Isotopic evidence for large gaseous nitrogen losses from tropical rainforests

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The nitrogen isotopic composition (15N/14N) of forested ecosystems varies systematically worldwide. In tropical forests, which are elevated in 15N relative to temperate biomes, a decrease in ecosystem 15N/14N with increasing rainfall has been reported. This trend is seen in a set of well-characterized Hawaiian rainforests, across which we have measured the 15N/14N of inputs and hydrologic losses. We report that the two most widely purported mechanisms, an isotopic shift in N inputs or isotopic discrimination by leaching, fail to explain this climate-dependent trend in 15N/14N. Rather, isotopic discrimination by microbial denitrification appears to be the major determinant of N isotopic variations across different climates. In the driest climates, the 15N/14N of total dissolved outputs is higher than that of inputs, which can only be explained by a 14N-rich gas loss. In contrast, in the wettest climates, denitrification completely consumes nitrate in local soil environments, thus preventing the expression of its isotope effect at the ecosystem scale. Under these conditions, the 15N/14N of bulk soils and stream outputs decrease to converge on the low 15N/14N of N inputs. N isotope budgets that account for such local isotopic underexpression suggest that denitrification is responsible for a large fraction (24–53%) of total ecosystem N loss across the sampled range in rainfall.

Fig. 1. Conceptual model of controls on forest 15N/14N; soil N and plant N are boxed. I, nitrogen input flux to the forest; H and G, hydrologic leaching and gaseous fluxes, respectively; U and R, plant fluxes (uptake and return, respectively). eU, eH, and eI are effective (i.e., expressed) isotope effects for plant uptake and external hydrologic and gaseous losses, respectively. (e in ‰) = (15/14)% - 1, where k is the rate constant. Under steady-state conditions, N inputs into plants must balance the losses from it. Hence, the same flux-weighted 15N that plants take up (I) is returned to the soil (R), resulting in no net change in soil 15N by plant uptake processes. By contrast, losses of N from the external environment that fractionate against soil N isotopes (i.e., eI or eH) can lead to an elevation in soil 15N relative to inputs. Consequently, at steady state, soil N isotopes can be modeled independently of plants: 15Ntotal = 15Ninput + eH(H + G) + eG(G/H + G). In this study, we test which of the following best explains the soil 15N variation across a rainfall sequence of tropical forests: (i) the 15N of inputs, (ii) N isotope discrimination during hydrologic leaching (eH), or (iii) discrimination during gaseous N loss (eG).

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Abbreviations: TDN, total dissolved N; MAP, mean annual precipitation.

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Results and Discussion

We measured over 4 years (14 events distributed across seasons and hydrological conditions) the $^{15}$N/$^{14}$N of NO$_3^-$ and of total dissolved N (TDN) in precipitation and cloud water inputs, in stream water losses, and in soil waters and extracts. In Fig. 1 we show that the assumption of steady state N pools permits us to exclude effects of plant N uptake, because this process only impacts bulk soil N isotopes under transient conditions such as net N accumulation (7). On the basis of previous work (11), we assume that the N contributed by biological N$_2$ fixation has a $^{15}$N/$^{14}$N close to that of air [i.e., its $\delta^{15}$N is 0‰, where $\delta^{15}$N (per mil, ‰, vs. air) = ($^{15}$N/$^{14}$N$_{\text{sample}}$/$^{15}$N/$^{14}$N$_{\text{air}}$ - 1)×1,000]. Combining these constraints on N isotope budgets with the isotope data for bulk soils and plants from Schuur and Matson (6), we test the competing hypotheses for the elevation of the $^{15}$N/$^{14}$N in these forests relative to atmospheric N$_2$ and for the observed $^{15}$N/$^{14}$N decrease in soil and plant N with increasing MAP (Fig. 1).

Elimination of Two Competing Hypotheses. Neither the forms of N in bulk deposition nor its $^{15}$N/$^{14}$N changed systematically with rainfall (Fig. 2 d and f). Concentrations of all dissolved N forms were exceedingly low (<3 µmol per liter) in the rainfall but did not change in relative contribution across the sequence (Fig. 2 f). The $^{15}$N/$^{14}$N of TDN in deposition was statistically indistinguishable from 0‰ across the sequence (one-tailed t test, $P > 0.15$; $n = 42$) and thus similar to the isotopic ratio of N$_2$ fixation (indicated by the dashed line in Fig. 2 d). In addition, cloud water $^{15}$N/$^{14}$N did not differ significantly from either bulk deposition or N$_2$ fixation (one-tailed t test, $P > 0.10$; $n = 6$) (Fig. 2 f). Thus, we found no evidence for our first hypothesis: that a trend in the $^{15}$N/$^{14}$N of N inputs explains the decline in soil $^{15}$N/$^{14}$N with rainfall.
The $^{15}\text{N}/^{14}\text{N}$ of bulk soil (and thus of forest-integrated N) was substantially elevated relative to inputs across all but the two wettest forests (Fig. 3a). Such a difference between inputs and internal pools requires a pathway of preferential $^{14}\text{N}$ loss from forests receiving $\geq 4,050$ mm of MAP, which acts to elevate forest $^{15}\text{N}/^{14}\text{N}$ above the $^{15}\text{N}/^{14}\text{N}$ of the inputs to these forests (Fig. 1). However, we found no evidence for our second hypothesis: that the trend in soil $^{15}\text{N}/^{14}\text{N}$ was caused by decreasing isotopic fractionation of N leaching losses with increasing rainfall (3–8, 11). The forms of dissolved N in stream water losses changed sharply with rainfall: $\text{NO}_3$ dominated in streams draining drier forests ($\leq 2,750$ mm of MAP), whereas DON (TDN minus inorganic N) was the most important form of N in streams draining wet forests (Fig. 2c). This pattern indicates that $\text{NO}_3$ accumulates in the drier forests to the point where $\text{NO}_3$ leaching occurs, whereas only DON leaks from the wet forest ecosystems. Despite this shift in N forms, the $^{15}\text{N}/^{14}\text{N}$ of stream water TDN changed in a manner similar to the trend in bulk soils (Figs. 2a and 3a). In fact, we found only slight differences in $^{15}\text{N}/^{14}\text{N}$ between stream losses and soils, most notably in forests with $\leq 2,500$ mm of MAP, where the $^{15}\text{N}$ of stream water TDN was 1–2‰ lower than soils. This isotopic difference between streams and soils, if anything, should cause an increase (rather than the observed decrease) in $^{15}\text{N}/^{14}\text{N}$ forest soils with increasing MAP.

Given that these forests can be assumed to be in steady state with respect to total N inputs and losses (14, 15), the $^{15}\text{N}/^{14}\text{N}$ of total losses must balance that of total inputs (Fig. 1). The $^{15}\text{N}/^{14}\text{N}$ ratios of leaching losses were consistently higher.

Fig. 3. Nitrogen isotope ratios and chemistry in rainforest soils. (a) $^{15}\text{N}/^{14}\text{N}$ of the top 50 cm of bulk soil (14). (b and c) $^{15}\text{N}/^{14}\text{N}$ of TDN (b) and of $\text{NO}_3$ (c) in soil solution from a 35-cm depth. (d) Soil solution N species. Symbols are the same as in Fig. 2. (e) Relationship between soil solution nitrate $^{15}\text{N}/^{14}\text{N}$ and $^{18}\text{O}/^{16}\text{O}$ across all sites. (f) $^{15}\text{N}/^{14}\text{N}$ of $\text{NO}_3$ vs. the natural logarithm of soil $\text{NO}_3$ concentration in a series of cores designed to eliminate processes other than denitrification, such as nitrification (by addition of N serve) and plant uptake (by removal of plant roots) (see Supporting Materials and Methods).
both $^{15}$N and $^{18}$O during NO$_3^-$ found in terrestrial systems only when denitrification contributes to the estimated initial NO$_3^-$ concentration. We estimated the initial NO$_3^-$ concentration and $^{15}$N/$^{14}$N as follows: for the incubation experiment (see Supporting Materials and Methods and Fig. 3f), we used the highest extractable NO$_3^-$ concentration and its corresponding $^{15}$N/$^{14}$N; for soil extracts, we used the mean of the five highest NO$_3^-$ concentrations and their corresponding $^{15}$N/$^{14}$N; for soil water, we used the mean of the five highest soil water NO$_3^-$ concentrations and their corresponding $^{15}$N/$^{14}$N; for streams, we used the mean of the five highest stream water NO$_3^-$ concentrations and their corresponding $^{15}$N/$^{14}$N. The organism-level isotope effect line was calculated using an approximate form of the Rayleigh equation, $\delta^{15}$NO$_3^-$ = $\delta^{15}$NO$_3^{initial}$ - $e(\ln f)$ (20), and an intrinsic $e$ of 29% (23, 24).

Proof of Denitrification. Examination of the isotope ratios of NO$_3^-$ and TDN in local soil solutions and extracts indicates that soil denitrification was an important process in these forest soils. In both soil extracts and lysimeter samples, the $^{15}$N of NO$_3^-$ was substantially elevated above the $\approx 0%e$ average input (Fig. 3c and Fig. 6), which is published as supporting information on the PNAS web site; see also ref. 16). The $^{15}$N/$^{14}$N of soil water TDN (Fig. 3b) tracked this $^{15}$N enrichment in NO$_3^-$, with highest values and greatest range in the $^{15}$N/$^{14}$N in the wetter forests. This similarity in $^{15}$N/$^{14}$N between NO$_3^-$ and TDN indicates that dissimilatory microbial reduction of NO$_3^-$ to NH$_4^+$ (17) or some other aspect of internal cycling could not explain the local $^{15}$N enrichment in NO$_3^-$. Previous work indicates that, under the nutrient conditions that characterize these forests, plant uptake of dissolved inorganic N does not appear to discriminate between the N isotopes (11).

$^{15}$N/$^{14}$N and $^{18}$O/$^{16}$O ratios of NO$_3^-$ in soil solutions were highly correlated ($R^2 = 0.76; n = 44; P < 0.0001$) within and across forests (Fig. 3e). Such a correlation has previously been found in terrestrial systems only when denitrification contributes actively to N loss (18, 19); it is caused by discrimination against both $^{15}$N and $^{18}$O during NO$_3^-$ reduction. Moreover, the slope of the relationship (0.66) was similar to that associated with denitrification in groundwater systems (0.49–0.67) (18, 19).

Soil extracts (2 M KCl) indicated that the $^{15}$N/$^{14}$N ratios of extracted NO$_3^-$ were markedly elevated relative to those of extracted NH$_4^+$ in all but the shallowest forest soils (16). Without denitrification, NO$_3^-$ that accumulates in soils would be lower in $^{15}$N/$^{14}$N than NH$_4^+$ as a consequence of fractionation during nitrification of NH$_4^+$ to NO$_3^-$ (9). Moreover, the soil extracts revealed decreasing NO$_3^-$ concentrations and sharply increasing $^{15}$N/$^{14}$N ratios from shallow to deep soils (Fig. 6). The most dramatic trends occurred in the wettest sites, where profiles of soil O$_2$ availability (6) show sharp declines with depth, approaching levels that favor anaerobic metabolism. To our knowledge, our measurement of a $\delta^{15}N$ of 180‰ for NO$_3^-$ extracted from soil at a 35-cm depth in the 4,050-mm MAP forest is the highest $\delta^{15}$N ever reported from a natural soil system. This parallel decline in NO$_3^-$ concentration, increase in the $^{15}$N/$^{14}$N and $^{18}$O/$^{16}$O of NO$_3^-$, and decrease in O$_2$ availability with soil depth is highly suggestive of active denitrification (16).

Finally, the presence of measurable NO$_3^-$ in soil extracts and soil solutions from wet forests (>2,750 mm; Fig. 3d), coupled with the virtual absence of NO$_3^-$ in streams draining the same forests (Fig. 2c), suggests that denitrification occurred along the path of water flow from local soil solutions to watershed streams.

Scale Dependence of Isotopic Imprint. Although denitrification raises the $^{15}$N/$^{14}$N of soil N in our forests relative to atmospheric inputs, we have not yet resolved why this isotope ratio in soil organic matter decreases systematically with rainfall, especially because the greatest $^{15}$N enrichment of NO$_3^-$ and TDN in soil waters and soil extracts is observed in the wettest forests. We must consider how the isotope effect of denitrification, a local process, is expressed at the largest scales, in the streams and bulk soils. Partial consumption of NO$_3^-$ (“open-system kinetics”) leaves behind $^{15}$N-enriched NO$_3^-$, which can diffuse out of the

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**Fig. 4.** Isotopic expression of denitrification across sampling scales. The x axis is $f$, the fraction of nitrate remaining, which is the ratio of measured NO$_3^-$ to the estimated initial NO$_3^-$ concentration. The y axis is $\delta^{15}$NO$_3^-$ (% vs. air).

"Wet" ≤ "Dry"
zone of ongoing denitrification and enter the larger plant–soil N cycle. Complete NO$_3^-$ consumption (“closed-system kinetics”) would cause underexpression of the isotope effect at larger scales (21, 22), because little or no $^{15}$N-rich NO$_3^-$ would escape the zone of denitrification to elevate the $^{15}$N/$^{14}$N of the larger plant–soil system.

We found evidence of such scale dependence of isotope effect expression in our sites. When we plot $\delta^{15}$N vs. the fraction of NO$_3^-$ consumed locally (see the legend of Fig. 4 for the calculation method), we find that the expressed isotope effect of denitrification decreased with increasing scale: the theoretical organism-level isotope effect (23, 24) (black line in Fig. 4) > local soil solution extracts (circles and squares) > water intercepted below rooting zones (triangles) > first-order watershed streams (inverted triangles). Moreover, the scale-dependent isotope effect underexpression was greatest in the wettest sites. Soil extracts and soil waters showed the greatest (and most variable) $^{15}$N enrichment of NO$_3^-$ and TDN in the wettest sites (Figs. 3b, c, e, and f and 6), but the stream waters and bulk soils, which integrate over the ecosystem-scale N budget (Fig. 1), displayed little to no $^{15}$N enrichment relative to inputs in these sites (Figs. 2a and b and 3a). The absence of detectable NO$_3^-$ in streams from the wettest forest provides a final indication that denitrification consumed NO$_3^-$ locally, preventing isotopic expression at the watershed scale. We conclude that increasingly complete NO$_3^-$ consumption by denitrification causes the observed decline in ecosystem $^{15}$N/$^{14}$N with increasing rainfall.

**An Isotope-Balance Approach for Reconstructing Pathways of N Loss.** We apply an isotope-balance approach to partition the relative magnitudes of gaseous vs. hydrologic N losses across forests. We present two calculations, which derive from the same mass-balance equation but differ in the degree to which they incorporate the above findings of isotopic underexpression (see the legend of Fig. 5; see also Supporting Materials and Methods, which is published as supporting information on the PNAS web site).

In the most conservative case, we assume no isotopic underexpression of denitrification [i.e., the ecosystem-level isotope effect is taken as 20‰ (23, 24)], which allows us to place a lower bound on the proportion of N loss by means of denitrification. This calculation (Fig. 5a) shows that denitrification constitutes $\approx$20% (and hydrological losses constitute $\approx$80%) of total N losses in sites with <4,050 mm of MAP. However, the fraction of N lost to denitrification drops to 0% in the 4,050-mm MAP site because, as discussed above, the organism-level isotope effect of denitrification is not expressed in streams draining our wettest forests. Given our evidence for underexpression of the denitrification isotope effect (Fig. 4), we believe that this calculation shows the lower limit of the true importance of gaseous N loss in the wettest sites.

In the second calculation, we seek to account for local isotopic underexpression of denitrification by using the $^{15}$N/$^{14}$N of NO$_3^-$ from soil solution extracts collected from beneath the plant-rooting zone, coupled with an empirically determined denitrification isotope effect to derive the $^{15}$N of gaseous N loss (see the legend of Fig. 5). The field-calibrated isotope effect was intended to match the degree of underexpression observed at scales of soil waters: we used in situ cores, removing the effects of plant N uptake (by excluding plant roots) and nitrification (by additions of N serve, i.e., nitrapyrin) (Supporting Materials and Methods). The $^{15}$N/$^{14}$N of NO$_3^-$ in these core incubations displayed a strong relationship of $^{15}$N enrichment with the logarithm of the NO$_3^-$ consumed, consistent with consumption of a closed NO$_3^-$ pool (ref. 20 and Fig. 3f). The slope (13.2‰) of this relationship ($n = 13; R^2 = 0.97; P < 0.0001$) identifies an empirical isotope effect that is lower than the organism-level isotope effect of (of $\approx$20‰; refs. 23 and 24); this result offers further evidence of heterogeneity in isotope expression within our soils.

Applying this empirical isotope effect to the soil water TDN-$^{15}$N data (as opposed to the stream TDN-$^{15}$N data in the calculation), we calculate that denitrification accounts for 24–53% of total N losses across our forests (Fig. 5a, circles connected by solid line). The estimates for the three driest sites ($\approx$25%) are almost identical to the stream-based approach. However, for the 3,350- and 4,050-mm MAP sites, denitrification increased substantially above the stream-based estimates to $\approx$50% of total N losses.

By combining these calculations with estimates of dissolved N losses, we can derive total fluxes of both gaseous and hydrological N losses. In Fig. 5b we show that total gas N fluxes ($\approx$2–9 kg of N per hectare per year) are appreciable when compared with the range of total N inputs (including biological N$_2$ fixation, rain, and cloud water deposition) of $\approx$6 kg of N per hectare per year in Hawaiian forests which are not immediately downwind of active volcanoes (25, 15). In the drier forests, our fluxes do not differ
substantially from short-term, chamber-based measures of oxidized N (Fig. 5b; ref. 26 and P. A. Matson, personal communication). However, in wet forests, our estimates are ~10 times higher than chamber-based measurements. This difference could be caused by episodic denitrification (e.g., linked to rainfall events) that is not captured by chamber methods. Alternatively, and perhaps more likely, denitrification is converting almost all of the NO₂ to N₂ in the wettest sites, consistent with expectations for denitrification under low-O₂ conditions (27).

Conclusions

We conclude that the pattern of decreasing ecosystem ¹⁵N/¹⁴N with increasing rainfall cannot be explained by changes in N inputs (9, 10) or preferential leaching of ¹⁴N to streams (3–8, 11). Rather, gaseous N loss is the major influence on forest ¹⁵N/¹⁴N across the climate gradient, with denitrification acting as a dominant pathway. In the drier sites, incomplete nitrate consumption by denitrification causes the elevation of bulk soil ¹⁵N/¹⁴N relative to atmospheric N₂ and the measured N inputs to the forests. In the wettest sites, complete denitrification prevents its isotopic expression at larger scales, explaining why the ¹⁵N/¹⁴N ratios of bulk soil N and stream losses decrease with rainfall and converge on the ¹⁵N/¹⁴N ratios of the inputs. Our findings provide evidence that denitrification can be a major vector of N loss from tropical rainforests (i.e., ~24–53% of total N loss), with a large impact in overall forest N balances. To the extent that these findings apply generally to tropical forests, they identify a poorly resolved pathway in the global N budget.

Methods

We collected bulk deposition in polypropylene funnels connected with silicon tubing to high-density polyethylene bottles. We sampled cloud water by an active collector mounted on a telescoping tower 15 m above the ground (25). We collected soil water from silica gel lysimeters at a 35-cm depth by using slight vacuum pressure (32 cm of Hg) over 72 h (14). Streams were sampled with syringes. Samples were immediately filtered through precleaned glass fiber filters (Gelman A/E, 1.0-μm nominal pore size). Chemical methods for the following were as described in ref. 14: ion chromatography for NO₃⁻, colorimetry for NH₄⁺, and persulfate oxidation followed by colorimetry or furnace combustion for TDN. The ¹⁵N/¹⁴N and ¹⁸O/¹⁰O ratios of NO₃ were analyzed by using the denitrifier method (28, 29). ¹⁵N/¹⁴N of TDN was analyzed by persulfate oxidation followed by the denitrifier method (30). (See Supporting Materials and Methods.)

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