Science of the Total Environment 633 (2018) 1078–1088

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

High nitrogen isotope fractionation of nitrate during denitrification in four forest soils and its implications for denitrification rate estimates

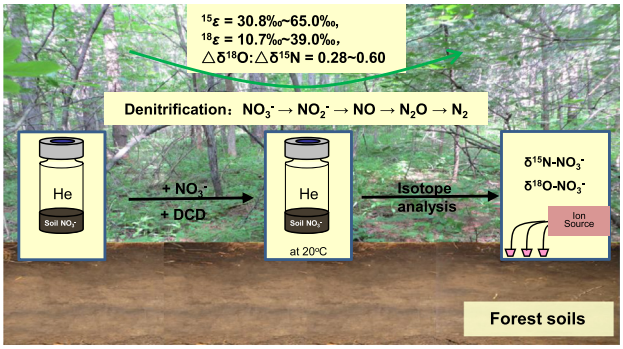
Ang Wang [a,b,f,g], Yunting Fang [a,f,*], Dexiang Chen [b,**], Oliver Phillips [c], Keisuke Koba [d], Weixing Zhu [a,e], Jiaojun Zhu [a,f]

[a] CAS Key Laboratory of Forest Ecology and Management, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110164, China
[b] Research Institute of Tropical Forestry, Chinese Academy of Forestry, Guangzhou 510520, China
[c] School of Geography, University of Leeds, Leeds, UK
[d] Center for Ecological Research, Kyoto University, Shiga 520-2113, Japan
[e] Department of Biological Sciences, Binghamton University, The State University of New York, Binghamton, NY 13902, USA
[f] Qingyuan Forest CERN, Chinese Academy of Sciences, Shenyang 110016, China
[g] College of Resources and Environment, University of Chinese Academy of Sciences, Beijing 100049, China

HIGHLIGHTS

- N and O isotope effects of forest soils during denitrification were determined.
- N isotope effect was unexpectedly higher than the reported range of previous studies.
- The ratio of $\Delta\delta^{18}O$:$\Delta\delta^{15}N$ is lower relative to the reported range in previous studies.
- Gaseous N losses may be overestimated for terrestrial ecosystems in previous studies.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 9 November 2017
Received in revised form 21 March 2018
Accepted 22 March 2018
Available online 28 March 2018

Editor: Ouyang Wei

Keywords:
Denitrification
N-isotope fractionation
O-isotope fractionation
$\Delta\delta^{18}O$:$\Delta\delta^{15}N$
Forest soils
Gaseous N losses

ABSTRACT

Denitrification is a major process contributing to the removal of nitrogen (N) from ecosystems, but its rate is difficult to quantify. The natural abundance of isotopes can be used to identify the occurrence of denitrification and has recently been used to quantify denitrification rates at the ecosystem level. However, the technique requires an understanding of the isotopic enrichment factor associated with denitrification, which few studies have investigated in forest soils. Here, soils collected from two tropical and two temperate forests in China were incubated under anaerobic or aerobic laboratory conditions for two weeks to determine the N and oxygen (O) isotope enrichment factors during denitrification. We found that at room temperature (20 °C), NO_3^- was reduced at a rate of 0.17 to 0.35 μg N g^{-1} h^{-1}, accompanied by the isotope fractionation of N ($^{15}\varepsilon$) and O ($^{18}\varepsilon$) of 31‰ to 65‰ (48.3 ± 2.0‰ on average) and 11‰ to 39‰ (18.9 ± 1.7‰ on average), respectively. The N isotope effects were, unexpectedly, much higher than reported in the literature for heterotrophic denitrification (typically ranging from 5‰ to 30‰) and in other environmental settings (e.g., groundwater, marine sediments and agricultural soils). In addition, the ratios of $\Delta\delta^{18}O$:$\Delta\delta^{15}N$ ranged from 0.28 to 0.60 (0.38 ± 0.02 on average), which were lower than the canonical ratios of 0.5 to 1 for denitrification reported in other terrestrial and freshwater systems. We suggest that the isotope effects of

* Correspondence to: Y. Fang, Institute of Applied Ecology, Chinese Academy of Sciences, 72 Wenhua Road, Shenyang 110016, China.
** Corresponding author.
E-mail addresses: fangyt@iae.ac.cn (Y. Fang), dexiangchen@ritf.ac.cn (D. Chen).

https://doi.org/10.1016/j.scitotenv.2018.03.261

denitrification for soils may vary greatly among regions and soil types and that gaseous N losses may have been overestimated for terrestrial ecosystems in previous studies in which lower fractionation factors were applied.

1. Introduction

Nitrogen (N) is an essential element that limits primary production in many forest ecosystems (Vitousek and Howarth, 1991). Anthropogenic emissions of reactive N from fossil fuel combustion and modern agriculture have greatly increased the amount of N deposition into the environment (Gruber and Galloway, 2008). The increased input of N has caused an N excess in certain terrestrial ecosystems, leading to soil acidification and changes in ecosystem structure and function. In soil, denitrification (the stepwise reduction of NO_3^- to NO_2^-, NO, N_2O and N_2) has long been considered a major process of N removal. However, this process is poorly resolved in N cycling studies (Vitousek and Howarth, 1991) because the rate is difficult to quantify at the ecosystem level due to limitations associated with conventional methods, including the intrusive nature of soil sampling, uncertainties in scaling up from local measurements, and the high background N_2 concentration (Groffman et al., 2006).

The natural abundance of stable isotope ratios of nitrogen ($^{15}N/^{14}N$) and oxygen ($^{18}O/^{16}O$) in nitrate (NO_3^-) have been used to evaluate the sources of and biogeochemical transformations acting on NO_3^- (Granger and Wankel, 2016). Microbial denitrifiers exert a large isotope discrimination against ^{15}N and ^{18}O during denitrification, such that the remaining NO_3^- is simultaneously enriched in ^{18}O and ^{15}N at a ratio from 0.5 to 1 (henceforth referred to as $\Delta\delta^{18}O{:}\Delta\delta^{15}N$) (Granger and Wankel, 2016; Kendall et al., 2007; Wunderlich et al., 2012). Thus, the $^{15}N/^{14}N$ and $^{18}O/^{16}O$ ratios in NO_3^- have been used to detect denitrification. The degree of discrimination expressed by the isotope effect, ε, is defined as $\varepsilon = ({}^{light}k/{}^{heavy}k - 1)$ (reported in per mil, ‰). Recently, the $^{15}N/^{14}N$ ratio has been used to quantify denitrification rates at the ecosystem level (Fang et al., 2015; Houlton and Bai, 2009; Houlton et al., 2006). Based on the ^{15}N enrichment in soil and water, including those of total dissolved N, bulk soil N and NO_3^-, several studies have suggested that denitrification is much more important than previously thought, accounting for 24% to 86% of total N losses (denitrification plus nitrate leaching) from unmanaged terrestrial ecosystems such as forests (Fang et al., 2015; Houlton and Bai, 2009; Houlton et al., 2006).

However, the use of ^{15}N natural abundance to quantify denitrification rates and to constrain N transformation relies heavily on detailed knowledge of the denitrification process and the isotope fractionation involved. The N isotope effect ($^{15}\varepsilon$) varies greatly with environmental and experimental conditions and has been reported to range from 5‰ to 40‰ in pure culture studies of heterotrophic denitrifying bacteria (Barford et al., 1999; Dabundo, 2014; Delwiche and Steyn, 1970; Frey et al., 2014; Granger et al., 2008; Hosono et al., 2015; Karsh et al., 2012; Knöeller et al., 2011; Kritee et al., 2012; Treibergs and Granger, 2016; Wunderlich et al., 2012) and in open ocean systems (Brandes et al., 1998; Cline and Kaplan, 1975; Sigman et al., 2005; Sigman et al., 2003; Voss et al., 2001), from 0‰ to 18‰ in continental sediments (Brandes and Devol, 1997; Brandes and Devol, 2002; Dähnke and Thamdrup, 2015; Kessler et al., 2014), from 5‰ to 30‰ in groundwater (Aravena and Robertson, 1998; Böttcher et al., 1990; Fukada et al., 2003; Lehmann et al., 2003; Mariotti et al., 1988; Mengis et al., 1999; Smith et al., 1991; Vogel et al., 1981; Wenk et al., 2014), and from 2‰ to 50‰ in agricultural soils (Blackmer and Bremner, 1977; Chien et al., 1977; Grabb et al., 2017; Lewicka-Szczebak et al., 2014; Lewicka-Szczebak et al., 2015; Mariotti et al., 1981; Mariotti et al., 1982; Mathieu et al., 2007; Well and Flessa, 2009). Estimations of the denitrification rate at the ecosystem level are sensitive to $^{15}\varepsilon$, and assigning different $^{15}\varepsilon$ values results in different denitrification rates. For example, when the average $^{15}\varepsilon$ of 20‰ from a pure culture of denitrifying bacteria was used, denitrification was estimated to account for 28% of N loss from unmanaged terrestrial ecosystems; however, when a $^{15}\varepsilon$ of 16‰ was observed for several native soil denitrifier communities, the denitrification contribution increased to 36% (Houlton and Bai, 2009).

Despite numerous studies of the N isotope effect performed using pure cultures of heterotrophic denitrifying bacteria (Granger et al., 2008; Treibergs and Granger, 2016, and references therein), groundwater (Lehmann et al., 2003; Wenk et al., 2014, and references therein), sediments (Dähnke and Thamdrup, 2015; Kessler et al., 2014, and references therein), and agricultural soils (Grabb et al., 2017; Lewicka-Szczebak et al., 2014; Lewicka-Szczebak et al., 2015; Mariotti et al., 1982; Mathieu et al., 2007; Well and Flessa, 2009), only four studies have examined forest soils (Houlton et al., 2006; Menyailo and Hungate, 2006; Perez et al., 2006; Snider et al., 2009). Furthermore, there is no report on the dynamics of the coupled N and O isotope trajectory during denitrification for forest soils via direct measurements of the N- and O-isotopes of NO_3^-. In addition, different environments have different slopes of $\Delta\delta^{18}O{:}\Delta\delta^{15}N$; when plotting $\delta^{18}O$ over $\delta^{15}N$ of the residual NO_3^-, a range from 0.47 to 0.89 was found in terrestrial environments and freshwater systems (Böttcher et al., 1990; Fukada et al., 2003; Lehmann et al., 2003; Wenk et al., 2014), a value of 1.25 in marine environments (Sigman et al., 2005), and a range from 0.33 to 1.02 in pure cultures of heterotrophic denitrifying bacteria (Dabundo, 2014; Frey et al., 2014; Granger et al., 2008; Hosono et al., 2015; Karsh et al., 2012; Knöeller et al., 2011; Kritee et al., 2012; Treibergs and Granger, 2016; Wunderlich et al., 2012). Thus, it is critical to determine the coupled N- and O-isotope discrimination in forest soils during denitrification.

To advance our understanding of isotopic fractionation during denitrification in forest soils and reduce the uncertainty in denitrification estimates, in this study, we selected four forest soils—two from temperate forests and two from tropical forests—to investigate the isotope fractionation of N and O of NO_3^- during denitrification by native soil microbial communities as well as the relationship between N and O isotopes ($\Delta\delta^{18}O{:}\Delta\delta^{15}N$).

2. Methods and materials

2.1. Study sites

Our two tropical forest ecosystems, a primary forest (PF, 18°43′47″N, 108°53′23″E, 893 m a.s.l.) and a secondary forest (SF, 18°44′41″N, 108°50′57″E, 935 m a.s.l.), are located in the Jianfengling (JFL) National Natural Reserve (Chinese Ecosystem Research Network, CERN) on Hainan Island, southern China. The tropical forests have a tropical monsoon climate with an annual precipitation of 2449 mm (>80% of which falls during May to October) and an annual average temperature of 19.8 °C (Chen et al., 2010). The primary forest has never been disturbed by human activities and is dominated by *Mallotus hookerianus*, *Gironniera subaequalis*, *Cryptocarya chinensis* and *Cyclobalanopsis patelliformis*. The soil is an acidic (pH = 4.2) lateritic yellow soil, and the soil texture is sandy clay with 57.1% sand, 18.2% silt, and 24.7% clay (Fang et al., 2004; Luo et al., 2005). The secondary forest was developed on a clear-cut site (1960–1970s) dominated by *Castanopsis tonkinensis*, *Schefflera octophylla*, *Psychotria rubra* and *Blastus cochinchinensis*. The soil is an acidic (pH = 4.1) lateritic yellow soil, and the soil texture is loamy clay with 53.8% sand, 12.1% silt, and 34.1% clay (Fang et al., 2004; Luo et al., 2005).

The two temperate forests, a larch forest dominated by *Larix olgensis* (LF, 41°50′58″N, 124°56′18″E, 625 m a.s.l.) and a mixed forest (MF, 41°50′48″N, 124°56′01″E, 640 m a.s.l.), are both located in the Qingyuan (QY) Forest (CERN) in northeastern China. The temperate forests have a continental temperate monsoon climate with an annual precipitation of 811 mm (with >80% falling during June, July and August) and an annual average temperature of 4.7 °C (Zhu et al., 2007). The mixed forest was developed from a clear-off following a large fire (1950s) and is dominated by *Quercus mongolica*, *Juglans mandshurica* and *Phellodendron amurense*. The larch forest is a 44-year-old stand dominated by *Larix olgensis*. The two temperate forests have acidic (pH = 5.2) brown soils and soil texture is clay loam with 25.6% sand, 51.2% silt, and 23.2% clay (Yang et al., 2010).

2.2. Soil sampling and laboratory incubation

In May 2015, we collected 0–10 cm mineral soil from all four forests. In each forest, we established three plots (20 m × 20 m) randomly, and the soils collected from individual plots (six cores taken at six random locations in each plot) were composited into one soil sample (defined as plot-level composite soil), such that we collected three plot-level composite soils for each forest. In May 2016, we resampled soils from the same plots of the tropical primary forest and the temperate mixed forest using the same method. The soils were placed in sterile plastic bags, sealed and transported to the laboratory in the Jianfengling or Qingyuan research stations on ice. In the laboratory, soil was passed through a 2-mm-mesh sieve to remove roots and other visible fragments. For each soil sample, one part was used for incubation and another was used for later analysis of NH_4^+ and NO_3^- concentrations. Subsamples of the soil were also air dried for the analysis of total C and N concentrations.

In 2015, four forest-level composite soils (i.e., three plot-level composite soils in each forest were combined into one forest-level composite soil) were incubated in the laboratory. For each forest soil, we prepared 17 glass vials (50 mL, Chromacol, 125 × 20-CV-P210); two were used for the measurement of initial conditions, and 15 were used for incubation (i.e., five replicates for each sampling day). For the 15 replicate glass vials, approximately 10 g fresh soil was added to each vial and then amended with 4 mL 3.57 mmol L^{-1} $NaNO_3$ (equal to addition of 20 μg N g^{-1} soil) and 0.1 mL 5% dicyanodiamine (DCD, a nitrification inhibitor), which was prepared using N_2-purged sterile deionized water. The final soil moisture in each vial was adjusted to exceed 100% of the water-filled pore space (WFPS). DCD was used to inhibit nitrifying bacteria to minimize the effect of NO_3^- produced from nitrification. The vials were immediately capped tightly with grey butyl septa (Chromacol, 20-B3P, No. 1132012634) and aluminium crimp seals (ANPEL Scientific Instrument (Shanghai) Co. Ltd., 6G390150). Each vial was vacuumed and flushed with ultra-high-purity He for 5 min (100 mL min^{-1}) so that the soil was under strictly anaerobic conditions. The vials were shaken gently, and the resulting soil slurries were incubated at 20 °C for 3, 7, 14 days. The incubation was terminated by injecting 0.5 mL of a 7 M $ZnCl_2$ solution. Five vials at each sampling day were removed to analyse the NO_3^- concentration and the ^{15}N and ^{18}O abundance of residual NO_3^-.

In 2016, two forest-level composite soils (collected in 2016) were further selected to test the effect of initial NO_3^- concentration, DCD presence and O_2 presence in the headspace on the N and O isotopes. The incubations used methods similar to those used in 2015, with the exception that vials received different treatments, as follows: (A) Initial NO_3^- concentration. Soil samples were divided into three parts: one part was amended with 4 mL 3.57 mmol L^{-1} $NaNO_3$ (equivalent to an addition of 20 μg N g^{-1} soil), one part was amended with 4 mL 1.79 mmol L^{-1} $NaNO_3$ (equivalent to an addition of 10 μg N g^{-1} soil), and one part was amended with 4 mL deionized water (control). Then, the vials were incubated under strictly anaerobic conditions at 20 °C as described above. (B) DCD and O_2 presence. Soil samples were divided into four parts: two parts were amended with 0.1 mL 5% DCD and incubated under anaerobic or aerobic conditions, and the other two parts were left without DCD and incubated under anaerobic or aerobic conditions. Then, the vials were incubated at 20 °C as described above. The soil samples collected in 2016 were also incubated at 4 °C under anaerobic conditions, and compared with those incubated at 20 °C to evaluate the effect of incubation temperature. However, NO_3^- was consumed slowly at 4 °C (only 7%–25% of the initial NO_3^-), preventing a valid isotope fractionation calculation (Fig. S1). Although the valid isotope fractionation could not be calculated, the results of $^{15}\varepsilon$ at 4 °C can be found in Table S3. In addition, in 2016, three plot-level composite soils from each forest were incubated using the same method. There were three replicates at each sampling for each treatment. Details of these treatments are provided in Tables 1 and 2.

The soils before and after incubation were extracted with 2 M KCl solution in a soil/solution ratio of 1:4. The KCl was pre-combusted at 450 °C for 48 h, and NO_3^-, NO_2^- and NH_4^+ were not detected in the KCl solution. Ammonium concentrations in the extracts were determined using the indophenol blue method followed by colourimetry. Nitrate concentrations were determined after hydrazine sulfate reduction to nitrite (NO_2^-), followed by diazamine coincidence spectrophotometry. Nitrite concentrations were determined using diazamine coincidence spectrophotometry. All analyses were performed on a Smartchem instrument 200 (Westco Scientific Instruments, Inc., Italy) (Buffen et al., 2014; Talbot et al., 2014). Air-dried soils were used to determine total N and C concentrations using a Vario micro-elemental analyser (Elementar Analysen systeme GmbH, Germany). Soil pH was determined with a glass electrode in a 1:2.5 soil/water suspension. Soil water content was calculated according to the weight change after drying for 24 h at 105 °C.

2.3. Isotope analysis

The air-dried soils were ball milled and analysed for C and N concentrations and $\delta^{15}N$ using an elemental analyser (Elementar Analysen Systeme GmbH, Germany) coupled to an isotope ratio mass spectrometer (Elementar Analysen Systeme GmbH, Germany; IsoPrime100, IsoPrime Limited, UK). Calibrated DL-alanine ($\delta^{15}N = -1.7‰$), glycine ($\delta^{15}N = 10.0‰$), and histidine ($\delta^{15}N = -8.0‰$) were used as internal standards to correct for $\delta^{15}N$ analysis. The analytical precision for $\delta^{15}N$ was 0.2‰. The $\delta^{15}N$ of the sample relative to the standard (atmospheric N_2) was expressed as following:

$$\delta^{15}N = \left(\left({}^{15}N/{}^{14}N \right)_{sample} / \left({}^{15}N/{}^{14}N \right)_{standard} - 1 \right) * 1000$$

Concentrations of residual NO_3^- in soils were measured using the method described above. The N and O isotopic compositions of residual NO_3^- were determined using the modified azide method (Tu et al., 2016). Briefly, Cd powder was used to reduce NO_3^- to NO_2^-, and then NO_2^- was reduced to N_2O by HN_3. The produced N_2O was determined using an automated purge and cryogenic trap system coupled to isotope ratio mass spectrometer (PT-IRMS), which included a continuous-flow IRMS (IsoPrime100, IsoPrime Limited, UK) and a 112-slot autosampler (Gilson GX-271, IsoPrime Limited, UK) with a cryo-focusing unit (Trace Gas Preconcentrator, IsoPrime Limited, UK). In our study, the N_2O peak of the reagent blank was approximately 4% of the standards (Table S1). To eliminate the influence of the reagent blank and any drift during IRMS isotope analysis, four standards (IAEA-NO-3, USGS32, USGS34 and USGS35) were used to correct for samples according to the mixing model (Tu et al., 2016) (Table S1, Fig. S2). According to this modified method, the analytical precision of the $\delta^{15}N$ and $\delta^{18}O$ values was 0.2‰ and 0.5‰, respectively.

In addition, for the 2016 incubation with different initial NO_3^- concentrations, we transferred the headspace gas using a gas-tight syringe to a newly vacuumed vial after incubation was terminated by injecting

Table 1
Estimates of the nitrogen isotope effect ($^{15}\varepsilon$), oxygen isotope effect ($^{18}\varepsilon$) and ratio of O and N isotopic fractionation ($\Delta\delta^{18}O{:}\Delta\delta^{15}N$) during denitrification. Forest-level composite soils collected from 2015 and 2016. Values are means ± 1 SE.

Year	Forest type	Temperature (°C)	O_2	Nitrification inhibitor	Added NO_3^- (μg N g^{-1})	$^{15}\varepsilon$ (‰)	$^{18}\varepsilon$ (‰)	$\Delta\delta^{18}O{:}\Delta\delta^{15}N$	k_1[§]	N
2015	JFL-PF	20	Anaerobic	Yes	20	44.1 ± 1.6	12.7 ± 0.7	0.29 ± 0.07	0.0116	7
	JFL-SF	20	Anaerobic	Yes	20	45.8 ± 2.8	13.0 ± 7.7	0.29 ± 0.02	0.0128	7
	QY-MF	20	Anaerobic	Yes	20	51.4 ± 2.7	16.7 ± 1.0	0.32 ± 0.01	0.0120	7
	QY-LF	20	Anaerobic	Yes	20	40.7 ± 1.8	15.7 ± 1.0	0.38 ± 0.02	0.0110	12
2016	JFL-PF	20	Anaerobic	Yes	20	39.5 ± 8.1	13.6 ± 3.9	0.34 ± 0.07	0.0094	6
	JFL-PF	20	Anaerobic	Yes	10	34.3 ± 3.2	10.7 ± 0.7	0.31 ± 0.03	0.0189	6
	JFL-PF*	20	Anaerobic	Yes	0					
	JFL-PF	20	Anaerobic	No	20	30.8 ± 0.9	12.3 ± 0.5	0.40 ± 0.02	0.0121	6
	JFL-PF	20	Aerobic	Yes	20	43.7 ± 8.7	19.8 ± 4.1	0.43 ± 0.05	0.0016	12
	JFL-PF	20	Aerobic	No	20	52.7 ± 7.7	24.8 ± 4.2	0.46 ± 0.04	0.0014	12
	QY-MF	20	Anaerobic	Yes	20	65.0 ± 2.1	19.5 ± 1.5	0.30 ± 0.02	0.0041	12
	QY-MF	20	Anaerobic	Yes	10	52.7 ± 1.4	15.0 ± 0.9	0.28 ± 0.02	0.0070	12
	QY-MF	20	Anaerobic	Yes	0	45.7 ± 5.8	23.3 ± 1.7	0.47 ± 0.05	0.0050	9
	QY-MF	20	Anaerobic	No	20	57.7 ± 2.2	19.5 ± 1.4	0.34 ± 0.01	0.0045	12
	QY-MF	20	Aerobic	Yes	20	58.3 ± 13.9	39.0 ± 8.2	0.60 ± 0.07	0.0007	12
	QY-MF	20	Aerobic	No	20	64.4 ± 7.5	38.0 ± 3.2	0.56 ± 0.05	0.0008	12

* Nitrate isotopes could not be determined due to low nitrate concentration; therefore, isotope fractionation factors were not calculated.
§ $k_1 = \ln([NO_3^-]/[NO_3^-]_{initial})/t$. t is given in hours.

0.5 mL 7 M $ZnCl_2$ solutions. Then, the $\delta^{15}N$-N_2O in the headspace gas was determined via the same automated PT-IRMS. The $\delta^{15}N$ of the sample was relative to the standard (atmospheric N_2). We used ambient N_2O to correct the $\delta^{15}N$-N_2O for samples ($\delta^{15}N$ of ambient N_2O was determined to be 8.2‰ in our laboratory (the laboratory air was collected and the $\delta^{15}N$ of N_2O was determined with an automated PT-IRMS, as with the incubated samples) while the average value reported was 6.7‰) (Harris et al., 2017; Kim and Craig, 1990).

2.4. Calculation of isotope fractionation

Estimates of N- and O-isotope effects ($^{15}\varepsilon$ and $^{18}\varepsilon$, respectively) were calculated by fitting the $\delta^{15}N$ and $\delta^{18}O$ of NO_3^- to the following linear equations (Mariotti et al., 1981):

$$\delta^{15}N = \delta^{15}N_{initial} - {}^{15}\varepsilon \ \ln\left([NO_3^-]/[NO_3^-]_{initial}\right) \quad (1)$$

$$\delta^{18}O = \delta^{18}O_{initial} - {}^{18}\varepsilon \ \ln\left([NO_3^-]/[NO_3^-]_{initial}\right) \quad (2)$$

We found that in some cases, during the incubation, NO_3^- was almost completely consumed after several days, with its concentration later increasing slightly, and both the ^{15}N and ^{18}O abundance of the remaining NO_3^- substantially decreased. This observation was also reported in previous studies (Granger et al., 2008; Kritee et al., 2012). For example, on the 14th day of the incubation in 2015, NO_3^- concentrations slightly increased in three of the four forest soils (from 0.13 ± 0.02 μg N g^{-1} in the 7th day to 0.65 ± 0.03 μg N g^{-1} in the 14th day, $P < 0.05$), while both ^{15}N and ^{18}O values of NO_3^- decreased by 6.9‰ to 40.6‰ (Fig. S3). Heterotrophic nitrification (not affected by the autotrophic nitrifier inhibitor) may be responsible for the low production of NO_3^-. NO_3^- production may have also occurred in other periods of the incubation. In those cases, the results in the later part of the incubation were excluded from the isotope effect calculation (Figs. 2 to 5).

In addition, previous studies of soils determined the N isotope effect during denitrification using the difference between the substrate ($\delta^{15}N_{substrate}$) and product ($\delta^{15}N_{product}$) (Grabb et al., 2017; Lewicka-Szczebak et al., 2014; Lewicka-Szczebak et al., 2015; Mathieu et al., 2007; Menyailo and Hungate, 2006; Perez et al., 2006; Snider et al., 2009; Well and Flessa, 2009). According to Mariotti et al. (1981), when $\delta^{15}N_{substrate}$ is small with regard to 1000, $^{15}\varepsilon$ can be calculated using the following equation:

$$^{15}\varepsilon = \delta^{15}N_{substrate} - \delta^{15}N_{product} \quad (3)$$

However, when s substantial amount of NO_3^- substrate is consumed, Eq. (3) does not hold and $^{15}\varepsilon$ can be calculated using the following equation:

$$\delta^{15}N_{product} = \delta^{15}N_{substrate} - {}^{15}\varepsilon f \ln f/(1-f) \quad (4)$$

where $f = [NO_3^-]/[NO_3^-]_{initial}$.

To compare our results to those of previous studies, we calculated the N isotope effect of denitrification using the ^{15}N natural abundance of product N_2O. We used the mean $\delta^{15}N$ value of NO_3^- at the beginning of experiment to estimate the $\delta^{15}N$ of the substrate. At the same time, we calculated the N isotope effect using Eq. (4) (in 14 days) and Eq. (3) (in 3 days) using the mean value as $^{15}\varepsilon$.

2.5. Statistical analysis

All analyses were conducted using SPSS software (version 19.0; SPSS Inc., Chicago, IL, U.S.A.). One-way ANOVA was conducted to examine the differences in the investigated soil property variables among forests. Pearson correlation analysis was performed to examine the correlation between N and O isotopes. Statistically significant differences were set at a *P*-value of 0.05 unless otherwise stated.

Table 2
Estimates of the nitrogen isotope effect ($^{15}\varepsilon$), oxygen isotope effect ($^{18}\varepsilon$) and ratio of O and N isotopic fractionation ($\Delta\delta^{18}O{:}\Delta\delta^{15}N$) during denitrification. Plot-level composite soils collected from 2016. Values are means ±1 SE.

	Forest type	Temperature (°C)	O_2	Nitrification inhibitor	Added NO_3^- (μg N g^{-1})	$^{15}\varepsilon$ (‰)	$^{18}\varepsilon$ (‰)	$\Delta\delta^{18}O{:}\Delta\delta^{15}N$	k_1	N
2016	JFL-PF	20	Anaerobic	Yes	20	42.3 ± 2.6	15.3 ± 1.7	0.36 ± 0.05	0.0112	6
	JFL-PF	20	Anaerobic	Yes	20	41.1 ± 6.7	14.2 ± 2.4	0.33 ± 0.07	0.0089	6
	JFL-PF	20	Anaerobic	Yes	20	42.1 ± 3.0	12.1 ± 3.2	0.29 ± 0.07	0.0087	6
	QY-MF	20	Anaerobic	Yes	20	54.0 ± 2.7	21.4 ± 1.7	0.40 ± 0.02	0.0045	12
	QY-MF	20	Anaerobic	Yes	20	54.8 ± 2.4	20.0 ± 1.4	0.37 ± 0.01	0.0046	12
	QY-MF	20	Anaerobic	Yes	20	53.3 ± 4.2	21.2 ± 2.7	0.41 ± 0.02	0.0035	12

3. Results

3.1. Soil properties

All forest soils examined were acidic, with pH values ranging from 4.1 to 5.3 (Table S2), and the pH of the tropical forest soils (JFL-PF and JFL-SF, averaging 4.1 and 4.2, respectively) was significantly lower than that of the temperate forest soils (QY-LF and QY-MF, averaging 5.1 and 5.3, respectively) ($P < 0.05$). Total C and total N concentrations varied from 1.9% to 4.5% and 0.18% to 0.46%, respectively, and were approximately twice as high in the temperate mixed forest soils as in the other three forest soils. Soil C/N ratios were similar among all four forests (Table S2).

3.2. Nitrate N- and O-isotope fractionation under anaerobic conditions

When forest-level composite soils were incubated under anaerobic conditions, NO_3^- concentrations quickly decreased, and NO_3^- was almost completely consumed within 14 days in all forest soils except the temperate mixed forest soil in 2016 (Fig. 1). The NO_3^- reduction rate ranged from 0.17 to 0.35 μg N g^{-1} h^{-1} (on average 0.26 ± 0.02 μg N g^{-1} h^{-1}) in the first 3 days (in temperate forest soils in 2015 and all tropical forest soils) or 14 days (in temperate mixed forest soils in 2016). Nitrite was not detected or was near the detection limit (0.02 mg L^{-1}) for all forest soils during the entire incubation, while NH_4^+ slightly increased over time in all forest soils (Fig. S4).

With NO_3^- consumption, the $\delta^{18}O$ and $\delta^{15}N$ of the residual NO_3^- increased (Figs. S5 and S6). As predicted by the Rayleigh model, there were significant linear relationships between the $\delta^{18}O$ and $\delta^{15}N$ values against the natural logarithm of the fraction of remaining NO_3^-. The slopes of the lines approximate the N- and O-isotope effect ($^{18}\varepsilon$ and $^{15}\varepsilon$). Nitrogen isotope effects ($^{15}\varepsilon$) spanned a broad range, between 30.8‰ and 65.0‰ (on average 42.3 ± 4.7‰), and $^{18}\varepsilon$ ranged from 10.7‰ to 23.3‰ (on average 15.6 ± 1.1‰) in the studied forest soils (Figs. 2 to 4, Table 1). The slopes of the $\Delta\delta^{18}O$:$\Delta\delta^{15}N$ ratio of the four forest soils had a narrow range, between 0.29 and 0.47, with a mean ratio of 0.34 ± 0.02 (Figs. 2 to 4, Table 1). These variations in $^{15}\varepsilon$ were found to correspond to the incubation conditions, e.g., initial NO_3^- concentrations and DCD (Table 1). An increase in the initial NO_3^- concentrations induced an increase in $^{15}\varepsilon$, from 34.3‰ to 39.5‰ in the tropical primary forest (JFL-PF) soils and from 45.7‰ to 65.0‰ in the temperate mixed forest (QY-MF) soils (Table 1). In addition, with the same initial NO_3^- concentrations, nitrogen isotope effects ($^{15}\varepsilon$) exhibited a greater $^{15}\varepsilon$ when soils were incubated with DCD (Table 1).

In addition, when the plot-level composite soil was incubated with the addition of 20 μg N g^{-1} soil, the NO_3^- reduction rate was on average 0.30 ± 0.02 μg N g^{-1} h^{-1} (in the first 3 days) and 0.22 ± 0.03 μg N g^{-1} h^{-1} (in 14 days) for the tropical primary forest and the temperate mixed forest, respectively, similar to the rates at the forest level (Fig. 1). With decreasing NO_3^- concentration, the $\delta^{18}O$ and $\delta^{15}N$ of the residual NO_3^- also increased (Figs. S5 and S6). The average $^{18}\varepsilon$ was 13.9 ± 0.9‰ and 20.9 ± 0.4‰, and the average $^{15}\varepsilon$ was 41.8 ± 0.4‰ and 54.0 ± 0.4‰ for the tropical primary forest and the temperate mixed forest soils, respectively (Fig. 5, Table 2). The $\Delta\delta^{18}O$:$\Delta\delta^{15}N$ ratios were 0.33 ± 0.2 and 0.39 ± 0.1, respectively (Fig. 5, Table 2).

Under anaerobic conditions, in certain cases, NO_3^- was almost completely consumed after several days (7 days or 14 days), and when the residual NO_3^- amount was less than one-tenth of the initial level, the $\delta^{18}O$ and $\delta^{15}N$ of the residual NO_3^- began to fall below the values expected from a constant $^{18}\varepsilon$ and $^{15}\varepsilon$ (Figs. 2 to 5, S4 and S5).

3.3. Nitrate N- and O-isotope fractionation under aerobic conditions

When forest-level composite soils were incubated under aerobic conditions (headspace filled with air), the NO_3^- reduction rate was on average 0.06 ± 0.00 μg N g^{-1} h^{-1} (in 14 days) for the tropical primary forest and the temperate mixed forest, much lower than under anaerobic conditions (Fig. 1). Nitrite was not detected or was near the detection limit in the two forest soils over the entire incubation, while NH_4^+ slightly increased over time in all forest soils (Fig. S4).

With decreasing NO_3^- concentration, the $\delta^{18}O$ and $\delta^{15}N$ of the residual NO_3^- also increased but in a narrow range compared to that under anaerobic conditions (Figs. S5 and S6). When soils were incubated with DCD, $^{15}\varepsilon$ was 43.7‰ and 58.3‰, and $^{18}\varepsilon$ was 19.8‰ and 39.0‰ for the tropical primary forest and the temperate mixed forest soils, respectively (Fig. 4, Table 1). The $\Delta\delta^{18}O$:$\Delta\delta^{15}N$ ratios were 0.43 and 0.60,

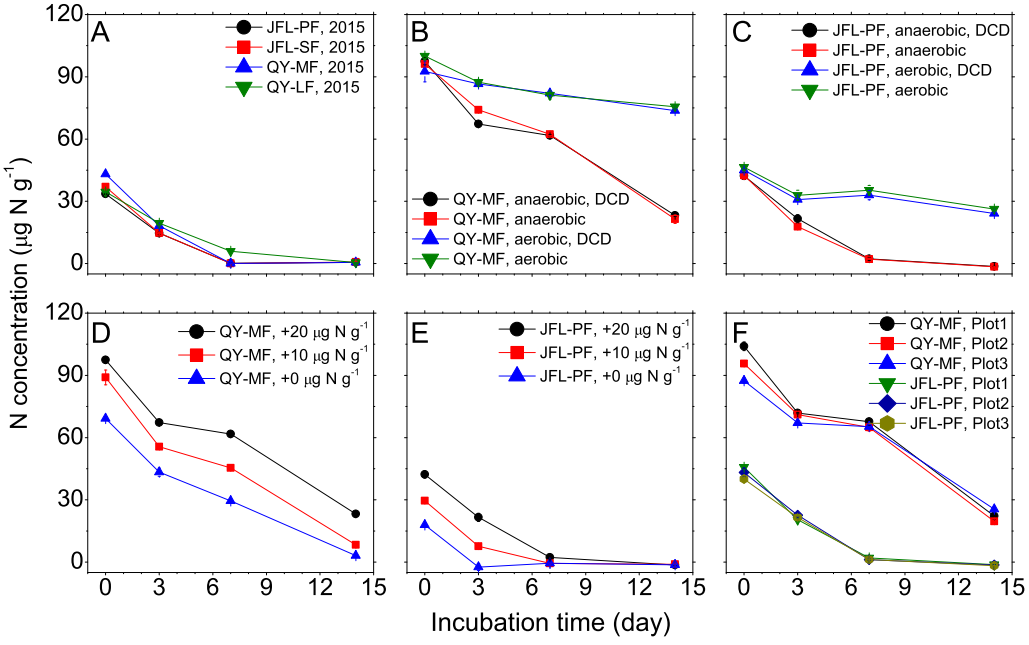


Fig. 1. Changes in the concentration of nitrate (NO_3^-) during the incubation of four forest soils (mean value ± 1 standard deviation, n = 3, except n = 2–5 in soil collected in 2015). (A) Forest-level composite soils sampled in 2015; (B, C, D and E) Forest-level composite soils resampled in 2016 under different amendments; (F) Plot-level composite soils sampled in 2016. Note: JFL-PF represents the tropical primary forest; JFL-SF represents the tropical secondary forest; QY-MF represents the temperate mixed forest; QY-LF represents the temperate larch forest.

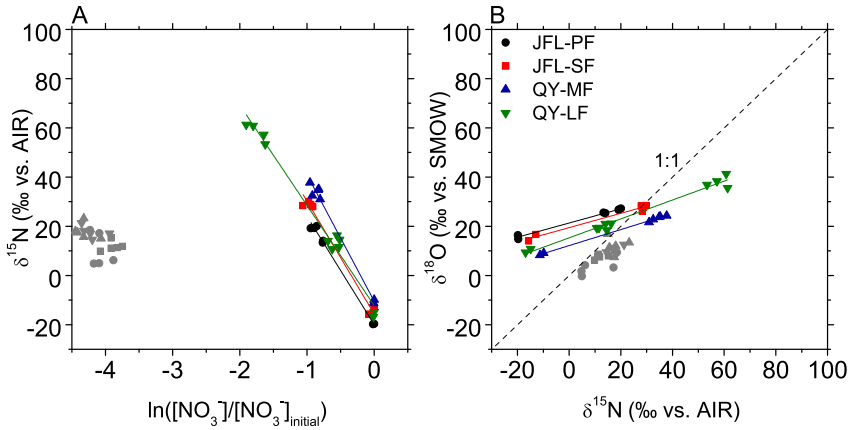


Fig. 2. Nitrogen isotope effect ($^{15}\varepsilon$) and the ratio of O and N isotopic fractionation ($\Delta\delta^{18}O{:}\Delta\delta^{15}N$) during denitrification measured in 2015. (A) NO_3^- $\delta^{15}N$ vs. $\ln([NO_3^-]/[NO_3^-]_{initial})$; (B) NO_3^- $\delta^{18}O$ plotted against the corresponding $\delta^{15}N$. Note: Forest-level composite soils collected in 2015 from two temperate and two tropical forests were incubated at room temperature (approximately 20 °C) with the addition of 20 μg NO_3^--N g^{-1} soil. All *P*-values are <0.05 based on linear regressions.

respectively (Fig. 4, Table 1). When soils were incubated without DCD, $^{15}\varepsilon$ was 52.7‰ and 64.4‰ and $^{18}\varepsilon$ was 24.8‰ and 38.0‰ for the tropical primary forest and the temperate mixed forest soils, respectively (Fig. 4, Table 1), and the $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ ratios were 0.46 and 0.56, respectively (Fig. 4, Table 1).

3.4. N- and O-isotope effect of denitrification determined by N_2O

The N_2O produced by denitrification was ^{15}N depleted at the beginning of incubation in all forest soils and all treatments, ranging from −21.6‰ to −52.6‰ (Table 3). However, the $\delta^{15}N$ of N_2O increased to −17.9‰ to −6.6‰ on the 14th day (Table 3). According to Eq. (3), $^{15}\varepsilon$ for the first 3 days ranged from 21.0‰ to 50.4‰ (on average 38.5 ± 8.9‰) and from 55.5‰ to 66.6‰ (on average 60.7 ± 3.2‰) for the tropical primary forest soils and temperate mixed forest soils, respectively. In addition, $^{15}\varepsilon$ for the entire 14-day incubation was calculated using Eq. (4), ranging from 38.4‰ to 77.4‰ (on average 60.0 ± 11.4‰) and 65.1‰ to 74.0‰ (on average 70.0 ± 2.6‰) for the primary forest soils and mixed forest soils, respectively.

4. Discussion

4.1. N isotope fractionation during denitrification

Our study shows that the apparent N isotope effect ($^{15}\varepsilon$) during denitrification under strictly anaerobic conditions and at room temperature for soils from the four study forests ranged from 30.8‰ to 65.0‰ (on average 48.3 ± 2.0‰, Tables 1 and 2). Our results are, unexpectedly, largely outside of the reported range of previous studies (Fig. 6); for example, values of 6‰ to 33‰ were reported for temperate agricultural soil (Blackmer and Bremner, 1977; Chien et al., 1977; Mariotti et al., 1982); 13‰ for the field incubation of Hawaiian tropical forest soils

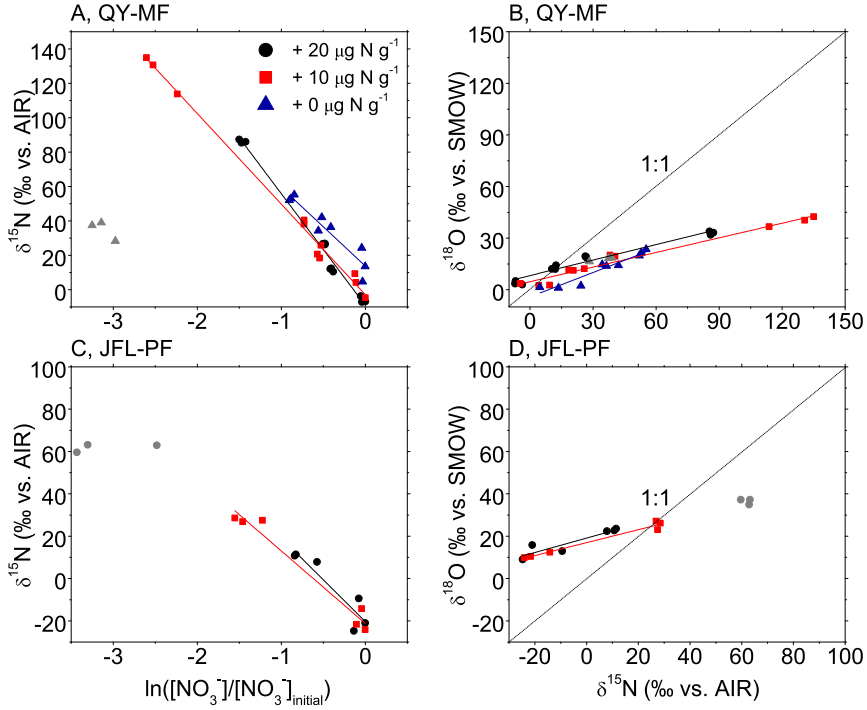


Fig. 3. Effects of initial nitrate concentration on the nitrogen isotope effect ($^{15}\varepsilon$) and the ratio of O and N isotopic fractionation ($\Delta\delta^{18}O{:}\Delta\delta^{15}N$) during denitrification. (A) NO_3^- $\delta^{15}N$ vs. $\ln([NO_3^-]/[NO_3^-]_{initial})$; (B) NO_3^- $\delta^{18}O$ plotted against corresponding $\delta^{15}N$. Note: Forest-level composite soils collected in 2016 from temperate forests and tropical forests were incubated at room temperature with the addition of 20, 10 or 0 μg N g^{-1}. All *P*-values are <0.05 based on linear regressions.

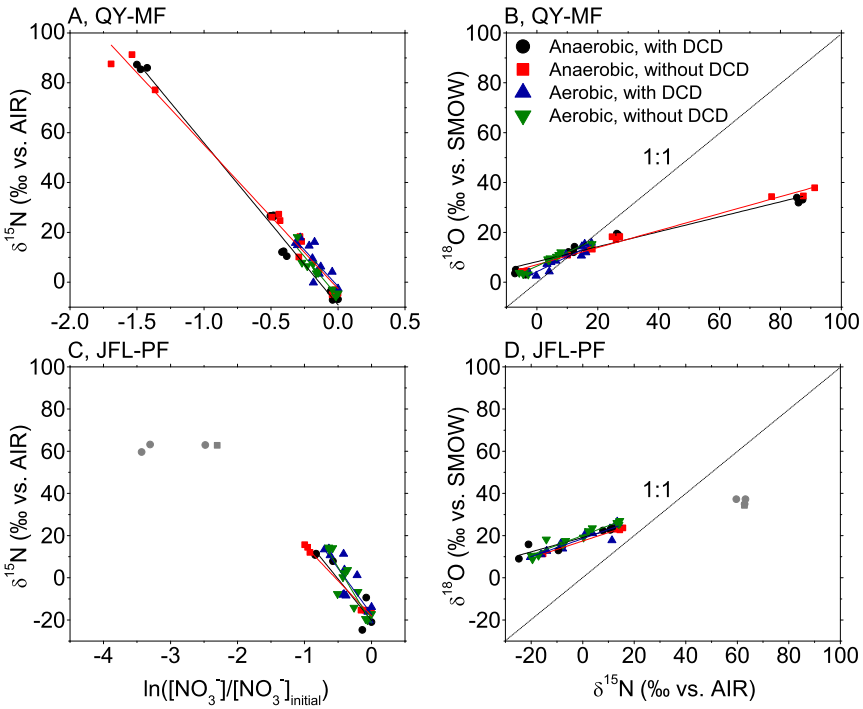


Fig. 4. Effects of nitrification inhibitor and oxygen on the nitrogen isotope effect ($^{15}\varepsilon$) and the ratio of O and N isotopic fractionation ($\Delta\delta^{18}O{:}\Delta\delta^{15}N$) during denitrification. (A) NO_3^- $\delta^{15}N$ vs. $\ln([NO_3^-]/[NO_3^-]_{initial})$. (B) NO_3^- $\delta^{18}O$ plotted against the corresponding $\delta^{15}N$. Note: Forest-level composite soils collected in 2016 from the temperate forest and the tropical forest were incubated under anaerobic or aerobic conditions with or without nitrification inhibitor DCD. All *P*-values are <0.05 based on linear regressions.

(Houlton et al., 2006); 10‰ to 45‰ for three tropical forests in the Brazilian Amazon (Perez et al., 2006); 20‰ to 29‰ for two temperate forests in central Ontario, Canada (Snider et al., 2009); and 24‰ to 29‰ for two boreal forests in Krasnoyarsk, Russia (Menyailo and Hungate, 2006). Our values are also higher than the $^{15}\varepsilon$ reported for many other environments, e.g., 5‰ to 30‰ in pure culture studies of heterotrophic denitrifying bacteria (Granger et al., 2008; Treibergs and Granger, 2016, and references therein) and 18‰ for permeable sediments (Kessler et al., 2014). We also determined the N_2O produced during the 2016 incubation and found that the $^{15}\varepsilon$ of NO_3^- to N_2O calculated by $\delta^{15}N\text{-}NO_3^-$ and $\delta^{15}N\text{-}N_2O$ was, on average, 49‰ and 65‰, respectively (Table 3). These results were also at the upper limit of other soil

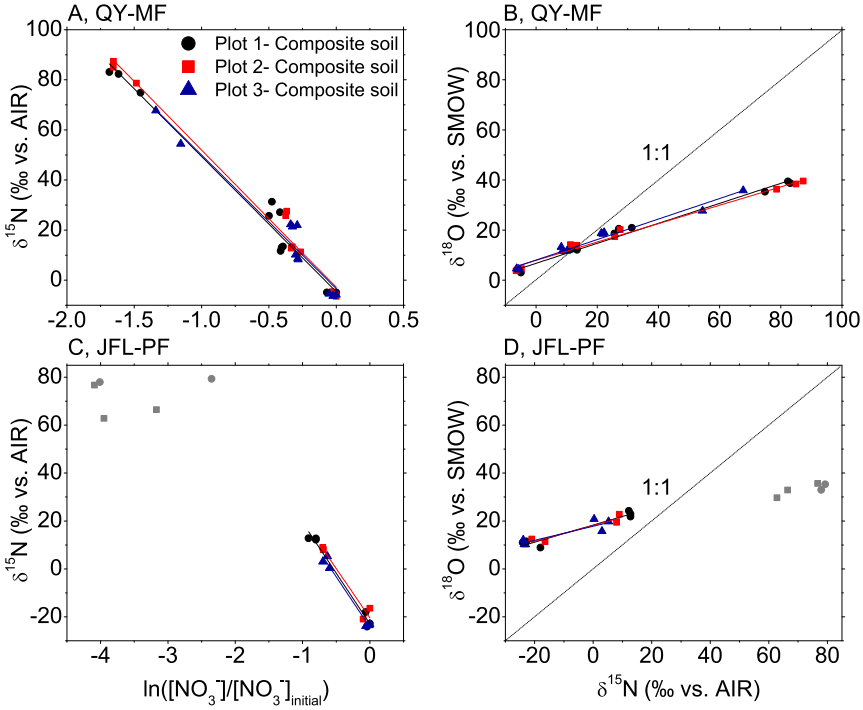


Fig. 5. Nitrogen isotope effect ($^{15}\varepsilon$) and the ratio of O and N isotopic fractionation ($\Delta\delta^{18}O{:}\Delta\delta^{15}N$) during denitrification. (A) NO_3^- $\delta^{15}N$ vs. $\ln([NO_3^-]/[NO_3^-]_{initial})$.; (B) NO_3^- $\delta^{18}O$ plotted against the corresponding $\delta^{15}N$. Note: Plot-level composite soils collected in 2016 from the temperate forest and the tropical forest were incubated at room temperature (approximately 20 °C) with the addition of 20 μg NO_3^--N g^{-1} soil. All *P*-values are <0.05 based on the linear regressions.

Table 3
Calculation of N isotope effects during denitrification based on Eqs. (3) and (4) using $\delta^{15}N$ of N_2O and remaining NO_3^-. Forest-level composite soils were incubated in 2016.

Forest type	Temperature (°C)	Nitrification inhibitor	Added NO_3^- (μg N g^{-1})	Incubation time (day)	$\delta^{15}N$-N_2O	$^{15}\varepsilon$ (‰) according to Eq. (4)[a]	$^{15}\varepsilon$ (‰) according to Eq. (3)[b]
JFL-PF	20	Yes	20	3	−48.2 ± 0.0		44.0
JFL-PF	20	Yes	20	7	−37.3 ± 0.9		
JFL-PF	20	Yes	20	14	−17.9 ± 0.4	38.4 ± 12.4	
JFL-PF	20	Yes	10	3	−47.0 ± 0.0		50.4
JFL-PF	20	Yes	10	7	−24.0 ± 5.1		
JFL-PF	20	Yes	10	14	−13.0 ± 0.3	64.2 ± 19.9	
JFL-PF	20	Yes	0	3	−21.6 ± 0.5		21.0
JFL-PF	20	Yes	0	7	−16.5 ± 0.1		
JFL-PF	20	Yes	0	14	−2.2 ± 6.2	77.4 ± 93.8	
QY-MF	20	Yes	20	3	−52.6 ± 0.4		55.5
QY-MF	20	Yes	20	7	−42.5 ± 0.1		
QY-MF	20	Yes	20	14	−22.5 ± 0.1	70.4 ± 7.4	
QY-MF	20	Yes	10	3	−48.0 ± 0.3		60.0
QY-MF	20	Yes	10	7	−31.0 ± 5.1		
QY-MF	20	Yes	10	14	−10.0 ± 1.4	65.1 ± 13.6	
QY-MF	20	Yes	0	3	−40.8 ± 0.1		66.6
QY-MF	20	Yes	0	7	−27.1 ± 0.3		
QY-MF	20	Yes	0	14	6.6 ± 0.1	74.0 ± 2.5	

[a] $^{15}\varepsilon$ was calculated with Eq. (4) for 14 days.
[b] $^{15}\varepsilon$ was calculated with Eq. (3) for the first 3 days.

studies determined via the same approach for agricultural soils (2‰ to 55‰) (Grabb et al., 2017; Lewicka-Szczebak et al., 2014; Lewicka-Szczebak et al., 2015; Mathieu et al., 2007; Well and Flessa, 2009) and forest soils (10‰ to 45‰) (Menyailo and Hungate, 2006; Perez et al., 2006; Snider et al., 2009).

Although N isotope enrichment factors ($^{15}\varepsilon$) observed in the present study were higher than previously observed, the range of values we observed (31‰ to 65‰, Tables 1 and 2) was nevertheless within that predicted by theory. According to the efflux model proposed by Kohl and Shearer (1978), the $^{15}\varepsilon_{organism}$ (at the organism level), as measured in our study, integrates the isotope effect that occurs during the uptake step from the medium to the cell through the cell membrane ($^{15}\varepsilon_{uptake}$) and the isotope effect that occurs during NO_3^- reduction within the cell ($^{15}\varepsilon_{intrinic}$). The uptake step will present a low isotopic fractionation. For a case of simple diffusion, this fractionation depends on the square root of the mass ratio of the two isotopic species; in this case, $^{15}NO_3^-$ and $^{14}NO_3^-$ for N isotope effect (resulting in a $^{15}\varepsilon_{uptake}$ of 8‰). The NO_3^- reduction step is the rate-limiting and irreversible step that involves enzymatic breakage of the N—O bond, which results in a large isotope fractionation of 65.9‰ at 25 °C (Urey, 1947). Thus, the maximum $^{15}\varepsilon_{organism}$ will be approximately 74‰, higher than the highest record in our study of forest soils.

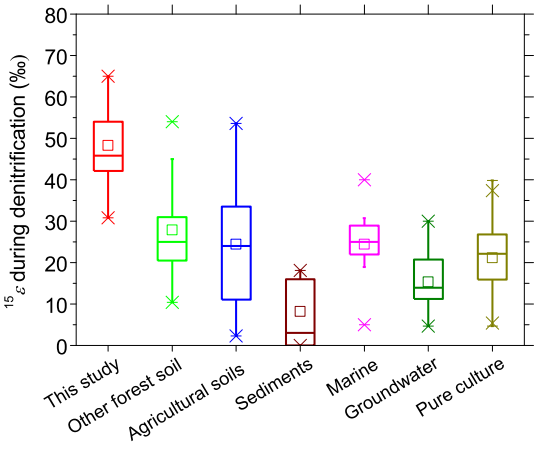


Fig. 6. Variations in $^{15}\varepsilon$ of denitrification under different environmental and experimental conditions.

The initial NO_3^- concentration has been shown to affect the isotope effect of denitrification (Mariotti et al., 1982). When the initial NO_3^- concentration is low, NO_3^- reduction is relatively more complete and NO_3^- uptake is the rate-limiting step, which may cause a negligible isotope fractionation (Granger et al., 2004; Granger et al., 2008). In this case, $^{15}\varepsilon$ was low due to the low efflux/uptake ratio (Wenk et al., 2014). However, with increased initial NO_3^- concentration, NO_3^- reductase was no longer sufficient to sustain maximal reduction rates (i.e., the enzymatic step became the rate-limiting step), and the intrinsic isotope fractionation could be nearly fully expressed in the environment (i.e., higher $^{15}\varepsilon$) (Mariotti et al., 1982; Wenk et al., 2014). Our results were consistent with this interpretation. We found that initial NO_3^- concentration was positively correlated with $^{15}\varepsilon$, i.e., a higher initial NO_3^- concentration was associated with a higher fractionation (Table 1). The treatments with 20 μg NO_3^--N g^{-1} soil had a higher $^{15}\varepsilon$ (39.5‰ and 65.0‰ for the tropical primary forest and the temperate mixed forest soils, respectively) than treatments with 10 μg NO_3^--N g^{-1} soil ($^{15}\varepsilon$ was 34.3‰ and 52.7‰, respectively) and those without N addition ($^{15}\varepsilon$ was 45.7‰ for temperate mixed forest).

Previous studies also showed that $^{15}\varepsilon$ was affected by temperature and the amount of available organic carbon (Maggi and Riley, 2015; Mariotti et al., 1982; Wunderlich et al., 2012). These effects primarily arise because denitrification rates are regulated by temperature and the quantity of electron donors. Mariotti et al. (1982) reported that $^{15}\varepsilon$ was highly correlated with denitrification rate for the studied agricultural soil, with $^{15}\varepsilon$ exponentially decreasing with an increasing denitrification rate (expressed as rate constant k_1) (Mariotti et al., 1988). Similar to this finding, across all forest soils in the present study, $^{15}\varepsilon$ was also found to exponentially decrease with increasing denitrification rate (Fig. 7). However, our results were still greater than the values observed in agricultural soil incubation with the same denitrification rate (Blackmer and Bremner, 1977; Chien et al., 1977; Mariotti et al., 1982).

In addition, the denitrifying bacterial communities and/or availability of nutrients and dissolved organic matter impacted by $^{15}\varepsilon$ may differ across soil types. Different forest soils may have distinct denitrifying bacterial communities, or if the bacterial communities are similar, they may have a different isotopic effect due to differential enzymatic isotope expression. Studies have shown that denitrifying communities were related to soil C/N ratios (Rich et al., 2003) and vegetation types (Menyailo, 2007). For example, the $^{15}\varepsilon$ in the tropical primary forest was always lower than that in the temperate mixed forest even when they received the same nutrient and temperature treatments

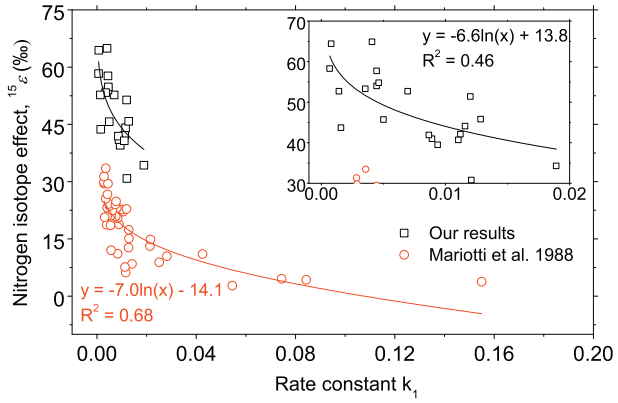


Fig. 7. Relationship between N isotopic effect ($^{15}\varepsilon$) and isotopic rate constant k_1 (first order) in our study in comparison with previous studies.

(Table 1). Additionally, we found that $^{15}\varepsilon$ (51.4‰) for the temperate mixed forest soils in 2015 was lower than that in 2016 (65.0‰), which had a different initial NO_3^- concentration and denitrification rate (Table 1, Fig. 1). This finding may provide further evidence that the N isotope effect of denitrification was correlated with the initial NO_3^- concentration and denitrification rate (Mariotti et al., 1982).

It remains unclear why $^{15}\varepsilon$ was higher for our study forests than for the other forest soils reported in previous studies. We propose two potential mechanisms. First, the method using ^{15}N depletion of N_2O relative to NO_3^- may underestimate $^{15}\varepsilon$ because N_2O is likely to be further reduced to N_2, such that the remaining N_2O becomes more ^{15}N enriched than it should be, although this was not the case in the forest soils in the present study, where 25% to 60% of produced N_2O was shown to be further reduced to N_2 under anaerobic conditions (Xi, 2016). This method has been used in three of the four previous studies for forest soils to determine $^{15}\varepsilon$ during denitrification (Menyailo and Hungate, 2006; Perez et al., 2006; Snider et al., 2009). Second, relatively complete NO_3^- reduction may explain the low $^{15}\varepsilon$ observed in the first 10 cm mineral soil in Hawaiian tropical forests, which results in an underexpression of N isotope effects (Houlton et al., 2006). Partial NO_3^- consumption ("open-system kinetics") leaves behind ^{15}N-enriched NO_3^-, which can diffuse out of the zone of ongoing denitrification, while complete NO_3^- consumption ("closed-system kinetics") would cause underexpression of the isotope effect because little or no ^{15}N-rich NO_3^- would escape. In fact, denitrification in the soil below a depth of 10 cm should exhibit a high N isotope effect (60‰, comparable to our results) across the Hawaiian tropical forests; therefore, the relationship between $^{15}N/^{14}N$ of NO_3^- and $\ln([NO_3^-])$ in the profile can be appropriately fitted (Houlton, 2005).

4.2. Oxygen isotope fractionation and $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ ratio in NO_3^- during denitrification

Unlike the large N-isotope effect, the O-isotope effect ($^{18}\varepsilon$) in our forest soils was small. The mean value of $^{18}\varepsilon$ was 16.2 ± 0.9‰, comparable to other studies, e.g., 5‰ to 24‰ in the pure culture studies of heterotrophic denitrifying bacteria (Dabundo, 2014; Frey et al., 2014; Granger et al., 2008; Hosono et al., 2015; Karsh et al., 2012; Knöeller et al., 2011; Kritee et al., 2012; Treibergs and Granger, 2016; Wunderlich et al., 2012), 14.2‰ in permeable sediments (Kessler et al., 2014), and 7‰ to 18‰ in groundwater (Böttcher et al., 1990; Fukada et al., 2003; Mengis et al., 1999; Wenk et al., 2014). As in the $\delta^{15}N$ of the residual NO_3^-, $\delta^{18}O$ also began to fall below the values expected from a constant $^{18}\varepsilon$ when the residual NO_3^- amount was less than one-tenth of the initial value (Figs. 2 to 5).

Due to the relatively higher $^{15}\varepsilon$, the ratio of $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ was lower, at 0.34 ± 0.01 across all forest soils. This ratio is significantly lower than the ratio of 1 for denitrification bacteria (which is expected because of the stronger ^{15}N effect but similar ^{18}O effect). The ratios in the present study are also lower relative to the reported range for terrestrial environments (0.47 to 0.89) (Böttcher et al., 1990; Fukada et al., 2003; Lehmann et al., 2003; Wenk et al., 2014), marine environments (1.25) (Sigman et al., 2005) and the pure culture of heterotrophic denitrifying bacteria (0.33 to 1.02) (Dabundo, 2014; Frey et al., 2014; Granger et al., 2008; Hosono et al., 2015; Karsh et al., 2012; Knöeller et al., 2011; Kritee et al., 2012; Treibergs and Granger, 2016; Wunderlich et al., 2012). In addition, our result was lower than the ratio of $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ in soil water and stream water from tropical forest studies (1.11–1.54 and 0.66, respectively) (Fang et al., 2015; Houlton et al., 2006), but similar to the value for a temperate forest (0.30) (Fang et al., 2015).

One possible explanation for the lower ratio of $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ may be related to the different fractionation of N and O isotopes via internal enzymatic reduction. During denitrification, there are two types of dissimilatory NO_3^- reductases: bacterial membrane-bound NO_3^- reductase (Nar) and periplasmic NO_3^- reductase (Nap). Evidence suggested that the differences in the catalytic steps between Nar and Nap would be responsible for the lower ratio of $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ under anaerobic conditions (Frey et al., 2014; Wenk et al., 2014). Nap can split not only light O but also heavy O, leading to a slower enrichment of ^{18}O than of ^{15}N in NO_3^- (Frey et al., 2014); thus, Nap enzymes may be responsible for the relatively low $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ ratio. Previous studies of a pure culture of heterotrophic denitrifying bacteria and in Lake Lugano were consistent with this finding (Frey et al., 2014; Granger et al., 2008; Treibergs and Granger, 2016; Wenk et al., 2014). Moreover, under NO_3^--limited conditions, Nap can be essential for NO_3^- reduction due to its higher affinity for NO_3^-, and the relative abundance of NapA genes has been shown to increase with decreasing NO_3^- concentrations (Dong et al., 2009).

Another potential explanation for the lower $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ ratio is the O isotope exchange between NO_2^- and water and the production of NO_3^- via NO_2^- oxidation (NXR) through anaerobic ammonium oxidation (anammox) (Granger and Wankel, 2016). Granger and Wankel (2016) recently proposed a numerical model of NO_3^- isotope dynamics and suggested that when the NXR/NAR ratio was low (i.e. NO_2^- was mostly reduced to NO rather than oxidized to NO_3^-), the $\delta^{15}N$ of NO_2^- was relatively more ^{15}N enriched and the NO_3^- produced by NO_2^- oxidation was further ^{15}N enriched due to the inverse isotope effect (Casciotti, 2009). In contrast, the $\delta^{18}O$ of NO_3^- produced by NO_2^- oxidation would decrease due to the O-isotope exchange between NO_2^- and water and the incorporation of O from water into NO_3^- (Fang et al., 2012; Kool et al., 2009; Wunderlich et al., 2013). The anammox process will increase $\delta^{15}N$ of residual NO_3^- and lower the $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ ratio. Wunderlich et al. (2013) also found that a redox NO_2^- and NO_3^- cycle driven by nitrite oxidoreductase (Nxr) may occur under moderate pH and anaerobic conditions, resulting in modification of the $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ ratio during denitrification. Moreover, O-isotope exchange is regulated by pH, with higher rates of isotope exchange at lower pH values (Kaneko and Poulson, 2013). In our study, soil pH, which ranged from 4.1 to 5.3, was positively correlated with $^{18}\varepsilon$ ($R^2 = 0.83$, $P < 0.05$, Fig. S7), which suggests that pH may affect the extent of O-isotope exchange and the $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ ratio.

4.3. Implications for estimating denitrification rates

Our study revealed an unexpectedly high N isotope effect during denitrification, with an average value of 48‰ across all experiments. In previous ecosystem studies that used a $^{15}\varepsilon$ of 16‰ to estimate the effects of denitrification on isotopes, the denitrification was estimated to account for 35% of total N losses from global unmanaged terrestrial ecosystems and for 48% to 86% for the six studied forests in southern China and central Japan (Fang et al., 2015; Houlton and Bai, 2009). However, if we instead apply our averaged value of 48‰ during denitrification, we find that the proportion of denitrification in total N losses would decrease to 12% for the Houlton and Bai (2009) study and to 26% to 68% for the Fang et al. (2015) study. Our new estimates indicate that the

contribution of denitrification to N losses might have been considerably overestimated for soils bearing a high N isotope fractionation in previous studies. However, the extent to which these findings are representative of ecosystems at a larger scale is uncertain due to soil spatial heterogeneity. Therefore, it will be important to confirm whether this observed high N isotope fractionation is indeed typical for forest ecosystems worldwide.

5. Conclusion

In this study, soils from two tropical and two temperate forests in China were incubated under both anaerobic and aerobic conditions to determine the isotope fractionation of N and O of NO_3^- during denitrification as well as the ratio of $\Delta\delta^{18}O{:}\Delta\delta^{15}N$. We found that the N isotope effects were, unexpectedly, much higher than the reported range of heterotrophic denitrification and other environmental settings (e.g., groundwater, marine sediments and agricultural soils), ranging from 31‰ and 65‰ in the studied forest soils. These variations in $^{15}\varepsilon$ were found to correspond to the incubation conditions, e.g., initial NO_3^- concentrations and DCD. However, the O-isotope effect ($^{18}\varepsilon$) of our forest soils was comparable to that in other studies, ranging from 11‰ to 39‰. In addition, the ratios of $\Delta\delta^{18}O{:}\Delta\delta^{15}N$ ranged from 0.28 to 0.60, which were lower than the canonical ratios of 0.5 to 1 for denitrification bacteria and other terrestrial environments. We suggest that the isotope effect of denitrification for soils may vary greatly with region and soil type. Furthermore, our new estimates for denitrification rates indicate that the contribution of denitrification to N loss might have been considerably overestimated for soils in previous studies in which lower fractionation factors were used.

Acknowledgements

This work was financially supported by the National Key Research and Development Program of China (grant number 2016YFA0600802); the Strategic Priority Research Program of Chinese Academy of Sciences (grant number XDB15020200); the National Natural Science Foundation of China (grant numbers 31370464, 31422009 and 41773094); the Key Research Program of Frontier Sciences of Chinese Academy of Sciences (grant number QYZDB-SSW-DQC002); the Hundred Talents Program of Chinese Academy of Sciences (grant number Y1SRC111J6) and the Key Laboratory of Tropical Forest Ecology of Chinese Academy of Sciences (grant number 09KF001B04). The work was also jointly supported by the Jianfengling National Key Field Station. We are grateful to Dr. Sarah A. Batterman for suggestions for the organization of the manuscript, the presentation of results and the novelty of the study as well as numerous edits of the language. Constructive comments by reviewers and the editor have greatly improved the quality of this paper. The authors declare no competing financial interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.03.261.

References

Aravena, R., 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. https://doi.org/10.1111/j.1745-6584.1998.tb02104.x.

Barford, C.C., Montoya, J.P., Altabet, M.A., Mitchell, R., 1999. Steady-state nitrogen isotope effects of N_2 and N_2O production in Paracoccus denitrificans. Appl. Environ. Microbiol. 65, 989–994.

Blackmer, A.M., Bremner, J.M., 1977. Nitrogen isotope discrimination in denitrification of nitrate in soils. Soil Biol. Biochem. 9:73–77. https://doi.org/10.1016/0038-0717(77)90040-2.

Böttcher, J., Strebel, O., Voerkelius, S., Schmidt, H.L., 1990. Using isotope fractionation of nitrate nitrogen and nitrate oxygen for evaluation of microbial denitrification in a sandy aquifer. J. Hydrol. 114:413–424. https://doi.org/10.1016/0022-1694(90)90068-9.

Brandes, J.A., Devol, A.H., 1997. Isotopic fractionation of oxygen and nitrogen in coastal marine sediments. Geochim. Cosmochim. Acta 61:1793–1801. https://doi.org/10.1016/s0016-7037(97)00041-0.

Brandes, J.A., Devol, A.H., 2002. A global marine-fixed nitrogen isotopic budget: implications for Holocene nitrogen cycling. Glob. Biogeochem. Cycles 16. https://doi.org/10.1029/2001gb001856.

Brandes, J.A., Devol, A.H., Yoshinari, T., Jayakumar, D.A., Naqvi, S.W.A., 1998. Isotopic composition of nitrate in the central Arabian Sea and eastern tropical North Pacific: a tracer for mixing and nitrogen cycles. Limnol. Oceanogr. 43:1680–1689. https://doi.org/10.4319/lo.1998.43.7.1680.

Buffen, A.M., Hastings, M.G., Thompson, L.G., Mosley-Thompson, E., 2014. Investigating the preservation of nitrate isotopic composition in a tropical ice core from the Quelccaya Ice Cap, Peru. J. Geophys. Res. Atmos. 119:2674–2697. https://doi.org/10.1002/2013jd020715.

Casciotti, K.L., 2009. Inverse kinetic isotope fractionation during bacterial nitrite oxidation. Geochim. Cosmochim. Acta 73:2061–2076. https://doi.org/10.1016/j.gca.2008.12.022.

Chen, D., Li, Y., Liu, H., Xu, H., Xiao, W., Luo, T., Zhou, Z., Lin, M.X., 2010. Biomass and carbon dynamics of a tropical mountain rain forest in China. Sci. China Life Sci. 53:798–810. https://doi.org/10.1007/s11427-010-4024-2.

Chien, S.H., Shearer, G., Kohl, D.H., 1977. The nitrogen isotope effect associated with nitrate and nitrite loss from waterlogged soils. Soil Sci. Soc. Am. J. 41:63–69. https://doi.org/10.2136/sssaj1977.03615995004100010021x.

Cline, J.D., Kaplan, I.R., 1975. Isotopic fractionation of dissolved nitrate during denitrification in the eastern tropical north pacific ocean. Mar. Chem. 3:271–299. https://doi.org/10.1016/0304-4203(75)90009-2.

Dabundo, R., 2014. Nitrogen Isotopes in the Measurement of N_2-fixation and the Estimation of Denitrification in the Global Ocean. Master's Theses. 703.

Dähnke, K., Thamdrup, B., 2015. Isotope fractionation and isotope decoupling during anammox and denitrification in marine sediments. Limnol. Oceanogr. 61:610–624. https://doi.org/10.1002/lno.10237.

Delwiche, C.C., Steyn, P.L., 1970. Nitrogen isotope fractionation in soils and microbial reactions. Environ. Sci. Technol. 4:929–935. https://doi.org/10.1021/es60046a004.

Dong, L.F., Smith, C.J., Papaspyrou, S., Stott, A., Osborn, A.M., Nedwell, D.B., 2009. Changes in benthic denitrification, nitrate ammonification, and anammox process rates and nitrate and nitrite reductase gene abundances along an estuarine nutrient gradient (the Colne estuary, United Kingdom). Appl. Environ. Microbiol. 75:3171–3179. https://doi.org/10.1128/aem.02511-08.

Fang, Y.T., Koba, K., Makabe, A., Takahashi, C., Zhu, W.X., Hayashi, T., Hokari, A., Urakawa, R., Bai, E., Houlton, B.Z., Xi, D., Zhang, S.S., Matsushita, K., Tu, Y., Liu, D.W., Zhu, F.F., Wang, Z., Zhou, G., Chen, D., Makita, T., Toda, H., Liu, X., Chen, Q., Zhang, D., Li, Y., Yoh, M., 2015. Microbial denitrification dominates nitrate losses from forest ecosystems. Proc. Natl. Acad. Sci. U. S. A. 112:1470–1474. https://doi.org/10.1073/pnas.1416776112.

Fang, Y.T., Koba, K., Makabe, A., Zhu, F.F., Fan, S.Y., Liu, X.Y., Yoh, M., 2012. Low delta O-18 values of nitrate produced from nitrification in temperate forest soils. Environ. Sci. Technol. 46:8723–8730. https://doi.org/10.1021/es300510r.

Fang, J., Li, Y., Zhu, B., Liu, G., Zhou, G., 2004. Community structures and species richness in the montane rain forest of Jianfengling, Hainan Island, China. Biodivers. Sci. 12, 29–43.

Frey, C., Hietanen, S., Jurgens, K., Labrenz, M., Voss, M., 2014. N and O isotope fractionation in nitrate during chemolithoautotrophic denitrification by Sulfurimonas gotlandica. Environ. Sci. Technol. 48:13229–13237. https://doi.org/10.1021/es503456g.

Fukada, T., Hiscock, K.M., Dennis, P.F., Grischek, T., 2003. A dual isotope approach to identify denitrification in groundwater at a river-bank infiltration site. Water Res. 37:3070–3078. https://doi.org/10.1016/s0043-1354(03)00176-3.

Grabb, K.C., Buchwald, C., Hansel, C.M., Wankel, S.D., 2017. A dual nitrite isotopic investigation of chemodenitrification by mineral-associated Fe(II) and its production of nitrous oxide. Geochim. Cosmochim. Acta 196:388–402. https://doi.org/10.1016/j.gca.2016.10.026.

Granger, J., Sigman, D.M., Lehmann, M.F., Tortell, P.D., 2008. Nitrogen and oxygen isotope fractionation during dissimilatory nitrate reduction by denitrifying bacteria. Limnol. Oceanogr. 53:2533–2545. https://doi.org/10.4319/lo.2008.53.6.2533.

Granger, J., Sigman, D.M., Needoba, J.A., Harrison, P.J., 2004. Coupled nitrogen and oxygen isotope fractionation of nitrate during assimilation by cultures of marine phytoplankton. Limnol. Oceanogr. 49:1763–1773. https://doi.org/10.4319/lo.2004.49.5.1763.

Granger, J., Wankel, S.D., 2016. Isotopic overprinting of nitrification on denitrification as a ubiquitous and unifying feature of environmental nitrogen cycling. Proc. Natl. Acad. Sci. U. S. A. 113:E6391–E6400. https://doi.org/10.1073/pnas.1601383113.

Groffman, P.M., Altabet, M.A., Böhlke, J., Butterbach-Bahl, K., David, M.B., Firestone, M.K., Giblin, A.E., Kana, T.M., Nielsen, L.P., Voytek, M.A., 2006. Methods for measuring denitrification: diverse approaches to a difficult problem. Ecol. Appl. 16:2091–2122. https://doi.org/10.1890/1051-0761(2006)016[2091:MFMDDA]2.0.CO;2.

Gruber, N., Galloway, J.N., 2008. An Earth-system perspective of the global nitrogen cycle. Nature 451:293–296. https://doi.org/10.1038/nature06592.

Harris, E., Emmenegger, L., Mohn, J., 2017. Using isotopic fingerprints to trace nitrous oxide in the atmosphere. CHIMIA Int. J. Chem. 71, 46.

Hosono, T., Alvarez, K., Lin, I.T., Shimada, J., 2015. Nitrogen, carbon, and sulfur isotopic change during heterotrophic (*Pseudomonas aureofaciens*) and autotrophic (*Thiobacillus denitrificans*) denitrification reactions. J. Contam. Hydrol. 183:72–81. https://doi.org/10.1016/j.jconhyd.2015.10.009.

Houlton, B.Z., 2005. Isotopic Evidence for the Climate Dependence of Nitrogen Cycles Across Old Tropical Rainforests, Mt. Haleakala, Hawaii (Doctoral dissertation).

Houlton, B.Z., Bai, E., 2009. Imprint of denitrifying bacteria on the global terrestrial biosphere. Proc. Natl. Acad. Sci. U. S. A. 106:21713–21716. https://doi.org/10.1073/pnas.0912111106.

Houlton, B.Z., Sigman, D.M., Hedin, L.O., 2006. Isotopic evidence for large gaseous nitrogen losses from tropical rainforests. Proc. Natl. Acad. Sci. U. S. A. 103:8745–8750. https://doi.org/10.1073/pnas.0510185103.

Kaneko, M., Poulson, S.R., 2013. The rate of oxygen isotope exchange between nitrate and water. Geochim. Cosmochim. Acta 118:148–156. https://doi.org/10.1016/j.gca.2013.05.010.

Karsh, K.L., Granger, J., Kritee, K., Sigman, D.M., 2012. Eukaryotic assimilatory nitrate reductase fractionates N and O isotopes with a ratio near unity. Environ. Sci. Technol. 46:5727–5735. https://doi.org/10.1021/es204593q.

Kendall, C., Elliott, E.M., Wankel, S.D., 2007. Tracing anthropogenic inputs of nitrogen to ecosystems. In: Michener, R.H., Lajtha, K. (Eds.), Stable Isotopes in Ecology and Environmental Science. 2007. Blackwell Publishing, pp. 375–449.

Kessler, A.J., Bristow, L.A., Cardenas, M.B., Glud, R.N., Thamdrup, B., Cook, P.L.M., 2014. The isotope effect of denitrification in permeable sediments. Geochim. Cosmochim. Acta 133:156–167. https://doi.org/10.1016/j.gca.2014.02.029.

Kim, K.R., Craig, H., 1990. Two-isotope characterization of N_2O in the Pacific Ocean and constraints on its origin in deep water. Nature 347:58–61. https://doi.org/10.1038/347058a0.

Knöeller, K., Vogt, C., Haupt, M., Feisthauer, S., Richnow, H.H., 2011. Experimental investigation of nitrogen and oxygen isotope fractionation in nitrate and nitrite during denitrification. Biogeochemistry 103:371–384. https://doi.org/10.1007/s10533-010-9483-9.

Kohl, D., Shearer, G., 1978. Isotope effects in metabolic studies. Recent Developments in Mass Spectrometry in Biochemistry and Medicine. Springer, pp. 623–640.

Kool, D.M., Müller, C., Wrage, N., Oenema, O., Van Groenigen, J.W., 2009. Oxygen exchange between nitrogen oxides and H_2O can occur during nitrifier pathways. Soil Biol. Biochem. 41:1632–1641. https://doi.org/10.1016/j.soilbio.2009.05.002.

Kritee, K., Sigman, D.M., Granger, J., Ward, B.B., Jayakumar, A., Deutsch, C., 2012. Reduced isotope fractionation by denitrification under conditions relevant to the ocean. Geochim. Cosmochim. Acta 92:243–259. https://doi.org/10.1016/j.gca.2012.05.020.

Lehmann, M.F., Reichert, P., Bernasconi, S.M., Barbieri, A., McKenzie, J.A., 2003. Modelling nitrogen and oxygen isotope fractionation during denitrification in a lacustrine redox-transition zone. Geochim. Cosmochim. Acta 67:2529–2542. https://doi.org/10.1016/s0016-7037(03)00085-1.

Lewicka-Szczebak, D., Well, R., Bol, R., Gregory, A.S., Matthews, G.P., Misselbrook, T., Whalley, W.R., Cardenas, L.M., 2015. Isotope fractionation factors controlling isotopocule signatures of soil-emitted N_2O produced by denitrification processes of various rates. Rapid Commun. Mass Spectrom. 29:269–282. https://doi.org/10.1002/rcm.7102.

Lewicka-Szczebak, D., Well, R., Köster, J.R., Fuß, R., Senbayram, M., Dittert, K., et al., 2014. Experimental determinations of isotopic fractionation factors associated with N_2O production and reduction during denitrification in soils. Geochim. Cosmochim. Acta 134:55–73. https://doi.org/10.1016/j.gca.2014.03.010.

Luo, T., Li, Y., Chen, D., Lin, M., Sun, Y., 2005. A study on the soil properties of dacrycarpus imbricatus Bl. plantation in tropical mountainous region in Hainan Island. Nat. Sci. J. Hainan Univ. 23, 340–346.

Maggi, F., Riley, W.J., 2015. The effect of temperature on the rate, affinity, and 15N fractionation of NO_3^- during biological denitrification in soils. Biogeochemistry 124: 235–253. https://doi.org/10.1007/s10533-015-0095-2.

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

Mariotti, A., Germon, J., Leclerc, A., 1982. Nitrogen isotope fractionation associated with the $NO_2^- \rightarrow N_2O$ step of denitrification in soils. Can. J. Soil Sci. 62:227–241. https://doi.org/10.4141/cjss82-027.

Mariotti, A., Landreau, A., Simon, B., 1988. ^{15}N isotope biogeochemistry and natural denitrification process in groundwater: application to the chalk aquifer of northern France. Geochim. Cosmochim. Acta 52:1869–1878. https://doi.org/10.1016/0016-7037(88)90010-5.

Mathieu, O., Leveque, J., Henault, C., Ambus, P., Milloux, M.J., Andreux, F., 2007. Influence of ^{15}N enrichment on the net isotopic fractionation factor during the reduction of nitrate to nitrous oxide in soil. Rapid Commun. Mass Spectrom. 21:1447–1451. https://doi.org/10.1002/rcm.2979.

Mengis, M., Schiff, S.L., Harris, M., English, M.C., Aravena, R., Elgood, R.J., MacLean, A., 1999. Multiple geochemical and isotopic approaches for assessing ground water NO_3^- elimination in a riparian zone. Ground Water 37:448–457. https://doi.org/10.1111/j.1745-6584.1999.tb01124.x.

Menyailo, O.V., 2007. The influence of tree species on the biomass of denitrifying bacteria in gray forest soils. Eurasian Soil Sci. 40:302–307. https://doi.org/10.1134/s1064229307030088.

Menyailo, O.V., Hungate, B.A., 2006. Stable isotope discrimination during soil denitrification: Production and consumption of nitrous oxide. Glob. Biogeochem. Cycles 20 n/a-n/a. https://doi.org/10.1029/2005gb002527.

Perez, T., Garcia-Montiel, D., Trumbore, S., Tyler, S., Camargo, P.D., Moreira, M., Piccolo, M., Cerri, C., 2006. Nitrous oxide nitrification and denitrification ^{15}N enrichment factors from Amazon forest soils. Ecol. Appl. 16:2153–2167. https://doi.org/10.1890/1051-0761(2006)016[2153:NONADN]2.0.CO;2.

Rich, J.J., Heichen, R.S., Bottomley, P.J., Cromack, K., Myrold, D.D., 2003. Community composition and functioning of denitrifying bacteria from adjacent meadow and forest soils. Appl. Environ. Microbiol. 69:5974–5982. https://doi.org/10.1128/aem.69.10.5974-5982.2003.

Sigman, D.M., Granger, J., DiFiore, P.J., Lehmann, M.M., Ho, R., Cane, G., van Geen, A., 2005. Coupled nitrogen and oxygen isotope measurements of nitrate along the eastern North Pacific margin. Glob. Biogeochem. Cycles 19. https://doi.org/10.1029/2005gb002458.

Sigman, D.M., Robinson, R., Knapp, A.N., van Geen, A., McCorkle, D.C., Brandes, J.A., Thunell, R.C., 2003. Distinguishing between water column and sedimentary denitrification in the Santa Barbara Basin using the stable isotopes of nitrate. Geochem. Geophys. Geosyst. 4. https://doi.org/10.1029/2002gc000384.

Smith, R.L., Howes, B.L., Duff, J.H., 1991. Denitrification in nitrate-contaminated groundwater: occurrence in steep vertical geochemical gradients. Geochim. Cosmochim. Acta 55:1815–1825. https://doi.org/10.1016/0016-7037(91)90026-2.

Snider, D.M., Schiff, S.L., Spoelstra, J., 2009. $^{15}N/^{14}N$ and $^{18}O/^{16}O$ stable isotope ratios of nitrous oxide produced during denitrification in temperate forest soils. Geochim. Cosmochim. Acta 73:877–888. https://doi.org/10.1016/j.gca.2008.11.004.

Talbot, J.M., Bruns, T.D., Taylor, J.W., Smith, D.P., Branco, S., Glassman, S.I., Erlandson, S., Vilgalys, R., Liao, H.L., Smith, M.E., Peay, K.G., 2014. Endemism and functional convergence across the North American soil mycobiome. Proc. Natl. Acad. Sci. U. S. A. 111: 6341–6346. https://doi.org/10.1073/pnas.1402584111.

Treibergs, L.A., Granger, J., 2016. Enzyme level N and O isotope effects of assimilatory and dissimilatory nitrate reduction. Limnol. Oceanogr. https://doi.org/10.1002/lno.10393.

Tu, Y., Fang, Y., Liu, D., Pan, Y., 2016. Modifications to the azide method for nitrate isotope analysis. Rapid Commun. Mass Spectrom. 30:1213–1222. https://doi.org/10.1002/rcm.7551.

Urey, H.C., 1947. The Thermodynumic properties of isotopic substances. J. Chem. Soc. 562–581.

Vitousek, P.M., Howarth, R.W., 1991. Nitrogen limitation on land and in the sea: how can it occur? Biogeochemistry 13, 87–115.

Vogel, J.C., Talma, A.S., Heaton, T.H.E., 1981. Gaseous nitrogen as evidence for denitrification in groundwater. J. Hydrol. 50:191–200. https://doi.org/10.1016/0022-1694(81)90069-X.

Voss, M., Dippner, J.W., Montoya, J.P., 2001. Nitrogen isotope patterns in the oxygen-deficient waters of the Eastern Tropical North Pacific Ocean. Deep-Sea Res. I Oceanogr. Res. Pap. 48:1905–1921. https://doi.org/10.1016/s0967-0637(00)00110-2.

Well, R., Flessa, H., 2009. Isotopologue signatures of N2O produced by denitrification in soils. J. Geophy. Res. Biogeosci. 114 n/a-n/a. https://doi.org/10.1029/2008jg000804.

Wenk, C.B., Zopfi, J., Blees, J., Veronesi, M., Niemann, H., Lehmann, M.F., 2014. Community N and O isotope fractionation by sulfide-dependent denitrification and anammox in a stratified lacustrine water column. Geochim. Cosmochim. Acta 125:551–563 DOI: 10.1029/2008jg000804. https://doi.org/10.1016/j.gca.2013.10.034.

Wunderlich, A., Meckenstock, R., Einsiedl, F., 2012. Effect of different carbon substrates on nitrate stable isotope fractionation during microbial denitrification. Environ. Sci. Technol. 46:4861–4868. https://doi.org/10.1021/es204075b.

Wunderlich, A., Meckenstock, R.U., Einsiedl, F., 2013. A mixture of nitrite-oxidizing and denitrifying microorganisms affects the $\delta^{18}O$ of dissolved nitrate during anaerobic microbial denitrification depending on the $\delta^{18}O$ of ambient water. Geochim. Cosmochim. Acta 119:31–45. https://doi.org/10.1016/j.gca.2013.05.028.

Xi, D., 2016. Gaseous Nitrogen Emissions from Forest Soils (Doctoral dissertation).

Yang, K., Zhu, J.J., Yan, Q.L., Sun, O.J., 2010. Changes in soil P chemistry as affected by conversion of natural secondary forests to larch plantations. For. Ecol. Manag. 260: 422–428. https://doi.org/10.1016/j.foreco.2010.04.038.

Zhu, J., Mao, Z., Hu, L., Zhang, J., 2007. Plant diversity of secondary forests in response to anthropogenic disturbance levels in montane regions of Northeastern China. J. For. Res. 12:403–416. https://doi.org/10.1007/s10310-007-0033-9.