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Stable nitrogen isotope patterns of trees and soils altered by long-term nitrogen and phosphorus addition to a lowland tropical rainforest

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Abstract Foliar nitrogen (N) isotope ratios ($\delta^{15}N$) are used as a proxy for N-cycling processes, including the "openness" of the N cycle and the use of distinct N sources, but there is little experimental support for such proxies in lowland tropical forest. To address this, we examined the $\delta^{15}N$ values of soluble soil N and canopy foliage of four tree species after 13 years of factorial N and P addition to a mature lowland rainforest. We hypothesized that N addition would lead to ^{15}N-enriched soil N forms due to fractionating losses, whereas P addition would reduce N losses as the plants and microbes adjusted their stoichiometric demands. Chronic N addition increased the concentration and $\delta^{15}N$ value of soil nitrate and $\delta^{15}N$ in live and senesced leaves in two of four tree species, but did not affect ammonium or dissolved organic N. Phosphorus addition significantly increased foliar $\delta^{15}N$ in one tree species and elicited significant N × P interactions in two others due to a reduction in foliar $\delta^{15}N$ enrichment under N and P co-addition. Isotope mixing models indicated that three of four tree species increased their use of nitrate relative to ammonium following N addition, supporting the expectation that tropical trees use the most available form of mineral N. Previous observations that anthropogenic N deposition in this tropical region have led to increasing foliar $\delta^{15}N$ values over decadal time-scales is now mechanistically linked to greater usage of ^{15}N-enriched nitrate.

Keywords Ecosystem ecology · Gigante Fertilization Experiment · Mass balance mixing models · Panama · Stoichiometry

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Introduction

Stable N isotope ratios (^{15}N:^{14}N expressed as $\delta^{15}N$) provide a non-destructive tracer that integrates plant

responses to their abiotic and biotic environments through time (Dawson et al. 2002). Several studies have demonstrated a relationship between ecosystem $\delta^{15}N$ values and the "openness" of the N cycle (e.g. a ratio of production and demand to loss). For instance, $\delta^{15}N$ measurements have been used to trace pathways of forest N losses in gaseous form (N_2O and N_2) or in leachate [NO_3^- and dissolved organic N (DON)] at both local (Houlton et al. 2006; Brookshire et al. 2012; Koehler et al. 2009) and regional scales (Bai and Houlton 2009). High rates of N loss influence $\delta^{15}N$ in the remaining soil pools in part through the strongly fractionating process of denitrification (Pérez et al. 2000) which preferentially consumes ^{14}N and can yield relatively ^{15}N-enriched soil nitrate remaining in soil solutions (reviewed in Hobbie and Ouimette 2009). This is a main mechanism whereby a "loosening" of the N cycle leads to enriched soil and plant $\delta^{15}N$ values. The ability of foliar $\delta^{15}N$ to act as an N cycling proxy has also enabled detection of regional increases in N availability due to agricultural intensification (Park et al. 2011), succession (Wang et al. 2007; Hobbie et al. 2005), afforestation (Davidson et al. 2007), and atmospheric deposition (Hietz et al. 2011).

Others studies have shown that plant $\delta^{15}N$ values trace uptake of specific N forms, even in N-rich tropical rainforests. For example, plant $\delta^{15}N$ values reflected a community-wide switch between two mineral N sources along a precipitation gradient in Hawaii (Houlton et al. 2006; Schuur and Matson 2001) and among spatially distinct N sources in litter and mineral soils of French Guiana (Schimann et al. 2008). Experimental additions of ^{15}N-enriched tracers have shown that plants prefer mineral over organic forms of N, even in N limited ecosystems (Harrison et al. 2007; Ashton et al. 2008), and that local adaptations to growth conditions in part determine N uptake rates and preferences for ammonium versus nitrate (Wang and Macko 2011; Andersen and Turner 2013).

The complexity of the tropical forest N cycle, with high rates of biological activity, adds to the difficulty in assessing the influence of perturbations (Hedin et al. 2009) and isolating the dominant controls on plant and soil $\delta^{15}N$ values (Pardo and Nadelhoffer 2010). Foliar and total soil $\delta^{15}N$ values show strong latitudinal gradients (Martinelli et al. 1999; Amundson et al. 2003), promoted by enhanced loss of isotopically light forms of N (Pérez et al. 2006) and more rapid microbial processing at lower latitudes (Kramer et al. 2003; Dijkstra et al. 2008). Once thought common to industrialized regions alone, isotopic signals of atmospheric N deposition have now been detected in plant tissue from tropical regions as well (Hietz et al. 2010, 2011). However, experimental evidence in support of the use of plant, soil, or (collectively) ecosystem $\delta^{15}N$ values as indicators of a changing N cycle in tropical forests remains scarce. In particular, no study has experimentally examined the link between N and P availability and the $\delta^{15}N$ values of extractable N and plants in a mature lowland tropical forest.

We used the longest running fertilization experiment in lowland tropical forest to test hypotheses linking nutrient availability to foliar $\delta^{15}N$. We measured $\delta^{15}N$ values in soluble soil N pools, leaves, and litterfall, beneath four locally common tree species following 13 years of factorial N and P fertilization. Previous studies in the experiment showed complexity in growth responses of the plant community: P addition increased fine litterfall, potassium addition reduced fine root growth and influenced seedling growth, and N addition in conjunction with potassium ameliorated otherwise stand-wide trends towards slower relative growth rates (Wright et al. 2011; Santiago et al. 2012). Further, species-specific responses in leaf N and P accumulation were observed among four common tree species (Mayor et al. 2014). We hypothesized that chronic N addition would enrich soluble $\delta^{15}N$ values due to greater N losses, and that this signal would be seen in the foliage of all tree species examined. In contrast, we hypothesized that P addition would lead to depletion of plant $\delta^{15}N$ values due to a tightening of the N cycle as plants and microbes sought to rebalance stoichiometric demands of growth and metabolism (Niklas et al. 2005). We specifically examined: (1) whether chronic N and P addition affected $\delta^{15}N$ values of extractable soil nitrate, ammonium, and dissolved organic N; (2) whether changes in $\delta^{15}N$ values of soil N pools correspond to $\delta^{15}N$ values of canopy foliage; (3) whether N resorption from litterfall and translocation within plants could contribute to altered foliar $\delta^{15}N$; and, (4) the extent to which altered N and P availability influence the forms of N used by tropical trees.

Methods

Site description and experimental design

The Gigante Fertilization Experiment occupies a 38.4 ha plot of mature (>200 years) tropical moist

forest on the Gigante peninsula in the Barro Colorado Natural Monument, Panama. Elevation is 25–61 m a.s.l., mean annual temperature is 27 ± 0.1 °C, and mean annual rainfall is 2,650 ± 146 mm (1995–2007) with a pronounced 4 month dry season from December to April (Wright et al. 2011). The site is underlain by Miocene basalt with soils classified as Oxisols and Inceptisols on higher and lower parts of the landscape, respectively, with clay contents of ~70 % (Turner et al. 2013). Soil extractions from control plots indicated very low concentrations of readily available phosphate (<1 mg P kg^{-1}) determined from Mehlich-3 solutions or anion-exchange membrane extractions (Turner et al. 2013).

Beginning in June 1998 a factorial NPK and a second micronutrient fertilization experiment were established in thirty-six 40 × 40 m plots. All treatments were replicated once in each of four blocks along the elevation gradient to control for spatial variation in soil properties (Yavitt et al. 2009). Fertilizer applications occurred four times a year, evenly spaced within the rainy season (May to November; Supplementary Fig. S1). The current study sampled from sixteen plots in a factorial N × P design (i.e. four plots each of control, +N, +P, and +N + P), with total annual applications of 125 kg N ha^{-1} $year^{-1}$ as urea ($(NH_2)_2CO$) and 50 kg P ha^{-1} $year^{-1}$ as triple superphosphate ($Ca(H_2PO_4)_2H_2O$). Annual rates of N addition were comparable to the annual inputs from litterfall (Yavitt et al. 2011). The $\delta^{15}N$ value of the urea fertilizer was −2.2 ‰.

Plant sampling

Four tree species were selected from within the Gigante Fertilization Experiment because at least two mature individuals were present in 95 % of the 16 × 4 species-by-plot combinations (J. Wright, unpublished data; Supplementary Table S1). Sun exposed canopy leaves of the medium sized canopy tree *Alseis blackiana* (Rubiaceae), the medium sized sub-canopy tree *Heisteria concinna* (Olacaceae), and the large canopy tree *Tetragastris panamensis* (Burseraceae) were obtained in March 2011 (i.e. mid dry season) using steel ammunition. All four species associate with arbuscular mycorrhizal fungi (M. Sheldrake, unpublished data), thus accounting of ectomycorrhizal fractionation is not necessary (Hobbie and Högberg 2012).

Freshly senesced litter was collected once every 2 weeks from 1 m^2 mesh litterfall traps located beneath target species between November 2010 and March 2011 (i.e. late wet season to mid dry season). Fresh and senesced fronds of a medium sized, clonal, sub-canopy palm, *Oenocarpus mapora* (Arecaceae) were obtained in April 2011 with a pole cutter. Leaflets of each *O. mapora* were taken from a minimum of nine fronds originating from three separate shoots and composited by tree (fresh) or plot (senesced). Leaf litterfall N concentration and $\delta^{15}N$ values were measured to assess whether N resorption during leaf senescence alters litter $\delta^{15}N$ values.

All leaf tissue (excluding petioles) was cleaned of epiphylls and damaged tissue before being dried at 60 °C for 48 h, and ground to a fine powder. Isotope analyses were performed using a Flash 1112 Series Elemental Analyzer coupled through a Conflo III interface to a Delta V Advantage continuous flow isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany) at the Smithsonian Tropical Research Institute's Stable Isotope Laboratory. Stable isotope abundances are reported as: $\delta^{15}N = (R_{sample}/R_{standard} - 1) \times 1000$, where $R = {}^{15}N:{}^{14}N$ ratio of the sample or standard. Based on three internal standards, run error rates were 0.05 ‰ and analytical precision for leaf samples analyzed in triplicate had an average standard error of 0.06 ‰.

Soil nutrient sampling

Ion exchange resin bag incubations were used to compare in situ accumulation rates of soluble nitrate, ammonium, and phosphate (Giblin et al. 1994; Schuur and Matson 2001) and to provide a time-integrated source of $\delta^{15}N$ values from mineral N. During three time periods (March 24–April 21, June 15–July 16, and September 22–October 19; hereafter referred to as early, mid, and late rainy season; Supplementary Fig. S1), eight mesh bags (220 μm mesh, 40 cm^2) containing either 3 g of anion or cation exchange resins (Biorad®, AG1/50W-X8, #140/142-1421) were inserted at a 45° angle 5–10 cm deep at four random locations in each of the 16 plots. Due to logistical constraints associated with large sample sizes, $\delta^{15}N$ values for both NO_3^- and NH_4^+ were fully replicated from the mid rainy season resin bag incubations, partially replicated (half the treatment plots) for NO_3^- in the early rainy season, and fully replicated for NO_3^-

only in the late rainy season. The rainy season is a period of high biological activity, associated with leaf flushes (J. Wright, pers. obsv.) and increases in microbial N and P (Turner and Wright 2014). After removal from the field, resin bags were rinsed with deionized water to remove adhering soil, extracted with 100 mL of weakly-acidified NaCl (Giblin et al. 1994), filtered through Whatman GF/A filters, and immediately frozen at −36 °C prior to colorimetric and isotopic analyses.

Five soil cores were collected to 10 cm depth from each plot in January 2011 using a 2.5 cm diameter soil corer. Within 8 h of collection, roots, stones, and mesofauna were removed and subsamples of each homogenized core (~10 g fresh soil) were extracted in 50 mL of 2 M KCl on a shaker table for 1 h, filtered (GF/A), and immediately frozen at −36 °C until colorimetric determination of nitrate + nitrite (NO_3^- + NO_2^-), ammonium (NH_4^+), or phosphate (PO_4^{-3}) on a Lachat QuikChem 8500 (Hach Ltd., Loveland, CO, USA). Diluted (0.5 M salt) aliquots (1.5 mL) of total dissolved N (TDN) and cation resin ammonium extracts were converted to nitrate using potassium peroxodisulfate thermo-oxidation (Cabrera and Beare 1993; Doyle et al. 2004), and converted to N_2O gas for determination of $\delta^{15}N$ and $\delta^{18}O$ values using anaerobic cultures of *Pseudomonas aureofasciens* denitrifying bacteria (Sigman et al. 2001; Knapp et al. 2005). This yielded $\delta^{15}N$ values for TDN in KCl extracts, $\delta^{15}N$ of ammonium in cation resin extracts, and $\delta^{15}N$ and $\delta^{18}O$ values in both KCl and anion resin nitrate extracts. Denitrified samples were analyzed using a GC-Pal gas sampler coupled to a Thermo-Finnigan continuous flow isotope ratio mass spectrometer at the University of Florida. Run error rates for ^{15}N and ^{18}O were typically better than 0.2 ‰ as compared to NIST N-3 (National Institute of Standards and Technology) and USGS #32, 34, and 35 standards (IAEA 2004), respectively. To assure 100 % oxidation efficiency of the NH_4^+ and TDN samples, a range of standards (glycine, and amino caproic acid) were oxidized with samples (concentrations of 1, 5, and 10 mg N L^{-1}, representing the range of sample concentrations). Resulting reagent blanks derived from purified (recrystallized three times) ACS-grade peroxodisulfate ($K_2S_2O_8$) solutions were removed from each resulting $\delta^{15}N$ value on a per batch basis (Knapp et al. 2005). The analytical precision of oxidized samples duplicated between runs was on average different by 1.29 ‰ ($n = 12$) and 0.49 ‰ ($n = 10$) for NH_4^+ and TDN, respectively. This analytical variability, although larger than unoxidized standards, was deemed inconsequential to final plot means because of independent replicate measurements of each N form in each plot and the use of standard deviations in mass balance estimations.

The concentration of DON was calculated as the difference between TDN and mineral N (nitrate + ammonium) and $\delta^{15}N_{DON}$ was calculated using the following mass balance equation:

$$\delta^{15}N_{DON} = \left(\delta^{15}N_{TDN} \times [TDN] - \left(\delta^{15}N_{NH_4} \times \left[NH_4^+\right] + \delta^{15}N_{NO_3} \times [NO_3]\right)\right) \quad (1)$$

where the N concentrations and the $\delta^{15}N_{TDN}$ were determined from 2 M KCl extractions and $\delta^{15}N$ values of ammonium and nitrate were measured from fully-replicated mid-rainy season resin bag incubations. On average, DON pools were ~12 times greater than mineral N and were therefore relatively robust against variability in mineral N $\delta^{15}N$ values. Resin accumulated ion $\delta^{15}N$ values were preferred for this mass balance calculation because of: their demonstrated temporal stability (Templer and Weathers 2011); their ability to concentrate ammonium in situ, rather than using the high pH diffusion technique with associated risks of fractionation during volatilization losses; and, their month-long incubation eliminates some uncertainty associated with spatially and temporally episodic mineral N production and loss processes characteristic of tropical soils. It should be noted, however, that resin and salt extractions may sample slightly different pools of labile nitrate, partially as a function of the anion exchange capacity of soils (Lohse and Matson 2005), but this remains to be determined.

Data analysis

Mixed effect models were fitted for leaf and soil metrics using fixed treatments represented as dummy variables of either +N or +P addition to assess nutrient specific treatment effects and interactions. To account for plot-level variance, Plot was fitted as a random effect using the function lmer in the package lme4 version 0.999999-0 (Bates et al. 2012) in R version 2.15. We used Markov Chain Monte Carlo sampling to test significance in each model using the

mcmcsamp function because this is the least controversial method for testing hypotheses in mixed effect models (Baayan et al. 2008). Models were evaluated by checking for symmetry and normality in posterior distribution of parameters derived from 10,000 samples. Significance of fixed effects was determined with p-values derived from ancillary functions calculated from the posterior distribution of the parameters (p_{MCMC}) using the pvals.fnc function from the languageR package version 1.4 as these are conservative with respect to sample sizes (Baayen 2011). For leaf litterfall $\delta^{15}N$ and estimated proportional N use derived from mixing models (discussed below), which lack within-plot replication, linear models were fit using the lm function in R with the four blocks included as a fixed effect because fitting random effects with fewer than five levels is not recommended (Bolker et al. 2009).

To explore the relationship between $\delta^{15}N$ values in plant tissue and available soil N, we used an isotope mixing model approach similar to (Houlton et al. 2007). Initial underdetermined models assessed whether foliar $\delta^{15}N$ values were interpretable as mixtures of all three measured N sources (DON, NH_4^+, NO_3^-) across all species and plots with or without N fertilization using the optim package in R. These initial underdetermined models fitted an uptake parameter assuming source contributions were proportional to soil N availability. Several measures of soil N availability were used, including the 2 M KCl and resin bag values reported here and monthly measures taken after a decade of fertilization (Turner et al. 2013). Despite the statistical power gained with this approach, these general models were very poor predictors (based on root mean square error) of plant $\delta^{15}N$ values because leaves were on average 9 ‰ more depleted than DON. There was therefore no reason to include DON as a potential source in this system, as the modeled proportional contributions would have been <0.01.

As a result of the elimination of DON as a potential source, a better constrained two-source mixing model termed IsoError (Phillips et al. 2005) was used on a plot level instead. This Excel-based model propagates plot-scale variances associated with plant and soil measurements as it solves for the proportional fraction (f) of mineral N used by each species following mass balance mixing models:

$$1 = f_{NO_3} + f_{NH_4} \quad (2)$$

$$*\delta^{15}N_{foliage} = \delta^{15}N_{NO_3} \times f_{NO_3} + \delta^{15}N_{NH_4} \times f_{NH_4} \quad (3)$$

$$*\delta^{15}N_{foliage} = \delta^{15}N_{foliage} - 0.89\ ‰ \quad (4)$$

The $*\delta^{15}N_{foliage}$ values were adjusted using fractionation values obtained from paired leaf and excavated fine root samples taken from seven other Panamanian tree species. Averages from that study suggested a regional enrichment factor of 0.89 ‰ ($n = 31$ adult trees from seven spp. on three soil types; unpublished data) is more appropriate than the extra-tropical 2.0 ‰ value used elsewhere (Houlton et al. 2007; Handley et al. 1993). This fractionation value accounts for the combined effects of internal plant N allocation and the minor fractionation associated with the transfer of N by arbuscular mycorrhizal fungi (Shearer and Kohl 1986; Handley et al. 1993). In seven of the 61 solvable models (11 % of total) species × plot solutions included nitrate proportions that exceeded the 100 % upper fractional limit of N use. Because these violations were small and unidirectional (average of 0.15 units above (1) we forced these nitrate fractional contributions to '1', read as 100 % of tree N use, for the sake of graphical analyses. Omission of these solutions did not alter statistical conclusions and both model parameters and outputs as well as the primary data are reported in Supplementary Tables S1–S4.

Earlier studies in Panama found very little fine root biomass >0.2 m deep (Yavitt and Wright 2001), and the high densities of roots located in surface soils were unaltered by N or P (Yavitt et al. 2011). This suggests minimal access to deeper pools of N. However, previous observations found soil nitrate at depth to be as enriched as our DON values (Koehler et al. 2012). As such, their incorporation into modeling efforts would place plant $\delta^{15}N$ end members outside of soil source mixtures (comparable to DON sources), requiring a large (and unsupported by literature estimates) fractionation magnitude to force plant $\delta^{15}N$ values into solvable ranges for mixing models. As both nitrate and ammonium concentrations also drop precipitously with depth (Koehler et al. 2012), the solutions presented from surface N sources are therefore likely reasonable approximations of N accessed by these tree species.

Table 1 Soil mineral nutrient concentrations after 13 years of fertilization

	C	N	NP	P
2 M KCl (μg g^{-1})				
[NH_4^+]	2.20 ± 0.80	1.44 ± 0.14	1.56 ± 0.65	2.47 ± 0.48
[NO_3^-]	4.18 ± 0.81	4.98 ± 1.07	5.44 ± 0.85	3.32 ± 0.44
[DON]	72.1 ± 10.7	85.1 ± 9.50	70.0 ± 11.8	83.2 ± 15.1
Exchange resin (μg^{-1} mL^{-1} g^{-1} day)				
Early [NH_4^+]	1.19 ± 0.28	0.84 ± 0.16	0.43 ± 0.06	0.83 ± 0.45
Mid [NH_4^+]	1.28 ± 0.26	2.53 ± 1.01	1.76 ± 0.49	1.13 ± 0.20
Late [NH_4^+]	2.01 ± 0.22	2.69 ± 0.49	1.36 ± 0.25*	2.44 ± 0.31
Early [NO_3^-]	4.83 ± 2.01	**10.21 ± 3.43**	**9.23 ± 1.02**	5.42 ± 2.33
Mid [NO_3^-]	13.27 ± 5.09	**27.86 ± 4.12**	**15.96 ± 2.09**	6.38 ± 1.83
Late [NO_3^-]	14.27 ± 5.90	**42.98 ± 10.63**	**13.39 ± 2.41***	9.97 ± 1.89
Early [PO_4^-]	0.02 ± 0.01	0.03 ± 0.02	**1.28 ± 0.18**	**1.47 ± 0.59**
Mid [PO_4^-]	0.45 ± 0.23	1.37 ± 1.10	**4.44 ± 1.19**	**3.62 ± 0.57**
Late [PO_4^-]	0.08 ± 0.03	0.02 ± 0.01	**4.12 ± 0.87**	**4.45 ± 1.56**

2 M KCl extractions and in situ ion exchange resin bags during early, mid, and late periods of the 2011 rainy season, the thirteenth year of fertilization. Bold values represent a significant N or P addition effect ($p \leq 0.10$) except where there was a significant N × P interaction (indicated with an * $p \leq 0.10$)

Results

The response of soil nutrient and $\delta^{15}N$ values to N and P fertilization

Soil nitrate concentrations from in situ ion exchange resin bags increased across all three rainy season deployments in response to N addition, although the increase was only marginally significant in the initial incubation ($p_{MCMC} = 0.09$, 0.002, 0.0001, respectively; Table 1). Similarly, P addition increased resin adsorbed phosphate across all three rainy season measurements ($p_{MCMC} = 0.055$, 0.007, 0.0008, respectively; Table 1). Only resin nitrate concentrations from the late rainy season exhibited an N × P interaction ($p_{MCMC} = 0.025$) due to lower nitrate concentrations in the +N+P treatment than in control plots. In contrast, there were no significant effects of N or P addition on resin ammonium for the same three rainy season deployments or on concentrations of any N form measured in the KCl extracts.

Soil nitrate $\delta^{15}N$ values obtained from the KCl extraction and the fully replicated mid-rainy season resin incubation were significantly enriched following N addition ($p = 0.004$, 0.025, respectively; Table 2). There was also a marginally significant N × P interaction in the mid-rainy season resin nitrate $\delta^{15}N$ values ($p_{MCMC} = 0.058$) due to $\delta^{15}N$ values in the +N+P treatment being lower than the +N treatment. Soil $\delta^{15}N$ of DON was significantly greater than ammonium ($p < 0.001$, paired Wilcoxon signed-rank test), but neither values were altered by fertilization ($p > 0.05$). The $\delta^{15}N$ and $\delta^{18}O$ of soil nitrate from salt extracts were positively correlated with one another in +N and +N+P treatments ($r^2 = 0.63$, $p < 0.001$, $n = 17$, and 0.44, $p < 0.001$, $n = 18$, respectively) but were not correlated in the control or +P treatments ($p > 0.2$; Supplementary Fig. S2).

Response of foliar and litterfall $\delta^{15}N$ values to N and P addition

Foliar $\delta^{15}N$ values responded significantly to N fertilization in two of the tree species; the overall N addition effect increased foliar $\delta^{15}N$ values in *A. blackiana* ($p_{MCMC} = 0.017$), and *H. concinna* became enriched in response to both N and P additions ($p_{MCMC} = 0.023$, 0.017). Despite having no overall N or P effect in *O. mapora* and *T. panamensis*, foliar $\delta^{15}N$ values of these trees exhibited significant or marginally significant N × P interactions owing to distinctively higher $\delta^{15}N$ values in the +N relative to +N+P plots ($p_{MCMC} = 0.018$, 0.069, respectively;

Table 2 Soil nitrogen $\delta^{15}N$ values after 13 years of fertilization

$\delta^{15}N$ (‰)	C	N	NP	P
2 M KCl NO_3^-	−0.5 ± 0.5	**3.6 ± 1.4**	**2.2 ± 1.2**	0.1 ± 1.4
2 M KCl DON	9.6 ± 0.4	9.6 ± 0.6	9.0 ± 0.6	8.9 ± 0.1
Early resin NO_3^-	−1.0 ± 0.7	0.1 ± 1.7	−1.1 ± 0.1	−0.1 ± 0.5
Mid resin NO_3^-	−0.8 ± 0.7	**0.0 ± 1.0**	**−1.4 ± 0.7***	−1.2 ± 0.7
Mid resin NH_4^+	3.9 ± 1.2	5.6 ± 0.1	4.4 ± 0.8	3.6 ± 1.1

2 M KCl extractions and in situ ion exchange resin bags during early and mid-rainy season incubation periods of the 2011 rainy season, the thirteenth year of fertilization. The $\delta^{15}N$ values of soil N forms were measured using the denitrifier method and were fully replicated for both ions across all 16 plots only for the mid-season resin bags. Bold values represent a significant N or P addition effect ($p < 0.05$) except where there was a significant N × P interaction (indicated with an * $p < 0.10$)

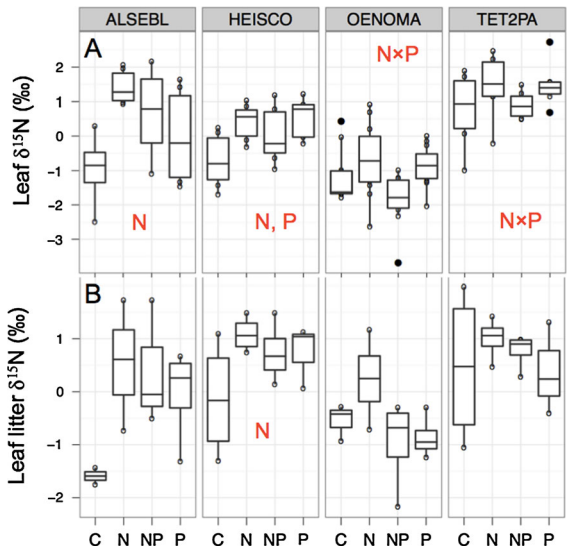

Fig. 1 Leaf and litter $\delta^{15}N$ values from four locally common tree species in the 13th year of fertilization. Fertilization treatments were nitrogen (N), phosphorus (P), or both (NP). Tree species: ALSEBL, *Alseis blackiana*; HEISCO, *Heisteria concinna*; OENOMA, *Oenocarpus mapora*; TET2PA, *Tetragastris panamensis*. *Red letters* indicate significant ($p \leq 0.05$) overall N or P fertilization effects or N × P interactions where N effects were contingent upon P co-addition. (Color figure online)

Fig. 1a). These changes to foliar $\delta^{15}N$ values were not accompanied by changes in leaf N concentrations (Mayor et al. 2014) nor was variability within species significantly altered by treatments ($p > 0.05$, Levene's test). Of the species by treatment combinations, the largest range of foliar $\delta^{15}N$ values was found for *A. blackiana* in +N+P and +P plots (2.66 and 3.04 ‰, respectively), and *T. panamensis* in the +N plots (2.56 ‰). The range of all other species by treatment combinations was 0.59–1.91 ‰.

As for leaves, the overall effect of N addition increased leaf litterfall $\delta^{15}N$ values significantly in *H. concinna* ($p = 0.011$) and non-significantly in *A. blackiana* (Fig. 1b). Leaf $\delta^{15}N$ values, hypothesized to be susceptible to continued accumulation of ^{15}N-enriched N during leaf N resorption (Evans 2001), were unrelated to calculated N resorption efficiencies ((leaf [mg g^{-1}] − leaf litter [mg g^{-1}]) / leaf [mg g^{-1}]) × 100 (Killingbeck 1996) across treatments (Mayor et al. 2014). Furthermore, leaf litter was not uniformly ^{15}N-enriched in *A. blackiana* and *H. concinna* in N addition treatments, as would be expected if resorption of ^{14}N contributed to green leaf enrichment. Rather, the average difference from leaf to litter was 0.44 ‰ in *A. blackiana* ($n = 7$) and −0.49 ‰ in *H. concinna* ($n = 7$), suggesting altered resorption dynamics did not contribute to green leaf ^{15}N-enrichment. The resorption efficiency of $\delta^{15}N$ was not significantly altered by nutrient addition in any species (data not shown), and is not discussed further.

^{15}N-based mixing model solutions

Of 64 potential models (four species × four plots per treatment × four treatments), 61 IsoError solutions were obtained due to the absence of one tree species in three plots (Supplementary Table S2). Of those, 89 % were solvable using the adjusted foliar (Eq. 4) and resin extracted soil N $\delta^{15}N$ values. As seen in Fig. 2, average mineral N contributions suggest that trees did not use proportionally greater amounts of either mineral N form (e.g. average NO_3^- to NH_4^+ use ratio ≈ 1) in control and P addition plots. However, *T. panamensis* and *O. mapora* exhibited slight trends toward greater proportional use of nitrate and ammonium, respectively (Fig. 2). In N addition treatments,

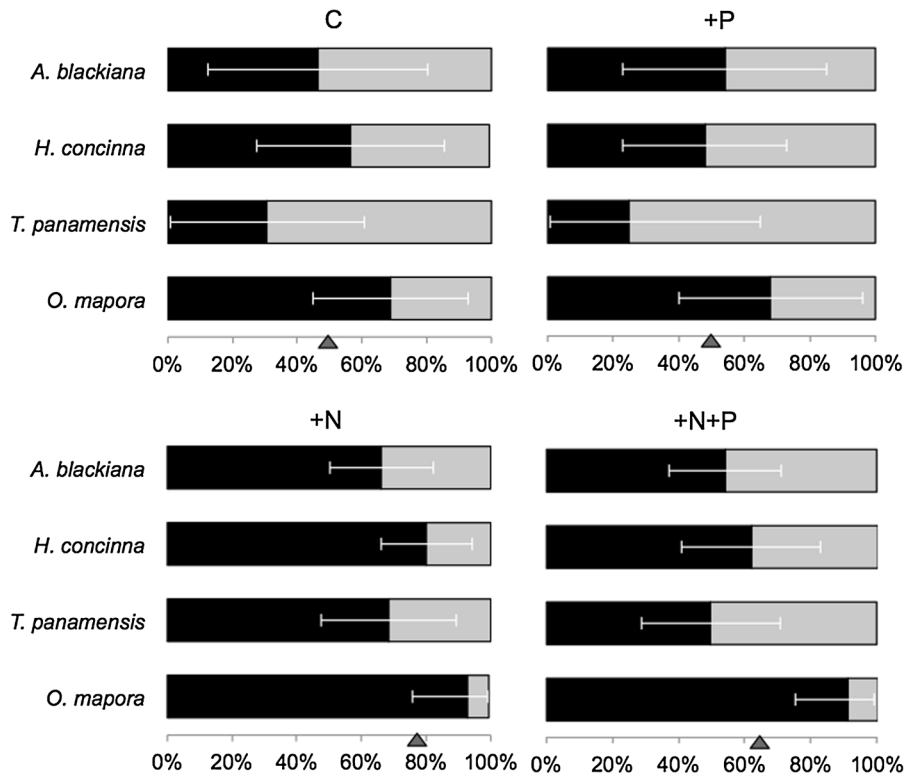

Fig. 2 Average ^{15}N-based mixing model solutions estimating each tree species' proportional use (as % of total leaf N) of inorganic N sources following 13 years of fertilization. *Error bars* are the standard error from a mean of means from treatment replicates. Nitrate, *black*; ammonium, *grey*. *Arrows* mean nitrate use. (Color figure online)

however, proportional use of nitrate increased in all species, significantly so in *H. concinna* ($p = 0.062$), *T. panamensis* ($p = 0.021$), and *O. mapora* ($p = 0.015$) calculated separately, and across all species when analyzed together ($p = 0.027$). Nitrate use was slightly reduced under P co-addition, but not enough to elicit an N × P interaction.

Across treatments, average contributions of soil nitrate were positively related to all three in situ metrics of soil nitrate availability ($r^2 = 0.22$, 0.45, 0.51; $n = 16$, 16, and 15, respectively; Fig. 3), but not ammonium. More specifically, mixing model solutions suggest that N addition caused three of the four tree species to increase proportional use of soil nitrate by threefold in +N versus –N treatments ($f_{NO_3}/f_{NH_4} = 3.7$ vs. 1.17, respectively; Supplementary Table S3). Time-integrated metrics of bioavailable soil N data exhibited similar, but lower fractional increases in $[NO_3^-]$:$[NH_4^+]$ ratios (13.26 vs. 6.3 in +N vs. –N treatments, respectively). Incorporation of these relative concentrations as weightings (see Eq. [13] in Phillips and Koch (2002)) in mass balance models increased proportional nitrate use estimates across all plots, thus diminishing the relative +N addition effect from a 40 % increase in nitrate use to 15 % (Supplementary Table S3).

Discussion

The effects of altered N cycling on soil δ^{15}N

Nitrogen addition more than doubled nitrate accumulated on ion exchange resins and this nitrate pool was ^{15}N-enriched relative to plots that did not receive N. In contrast, N addition did not affect the concentration or δ^{15}N values of ammonium or DON (Table 1, 2), nor were total soil N concentrations or δ^{15}N values modified following 8 years of N addition (Koehler et al. 2009). The increased concentrations of extractable nitrate, but not ammonium, in N addition plots were in agreement with a previous study 4 years after the experiment began (Yavitt et al. 2011) and in a monthly sampling effort in year ten (Turner et al. 2013). The increased concentration of nitrate, but not ammonium, is likely caused by rapid dissolution of the urea fertilizer and nitrification of resulting ammonium. This is in agreement with observations in the four +N

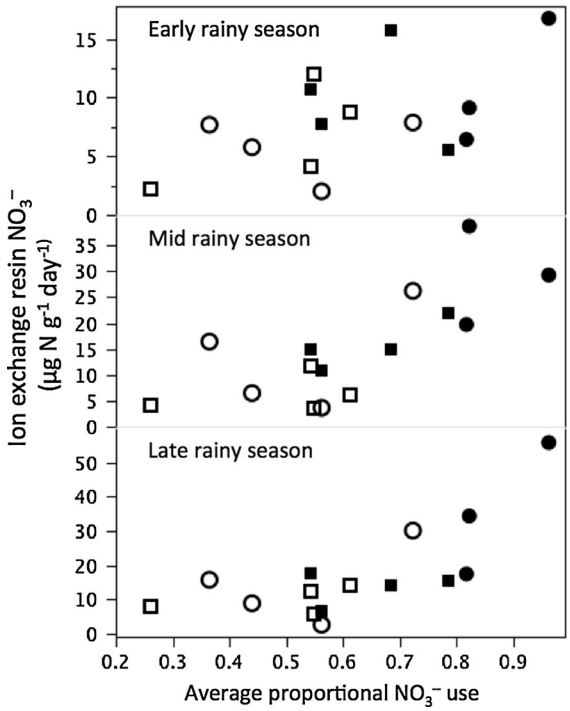

Fig. 3 Relationship between foliar ^{15}N-based mixing model solutions of nitrate use and nitrate concentrations of in situ ion exchange resin bags during the 2011 rainy season. Higher proportional use of nitrate corresponded to higher nitrate concentrations at all time periods ($r^2 = 0.22$, 0.45, 0.51; $n = 16$, 16, and 15, respectively; $n = 16$, respectively). Axes reversed for graphical purposes. Each symbol is the average of model solutions for four tree species (one outlier from late rainy season omitted) across control (*open circle*), +P (*open square*), +N (*filled circle*), and +N+P (*filled square*) treatments

plots that 9 years of N addition increased mineralization, nitrification, and denitrification rates in this experiment (Corre et al. 2010).

Isotopic evidence from this and a previous study (Koehler et al. 2012) suggest that increased trace gas losses resulting from the greater soil nitrate concentrations contributed to the observed ^{15}N-enrichment of the soil nitrate pool. The remaining nitrate pool becomes ^{15}N- and ^{18}O-enriched because the lighter isotopomers are enzymatically favored by denitrifiers (Pérez et al. 2006; Wunderlich et al. 2013). In the Gigante Fertilization Experiment, positive correlations between $\delta^{15}N$ and $\delta^{18}O$ values of soil nitrate following N addition provide evidence for the imprint of gaseous losses on soil nitrate (Supplementary Fig. S2), and isotopomer maps found clear imprints of N_2O production from denitrification rather than nitrification (Fig. 5 in Koehler et al. 2012). Positive correlations between $\delta^{15}N$ and $\delta^{18}O$ values of soil nitrate are due to fractionation of both isotopes during denitrification (Houlton et al. 2006; Pörtl et al. 2007; Park et al. 2011), a small-scale process that translates to regional and globally enriched tropical soil $\delta^{15}N$ values in modeling exercises (Bai and Houlton 2009; Bai et al. 2011). That nitrous oxide emissions are stimulated by N additions to tropical forests is in agreement with previous studies (Hall and Matson 2003; Lohse and Matson 2005; Corre et al. 2010; Lu et al. 2011) and confirms the theorized mechanism accounting for regional enrichment of canopy leaf $\delta^{15}N$ values in Panama over decadal time scales (Hietz et al. 2011).

Urea volatilization, a potentially fractionating process that could temporarily cause ^{15}N-enrichment of ammonium pools (Marshal et al. 2007; Khanif 1992), was unlikely in our system for several reasons. First, wet season ammonium was not isotopically enriched in this or a previous study (Koehler et al. 2012). Second, ammonia volatilization is typically observed under low rainfall conditions (Bouwmeester et al. 1985) where urea is not given a chance to dissolve, disassociate, and diffuse into the subsoil (Christianson et al. 1993). Third, ammonia volatilization occurs under basic soil pH conditions. None of these conditions characterize this experiment; urea was dissolved prior to wet season application, and its addition lowered soil pH values relative from 5.25 in control plots to 4.47 in +N and 4.85 in +N+P plots ($p < 0.001$; Turner et al. 2013).

The effects of altered P cycling on soil $\delta^{15}N$ values

Phosphorus addition increased resin phosphate regardless of N co-addition (Table 1), which agrees with previous findings using different extraction methods (Turner et al. 2013; Yavitt et al. 2011). Interestingly, P addition also influenced soil nitrate $\delta^{15}N$ indirectly, as indicated by the N × P interaction for the fully replicated mid-rainy season resin nitrate (Table 2). This was caused by the loss of ^{15}N-enrichment in plots receiving both N and P. The observed ^{15}N-enrichment under N addition, but not under N and P co-addition, suggests P co-addition directly or indirectly reduced fractionating losses caused by N addition, perhaps through an increase in microbial competition for soil nitrate prior to denitrification or a decrease in

nitrification limiting nitrate supply to denitrifiers. Support for the first possibility includes the observation that microbial biomass was increased by P addition after 10 years of treatments (Turner et al. 2013). Support for the second possibility are observations following 11 years of N-addition to P-limited Hawaiian rainforest, where overall N-oxide emissions were more strongly related to rates of nitrification than of denitrification (Hall and Matson 2003).

The influence of altered soil nutrient availability on leaf $\delta^{15}N$ values

The variation in foliar $\delta^{15}N$ values in control plots (maximum range = 4.37 ‰, 90 % quantile range = 3.27 ‰) was similar to or smaller than more exhaustive taxonomic sampling from other lowland tropical forests (Koba et al. 2010; Guehl et al. 1998; Martinelli et al. 1999), but equivalent to the 90 % quantile range of exhaustive ($n = 213$ species) sampling of "sun-leaves" on nearby Barro Colorado Island (maximum range = 9.38 ‰, whereas 90 % quantile range = 3.31 ‰; subset of data from Hietz et al. 2011).

Of the four locally common tree species, *A. blackiana* and *H. concinna* produced ^{15}N-enriched leaf material in N addition plots (Fig. 1a), a pattern that parsimoniously suggests access to the enriched nitrate pool in the same treatments. Foliar N concentrations, as detailed in a companion manuscript, were unresponsive to N addition in all four of these tree species (Mayor et al. 2014). However, P addition also influenced foliar $\delta^{15}N$ values: *H. concinna* leaves were enriched in response to P addition, and the trend toward ^{15}N-enrichment in *O. mapora* and *T. panamensis* leaves was suppressed in +N+P plots (Fig. 1a). These N × P interactive patterns were similar to the suppression of nitrate ^{15}N-enrichment in +N+P plots (Table 2). Collectively, these patterns suggest that the use of foliar $\delta^{15}N$ to indicate the openness of a tropical forest N cycle depends in part on how soil N cycling and plant mineral N use respond to P availability. In particular, $\delta^{15}N$ values from tropical trees growing on soils relatively high in P may be less isotopically sensitive to N deposition than trees growing on relatively P poor soils. Similarly, 2 years of P addition to an arctic ecosystem increased foliar $\delta^{15}N$ values significantly in two common plant species, but not in two rare species; a pattern attributed to increased N mineralization following P addition (Giblin et al. 1991, Nadelhoffer et al. 1996).

Similar to canopy foliage, litterfall $\delta^{15}N$ values increased in response to both N and P additions in *A. blackiana* and *H. concinna*, although only significantly so for *H. concinna* in N addition plots, in part due to composited sampling reducing within-plot statistical power. In a previous litterfall study, community-wide fine litterfall was approximately 1 ‰ enriched after ten years of N addition (Corre et al. 2010). These results demonstrate: (i) a clear pathway by which the isotopic imprint of some tree species can feedback to soils as annual N application rates were comparable to annual litterfall inputs (Yavitt et al. 2011); (ii) the importance of sampling litterfall from more than one species unless it is predetermined to be representative of the wider community (i.e. *H. concinna*); and, (iii) that litterfall may be a suitable substitute for relative comparisons when sampling canopy foliage is impossible to obtain.

The $\delta^{15}N$ based mass balance models indicated that the proportional use of nitrate increased in accordance with increased nitrate concentrations following chronic N addition (Fig. 3; Supplementary Table S2). This interpretation was supported by simple observations of enrichment patterns as well as statistical probabilities derived from mass balance mixing models based on $\delta^{15}N$ values alone and with respective mineral N concentrations as weightings. Including nutrient weightings, however, diminished the magnitude of change from a 40 % relative increase in nitrate use following N addition to a 15 % increase (Supplementary Table S3). While use of concentration weightings from time-integrated resins has certain intuitive appeal, their acceptance requires two main assumptions. First, the resin nitrate to ammonium ratios must be seen as a precise metric of their respective bioavailability experienced by trees. Second, any a priori bias toward one mineral N form, such as through transporter affinities or ion-influx interactions between [NO_3^-] and [NH_4^+] during N absorption (Glass et al. 2002; Bassirirad 2000), must be accounted for. Regardless, both approaches indicated that tropical trees increased proportional use of nitrate in response to its greater relative availability resulting from chronic N addition. As a result, there was no strong evidence that preference for ammonium, for instance, was retained irrespective of its relative

availability. This finding is in agreement with previous observations that plants track nitrate availability in lower latitude ecosystems (Houlton et al. 2007; Wang and Macko 2011; Kahmen et al. 2008; Takebayashi et al. 2010).

Although tree use of DON has been shown using $\delta^{15}N$ data from montane and high-latitude ecosystems (Averill and Finzi 2011; Mayor et al. 2012), there was no isotopic evidence suggesting the tropical trees used DON in our system or along a precipitation gradient in Hawaiian tropical montane forest (Houlton et al. 2007). Although use of ^{15}N-labeled amino acids has been demonstrated in tropical montane palms (Andersen and Turner 2013), appreciable use of such compounds is likely not beneficial to plants growing on soils with highly mobile mineral nitrogen concentrations. Indeed, although data from tropical forests are exceedingly rare, studies from a variety of montane tropical forest plants suggests that mineral N forms are often preferred over amino acids even when provided in concert at comparable concentrations (Andersen K, Mayor J, Turner B, unpublished data). Furthermore, the predominance of arbuscular mycorrhizal associations among most tropical tree species (including those in this study) precludes the enzymatic advantage described for ectomycorrhizal associations that retain the capacity for the degradation of various organic N compounds in N-limited ecosystems (Talbot and Treseder 2010).

Conclusion

Tropical regions account for 71 % of global N losses from unmanaged land and the continued gaseous loss of isotopically light nitrous oxides leaves an isotopic imprint on the residual nitrate pool from which it is derived (Bai et al. 2011). Therefore, the mechanism used to account for the gradual increase in archived plant $\delta^{15}N$ samples seen in tropical forests at decadal timescales (Hietz et al. 2011) is now empirically supported. This study, from the longest running fertilization experiment in lowland tropical forest, demonstrates that despite chronic addition of isotopically depleted urea, rapid rates of N cycling resulted in relatively higher concentrations of nitrate that comprised greater proportions of tree N use. Surprisingly, the relative availability of P also altered the isotopic imprint in both soils and plants, possibly through increased microbial demand for N under P addition. Coexistence of the tree species examined here appear to not be linked to specialization on N forms; instead, the sampled plant community appears to prefer roughly equal proportions of ammonium and nitrate under ambient conditions, and continued anthropogenic inputs of N will likely result in accelerated N cycling, and increased use of nitrate by all species.

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