Controls Over Leaf Litter and Soil Nitrogen Fixation in Two Lowland Tropical Rain Forests

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ABSTRACT

Global comparisons suggest that rates of N fixation in tropical rain forests may be among the highest on earth. However, data supporting this contention are rare, and the factors that regulate N fixation within the biome remain largely unknown. We conducted a full-factorial (N x P) fertilization experiment in two lowland tropical rain forests in Costa Rica to explore the effects of nutrient availability on rates of free-living N fixation in leaf litter and soil. P fertilization significantly increased N fixation rates in both leaf litter and soil, and the effect was dependent on sampling date. Fertilization with N did not affect rates of N fixation at any time. In addition, variation in N fixation rates measured in unfertilized plots at four sampling time points suggested seasonal variability in N fixation: leaf litter N fixation ranged from 0.36 kg/ha/yr in the dry season to 5.48 kg/ha/yr in the wet season. Soil N fixation showed similar patterns ranging from a dry season low of 0.26 kg/ha/yr to a wet season high of 2.71 kg/ha/yr. While the observed temporal variability suggests potential climatic control over free-living N fixation in these forests, data suggest that neither soil nor leaf litter moisture alone regulate N fixation rates. Instead, we hypothesize that a combination of ample C availability, low leaf litter N:P ratios, and high rainfall coincide during the latter portions of the rainy season and drive the highest free-living N fixation rates of the year.


Key words: Costa Rica; fertilization; free-living; phosphorus; tropical wet forest.
METHODS

SITE DESCRIPTIONS.—The study was conducted in two adjacent mature rain forests (lying within the tropical wet lowland forest bioclimate [Holdridge et al. 1971]) on the Osa Peninsula in southwestern Costa Rica (8°43' N, 83°37' W). At these sites, mean annual temperature is approximately 26°C ± 1.5°C (Kappelle et al. 2002) and monthly rainfall data collected at the Sirena Biological Station (~25 km from the sites) show a regional mean annual rainfall of 5578 mm/yr ± 773 mm/yr (2001-2005; A. Vega, unpublished data). In addition, like many tropical rain forests (Gentry 1990, Kricher 1999), those on the Osa Peninsula experience a dry season. The Sirena rainfall data show that the dry season on the Osa Peninsula (defined here as months receiving < 200 mm of rainfall) consistently occurs from December through March. Litter trap data from the forest sites of this study also show that roughly half of the annual litterfall occurs during the dry season months (Cleveland & Townsend 2006).

The two forest sites are less than a few hundred meters apart, have high plant diversity and similar species composition, and experience identical climate. Both sites are stratified, closed canopy forests containing common canopy species (e.g., *Bromus utile* [Kunth] Oken. [Moraceae], *Carapa costaricensis* Donn. Sm. [Carpo­caceae], *Hieronyma alchorneoides* Fr. Allem. [Phyllanthaceae], *Schizolobium parahybum* [Vell.] S.F. Blake [Fabaceae], and *Van­tanea barbourii* Standl. [Humiriaceae]) and understory species (e.g., *Bromelia spp.* [Bromeliaceae] and *Geziono spp.* [Areaceae]). However, the soil type at the two sites differs: one contains a highly weathered, P-poor Ultisol (Typic Tropohumult; Pérez et al. 2002, hereafter Ultisol site) that was formed from Quaternary alluvial deposits. The Ultisol soil has 76.2 percent clay content, 6.2 ± 1.7 percent total C, and significantly greater stocks of total P (1051.4 ± 43.1 Mg/g soil; P  <  0.001) than the Ultisol site (see Cleveland et al. [2002] and Bern et al. [2005] for complete soil descriptions). The Ultisol and Mollisol soils at the sites represent the dominant soil orders found in this area (Perez et al. 2002).

EXPERIMENTAL DESIGN.—Labile P was defined as the sum of the resin- and bicarbonate-extractable P fractions of the Hedley fractionation (Tieszen & Moir 1993) for the top 10 cm of soil, and significantly different labile P concentrations between the soil at the Ultisol site and the relatively P-rich Mollisol site (Table 1; Cleveland et al. 2006) allowed us to test the effects of in situ P availability on forest floor N fixation. In addition, we used a set of N and P fertilization plots on each soil type to investigate the role of N and P availability in regulating N fixation at each site separately. Each site included forty 5 m × 5 m plots fertilized with N (+N) and P (+P) in a half-factorial design: 10 +N plots, 10 +P plots, 10 +N+P plots, and 10 control plots that received no fertilization.

The fertilization plots received nutrient amendments for 3 yr prior to the beginning of this study, and fertilization continued throughout the course of the experiment. Fertilizer was applied twice per year by hand-broadcasting N (as NH₄NO₃) or P (as KH₂PO₄) at a rate of 150 kg N/ha/yr or 150 kg P/ha/yr. +N+P plots received 150 kg N/ha/yr and 150 kg P/ha/yr. Owing to the high P sorption capacity of the Ultisol soil (Oberson et al. 1997, Townsend et al. 2002), and to ensure the removal of nutrient constraints, fertilization plots received N and P at a 1:1 ratio. Soil analyses showed that plots fertilized with P (+P and +N+P plots) had significantly higher concentrations of labile P compared with control and +N plots for both the Ultisol and Mollisol sites (P  <  0.05 for each; Table 1). Similarly, plots fertilized with N (+N and +N+P plots) had significantly higher soil inorganic N concentrations (defined as the sum of 2 M KCl-extractable nitrate [NO₃⁻] and ammonium [NH₄⁺]) compared with control and +P plots for both the Ultisol and Mollisol sites (P  <  0.001 for each; Table 1).

We used the acetylene reduction assay (ARA; Hardy et al. 1968) to measure leaf litter and soil N fixation rates. In addition, to investigate the possibility of temporal variation in N fixation rates and controls, we measured N fixation rates in all plots at both sites four times throughout a single year: (1) January (dry season); (2) April (transition from the dry to the wet season); (3) June (mid wet season); and (4) September (late wet season).

TABLE 1. Soil labile P and inorganic N content (0–10 cm) of the Ultisol and Mollisol sites.⁸

<table>
<thead>
<tr>
<th></th>
<th>Ultisol</th>
<th>Mollisol</th>
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</thead>
<tbody>
<tr>
<td>Labile P (µg/g soil)</td>
<td>Control 19.4 ± 5.3***</td>
<td>25.1 ± 7.6***</td>
</tr>
<tr>
<td></td>
<td>+N 25.5 ± 2.7⁸</td>
<td>33.4 ± 1.7⁸</td>
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<tr>
<td></td>
<td>+P 67.7 ± 13.8⁸</td>
<td>116.8 ± 40.0⁸</td>
</tr>
<tr>
<td></td>
<td>+N+P 107.4 ± 33.5⁸</td>
<td>146.2 ± 34.3⁸</td>
</tr>
<tr>
<td>Inorganic N (µg/g soil)</td>
<td>Control 73.7 ± 14.6***</td>
<td>49.0 ± 10.4***</td>
</tr>
<tr>
<td></td>
<td>+N 308.1 ± 187.0⁸</td>
<td>113.7 ± 43.7⁸</td>
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<tr>
<td></td>
<td>+P 85 ± 37.4⁴</td>
<td>54.1 ± 52.0⁸</td>
</tr>
<tr>
<td></td>
<td>+N+P 264.9 ± 350.9⁸</td>
<td>195.8 ± 91.1⁸</td>
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</tbody>
</table>

⁸Values are means ± 1 SD.

**P < 0.01; ***P < 0.001 represent significant differences between control plots of the Ultisol and Mollisol sites. Significant differences among the fertilization plots within a single site are denoted by differing lowercase letters within a column.

SAMPLE COLLECTION AND ANALYSES.—During each sampling event, two leaf litter and two soil samples were randomly collected from each of the 80 fertilization plots (40 at the Ultisol site and 40 at the Mollisol site), and the two sample values from any plot were averaged to obtain a single leaf litter and single soil value for each plot. Leaf litter was hand-collected near the surface of the soil and placed into 55-mL clear acrylic tubes. After gently removing all litter from the soil surface, soils were sampled as intact cores using identical 55 mL, 2.54-cm-diam acrylic tubes inserted to a depth of 1 cm. All methods for estimating rates of N fixation have some limitations.
After the incubation, sample headspaces were mixed, subsampled, vented to the atmosphere. All samples were left overnight to incubate were also converted into N fixation rates (on both a mass and an area basis). We applied a literature conversion factor of 3.9 moles of acetylene reduced/gram dry mass of sample/hour of incubation. However, to facilitate comparison with other published estimates, and to present N fixation rates as nanomoles (10^-9 moles) of acetylene reduced/gram dry mass of sample/hour of incubation. Consequently, to calculate the one-way analysis of variances (ANOVARs) were used to assess the effects of individual factors. Multiple comparisons within a factor were analyzed using Tukey’s post-hoc analysis. We hypothesized that the relative moisteres of the leaf litter and of the soil would positively correlate with rates of free-living N fixation. Thus, for each site we performed linear regression analyses of control plot leaf litter and soil N fixation rates and their respective moisteres. All analyses were conducted using SPSS 11.0.4 software (SPSS, Chicago, IL, U.S.A.).

RESULTS

GLM analysis showed a significant interaction between the date of sampling and P fertilization factors for both leaf litter (P = 0.028) and soil (P = 0.040). There was also a significant interaction between the date of sampling and the site factors for leaf litter and soil (P < 0.001 for each). No other interactions between factors were significant.

NUTRIENT EFFECTS.— P fertilization significantly increased N fixation rates of leaf litter and soil samples for both sites; however, the effect of fertilization with P was dependent on the sampling date (Table 2). In general, the effect of fertilization with P at both sites was strongest during the wet season months of June and September (Table 2). In contrast, fertilization with N had no effect on rates of N fixation, regardless of the sampling date (Table 2).

SAMPLING DATE TRENDS.—Our results suggest that N fixation rates in leaf litter and soil (P < 0.001 for each) differed significantly across sampling dates in the control plots at the Ultisol site (Fig. 1). Rates of N fixation in the Ultisol leaf litter were significantly higher in the wet months of June (0.78 mg/g/yr) and September (0.74 mg/g/yr) than they were during the dry season month of January (0.23 mg/g/yr). The April (transition from dry to wet season) fell between the dry and wet season values (0.44 mg/g/yr). Likewise, N fixation rates in the Ultisol soil were significantly higher in June (0.007 mg/g/yr) and September (0.009 mg/g/yr) than in January (0.002 mg/g/yr) or April (0.001 mg/g/yr).

Similarly, both the Mollisol site leaf litter and soil N fixation rates were significantly different among sampling dates (P < 0.001 for each; Fig. 1). September leaf litter had the highest rates of N fixation (1.03 mg/g/yr), followed by June and April rates (0.202 mg/g/yr and 0.15 mg/g/yr, respectively), and January leaf litter N fixation (0.09 mg/g/yr) was the lowest of the year. Similarly in the Mollisol soil, N fixation rates were highest in September (0.007 mg/g/yr), lower in June (0.002 mg/g/yr), and N fixation rates were lowest in January and April (0.0008 mg/g/yr and 0.0005 mg/g/yr, respectively).

SAMPLE MOISTURE EFFECTS.—There was no significant relationship between leaf litter N fixation rates and leaf litter percent moistures in the Ultisol plots (P = 0.674; R² = 0.005). There was a significant relationship between the Mollisol leaf litter moistures and rates of...
TABLE 2. Effects of fertilization on acetylene reduction rates in the leaf litter and soil of Ultisol and Mollisol sites.  

<table>
<thead>
<tr>
<th>Month</th>
<th>Ultisol litter</th>
<th></th>
<th>Mollisol litter</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>+N</td>
<td>+P</td>
<td>+N+P</td>
<td>Control</td>
<td>+N</td>
<td>+P</td>
</tr>
<tr>
<td>January</td>
<td>3.71 (1.33)</td>
<td>6.32 (1.83)</td>
<td>10.62 (4.1)</td>
<td>3.12 (0.91)</td>
<td>1.40 (0.66)</td>
<td>0.99 (0.49)</td>
<td>1.09 (0.44)</td>
</tr>
<tr>
<td>April</td>
<td>7.19 (2.2)</td>
<td>2.33 (1.11)</td>
<td>5.39 (2.24)</td>
<td>7.22 (2.09)</td>
<td>2.36 (1.95)</td>
<td>1.87 (0.82)</td>
<td>3.35 (1.23)</td>
</tr>
<tr>
<td>June</td>
<td>12.34 (2.77)</td>
<td>14.16 (3.45)</td>
<td>21.45 (6.02)</td>
<td>24.22 (17.5)</td>
<td>3.43 (1.47)</td>
<td>6.04 (4.50)</td>
<td>12.16 (3.8)</td>
</tr>
<tr>
<td>September</td>
<td>12.02 (4.09)</td>
<td>10.44 (3.96)</td>
<td>36.49 (12.2)</td>
<td>24.03 (8.36)</td>
<td>16.37 (5.41)</td>
<td>10.14 (3.30)</td>
<td>41.29 (9.93)</td>
</tr>
<tr>
<td></td>
<td>Ultsol soil</td>
<td></td>
<td>Mollsol soil</td>
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<td>Ultsol soil</td>
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<td>Mollsol soil</td>
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<tr>
<td>January</td>
<td>0.035 (0.016)</td>
<td>0.071 (0.056)</td>
<td>0.026 (0.008)</td>
<td>0.042 (0.026)</td>
<td>0.013 (0.005)</td>
<td>0.004 (0.001)</td>
<td>0.003 (0.001)</td>
</tr>
<tr>
<td>April</td>
<td>0.016 (0.009)</td>
<td>0.008 (0.002)</td>
<td>0.011 (0.001)</td>
<td>0.010 (0.002)</td>
<td>0.008 (0.003)</td>
<td>0.005 (0.001)</td>
<td>0.011 (0.002)</td>
</tr>
<tr>
<td>June</td>
<td>0.116 (0.011)</td>
<td>0.113 (0.016)</td>
<td>0.14 (0.014)</td>
<td>0.177 (0.073)</td>
<td>0.031 (0.006)</td>
<td>0.039 (0.014)</td>
<td>0.076 (0.017)</td>
</tr>
<tr>
<td>September</td>
<td>0.151 (0.015)</td>
<td>0.150 (0.019)</td>
<td>0.182 (0.020)</td>
<td>0.183 (0.027)</td>
<td>0.115 (0.02)</td>
<td>0.107 (0.012)</td>
<td>0.103 (0.01)</td>
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</table>

*Values are means (expressed in nmol C\textsubscript{2}H\textsubscript{2} reduced/gram of dry sample/hour of incubation) with standard errors in parentheses.

*P < 0.05; **P < 0.01 represent significant differences in leaf litter or soil rates among fertilization treatments for each sampling date and site independently. Significance of P fertilization is represented on both the +P and +N+P plots.

Leaf litter N fixation (P = 0.042; R\textsuperscript{2} = 0.124). There was also a significant relationship between soil moisture and soil N fixation rates in the Ultisol plots (P < 0.01; R\textsuperscript{2} = 0.180) and the Mollisol plots (P = 0.036 R\textsuperscript{2} = 0.139).

SITE AND SAMPLE TYPE EFFECTS.—Regardless of site or sampling date, rates of N fixation (on a mass basis) were several orders of magnitude greater in leaf litter than in soil (Fig. 1). Both leaf litter and soil N fixation rates tended to be higher in the P-poor Ultisol...
site, though not in all sampling periods (Fig. 1). Control plot Ultisol leaf litter displayed significantly higher N fixation rates than control plot Mollisol leaf litter (January \( P = 0.011 \); April \( P = 0.016 \); June \( P = 0.002 \)) for all but the late wet season September samples, when there was no significant difference between sites \( (P = 0.848; \) Fig. 1). Ultisol soil also had significantly higher N fixation rates than Mollisol soil, though the difference is variable with time (Fig. 1). Ultisol soil had significantly higher rates of N fixation in June \( (P < 0.001 \) and marginally higher rates in January \( (P = 0.057 \), but April \( (P = 0.428 \) and September \( (P = 0.212 \) rates were not different.

**DISCUSSION**

With > 5000 mm of precipitation a year, our sites receive more rain­fall than many tropical rain forests (Gentry 1990, Kricher 1999), but our results corroborate other data showing high rates of free-living N fixation for this biome (Jordan et al. 1982, Maheswaran & Gunatilleke 1990). Moreover, the unfertilized N fixation rates we measured were more than four times greater than free-living leaf litter and soil rates typical of temperate forests (Grant & Binkley 1987, Heath et al. 1988, Hendrickson 1990), suggesting that significant amounts of biologically fixed N enter this rain forest ecosystem via free-living N fixation (Fig. 2).

In these tropical rain forest sites, high rates of free-living N fixation occur in spite of the fact that (1) P availability has been shown to limit the process (Eisele et al. 1989); (2) these soils are characterized as P-poor and highly weathered (Bern et al. 2005); and (3) P limits other microbial processes in these sites (Cleveland et al. 2002, Cleveland & Townsend 2006). Larger-scale fertilization studies in tropical ecosystems have shown that adding P alters the live-leaf chemistry of plants within the fertilization plots, and thus changes the decomposability of leaves falling on the forest floor. Accordingly, the C availability of the leaf litter is changed, and P fertilization indirectly stimulates free-living N fixation through changes in C cycling (Crews et al. 2000, Vitousek & Hobbie 2000). At our sites, the small plot size \((5 \text{ m} \times 5 \text{ m})\) allowed for a more direct assessment of the effects of fertilization on forest floor processes, because plots were too small to alter the chemistry of leaves falling from the canopy. Data from our fertilization plots suggest that increases in P availability more directly stimulated N fixation in these rain forests, and that low P availability may limit both leaf litter and soil N fixation, at least during some portions of the year (Table 2).

Contrary to our expectations, N fertilization did not suppress rates of N fixation (Table 2). Although N is relatively abundant in many tropical rain forests (Martinek et al. 1999), recent data suggest that exceptionally wet forests—such as the ones studied here—can show signs of N limitation, even in ecosystems on highly weathered soils (Ilsstedt & Singh 2005). The fact that added N did not suppress N fixation in the +N plots, that overall rates in control plots were relatively high, and that N additions significantly increased soil respiration and root biomass (Cleveland & Townsend 2006) suggests that N availability may not consistently exceed demand at these sites.

We also observed variations in unfertilized rates of N fixation sampled at different times during the year. In both control plot leaf litter and soil, N fixation was severalfold greater in the wet season months of June and September than it was during the relatively dry month of January (Figs. 1 and 2). At first glance, the observed annual variation makes intuitive sense: while temperature at the site remains relatively constant throughout the year, average rainfall increases from April through October, such that the September sampling period coincides with the wettest time of the year. Given the sensitivity of the nitrogenase enzyme to oxygen (Nohrstedt 1983, Hicks et al. 2003), the high rates of N fixation observed in September could be directly related to an increase in the proportion of anoxic microsites that may result from several months of heavy rainfall (Silver et al. 1999, Schuur 2001).

Surprisingly, however, the relationships between soil or leaf litter moisture content and N fixation rates did not suggest that moisture content was the dominant control over N fixation. There were significant relationships between soil moisture and soil N fixation rates for both the Ultisol and Mollisol sites \((P < 0.01 \text{ and } P < 0.05, \text{ respectively})\), but those relationships only explained a fraction of the variability in the N fixation data \((R^2 = 0.180 \text{ and } 0.139, \text{ respectively})\). Moreover, leaf litter N fixation values (on a mass basis) were far higher than those in soil (Fig. 1). However, there was no significant effect of leaf litter moisture on N fixation at the Ultisol site and, though significant, the variability in leaf litter moisture at the Mollisol site did not explain a high percentage of the variability in N fixation rates \((R^2 = 0.124)\). Total moisture values do not necessarily indicate the amount of anoxic microsites that would favor N fixation, but the lack of a strong relationship between moisture

![FIGURE 2. Area-based estimates of N fixation rates by month for the leaf litter and soil of the Ultisol and Mollisol control plots. Rates represent means of samples collected in the control plots in January (dry season), April (transition from the dry to wet season), June (mid wet season), and September (late wet season) of a single year.](image-url)
content and N fixation suggests the possibility of seasonal controls over N fixation that are not solely driven by moisture content.

We hypothesize that the high rates of N fixation observed in June (mid wet season) and September (late wet season) may be partially driven by interactions between seasonal changes in labile C availability, N demand, and water availability. The short dry season at our sites is typified by a large input of leaf litter to the forest floor (Cleveland & Townsend 2006). Once the rainy season begins in earnest (April), ample daily rainfall provides a vehicle for the movement of substantial pulses of readily decomposable, dissolved organic C (DOC) from the litter layer, driving annual-high rates of soil respiration (Cleveland & Townsend 2006). The N-fixing microbial community in the litter layer and the surface soil is likely dominated by heterotrophic microorganisms (Sprent & Sprent 1990), thus one might expect a similar seasonal pattern for N fixation at the forest floor. However, soil respiration peaked at the beginning of the rainy season (April), while the highest N fixation rates were observed much later in the rainy season (Fig. 1).

While the exact mechanisms remain unclear, the offset between the timing of peak soil respiration and N fixation may be a consequence of seasonal variations in relative N availability and demand. While standing litter C pools are highest early in the wet season (Cleveland & Townsend 2006), leaf litter N:P values are also at an annual high, but slowly decline to annual lows at the end of the rainy season (Fig. 3; C. Cleveland, unpublished data). The decline in leaf litter N:P ratios as the rainy season progresses likely results from high P immobilization rates during decomposition in this P-poor ecosystem, as well as from steady N loss rates (relative to P) through the year. Wood et al. (2005) showed the same pattern in another Costa Rican rain forest, where the N:P value of leaf litter was a function of the amount of rainfall prior to sampling, with lower N:P ratios consistently following greater rainfall. Lower N:P values are known to drive higher rates of N fixation in other ecosystems (Eisele et al. 1989), and litter-layer fixation has been shown to vary in concert with the extent to which decomposition is limited by N (Vitousek & Hobbie 2000). Because the soluble fraction of decomposing litter remains relatively constant throughout the rainy season at these sites (Cleveland et al. 2006), persistent C availability combined with declining N:P ratios through the rainy season suggest a conceptual model for forest floor N fixation in which reasonable C availability and favorable N:P ratios co-occur in the latter portion of the rainy season to drive the highest rates of N fixation.

Finally, while P additions did stimulate N fixation rates in all sites, unfertilized rates were higher at the Ultisol site than the Mollisol site (Fig. 1), despite the fact that labile P pools are greater in the latter soil type (Table 1). Rates of litter mass loss were also significantly greater at the Ultisol site than at the Mollisol site (Cleveland et al. 2006), and the higher decomposition rates may fuel higher N fixation rates (cf. Vitousek & Hobbie 2000). In addition, although sample moisture did not appear to be the only control over N fixation within a site, percent moisture was significantly related to soil N fixation rates at both sites and to leaf litter N fixation rates at the Mollisol site. Both soil and leaf litter percent moistures were consistently higher at the Ultisol site (data not shown) and site-specific hydrology could influence overall N fixation rates. Regardless of the cause, the higher rates of N fixation at the Ultisol site suggest that the absolute availability of P does not exclusively control N fixation in this system.

We stress that the data presented here are from rain forest sites that receive relatively high levels of annual rainfall, and our data only represent free-living leaf litter and soil N fixation. Within these ecosystem components, the observed rates of N fixation are notably high, and therefore may be an important source of N to this ecosystem (Fig. 2). High rates of free-living N fixation are more likely to be important to the N economy of relatively wet tropical forests (such as those studied here), as they are more likely to experience high energy availability (as available C; Malhi et al. 1999, Cleveland et al. 2006), frequent periods of low oxygen availability (Silver et al. 1999, Schuur 2001), and high N loss rates through denitrification (Hall & Marston 2003) and leaching (Lewis 1986). In addition, recent studies suggest that, although leguminous trees are relatively common in tropical rain forests, many may not be fixing significant amounts of N (Gehring et al. 2005). Taken together, these factors emphasize the potential importance of free-living N fixation in sustaining ecosystem N availability in tropical rain forests. However, the considerable temporal variability in our data also highlights the complexity of N fixation and the factors that govern it at these rain forest sites. Here we present a framework that may help understand and predict forest floor N inputs, but those still account for only a portion of total potential N fixation in any tropical forest.

\[\text{FIGURE 3. N:P ratios (total nutrient concentrations on a mass basis) of leaves decomposing on the forest floor of the Ultisol and Mollisol sites. Values represent means (± 1 SE) and data are from a litterbag decomposition experiment described in Cleveland et al. (2006). Months begin in May and are represented by the first letter of the month's name. Data for Mollisol litter in December were unavailable.}\]
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LITERATURE CITED


