*Agric. Chem. Biotechnol.* **44**(3), 135-139 (2001)

# Increase in $\delta^{15}N$ of Nitrate through Kinetic Isotope Fractionation Associated with Denitrification in Soil

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Received August 6, 2001

**To observe the changes in isotopic composition ($\delta^{15}N$) of $NO_3^-$ during denitrification, an incubation experiment using soil treated with nitrification inhibitor (2-chloro-6-trichloromethyl-pyridine) under water-saturated condition was conducted for 153 h. The $NO_3$-N concentration decreased from 73.3 to 20.6 mg $kg^{-1}$ during the incubation period, with denitrification rate constant of 0.00905 $h^{-1}$, and $\delta^{15}N$ values of $NO_3$-N increased from +0.9 to +25.5‰ with decreasing the $NO_3$-N concentration. The increase in the $\delta^{15}N$ values of $NO_3$-N is due to kinetic isotope fractionation, which always results in $^{15}N$ enrichment of the substrate. The isotopic fractionation factor calculated in this study was 1.0196, an indication that 1.96% more $^{14}NO_3^-$ reacted at a given time interval than a comparable number of $^{15}NO_3^-$. The $\delta^{15}N$ values measured through the incubation study showed a good agreement with the results calculated from the Fochts isotope fractionation model. Our results suggest that when the $\delta^{15}N$ of $NO_3^-$ is used for tracing the fate of N, the kinetic isotope fractionation associated with denitrification must be taken into consideration.**

**Key words:** *denitrification, focht model, isotope fractionation, natural $^{15}N$ abundance, nitrate.*

In view of plant nutrition and environmental contamination, nitrate ($NO_3^-$) is the most important N form in the agro-ecosystem. In paddy soil, $NO_3$-N forms in the thin aerobic soil layer just below the soil-water interface and diffuses into the anaerobic soil layer below. It is denitrified into $N_2$ and $N_2O$ gaseous forms under the anaerobic condition, which are then lost to the atmosphere.[1)] On the other hand, nitrate is the main N form directly available for plant uptake in upland soil. Nitrate, the most commonly identified pollutant in groundwater, easily leaches into groundwater without any interaction such as adsorption or precipitation with soil materials[2)] after a storm event through unsaturated zone.[3)]

Natural isotope abundance of $NO_3$-N ($\delta^{15}N$) has not only been used for tracing the fate of fertilizer or manure N in agro-ecosystem,[4-7)] but also for determining the sources of nitrate in groundwater.[8-12)] This is possible because various nitrate sources have distinct isotopic signatures from that of atmospheric $N_2$ (0‰) depending on their mechanism of formation. Chemical fertilizer sources have $\delta^{15}N$ values similar to that of atmospheric $N_2$, while soil organic N signatures are slightly heavier (4~9‰) and livestock wastes are considerably heavier (>9‰).[13)] However, the $\delta^{15}N$ values of $NO_3^-$ in the agro-ecosystem slightly deviates from the original values of the sources through isotopic fractionation accompanying $NO_3^-$ formation and transformation.[14)] Non-conservative behavior in the $\delta^{15}N$ values of $NO_3^-$ is most often due to the kinetic isotope fractionation, which always results in $^{15}N$ enrichment of the substrate and depletion of the product because of the tendency of molecules bearing the lighter isotope to react somewhat faster than those with the heavier isotope.[15)] For instance, during nitrification, the light isotope ($^{14}N$) is preferentially incorporated into $NO_3^-$, and low $\delta^{15}N$ value of $NO_3^-$ is observed.[14)] In contrast, denitrification results in a marked enrichment in the $^{15}N$ content of the remaining $NO_3^-$.[16, 17)]

In general, denitrification takes place in an anaerobic environment under water-saturated condition. However, some anaerobic microsites permitting denitrification are still exist under water-unsaturated condition.[18)] Therefore, understanding of the isotopic fractionation associated with denitrification is necessary for the use of $\delta^{15}N$ of $NO_3^-$ in tracing the fate of N derived from fertilizer or manure wastes. Kinetic isotope fractionations are often described by $\alpha$ (isotope fractionation factor), which relates to the ratio between the rates, $k_{14N}$ and $k_{15N}$, of a process for the light and heavy N isotopes, respectively:

$$\alpha = k_{14N} / k_{15N}. \quad (1)$$

Delwiche and Steyn[14)], using *Pseudomonas*, found an isotopic fractionation factor of 1.02 for denitrification. However, the isotopic fractionation factor in a complex soil system might well be different from that observed in pure cultures of a single bacterial genus. A few studies have been conducted to observe $\alpha$ for denitrification in soil.[19, 20)] Chien *et al.*[19)] and Mariotti *et al.*[20)] reported that the *in vivo* $\alpha$ values for denitrification were 1.0191 and 1.0292, respectively. Since the isotopic fractionation factor is dependent on the species

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distribution of microorganisms, which are responsible for denitrification,[21] $\alpha$ is different from soil to soil.

In this study, an increase in $\delta^{15}N$ of $NO_3^-$ through denitrification was observed, and the isotopic fractionation factor associated with denitrification in soil was investigated. Furthermore, a theoretical fractionation model for single reactions developed by Focht[22] was evaluated using denitrification rate constant and the isotopic fractionation factor.

## Materials and Methods

**Incubation.** For incubation study, soil was collected from a field at experimental station of Seoul National University, air-dried, and passed through a 2-mm sieve. The soil was classified as coarse loamy, Typic Dystrudepts, containing 13.8 g C $kg^{-1}$ and 1.3 g N $kg^{-1}$, and the pH ($H_2O$) was 5.8. Two milliliters of 2-chloro-6-(trichloromethyl)-pyridine (nitrification inhibitor) suspension (1000 mg $l^{-1}$) was added to 40 g of dried soil in a 250-ml flask, and the water content was adjusted to nearly saturated conditions (0.37 kg $kg^{-1}$). We established that the amount of nitrification inhibitor was sufficient through a previous experiment. Twenty-one flasks containing the samples were prepared for 7 time-intervals with triplication. In order to eliminate the indigenous nitrates and to increase microbial population, soil was preincubated at 27°C in the dark for 7 d. After preincuation, 1 m*l* of solution containing 3 mg $KNO_3$-N (+0.8‰) was added to the soil, and the samples were mixed homogeneously with a spatula. The soils were adjusted to a water content of 0.40 kg $kg^{-1}$ for water saturation condition. The flasks were covered with caps, which have holes for aeration, to minimize water loss and then incubated at 27°C in the dark. Soil was sampled periodically for up to 153 h.

**Analysis of concentration and $\delta^{15}N$ of $NO_3$-N.** Nitrate was extracted from the soil with 100 m*l* of 2 M KCl solution. For the $NO_3$-N concentrations, 30 m*l* of the extracts were steam distilled using MgO and Devarda alloy.[22] Residual extracts were used for $\delta^{15}N$ analysis. The $NO_3^-$ in the extracts were steam-distilled and collected into $H_2SO_4$ instead of $H_3BO_3$ trap. Ethanol was used to clean the steam distillation apparatus between each distillation to prevent isotopic cross-contamination.[23] After adjusting the $NH_4^+$ solution to pH 2~3 using 0.1 N $H_2SO_4$ or 0.1 N NaOH, water in the solution was evaporated to dryness under an infra-red lamp. The fine powder (ammonium sulfate) of the dried sample was analyzed for $\delta^{15}N$ through a combustion method[24] using a stable isotope ratio mass spectrometer of continuous-flow type (Isoprime-EA, Micrmass, UK). The $\delta^{15}N$ is expressed in parts per thousand deviation from the atmospheric $N_2$ as defined by the following equation:

$$\delta^{15}N\ (‰) = [(R_{sample} / R_{standard}) - 1] \times 1000 \quad (2)$$

where $R_{sample}$ and $R_{standard}$ are the $^{15}N/(^{14}N+^{15}N)$ ratios of the sample and standard, respectively. The accuracy and reproducibility of analysis checked with a reference material (RM 8548: IAEA-N2) from International Atomic Energy Agency were better than 0.4 and 0.2‰, respectively.

**Isotopic fractionation factor.** The 'Rayleigh' equation was used to obtain isotopic fractionation factor ($\alpha_{s/p}$) as follows:

$$\left(\frac{1}{\alpha_{s/p}} - 1\right)\ln f = \ln\left(\frac{10^{-3}\delta_s + 1}{10^{-3}\delta_{s,o} + 1}\right) \quad (3)$$

where f is the unreacted fraction of substrate ($NO_3$-N) at time t, and $\delta_{s,o}$ and $d_s$ are the $\delta^{15}N$ of $NO_3$-N at times 0 and t, respectively.[20]

## Results

**Changes in the concentration and $\delta^{15}N$ of $NO_3$-N.** The $NO_3$-N concentration decreased progressively from 73.3 mg $kg^{-1}$ at 0 h to 20.6 mg $kg^{-1}$ at 153 h after incubation (Fig. 1). Although enzymatic reactions follow Michaelis-Menten kinetics, first-order kinetics are closely approximated where substrate concentration is small with respect to the Michaelis constant, $K_m$. First-order kinetics are described as follows:

$$\ln(C/C_0) = -kt \quad (4)$$

where C and $C_0$ are the concentration of substrate at times 0 and t, respectively, and $k$ is the rate constant. Thus, a plot of $\ln(C/C_0)$ versus $t$ gives a straight line having slope $-k$ (Fig. 2). The rate constant was 0.00905 $h^{-1}$, and the denitrification rate calculated based on all incubation periods was 8.616 mg N $kg^{-1}$ $h^{-1}$. The $\delta^{15}N$ values of $NO_3$-N increased from +0.9 to +25.5‰ during the same incubation period in accordance with the decrease in $NO_3$-N concentration (Fig. 1).

**Isotopic fractionation factor.** Data for calculation of isotopic fractionation factor are summarized in Table 1. Using data in Table 1, changes in isotopic composition of $NO_3$-N as a function of ln(f) during denitrification are plotted in Fig. 3. The isotopic fractionation factor ($\alpha_{s/p}$ = 1.0196) was calculated

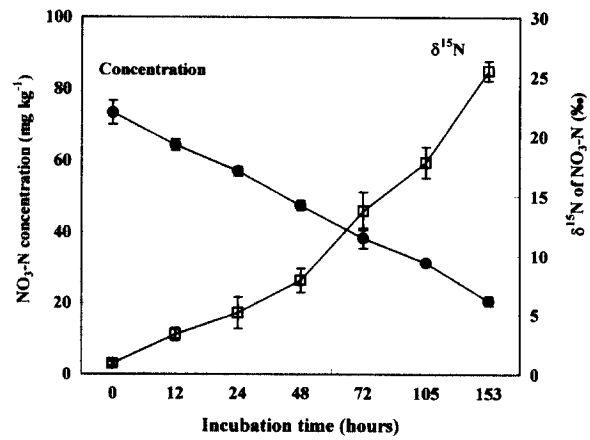


**Fig. 1. Changes in concentrations and $\delta^{15}N$ values of $NO_3$-N during incubation.** Vertical bars represent standard deviation of the means (n=3).

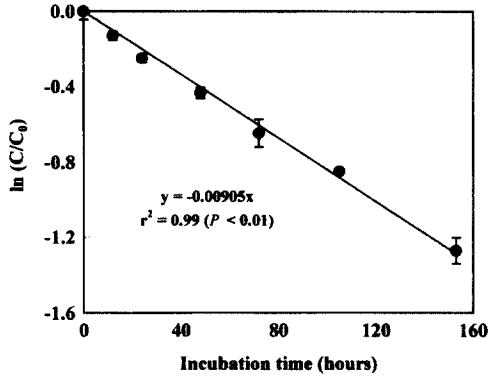


**Fig. 2. Semilogarithmic plot of $NO_3$-N concentration as a function of time for a first-order reaction.** $C_0$ and C are the $NO_3$-N concentration at times 0 and t, respectively. The absolute value (0.00905) of slope is the denitrification rate constant. Vertical bars represent standard deviation of the means (n=3).

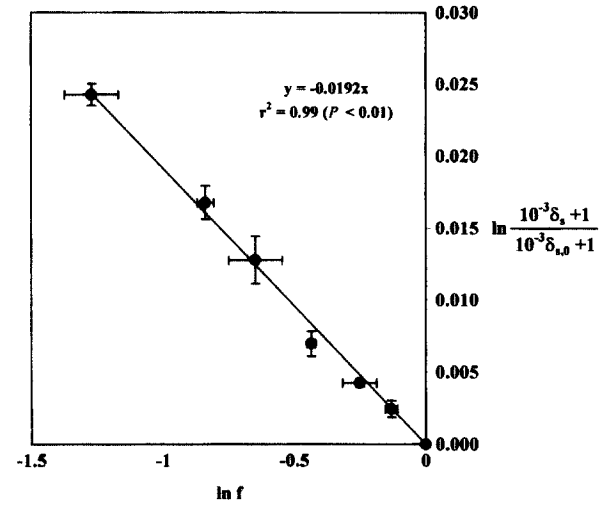


**Fig. 3. Changes in the isotopic composition of $NO_3$-N as a function of ln(f) during denitrification.** f is the unreacted fraction of $NO_3$-N at time t, and $\delta_{s,0}$ and $\delta_s$ are the $\delta^{15}N$ of $NO_3$-N at times 0 and t, respectively. Vertical and horizontal bars represent standard deviation of the means (n=3).

from the slope ($1/\alpha_{s/p} - 1$) of the straight line. A $\alpha_{s/p}$ value of 1.0196 indicates that 1.96% more $^{14}N$-$NO_3^-$ reacted in a given time interval than a comparable number of $^{15}N$-$NO_3^-$.

## Discussion

Figure. 1 shows the $^{15}N$-enrichment of residual $NO_3$-N during denitrification. Many hydrogeological investigations report that distinctions between groundwater nitrate derived from human/animal waste and that derived from agricultural sources, such as inorganic fertilizers or mineralized soil organic nitrogen, can be made on the basis of $\delta^{15}N$ values.[8-12] The widely accepted $\delta^{15}N$ ranges for chemical fertilizers are between −3 and +2‰, for natural soil $NO_3^-$ between +2 and +8‰, and for human or animal waste $NO_3^-$ between +10 and +20‰.[25] However, the results of this study suggest that if considerable denitrification of $NO_3^-$ derived from chemical fertilizer occurs, the source identification of $NO_3^-$ based on the $\delta^{15}N$ may lead to misinterpretation. Although a continuous increase in $\delta^{15}N$ of $NO_3^-$ is not likely to occur in natural system since $NO_3^-$ is involved in various N transformation processes, it is necessary to understand the isotopic fractionation through denitrification for tracing the fate of fertilizer or manure N in the agro-ecosystem.

Focht[21] developed an isotopic fractionation model of first order reactions of nitrogen under limited substrate, based on the rate constant ($k$) and isotopic fractionation factor ($\alpha$) as follows:

$$(1 + 10^{-3}\delta_s) = (1 + 10^{-3}\delta_{s,0})\exp\left(kt\frac{\alpha - 1}{\alpha}\right) \quad (5)$$

The model results were in well-agreement with the measured $\delta^{15}N$ values (Fig. 4). This result shows that the reactions involved with $NO_3^-$ other than denitrification, such as nitrification and the reduction of $NO_3^-$ to $NH_4^+$ or organic forms, were not significant in the soil, since these reactions also affect the $^{15}N$ content of $NO_3^-$ pool and the Focht model used in this

**Table 1. Data for calculation of isotopic fractionation factor associated with denitrification.**

| Time (hr) | $NO_3$-N (mg $kg^{-1}$) | $\delta^{15}N$ of $NO_3$-N (‰) | f | ln f | $\ln\left(\frac{10^{-3}\delta_s + 1}{10^{-3}\delta_{s,0} + 1}\right)$ |
|---|---|---|---|---|---|
| 0 | 73.3 | 0.9 | 1.00 | 0.00 | 0.0000 |
| 12 | 64.4 | 3.4 | 0.88 | -0.13 | 0.0025 |
| 24 | 57.0 | 5.2 | 0.78 | -0.25 | 0.0043 |
| 48 | 47.4 | 7.9 | 0.65 | -0.44 | 0.0070 |
| 72 | 38.3 | 13.8 | 0.52 | -0.65 | 0.0128 |
| 105 | 31.3 | 17.8 | 0.43 | -0.84 | 0.0168 |
| 153 | 20.6 | 25.5 | 0.28 | -1.27 | 0.0243 |

Values are the means of triplication.
f is the unreacted fraction of substrate ($NO_3$-N) at time t.
$\delta_{s,0}$ and $\delta_s$ are the $\delta^{15}N$ of $NO_3$-N at times 0 and t, respectively.

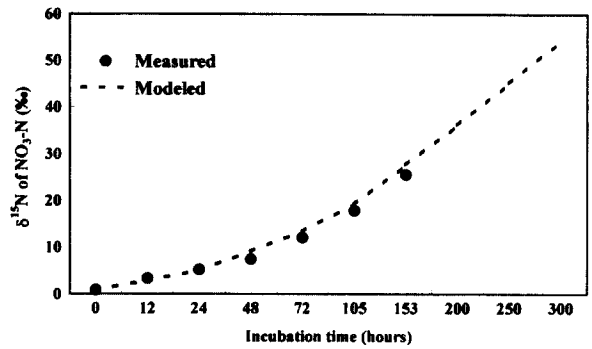


**Fig. 4. Measured and modeled $\delta^{15}N$ values of $NO_3$-N.**

study was developed for unidirectional single reactions. Using the Focht model, the $\delta^{15}N$ values of $NO_3$-N at the prolonged incubation time could be calculated. For example, the $\delta^{15}N$ value was +54.4‰ at 300 h after incubation when the $NO_3$-N concentration was 4.6 mg $kg^{-1}$, which was calculated using Equation (4).

In conclusion, the increase in the $\delta^{15}N$ values of $NO_3$-N during denitrification strongly suggests that isotope fractionation must be considered when the $\delta^{15}N$ values of $NO_3$-N are used for tracing N derived from chemical fertilizer or manure, especially under an anaerobic environment. However, the dependence of $\delta^{15}N$ values of $NO_3$-N on denitrification can be used to investigate the degree of denitrification in soils, i.e. the $\delta^{15}N$ values of $NO_3$-N may be an indicator for denitrification.

**Acknowledgments.** The authors thank graduate fellowship provided by the Ministry of Education through the Brain Korea 21 Project, and to NICEM, SNU for allowing the use of a stable isotope ratio mass spectrometer.

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