

Relationships among phosphorus, molybdenum and free-living nitrogen fixation in tropical rain forests: results from observational and experimental analyses

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Abstract Biological nitrogen (N) fixation is the primary source of “new” N to unmanaged ecosystems, and recent analyses suggest that terrestrial N inputs via free-living N fixation may be more important than previously assumed. This may be particularly true in some tropical rain forests, where free-living fixation could outpace symbiotic N fixation to represent the dominant source of new N inputs. However, our understanding of the controls over free-living N fixation in tropical rain forests remains poor, which directly constrains our ability to predict how N cycling will respond to changing environmental conditions. Although both phosphorus (P) and molybdenum (Mo) availability have been shown to limit free-living N fixation rates in the tropics, few studies have simultaneously explored P versus Mo limitation or the

potential importance of P × Mo interactions. Here, an archived set of foliar, litter, and soil samples from a Costa Rican tropical rain forest provided an opportunity to simultaneously assess the relative strength of P versus Mo relationships with free-living N fixation rates. We also conducted a short-term, full-factorial (P × Mo) litter incubation experiment to directly assess nutrient limitation, allowing us to explore P and Mo controls over free-living N fixation rates using both observational and experimental approaches. We previously showed that N fixation rates were positively correlated with P concentrations in all substrates and, using the archived samples, we now show that Mo concentrations correlated with N fixation only in canopy leaves (where total Mo concentrations were extremely low). Likewise, fertilization with P alone (and not Mo) stimulated leaf litter N fixation rates. Thus, our results suggest that P availability dominantly controls free-living N fixation at this site, and when taken with data from other studies, our results suggest that attempts to identify “the nutrient” that limits N fixation in “the tropics” may be misguided. Rather, nutrient controls over free-living N fixation appear to be more nuanced—and the true nature of nutrient limitation to N fixation likely varies over a variety of scales across the vast tropical rain forest biome.

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Introduction

Nitrogen (N) availability strongly regulates ecosystem structure and function (Howarth et al. 1988; Aber et al. 1989; Vitousek et al. 1997; Elser et al. 2007), and N fixation represents the largest natural source of new N to the majority of unmanaged ecosystems (Galloway et al. 2004). As a result, identifying the factors that control this important process is critical for predicting a suite of ecosystem processes—including net primary productivity (NPP), decomposition and community composition—both now and into the future (Vitousek and Howarth 1991; Vitousek et al. 2002; Hungate et al. 2003; van Groenigen et al. 2006; Hedin et al. 2009; Menge et al. 2009). While “biological N fixation” broadly refers to the process through which N-fixing organisms reduce atmospheric dinitrogen gas (N_2) to reactive and biologically available forms, in practice, N fixation reflects N inputs via two distinct pathways: symbiotic and free-living. Symbiotic N fixation is often defined as the biological reduction of N_2 occurring via structured mutualistic relationships between microorganisms (e.g., Rhizobia) and plant roots (e.g., legumes), while free-living N fixation (also called nonsymbiotic and asymbiotic N fixation) refers to fixation that occurs outside of these associations (e.g., heterotrophic N fixation in leaf litter and soil).

Very high rates of symbiotic N fixation observed in some sites (Cleveland et al. 1999) have led to the conclusion that symbiotic fixation may be the dominant biological source of new N. However, recent analyses indicate that free-living N fixation may be more important than previously thought, and in some ecosystems may represent the principal source of newly fixed N (Reed et al. 2011). For example, although putative N-fixing species are relatively abundant in many late-successional tropical forests, it appears that relatively little of that potential N fixation capacity is realized in practice (e.g., Barron et al. 2011). Thus, in many tropical forests, N inputs via free-living N-fixers, which are ubiquitous and responsive across space and time, could be critical for meeting high N demands and loss rates (Lewis et al. 1999; Hedin et al. 2003, 2009; Houlton et al. 2006; Brookshire et al. 2012).

Free-living N fixation may indeed be an important if not dominant source of new N to ecosystems (Cleveland et al. 1999; Reed et al. 2011), yet our understanding of the controls over free-living N fixation rates in tropical forests remains relatively

poor (Cleveland et al. 2010). A number of biotic and abiotic factors—including temperature, species composition and nutrient availability—affect N fixation rates (Sprent and Sprent 1990; Vitousek and Howarth 1991; Vitousek and Field 1999; Vitousek et al. 2002; Hicks et al. 2003; Cusack et al. 2009). In particular, experimental work suggests the importance of phosphorus (P) and/or molybdenum (Mo) availability in regulating the process (Eisele et al. 1989; Silvester 1989; Pearson and Vitousek 2002; Hungate and 2004; Reed et al. 2007a, b; Benner et al. 2007; Barron et al. 2009; Wurzbürger et al. 2012).

Studies addressing nutrient controls over free-living N fixation in tropical forests have focused on P and/or Mo for several reasons. First, many tropical forests occur on highly weathered soils depleted of rock-derived nutrients, including P and Mo (Sanchez et al. 1982; Vitousek and Sanford 1986; Palm et al. 2007). Next, while the dominant tropical soil orders are generally nutrient-poor, we see remarkable heterogeneity in potential nutrient availability of elements such as P and Mo, even on highly weathered soils (Townsend et al. 2008). Third, the nutrient requirements of N fixation imply that P and/or Mo limitation of the process could be common: N fixation requires both a great deal of energy (provided in the form of adenosine tri-phosphate (ATP); Gutschick 1981) and an oxygen-poor environment at the site of fixation. The ATP and cellular attributes needed by N-fixing organisms to create these conditions use comparatively large amounts of P. Accordingly, the relatively high P demand of N-fixers creates the potential for P limitation to N fixation, and multiple studies have shown that P additions stimulate the process (Eisele et al. 1989; Smith 1992; Benner et al. 2007; Reed et al. 2007a, b). Similarly, Mo is a critical component of the most common form of nitrogenase (the enzyme complex N-fixing organisms use to reduce N_2 ; Bishop et al. 1982; Madigan et al. 2003) and studies have suggested Mo limitation to free-living N fixation rates (Silvester 1989, Barron et al. 2009, Wurzbürger et al. 2012).

Despite strong theoretical justification for the hypothesized P and/or Mo controls, our understanding of when and where these nutrients—either alone or in combination—regulate free-living N fixation in tropical forests remains unclear. In part, this stems from an incomplete understanding of how Mo cycles in terrestrial ecosystems, how Mo interacts with other biogeochemical cycles, and methodological difficulties associated with assessing Mo availability (Wichard et al. 2009). Further,

attempts to assess Mo controls over terrestrial N fixation and direct comparisons of P versus Mo limitation are rare and show variable results, with some data suggesting P limitation, others Mo limitation, and some suggesting co-limitation by P and Mo (e.g., Vitousek & Hobbie 2000; Barron et al. 2009; Wurzbürger et al. 2012). Finally, when considering P versus Mo limitation, it is important to consider that Barron et al. (2009) showed Mo can be a contaminant in commercial P fertilizer, and that stimulation of N fixation after additions of P fertilizer may actually reflect fixation responses to Mo. In effect, the Barron et al. (2009) results cast doubt on the interpretation of some past work showing P limitation of N fixation by suggesting that if Mo was not explicitly considered, the presumed P responses could actually reflect responses to Mo.

Given the tremendous uncertainty regarding the relative roles of P and Mo in N fixation, we took advantage of an existing set of archived samples and analyses that previously focused solely on P controls over N fixation (Reed et al. 2008) to address P versus Mo controls. Previously, we investigated how tree species-specific variations in foliar and soil carbon (C), N and P concentrations were related to free-living N fixation rates on rain forest leaves, leaf litter, and soil (Reed et al. 2008). Results showed that natural variations in P concentrations were positively correlated with N fixation rates in all substrates measured, corroborating a field fertilization study at the same site suggesting P limitation (Reed et al. 2007b). However, in light of the results of Barron et al. (2009), we re-analyzed our archived samples for Mo concentrations and performed a short-term fertilization incubation experiment using Mo-free P and P-free Mo. We hypothesized that both P and Mo would play a role in regulating free-living N fixation at our site, and predicted that: (1) N fixation rates in substrates with relatively more Mo or less P would correlate more strongly with P than Mo and vice versa; and (2) fertilization with both P and Mo would stimulate leaf litter N fixation rates and rates would be highest when the two nutrients were added in concert.

Methods

Site description

The study was conducted in a mature lowland tropical wet forest (Holdridge et al. 1971) located on the Osa

Peninsula in southwest Costa Rica (8°43' N, 83°37' W). Mean annual temperature at the site is 26 ± 1.5 °C and rainfall averages $\sim 5,000$ mm year⁻¹. Soil is classified as an Ultisol (Berrange and Thorpe 1988; Bern et al. 2005) and the forest is a highly diverse (100–200 tree species ha⁻¹; Kappelle et al. 2002), closed canopy rain forest that includes many common neo-tropical tree species. More detailed descriptions of other site characteristics and of temporal and spatial variation in N fixation rates are available elsewhere (Cleveland and Townsend 2006; Reed et al. 2007b, 2008).

Sample collection

We collected an initial set of samples in 2006 to investigate how tree species variation in C, N and P concentrations may lead to species-specific differences in rates of free-living N fixation (described in Reed et al. 2008). Briefly, we sampled eight individuals from six tree species (*Brosimum utile* Kunth Oken. (Moraceae); *Caryocar costaricense* Donn. Sm. (Caryocaraceae); *Manilkara staminodella* Gilly. (Sapotaceae); *Qualea paraensis* Ducke (Vochysiaceae); *Schizolobium parahybum* Vell. S.F. Blake (Fabaceae); and *Symphonia globulifera* L.f. (Clusiaceae); 48 trees total). These species were selected because they are common in the study site, they are members of widely distributed neo-tropical families, and foliar N and P concentrations vary significantly among the species (Townsend et al. 2007). From each of the 48 trees, we collected live canopy foliage, bulk leaf litter, and topsoil (0–2 cm depth; collected within a 4 m radius of the base of each tree). We used these 144 samples (48 trees with 3 forest layers for each) for the subsequent assessment of natural variation in N fixation and P (Reed et al. 2008) and Mo concentrations (this study).

Live canopy leaves were collected using a 12-gauge shotgun; sunlit leaves were shot from the canopy of each tree and were caught by hand as they fell to the ground. Bulk forest floor leaf litter (not selecting for leaves of particular species) was hand-collected beneath the crown of each of the 48 trees using a stratified random design within a 4 m radius of the base of each tree. Soils were sampled within the same tree-specific rings to a depth of 2 cm (first removing leaf litter), and all soils were collected as intact cores using 55 ml, 2.5 cm diameter clear acrylic tubes.

Natural variation in N fixation rates

All samples were collected and analyzed for N fixation rates on the same day. Canopy leaves were misted with 1 ml of deionized (DI) water before analysis, but the moisture contents of bulk leaf litter and soil were not manipulated. We used the acetylene (C_2H_2) reduction assay (ARA; Hardy et al. 1968; Belnap 1996) to determine rates of free-living N fixation in the foliage, litter and soil samples. Samples (~ 1 g foliage or leaf litter and ~ 10 g soil) were sealed in 55 ml clear acrylic tubes, injected with enough acetylene to create a 10 % headspace by volume (through a lid fitted with a septum), and vented to the atmosphere. All samples were incubated for 16 h in situ on the forest floor, receiving ambient light and temperature during both daylight and nighttime hours. After incubation, sample headspaces were mixed, subsampled, and injected into pre purged Vacutainer tubes (first baked for 48 h at 70 °C to eliminate ethylene off gassing; Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). Samples were sealed with a silicone sealant and returned to the University of Colorado, Boulder for analysis.

In the laboratory, ethylene (C_2H_4) concentrations were measured using a gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID; 330 °C) and a Poropak N column (110 °C; Supelco, Bellefonte, Pennsylvania, USA). We also incubated acetylene blanks during the field incubation to assess background levels of ethylene in the acetylene gas. Background ethylene concentrations made up a small percentage of the total ethylene produced by leaf litter and soil but, because of low canopy N fixation rates, did comprise a significant percentage of the ethylene produced by canopy leaves. However, blank ethylene concentrations were quite consistent (<3 % variation among tubes), and thus an average calculated background ethylene value was subtracted from all sample values before analysis. Controls for ethylene production in the absence of acetylene were also determined and were consistently small compared with acetylene reduction rates. The dry mass and moisture content of each sample was determined gravimetrically after oven drying for 48 h (70 °C for foliage and litter, 105 °C for soil). Rates of acetylene reduction were calculated in units of nanograms (10^{-9} g) of C_2H_4 produced per gram dry mass of sample per hour of incubation.

Chemical analyses

Foliar and soil C, N, and P analysis methods are described in Reed et al. (2008). Briefly, total foliar and soil C and N were measured using a Carlo Erba Elemental Analyzer 1110 (Lakewood, New Jersey, USA). Total foliar and soil P concentrations were determined by performing a sulfuric acid/hydrogen peroxide digest and assessing P concentrations colorimetrically using an ascorbic acid molybdate analysis (Kuo 1996) on an Alpkem autoanalyzer (OI Analytical, College Station, Texas, USA). To measure total Mo concentrations, all foliage and soil samples were air-dried, ground to a fine powder using a mortar and pestle, and oven-dried for 48 h. For each sample, ~ 0.25 g of foliage or ~ 0.5 g of soil were weighed into a 75 ml digestion tube and mixed with 10 ml of 35 % trace metal grade HNO_3 . Samples were heated to 150 °C, boiled for 20 min, cooled, and 2 ml of H_2O_2 were added. Samples were returned to 150 °C and allowed to boil for 20 min. The H_2O_2 additions and boiling were repeated until all color was gone from samples. Samples were allowed to cool and were diluted with Mo-free DI water. Standards [reference peach leaves and reference soil; National Institute of Standards and Technology (Gaithersburg, Maryland, USA)] were also digested and analyzed to ensure complete digestion efficiency (data not shown). Total Mo concentrations were measured with an inductively coupled plasma-mass spectrometer (ICP/MS; Perkin Elmer Elan DRC-e, Waltham, Massachusetts, USA) by the University of Colorado's Laboratory for Environmental and Geological Studies (Boulder, Colorado, USA).

Fertilization experiment and N fixation rates

In June 2009, we conducted a short-term fertilization experiment using a fresh set of samples collected from the same study site to directly compare the effects of Mo versus P additions on free-living N fixation rates. Other leaf litter N fixation research has suggested a strong connection between the responses of short-term incubations with those from long-term field fertilizations (Barron et al. 2009). We used bulk leaf litter as our substrate both because litter showed the highest mass-based rates of N fixation (Reed et al. 2008) and because a tropical rain forest study in Panama found

Mo limitation in the leaf litter layer (O_i horizon; Barron et al. 2009).

Leaf litter was collected from a 50×50 m plot at the site, mixed by hand, and for each sample ~ 2.5 g of leaf litter was added to a 100 ml clear acrylic container. There were eight, ~ 2.5 g leaf litter samples for each treatment (control, +Mo, +P, and +Mo+P) for a total of 32 samples. Fertilization treatments were added using certified P-free Mo and Mo-free P reagents (Alltech Inc., Nicholasville, Kentucky, USA), and fertilization concentrations were determined using natural concentrations as a guide. For +P treatments, we added $1 \times$ the average amount of total P found in bulk litter ($844 \mu\text{g P g}^{-1}$; Reed et al. 2008; Table 1), and to +Mo treatments we added $1 \times$ the average amount of total Mo found in bulk litter (297 ng Mo g^{-1} ; Table 1). This represents more Mo per unit mass than all concentrations used on litter in Silvester (1989), $\sim 7 \times$ more Mo than the 'low' Mo concentration and a little over half the 'high' concentration in Barron et al. (2009), and slightly less than half of the Mo added in Wurzbürger et al. (2012). Mo was added as P-free Na_2MoO_4 and P was added as Mo-free NaH_2PO_4 (Alltech, Inc.). All samples were misted with 2.4 ml of an appropriate solution (control = DI water; +Mo = $309.4 \text{ ng Mo ml}^{-1}$; +P = $879.2 \mu\text{g P ml}^{-1}$; +Mo +P = $309.4 \text{ ng Mo ml}^{-1}$ and $879.2 \mu\text{g P ml}^{-1}$), sealed, injected with enough acetylene to create a 10 % C_2H_2 headspace, vented to the atmosphere, and allowed to incubate at the site for 16 h. We also incubated two sets of blanks: one set of containers received leaf litter and no acetylene (to assess any sample production of ethylene) and another set received acetylene but no leaf litter (to assess the amount of ethylene present in the acetylene). As described above, headspace gas samples were collected, transported to the University of Montana in sealed Vacutainer tubes (Becton, Dickinson and Company), and assessed for C_2H_4 concentrations using a gas chromatograph (Shimadzu).

Statistics

All data were tested for normality and homoscedasticity (using Levene's test for the equality of variances); if either assumption was violated, data were ln-transformed before analysis. Differences among rates of N fixation and among chemical properties, both along the vertical forest profile (canopy to soil) and between

species within a profile layer, were determined using analyses of variance (ANOVA). To examine variation along the vertical profile, data were grouped by position in the profile and species identity was ignored. To explore variation among tree species, data were analyzed separately for each layer of the profile and data were grouped according to species identity. Relationships between rates of N fixation and leaf or soil nutrient concentrations were determined using bivariate correlation analyses. For canopy samples, both P and Mo were significantly correlated with N fixation rates and thus we used multiple regression analyses with partial correlations to elucidate the relationship of N fixation with P versus the relationship with Mo. Partial correlations estimate the amount of N fixation variation which was not explained by variation in a first variable (e.g., P concentration) that could be attributable to the variation in a second variable (e.g., Mo concentration). For the fertilization experiment, N fixation rates were grouped by fertilization treatment and assessed using ANOVA. Multiple comparisons within ANOVAs were analyzed using LSD post hoc analyses. All data were analyzed using SPSS v. 20 (Chicago, Illinois, USA).

Results

Nutrient concentration variation along the vertical forest canopy-to-soil profile

Total Mo concentrations increased along the vertical profile from canopy leaves to surface soils ($P < 0.001$). Averaged across all species, canopy leaves had the lowest Mo concentrations ($29.7 \pm 2.2 \text{ ng g}^{-1}$), bulk leaf litter collected from the forest floor had significantly higher Mo concentrations ($296.5 \pm 44.8 \text{ ng g}^{-1}$), and soil maintained the highest concentrations of total Mo ($7,264.9 \pm 463.5 \text{ ng g}^{-1}$; Fig. 1). In contrast, P concentrations declined along the vertical profile ($P < 0.001$): Canopy leaves had the highest average concentrations of total P ($1,133 \pm 183 \mu\text{g g}^{-1}$), leaf litter had significantly lower P concentrations ($844 \pm 131 \mu\text{g g}^{-1}$), and soil had the lowest concentrations of total P ($557 \pm 45 \mu\text{g g}^{-1}$; Fig. 1; Cleveland et al. 2002). Accordingly, P:Mo ratios decreased from the canopy to the forest floor, ranging from 37,767 in canopy leaves to 2,842 in leaf litter to 77 in topsoil (Fig. 1; Table 1).

Table 1 Total C, N, P, and Mo concentrations and P:Mo ratios (on a mass basis) for unmanaged tropical ecosystems

Substrate	Site	%C	%N	Total P ($\mu\text{g g}^{-1}$)	Mo (ng g^{-1})	P:Mo	Reference
Canopy leaves	Costa Rica [∞] Ultisol (wet)	45.67 ± 0.81	1.48 ± 0.09	1,133 ± 183	30 ± 2	37,767	C, N, and P from Reed et al. (2008); Mo from this study
	Ghana Ferralsol (wet)				50		Bowell and Ansah (1993)
	Ghana Ferralsol (dry)				30		Bowell and Ansah (1993)
Leaf litter	Costa Rica Ultisol (wet)	44.63 ± 1.09	1.38 ± 0.14	844 ± 131	297 ± 45	2,842	C, N, and P from Reed et al. (2008); Mo from this study
	Panama Oxisol (early wet)	40.99 ± 1.87	1.44 ± 0.07	397 ± 28	53 ± 7	7,491	Barron et al. (2009)
	Hawai'i (Kaniku)		0.47 ± 0.02	300 ± 20	450 ± 140	667	Vitousek and Hobbie (2000)
	Hawai'i (Pahoehoe) Histosol		0.27 ± 0.01	160 ± 10	230 ± 110	696	Vitousek and Hobbie (2000)
	Ghana Ferralsol (wet)				250		Bowell and Ansah (1993)
	Ghana Ferralsol (dry)				120		Bowell and Ansah (1993)
Soil	Costa Rica Ultisol (wet)	11.39 ± 0.98	0.56 ± 0.06	557 ± 45	7,265 ± 464	77	C, N, and P from Cleveland et al. (2002); Mo from this study
	Brazil Acru toxes	2.49	0.142	388	3,460	112	Lilienfein et al. (2000)
	Ghana Ferralsol (wet)				6,520 ± 2,710		Bowell and Ansah (1993)
	Ghana Ferralsol (dry)				5,210 ± 3,720		Bowell and Ansah (1993)

Values represent means ± 1 SE and [∞] is the average of 6 species of rain forest tree ($n \leq 36$ for each measure). If reported, the season (dry or wet) when samples were collected is provided

Nutrient concentration variation among species

Position along the vertical forest gradient (i.e., from the canopy to the forest floor) was one source of differences in substrate nutrient content, but our data also showed substantial species-level variability in foliar chemistry in this diverse forest. Mo concentrations varied significantly among species: for the six tree species analyzed, Mo concentrations varied among canopy leaves ($P = 0.027$), leaf litter

($P = 0.049$), and topsoil ($P < 0.001$) (Table 1). Canopy leaf Mo concentrations ranged from 13.1 to 63.2 ng g^{-1} , leaf litter Mo concentrations ranged from 47.1 to 857.8 ng g^{-1} and soil Mo concentrations ranged from 1,976.6 to 14,112.3 ng g^{-1} (Table 1). In addition, there were some consistent relationships between Mo concentrations of samples collected from different positions along the vertical canopy-to-soil profile: Foliar canopy Mo concentrations were significantly correlated with soil Mo concentrations

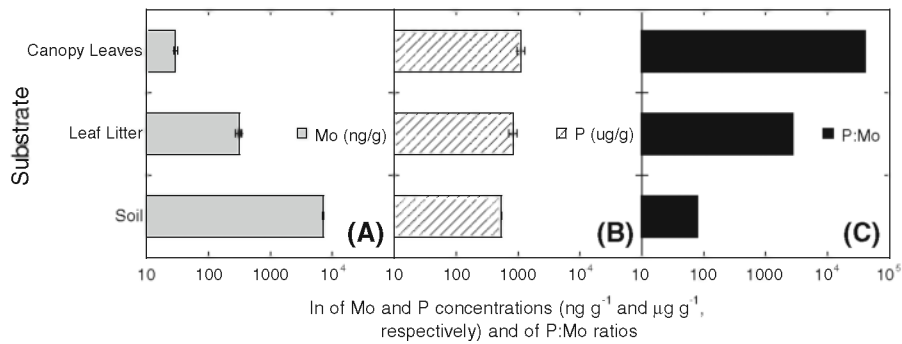


Fig. 1 Total **A** Mo (ng g^{-1}) and **B** P ($\mu\text{g g}^{-1}$) concentrations and **C** P:Mo ratios (on a mass basis) along a vertical profile in a Costa Rica rain forest. Concentrations and ratios are significantly different ($P < 0.001$) among the three vertical positions.

($P < 0.002$; $R = 0.342$), but neither were correlated with leaf litter Mo concentrations.

Relationships between N fixation rates and nutrient concentrations

We found no significant correlations between N fixation rates and Mo concentrations in leaf litter or soil samples (Table 2). However, in the canopy—where Mo concentrations were lowest—foliar N fixation rates and foliar Mo concentrations were significantly correlated ($P = 0.024$; $R = 0.377$; Table 2; Fig. 2). In contrast, N fixation rates and P concentrations were significantly correlated for all substrates measured (Table 2; Fig. 2; $P < 0.05$ for each; Reed et al. 2008). Using multiple regression analysis we found that, for leaf litter and soil, including Mo did not improve the predictive capacity of the N fixation model beyond results obtained using P alone. However, in canopy leaves, Mo and P together explained more variation in foliar N fixation than when including either nutrient alone. R^2 values for regressions using only P or Mo suggested that canopy fixation was most strongly correlated with P (explaining 53 % of total variance; $P < 0.001$) and less with Mo (14 %; $P = 0.024$; Table 2), and explanatory power was highest when both element concentrations were included in the multiple regression model ($R^2 = 0.611$; $P < 0.001$). We performed a partial correlation analysis within the multiple regression analyses and the results suggested that variation in P accounted for 34 % and variation in Mo accounted for 18 % of the variation in N fixation rates

Mo concentrations increase from canopy leaves to topsoil, and P concentrations and P:Mo ratios decline. Total soil P values are from Cleveland et al. (2002) and canopy and leaf litter total P concentrations are from Reed et al. (2008)

explained by P and Mo (R_{partial} of 0.58 and 0.42 for P and Mo, respectively; $P < 0.01$ for each; Table 2). Relationships between the concentrations of other measured elements (C and N) and N fixation rates were always weaker than those observed for P or Mo (data not shown).

Table 2 Bivariate correlations of N fixation rates with P and Mo concentrations for canopy leaves, leaf litter, and soil

Substrate	P concentration R/P	Mo concentration R/P
Canopy leaves		
N fixation rates	0.728/0.000	0.377/0.024
P concentration		0.167/0.322
R_{partial}	0.58	0.42
Leaf litter		
N fixation rates	0.429/0.020	-0.043/0.340
P concentration		-0.207/0.344
Soil		
N fixation rates	0.419/0.008	0.282/0.101
P concentration		0.605/0.088

For each group, values shown are Pearson correlations coefficients and P values (R/P). Significant correlations ($\alpha = 0.05$) are shown in bold. For the canopy layer where both P and Mo concentrations significantly correlated with N fixation rates, multiple regression with partial correlation analyses were used to partition explanatory power. Shown are the partial correlation coefficients (R_{partial}) for the relative importance of P versus Mo variation in explaining the variation in N fixation explainable by P and Mo concentrations. Values comparing P concentrations to N fixation rates are from Reed et al. (2008)

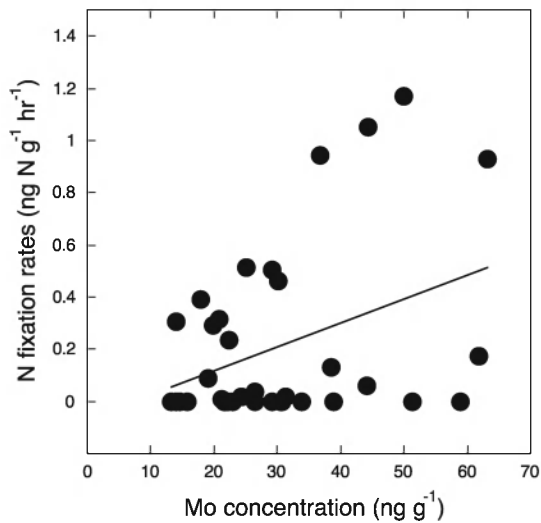


Fig. 2 Linear relationship between canopy leaf N fixation rates ($\text{ng N g}^{-1} \text{h}^{-1}$) and total Mo (ng g^{-1}) concentrations in a Costa Rican rain forest. Linear regression analysis showed a significant positive relationship between N fixation and foliar Mo concentration ($P = 0.024$; $R^2 = 0.142$)

Fertilization experiment

To directly compare Mo versus P effects on free-living N fixation, we conducted an in situ incubation experiment in which we added both Mo and P to litter samples in a full-factorial design. Given recent evidence suggesting that Mo contamination of P fertilizer is common (Barron et al. 2009), we took care to purchase and use Mo-free P and P-free Mo. Fertilization with P alone significantly stimulated N fixation rates in leaf litter ($P = 0.037$; P fertilized rates were nearly twice the control rates), but fertilization with Mo did not affect N fixation rates (Fig. 3). Additionally, simultaneous +Mo+P additions did not elicit significantly higher N fixation rates than additions of P alone (Fig. 3).

Discussion

Answers to questions of how, when, where and what nutrients limit N fixation in tropical forests are exceedingly rare, but two studies in Panama have shown that Mo and P may interact to regulate free-living N fixation there. First, Barron et al. (2009) used a set of fertilization experiments to show that low Mo availability limited leaf litter N fixation rates in a

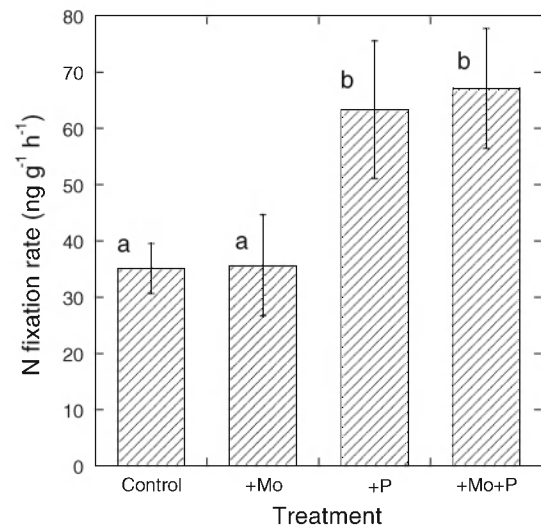


Fig. 3 N fixation rates ($\text{ng g}^{-1} \text{h}^{-1}$) in leaf litter samples fertilized with: DI water (control); 297 ng g^{-1} Mo (+Mo); $844 \mu\text{g g}^{-1}$ P (+P); or 297 ng g^{-1} Mo and $844 \mu\text{g g}^{-1}$ P (+Mo+P). N fixation rates varied significantly among treatments, and rates from samples fertilized with +P and +Mo+P were significantly higher than control and +Mo rates. Values represent means ± 1 SE

Panamanian tropical forest. In the same study, data showed that Mo can be present (as a contaminant) in commercial P fertilizer, casting some doubt on interpretations of results from some previous fertilization experiments reporting P limitation of free-living N fixation. Second, Wurzbürger et al. (2012) showed that, at their Panamanian forest sites, the extent of Mo versus Mo/P co-limitation depended on soil P availability, such that N fixation on soils with low P availability were P/Mo co-limited, while leaf litter fixation on soils with high P were limited by Mo alone.

In a Costa Rican tropical forest, we had conducted an in situ nutrient manipulation experiment that led us to conclude that low P availability strongly limited leaf litter and soil N fixation at this site (Reed et al. 2007b). However, in light of the Barron et al. (2009) result suggesting the possibility of trace Mo contamination effects, we used samples archived from an exploration of natural variations in P concentration and free-living N fixation rates (Reed et al. 2008) to assess possible relationships between Mo concentrations and N fixation. Our new analysis showed that while P concentrations positively correlated with N fixation rates in all substrates collected along the

vertical profile (Table 2; Reed et al. 2008 $P < 0.02$ for each), total Mo concentrations correlated with N fixation rates only in canopy leaves (Table 2; $P = 0.024$). In particular, our analysis suggested that rates of canopy N fixation were best explained using a model that included both P and Mo concentrations, explaining 61 % of the variation in canopy N fixation rates, and that both P and Mo were significantly related to N fixation rates (Table 2). Thus, in the canopy, where Mo concentrations were relatively low and C:Mo and P:Mo ratios were relatively high, the data suggest that both P and Mo play a significant role in regulating the process (Table 2; Fig. 2). However, variation in canopy foliar P explained a larger proportion of variation in canopy fixation, and in all other substrates measured, P alone was the dominant control. Taken together, the data suggest free-living N fixation rates in the Costa Rican forest are much more strongly controlled by P availability, and that Mo plays a subtle role.

The observed relationship between total P, Mo and N fixation in canopy leaves (where Mo concentrations were the lowest) makes sense in the context of nutrient stoichiometry, as rates of N fixation likely reflect the availability of the most limiting nutrient (Vitousek et al. 2002). Consistent with this idea, in Panama (where Mo and not P limited leaf litter N fixation rates) litter Mo concentrations were six times lower than in litter Mo concentrations in the Costa Rican site, again suggesting that Mo limitation is more likely to occur in sites (or on substrates) where Mo concentrations are low relative to other essential nutrients (Table 1; Barron et al. 2009). The data from Wurzbürger et al. (2012) strongly support this idea. However, it is also important to note that, even when combined, natural variation in nutrient concentrations only explained a portion of the variability in free-living N fixation rates (Table 2; Fig. 2), suggesting that other factors (e.g., C availability, substrate moisture, variance and/or errors introduced by our measurement and experimental techniques; Vitousek and Hobbie 2000, Reed et al. 2007b) also exert control and interact with nutrient availability to regulate free-living N fixation.

Simple correlations between substrate nutrient concentrations and N fixation rates (or lack thereof; Table 2; Fig. 2) cannot resolve questions of nutrient limitation, but the fertilization experiment we conducted does provide direct evidence that P, but not Mo, limits leaf litter N fixation in the Costa Rican rain

forest (Fig. 3; Table 2). Contrary to our predictions, data from the Mo \times P fertilization showed that leaf litter N fixation rates responded solely to P additions, suggesting that Mo did not limit N fixation even when P was supplied in excess (+Mo+P treatment; Fig. 3). These data contrast with results from the Barron et al. (2009) and Wurzbürger et al. (2012) fertilization and incubation studies, which showed that Mo or +Mo+P (and never +P alone) limited leaf litter N fixation in a set of tropical forests in Panama.

The Panama sites studied by Barron et al. (2009) and the Costa Rica site are climatically similar, high diversity forests that occupy a similar region of the neo-tropics (the sites are <500 km apart). In addition, they are both on highly weathered soils, yet the nature of nutrient limitation of free-living N fixation varies between these sites. How do we explain this difference? First, despite the fact that P and Mo are both rock-derived elements, the relative availability of both varies within and among tropical rain forest sites, even on those that generally occur on highly weathered soils. For example, there were large differences in total Mo and P concentrations among positions along the vertical profile in Costa Rica, and the elements did not vary across this spatial scale in the same way: On a mass basis, total P concentrations were highest in the canopy and lowest in the soil, while total Mo concentrations were highest in the soil and lowest in the canopy (Fig. 1). This opposing pattern could lend insight into nutrient limitation at a larger scale. In particular, as described above, support for relationships between Mo and N fixation only emerged in the portion of the ecosystem where Mo concentrations were at their lowest, and P:Mo ratios highest (i.e., in the canopy; Figs. 1, 2; Table 1). More broadly, these results highlight the potential for tree species-driven differences in nutrient cycling to have significant effects on N inputs via fixation (sensu Reed et al. 2008). While canopy N fixation rates in Costa Rica are relatively low, species-specific differences in Mo could more strongly regulate N fixation in more Mo-depleted sites such as Panama (Barron et al. 2009), ultimately affecting small-scale variation in rates of other ecosystem processes via changes to N cycling. For example, through their effects on N fixation rates, among species differences in foliar and soil nutrient concentrations (John et al. 2007; Townsend et al. 2007; Reed et al. 2008) could create small-scale gradients in N availability that drive observed species

variation in other ecosystem processes (Menyailo and Hungate 2003; McNamara et al. 2008; van Haren et al. 2010).

Next, the potentially varying effects of nutrient fertilization on N-fixer community structure may also help explain site-specific differences in the nature of nutrient limitation. For example, the concentrations of Mo and P added often vary significantly among fertilization experiments, and the concentration of the nutrient additions could affect N-fixing communities in ways that impact the responses. It is clear that fertilization can rapidly alter free-living N-fixing community composition (Reed et al. 2010, 2011). If high nutrient inputs select for particular organisms or microbial communities that are normally rare, results from nutrient enrichment experiments could provide misleading information about nutrient constraints over N fixation in natural ecosystems. Similarly, deeper assessments of N-fixing community composition in unmanipulated forest substrates could help clarify questions about the relative limitations posed by P or Mo. For example, if a site is dominated by an N-fixing community that uses Mo-free nitrogenase to fix N₂ (instead using nitrogenase with iron or vanadium; Raymond et al. 2004), considering Mo limitation to N fixation rates might be immaterial. Thus, the data we do have suggest that studies clarifying the role of community structure in regulating responses to fertilization could be particularly valuable.

Finally, the challenges of measuring biologically relevant forms of Mo directly, combined with a relatively poor understanding of how Mo cycles and the factors that influence Mo cycling in litter and soils all encumber attempts to assess nutrient limitation of N fixation in any site. For example, most previous studies (including this one) have assessed total Mo concentrations and comparisons between extractable Mo pools and N fixation rates are rare (but see Wurzburger et al. 2012). Nevertheless, the relationship between total Mo and available Mo is likely complex, and total Mo concentrations may not accurately predict Mo availability, especially across broad geographical gradients. Similarly, soils and leaf litter may vary considerably in their capacity to bind Mo (e.g., across edaphic gradients), thus generating differences in the size of the biologically available Mo pool within and between sites (Wichard et al. 2009; Wurzburger et al. 2012).

Unfortunately, very few other studies have attempted to explicitly assess and compare P versus Mo controls over free-living N fixation using direct nutrient additions. Vitousek and Hobbie (2000) did investigate micro-nutrient (a mixture of micro-nutrients including Mo) versus P controls over free-living N fixation in a set of tropical rain forest sites in Hawai'i. We explored their data for N fixation rates from a common litter that was fertilized over the short-term (<1 year), which is the component of their experiment analogous to the incubation experiments described here. Their data showed that, when native leaf litter (collected outside of fertilization plots or from control plots) was decomposed in fertilized plots, P fertilization alone stimulated N fixation rates in litter from one forest. In a second forest, however, litter N fixation rates only increased when P and micro-nutrients were added in combination (Fig. 4). Although trace Mo contamination of P fertilizer could confound the interpretation of some N fixation fertilization studies (sensu Barron et al. 2009), the Vitousek and Hobbie (2000) experiment used a full-factorial design, and thus discriminating between the effect of P and micro-nutrients is more straightforward. In particular, in the forest where N fixation responded to P but not to micro-nutrient additions, it seems reasonable to conclude that N fixation was limited by P and not by micronutrients (including Mo). Additionally, natural litter Mo concentrations varied in these two Hawai'ian forests (0.45 ± 0.14 vs. $0.23 \pm 0.11 \mu\text{g Mo g}^{-1}$; Vitousek and Hobbie 2000; Table 1), such that N fixation on the high Mo leaf litter responded to P fertilization, while the leaf litter with lower Mo concentrations responded solely to additions of micro-nutrients and P (Fig. 3). It is important to note that Vitousek and Hobbie (2000) suggested that litter quality played a larger role than nutrients in regulating leaf litter N fixation, as well as that plot differences in pH could have played a role in the responses. In addition, we did not include data from litter that was collected from fertilized plots and subsequently added to other plots [due to the variation in other aspects of litter chemistry and the unnaturally high Mo concentrations compared with those we could find in the literature (Table 1)]; however, it's still important to consider that these data did not show such straightforward patterns.

Nevertheless, a recent survey of Panamanian tropical forests strongly supports the idea that natural variation in nutrient concentrations could help regulate what limits free-living N fixation (Wurzburger et al. 2012).

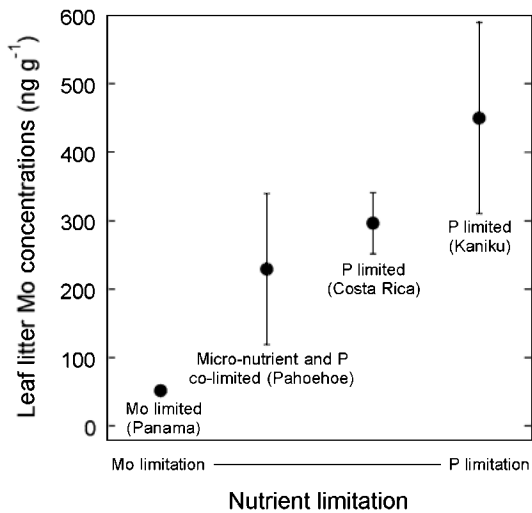


Fig. 4 Relationship between the nature of nutrient limitation (in this case, micro-nutrients vs. P limitation) and total Mo concentrations ± 1 SE (ng g^{-1}) for leaf litter from four tropical rain forests (Panama, Costa Rica, and two in Hawai‘i). Relationships were determined using data from studies that simultaneously, and using a full-factorial design, assessed responses to micro-nutrient and P additions. N fixation rates in the site with the lowest concentration of Mo were Mo limited (Barron et al. 2009), N fixation rates in the site with intermediate Mo concentrations were co-limited by micro-nutrients and P (Vitousek and Hobbie 2000), and N fixation rates in the two sites with the highest Mo concentrations were P limited (this study and Vitousek and Hobbie 2000). Data highlighted from Barron et al. (2009) and from this study are from short-term fertilization incubations and data from Vitousek and Hobbie (2000) represent N fixation assessments on common litter from the Kaniku and Pahoehoe sites allowed to decompose in fertilization plots (P \times micronutrients in full-factorial design)

This study suggests that variation in P availability can regulate whether soil O horizon N fixation is Mo or Mo + P limited (Wurzburger et al. 2012). Taken together, data from Costa Rica, Panama, and Hawai‘i suggest that extant nutrient concentrations may help predict variation in the nature of nutrient limitation to free-living N fixation (Fig. 4). Nevertheless, these are the only data we could find in the literature from experiments that simultaneously addressed the control of macro- and micro-nutrient limitation to free-living N fixation, and more data are clearly needed before overarching conclusions can emerge.

The paucity of data from direct nutrient manipulation experiments limits our ability to draw firm conclusions about general patterns in P versus Mo limitation of N fixation in tropical forests. However, attempts to identify “the nutrient” that limits N fixation in “the

tropics” belie the heterogeneity of the tropical forest biome. For example, multiple lines of evidence show the exceptional heterogeneity of tropical forests in a number of factors that are relevant to nutrient cycling (e.g., Townsend et al. 2008)—including notable variation in soil type, rainfall amount and timing, biotic storage of nutrients, and landscape stability (sensu Porder et al. 2007). Acknowledging such variation is important and would imply that the true nature of nutrient limitation to N fixation varies among specific sites, over a variety of spatial scales, and certainly across the vast tropical rain forest biome. This new and more nuanced hypothesis is consistent with prior work (Vitousek and Hobbie 2000; Reed et al. 2008; Barron et al. 2009) and with new mechanistic models (Wurzburger et al. 2012), which as a whole suggest that both P and micro-nutrients play important roles in regulating new inputs of N to tropical forests. Our work also supports the idea that assessments of P and Mo pools and their relative abundance could improve predictive capabilities regarding limitations to free-living N fixation (Wurzburger et al. 2012). What is also clear, however, is that more robust conclusions and prognostic capabilities will first require additional data on how N fixation is regulated in a much broader range of tropical forests, as well as improvements in our ability to quantify N fixation itself (sensu Cleveland et al. 2010).

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