SYNTHESIS AND EMERGING IDEAS

Using indirect methods to constrain symbiotic nitrogen fixation rates: a case study from an Amazonian rain forest

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Abstract Human activities have profoundly altered the global nitrogen (N) cycle. Increases in anthropogenic N have had multiple effects on the atmosphere, on terrestrial, freshwater and marine ecosystems, and even on human health. Unfortunately, methodological limitations challenge our ability to directly measure natural N inputs via biological N fixation (BNF)—the largest natural source of new N to ecosystems. This confounds efforts to quantify the extent of anthropogenic perturbation to the N cycle. To address this gap, we used a pair of indirect methods—analytical modeling and N balance—to generate independent estimates of BNF in a presumed hotspot of N fixation, a tropical rain forest site in central Rondônia in the Brazilian Amazon Basin. Our objectives were to attempt to constrain symbiotic N fixation rates in this site using indirect methods, and to assess strengths and weaknesses of this approach by looking for areas of convergence and disagreement between the estimates. This approach yielded two remarkably similar estimates of N fixation. However, when compared to a previously published bottom-up estimate, our analysis indicated much lower N inputs via symbiotic BNF in the Rondônia site than has been suggested for the tropics as a whole. This discrepancy may reflect errors associated with extrapolating bottom-up fluxes from plot-scale measures, those resulting from the indirect analyses, and/or the relatively low abundance of legumes at the Rondônia site. While indirect methods have some limitations, we suggest that until the technological challenges of directly measuring N fixation are overcome, integrated approaches that employ a combination of model-generated and empirically-derived data offer a promising way of constraining N inputs via BNF in natural ecosystems.

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Introduction

How much have human activities altered the global nitrogen (N) cycle? Estimates now suggest that the creation of reactive N via fossil fuel combustion, fertilizer production and the cultivation of N-fixing crops rivals N inputs from all natural processes combined (Vitousek et al. 1997; Galloway et al. 2004). The increase in human-derived N has been implicated in a range of environmental problems including widespread declines in forest health, changes in species diversity and composition, and eutrophication of aquatic ecosystems (Schulze 1989; Vitousek et al. 1997; Galloway et al. 2003). However, the true extent to which human activities have perturbed the global N cycle is unknown, owing largely to uncertainties in estimates of the amount fixed N that enters the biosphere via biological N fixation (BNF).

In natural ecosystems, BNF is the largest potential source of "new" N—entering either through symbiotic relationships (e.g., between legumes and Rhizobia) or by free-living microorganisms. Empirical measurements of natural BNF in terrestrial ecosystems are technically difficult to make. As a result, current estimates remain largely unresolved, limiting our ability to compare anthropogenic versus natural N inputs. For example, published estimates of terrestrial BNF suggest that it may vary by more than a factor of two within any single biome, and by more than a factor of three (i.e., 80–250 Tg N year^{-1}) at the global scale (e.g., Cleveland et al. 1999). This uncertainty undermines efforts to assess human impacts on the global N cycle, fundamentally challenging the biogeosciences (Vitousek et al. 2002).

Two things in particular limit our ability to accurately quantify BNF. First, we lack a method to directly measure symbiotic N fixation rates (e.g., Warembourg 1993). High background concentrations of N_2 in the atmosphere make it impossible—at least using current technology—to detect small changes in N_2 concentration resulting from N fixation. As a result, rates are most frequently assessed using indirect approaches (e.g., ^{15}N and ARA) that may

or may not provide accurate estimates (Warembourg 1993, Gehring et al. 2005). Second, symbiotic BNF is both spatially and temporally heterogeneous, owing to patchy distributions of potential N-fixers (ter Steege et al. 2006) and phylogenetic constraints to nodulation and N fixation (Sprent 2005). For example, N fixation is widespread throughout the Fabaceae, but the predisposition to fix N is not expressed equally among all sub-families (Sprent and Parsons 2000). In the Caesalpiniaceae subfamily, only ~20% of the species examined to date appear capable of N fixation (Allen and Allen 1981; Sprent 2005). Yet, as is the case with most subfamilies, there are numerous genera within the Caesalpiniaceae for which the potential for nodulation has not been well established (Sprent 2005). In addition, even in species with the ability to nodulate, the presence of nodules does not necessarily guarantee N fixation, making it difficult to infer N fixation rates from nodule abundance (Cleveland et al. 1999).

Finally, ^{15}N isotopic methods have been used successfully to assess symbiotic BNF rates in agricultural species growing in monoculture, but they often fail when applied to heterogeneous ecosystems where source ^{15}N and isotopic fractionations significantly alter N pools and fluxes (Houlton et al. 2007). As a result of these difficulties our understanding of N fixation rates in natural ecosystems remains poor. This situation thus begs the question, how do we understand a fundamental ecological process (like N fixation) when we lack the ability to measure it?

Indirect methods such as simulation modeling and analyses of ecosystem N balances may offer useful alternative approaches for constraining N fixation rates at the watershed scale. For example, N balance approaches have been used to estimate nutrient fluxes in a range of sites and contexts (e.g., Chestnut et al. 1999; Vitousek et al. 2002; Binkley et al. 2004), and models have been used to predict N fixation as a function of forest succession (Rastetter et al. 2001) and at the global scale (Houlton et al. 2008). Thus, in the absence of methods to directly assess symbiotic N fixation rates, we suggest that indirect approaches may offer a reasonable way forward for estimating BNF beyond the plot-level scale.

Here, by way of example, we used both synthetic modeling and N balance approaches to generate

independent estimates of BNF at a tropical rain forest site in Rondônia in the Amazon Basin. We focused on a tropical rain forest site for several reasons. First, while tropical rain forests occupy only ≈13% of global land area (Cramer et al. 1999), they represent a hotspot of putative N-fixers in the Fabaceae, and estimates suggest that the tropical rain forest biome may account for up to 25% of natural terrestrial BNF (Cleveland et al. 1999). Second, empirical data describing natural N fixation rates in tropical forests are rare, and large-scale estimates of tropical N fixation are based on a handful of instantaneous, plot-scale estimates extrapolated to the biome scale. Third, while N fixation is presumed to be high in tropical rain forests, a number of recent studies have questioned this conclusion (e.g., Gehring et al. 2005). Fourth, since the potential for substantial natural N fixation appears high in lowland tropical forests, further constraining N inputs via N fixation is a necessary next step in evaluating how human activities have altered the global N cycle. Finally, the study site in the Amazon Basin is well studied, and thus a wealth of empirically-derived biogeochemical data necessary for generating estimates of BNF using both the modeling and mass balance approaches were available.
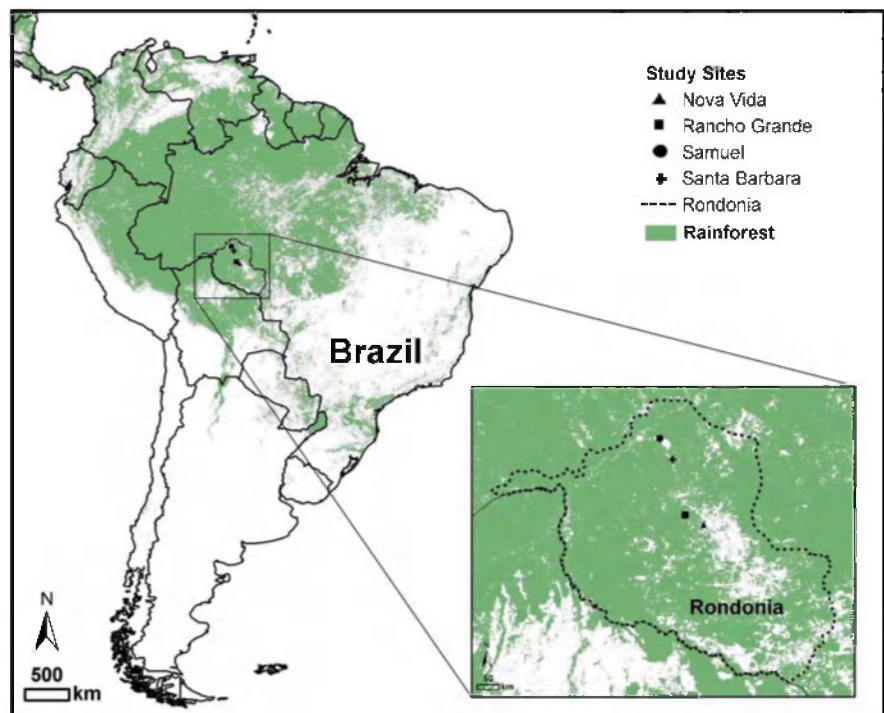
Methods

Study sites

To generate both modeled and mass-balance estimates of N fixation, we synthesized data from several primary rain forest sites in central Rondônia in the Amazon Basin (Fig. 1). These were Fazenda (Ranch) Nova Vida (10°30′S, 62°30′W), Rancho Grande (10°18′S, 62°52′W), and a site in Santa Bárbara (9°11′S, 63°07′W) (Fig. 1). Detailed descriptions of the sites can be found elsewhere (Kauffman et al. 1995; Neill et al. 1997; Germer et al. 2007), but all are in primary tropical forests, are similar topographically and climatologically, and soils at all the study sites are classified as Ultisols (Table 1). We also obtained data on legume abundance from a study conducted at the nearby Samuel Ecological Reserve in northern Rondônia (Martinelli et al. 2000). For the purposes of this analysis, data from these adjacent sites were

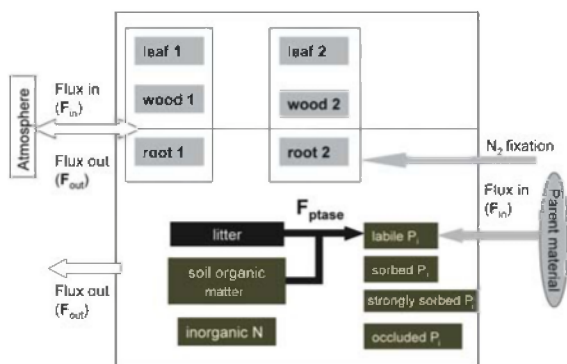Fig. 1 Location of Rondônia and the primary forest study sites

Table 1 Climatologic, topographic and edaphic characteristics of the study sites

	Site			
	Nova Vida	Rancho Grande	Santa Bárbara	Samuel Reserve
Mean annual temperature (°C)	25	27	26	NA
Mean annual precipitation (mm)	2200	2300	2100	2300
Dry season	May–Sept	July–Sept	June–Sept	June–Sept
Elevation (m)	150	143	NA	NA
Soil type	Ultisols	Ultisols	Oxisols/ Ultisols	Oxisols

combined to generate results that describe a single, representative primary forest site in Rondônia.

Modeled estimates of BNF

Several models have attempted to examine patterns and regulation of N fixation on land (Vitousek and Field 1999; Vitousek et al. 2002). Most recently, Houlton et al. (2008) applied the resource optimization paradigm (Rastetter et al. 2001; Wang et al. 2007) to simulate N fixation among diverse terrestrial ecosystems and at the global scale. This model, which is described in detail in Wang et al. (2007) and Houlton et al. (2008) and depicted in Fig. 2 consists of multiple pools of C, N and P in soils and in N-fixing and non-fixing plants, and fully couples carbon (C), N and phosphorus (P) interactions

Fig. 2 Schematic diagram of the analytical model used in this analysis. The large *square box* represents the spatial domain of the Rondônia primary forest, within which plant pools (*light grey boxes*, one set that represents plants that fix N and another that does not) and corresponding litter and soil pools (*dark grey boxes*) exist. Exchanges between the atmosphere, hydrosphere, and geosphere are indicated by arrows. Biochemical P mineralization rate (F_{Ptase}) represents the flow of organic P from litter to the labile P pool. Reproduced from Wang et al. (2007) by permission of the American Geophysical Union

according to widely accepted conceptual models of ecosystem nutrient dynamics (Walker and Syers 1976; McGill and Cole 1981).

Briefly, N-fixing and non N-fixing plants are identical in the model, except for the capacity to fix N. Plants compete for light aboveground and nutrients belowground, with competitive outcomes proportional to net primary production (NPP) yield. In maximizing NPP, the model considers optimal strategies in acquiring N (N uptake versus N fixation) and P (phosphatase enzymes versus uptake of microbially-mineralized P), and in C allocation (e.g., to shoots versus roots) in a given environment and parameter space. To simulate the widely known energetic constraints to N fixation, the model assumes a cost of 10 g of C per g of N fixed (Gutschick 1981; Vitousek and Field 1999). Thus, while allocation of C is initially the same among fixers and non-fixers, the differential C cost of these two pathways ultimately modifies the investment in belowground pools. In addition, maximum achievable rates of N fixation are forced by an empirically derived relationship between nitrogenase activity and temperature (Houlton et al. 2008). For P acquisition, the model assumes a requirement of 15 g of N per g of plant P captured via phosphatase pathways (i.e., organic P-hydrolyzing enzymes), consistent with conceptual models and empirical studies (Olander and Vitousek 2000; Treseder and Vitousek 2001).

For our analysis, we parameterized the model using a combination of data from either Nova Vida or Santa Barbara (Table 2). The inputs to the model included monthly mean air temperature, NPP as estimated by the CASA model (Potter et al. 2001), and N and P inputs of 1.13 g N m^{-2} year^{-1} (Table 5) and 0.002 g P m^{-2} year^{-1}, respectively (Houlton et al. 2008). The model was calibrated by scaling maximum uptake rates of C, N and P until the

Table 2 Carbon (C) pools, nitrogen (N) and phosphorus (P) concentrations and other data used to parameterize the model

Parameter	Value	Units	Reference
C pools			
Foliar biomass	3.13	Mg/ha	Kauffman et al. (1995)
Woody biomass	66.35	Mg/ha	Kauffman et al. (1995)
Root biomass	6.22	Mg/ha	Kauffman et al. (1995)
Standing litter biomass	5.5	Mg/ha	Kauffman et al. (1995)
Soil C	87	Mg/ha	Hughes et al. (2002)
N concentrations			
Foliar N	23.32	mg/g	Kauffman et al. (1995)
Wood N	5.23	mg/g	Kauffman et al. (1995)
Root N	18.97	mg/g	Kauffman et al. (1995)
Litter N	18.91	mg/g	Kauffman et al. (1995)
Soil N			
Total	0.58	mg/g	Hughes et al. (2002)
Mineral	3.1	µg/g	Garcia-Montiel et al. (2001)
P concentrations			
Foliar P	0.92	mg/g	Kauffman et al. (1995)
Wood P	0.16	mg/g	Kauffman et al. (1995)
Root P	0.50	mg/g	Kauffman et al. (1995)
Litter P	0.65	mg/g	Kauffman et al. (1995)
Soil P			
Labile P	4.4	µg/g	Garcia-Montiel et al. (2000)
Organic P	13.8	µg/g	Garcia-Montiel et al. (2000)
Sorbed P	6.1	µg/g	Garcia-Montiel et al. (2000)
Occluded P	14.9	µg/g	Garcia-Montiel et al. (2000)
Residual P	28.0	µg/g	Garcia-Montiel et al. (2000)
Fluxes			
N deposition	5.3	kg/ha	Germer et al. (2007)
Net primary production (NPP)	1247	g/m^2/year	Potter et al. (2001)
Other			
Leaf Area Index (LAI)	4.6	m^2/m^2	Potter et al. (2001)
Legume abundance	13	%	Martinelli et al. (2000)
Potential N-fixer abundance	5.2	%	This study

amount of soil C, N and P at steady state agreed with the data in Table 2. The model was then run to steady state, providing an estimate of N fixation rates under quasi-equilibrium conditions.

Rates of symbiotic N fixation were modeled using the following equation:

$$F_{N,fix} \equiv v_{fix} f_{fix} C_{fix,r} \qquad (1)$$

where, v_{fix} is the rate of N_2 fixed per unit of nodulated root ([gN gC^{-1}] year^{-1}), f_{fix} is the fraction of nodulated root biomass, and $C_{fix,r}$ is the total root C pool. The second two terms on the right hand side of (1) are simulated by the model, whereas v_{fix} is a parameter

that can be adjusted to force the model to known abundance patterns of symbiotic N-fixers at a given site. This approach assumes that the biomass of fixers and non-fixers is proportional to their contributions to NPP; in addition, we assume that f_{fix} is a long-term integrative (i.e., steady-state) mean, as opposed to considering fine-scale temporal dynamics in this parameter.

We estimated symbiotic N-fixer abundance in the Rondônia primary forest from empirically measured abundances of Fabaceae species in the forest at the Samuel Ecological Reserve (Samuel) (Martinelli et al. 2000) and from information on known nodulation

patterns of species within the Fabaceae. Namely, legumes represent 13% of total tree species abundance in the Samuel forest (Martinelli et al. 2000). Seventy-five percent of the legumes surveyed at the site occupy genera within the Caesalpiniaceae subfamily, and ~20% of the Caesalpiniaceae surveyed to date have the capacity to nodulate (Sprent 2005). We therefore estimate that potentially N-fixing Caesalpiniaceae species represent 1.95% of the trees at Samuel. Species within the Mimosaceae make up the balance (25%) of the legumes at Samuel. Data on nodulation of the Mimosaceae species at the site were unavailable, so we assumed that 100% of the Mimosaceae species at Samuel have the capacity to nodulate and fix N (representing 3.25% of the total species at Samuel). Assuming that species abundance is proportional to biomass, we estimate that a total of 5.2% of the biomass at Samuel consists of trees with the capacity to fix N. Therefore, for our simulations of N fixation rates, we adjusted v_{fix} until the modeled abundance of symbiotic N-fixers agreed with this measured estimate, holding all other parameters constant. For more information on the sensitivity of the model to input parameters, please refer to Wang et al. (2007).

N balance estimates of BNF

If an ecosystem is at steady state with respect to N, then N inputs via symbiotic BNF can be assessed from net differences between all other measured N inputs and outputs. Similar mass-balance approaches have be used successfully to generate nearly complete input–output budgets for non-gaseous elements in small watersheds (Likens and Borman 1995), and comprehensive ecosystem N balances have been estimated by quantifying N inputs, hydrologic N losses, N pools, N transformations and N fluxes (e.g., McDowell and Asbury 1994; Neill et al. 2001, 2006; Chaves et al. 2007).

While the assumption of steady state is implicit in this approach, we believe this is reasonable in this primary forest site. First, while atmospheric N deposition in this region is high relative to the Amazon as a whole (L. Lara, personal communication), this is a recent phenomenon (Germer et al. 2007). In addition, the site has remained free from catastrophic fires and other large-scale or long-term disturbances that would more fundamentally alter the N cycle and thus more seriously violate the steady state assumption.

N inputs

Together, symbiotic N fixation, free-living N fixation and atmospheric deposition are the dominant sources of external N to the forests of Amazon Basin. In some ecosystems, geological weathering can also contribute sizable N inputs (e.g., Dahlgren 1994). However, this is likely negligible in the Rondônia primary forest because the low-N parent materials that dominate the site have weathered significantly past the point at which local rocks contribute substantial nutrients (Sanchez et al. 1982).

We obtained estimates of bulk atmospheric (total N) deposition at Rancho Grande from the literature. Next, compared to symbiotic N fixation, free-living N fixation can be measured with relative confidence using current methods. Free-living N fixation has been observed in multiple substrates including leaf litter, soil, decaying wood, and on plant leaves, and larger-scale rates have been estimated in a variety of ecosystems (Cleveland et al. 1999; Son 2001). Empirical measurements of free-living N fixation—defined here as any N fixation not occurring via a structured symbiotic relationship between Rhizobia and legume roots—were not available from any of the sites in Rondônia, and were therefore calculated from published estimates of free-living N fixation data obtained from a range of sites throughout the humid neotropics (Table 3). If multiple components of free-living N fixation were assayed at the same site (e.g., leaf litter, decaying wood), those values were added to generate a single estimate (Table 3). Similarly, if N fixation was assayed multiple times on the same substrate at the same site, we created a single-site average of data. All free-living N fixation rate data used in our analyses were originally obtained using the acetylene reduction assay (Burns and Hardy 1975).

N losses

N can be lost from ecosystems via erosion, fire, or biomass removal, but gaseous emissions and export to streams are the principal mechanisms of N loss from primary tropical rain forests (Chestnut et al. 1999; Melillo et al. 2001; Markewitz et al. 2004; Neill et al. 2006). Hydrological N losses—N lost along the continuum from soils to streams—were calculated from the literature, with an emphasis on data obtained

Table 3 Rates of free-living N fixation in tropical forests. All rates (kg N fixed ha^{-1} year^{-1}) were obtained using the acetylene reduction assay. Nitrogen fixation rate averages are provided for each substrate, and averages within each substrate compartment were added to generate the "total estimated flux" of N via free-living N fixation

Substrate	Site	Rate (kg N ha^{-1} year^{-1})	Reference
Canopy epiphytes	Columbia	1.5	Forman (1975)
	Brazil	<1	Jordan et al. (1983)
	Costa Rica	3	Freiberg (1998)
	Costa Rica	<0.1	Reed et al. (2008)
Estimated flux		1	
Litter layer	Brazil	8	Jordan et al. (1983)
	Hawaii	2.8	Vitousek (1994)
	Hawaii	<1	Russell and Vitousek (1997)
	Hawaii	4.9	Ley and D'Antonio (1998)
	Hawaii	1	Crews et al. (2000), Matzek and Vitousek (2003)
	Hawaii	2.4	Crews et al. (2001)
	Costa Rica	3.5	Reed et al. (2007)
Estimated flux		3	
Soil	Brazil	1.3	Sylvester-Bradley et al. (1980)
	Brazil	3	Jordan et al. (1983)
	Costa Rica	3	Reed et al. (2007)
Estimated flux		2	
Total estimated flux		6	

Table 4 Summary of literature-derived NO and N$_2$O emissions from primary tropical rain forests in Rondônia. Calculated averages for NO and N$_2$O losses were added to generate the total estimated N trace gas flux estimate

Trace gas losses	Rate (kg N ha^{-1} year^{-1})	Season	Reference
NO	2.4		Garcia-Montiel et al. (2003)
	2.2		Steudler et al. (2002)
	0.6	Wet	Garcia-Montiel et al. (2001)
	2.8	Dry	Garcia-Montiel et al. (2001)
	1.4		Neill et al. (2005)
Estimated NO flux	1.9		
N$_2$O	2.1		Garcia-Montiel et al. (2004)
	6.2	Wet	Garcia-Montiel et al. (2001)
	0.0	Dry	Garcia-Montiel et al. (2001)
	3.2	Wet	Neill et al. (2005)
	2.0	Dry	Neill et al. (2005)
Estimated N$_2$O flux	2.6		
Total estimated N trace gas flux	4.6		

from the forest at Rancho Grande. We estimated N losses from hydrologic fluxes leading from surface soils (20 cm) to streams (8.7 kg N ha^{-1} year^{-1}) based on Chaves et al. (2009; Table 4). It is possible that deep soil storage or adsorption accounted for a portion of these losses, and we assumed that this N was not available to plants (representing a net loss of N) and therefore had to be balanced by BNF. N losses as NO and N$_2$O were estimated from published data obtained from the Rondônia sites (Table 4). Data collected multiple times at the same site were averaged to create a single N$_2$O or NO flux (Table 4).

As is the case with N fixation, direct measurements of gaseous soil N losses as N_2 are hindered by methodological limitations (Davidson and Seitzinger 2006; Groffman et al. 2006). Soil N_2O and NO fluxes likely represent two important N losses from most tropical rain forests (e.g., Matson and Vitousek 1990; Matson et al. 1999), but measured fluxes of NO and N_2O still underestimate total gaseous N losses because they do not account for N_2 efflux during denitrification. Measurements of N_2 emissions from tropical forest soils are virtually nonexistent (and the true extent of gaseous N losses remain a major source of uncertainty in Amazon forest N budgets), but several studies highlight the potential for very high N_2 losses (e.g., Houlton et al. 2006).

Thus, we estimated total $N_2O + N_2$ production directly using a soil incubation experiment. Acetylene (C_2H_2) blocks the reduction of N_2O to N_2 (Yoshinari and Knowles 1976; Tiedje 1982), and the difference between treatments (i.e., with or without C_2H_2) provides an estimate of the N released as N_2 during denitrification (and hence total N trace gas losses, but see Groffman et al. (2006)). Twenty Rondônia forest soil samples obtained from the surface (0–5 cm) were sieved to 4 mm, placed in airtight jars, and incubated under one of three conditions: (1) at ambient dry season moisture; (2) amended with 1.0 µg NO_3^-–N g dry soil^{-1} and water to field capacity; and (3) amended with 1.0 µg NO_3^-–N g dry soil^{-1} and water to field capacity plus 10 kPa acetylene. Nitrous oxide in the jars was periodically sampled using 20 ml gastight syringes, and concentrations of N_2O were measured on a gas chromatograph equipped with an electron capture detector (ECD; Shimadzu Mini-2, Shimadzu Corp, Kyoto, Japan). Fluxes were calculated as N_2O produced per unit time and normalized per gram of dry soil. While this laboratory experiment did not reflect actual field conditions, it does indicate the *potential* for N_2 emissions from soils at this site.

Results

Modeled estimates of BNF

Assuming that 5.2% of the biomass in the Rondônia primary forest site fixes N (See Methods), our model-based analysis of symbiotic N fixation (i.e., forcing symbiotic N-fixers' NPP to 5.2% of the total) suggests a symbiotic N fixation input of 6.8 kg N ha^{-1} year^{-1}.

N balance estimates of BNF

N inputs

Empirical measurements indicate that bulk N deposition inputs to the region (Rancho Grande) are 5.3 kg N ha^{-1} year^{-1}, with inputs dominated by NH_4^+ (Germer et al. 2007). We also estimate that free-living N fixation in tropical forest canopies, decomposing organic matter, and soils contribute an additional 6 kg N ha^{-1} year^{-1} to the Amazon Basin, including the Rondônia tropical forest site (range = 1–8 kg N ha^{-1} year^{-1}; Table 3). Because no single study accounted for all possible sources of free-living N fixation, we believe this value represents a conservative estimate. Thus we estimate total N inputs to the watershed (excluding symbiotic N fixation) of 11.3 kg N ha^{-1} year^{-1} via the combined processes of atmospheric deposition (5.3 kg N ha^{-1} year^{-1}) and free-living N fixation (6.0 kg N ha^{-1} year^{-1}).

N losses

The low pH (4.9) of the Nova Vida rain forest soils indicates that ammonia volatilization (pKa = 9.3) is negligible. Losses of N via NO and N_2O at the site are 4.5 kg N ha^{-1} year^{-1}. In our incubations, N_2O fluxes with acetylene averaged 0.29 ± 0.04 µg N_2O–N h^{-1}, and N_2O fluxes without acetylene averaged 0.14 ± 0.04 µg N_2O–N h^{-1}. Thus, we estimate that N_2 emissions equal losses as N_2O (i.e., 2.6 kg N ha^{-1} year^{-1}), resulting in a total gaseous loss of 7.1 kg N ha^{-1} year^{-1} (Tables 4, 5).

We estimate a net decrease (N loss) of 8.7 kg N ha^{-1} year^{-1} via hydrologic flowpaths from soil solution to groundwater (Chaves et al. 2009). Thus, we estimate total watershed N losses of 15.8 kg ha^{-1} year^{-1}, which includes total N gas losses of 7.1 kg N ha^{-1} year^{-1}, and total hydrologic flowpath losses of 8.7 kg N ha^{-1} year^{-1} (Chaves et al. 2009). Using the mass balance approach, we therefore estimate that symbiotic N fixation contributes 4.5 kg N ha^{-1} year^{-1} to the Rondônia site (Table 5).

Table 5 Estimated N fluxes in the Rondônia primary forest calculated using the N balance and modeling approaches, compared to the bottom-up estimate of Cleveland et al. (1999). N balance data represent the mean of published or derived values, with potential ranges based on variation among individual components in Tables 3 and 4 (in parentheses). Unless otherwise noted, data are reported with units of kg N ha^{-1} year^{-1}

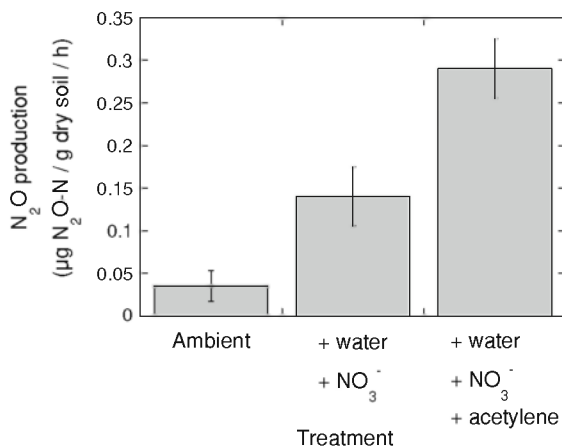| Source	External input	External output
N balance estimate		
N inputs		
Precipitation (bulk)	5.3	
Weathering (geological)	0	
N fixation (free-living)	6 (2.4–14)	
Total estimated N inputs	11.3 (7.7–19.3)	
N losses		
Gaseous N losses		
N$_2$O		2.6 (2.0–6.2)
NO		1.9 (0.6–2.8)
N$_2$		2.6
Total gaseous N losses		7.1 (5.2–11.6)
Deep soil/hydrologic flowpath N losses		8.7
Total estimated N losses		15.8 (13.9–20.3)
Symbiotic N fixation	4.5	
Modeled estimate		
Simulated symbiotic N fixation	6.8	
Databased estimate		
Range N fixation (Cleveland et al. 1999)	14–36	
Mean N fixation (Cleveland et al. 1999)	25.4	

Discussion

We used two independent, indirect approaches to estimate symbiotic N fixation in the forest site in Rondônia, with remarkably consistent results (Table 5). Our analysis suggests rates of BNF in this tropical rain forest site (i.e., 4–7 kg N ha^{-1} year^{-1}) that are lower than existing estimates for the tropical forest biome as a whole [e.g., 15–53 kg N ha^{-1} year^{-1} (Cleveland et al. 1999), 25 kg N ha^{-1} year^{-1} (Houlton et al. 2008)] (see Table 5). This makes sense: the Rondônia site occupies the dry end of the rainfall spectrum for tropical rain forests and the abundance of N-fixing legumes is relatively low compared to "average" Amazonian conditions (ter Steege et al. 2006). In wetter sites with more rapid N cycling rates and higher potential N losses, BNF rates may be significantly higher than those estimated here; without significant inputs of new N via BNF, high N losses would quickly deplete the N pools in productive, mature rain forest sites. In addition, relatively high N inputs via atmospheric deposition in the Rondônia site may actually be down-regulating current N fixation rates (Vitousek et al. 2002).

Our analysis also highlights the potential for N-fixer abundance patterns to inform fluxes of N fixation at ecosystem scales. By forcing our model to field estimates for N-fixer abundance in the Rondônia site, we found strong agreement between symbiotic N fixation rates—based on modeling—and the "missing N" in our watershed—based on N flux estimates. This suggests an intriguing possibility: that putative N-fixer abundance data, coupled with modeling, may be used to improve quantitative estimates of N fixation at large geographic scales. Although abundance data are not available for all (or even most) watershed sites, they are much easier to generate than many direct flux measurements. We suggest that this approach could be applied at other sites to examine potential connections between fixation and abundance patterns of putative N-fixers in the tropics.

While these results illustrate the potential utility of indirect approaches for constraining estimates of BNF at the watershed (and perhaps larger) scales, each approach has limitations that must be considered when interpreting the results. In terms of the model, there is an implicit assumption that we have sufficient

understanding of controls over N fixation to justify reasonable predictions using calibrated simulations. However, the costs of N fixation and P acquisition in the model are estimated and could benefit from additional field-studies and tests; the mechanisms regulating these "costs" are critical for accurately predicting rates of N fixation rates in terrestrial ecosystems (Vitousek and Field 1999; Rastetter et al. 2001; Houlton et al. 2008). Moreover, controls such as trace metal availability (Barron et al. 2008) and herbivory (Vitousek and Howarth 1991) are not currently incorporated in the model; future work demonstrating the importance of these types of controls on fixation may help to advance modeling analyses further.

Similar to the model, there are uncertainties in the N balance approach that could lead to systematic errors in estimates of symbiotic N fixation. First, direct field-based measurements of N_2 fluxes do not exist, though N_2 is likely to account for considerable losses of gaseous N from tropical forests (Robertson and Tiedje 1988; Houlton et al. 2006). In our lab assays, measured N_2 losses were approximately equal to those as N_2O (Fig. 3). However, high spatial and temporal variability in N_2 versus N_2O production during denitrification emphasizes the need to comprehensively assess these ratios (using C_2H_2, for example) in both space and time, which remains another major challenge to biogeochemistry (Groffman et al. 2006). Next, the "deep soil/hydrologic flowpath N" term (Table 5) presents a dilemma for calculating total ecosystem N losses. Based on data collected at Rancho Grande, we estimate potential maximum N losses via hydrological flowpaths of 8.7 kg N ha^{-1} year^{-1} (Table 5). Of that total, we estimate that 1.5 kg N ha^{-1} year^{-1} is exported in streams. Of the remaining 7.2 kg N ha^{-1} year^{-1}, we assume that some is lost via denitrification in zones not systematically sampled in soil trace gas studies (e.g., riparian areas) or via abiotic storage in deep soils. However, while root biomass is typically concentrated at soil depths above 1 m, any plant N uptake via roots at depths >1 m would move our mass-balance derived N loss estimate (and hence our N fixation estimate) downward. Finally, an assumption of steady state (with respect to N) is implicit in the mass balance approach. In the Rondônia site, we believe this assumption is probably valid, but in recently disturbed sites or sites with a long history of chronic N deposition, for example, the steady state assumption may not be, thus limiting the utility of the mass balance approach.

In the decade since Cleveland et al. (1999) highlighted both the potential magnitude and uncertainty of tropical forest BNF, little progress has been made improving our understanding of the process. Doing so has become increasingly important, as human activities are now fundamentally altering the N cycle in many tropical forests (Martinelli et al. 2006). More accurate direct estimates of BNF will only be achieved when we overcome methodological limitations of measuring N fixation, just as more robust watershed-level estimates of N losses require improved methods for measuring gaseous N fluxes from soils and along hydrologic flowpaths. However, in the absence of a direct method for measuring symbiotic N fixation, we suggest that employing a strategy that combines modeling and empirical techniques—including analyses of natural abundance ^{15}N pools (see Houlton et al. 2006), potential symbiotic N-fixer abundances (e.g., Martinelli et al. 2000), free-living N fixation rates (e.g., Reed et al. 2007), trace gas fluxes (e.g., Garcia-Montiel et al. 2003), and N losses via hydrologic pathways (e.g., Chaves et al. 2007)—in a series of small watersheds and in a range of biome types—offers an opportunity to further

Fig. 3 Results of a laboratory incubation using soil from a primary forest in Rondônia. Treatments were: (1) soil incubated at ambient dry season moisture (ambient); (2) soil amended with 1.0 μg NO_3^-–N g^{-1} dry soil and water to field capacity (+ water + NO_3^-); and (3) soil amended with 1.0 μg NO_3^-–N g^{-1} dry soil and water to field capacity plus 10 kPa acetylene (+ water + NO_3^- + acetylene). Error bars are ±1SE

reduce uncertainties surrounding rates of and controls over BNF. We view the application of indirect methods to estimate N fixation as a useful bridge to the day when direct measurements of BNF at multiple scales are possible.

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