Pergamon

Geochimica et Cosmochimica Acta, Vol. 65, No. 16, pp. 2743–2756, 2001

0016-7037/01 $20.00 + .00

PII S0016-7037(01)00612-3

The oxygen isotope composition of nitrate generated by nitrification in acid forest floors

BERNHARD MAYER,[1,†,*] SANDRA M. BOLLWERK,[1] TIM MANSFELDT,[2] BIRGIT HÜTTER,[2] and JAN VEIZER[1,3]

[1]Institut für Geologie, Mineralogie und Geophysik, Lehrstuhl für Sediment und Isotopengeologie, Ruhr-Universität Bochum, D-44780 Bochum, Germany

[2]Arbeitsgruppe Bodenkunde und Bodenökologie, Fakultät für Geowissenschaften, Ruhr-Universität Bochum, D-44780 Bochum, Germany

[3]Ottawa-Carleton Geoscience Center, University of Ottawa, Ottawa, Ontario, Canada K1N 6N5

(*Received July* 18, 2000; *accepted in revised form March* 19, 2001)

Abstract—The oxygen isotope composition of nitrate is used increasingly for identifying the origin of nitrate in terrestrial and aquatic ecosystems. This novel isotope tracer technique is based on the fact that nitrate in atmospheric deposition, in fertilizers, and nitrate generated by nitrification in soils appear to have distinct oxygen isotope ratios. While the typical ranges of $\delta^{18}O$ values of nitrate in atmospheric deposition and fertilizers are comparatively well known, few experimental data exist for the oxygen isotope composition of nitrate generated by nitrification in soils. The objective of this study was to determine $\delta^{18}O$ values of nitrate formed by microbial nitrification in acid forest floors.

Evidence from laboratory incubation experiments and field studies suggests that during microbial nitrification in acid forest floor horizons, up to two of the three oxygen atoms in newly formed nitrate are derived from water, particularly if ammonium is abundant and nitrification rates are high. It was, however, also observed that in ammonium-limited systems with low nitrification rates, significantly less than two thirds of the oxygen in newly formed nitrate can be derived from water oxygen, presumably as a result of heterotrophic nitrification. It can be concluded from the presented data that the $\delta^{18}O$ values of nitrate formed by microbial nitrification in acid forest floors typically range between +2 and +14‰, assuming that soil water $\delta^{18}O$ values vary between −15 and −5‰. Hence, oxygen isotope ratios of nitrate formed by nitrification in forest floors are usually distinct from those of other nitrate sources such as atmospheric deposition and synthetic fertilizers and, therefore, constitute a valuable qualitative tracer for distinguishing among these sources of nitrate. A quantitative source apportionment appears, however, difficult because of the wide range of $\delta^{18}O$ values, particularly for atmospheric nitrate deposition and for nitrate from microbial nitrification. *Copyright © 2001 Elsevier Science Ltd*

1. INTRODUCTION

Human activity has greatly altered nitrogen cycling in terrestrial and aquatic ecosystems (e.g., Kinzing and Socolow, 1994; Schlesinger, 1997; Vitousek et al., 1997). Increased use of nitrogen-containing organic and inorganic fertilizers (Smil, 1999) and elevated atmospheric nitrogen deposition in many regions, particularly in the northern hemisphere (Benkovitz et al., 1996), have caused increasing nitrate fluxes in soil solutions, groundwater, and surface waters (e.g., Turner and Rabalais, 1991). This affects not only groundwater quality, but can also lead to eutrophication of freshwater, estuaries, and shelf seas.

Identifying sources of nitrate in the environment is important to elucidate the causes of increased nitrate fluxes in aquatic ecosystems. Isotopic techniques have been utilized successfully for this purpose for more than three decades (e.g., Hübner, 1986), initially focusing exclusively on nitrogen isotope ratios. However, interpretation of nitrogen isotope ratios has not always led to unequivocal results, since many sources of nitrate have wide and overlapping ranges of $\delta^{15}N$ values (e.g., Gormly and Spalding, 1979; Kreitler, 1979). Furthermore, isotopic fractionation effects during nitrogen transformation processes, such as nitrification, ammonia volatilization, and denitrification (Delwiche and Steyn, 1970), further complicate the interpretation of $\delta^{15}N$ values of nitrate in soil solutions, groundwater, and surface water.

Recently, techniques have been developed to analyze the oxygen isotope ratio of nitrate (e.g., Amberger and Schmidt, 1987; Revesz et al., 1997; Silva et al., 2000; Bräuer and Strauch, 2000) and this tool is used increasingly for describing nitrogen cycling in terrestrial and aquatic ecosystems (e.g., Durka et al., 1994; Wassenaar, 1995; Aravena and Robertson, 1998; Cey et al., 1999; Mengis et al., 1999). It has been demonstrated that fertilizer nitrate has remarkably constant $\delta^{18}O$ values of +22 ± 3‰ (Amberger and Schmidt, 1987; Voerkelius, 1990; Wassenaar, 1995). Atmospheric nitrate is usually characterized by $\delta^{18}O$ values between +25 and +75‰ (Durka et al., 1994; Kendall, 1998). In soils, nitrogen occurs predominantly in organic binding form as a result of immobilization of ammonium (NH_4^+) and nitrate (NO_3^-) or N_2 fixation. The process by which organic nitrogen compounds are reconverted to nitrate is usually termed mineralization and consists of ammonification ($N_{org} \rightarrow NH_4^+$) and nitrification ($NH_4^+ \rightarrow NO_3^-$). During the latter process, three oxygen atoms are introduced into the newly formed nitrate molecule. Laboratory culture experiments with *Nitrosomonas* and *Nitrobacter agilis*, conducted under neutral pH conditions, showed that during chemolithoautotrophic nitrification two oxygen atoms of the newly formed nitrate are derived from water oxygen (Aleem et

*Author to whom correspondence should be addressed (bernhard@geo.ucalgary.ca).

† *Present address:* University of Calgary, Departments of Geology & Geophysics and Physics & Astronomy, 2500 University Drive NW, Calgary, Alberta, Canada T2N 1N4.

Table 1. Some chemical and isotopic properties of the O horizon material (Of and Oh) from the investigated Gleyic Cambisols.

Stand	Forest Floor	Thickness [cm]	pH[a]	TOC[b] [g kg^{-1}]	N [g kg^{-1}]	C/N	$\delta^{15}N_{total}$ [‰]
Deciduous (beech/oak)	Mor	5	3.3	238	10	24	−4.8
Coniferous (spruce)	Raw humus	5	3.2	325	10	32	−2.1

[a] 0.01 M $CaCl_2$.
[b] TOC (total organic carbon).

al., 1965; Anderson and Hooper, 1983; Hollocher, 1984; Yoshinari and Wahlen, 1985), with the third oxygen atom incorporated from atmospheric O_2. Atmospheric O_2 has a $\delta^{18}O$ value of + 23.5‰ (Kroopnick and Craig, 1972; Horibe et al., 1973). The $\delta^{18}O$ value of soil seepage water can vary from $<$ −20‰ to $>$ +5‰, depending on location and environmental conditions (Gat, 1996). Based on the assumption that two oxygen atoms in the nitrate molecule are derived from water and one from atmospheric O_2, and assuming no isotope fractionation during incorporation of oxygen into the newly formed nitrate molecule, Amberger and Schmidt (1987), Voerkelius (1990), and Durka et al. (1994) hypothesized that nitrate formed by nitrification in soils should have $\delta^{18}O$ values between −2 and +6‰. If confirmed, nitrate generated by nitrification in soils would have distinct oxygen isotope ratios, markedly different from those of nitrate in atmospheric deposition and in nitrate-containing fertilizers.

It is noteworthy that few experimental data have been published for the oxygen isotope composition of nitrate derived from microbial nitrification in water-unsaturated soils (Amberger, 1987; Voerkelius, 1990; Kendall, 1998). This is particularly true for acid forest soils, where pH values are commonly $<$4 and thus markedly different from those of most laboratory experiments (e.g., Focht and Verstraete, 1977; Hollocher, 1984). Also, there is evidence that chemolithoautotrophic nitrification is not the only nitrification pathway in acid forest soils (Killham, 1986; Gundersen and Rasmussen, 1990; Frank, 1996; Pedersen et al., 1999; Simek, 2000). Thus, it is not clear whether the above-described theoretical assumptions for calculating typical $\delta^{18}O$ values of nitrate derived from nitrification are applicable to the formation of nitrate in acid forest soils. The few previous experimental attempts to determine the oxygen isotope composition of nitrate derived from microbial nitrification in water-unsaturated soils yielded quite contrasting results. Soil incubation experiments conducted by Amberger (1987) indicated that nitrate produced by nitrification should have negative $\delta^{18}O$ values, whereas Voerkelius (1990) reported $\delta^{18}O$ values of 0 ± 2‰ for nitrification nitrate generated in soil column experiments. More recent soil incubation studies yielded $\delta^{18}O_{nitrate}$ values of between +14 and +17‰ (Kendall, 1998; Burns and Kendall, in review).

The objective of this study was to experimentally determine the oxygen isotope composition of nitrate that was produced exclusively by nitrification in two acid forest floors. This objective was met by analyzing the $\delta^{18}O$ values of nitrate in percolation water from a laboratory incubation experiment using O horizons from two different forest sites in northwestern Germany. Laboratory results were then compared with isotopic compositions of nitrate in forest floor solutions from these two field sites.

2. MATERIALS AND METHODS

2.1. Field Studies

Field investigations were conducted in east-central North-Rhine Westphalia, Germany (51°44′ N, 7°49′ E) at two adjacent forest sites. One site is stocked with beech (*Fagus sylvatica*) and oak (*Quercus robur*), the second site with Norway spruce (*Picea abies*). Soils have developed in a thin layer of sandy loess overlaying glacial till, which covers Upper Cretaceous limestone. Both the compact till and the argillaceous limestone act as a water-restrictive layer causing perched water tables in the lower mineral soil. According to FAO (1990), both soils can be classified as Gleyic Cambisols. The organic topsoil horizon at the deciduous site is classified as mor, at the coniferous site as raw humus. The raw humus had a higher C content and a wider C : N ratio than the mor (Table 1). Both forest floors were acidic with pH values ranging between 3.2 and 3.3.

Throughfall and forest floor solutions were collected weekly from April 1994 to March 1997. Throughfall was collected using 16 cm diameter polyethylene (PE) funnels, which were installed 1 m above the forest floor in five replicates per site. They were connected with PE tubes to 2.5 L glass bottles acting as storage containers placed in the soils. The funnels were covered with PE screens to minimize contamination. The forest floor solutions were obtained by stainless steel zero-tension lysimeters installed underneath the O horizons at both sites. Forest floor solutions were sampled from three lysimeters per site.

2.2. Laboratory Experiment

Forest floor for the laboratory experiment was sampled at both sites in November 1996. O horizon material (Of and Oh) was collected from 10, 15 × 15 cm plots. After roots and branches were removed, the O horizons were manually mixed and homogenized. An aliquot was ground and oven dried at 30°C for subsequent chemical and isotope analyses.

The laboratory incubation experiment was performed according to Stanford and Smith (1972). The field-moist O horizon material was placed in five replicates per soil type into 1000 mL Nalgene percolation containers. The tops of the containers were covered with glass wool to prevent evaporation and ensure regular distribution of the percolation water, but to maintain free exchange with atmospheric O_2. On the first day of the experiment, the containers with raw humus were percolated

with 350 mL and those with mor were irrigated with 250 mL of deionized water to exchange the initial soil water and to adjust the water content to ~50% of water holding capacity. The percolation containers were subsequently stored in darkness at 15°C. Over a period of 4 months, the containers were irrigated weekly with 750 mL deionized water. Percolation water was removed by applying a moderate vacuum for 1 h after each of the 16 irrigation events. The solutions were membrane filtered with prewashed 0.45 μm cellulose acetate filters and subsequently used for chemical and isotope analyses.

Disturbed, mixed, and homogenized O horizon material, but no mineral soils, were used for the laboratory incubation experiments to avoid or minimize the establishment of anoxic microsites. High water contents in the incubated O horizons were limited to periods of <1 h after each irrigation event. For similar experimental conditions, Koopmans et al. (1995) demonstrated that denitrification was negligible. In our experiment, denitrification rates were not measured but are believed to have been minimal, since the isotopic composition of nitrate in the percolation water of our experiments provided no evidence for denitrification, that is, simultaneously increasing $\delta^{15}N_{nitrate}$ and $\delta^{18}O_{nitrate}$ values.

For both forest floors, each of the five replicates was irrigated with deionized water with a markedly different $\delta^{18}O$ value: −8.3‰ (treatment 1), +1.9‰ (treatment 2), +10.6‰ (treatment 3), +28.1‰ (treatment 4), and +59.3‰ (treatment 5). The irrigation water was produced by mixing double-deionized water with different quantities of ^{18}O enriched water, i.e., 2% $H_2^{18}O$ (Enritech Enrichment Technologies LTD).

2.3. Chemical Measurements

2.3.1. Forest floor

Total organic carbon was determined by dry combustion of the O horizon material at 1200°C. The evolved CO_2 was absorbed in an alkaline solution and detected by coulometry (TR 3600 Deltronik). Total nitrogen was measured by the Kjeldahl technique. pH values were determined potentiometrically in 0.01 *M* $CaCl_2$ solution (10 mL forest floor, 25 mL solution).

2.3.2. Throughfall, forest floor solutions, and percolation solutions

Nitrate and chloride concentrations were measured by ion chromatography (Dionex D500) using electrical conductivity (Ion Pac AS 12 A, precision ±1%). Ammonium was analyzed spectrophotometrically (UV/VIS Spectrometer Lambda 2, Perkin Elmer) as indophenole blue at 665 nm (DEV, 1983; precision ±2%).

2.4. Isotope Measurements

$\delta^{15}N$ and $\delta^{18}O$ values of nitrate were determined using a modified version of the method described by Silva et al. (2000). Water samples from the laboratory incubation experiment (400–800 mL, 3–50 mg NO_3^- L^{-1}) and from the field study (30–3000 mL, 1–160 mg NO_3^- L^{-1}) were passed through a cation exchange resin (2 mL of 50W-X4, H^+-form, Bio-Rad) at a rate of 5 mL min^{-1}. Subsequently, the acidified solutions were passed through 2 mL of XAD-7 resin (Fluka) to remove the high molecular dissolved organic carbon (DOC) fraction, since oxygen in DOC potentially compromises oxygen isotope ratio determinations on $AgNO_3$. Finally the solutions were passed through an anion exchange resin (2 mL AG 1-X8-resin, Cl^--form, Bio-Rad), which quantitatively retained dissolved nitrate, sulfate, and potentially, phosphate. Subsequently, the ion exchange resins were rinsed with 20 mL deionized water. Anion exchange resins containing nitrate were stored at 5°C in darkness until further processing.

Nitrate, sulfate, and phosphate were eluted from the anion exchange resins into a 60 mL beaker by percolating 15 mL 3 *M* HCl through the columns. Two mL of 0.2 *M* $BaCl_2$ solution were added to the HCl eluate to precipitate sulfate as $BaSO_4$ and phosphate as $Ba_3(PO_4)_2$. After 24 h, $BaSO_4$ and $Ba_3(PO_4)_2$ were removed by filtration (0.45 μm membrane filter). To remove excess Ba^{2+} from the remaining solution, the sample was passed again through a cation exchange resin (2 mL 50W-X4 resin, H^+-form, Bio-Rad). The remaining solution containing HNO_3 and HCl was neutralized with approximately 7.5 g pure Ag_2O (Merck). The resulting AgCl precipitate was removed by filtration (0.45 μm membrane filter) leaving only Ag^+ and NO_3^- in solution (Eqn. 1). The solutions were freeze-dried yielding a pure, dry $AgNO_3$ precipitate.

$$Ag_2O + HNO_3 + HCl \rightarrow Ag^+ + NO_3^- + H_2O + AgCl\downarrow \quad (1)$$

For oxygen isotope analyses on nitrate, 10 mg $AgNO_3$ was mixed with 2 mg pure graphite powder (Fluka). This mixture was placed in a 9 mm quartz tube, which was evacuated and flame sealed. The mixture was thermally decomposed at 860°C for 3 h, followed by slow cooling to ensure complete conversion of the nitrate-oxygen to CO_2 as described in Eqn. 2:

$$2AgNO_3 + 3C \rightarrow 2Ag + N_2 + 3CO_2 \quad (2)$$

The resulting CO_2 gas was cryogenically separated and analyzed mass spectrometrically on a Finnigan MAT delta S. Accuracy and precision of the measurements was assured by repeated analyses of international reference materials (IAEA-NO-3, KNO_3, $\delta^{18}O = +23.5 \pm 0.7$‰, $n = 24$) and two laboratory internal nitrate standards (KNO_3 from Riedel-de-Haen, $\delta^{18}O = +21.5 \pm 0.5$‰, $n = 31$; $AgNO_3$ from Degussa, $\delta^{18}O = +14.4 \pm 0.5$‰, $n = 16$). Additionally, both potassium nitrate standards were repeatedly dissolved in deionized water and the NO_3^- was subsequently converted to $AgNO_3$ as described above. Oxygen isotope ratios determined on $AgNO_3$ were +24.1 ± 0.9‰ ($n = 12$) for IAEA-NO-3, and +21.8 ± 1.0‰ ($n = 12$) for the Riedel-de-Haen standard, identical to those obtained for the original standard material within the analytical reproducibility of ±1.0‰ for $\delta^{18}O_{nitrate}$ determinations, including nitrate separation, gas preparation, and mass spectrometric measurements. The oxygen isotope ratios obtained for IAEA-NO-3 were also within the range of previously reported $\delta^{18}O$ values (between +22.7 and +25.3‰) for this reference material (Revesz et al., 1997; Kornexl et al., 1999; Bräuer and Strauch, 2000).

Nitrogen isotope ratios and total nitrogen contents of $AgNO_3$ precipitates and soil samples were determined by thermal de-

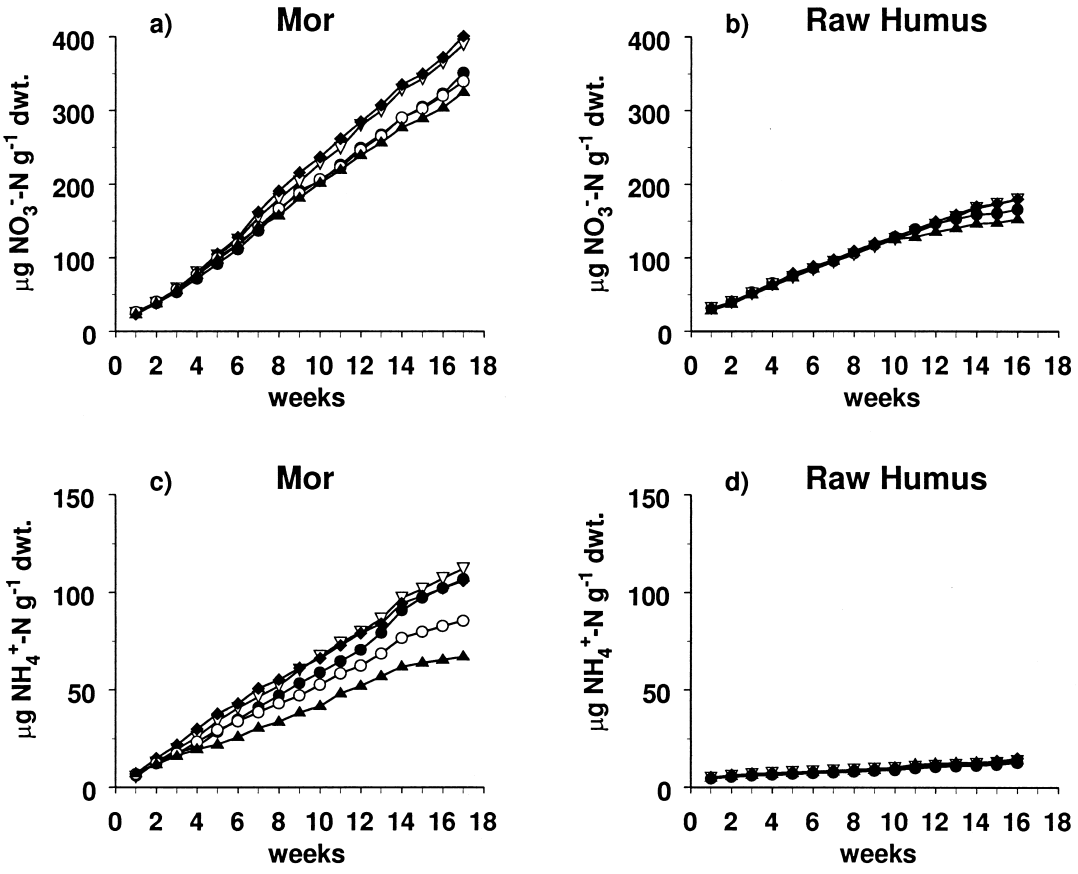

Fig. 1. Cumulative nitrate (a,b) and ammonium (c,d) concentrations (expressed in microgram N per gram dry soil) in percolates from the laboratory incubation experiment with the mor and raw humus. Note that the y-axes for nitrate and ammonium plots have different scales. Treatment 1 = solid circle; treatment 2 = open triangle; treatment 3 = solid diamond; treatment 4 = open circle; treatment 5 = solid triangle.

composition in an elemental analyzer (CE 1110) and subsequent isotope ratio mass spectrometry in continuous-flow mode using a Finnigan MAT delta C. Nitrogen contents of the $AgNO_3$ samples were typically ~8%, indicating that no major contaminants were present in the precipitate. $\delta^{15}N$ values for all samples were normalized against internationally accepted reference materials (IAEA N1, $\delta^{15}N = +0.43 \pm 0.07‰$, $n = 48$; IAEA N2, $\delta^{15}N = +20.41 \pm 0.12‰$, $n = 36$). The nitrogen isotope ratios of $AgNO_3$ generated from dissolved IAEA-NO-3 potassium nitrate were within $+4.34‰ \pm 0.29‰$ ($n = 12$) similar to the accepted value. Duplicate nitrogen isotope ratio determinations on $AgNO_3$ from field and laboratory nitrate samples were performed with a precision generally better than $\pm$ 0.3‰.

Isotopic analyses of $\delta^{18}O$ values from water were conducted using standard equilibration techniques (Epstein and Mayeda, 1953).

All stable isotope ratios are expressed in the usual delta per mil (‰) notation relative to the respective international standards:

$$\delta_{sample}\ (‰) = [(R_{sample} - R_{standard})/R_{standard}] * 1000 \qquad (3)$$

where R is the $^{15}N/^{14}N$ or $^{18}O/^{16}O$ ratio of the sample and standard, respectively. $\delta^{15}N$ values are reported with respect to AIR, and $\delta^{18}O$ values with respect to Standard Mean Ocean Water (V-SMOW).

3. RESULTS

3.1. Laboratory Experiment

3.1.1. Nitrogen mineralization

During the 16 weeks of the experiment, there was a continuous release of nitrate from the incubated O horizons (Figs. 1a,b). After the first percolation of the mor, an average of 24 $\pm$ 1 μg NO_3-N g^{-1} ($n = 5$) was detected in the seepage water. After each subsequent percolation, NO_3-N concentrations remained almost constant throughout the 16 weeks of the experiment, resulting in a cumulative net-release of 361 $\pm$ 33 μg NO_3-N g^{-1}. For the raw humus, an average of 30 $\pm$ 1 μg NO_3-N g^{-1} ($n = 5$) was detected in the initial percolation water. Thereafter, mean nitrate concentrations in the weekly percolates were generally <13 μg NO_3-N g^{-1}, resulting in an average cumulative net-release of 169 $\pm$ 13 μg NO_3-N g^{-1}. Nitrification rates can be assessed from the slopes of the curves in Figure 1a,b, assuming minimal denitrification. Nitrification

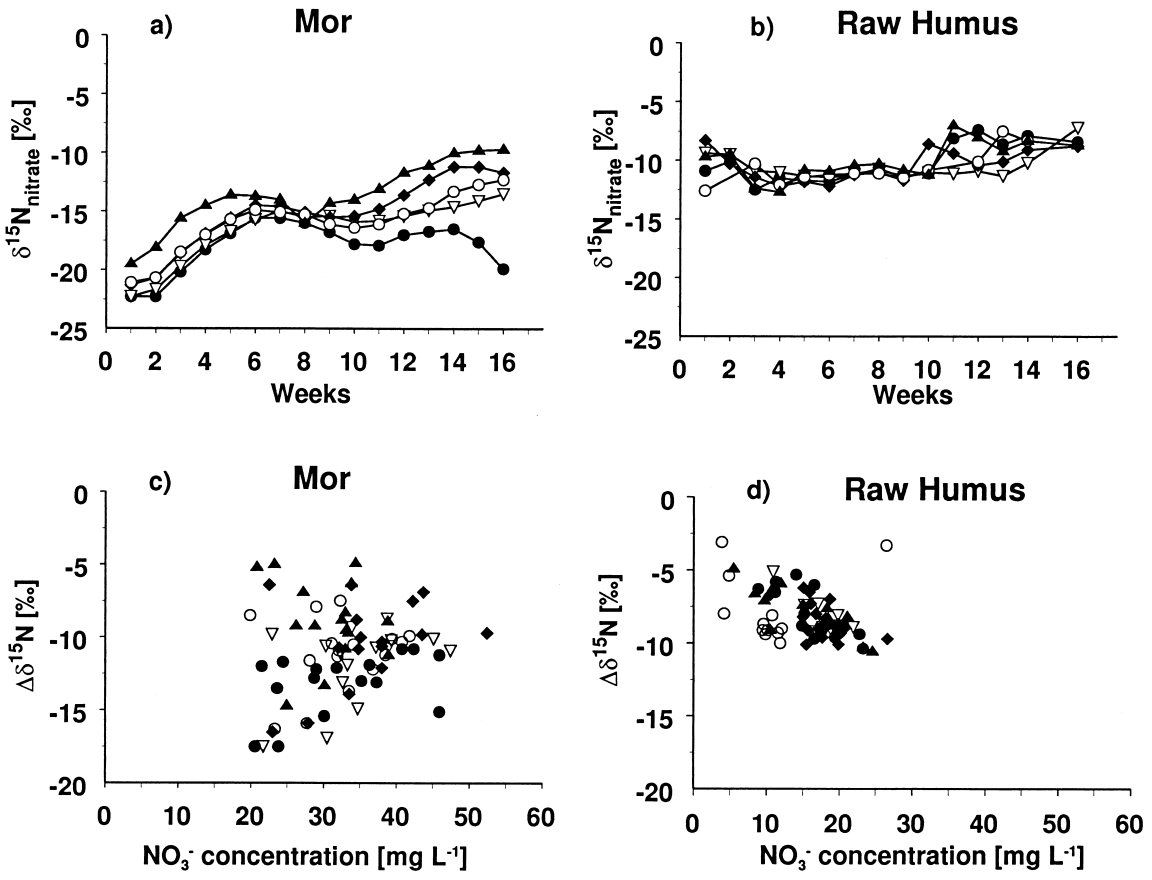

Fig. 2. $\delta^{15}N$ values of nitrate in the percolation water from the laboratory incubation experiments with the mor (a) and the raw humus (b). Also shown is the difference between the $\delta^{15}N$ values of total nitrogen in the incubated O horizons and those of nitrate in the percolates (expressed as $\Delta\delta^{15}N$ values) vs. the respective nitrate concentrations for the mor (c) and raw humus (d) experiments. Treatment 1 = solid circle; treatment 2 = open triangle; treatment 3 = solid diamond; treatment 4 = open circle; treatment 5 = solid triangle.

rates were on average 23 μg NO_3-N g^{-1} $week^{-1}$ (weeks 3–16) in the experiment with the mor. In the experiment with the raw humus, nitrification rates were initially 12 μg NO_3-N g^{-1} $week^{-1}$ (weeks 3–6). They decreased to 10 μg NO_3-N g^{-1} $week^{-1}$ (weeks 7–10), and finally to 7 μg NO_3-N g^{-1} $week^{-1}$ (weeks 11–16) throughout the experiment.

Significant differences in ammonium concentrations were observed for the percolation solutions from the incubated O horizons (Fig. 1c,d). After the first percolation, an average of 6 ± 2 μg NH_4-N g^{-1} ($n = 10$) had accumulated in the seepage water of the experiments with both forest floors. Subsequently, a constant net ammonium release with a rate of approximately 6 μg NH_4-N g^{-1} $week^{-1}$ was observed for the mor. In contrast, <1 μg NH_4-N g^{-1} had accumulated in the weekly percolates of the experiment with the raw humus. This resulted in cumulative net ammonium releases of 95 ± 17 μg NH_4-N g^{-1} ($n = 5$) from the mor, and 14 ± 1 μg NH_4-N g^{-1} ($n = 4$) from the raw humus, respectively.

3.1.2. Isotope ratios of total nitrogen in soil

The $\delta^{15}N$ values of total nitrogen in the O horizons after percolation were within -4.8 ± 0.3‰ ($n = 5$) for the mor and -2.1 ± 0.3‰ ($n = 5$) for the raw humus, identical to the $\delta^{15}N_{total}$ values of the soils at the beginning of the incubation experiment (Table 1). This is not surprising, since only 3.6% (mor) and 1.7% (raw humus) of the total soil nitrogen were released as ammonium or nitrate during the incubation experiments.

3.1.3. Nitrogen isotope ratios of nitrate

$\delta^{15}N$ values of nitrate in the percolation water for both experimental variants with five treatments are shown in Figure 2a (mor) and Figure 2b (raw humus). In the first percolates from the mor, the mean $\delta^{15}N$ value of nitrate was -21.3 ± 1.0‰ ($n = 5$). Within the following six weeks, the mean $\delta^{15}N_{nitrate}$ value increased to -14.7 ± 0.7‰. At the end of the experiment, most $\delta^{15}N_{nitrate}$ values increased further to -11.8 ± 1.5‰, with the exception of those in treatment 1. For the raw humus experiment, the mean $\delta^{15}N$ value of nitrate in the initial percolates was -10.5 ± 1.0‰. $\delta^{15}N_{nitrate}$ reached minimum values of approximately -13‰ after 4 weeks. Subsequently, the nitrogen isotope ratios increased constantly and approached a mean $\delta^{15}N$ value of -8.1 ± 1.0‰ at the end of the experiment.

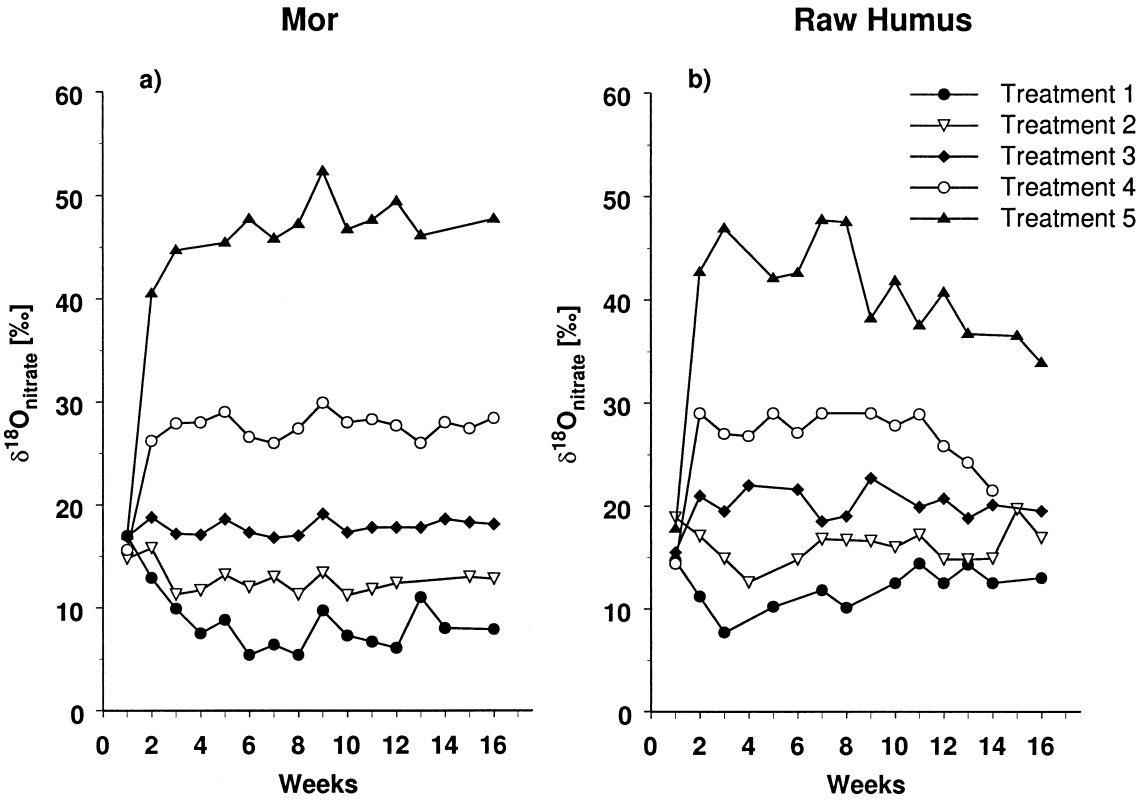

Fig. 3. $\delta^{18}O$ values of nitrate in the percolation water from the five respective treatments of the laboratory incubation experiments with the mor (a) and the raw humus (b).

The difference between the $\delta^{15}N$ values of total nitrogen in the incubated O horizons (Table 1) and those of nitrate in the percolates (Figs. 2a,b) is plotted as $\Delta\delta^{15}N$ values vs. the respective nitrate concentrations in Figures 2c (mor) and 2d (raw humus). For the experiment with the mor, an average $\Delta\delta^{15}N$ value of 11.1 ± 2.9‰ (n = 80) was observed. There was no significant correlation between $\Delta\delta^{15}N$ values and nitrate concentrations in the percolates (r = 0.20; n = 79). For the experiment with the raw humus, the highest $\Delta\delta^{15}N$ values (e.g., 9.8 ± 0.7‰, week 4, n = 5) were observed in the first 4 weeks of the experiment. Subsequently, $\Delta\delta^{15}N$ values decreased to 6.2 ± 0.7‰ (week 16, n = 4). In contrast to the mor experiment, a significant linear relation (r = 0.61; n = 72; $p <$ 0.0001) was observed between $\Delta\delta^{15}N$ values and nitrate concentrations in the percolates, with decreasing nitrate concentrations corresponding to lower $\Delta\delta^{15}N$ values (Fig. 2d).

3.1.4. Oxygen isotope ratios of water

For the first percolates, $\delta^{18}O_{water}$ values indicated that the percolation solutions consisted of a mixture of irrigation water and original soil water. After the second week of the experiment, the $\delta^{18}O$ values of the percolation water were, for both experimental variants (mor and raw humus) and the five respective treatments, identical to those of the irrigation solutions ($< \pm 1.0$‰ deviation), indicating that the replacement of the original soil water by the irrigation solutions was complete. Consequently, only data obtained for percolates of weeks three through 16 were used for the interpretation of results from the soil incubation experiments.

3.1.5. Oxygen isotope ratios of nitrate

In the first percolates, the mean $\delta^{18}O$ values of nitrate were with +17 ± 2‰ (n = 5) for the mor and +16 ± 2‰ (n = 5) for the raw humus, almost identical for the two experimental variants and five respective treatments (Fig. 3a,b). After the second percolation, $\delta^{18}O_{nitrate}$ values started to differ significantly, depending on the oxygen isotope ratios of the irrigation water. The lowest $\delta^{18}O_{nitrate}$ values (+5 to +15‰) were generally observed for nitrate formed in O horizons irrigated with treatment 1 ($\delta^{18}O_{water}$ = −8.0‰). The irrigation solution with the highest $\delta^{18}O_{water}$ value (+59.3‰) caused the highest $\delta^{18}O_{nitrate}$ values in the newly formed nitrate (+34 to +52‰) in both experimental variants.

In the experimental variants with the mor (Fig. 3a), after 4 weeks the oxygen isotope ratios of nitrate reached values of +8‰ (treatment 1), +12‰ (treatment 2), +17‰ (treatment 3), +28‰ (treatment 4), and +45‰ (treatment 5), respectively. Thereafter, the $\delta^{18}O$ values of the newly formed nitrate remained essentially constant. In the experimental variants with the raw humus (Fig. 3b), $\delta^{18}O_{nitrate}$ values of +9‰ (treatment 1), +13‰ (treatment 2), +22‰ (treatment 3), +27‰ (treatment 4), and +45‰ (treatment 5) were observed after 4 weeks. In the following 12 weeks of the experiment, the $\delta^{18}O_{nitrate}$

Table 2. Average ammonium and nitrate concentrations and average isotope compositions of nitrate and water-oxygen (± SD) in throughfall and forest floor solutions of a deciduous and a coniferous stand in North-Rhine Westphalia (number of samples given in brackets). Mean concentrations were calculated using data for weekly composite precipitation samples from the period April 1994 to March 1997. Throughfall for isotope analyses was obtained between January 1996 and June 1997 (weekly composite samples). Forest floor solutions for isotope analyses were sampled in April and May 1997.

Stand	Sampling Compartment	NH_4-N [mg L^{-1}]	NH_4-N/Cl	NO_3-N [mg L^{-1}]	NO_3-N/Cl	$\delta^{18}O_{water}$ [‰]	$\delta^{15}N_{nitrate}$ [‰]	$\delta^{18}O_{nitrate}$ [‰]
Deciduous	Throughfall	2.26 (117)[a]	1.48	1.06 (117)[a]	0.32	−8.3 ± 2.6 (26)	5.6 ± 3.2 (21)	39.5 ± 9.8 (18)
(beech/oak)	forest floor (mor)	3.70 (50)[a]	0.63	28.4 (66)[a]	4.81	−5.6 ± 1.5 (6)	−10.3 ± 1.7 (6)	10.1 ± 1.5 (6)
Coniferous	Throughfall	4.08 (119)[a]	0.89	3.38 (119)[a]	0.74	−8.1 ± 2.5 (27)	2.6 ± 2.7 (19)	36.1 ± 9.8 (20)
(spruce)	forest floor (raw humus)	4.66 (41)[a]	0.38	35.0 (46)[a]	2.84	−4.7 ± 0.4 (2)	−11.3 ± 0.3 (2)	23.0 ± 4.8 (2)

[a] Volume weighted concentration.

values increased slightly in treatments 1 (Δ = +4‰) and 2 (Δ = +4‰), remained almost constant in treatment 3 (Δ = −2‰), and decreased markedly in treatments 4 (Δ = −5‰) and 5 (Δ = −11‰).

3.2. Field Study

3.2.1. Chemical analyses

Concentration data for ammonium and nitrate in throughfall and forest floor solutions collected at a deciduous and a coniferous stand in North-Rhine Westphalia (Germany) are summarized in Table 2. A moderate increase in mean ammonium concentrations was observed in the forest floor solutions compared to throughfall at both sites. At the deciduous forest stand, nitrate concentrations in the forest floor solutions were by a factor of 27 higher than in throughfall. At the coniferous site, they were 10 times higher than in the throughfall. The NO_3^--N : Cl^- ratios increased from 0.3 in throughfall to 4.8 in the forest floor solutions at the deciduous stand, and from 0.7 (throughfall) to 2.8 (forest floor solutions) at the coniferous site. In contrast, NH_4^+-N : Cl^- ratios decreased significantly between throughfall and soil solutions at both sites (Table 2).

3.2.2. Isotope analyses

Weekly composite throughfall samples for isotope analyses were obtained between January 1996 and June 1997. Forest floor solutions for isotope analyses were sampled in April and May 1997. The mean $\delta^{18}O$ value of throughfall water was −8.3‰ (deciduous) and −8.1‰ (coniferous) at the two respective field sites in the 18-month observation period (Table 2). The mean $\delta^{18}O_{water}$ values of the forest floor solutions in April and May 1997 were −5.6‰ at the deciduous site and −4.7‰ at the coniferous site. These $\delta^{18}O$ values did not deviate considerably from those of throughfall in this time period (Grüter, 1997), and thus provide no evidence for significant evaporitic enrichment of the oxygen isotope ratios of the forest floor solutions.

The isotopic composition of nitrate in throughfall and forest floor solutions at both field sites is summarized in Table 2. Mean $\delta^{15}N$ values of nitrate in forest floor solutions were −10.3 ± 1.7‰ (n = 6) at the deciduous site and −11.3‰ (n = 2) at the coniferous site. These nitrogen isotope ratios of soil solution nitrate were ~16‰ (deciduous) and ~14‰ (coniferous) lower than those of throughfall nitrate (Table 2), and were also ~6‰ (deciduous) and ~9‰ (coniferous) lower than the $\delta^{15}N$ values of total nitrogen in the respective forest floors (Table 1). Mean $\delta^{18}O$ values of nitrate in throughfall of +39.5 ± 9.8‰ (n = 18) and +36.1 ± 9.8‰ (n = 20) were observed at the deciduous and coniferous forest stands, respectively (Table 2). The oxygen isotope ratios of nitrate in the forest floor solutions at the deciduous site varied between +7.8 and +12.0‰ (n = 6) and were on average 29‰ lower than those of throughfall nitrate. At the coniferous site, $\delta^{18}O_{nitrate}$ values in forest floor solutions were within +18.2 and +27.7‰ markedly higher than those at the deciduous site. The mean $\delta^{18}O$ value of nitrate in forest floor solutions at the coniferous site was 13‰ lower than that of throughfall nitrate.

4. DISCUSSION

The majority of the total nitrogen in soils is bound organically, predominantly in topsoil horizons. Since the O horizons (Of and Oh) used in the laboratory experiment contained nitrogen almost exclusively in organic binding form, the nitrate in the percolation water must have been generated by the process of microbial nitrification.

Chemolithoautotrophic nitrification is a microbially mediated multi-step oxidation of ammonium to nitrite and nitrate. This process is carried out by chemolithoautotrophic organisms via an inorganic pathway, in which ammonium is used as an energy source for bacteria of the genera *Nitrosomonas*, *Nitrosococcus*, *Nitrosospira*, *Nitrosolobus*, and *Nitrosovibrio* (Bock et al., 1991), according to the generalized Eqn. 4:

$$\underset{-3}{NH_4^+} \rightarrow \underset{-1}{NH_2OH} \xrightarrow{[NOH]} \underset{+3}{NO_2^-} \rightarrow \underset{+5}{NO_3^-} \qquad (4)$$

In the cytoplasm of the microorganisms, ammonium is oxidized by ammonia monooxygenase to NH_2OH and H_2O. Subsequently, enzyme-catalyzed (hydroxylamine oxidoreductase) reactions mediate the oxidation of NH_2OH to NO_2^- in the periplasm (Brock and Madigan, 1991) with enzyme-bound hydroxylamine (NOH) as a likely intermediate product (Hooper and Balny, 1982). Finally, the enzyme nitrite oxidase catalyzes the oxidation of nitrite to nitrate carried out by *Nitrobacter* (Brock and Madigan, 1991). It has been shown that NO and N_2O can be generated during chemolithoautotrophic nitrification either during the oxidation of ammonium (Ritchie and Nicholas, 1972; Blackmer et al., 1980; Anderson and Levine, 1986) or as a result of nitrite reduction in oxygen-

limited systems (Poth and Focht, 1985; Remde and Conrad, 1990).

Nitrification potentials in soils vary depending on environmental conditions, such as temperature, moisture, pH, chemical soil properties (including C:N ratios and ammonia supply), availability of nutrients, and microbial communities, among others. In the incubation experiment, the mor released considerably more nitrate than the raw humus (Fig. 1), presumably as a result of their different C:N ratios (Table 1), differences in bio-availability of C- and N-containing compounds, and possibly different microbial communities, since all other environmental conditions in the laboratory, including soil pH values, were almost identical for the two forest floors. While the nitrification rates were higher in the experiment with the mor (C/N = 24) and remained constant throughout the observation period (Fig. 1), the rates for the experiment with the raw humus (C/N = 32) were significantly lower and decreased with time. This indicates that the pool of available nitrogen for nitrifying microorganisms was more rapidly exhausted in the raw humus than in the mor. In contrast to the experiment with the mor where ammonium was constantly produced and available for nitrification (Fig. 1c), ammonium was not abundant throughout the experiment with the raw humus (Fig. 1d). It is possible that the ammonification potential of the raw humus with its C:N ratio of 32 was low, thereby limiting the supply of NH_4^+ available for nitrification, resulting in comparatively low nitrification rates.

Nitrogen isotope data are consistent with the above interpretation. Nitrogen isotope ratios of nitrate generated by nitrification are dependent on the $\delta^{15}N$ value of the nitrogen source and kinetic isotope effects during microbial nitrification. If organic soil nitrogen is the source of NH_4^+, the latter has typically a $\delta^{15}N$ value within a few per mil of that of soil organic nitrogen, since the ammonification process ($N_{org} \rightarrow NH_4^+$) does usually not proceed with large nitrogen isotope fractionation (e.g., Kendall, 1998). In contrast, the conversion of NH_4^+ to NO_2^- and NO_3^- can be accompanied by marked nitrogen isotope fractionation effects of > -30‰ (e.g., Delwiche and Steyn, 1970; Mariotti et al., 1981; Hübner, 1986). The extent of the overall nitrogen isotope fractionation during chemolithoautotrophic nitrification depends on which of the reaction steps is rate limiting and on the size of the substrate pool. In natural systems, the oxidation of nitrite to nitrate is usually rapid, and therefore is not the rate-determining step for the overall nitrification reaction (e.g., Kendall, 1998). Also, since this reaction step typically involves quantitative conversion of nitrite to nitrate, nitrogen isotope fractionation is expected to be minimal. Thus, the most likely step for nitrogen isotope fractionation is the slow oxidation of ammonium. Here, the size of the ammonium pool has a major influence on the extent of nitrogen isotope fractionation (Shearer and Kohl, 1993). With a large pool of available NH_4^+, the isotopic selectivity is generally large and the lighter isotope ^{14}N is preferentially converted into reaction products NO_2^- and NO_3^-, which can have $\delta^{15}N$ values up to 35‰ lower than that of the initial NH_4^+ pool (Mariotti et al., 1981). In contrast, the nitrogen isotope fractionation in NH_4^+ limited systems, where the conversion of organic nitrogen to NH_4^+ becomes rate limiting, is typically small (e.g., Feigin et al., 1974; Shearer et al., 1978).

A detailed interpretation of $\delta^{15}N$ values of nitrate in the percolates from the soil incubation experiments is beyond the scope of this paper and is somewhat hampered by the fact that the nitrogen isotope ratios of ammonium were not measured. Nevertheless, $\delta^{15}N_{nitrate}$ values of < -20‰ in the first percolates of the experimental variants with the mor are indicative of nitrification with a large pool of available NH_4^+, thus resulting in significant nitrogen isotope selectivity. Both ammonium concentrations (Fig. 1c) and $\Delta\delta^{15}N$ values (Fig. 2c) remained comparatively high throughout the experiment with the mor, indicating that NH_4^+ was not rate limiting. Consequently, high and constant nitrification rates were observed during the 16 weeks of the experiment. In contrast, low ammonium concentrations in the percolates (Fig. 1d) and decreasing $\Delta\delta^{15}N$ values with decreasing nitrate concentrations (Fig. 2d) were observed during the experiment with the raw humus. This is consistent with NH_4^+ becoming rate limiting throughout the experiment, causing decreasing nitrification rates and, consequently, low nitrate concentrations in the latter part of the experiment (Fig. 1b). Further potential explanations for the variability in the $\delta^{15}N_{nitrate}$ values in the percolates are discussed later in this section (see Fig. 5b).

The oxygen isotope composition of nitrate derived exclusively from nitrification processes has been determined previously only in few studies, which yielded variable results (Amberger, 1987; Voerkelius, 1990; Kendall, 1998; Burns and Kendall, in review). There is evidence from laboratory culture experiments that oxygen from H_2O and O_2 is incorporated into nitrate formed during chemolithoautotrophic nitrification. Anderson and Levine (1986) have shown that NO_2^- produced by microbial (chemolithoautotrophic) oxidation of NH_3 by *Nitrosomonas* derives one oxygen from H_2O and a second oxygen from O_2. It has been further reported that NO_3^- generated by microbial oxidation of NO_2^- by *Nitrobacter* derives the third oxygen from H_2O (Hollocher, 1984). Based on these studies, it has been suggested (Amberger and Schmidt, 1987; Voerkelius, 1990; Durka et al., 1994; Wassenaar, 1995; Böhlke et al., 1997; Kendall, 1998) that the oxygen isotope composition of nitrate generated by nitrification can be calculated as follows:

$$\delta^{18}O_{nitrate} = \tfrac{2}{3}(\delta^{18}O_{water} + \varepsilon_{water}) + \tfrac{1}{3}(\delta^{18}O_{O_2} + \varepsilon_{O_2}) \quad (5)$$

Based on Eqn. 5, and assuming negligible isotope fractionation during water (ε_{water}) and O_2 (ε_{O2}) incorporation, nitrate derived from nitrification would have $\delta^{18}O$ values between -2 and $+6$‰, since environmental water has typically $\delta^{18}O$ values between -15 and -5‰, and $\delta^{18}O$ of atmospheric O_2 is $+23.5$‰ (Kroopnick and Craig, 1972; Horibe et al., 1973). It has, however, been recently noted that $\delta^{18}O_{nitrate}$ values outside of this range may be expected if (1) the $\delta^{18}O$ value of H_2O used by the microorganisms is isotopically enriched (e.g., as a result of evaporation), (2) if the $\delta^{18}O$ value of O_2 used by the microorganisms is different from that of atmospheric O_2, (3) if there is significant isotope fractionation during the incorporation of oxygen from H_2O and O_2 into the newly formed nitrate, or (4) if the ratio of oxygen incorporation from H_2O and O_2 is not 2:1 (Aravena et al., 1993; Kendall et al., 1995; Wassenaar, 1995; Böhlke et al., 1997; Kendall, 1998).

To determine the ratio of oxygen incorporation from H_2O and O_2 into the newly formed nitrate, the $\delta^{18}O$ values of the

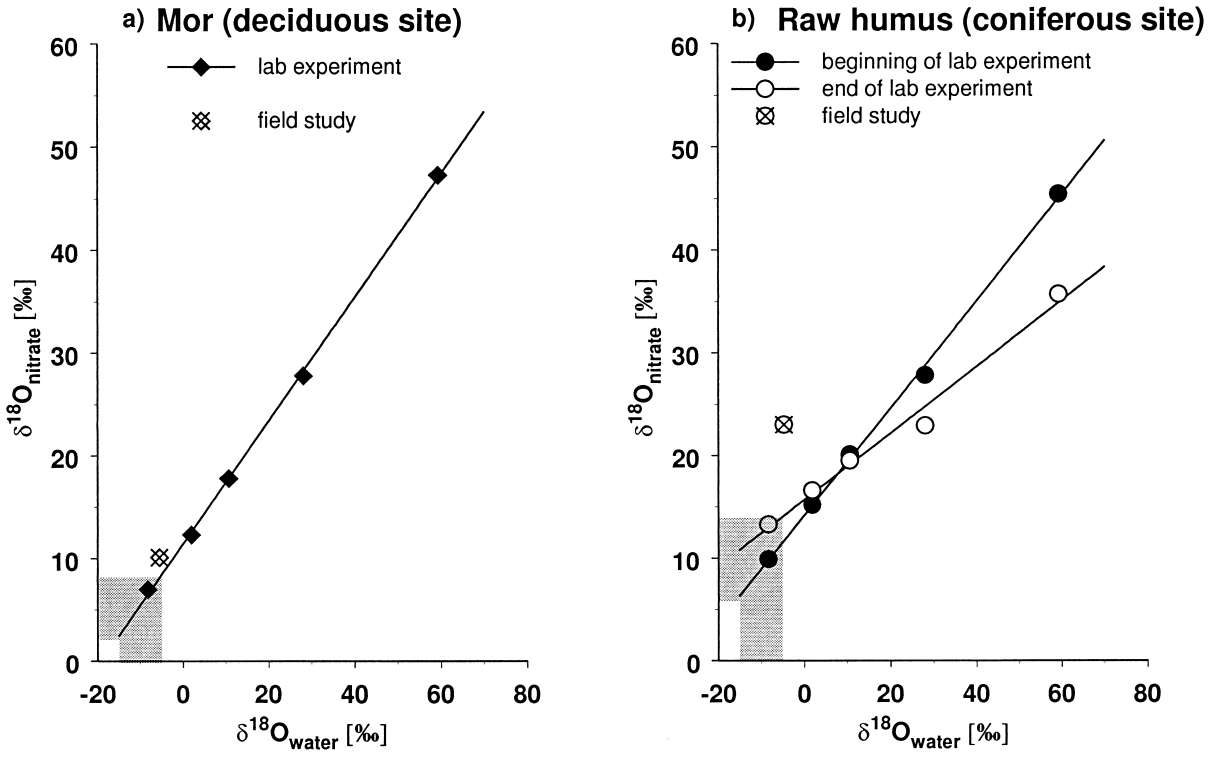

Fig. 4. Mean $\delta^{18}O$ values of seepage water nitrate vs. mean $\delta^{18}O$ values of the irrigation water in the incubation experiment with the mor from the deciduous site (a) and the raw humus from the coniferous site (b). The slopes of the regression lines indicate the percentage of water-oxygen incorporation into the newly formed nitrate. In the experiment with the mor, the slope was 0.60. In the experiment with the raw humus, the slope was 0.52 at the beginning of the experiment (weeks 3–8) and 0.32 at the end of the experiment (weeks 13–16). Average oxygen isotope compositions of water and nitrate from soil solutions obtained at the deciduous (a) and the coniferous (b) forest sites are also shown with separate (crossed) symbols. The shaded areas indicate the range of $\delta^{18}O$ values expected for nitrate formed during nitrification in soil solutions with $\delta^{18}O_{water}$ values between −15 and −5‰.

isotopically labeled irrigation water were plotted vs. the mean $\delta^{18}O$ values of nitrate generated by nitrification in the five experimental variants of both laboratory incubation experiments (Fig. 4). If indeed two oxygen atoms in the newly formed nitrate were derived from water, a linear correlation between $\delta^{18}O_{water}$ and $\delta^{18}O_{nitrate}$ values would be expected for the five experimental variants, and the slope of the regression line should be theoretically 0.67. In Figure 4a, the $\delta^{18}O$ values of the isotopically labeled irrigation water are plotted vs. the mean $\delta^{18}O$ values of nitrate (weeks 3–16) generated by nitrification in the five variants of the experiment with the mor. A highly significant linear relation ($r^2 = 0.999$; $p < 0.00001$; $n = 5$) was observed with a slope of the regression line of 0.60. This is close to the expected value and suggests that two oxygen atoms in the newly formed nitrate were derived from water throughout the experiment with the mor with its continuously high nitrification rates. For the experiment with the raw humus, the mean $\delta^{18}O$ values of the newly formed nitrate were also found to correlate linearly with the $\delta^{18}O$ values of the irrigation water in the five experimental treatments (Fig. 4b), but the slope of the regression line changed throughout the course of the incubation period. At the beginning (weeks 3–8), the slope of the regression line was 0.52 ($r^2 = 0.998$; $p < 0.0001$; $n = 5$). For the mean $\delta^{18}O_{nitrate}$ values at the end of the experiment (weeks 13–16), the slope decreased to 0.32 ($r^2 = 0.985$; $p = 0.0008$; $n = 5$), suggesting that only one oxygen in the nitrate molecule was derived from H_2O. In particular, the relations obtained from the experiment with the raw humus are markedly different from that expected according to Eqn. 5. For typical $\delta^{18}O$ values of soil seepage water between −15 and −5‰, results from the laboratory incubation experiments predict that oxygen isotope ratios of nitrate derived from nitrification would range between +2 and +8‰ for the mor, and between +6 and +14‰ for the raw humus (see shaded areas in Fig. 4). Potential explanations for this wide range of $\delta^{18}O_{nitrate}$ values are discussed below.

Throughout weeks 3 to 16 of the incubation study, the $\delta^{18}O_{water}$ values of the percolates were identical to those of the irrigation solutions in all experimental variants. Therefore, evaporitic enrichment of ^{18}O in the soil water during the experiment can be excluded as a potential explanation for $\delta^{18}O_{nitrate}$ values deviating from those expected according to Eqn. 5.

$\delta^{18}O$ values of soil O_2 were not determined during the incubation study, but it can be assumed that they were initially similar to those of atmospheric O_2 (+23.5‰), since the O horizon material had been mixed and homogenized before the experiments. The experimental design allowed for exchange of air into and out of the incubation containers, but it cannot be

ruled out that bacterial respiration might have influenced the oxygen isotope ratios of soil O_2 as the experiment progressed. Since the oxygen isotope fractionation factor for bacterial respiration is 1.015 (Lane and Dole, 1956; Guy et al., 1993), the remaining soil O_2 is expected to become enriched in ^{18}O as a result of this process. If indeed $\delta^{18}O$ values for soil O_2 had significantly increased with time, one would have expected increasing $\delta^{18}O_{nitrate}$ values during the experiment. This was the case for treatments 1 and 2 of the experiment with the raw humus, but constant or decreasing $\delta^{18}O_{nitrate}$ values were observed for the other eight experimental variants (Fig. 3). Although it is possible that the $\delta^{18}O$ values of soil O_2 were higher than +23.5‰ and might have varied throughout the experiment, this cannot explain the different slopes of the regression lines in Figure 4, if a 2:1 ratio of oxygen incorporation from H_2O and O_2 into the newly formed nitrate is assumed.

It was not possible to determine conclusively the extent of isotope fractionation during incorporation of oxygen from H_2O (ε_{water}) and O_2 (ε_{O2}) into the newly formed nitrate since the $\delta^{18}O$ values of soil O_2 were not measured, but there is reason to believe that these fractionation factors are comparatively small. Assuming negligible isotope fractionation during oxygen incorporation from H_2O and O_2 into the newly formed nitrate, it is possible to calculate the $\delta^{18}O$ value of soil O_2 based on the linear relations displayed in Figure 4. If the $\delta^{18}O_{water}$ value is chosen identical to that of atmospheric O_2 (+23.5‰), the regression equations predict $\delta^{18}O_{nitrate}$ values between +23 and +26‰. Depending on the respective proportions of oxygen incorporation from O_2 into the newly formed nitrate for the two different experiments, this suggests $\delta^{18}O$ values for soil O_2 between +23 and +29‰. This data range is typical for soil O_2 as described above. It is important to note that these considerations do not conclusively rule out isotopic fractionation during incorporation of oxygen from H_2O and O_2 into the newly formed nitrate, but if existing, these hypothetical isotope effects would have been of similar extent in all experimental variants of the laboratory incubation experiment. Therefore, neither isotopic fractionation during incorporation of oxygen from H_2O and O_2 into the newly formed nitrate, nor variations in the $\delta^{18}O$ values of soil O_2 or soil H_2O, provide a satisfactory explanation for the different slopes of the regression lines shown in Figure 4. Consequently, we suggest that the ratio of oxygen incorporation from H_2O and O_2 must have deviated from the previously assumed ratio of 2:1 (Eqn. 5) during the experiment with the raw humus. It is proposed that such deviations might occur in situations where chemolithoautotrophic nitrification is not the predominant nitrification pathway in soils.

It is generally believed that the activity and growth of chemolithoautotrophic nitrifiers become increasingly inhibited at low pH values (Focht and Verstraete, 1977; Lang, 1986). It has been, therefore, suggested that heterotrophic nitrification might be of considerable importance in acid forest soils (e.g., Focht and Verstraete, 1977; Killham, 1986; Gundersen and Rasmussen, 1990; Pedersen et al., 1999).

Heterotrophic nitrification is the microbial oxidation of both organic and inorganic nitrogen compounds, which does not entail an energy gain for the nitrifying bacteria (Killham, 1986; Bock et al., 1991; Simek, 2000). The exact reaction pathways and catalyzing enzymes involved in heterotrophic nitrification are poorly known. It has been suggested that heterotrophic nitrification follows an organic reaction pathway (Doxtrader, 1965; Focht and Verstraete, 1977), according to the following reaction:

$$\begin{array}{ccccccc} \text{R-NH}_2 & \rightarrow & \text{R-NH}_2\text{OH} & \rightarrow & \text{R-NO} & \rightarrow & \text{RNO}_2^- \rightarrow \text{NO}_3^- \\ -3 & & -1 & & +1 & & +3 \quad +5 \end{array} \qquad (6)$$

Some authors have also proposed an inorganic reaction pathway for the heterotrophic nitrification carried out, for example, by *Aspergillus flavus* (Lang, 1986; Paul and Clark, 1989; Killham, 1990).

$$\begin{array}{ccccccc} \text{NH}_4^+ & \rightarrow & \text{NH}_2\text{OH} & \rightarrow & \text{NOH} & \rightarrow & \text{NO}_2^- \rightarrow \text{NO}_3^- \\ -3 & & -1 & & +1 & & +3 \quad +5 \end{array} \qquad (7)$$

It has also been suggested that both pathways (Eqn. 6 and 7) might proceed in combination during heterotrophic nitrification (Prosser, 1989). It is further interesting to note that heterotrophic organisms can release NO_2^- and NO_3^- directly from their organic moiety (Wood, 1988; Wood, 1990). In case of NO_2^- release, two oxygen atoms in the newly formed nitrate are presumably derived from the organic nitrogen compound. The third oxygen atom would be incorporated during the oxidation of NO_2^- to NO_3^- and is typically derived from water (Hollocher, 1984). This reaction pathway is consistent with the results obtained at the end of the laboratory experiment with the raw humus, where the linear relation between $\delta^{18}O_{nitrate}$ and $\delta^{18}O_{water}$ values with a slope of 0.32 indicated that only one water oxygen was incorporated into the newly formed nitrate. It can be further speculated that in the case of a potential NO_3^- release directly from organic nitrogen compounds (Wood, 1988; Wood, 1990), no water oxygen would be incorporated into the newly formed nitrate, and the slope of the linear regression line between $\delta^{18}O_{water}$ and $\delta^{18}O_{nitrate}$ values would tend towards 0. Under these circumstances, one would expect that the $\delta^{15}N$ and $\delta^{18}O$ values of the newly formed nitrate might resemble those of nitrogen and oxygen in soil organic matter. Consistent with this hypothesis, $\delta^{15}N$ values of the newly formed nitrate increased toward those of soil nitrogen (−2.1‰), and $\delta^{18}O_{nitrate}$ displayed trends toward values around +20‰ as the experiment with the raw humus progressed (Fig. 5b). In contrast, throughout the experiment with the mor, $\delta^{15}N_{nitrate}$ values in the percolates were lower than those in the raw humus solutions, and $\delta^{18}O_{nitrate}$ values were constant (Fig. 5a).

Based on the above-described observations, we suggest that nitrate formed by nitrification in acid forest floors may obtain less than two thirds of its oxygen from water. Chemolithoautotrophic and heterotrophic nitrification are carried out by different microorganisms and can occur concurrently in soils (e.g., Pedersen et al., 1999). In the incubation experiment with the raw humus, ammonium concentrations in the percolates were generally low. Nitrification rates were initially relatively low and decreased further throughout the experiment. It is possible that heterotrophic nitrification became the predominant nitrification pathway as the experiment progressed, explaining water-oxygen proportions between 52% (beginning) and 32% (end) in the newly formed nitrate. In the experiment with the mor, ammonium was abundant and nitrification rates were consis-

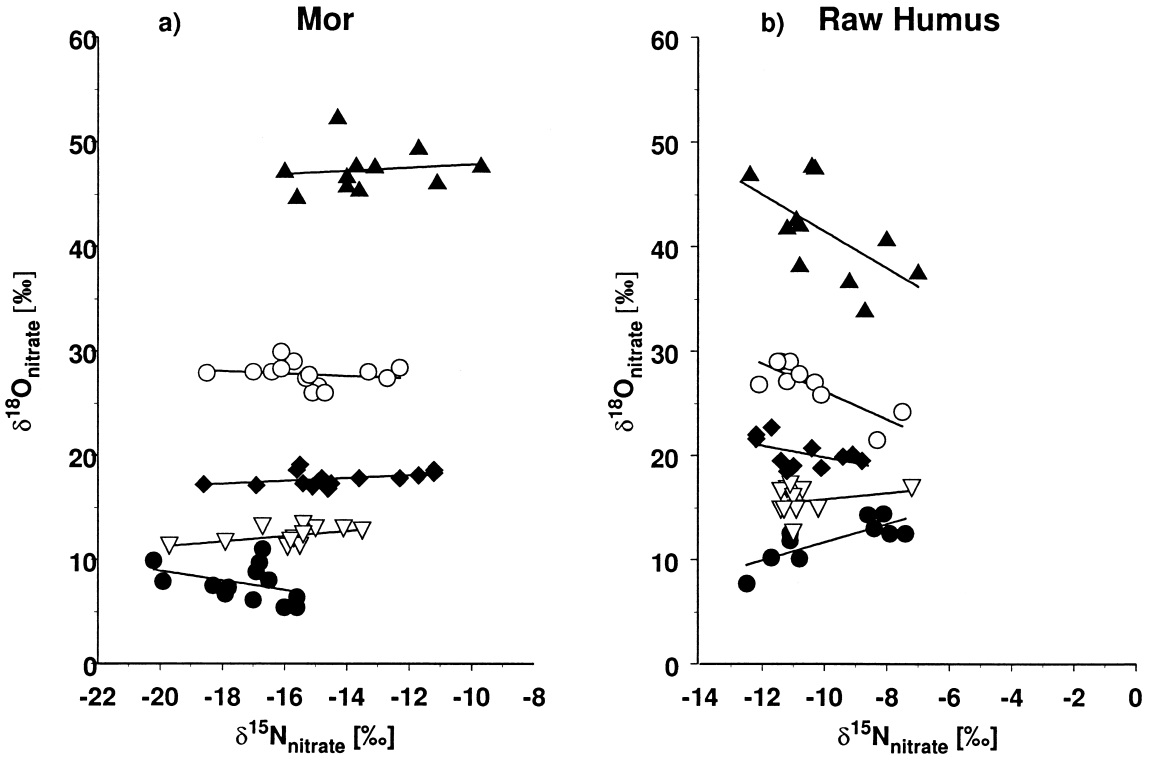

Fig. 5. $\delta^{15}N$ values vs. $\delta^{18}O$ values for nitrate in the percolation water from the five respective treatments of the laboratory incubation experiments with the mor (a) and the raw humus (b). The nitrate was derived from nitrification processes in the O horizons and was not significantly influenced by denitrification, since the latter process would have resulted in simultaneously increasing $\delta^{15}N_{nitrate}$ and $\delta^{18}O_{nitrate}$ values in all experimental variants.

tently high throughout the observation period. In this case, chemolithoautotrophic nitrification was presumably the dominant process with potentially minor nitrate contributions via heterotrophic nitrification. Therefore, the proportion of water oxygen in the newly formed nitrate was close to the expected value of 67% (Eqn. 5). Consequently, variations in the relative proportions of nitrate formation via the chemolithoautotrophic and the heterotrophic nitrification pathways are suggested as the most likely reason for the wide range of $\delta^{18}O_{nitrate}$ values observed during the soil incubation experiment.

Results from field studies support the interpretations derived from the laboratory incubation experiments. The significant increase of nitrate concentrations and the marked increase of NO_3^--N : Cl^- ratios in forest floor solutions compared to throughfall (Table 2) provides evidence that nitrate in the lysimeter solutions was formed predominantly in the forest floors, and that only a small proportion of the soil nitrate pool might have been derived from atmospheric nitrate deposition. Since the forest floor horizons contained nitrogen almost exclusively in organic binding form, the majority of the nitrate in the forest floor solutions must have been generated by microbial nitrification processes. At the deciduous site, the increase of the NO_3^--N : Cl^- ratios in the soil solutions, compared to those of the throughfall, was more pronounced (factor 15) than at the coniferous site (factor 4), suggesting that nitrification rates were higher in the mor at the deciduous stand than in the raw humus at the coniferous site. This finding is in excellent agreement with results from the laboratory experiments with O horizons from the two field sites. Ammonium was available in both forest floor solutions at concentrations similar to those of throughfall, but NH_4^+-N : Cl^- ratios decreased markedly between throughfall and forest floor solutions at both sites (Table 2). This indicates that NH_4^+ was consumed in both forest floors presumably by chemolithoautotrophic or heterotrophic nitrification.

$\delta^{15}N$ values of nitrate in the forest floor solutions at both field sites were more than 13‰ lower than those of throughfall nitrate (Fig. 6), providing further evidence that soil solution nitrate was not derived to a large extent from atmospheric deposition, but from microbial nitrification. The latter process proceeded with significant nitrogen isotope fractionation, since the $\delta^{15}N$ values of soil solution nitrate were within −10.3‰ (mor) and −11.3‰ (raw humus) markedly lower than the $\delta^{15}N$ values of total nitrogen in the mor (−4.8‰) and the raw humus (−2.1‰), respectively. This is consistent with a model, in which ammonium is converted to nitrate without exhausting the entire substrate (NH_4^+) pool, thus allowing the chemolithoautotrophic or heterotrophic nitrifying bacteria to preferentially metabolize ^{14}N. Because of the high concentrations yet comparatively low $\delta^{15}N$ values of soil solution nitrate, it is unlikely that denitrification had occurred and influenced the isotopic composition of seepage water nitrate at both field sites.

Since there is compelling evidence that nitrate in soil solutions obtained at both field sites was derived predominantly from microbial nitrification, it appears feasible to compare the oxygen isotope ratios observed in the laboratory experiments

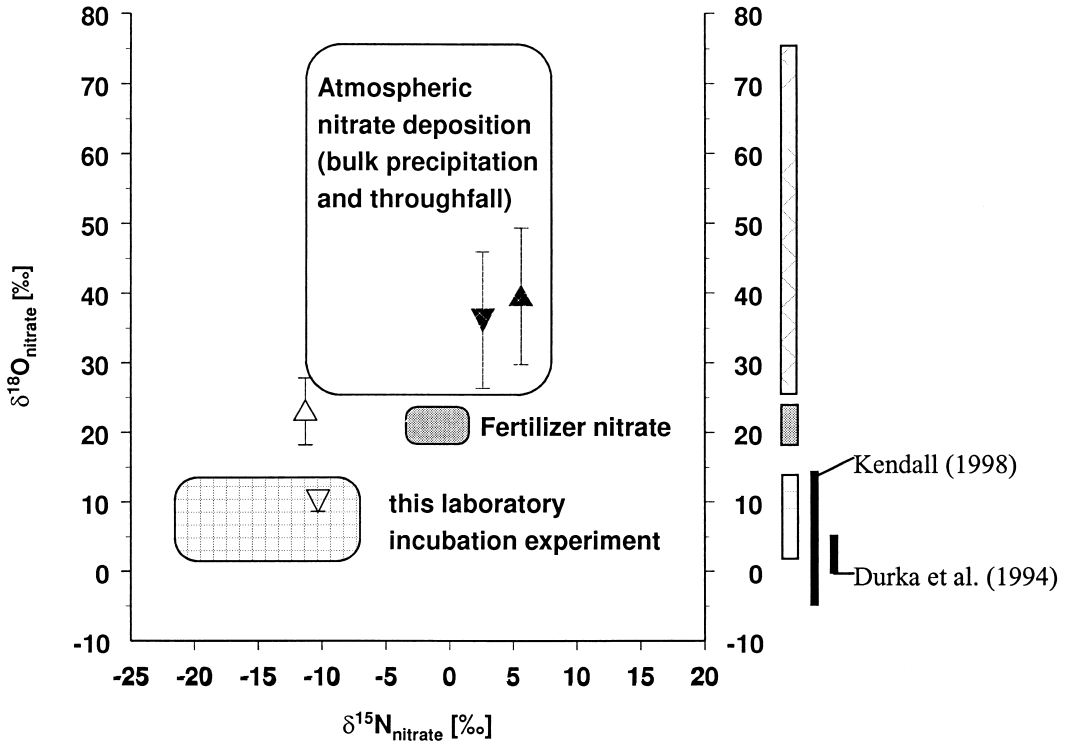

Fig. 6. Average $\delta^{15}N$ and $\delta^{18}O$ values of nitrate ($\pm$SD) in throughfall (solid triangles) and forest floor solution samples (open triangles) obtained from the deciduous (inverted triangles) and the coniferous field site (upright triangles). Also shown are the isotopic compositions of other sources of nitrate in terrestrial ecosystems and previously published ranges of oxygen isotope ratios of nitrate formed by nitrification in soils (Durka et al.; 1994; Kendall, 1998).

with those of the field samples. The mean $\delta^{18}O$ value for nitrate in the soil solutions at the deciduous site was $+10.1 \pm 1.5$‰ ($n = 6$), only marginally higher than expected according to the results from the laboratory incubation experiments (Fig. 4a). In the forest floor solutions of the coniferous site, the mean $\delta^{18}O$ value of nitrate was within $+23.0$‰ ($n = 2$), significantly higher than expected according to the results from the laboratory incubation experiments (Fig. 4b). It is interesting to note that the mean oxygen isotope ratios of soil solution nitrate from the deciduous and the coniferous sites differed by more than 10‰ (Fig. 6) although the two field sites were located less than 1 km apart. The considerable difference in the $\delta^{18}O$ values of soil solution nitrate from both sites cannot be explained by evaporitic enrichment of ^{18}O in the soil water, since the field samples were obtained under similar meteorological conditions in April and May 1997. Also, isotope fractionation during the incorporation of oxygen from H_2O or O_2 into the newly formed nitrate does not provide a satisfactory explanation, since these isotope effects are believed to be small and identical at both sites. Also, admixture of nitrate from atmospheric deposition to the soil nitrate pool cannot fully account for the observed differences in $\delta^{18}O_{nitrate}$ values at both sites, since the NO_3^--N : Cl^- ratios suggest that $<25\%$ of the soil solution nitrate was derived from atmospheric deposition (Table 2). One possible explanation for the markedly different $\delta^{18}O_{nitrate}$ values in the forest floor solutions of both sites is that the $\delta^{18}O$ values of soil O_2 might have been significantly higher in the raw humus at the coniferous site than in the mor at the deciduous site as a result of respiratory isotope fractionation leaving the remaining O_2 in semi-isolated soil pores enriched in ^{18}O (Kendall, 1998). We propose as an alternate explanation that significantly less than two thirds of the nitrate-oxygen in the soil solutions at the coniferous site was derived from water. Thus, differences in the nitrification pathways might have been responsible for the markedly different oxygen isotope ratios of soil solution nitrate at the deciduous and coniferous site, a hypothesis that is in good agreement with observations from the laboratory incubation experiments.

5. CONCLUSIONS

Nitrate derived from nitrification processes in forest floors can constitute a large proportion of the nitrate pool in soil seepage water, surface water, and groundwater. The ability to isotopically identify nitrate derived from microbial nitrification requires that its isotopic composition be distinct from that of other nitrate sources, such as atmospheric nitrate deposition and fertilizer nitrate. Evidence from soil incubation experiments and field studies suggests that during microbial nitrification in O horizons of forest floors, up to two of the three oxygen atoms in the newly formed nitrate are derived from water, particularly if NH_4^+ is abundant and nitrification rates are high. This is consistent with current knowledge about oxygen incorporation into nitrate during chemolithoautotrophic nitrification (Aleem et al., 1965; Anderson and Hooper, 1983; Hollocher, 1984). It was, however, also observed that in NH_4^+ limited systems with

low nitrification rates, significantly less than two thirds of the oxygen in newly formed nitrate was derived from water oxygen. This might indicate the occurrence of heterotrophic nitrification in acid forest floor horizons, during which incorporation of water oxygen into nitrate may be considerably lower than during chemolithoautotrophic nitrification. It is obvious from this study that a better understanding of (1) the various biochemical nitrification pathways and (2) the influence of microbial respiration on the $\delta^{18}O$ value of soil O_2 in forest floors and mineral soil horizons is essential for a more precise description of the underlying mechanisms that control the oxygen isotope ratios of nitrate from microbial nitrification. Nevertheless, the presented data suggest that variations in the relative proportions of nitrate formation via the chemolithoautotrophic and the heterotrophic nitrification pathways may be in part responsible for the relatively wide range of $\delta^{18}O$ values for nitrate formed by microbial nitrification reported in the literature (e.g., Durka et al., 1994; Kendall, 1998) and confirmed in this study (Fig. 6). Based on laboratory incubation experiments, we suggest that $\delta^{18}O$ values between +2 and +14‰ should be considered as typical for nitrate derived from nitrification processes in acid forest floors with soil water $\delta^{18}O$ values ranging between −15 and −5‰. This range of oxygen isotope ratios is distinct from those of nitrate-containing fertilizers and from atmospheric nitrate deposition (Fig. 6). The oxygen isotope composition of nitrate is, therefore, a useful qualitative tracer for distinguishing nitrate from atmospheric and pedospheric sources in forested catchments and also represents a potential tool for tracing nitrate-containing fertilizers in watersheds with agricultural land use. However, because of the wide range of $\delta^{18}O$ values for nitrate from atmospheric deposition and from soil nitrification (Fig. 6), a reliable quantitative apportionment of various nitrate sources contributing to seepage water, surface water, and groundwater that is based on the oxygen isotope ratios of nitrate alone (e.g., Durka et al., 1994) appears to be difficult, particularly if the nitrification pathways in the studied system are not known.

Acknowledgments—The help of Constanze Heinzer, Bernd Köhler, Timo Kluttig, Kai Gockel, Thilo Rennert, Heike Leyer, and Verena Fiedler (Ruhr-University, Bochum) in obtaining samples from the field experiment and in conducting the laboratory incubation experiments is gratefully acknowledged. Roland Grüter (Ruhr-University, Bochum) assisted in determining the isotopic composition of bulk precipitation and throughfall nitrate collected at the field sites. We thank N. E. Ostrom, C. Kendall, S. Silva, W. Durka, and two anonymous reviewers for their constructive criticism, which greatly helped in improving this manuscript.

Associate editor: N. E. Ostrom

REFERENCES

Aleem M. I. H., Hoch G. E., and Varner J. E. (1965) Water as the source of oxidant and reducant in bacterial chemosynthesis. *Biochemistry* **54,** 869–873.

Amberger A. (1987) Natürliche ^{15}N und ^{18}O Gehalte als Indikatoren für die Herkunft von Nitrat in Boden und Grundwasser. Ph.D. thesis, Technical University Munich.

Amberger A. and Schmidt H. L. (1987) Natürliche Isotopengehalte von Nitrat als Indikatoren für dessen Herkunft. *Geochim. Cosmochim. Acta* **51,** 2699–2705.

Anderson I. C. and Levine J. S. (1986) Relative rates of nitric oxide and nitrous oxide production by nitrifiers, denitrifiers, and nitrate respirers. *Appl. Environ. Microbiol.* **51,** 938–945.

Anderson K. K. and Hooper A. B. (1983) O_2 and H_2O are each the source of one NO_2^- produced from NH_3 by *Nitrosomas*. ^{15}N-NMR evidence. *FEBS Lett.* **65,** 236–240.

Aravena R., Evans M. L., and Cherry J. A. (1993) Stable isotopes of oxygen and nitrogen in source identification of nitrate from septic systems. *Ground Water* **31,** 180–186.

Aravena R. and Robertson W. D. (1998) Use of multiple isotope tracers to evaluate denitrification in ground water: Study of nitrate from a large-flux septic system plume. *Ground Water* **36,** 975–982.

Benkovitz C. M., Scholtz M. T., Pacyna J., Tarrason L., Dignon J., Voldner E. C., Spiro P. A., Logan J. A., and Graedel T. E. (1996) Global gridded inventories of anthropogenic emissions of sulfur and nitrogen. *J. Geophys. Res.* **101,** 29239–29253.

Blackmer A. M., Bremner J. M., and Schmidt E. L. (1980) Production of nitrous oxide by ammonia-oxidizing chemoautotrophic microorganisms in soils. *Appl. Environ. Microbiol.* **40,** 1060–1066.

Bock E., Koops H. P., Harms H., and Ahlers B. (1991) The biochemistry of nitrifying organisms. In *Variations in Autotrophic Life* (ed. J. M. Shively and L. L. Barton), pp. 171–200. Academic Press, London.

Böhlke J. K., Ericksen G. E., and Revesz K. (1997) Stable isotope evidence for an atmospheric origin of desert nitrate deposits in northern Chile and southern California, U.S.A. *Chem. Geol.* **136,** 135–152.

Bräuer K. and Strauch G. (2000). An alternative procedure for the ^{18}O measurement of nitrate oxygen. *Chem. Geol.* **168,** 283–290.

Brock T. D. and Madigan M. T. (1991) *Biology of Microorganisms*. Prentice-Hall, Englewood Cliffs, N.J.

Burns D. A. and Kendall C. (in review) Analysis of ^{15}N and ^{18}O to differentiate NO_3^- sources in runoff at two watersheds in the Catskill Mountains of New York. *Water Resour. Res.*

Cey E. E., Rudolph D. L., Aravena R., and Parkin G. (1999) Role of the riparian zone in controlling the distribution and fate of agricultural nitrogen near a small stream in southern Ontario. *J. Contam. Hydrol.* **37,** 45–67.

Delwiche C. C. and Steyn P. L. (1970) Nitrogen isotope fractionation in soils and microbial reactions. *Environ. Sci. Technol.* **4,** 929–935.

DEV. (1983) Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung. Verlag Chemie.

Doxtrader K. G. (1965) *Nitrification by heterotrophic microorganisms*. Ph.D. thesis, Cornell University.

Durka W., Schulze E. D., Gebauer G., and Voerkelius S. (1994) Effects of forest decline on uptake and leaching of deposited nitrate determined from ^{15}N and ^{18}O measurements. *Nature* **372,** 765–767.

Epstein S. and Mayeda T. (1953) Variation of O-18 content of waters from natural sources. *Geochim. Cosmochim. Acta* **4,** 213–224.

FAO. (1990) *Soil map of the world*. Food and Agriculture Organisation of the United Nations (FAO-UNESCO).

Feigin A., Shearer G., Kohl D. H., and Commoner B. (1974) The amount and nitrogen-15 content of nitrate in soil profiles from two central Illinois fields in a corn-soybean rotation. *Soil Sci. Soc. Am. Proc.* **38,** 465–471.

Focht D. D. and Verstraete W. (1977) Biochemical ecology of nitrification and denitrification. *Adv. Mirob. Ecol.* **1,** 135–214.

Frank C. (1996) Nitrifikation und N-Mineralisation in sauren und Dolomit-gekalkten Nadelwaldböden im Fichtelgebirge. *Bayreuther Forum Ökologie* **36,** 1–149.

Gat J. R. (1996) Oxygen and hydrogen isotopes in the hydrological cycle. *Annu. Rev. Earth Planet. Sci.* **24,** 225–262.

Gormly J. R. and Spalding R. F. (1979) Sources and concentrations of nitrate-nitrogen in ground water of the Central Platte Region, Nebraska. *Ground Water* **17,** 291–301.

Grüter R. (1997) Die Isotopenzusammensetzung von Nitrat und Sulfat aus Niederschlagsproben des Münsterlandes. M.Sc., Ruhr-University, Bochum.

Gundersen P. and Rasmussen L. (1990) Nitrification in forest soils: Effects from nitrogen deposition on soil acidification and aluminum release. *Rev. Environ. Contam. T.* **113,** 1–43.

Guy R. D., Fogel M. L., and Berry J. A. (1993) Photosynthetic fractionation of the stable isotopes of oxygen and carbon. *Plant Physiol.* **101,** 37–47.

Hollocher T. C. (1984) Source of the oxygen atoms of nitrate in the oxidation of nitrite by *Nitrobacter agilis* and evidence against a P-O-N anhydride mechanism in oxidative phosphorylation. *Arch. Biochem. Biophys.* **233,** 721–727.

Hooper A. B. and Balny C. (1982) Reaction of oxygen with hydroxylamine-oxidoreductase of *Nitrosomas*. *FEBS Lett.* **144,** 299–303.

Horibe Y., Shigehara K., and Takakuwa Y. (1973) Isotopic separation factors of carbon-dioxide-water system and isotopic composition of atmospheric oxygen. *J. Geophys. Res.* **78,** 2625–2629.

Hübner H. (1986) Isotope effects of nitrogen in the soil and biosphere. In *Handbook of Environmental Isotope Geochemistry: The Terrestrial Environment*, Vol. 2 (ed. P. Fritz and J. C. Fontes), pp. 361–425. Elsevier, Amsterdam.

Kendall C. (1998) Tracing nitrogen sources and cycling in catchments. In *Isotope Tracers in Catchment Hydrology* (ed. C. Kendall and J. J. McDonnell), pp. 521–576. Elsevier, Amsterdam.

Kendall C., Burns D. A., Silva S. R., Chang C. C. Y., and McMahon P. B. (1995) Sources of variation in the oxygen and nitrogen isotopic composition of nitrate in soils. *AGU Trans.* **76,** 210.

Killham K. (1986) Heterotrophic nitrification. In *Nitrification* (ed. J. I. Prosser), pp. 117–126. IRL Press, Oxford.

Killham K. (1990) Nitrification in coniferous forest soils. *Plant Soil* **128,** 31–44.

Kinzing A. P. and Socolow R. H. (1994) Human impacts on the nitrogen cycle. *Phys. Today*, Nov. 1994, 24–31.

Koopmans C. J., Lubrecht W. C., and Tietema A. (1995) Nitrogen transformations in two nitrogen saturated forest ecosystems subjected to an experimental decrease in nitrogen deposition. *Plant Soil* **175,** 205–218.

Kornexl B. E., Gehre M., Höfling R., and Werner R. A. (1999) On-line $\delta^{18}O$ measurement of organic and inorganic substances. *Rapid Commun. Mass Sp.* **13,** 1685–1693.

Kreitler C. W. (1979) Nitrogen-isotope ratio studies of soils and groundwater nitrate from alluvial fan aquifers in Texas. *J. Hydrol.* **42,** 147–170.

Kroopnick P. and Craig H. (1972) Atmospheric oxygen: Isotopic composition and solubility fractionation. *Science* **175,** 54–55.

Lane G. A. and Dole M. (1956) Fractionation of oxygen isotopes during respiration. *Science* **123,** 574–576.

Lang E. (1986) Heterotrophe und autotrophe Nitrifikation untersucht an Bodenproben von drei Buchenstandorten. *Göttinger Bodenkdl*. Ber. 89.

Mariotti A., Germon J. C., Hubert P., Kaiser P., Letolle R., Tardieux A., and Tardieux P. (1981) Experimental determination of nitrogen kinetic isotope fractionation: Some principles; illustration for the denitrification and nitrification processes. *Plant Soil* **62,** 413–430.

Mengis M., Schiff S. L., Harris M., English M. C., Aravena R., Elgood R. J., and MacLean A. (1999) Multiple geochemical and isotopic approaches for assessing ground water NO_3^- elimination in a riparian zone. *Ground Water* **37,** 448–457.

Paul E. A. and Clark F. E. (1989) *Soil Microbiology and Biochemistry*. Academic Press, San Diego.

Pedersen H., Dunkin K. A., and Firestone M. K. (1999) The relative importance of autotrophic and heterotrophic nitrification in a conifer forest soil as measured by N-15 tracer and pool dilution techniques. *Biogeochemistry* **44,** 135–150.

Poth M. and Focht D. D. (1985) ^{15}N kinetic analysis of N_2O production by *Nitrosomonas europaea*: An examination of nitrifier denitrification. *Appl. Environ. Microbiol.* **49,** 1134–1141.

Prosser J. I. (1989) Autotrophic nitrification in bacteria. *Adv. Microb. Physiol.* **30,** 125–181.

Remde A. and Conrad R. (1990) Production of nitric oxide in *Nitrosomonas europaea* by reduction of nitrite. *Arch. Microbiol.* **154,** 187–191.

Revesz K., Böhlke J. K., and Yoshinari T. (1997) Determination of $\delta^{18}O$ and $\delta^{15}N$ in nitrate. *Anal. Chem.* **69,** 4375–4380.

Ritchie G. A. and Nicholas D. J. D. (1972) Identification of the source of nitrous oxide produced by oxidative and reductive processes in *Nitrosomonas europaea*. *Biochem. J.* **126,** 1181–1191.

Schlesinger W. H. (1997) *Biogeochemistry: An Analysis of Global Change*. Academic Press, San Diego.

Shearer G. and Kohl D. (1993) Natural abundance of ^{15}N: Fractional contribution of two sources to a common sink and use of isotope discrimination. In *Nitrogen Isotope Techniques* (ed. R. Knowles and T. H. Blackburn), pp. 89–125. Academic Press, San Diego.

Shearer G., Kohl D., and Chien S. H. (1978) The nitrogen-15 abundance in a wide variety of soils. *Soil Sci. Soc. Am. J.* **42,** 899–902.

Silva S. R., Kendall C., Wilkinson D. H., Ziegler A. C., Chang C. C. Y., and Avanzino R. J. (2000). A new method for collection of nitrate from fresh water and the analysis of nitrogen and oxygen isotope ratios. *J. Hydrol.* **228,** 22–36.

Simek M. (2000). Nitrification in soil – terminology and methodology. *Rost. Vyroba* **46,** 385–395.

Smil V. (1999) Nitrogen in crop production: An account of global flows. *Global Biogeochem. Cy.* **13,** 647–662.

Stanford G. and Smith S. J. (1972) Nitrogen mineralization potentials of soils. *Soil Sci. Soc. Am. Proc.* **36,** 465–472.

Turner R. E. and Rabalais N. N. (1991) Changes in Mississippi River water quality this century. *Bioscience* **41,** 140–147.

Vitousek P. M., Aber J. D., Howarth R. W., Likens G. E., Matson P. A., Schindler D. W., Schlesinger W. H., and Tilman D. G. (1997) Human alteration of the global nitrogen cycle: Sources and consequences. *Ecol. Appl.* **7,** 737–750.

Voerkelius S. (1990) Isotopendiskriminierungen bei der Nitrifikation und Denitrifikation: Grundlagen und Anwendungen der Herkunfts-Zuordnung von Nitrat und Distickstoffmonoxid. TU, Munich.

Wassenaar L. I. (1995) Evaluation of the origin and fate of nitrate in the Abbotsford Aquifer using the isotopes of ^{15}N and ^{18}O in NO_3^-. *Appl. Geochem.* **10,** 391–405.

Wood P. M. (1988) Mono-oxygenase and free radical mechanisms for biological ammonia-oxidation. In *The Nitrogen and Sulphur Cycles*, Vol. 42 (ed. J. A. Cole and S. J. Ferguson), pp. 219–243, Cambridge University Press, Cambridge, New York.

Wood P. M. (1990) Autotrophic and heterotrophic mechanisms for ammonia oxidation. *Soil Use Manag.* **6,** 78–79.

Yoshinari T. and Wahlen M. (1985) Oxygen isotope ratios in N_2O from nitrification at a waste water treatment facility. *Nature* **317,** 349–350.