Nitrate is an important nitrogen source for Arctic tundra plants

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Plant nitrogen (N) use is a key component of the N cycle in terrestrial ecosystems. The supply of N to plants affects community species composition and ecosystem processes such as photosynthesis and carbon (C) accumulation. However, the availabilities and relative importance of different N forms to plants are not well understood. While nitrate (NO_3^-) is a major N form used by plants worldwide, it is discounted as a N source for Arctic tundra plants because of extremely low NO_3^- concentrations in Arctic tundra soils, undetectable soil nitrification, and plant-tissue NO_3^- that is typically below detection limits. Here we reexamine NO_3^- use by tundra plants using a sensitive denitrifier method to analyze plant-tissue NO_3^-. Soil-derived NO_3^- was detected in tundra plant tissues, and tundra plants took up soil NO_3^- at comparable rates to plants from relatively NO_3^--rich ecosystems in other biomes. Nitrate assimilation determined by ^{15}N enrichments of leaf NO_3^- relative to soil NO_3^- accounted for 4 to 52% (as estimated by a Bayesian isotope-mixing model) of species-specific total leaf N of Alaskan tundra plants. Our finding that in situ soil NO_3^- availability for tundra plants is high has important implications for Arctic ecosystems, not only in determining species compositions, but also in determining the loss of N from soils via leaching and denitrification. Plant N uptake and soil N losses can strongly influence C uptake and accumulation in tundra soils. Accordingly, this evidence of NO_3^- availability in tundra soils is crucial for predicting C storage in tundra.

Arctic tundra plants | nitrogen dynamics | plant nitrate | soil nitrate | stable isotopes

Nitrogen (N) is often the nutrient that most limits terrestrial plant growth, making plant N availability a key determinant of primary productivity in terrestrial ecosystems (1). Hence, improved knowledge of in situ plant N availability and consequent plant N use is crucial for better evaluating and predicting responses of vegetation to climate change and N loading (2, 3). However, the availability of N to terrestrial plants is difficult to evaluate using measurements of soil N because of strong plant–microbe and plant–plant competition for N and the resulting rapid turnover of soil N pools (4).

Arctic ecosystems are typically characterized by strong N limitation (1). Because of high carbon (C) stocks in permafrost soil and their sensitivity to environmental change, the Arctic C cycle has important implications for global C balance and C-climate feedbacks (5, 6). Although it remains difficult to budget N inputs in the Arctic, the Arctic biome is a potential sink for anthropogenic N pollutants (7). So far, long-term N addition experiments have revealed that elevated N inputs into Arctic tundra ecosystems change C accumulation and species diversity (5, 8, 9). Field observations and isotope labeling experiments provide evidence of how added N has altered the distribution, fate, biotic use, and losses of N in Arctic tundra ecosystems (10–15). These studies indicate that a better understanding of in situ N availability in Arctic ecosystems is important because C and N cycles are tightly coupled between the vegetation and soils, and elevated N loading can influence the Arctic's C balance (5, 16).

Nitrate (NO_3^-) is a common and pivotal plant-available N form in addition to ammonium (NH_4^+) and some forms of dissolved organic N (DON) (1). Until the 1990s, researchers underestimated the availability of soil NO_3^- to microbes because microbial uptake of NO_3^- often results in very low NO_3^- standing stock and low or negative net NO_3^- production (nitrification) rates in soil, even when gross nitrification rates are high (17–19). However, it remains undetermined how important soil NO_3^- is for plants because of inadequate understanding of in situ plant NO_3^- use. In Arctic tundra, NO_3^- availability can be increased by direct release from thawing permafrost, melting snow, and increased nitrification resulting from elevated N loading and warming

Significance

How terrestrial plants use N and respond to soil N loading is central to evaluating and predicting changing ecosystem structure and function with climate warming and N pollution. Here, evidence from NO_3^- in plant tissues has uncovered the uptake and assimilation of soil NO_3^- by Arctic tundra plants, which has long been assumed negligible. Soil NO_3^- contributed about one-third of the bulk N used by tundra plants of northern Alaska. Accordingly, the importance of soil NO_3^- for tundra plants should be considered in future studies on N and C cycling in Arctic ecosystems where C sequestration is strongly determined by N availability.

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temperatures (7, 14, 20). Elevated NO_3^- availability to tundra plants can change interspecific N competition and N-use strategies of tundra plants (9, 13, 21), potentially resulting in the spread of NO_3^--adapted species and altering the partitioning of above-ground vs. below-ground biomass (18, 22–24). These factors could alter CO_2 fixation by vegetation and the quantity and quality of litter inputs to the soil, which would then change microbial breakdown of soil C and the emission and uptake of greenhouse gases (5, 8, 25–27). Accordingly, soil NO_3^- availability and plant NO_3^- use have important implications for both N and C cycles in Arctic tundra.

Despite its potential importance, NO_3^- availability and the contribution of different N forms to plant N use have been unclear in Arctic tundra (21, 28). Four decades of research show that tundra plants rely on soil NH_4^+ and DON (e.g., direct uptake of free amino acids) to meet growth requirements for N (12, 21, 28–31). In contrast, researchers generally have considered plant NO_3^- use to be negligible in the Arctic for several reasons. First, NO_3^- concentrations in soils are often low or undetectable, and soil net nitrification rates seldom show positive values (*SI Appendix*, Figs. S1 and S2), presumably because of low temperature, low soil NH_4^+ availability, and low soil pH, together with high microbial N demand (32, 33). Second, plant-tissue NO_3^-, a common marker of plant NO_3^- uptake, is rarely detected in tundra plants with conventional analytical methods (11, 12, 34).

We argue that the importance of NO_3^- to plants in such seemingly low-NO_3^- Arctic tundra ecosystems remains an open question for several reasons. First, although extractable soil NO_3^- concentrations are typically low in Arctic tundra soils, NO_3^- is sometimes present in measurable amounts and contributes non-trivial fractions of total extractable N (TEN) stocks similar to high-NO_3^- ecosystems (*SI Appendix*, Fig. S2*B*). Second, rates of in situ NO_3^- reductase activity (NRA), which is inducible and reflects the enzymatic NO_3^- reduction occurring in plants, are measurable in tundra plants and are not distinct from NRA rates measured in plants at lower latitudes (*SI Appendix*, Fig. S3). Accordingly, the abilities of Arctic tundra plants to assimilate NO_3^- are comparable to those of plants in relatively NO_3^--rich ecosystems. Third, controlled experiments revealed that tundra plants took up NH_4^+ and NO_3^- at similar rates (9, 12, 29) or even took up NO_3^- at higher rates (33). Field ^{15}N application (7, 13, 31) and modeling results (35) confirmed that tundra plants can assimilate NO_3^-, NH_4^+, and amino acids. All these observations illustrate that NO_3^- is an important soil N source in Arctic tundra and that tundra plants can use NO_3^-. However, the relative importance of soil NO_3^- for plants in Arctic tundra ecosystems is unknown because we lack measures of in situ plant NO_3^- use and how it compares to that of plants in other NO_3^--poor or NO_3^--rich ecosystems.

Results and Discussion

Using the highly sensitive denitrifier method (detailed in *Materials and Methods*), we analyzed concentrations and stable isotope compositions of NO_3^- in tissues of dominant plant species in Alaskan tundra ecosystems. We then compared our results with those for plants from relatively high-N or high-NO_3^- ecosystems in lower-latitude regions (Figs. 1 and 2). Such comparisons of Arctic sites to non-Arctic sites, using both traditional and new methods, are important for understanding soil N cycling (particularly soil NO_3^- availability) and for placing the N uptake abilities of tundra plants into a broader context.

The Uptake of NO_3^- in Plants. The existence of NO_3^- in plant tissues is evidence for NO_3^- uptake from the soil or atmosphere because NO_3^- production in non-N_2 fixing plants is negligible under normal conditions (36–40). Although NO_3^- can be produced from the oxidation of nitric oxide (NO) both enzymatically and non-enzymatically in non-N_2 fixing plants (37–40), the rates are very low in natural environments (41–44), especially compared with the pool sizes of NO_3^- detected in plants of this study. Besides, while NO_3^- production by nonsymbiotic hemoglobin is possible in anoxic conditions (38, 39) and with high ambient NO concentrations (40), neither anoxic conditions nor high ambient NO applies to the present study.

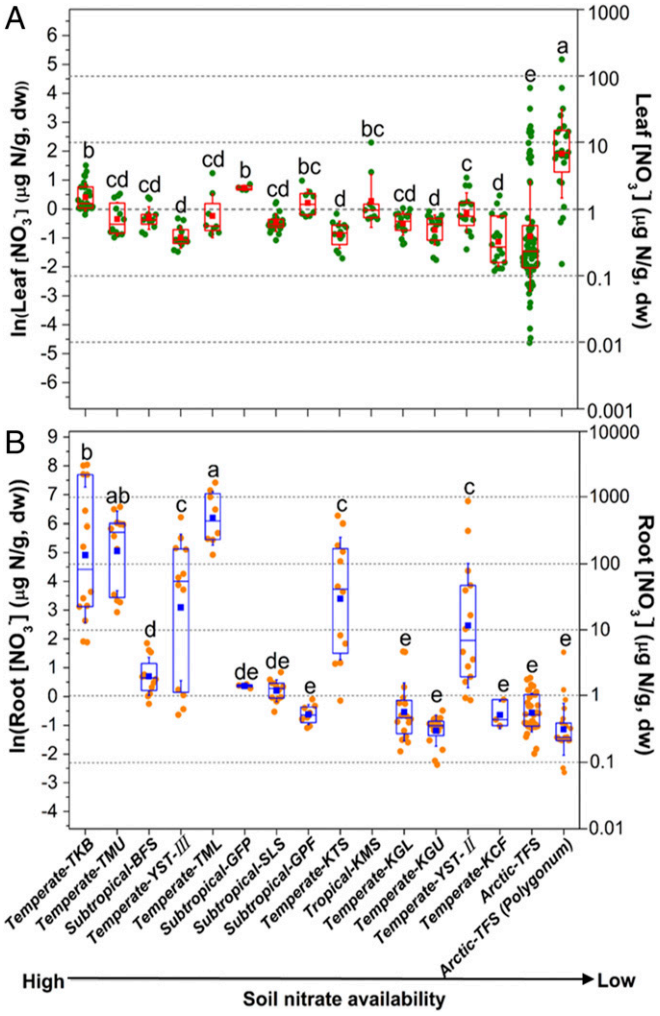

Fig. 1. Concentrations of NO_3^- in plant leaves (*A*) and roots (*B*) across different ecosystems. The box encompasses the 25th to 75th percentiles, and whiskers are the SD values. The line and square in each box mark the median and mean values of studied plants at each site, respectively. Unique letters above the boxes mark significant differences at the level of $P < 0.05$. Detailed site information, including site abbreviation definitions, and species-specific values are given in *SI Appendix*, Tables S1 and S2. dw, dry weight.

We detected unexpectedly high NO_3^- concentrations in leaves and roots of the tundra plant species studied (Fig. 1 and *SI Appendix*, Tables S1 and S2). First, of the 153 tundra plant samples analyzed, 143 had measurable NO_3^- concentrations (detailed in *Materials and Methods*). Some species (e.g., *Polygonum bistorta*) had higher foliar NO_3^- than low-latitude forest species, including those in high-NO_3^- environments (Fig. 1*A* and *SI Appendix*, Table S2). Second, ratios of leaf NO_3^- to soil NO_3^- and of root NO_3^- to soil NO_3^- were similar between tundra and lower-latitude ecosystems or even higher in tundra than in some lower-latitude ecosystems (*SI Appendix*, Fig. S4). These results provide evidence of high NO_3^- uptake of tundra plants despite much lower concentrations of NO_3^- in tundra soils. Thus, we conclude that tundra plants can take up NO_3^- as efficiently as plants from relatively NO_3^--rich ecosystems in other biomes. In addition, NO_3^- additions to soils enhanced leaf NO_3^- concentrations in most tundra plants (*SI Appendix*, Figs. S5 and S6). This result is evidence that plant NO_3^- uptake is responsive to soil NO_3^- variations in Arctic tundra ecosystems. Such responses and patterns of NO_3^- uptake among studied species are useful for interpreting

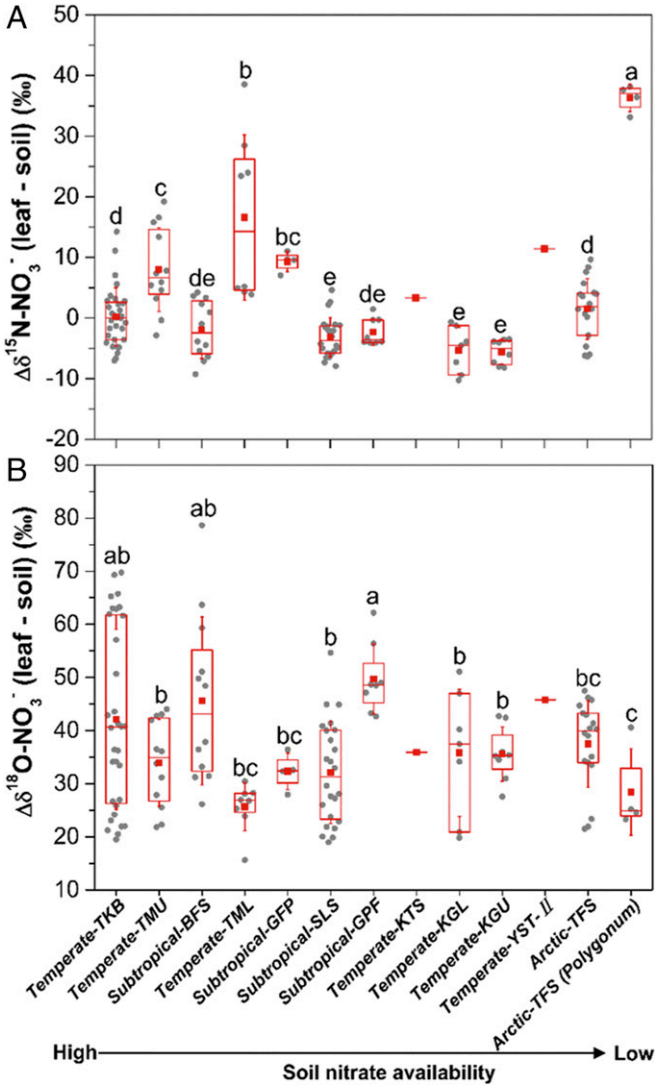

Fig. 2. Differences (Δ values) in $\delta^{15}N$ (*A*) and $\delta^{18}O$ (*B*) between leaf NO_3^- and soil NO_3^- across different ecosystems. The box encompasses the 25th to 75th percentiles, whiskers are the SD values, and the red line and red square in each box mark the median and mean values, respectively. Unique letters above the boxes indicate significant differences at the level of $P < 0.05$. The Δ values were calculated using replicate values of plant tissues minus mean values of soil in corresponding sites (*SI Appendix*, Fig. S8 and Table S1).

changes in functional traits and the structure of tundra plant communities in response to projected increases of soil NO_3^- with climate warming and elevated N deposition (1, 45).

The Sources of NO_3^- in Plants. We used the $\Delta^{17}O$ signatures of leaf NO_3^- ($\Delta^{17}O_{leaf}$) to verify the mixing of atmospheric-derived NO_3^- [$\Delta^{17}O_{atm} > 0$ per mille (‰) due to an enrichment in ^{17}O during photochemical oxidization of nitrogen oxides (NOx) by O_3] with soil-derived NO_3^- ($\Delta^{17}O_{soil} = 0‰$ because of no ^{17}O excess in atmospheric O_2 and soil H_2O molecules) (46–48). Leaf NO_3^- of *P. bistorta* showed no ^{17}O isotope anomaly ($\Delta^{17}O$ values = 0.0‰; *SI Appendix*, Fig. S7), indicating that the NO_3^- detected in this species was purely soil derived. Clearly, soil NO_3^- is available to, and taken up by, tundra plants.

In contrast, positive $\Delta^{17}O_{leaf}$ values in low-latitude forests (*SI Appendix*, Fig. S7) indicate the direct leaf absorption of atmospheric-derived NO_3^- ($\Delta^{17}O > 0‰$) or possibly the root uptake of NO_3^- at the surface soil with positive $\Delta^{17}O$ values (49). We used mean $\Delta^{17}O$ values of precipitation NO_3^- measured in the Tama-Kyuryo Field Museum forest in temperate Japan (TML) (see *SI Appendix*, Table S1 for descriptions of the forest sites used in this study) (49); in Guiyang in subtropical China (this study); and in Jianfengling forests in Hainan, tropical China (49) as $\Delta^{17}O_{atm}$ values in the studied temperate, subtropical, and tropical forests, respectively (*SI Appendix*, Fig. S7). We then estimated mixing ratios of atmospheric-derived NO_3^- ($\Delta^{17}O_{leaf}$:$\Delta^{17}O_{atm}$) for plants in lower-latitude ecosystems. The results showed that atmospheric-derived NO_3^- accounted for, on average, 35% (6 to 86%) of total leaf NO_3^- in measured samples from lower-latitude forests.

NO_3^- Assimilation in Plants. Higher $\delta^{15}N$ and $\delta^{18}O$ values in plant-tissue NO_3^- relative to source NO_3^- could provide new evidence for in situ plant NO_3^- assimilation because NO_3^- reduction via NO_3^- reductase would cause ^{15}N and ^{18}O enrichments in the unassimilated NO_3^- (2, 50–52). Accordingly, we calculated differences (Δ values) between isotopic values of tissue NO_3^- ($\delta^{15}N$ and $\delta^{18}O$) in each plant sample and mean values of soil NO_3^- in corresponding ecosystems (Fig. 2 and *SI Appendix*, Fig. S8).

In northern Alaska, $\delta^{15}N$ values of soil NO_3^- were 1.0‰ at Toolik Field Station (TFS) (see *SI Appendix*, Table S1) (21, 53) and 0.5 ± 4.7‰ at Barrow (54). Atmospheric-derived NO_3^- in snowmelt had lower $\delta^{15}N$ values of −4.8 ± 1.0‰ at Barrow (54) and much lower values of −8.6 ± 0.7‰ at a high Arctic site at Midtre Lovénbreen, Svalbard (55). Compared with $\delta^{15}N$ values of soil- or atmospheric-derived NO_3^- (*SI Appendix*, Fig. S8*A*), the higher $\delta^{15}N$ values of leaf NO_3^- in tundra of northern Alaska (positive $\Delta\delta^{15}N$ values; Fig. 2*A*) are evidence for in situ NO_3^- assimilation in tundra plants (Fig. 2*A*). The $\delta^{18}O$ values of NO_3^- produced in high-centered soil polygons averaged −4.4 ± 2.7‰ at Barrow (54). By comparison, distinctly higher $\delta^{18}O$ values of leaf NO_3^- than those of soil NO_3^- (positive $\Delta\delta^{18}O$ values; Fig. 2*B*) also provide evidence for in situ NO_3^- assimilation in tundra plants.

In non-Arctic sites, higher $\delta^{18}O$ values of leaf NO_3^- than those of a soil- and atmospheric-derived NO_3^- mixture (distributed on the mixing line; Fig. 3) indicated assimilation of the mixed NO_3^- pool in the studied plants. However, higher ^{18}O enrichments (*SI Appendix*, Fig. S8) might be due, in part, to contributions from high $\delta^{18}O$ values of atmospheric-derived NO_3^- (57). Major uncertainties existed in fractional contributions of atmospheric-derived NO_3^- in leaf NO_3^- because of limited $\Delta^{17}O$ data of leaf NO_3^- and lack of explicit $\Delta^{17}O$ values of atmospheric NO_3^-.

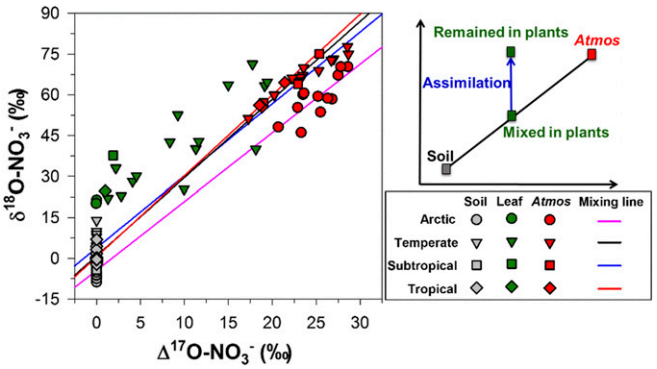

Fig. 3. $\Delta^{17}O$ vs. $\delta^{18}O$ plots of NO_3^- in soil, leaves, and atmospheric (*Atmos*, as precipitation or snow) deposition across different ecosystems. The mixing lines of Arctic and tropical sites ($y = 2.52x - 4.42$ and $y = 2.97x + 0.58$, respectively) were based on isotopic values of soil NO_3^- ($n = 18$) (54) and snowpack NO_3^- ($n = 12$) (56) at Barrow, and of soil NO_3^- ($n = 18$) and precipitation NO_3^- ($n = 3$) at Jianfengling in tropical China (49), respectively. The mixing line of temperate sites ($y = 2.64x + 3.82$) was based on isotopic values of soil NO_3^- at Japanese temperate sites ($n = 22$) and precipitation at TML ($n = 12$) in this study. The mixing line of subtropical sites ($y = 2.87x + 0.91$) was based on isotopic values of soil NO_3^- ($n = 29$) at subtropical sites and precipitation NO_3^- at Guiyang, China ($n = 3$) in this study. The $\Delta^{17}O$ of soil NO_3^- was assumed to be zero.

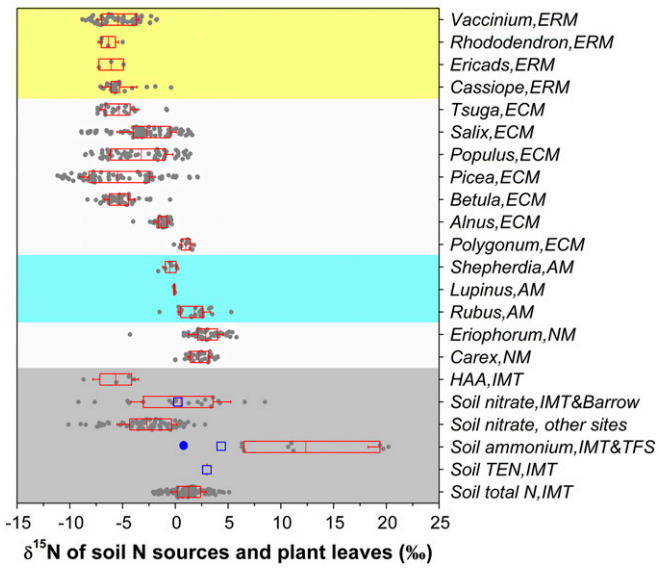

Fig. 4. $\delta^{15}N$ values of leaf total N and soil N sources of tundra plants in Alaska. AM, arbuscular mycorrhiza; ECM, ectomycorrhiza; ERM, ericoid mycorrhiza; NM, nonmycorrhiza. The box encompasses the 25th to 75th percentiles, and whiskers are the SD values. The line in each box marks the mean value. Plant $\delta^{15}N$ data were summarized from ref. 58 and those of *SI Appendix*, Fig. S9*B*. The empty squares show soil $\delta^{15}N$ data reported at IMT (53) and the blue-filled circle shows data at TFS (21). Soil $\delta^{15}N$-NO_3^- values of other sites are summarized from available data of non-Arctic sites in this study; soil $\delta^{15}N$-NO_3^- values at Barrow are cited from ref. 54.

Precipitation NO_3^- might not fully represent all atmospheric NO_3^- contributions to plant leaves; in addition, it is even more difficult to determine reasonable $\delta^{15}N$ and $\delta^{18}O$ end-member values of atmospheric-derived NO_3^- in plant leaves. Despite these problems, NO_3^- isotopes in plant tissues did provide information on plant NO_3^- sources and uptake in disturbed ecosystems.

Contributions of Soil NO_3^- to Total N in Tundra Plants. Compared with plants in relatively N-rich ecosystems, tundra plants showed a similar distribution of leaf total N concentrations but a much wider distribution of leaf total (bulk) $\delta^{15}N$ values (*SI Appendix*, Fig. S9). The wider distribution of leaf total $\delta^{15}N$ values arises because of the strong niche differentiation of N-use regimes among tundra plants (13, 58). However, $\delta^{15}N$ values of total N in tundra plants (−11.2 to 5.8‰ in Alaska) are generally lower than those of soil NH_4^+ [around 12.3 ± 3.6‰ (this study); 4.4 ± 0.9‰ (53); and 1.4 ± 0.5‰ (21)], although some DON components are ^{15}N depleted [around −5.7‰ for hydrolyzable amino acids (HAA) at Imnavait Creek (IMT) in northern Alaska; see *SI Appendix*, Table S1] (Fig. 4). This disparity between the $\delta^{15}N$ signatures of plant total N vs. soil N sources exists even when isotopic fractionations for NH_4^+ and HAA assimilation by mycorrhizal plants are considered. Given plant NO_3^- uptake and assimilation as indicated by NO_3^- in plant tissues, soil NO_3^- should be considered when using $\delta^{15}N$ methods to evaluate in situ contributions of soil N sources to total N of tundra plants.

Proportional contributions (*f*, expressed as a percentage) of soil NO_3^- to total N in tundra plants were estimated using $\delta^{15}N$ values of soil N (NO_3^-, NH_4^+, and HAA) and $\delta^{15}N$ values of leaf total N in a Bayesian isotope-mixing model [Stable Isotope Analysis in R (SIAR) (cran.r-project.org/web/packages/siar/index.html) (59)] (Fig. 5). The SIAR model uses a Bayesian framework to establish a logical prior distribution (60) for estimating *f* values, and then determines the probability distribution for the *f* values of each source (soil NO_3^-, NH_4^+, and HAA, in this study) to the mixture (total N of plant leaves, in this study). We contend that this approach provides reliable estimations of fractional contributions of different N sources to plant total N because the mixing model considers isotope effects during plant N uptake ($^{15}\varepsilon$ values hereafter) and variability in both source $\delta^{15}N$ values and plant $\delta^{15}N$ values (61).

In this study, the $\delta^{15}N$ values (mean ± SD) of soil NO_3^- at Barrow [0.5 ± 4.7‰ (54)], soil NH_4^+ at IMT and TFS (11.5 ± 8.4‰, this study and ref. 21), and soil HAA at IMT [−5.7 ± 2.2‰; (53)] were used as source $\delta^{15}N$ values. For nonmycorrhizal (NM) plants, leaf $\delta^{15}N$ values were mainly controlled by the $\delta^{15}N$ values and *f* values of source N (NO_3^-, NH_4^+, and HAA), assuming negligible isotope effects during the acquisition processes of source N from soil into NM plants (i.e., $^{15}\varepsilon = 0‰$). For mycorrhizal plants, the $^{15}\varepsilon$ values during the acquisition processes of soil N sources were calculated as the net differences of leaf $\delta^{15}N$ values between mycorrhizal and NM plants. The same $^{15}\varepsilon$ value was assumed for plant species associated with the same type of mycorrhiza and for N forms absorbed through the same type of mycorrhiza. In Alaskan tundra, the $^{15}\varepsilon$ values for plant species associated with arbuscular mycorrhizae (AM), ectomycorrhizae (ECM), and ericoid mycorrhizae (ERM) were estimated as net $\delta^{15}N$ differences from NM plants—that is, −5.0‰, −6.9‰, and −7.7‰, respectively (21, 62), which differed from the $^{15}\varepsilon$ values normalized for worldwide plants [−2.0‰, −3.2‰, and −5.9‰, respectively (63)]. Our $^{15}\varepsilon$ values (0‰ for NM plants, −5.0‰ for AM plants, −6.9‰ for ECM plants, and −7.7‰ for ERM plants)

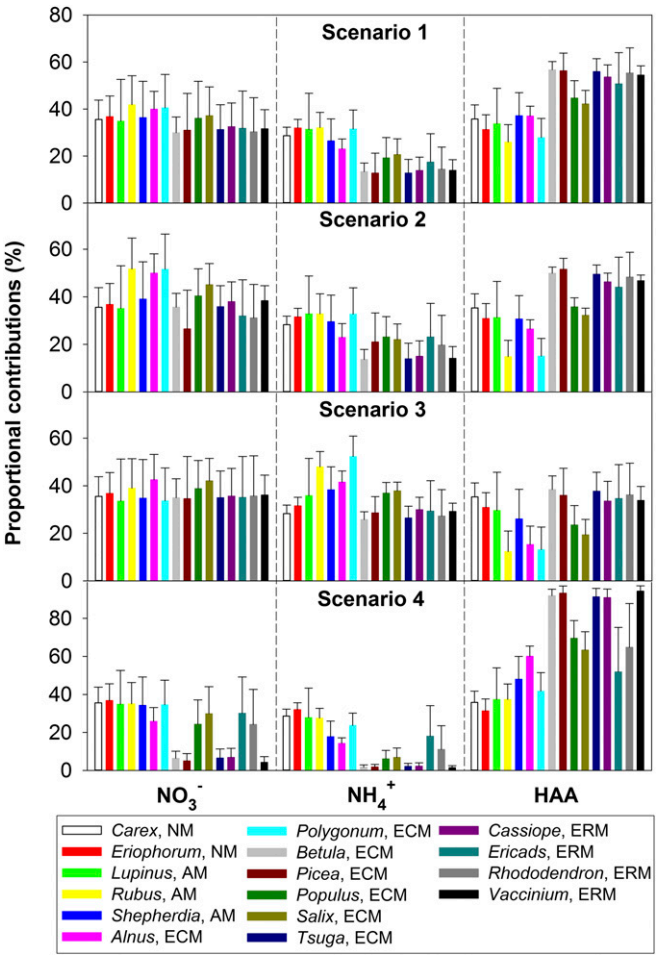

Fig. 5. Proportional contributions (mean ± SD) of soil NO_3^-, NH_4^+, and HAA to leaf total N of tundra plants in Alaska. The $^{15}\varepsilon$ values [0‰ for NM plants, −5.0‰ for AM plants, −6.9‰ for ECM plants, and −7.7‰ for ERM plants (21, 62)] were considered for NO_3^-, NH_4^+, and HAA (scenario 1); for NH_4^+ and HAA only (scenario 2); for HAA only (scenario 3); for none of NO_3^-, NH_4^+, and HAA (scenario 4).

were considered under four scenarios (scenario 1: for NO_3^-, NH_4^+, and HAA; scenario 2: for NH_4^+ and HAA only; scenario 3: for HAA only; and scenario 4: for none of NO_3^-, NH_4^+, and HAA) (Fig. 5). Estimates from natural ^{15}N evidence were that NO_3^- assimilation accounted for 4 to 52% of species-specific leaf total N (around one-third, on average) of Alaskan tundra plants (Fig. 5), thereby demonstrating the importance of soil NO_3^- relative to soil NH_4^+ and HAA for N use by many tundra plants. These findings also enhance understanding of N competition among plant species and between plants and microbes in Arctic tundra ecosystems, and how that may affect changes in species community composition and productivity with climate change and N pollution.

Materials and Methods

Study Sites and Sampling. To evaluate in situ NO_3^- uptake and assimilation in terrestrial plants in relation to NO_3^- availability, we selected 18 sites (see descriptions in *SI Appendix*, Table S1) across a distinct gradient of soil NO_3^- (*SI Appendix*, Fig. S2), including one tropical and four subtropical sites in southwestern China; nine temperate sites in central, southern, and western Japan; and four Arctic tundra sites in northern Alaska. Among them, Tsukuba Forest Experimental Watershed (TKB) and Tama-Kyuryo Field Museum upper slope (TMU) and lower slope (TML) (*SI Appendix*, Table S1) are characterized by high soil NO_3^- or N saturation (49, 64, 65), while the Arctic sites TFS, Sagavanirktok River Valley (SAG), and IMT (*SI Appendix*, Table S1) are characterized by unmeasurable nitrification rates and negligible soil NO_3^- and, thus, are assumed to be typically low-NO_3^- ecosystems (*SI Appendix*, Fig. S2). In total, 28 plant species in the above study sites were sampled for fine roots (roughly <5 mm in diameter and <20 cm in spatial distribution of soil depth) or mature sunlit leaves. The studied plants in each ecosystem include dominant indigenous species (*SI Appendix*, Table S1). The design of this study allows us to evaluate plant NO_3^- use at the species and ecosystem levels.

Soil N Analyses. Soil N concentrations and net N transformation rates (mineralization and nitrification) were measured as indices of potentially available NO_3^- for both plants and soil microbes. Information on soil types and samplings, N variables, and corresponding methods used for each ecosystem are summarized in *SI Appendix*, Table S1. Concentrations of NO_3^- and NH_4^+ in soil solutions, extracts of fresh soils, and extracts of incubated soils (for net N mineralization and net nitrification rates) were determined colorimetrically. TEN was digested to NO_3^- using alkaline persulfate digestion and its concentration measured as NO_3^- on the autoanalyzer (specified in *SI Appendix*, Table S1). In-house standards (alanine, glycine, and histidine) dissolved in corresponding extracts were used for calibrating the concentrations of TEN and estimating the effect of the N blank from reagents (the same as that described in ref. 65). The soil extractable organic N was calculated as the difference between soil TEN and extractable inorganic N.

$\delta^{15}N$ and $\delta^{18}O$ ratios of soil NO_3^- were determined using the denitrifier (*Pseudomonas aureofaciens*) method (described in refs. 65 and 66) that converts NO_3^- to nitrous oxide (N_2O) (67, 68). The calibration curve between measured isotope ratios of N_2O and those of NO_3^- was prepared using US Geological Survey (USGS)-32, USGS-34, USGS-35, and International Atomic Energy Agency (IAEA) NO_3 standards. Soil NH_4^+ in 100-mL extracts of IMT soil was separated onto glass filter papers (GF/D; Whatman) using the diffusion method (69), and then the NH_4^+ diffused on the filter papers was measured for $\delta^{15}N$ values on an elemental analyzer coupled with an isotope ratio mass spectrometer (EA-IRMS) (70) at The Ecosystems Center, Marine Biological Laboratory (Woods Hole, MA). IAEA-N_2 was run with the samples to check the accuracy of $\delta^{15}N$-NH_4^+ data. The analytical precision was better than 0.2‰ for $\delta^{15}N$-NO_3^-, 0.5‰ for $\delta^{18}O$-NO_3^-, and 0.5‰ for $\delta^{15}N$-NH_4^+. The respective natural abundances of ^{15}N and ^{18}O were reported as $\delta^{15}N$ and $\delta^{18}O$ values expressed in per mille units, as $\delta^{15}N$ or $\delta^{18}O = [(R_{sample}/R_{standard}) - 1] \times 1{,}000$, where $R = {}^{15}N/{}^{14}N$ or $^{18}O/^{16}O$ and standards are atmospheric N_2 and standard mean ocean water for N and O, respectively.

Plant N Analyses. Leaf total N concentrations and total $\delta^{15}N$ values of plant samples were analyzed using an EA-IRMS (detailed in *SI Appendix*, Table S1). The analytical precision for $\delta^{15}N$ was better than 0.2‰. The leaf NRA assay, which has been used to evaluate the NO_3^--reduction potential of tundra plants [expressed per either fresh or dry weight (58, 71)], was conducted for plants at pristine and control sites of IMT, SAG, TFS-MAT (moist acidic tundra), TFS-MNT (moist non-acidic tundra), and at fertilized plots of TFS-MAT (*SI Appendix*, Table S1 and Fig. S3 *A* and *B*). The method of leaf NRA determination was the same as that described in refs. 58, 72, and 73. The NRA data (only those uniformly reported in dry weight) of natural terrestrial plants in low-latitude ecosystems were compiled (*SI Appendix*, Fig. S3*C*) for comparing NRA levels between tundra and low-latitude ecosystems.

The concentrations and $\delta^{15}N$ and $\delta^{18}O$ of NO_3^- in plants were measured using the sensitive denitrifier method (67, 68) at the Tokyo University of Agriculture and Technology (TUAT; method details are described in refs. 74 and 75). In the present study, 1 of 7 root samples of *Eriophorum vaginatum* and 7 of 94 leaf samples of tundra plants showed measurable NO_3^- concentration as zero, including 5 of 15 *Sphagnum* samples, 1 of 8 *Cassiope tetragona* leaf samples, and 1 of 1 *Juniperus communis* leaf sample.

The $\Delta^{17}O$ values of NO_3^- in plant leaves were determined by combining bacterial reduction [i.e., denitrifier method (67, 68)] and the thermal decomposition method (76). First, NO_3^- in plant extracts was converted to N_2O using the denitrifier method (67, 68) at TUAT (method details are described in refs. 74 and 75). Next, the gold-tube conversion of bacteria-produced N_2O into N_2 and O_2 was conducted, and $\Delta^{17}O$ values (defined as $\Delta^{17}O = [(1 + \delta^{17}O)/(1 + \delta^{18}O)^{\beta}] - 1$, where the constant β is 0.5247; see refs. 76 and 77) were measured on a Finnigan Delta Plus Advantage IRMS (Thermo Fischer Scientific) at the University of Washington (method details are described in ref. 78). A laboratory standard courtesy of Greg Michalski, Purdue University, West Lafayette, IN [$NaNO_3$ with $\Delta^{17}O$ = 19.9‰ (79)] and several standards that mimic the 5% and 10% of atmospheric NO_3^- (i.e., $\Delta^{17}O$ = 1‰ and 2‰, respectively) were used to check the precision of low $\Delta^{17}O$ samples. The average SDs for replicate analyses of an individual sample were ±0.2‰ for $\Delta^{17}O$.

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1. Chapin FS, III, Matson PA, Vitousek PM (2011) *Principles of Terrestrial Ecosystem Ecology* (Springer, New York), 2nd Ed.
2. Bloom AJ, Burger M, Rubio Asensio JS, Cousins AB (2010) Carbon dioxide enrichment inhibits nitrate assimilation in wheat and *Arabidopsis*. *Science* 328:899–903.
3. Reich PB, Hobbie SE, Lee TD (2014) Plant growth enhancement by elevated CO_2 eliminated by joint water and nitrogen limitation. *Nat Geosci* 7:920–924.
4. Schimel JP, Bennett J (2004) Nitrogen mineralization: Challenges of a changing paradigm. *Ecology* 85:591–602.
5. Mack MC, Schuur EA, Bret-Harte MS, Shaver GR, Chapin FS, III (2004) Ecosystem carbon storage in Arctic tundra reduced by long-term nutrient fertilization. *Nature* 431:440–443.
6. Schuur EA, et al. (2015) Climate change and the permafrost carbon feedback. *Nature* 520:171–179.
7. Choudhary S, Blaud A, Osborn AM, Press MC, Phoenix GK (2016) Nitrogen accumulation and partitioning in a High Arctic tundra ecosystem from extreme atmospheric N deposition events. *Sci Total Environ* 554-555:303–310.
8. Shaver GR, et al. (2001) Species composition interacts with fertilizer to control long term change in tundra productivity. *Ecology* 82:3163–3181.
9. Hill PW, et al. (2011) Vascular plant success in a warming Antarctic may be due to efficient nitrogen acquisition. *Nat Clim Chang* 1:50–53.
10. Alexander V, Whalen SC, Klingensmith KM (1989) Nitrogen cycling in arctic lakes and ponds. *Hydrobiologia* 172:165–172.
11. Chapin FS, III, Shaver GR, Kedrowski RA (1986) Environmental controls over carbon, nitrogen and phosphorus fractions in *Eriophorum vaginatum* in Alaskan tussock tundra. *J Ecol* 74:167–195.
12. Chapin FS, III, Moilanen L, Kielland K (1993) Preferential use of organic nitrogen for growth by a nonmycorrhizal arctic sedge. *Nature* 361:150–153.

13. McKane RB, et al. (2002) Resource-based niches provide a basis for plant species diversity and dominance in arctic tundra. *Nature* 415:68–71.
14. Yano Y, Shaver GR, Giblin AE, Rastetter EB, Nadelhoffer KJ (2010) Nitrogen dynamics in a small arctic watershed: Retention and downhill movement of ^{15}N. *Ecol Monogr* 80:331–351.
15. Yano Y, Shaver GR, Rastetter EB, Giblin AE, Laundre JA (2013) Nitrogen dynamics in arctic tundra soils of varying age: Differential responses to fertilization and warming. *Oecologia* 173:1575–1586.
16. Hobbie SE, Nadelhoffer KJ, Högberg P (2002) A synthesis: The role of nutrients as constraints on carbon balances in boreal and arctic regions. *Plant Soil* 242:163–170.
17. Davidson EA, Hart SC, Firestone MK (1992) Internal cycling of nitrate in soils of a mature coniferous forest. *Ecology* 73:1148–1156.
18. Atkin OK (1996) Reassessing the nitrogen relations of arctic plants: A mini-review. *Plant Cell Environ* 19:695–704.
19. Stark JM, Hart SC (1997) High rates of nitrification and nitrate turnover in undisturbed coniferous forests. *Nature* 385:61–64.
20. Finger RA, et al. (2016) Effects of permafrost thaw on nitrogen availability and plant-soil interactions in a boreal Alaskan lowland. *J Ecol* 104:1542–1554.
21. Hobbie JE, Hobbie EA (2006) ^{15}N in symbiotic fungi and plants estimates nitrogen and carbon flux rates in Arctic tundra. *Ecology* 87:816–822.
22. Hobbie SE, Chapin FS, III (1998) The response of tundra plant biomass, aboveground production, nitrogen, and CO_2 flux to experimental warming. *Ecology* 79:1526–1544.
23. Scheurwater I, Koren M, Lambers H, Atkin OK (2002) The contribution of roots and shoots to whole plant nitrate reduction in fast- and slow-growing grass species. *J Exp Bot* 53:1635–1642.
24. Iversen CM, et al. (2015) The unseen iceberg: Plant roots in arctic tundra. *New Phytol* 205:34–58.
25. Weintraub MN, Schimel JP (2003) Interactions between carbon and nitrogen mineralization and soil organic matter chemistry in arctic tundra soils. *Ecosystems* 6: 129–143.
26. Weintraub MN, Schimel JP (2005) Nitrogen cycling and the spread of shrubs control changes in the carbon balance of Arctic tundra ecosystems. *Bioscience* 55:408–415.
27. Sistla SA, Asao S, Schimel JP (2012) Detecting microbial N-limitation in tussock tundra soil: Implications for Arctic soil organic carbon cycling. *Soil Biol Biochem* 55:78–84.
28. Schimel JP, Chapin FS, III (1996) Tundra plant uptake of amino acid and NH_4^+ nitrogen *in situ*: Plants complete well for amino acid N. *Ecology* 77:2142–2147.
29. Koch GW, Bloom AJ, Chapin FS, III (1991) Ammonium and nitrate as nitrogen sources in two *Eriophorum* species. *Oecologia* 88:570–573.
30. Kielland K (1994) Amino-acid absorption by arctic plants: Implications for plant nutrition and nitrogen cycling. *Ecology* 75:2373–2383.
31. Nordin AI, Schmidt K, Shaver GR (2004) Nitrogen uptake by arctic soil microbes and plants in relation to soil nitrogen supply. *Ecology* 85:955–962.
32. Chapin FS, III, Kedrowski RA (1983) Seasonal changes in nitrogen and phosphorus fractions and autumn retranslocation in evergreen and deciduous taiga trees. *Ecology* 64:376–391.
33. Harms TK, Jones JB, Jr (2012) Thaw depth determines reaction and transport of inorganic nitrogen in valley bottom permafrost soils: Nitrogen cycling in permafrost soils. *Glob Change Biol* 18:2958–2968.
34. Van Cleve K, Viereck LA (1981) *Forest Succession in Relation to Nutrient Cycling in the Boreal Forest of Alaska* (Springer, New York), pp 185–211.
35. Leadley PW, Reynolds JF, Chapin FS, III (1997) A model of nitrogen uptake by *Eriophorum vaginatum* roots in the field: Ecological implications. *Ecol Monogr* 67:1–22.
36. Hipkin CR, Simpson DJ, Wainwright SJ, Salem MA (2004) Nitrification by plants that also fix nitrogen. *Nature* 430:98–101.
37. Yamasaki H, et al. (2011) Nitric oxide synthase-like activities in plants. *Nitrogen Metabolism in Plants in the Post-Genomic Era*, Annual Plant Reviews, eds Foyer CH, Zhang H (Blackwell Publishing Ltd, West Sussex, UK), Vol 42, pp 103–125.
38. Mur LAJ, et al. (2013) Nitric oxide in plants: An assessment of the current state of knowledge. *AoB Plants* 5:pls052.
39. Limami AM, Diab H, Lothier J (2014) Nitrogen metabolism in plants under low oxygen stress. *Planta* 239:531–541.
40. Kuruthukulangarakoola GT, et al. (2017) Nitric oxide-fixation by non-symbiotic haemoglobin proteins in *Arabidopsis thaliana* under N-limited conditions. *Plant Cell Environ* 40:36–50.
41. Rockel P, Strube F, Rockel A, Wildt J, Kaiser WM (2002) Regulation of nitric oxide (NO) production by plant nitrate reductase in vivo and in vitro. *J Exp Bot* 53:103–110.
42. Perazzolli M, et al. (2004) Arabidopsis nonsymbiotic hemoglobin AHb1 modulates nitric oxide bioactivity. *Plant Cell* 16:2785–2794.
43. Neill S, et al. (2008) Nitric oxide evolution and perception. *J Exp Bot* 59:25–35.
44. Romero-Puertas MC, Sandalio LM (2016) Role of NO-dependent posttranslational modifications in switching metabolic pathways. *Adv Bot Res* 77:123–144.
45. Zaehle S (2013) Terrestrial nitrogen-carbon cycle interactions at the global scale. *Philos Trans R Soc Lond B Biol Sci* 368:20130125.
46. Michalski G, Scott Z, Kabiling M, Thiemens MH (2003) First measurements and modeling of $\Delta^{17}O$ in atmospheric nitrate. *Geophys Res Lett* 30:1870.
47. Michalski G, et al. (2004) Tracing atmospheric nitrate deposition in a complex semi-arid ecosystem using $\Delta^{17}O$. *Environ Sci Technol* 38:2175–2181.
48. Dejwakh NR, Meixner T, Michalski G, McIntosh J (2012) Using ^{17}O to investigate nitrate sources and sinks in a semi-arid groundwater system. *Environ Sci Technol* 46: 745–751.
49. Fang Y, et al. (2015) Microbial denitrification dominates nitrate losses from forest ecosystems. *Proc Natl Acad Sci USA* 112:1470–1474.
50. Liu XY, Koba K, Makabe A, Liu CQ (2014) Nitrate dynamics in natural plants: Insights based on the concentration and natural isotope abundances of tissue nitrate. *Front Plant Sci* 5:355.
51. Tcherkez G, Farquhar GD (2006) Viewpoint: Isotopic fractionation by plant nitrate reductase, twenty years later. *Funct Plant Biol* 33:531–537.
52. Carlisle E, Yarnes C, Toney MD, Bloom AJ (2014) Nitrate reductase ^{15}N discrimination in *Arabidopsis thaliana, Zeamays, Aspergillus niger, Picheaangusta*, and *Escherichia coli*. *Front Plant Sci* 3:195.
53. Yano Y, Shaver GR, Giblin AE, Rastetter EB (2010) Depleted ^{15}N in hydrolysable-N of arctic soils and its implication for mycorrhizal fungi-plant interaction. *Biogeochemistry* 97:183–194.
54. Heikoop JM, et al. (2015) Isotopic identification of soil and permafrost nitrate sources in an arctic tundra ecosystem. *J Geophys Res* 120:1000–1017.
55. Wynn PM, Hodson AJ, Heaton THE, Chenery SR (2007) Nitrate production beneath a high arctic glacier, Svalbard. *Chem Geol* 244:88–102.
56. Morin S, et al. (2012) An isotopic view on the connection between photolytic emissions of NO_x from the Arctic snowpack and its oxidation by reactive halogens. *J Geophys Res* 117:D00R08.
57. Kendall C, Elliott EM, Wankel SD (2007) Tracing anthropogenic inputs of nitrogen to ecosystems. *Stable Isotopes in Ecology and Environmental Science*, eds Michener R, Lajtha K (Blackwell Publishing, Oxford, UK), 2nd Ed, pp 375–449.
58. Nadelhoffer K, et al. (1996) ^{15}N natural abundances and N use by tundra plants. *Oecologia* 107:386–394.
59. Parnell A, Jackson A (2008) SIAR: Stable isotope analysis in R, Version 4.2. Available at cran.r-project.org/web/packages/siar/index.html. Accessed April 19, 2016.
60. Evans JSBT, Handley SJ, Perham N, Over DE, Thompson VA (2000) Frequency versus probability formats in statistical word problems. *Cognition* 77:197–213.
61. Moore JW, Semmens BX (2008) Incorporating uncertainty and prior information into stable isotope mixing models. *Ecol Lett* 11:470–480.
62. Hobbie EA, Hobbie JE (2008) Natural abundance of ^{15}N in nitrogen-limited forests and tundra can estimate nitrogen cycling through mycorrhizal fungi: A review. *Ecosystems* 11:815–830.
63. Craine JM, et al. (2009) Global patterns of foliar nitrogen isotopes and their relationships with climate, mycorrhizal fungi, foliar nutrient concentrations, and nitrogen availability. *New Phytol* 183:980–992.
64. Tabayashi Y, Koba K (2011) Heterogeneous atmospheric nitrogen deposition effects upon the nitrate concentration of stream waters in a forested mountain area. *Water Air Soil Pollut* 216:105–115.
65. Takebayashi Y, Koba K, Sasaki Y, Fang Y, Yoh M (2010) The natural abundance of ^{15}N in plant and soil-available N indicates a shift of main plant N resources to NO_3^- from NH_4^+ along the N leaching gradient. *Rapid Commun Mass Spectrom* 24:1001–1008.
66. Koba K, et al. (2012) ^{15}N natural abundance of the N lost from an N-saturated subtropical forest in southern China. *J Geophys Res* 117:G02015.
67. Sigman DM, et al. (2001) A bacterial method for the nitrogen isotopic analysis of nitrate in seawater and freshwater. *Anal Chem* 73:4145–4153.
68. Casciotti KL, Sigman DM, Hastings MG, Böhlke JK, Hilkert A (2002) Measurement of the oxygen isotopic composition of nitrate in seawater and freshwater using the denitrifier method. *Anal Chem* 74:4905–4912.
69. Koba K, Inagaki K, Sasaki Y, Takebayashi Y, Yoh M (2010) Nitrogen isotopic analysis of dissolved inorganic and organic nitrogen in soil extracts. *Earth, Life and Isotopes*, eds Ohkouchi N, Tayasu I, Koba K (Kyoto Univ Press, Kyoto), pp 17–37.
70. Fry B, et al. (1996) Cryoflow: Cryofocusing nanomole amounts of CO_2, N_2, and SO_2 from an elemental analyzer for stable isotopic analysis. *Rapid Commun Mass Spectrom* 10:953–958.
71. Atkin OK, Cummins WR (1994) The effect of root temperature on the induction of nitrate reductase activites and nitrogen uptake rates in arctic plant species. *Plant Soil* 159:187–197.
72. Koba K, et al. (2003) Natural ^{15}N abundance of plants and soil N in a temperate coniferous forest. *Ecosystems* 6:457–469.
73. Koyama L, Kielland K (2011) Plant physiological responses to hydrologically mediated changes in nitrogen supply on a boreal forest floodplain: A mechanism explaining the discrepancy in nitrogen demand and supply. *Plant Soil* 342:129–139.
74. Liu XY, et al. (2012) Preliminary insights into $\delta^{15}N$ and $\delta^{18}O$ of nitrate in natural mosses: A new application of the denitrifier method. *Environ Pollut* 162:48–55.
75. Liu XY, et al. (2013) Dual N and O isotopes of nitrate in natural plants: First insights into individual variability and organ-specific pattern. *Biogeochemistry* 114:399–411.
76. Kaiser J, Hastings MG, Houlton BZ, Röckmann T, Sigman DM (2007) Triple oxygen isotope analysis of nitrate using the denitrifier method and thermal decomposition of N_2O. *Anal Chem* 79:599–607.
77. Miller MF (2002) Isotopic fractionation and the quantification of ^{17}O anomalies in the oxygen three-isotope system: An appraisal and geochemical significance. *Geochim Cosmochim Acta* 66:1881–1889.
78. Costa AW, et al. (2011) Analysis of atmospheric inputs of nitrate to a temperate forest ecosystem from $\Delta^{17}O$ isotope ratio measurements. *Geophys Res Lett* 38: L15805–L15810.
79. Michalski G (2010) Purification procedure for delta $\delta^{15}N$, $\delta^{18}O$, $\Delta^{17}O$ analysis of nitrate. *Int J Environ Anal Chem* 90:586–590.