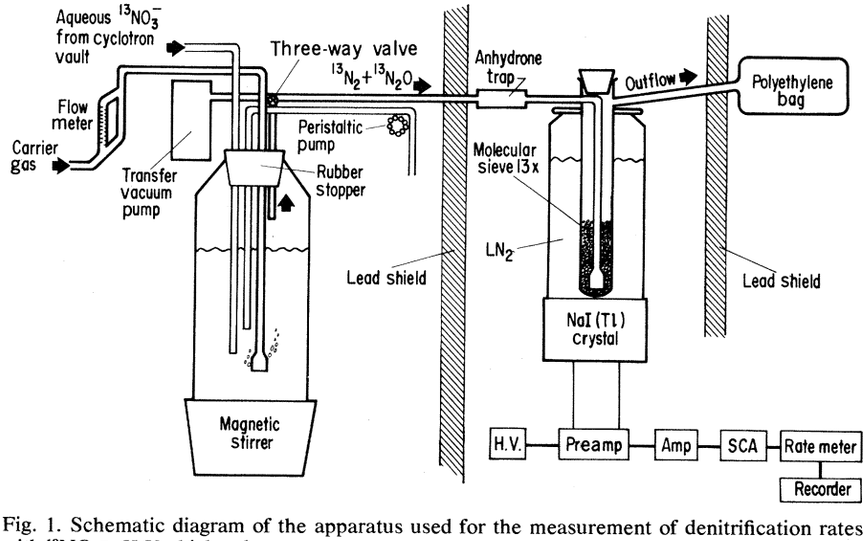

Fig. 1. Schematic diagram of the apparatus used for the measurement of denitrification rates with $^{13}NO_3^-$; *H.V.*, high voltage.

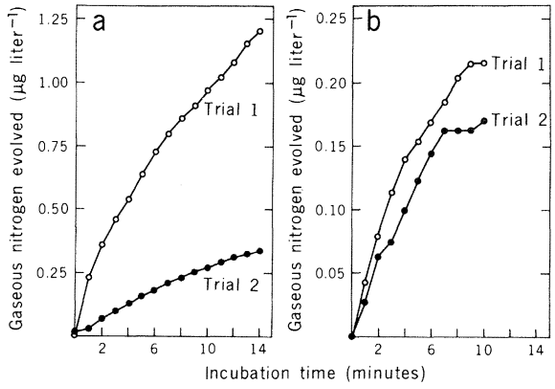

Fig. 2. Curves showing the quantity of N_2 and N_2O gases evolved during incubation. Values were calculated at 1-minute intervals from Eq. 1. (a) Experiment 1, successive trials (28°C) on 2 September 1975: trial 1, at the start of incubation the $^{13}NO_3^-$ activity was 10 mc and $[NO_3^-]$ was 201 μg of NO_3^--N per liter; trial 2, at the start of incubation the $^{13}NO_3^-$ activity was 13 mc and $[NO_3^-]$ was 75 μg of NO_3^--N per liter. (b) Experiment 2, successive trials (27°C) on 3 October 1975; trial 1, at the start of incubation the $^{13}NO_3^-$ activity was 1.6 mc and $[NO_3^-]$ was 82 μg of NO_3^--N per liter; trial 2, at the start of incubation the $^{13}NO_3^-$ activity was 3.9 mc and $[NO_3^-]$ was 79 μg of NO_3^--N per liter.

tained anaerobic conditions and a flow rate meter made it possible to monitor any flow interference downstream. A magnetic stirrer ensured proper outgassing of the N_2 and N_2O produced, and these gases were swept from the incubation bottle into a molecular sieve trap cooled by liquid nitrogen (LN_2). We used molecular sieve 13× (*13*), which adsorbs molecules with an effective diameter of less than 10 Å. The N_2, with a molecular diameter of < 4 Å, is trapped in the sieve apertures more readily at lowered temperatures (*14*). The molecular sieve material was placed in a glass finger trap which was partially immersed in a Dewar flask filled with LN_2. At LN_2 temperatures (−196°C), N_2O solidifies and N_2 is adsorbed in the molecular sieve lattices.

The radioactivity in the molecular sieve trap was monitored with a lead-shielded NaI(Tl) crystal detector (7.6 by 7.6 cm) coupled to an amplifier and a single-channel analyzer (SCA). The ^{13}N isotope decays by emission of positrons (β^+), which upon annihilation yield two 0.511-Mev gamma rays (γ). With the SCA window set to detect only γ emissions of this energy, the output logic pulse was used to drive a rate meter which supplied an analogue voltage to a chart recorder. Recorded radioactivity data, when corrected for decay (*15*), provided a graph of the accumulation of gaseous ^{13}N activity as a function of time. We calibrated the NaI(Tl) crystal detector by placing a ^{22}Na standard source in the Dewar flask at a position corresponding to the level at which the sintered glass tube terminates in the sieve material. Counts detected could then be expressed directly in microcuries. Effluent gas from the trap was collected in an inflatable polyethylene bag which was monitored by a second lead-shielded NaI(T1) crystal detector (7.6 by 7.6 cm). No measurable 0.511-Mev γ-rays were detected in the polyethylene bag, and we concluded that the molecular sieve trap was quantitatively removing gaseous ^{13}N activity.

The denitrification rate was calculated from the following equation (*16*):

$$D = \left(\frac{dN}{dt} + \lambda N \right) \frac{^{14}NO_3^-}{^{13}NO_3^-} \quad (1)$$

where D is the rate at which gaseous nitrogen is evolved (in micrograms per minute); dN/dt is the accumulation rate of gaseous ^{13}N activity (in microcuries per minute) in the trap; λN, the decay correction, is the amount of gaseous ^{13}N activity (in microcuries) multiplied by λ ($\lambda = 0.0693$ min^{-1}); $^{13}NO_3^-$ is the activity of $^{13}NO_3^-$ in the incubation bottle (in microcuries); and $^{14}NO_3^-$ is the amount of unlabeled NO_3^--N (in micrograms). We determined the $^{13}NO_3^-$ activity by counting the activity of an aliquot of waterlogged soil in an ion chamber and correcting for decay (*16*). The NO_3^--N was determined by the cadmium reduction and diazotization technique (*17*).

In each experiment we conducted two successive ^{13}N trials on a soil sample. After initial incubation with $^{13}NO_3^-$, we allowed the activity to decay, substituted a new molecular sieve trap, and then reintroduced another sample of radioactive $^{13}NO_3^-$ for a second trial. Solutions for Eq. 1 were calculated at 1-minute intervals during each incubation period (Fig. 2). Denitrification rates (in micrograms per liter per hour) calculated from the slopes of the curves (*18*) are 5.9 and 1.6 experiment 1) and 1.3 and 1.0 (experiment 2). During the time between successive trials in experiment 1, the NO_3^--N concentration in the waterlogged soil sample decreased from about 200 to 75 μg of NO_3^--N per liter as a result of denitrification and assimilation by microorganisms (*19*). The lowered rate in the second trial reflects this decrease in substrate concentration. Values for the NO_3^- concentration were nearly the same in both trials of experiment 2, and the rates are in close agreement.

Almost the entire inorganic nitrogen pool in waterlogged soils consists of NH_4^+-N (*20*). Except in the aerobic layer (or rhizosphere of rice roots), the mineralization of organic nitrogen cannot proceed beyond the NH_4^+ stage. This scheme is in accordance with the low concentrations (< 250 μg of NO_3^--N per liter) we detected and, coupled with our rate measurements, suggests that in waterlogged soils the denitrification rate is regulated by the supply of NO_3^-.

Moore and Schroeder estimated a "half saturation constant" for a mixed denitrifying population to be 80 μg of NO_3^--N (*21*), and half saturation constant values reported for cell-free dissimilatory nitrate reductases are > 200 μg of NO_3^--N per liter (*22*). In certain lake and ocean waters where NO_3^- concentrations are below enzyme saturation level, ^{15}N techniques may indicate a falsely high denitrification rate as a result of $^{15}NO_3^-$ enrichment. Denitrification rates of 34 μg of N per liter per day measured for anoxic bottom water of a brackish lake involved 18-fold enrichments of the sample NO_3^- content by $^{15}NO_3^-$ (*2*). Similarly, denitrification rates of 12 μg of N per liter per day obtained for anoxic hypolimnetic water of an island bay in the equatorial Pacific Ocean involved 75-fold enrichments (*23*). These values are probably significant overestimates of the actual denitrification rates in natural waters.

The accuracy of our technique is currently limited by the calibration of the two radioactivity detection systems. We used a standard ^{22}Na point source for calibration of the crystal detector; however, since the gaseous ^{13}N activity was collected over a diffuse area, the counting geometries are different. We checked the accuracy of our measurements by counting the activity of the molecular sieve trap in an ion chamber. When the crystal detector recorded an activity of 13.5 μc, the ion chamber detected 19.4 μc. The 30 percent difference in these determinations is reflected by errors of the same magnitude in the absolute rate measurements. Further refinements of the calibration technique and standardization of counting geometries are necessary for more exact determinations.

The production of millicurie quantities of $^{13}NO_3^-$, $^{13}N_2$, and $^{13}NH_3$ (*5*) offers a sensitive tracer technique for the investigation of those environmental transformations most important to global nitro-

gen cycling. The inconveniently short half-life of ^{13}N requires that field samples be returned to the cyclotron and has discouraged investigators from conducting ^{13}N radiotracer experiments. We are presently proceeding with plans to apply this ^{13}N technique to denitrification studies in anaerobic sediment and anoxic bottom water of Castle Lake, California. In order to determine actual in situ rates, we intend to carry out future experiments on undisturbed sediment cores and water samples without mixing or ^{13}N outgassing until sample incubation is complete.

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18. Rates for both experiments 1 and 2 (Fig. 2) are calculated from a linear regression over a 10-minute incubation period extrapolated to 1 hour.
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24. We thank G. Russell of the cyclotron group at Crocker Nuclear Laboratory for valuable technical assistance, R. Axler for help with design, Prof. J. Ingraham and B. Kimmel for helpful suggestions, and B. Jost and M. Smith for manuscript preparation. This research was supported by NSF grant BMS 72-02246-AO1 to Dr. C. R. Goldman.

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Atmospheric Carbon Tetrachloride: Another Man-Made Pollutant

Abstract. *On the basis of an analysis of historic worldwide emissions and removal mechanisms for carbon tetrachloride, a possible precursor for stratospheric ozone destruction, it has been demonstrated that the present atmospheric loading and distribution of carbon tetrachloride is primarily attributable to man-made emissions and no natural sources need be invoked to explain its presence in the atmosphere.*

In recent years, the release of chlorofluorocarbons into the atmosphere has received attention because of potential damage to the stratospheric O_3 layer (*1*). The commonly used refrigerants and aerosol propellants F-11 (CCl_3F) and F-12 (CCl_2F_2) are estimated to be present in the atmosphere in amounts approximately equal to their probable cumulative emissions (*1–5*). These observations have tended to confirm the anthropogenic nature of F-11 and F-12 and their tropospheric stability.

An equally important (and ubiquitous) compound present in the ambient air at concentrations similar to those of F-11 and F-12 is CCl_4 (*4–7*). Because of the extreme inertness of this compound in the troposphere, it has been proposed that CCl_4—like F-11 and F-12—may be a likely precursor of stratospheric O_3 destruction (*7*). Although the sources of F-11 and F-12 are known to be exclusively anthropogenic, the atmospheric budget of CCl_4 has been a subject of considerable uncertainty.

Lovelock and his co-workers (*2, 4*) suggested that CCl_4 must have a natural source. Their suggestions were based on two observations: (i) the current emissions of CCl_4 seem unlikely to account for the present atmospheric loading of CCl_4; and (ii) the global distribution of CCl_4 is more uniform than that of F-11. This report demonstrates, by an analysis of worldwide production-emission relationships of CCl_4, its possible secondary anthropogenic synthesis, and its atmospheric sinks, that the present biospheric loading and distribution of CCl_4 is consistent with cumulative anthropogenic emissions.

Curves 1, 2, and 3 (Fig. 1) show the production data for CCl_4 for the United States, Japan, and Western Europe, respectively. Data on U.S. production were available for blocks of time between 1914 and the present from a number of different sources (*8, 9*). The Japanese yearly production data were available from 1967 to 1973 (*10*); these data were found to be linear on a logarithmic scale, and the relationship has been extrapolated to 1940 to obtain curve 2 (Fig. 1). The production of CCl_4 in Western Europe was negligible before 1950 and did not reach the 1000–metric ton mark until 1954 (*11*). An estimate for the production of CCl_4 in Western Europe was available for the year 1973 (*12*). The total Western Europe production for other years was estimated at three times the value for the French or the United Kingdom production figures, which were available (*9, 11*).

The pattern of industrial CCl_4 usage has undergone significant changes over the last four decades. Before 1950, the major world market for CCl_4 was in the United States, where it was primarily used dispersively as an industrial solvent, dry-cleaning agent, fire extinguisher, grain fumigant, and in other miscellaneous applications. Since 1950, the world production of CCl_4 has kept pace with the production of chlorofluorocarbons, for which it is the principal reactant (*11, 13*). Figure 1 also shows the various types of dispersive uses of CCl_4 in the United States (*8, 9, 14*). Although the U.S. production of CCl_4 increased nearly tenfold between 1940 and 1972, the dispersive fraction declined from 84 to 7 percent of the total production (*14*). This decline is principally attributable to the recognition of the acute and chronic toxicity