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Key Points:

- Nitrogen additions cause delayed decreases in nitrogen fixation
- Phosphorus and molybdenum additions cause site-specific increases in nitrogen fixation
- Differences in soil and litter nutrient stoichiometry likely explain differences in responses to phosphorus and molybdenum fertilization

Supporting Information:

Supporting Information may be found in the online version of this article.

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Impact of Nutrient Additions on Free-Living Nitrogen Fixation in Litter and Soil of Two French-Guianese Lowland Tropical Forests

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Abstract In tropical forests, free-living Biological nitrogen (N) fixation (BNF) in soil and litter tends to decrease when substrate N concentrations increase, whereas increasing phosphorus (P) and molybdenum (Mo) soil and litter concentrations have been shown to stimulate free-living BNF rates. Yet, very few studies explored the effects of adding N, P, and Mo together in a single large-scale fertilization experiment, which would teach us which of these elements constrain or limit BNF activities. At two distinct forest sites in French Guiana, we performed a 3-year in situ nutrient addition study to explore the effects of N, P, and Mo additions on leaf litter and soil BNF. Additionally, we conducted a short-term laboratory study with the same nutrient addition treatments (+N, +N+P, +P, +Mo, and +P+Mo). We found that N additions alone suppressed litter free-living BNF in the field, but not in the short-term laboratory study, while litter free-living BNF remained unchanged in response to N+P additions. Additionally, we found that P and P+Mo additions stimulated BNF in leaf litter, both in the field and in the lab, while Mo alone yielded no changes. Soil BNF increased with P and P+Mo additions in only one of the field sites, while in the other site soil BNF increased with Mo and P+Mo additions. We concluded that increased substrate N concentrations suppress BNF. Moreover, both P and Mo have the potential to limit free-living BNF in these tropical forests, but the balance between P versus Mo limitation is determined by site-specific characteristics of nutrient supply and demand.

Plain Language Summary Nitrogen fixation by microorganisms is an important source of nitrogen for tropical forests. The controls over this process remain ambiguous but nutrient availability has been put forward as an important regulator. Especially nitrogen, phosphorus, and molybdenum have been shown to affect nitrogen fixation. In this experiment, we tested the effect of adding nitrogen, phosphorus, and molybdenum in different combinations to two mature tropical forest field sites over a period of 3 years. We found that phosphorus mainly causes nitrogen fixation to increase while nitrogen addition leads to a delayed decrease in nitrogen fixation. Molybdenum had a very site-specific effect and only affected nitrogen fixation in one of the two sites.

1. Introduction

Biological nitrogen (N) fixation (BNF) is a major natural source of new N to unmanaged terrestrial ecosystems worldwide (Cleveland et al., 1999; Reed et al., 2011; Vitousek et al., 2013) and in tropical evergreen forests, N input through free-living BNF has been found to vary between 0.1 and 60 kg N ha^{-1} yr^{-1} (Reed et al., 2011). During BNF, inert dinitrogen (N_2) is reduced to ammonia by N_2 fixing microorganisms,

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commonly called diazotrophs, that are ubiquitous in a variety of terrestrial environments including leaf litter and soils (Camenzind et al., 2018). BNF is catalyzed by nitrogenases and requires high adenosine triphosphate (ATP) consumption, rendering it energetically expensive (Gutschick, 1981). Moreover, maintaining an oxygen-free environment, which is necessary for nitrogenase functioning, also requires energy and thus ATP consumption (Robson & Postgate, 1980). Because of this dependence on ATP, the macronutrient phosphorus (P) plays an important role in the BNF metabolism. Tropical evergreen forests mostly occur on old and highly weathered soils with low P contents (Vitousek & Sanford, 1986; Walker & Syers, 1976), supporting the idea that low P limits BNF in these forests (Reed et al., 2011; Vitousek & Sanford, 1986). Conversely, because BNF is energetically expensive, the process is thought to be suppressed in diazotrophs when sufficient N is present in the immediate environment (Hedin et al., 2009; Menge et al., 2009), likely through inhibition of nitrogenase synthesis or activity caused by the presence of inorganic N (Cheng et al., 1999). Most tropical forests are rich in N (Houlton et al., 2006; Martinelli et al., 1999; Vitousek & Sanford, 1986), yet free-living BNF can still be high (Hedin et al., 2009; Reed et al., 2011; Sullivan et al., 2014). One explanation is that N bioavailability is not homogeneously distributed within forests and thus localized biogeochemical niches are created in which diazotrophs are decoupled from the abundant N and wherein free-living BNF is high (Menge & Hedin, 2009). Another explanation is that N can be perceived limiting for N-fixing microbial groups, in substrates that are relatively richer in other resources, such as carbon (C), even when N bioavailability is high. The litter layer is an example of low N availability compared to C, because senesced plant tissues can be poorer in N relative to C, compared to the stoichiometric optimum of heterotrophic microbes (Cherif & Loreau, 2007; Sterner & Elser, 2002). Heterotrophic diazotrophs may therefore fix N_2 to offset a perceived N deficit even in an N-rich system, as was shown by Dynarski et al. (2019) in temperate forests with high N supply.

Two recent meta-analyses on effects of nutrient addition experiments in forests around the world found that, overall, in tropical forests, litter and soil free-living BNF rates decreased after N addition and increased following P addition (Dynarski & Houlton, 2018; Zheng et al., 2019). However, they also found large differences between individual studies, where some studies report no changes in free-living BNF rates with N addition (Reed et al., 2007; Zheng et al., 2017), whereas others show a suppression following N (Cusack et al., 2009) or, in a temperate forest at least, P additions (Jean et al., 2013). Moreover, previous study in Hawaiian montane tropical forests showed that trace metals, including molybdenum (Mo) (Vitousek & Hobbie, 2000), could also stimulate BNF in soils. More recent studies indicate that additions of the micronutrient Mo stimulated free-living BNF in numerous terrestrial environments, including tropical forests (Dynarski & Houlton, 2018). The most common nitrogenases use Mo as an essential co-factor (Reed et al., 2011; Seefeldt et al., 2009) and Mo has long been considered to play an indispensable catalytic role for BNF (Raymond et al., 2004). However, there are examples of nitrogenases in which Mo is replaced by vanadium (V) or iron (Fe) (Pau, 1989) and recent studies suggest that these so-called Mo-independent nitrogenases may be more commonplace than previously thought (McRose et al., 2017; Zhang et al., 2016). In a Panamanian lowland tropical forest, both P and Mo stimulated soil free-living BNF, however, industrial P fertilizers often contain Mo as a hidden contaminant and when Mo-free P fertilizers were applied, soil BNF rates did not change (Barron et al., 2009). A follow-up study in Panama showed that indeed Mo constrained BNF in P-rich soils, while there was P and Mo co-limitation in P poor soils (Wurzburger et al., 2012). However, in nearby Costa Rica, free-living BNF in soil and leaf litter was shown to be constrained by P but not Mo (Reed et al., 2013). Hence, the role of P versus Mo limitation in tropical forests remains ambiguous, and even the effect of N additions on free-living BNF is not always clear.

Tropical BNF is severely understudied compared to temperate BNF, although the last two decades marked a noticeable increase in tropical studies. However, many of those studies were carried out in Central America and the inherent biogeochemical heterogeneity of tropical forests makes it difficult to extrapolate measurements and conclusions drawn from one forest to another (Townsend et al., 2008). As a consequence, more constrained estimates of BNF are needed to better model and predict this essential ecosystem process which helps regulate plant productivity and ecosystem C uptake. Moreover, to fully understand and quantify the anthropogenic changes to the N cycle, more measurements of key N cycle process such as BNF are needed (Fowler et al., 2013; Vitousek et al., 2013). It follows that more data are urgently needed to draw firm conclusions about general patterns in P versus Mo limitation of BNF in tropical forests. Nutrient addition

Table 1
Soil and Litter Nutrient Concentrations

	Litter		Soil	
Variable	Nouragues	Paracou	Nouragues	Paracou
C (%)	45.9 (0.6)	45.5 (0.3)	3.4 (0.2)	2.2 (0.1)
N(%)	1.25 (0.04)	1.13 (0.03)	0.24 (0.01)	0.15 (0.01)
P (mg/kg)	203 (9)	185 (10)	153 (25)	96 (7)
Mo (mg/kg)	1.2 (0.2)	2.5 (0.3)	1.2 (0.1)	0.8 (0.1)
Extractable N (mg/kg)	–	–	11.7 (0.6)	7.3 (0.5)
PBray (mg/kg)	–	–	1.4 (0.1)	1.8 (0.2)
Resin Mo (μg/kg)	–	–	2.1 (0.3)	5.5 (0.5)
Sand (%)	–	–	46 (6)	72 (2)
Clay (%)	–	–	29 (3)	12 (2)

Note. The mean concentrations of carbon (C), nitrogen (N), phosphorus (P), and molybdenum (Mo) are given for both soil and litter. Additionally, the concentrations of extractable N (sum of NH_4^+ and NO_3^- concentrations), P (Bray) and Mo (Resin Mo) and texture (percentage sand and clay) are given for the soil. Values in parentheses indicate standard errors (*SE*). $N = 60$ for nutrient concentrations, $N = 12$ for soil texture. Values were obtained from Van Langenhove et al. (2019). See Van Langenhove et al. (2019) for a description of the methods used to analyze these variables.

studies carried out in the lab, in the field or both are of particular interest to identify mechanisms involved in nutrient limitation of BNF (Dynarski & Houlton, 2018; Pajares & Bohannan, 2016).

Here, we present results of litter and soil BNF from two 3-year in situ nutrient addition experiments carried out in two French Guianese tropical forests with different soil characteristics. The more clayey Nouragues forest soil is richer in total N, P, and Mo compared to the sandier Paracou forest soil (Table 1). Nonetheless, soil extractable P and Mo is higher in Paracou than in Nouragues (Courtois et al., 2018; Van Langenhove et al., 2019). Since French Guiana, part of the Guiana Shield, has soils that are among the nutrient poorest of tropical South America (Hammond, 2005; Quesada et al., 2010), we expected strong responses of BNF rates to nutrient additions. The field experiments included factorial combinations of N, P, and Mo (+N, +N+P, +P, +Mo, and +P+Mo) additions, using industrial fertilizers, and BNF was measured before, after 1 year, and after 3 years of fertilizer additions. Additionally, because in situ environmental and microclimatic conditions are spatially variable, we also conducted a short-term nutrient addition experiment using soil and litter from one of the two forests. In this laboratory experiment, we tested the short-term effect of adding analytical grade N, P, and Mo in similar combinations as in the field to see if short-term fertilization under controlled conditions is comparable to the in situ situations.

Since litter nutrient concentrations were similar between both forest sites, we (I) hypothesized that, in both forests, litter BNF would increase following P and Mo additions, while N additions would lead to suppressed BNF rates. As compared to other tropical forests, the studied sites had low soil P contents, we expected (II) that P additions would stimulate BNF rates. However, because of the relative differences in soil P content between the sites, we additionally (III) hypothesized that in Nouragues soil BNF rates would show a smaller increase than Paracou soil BNF rates following the P additions. For Mo additions, we expected similar results, that in Nouragues where Mo is higher, the increase of soil BNF rates would be smaller than in Paracou. Because of the expected increase in BNF rate by P additions and decrease by N additions, we (IV) hypothesized that the addition of both nutrients together (+N+P) would cancel out and cause no change in BNF. Finally, since BNF is carried out by microorganisms that can turnover rapidly, we (V) hypothesized that BNF rates would immediately respond to increased nutrient availability and these rates would persist throughout the nutrient additions over the following years.

2. Methods

2.1. Site Description

The study was conducted at two primary forest sites in French Guiana, in the research stations of Paracou and Nouragues. Paracou is situated 15 km from the coast (5°15′N, 52°55′W), while Nouragues is located inland (4°05′N, 52°41′W). Annual rainfall quantities at both sites were similar, with Paracou receiving an average of 3,100 mm yr^{-1} for the period 2004–2015 (Aguilos et al., 2019) and Nouragues receiving an average of 2,990 mm $year^{-1}$ (Bongers et al., 2001). The mean annual air temperature is near 26°C for both sites (Bongers et al., 2001; Gourlet-Fleury et al., 2004). The French Guianese climate is characterized by a wet and a dry season due to the north/south movement of the Inter-Tropical Convergence Zone. The region receives heavy rains from December to July and has a dry period, typically characterized by less than 100 mm rainfall $month^{-1}$, from August to November (Aguilos et al., 2018). Both sites have over 200 tree species ha^{-1} and the most represented plant families are the Lecythidaceae, Fabaceae, Sapotaceae, and Chrysobalanaceae. Trees in Paracou are slightly smaller than in Nouragues, with an average canopy height between 30 and 35 m and emergent trees up to 45 m tall (Gourlet-Fleury et al., 2004), while in Nouragues average canopy height is 40–45 m and emergent trees are up to 60 m tall (Ho Tong Minh et al., 2016). Soils at the Paracou and Nouragues site are derived from the Bonidoro series, characterized by schist and sandstones and locally crossed by veins of pegmatite, aplite, and quartz. This Precambrian geological substrate is particularly low in total P content (Table 1) compared to the generally younger, nutrient-richer soils of western Amazonia (Grau et al., 2017; Hammond, 2005) and soils at both sites are classified as nutrient-poor Acrisols (FAO, 1998), although there are differences in soil N, P, and Mo between both sites (Table 1). Soils at Paracou range from loamy sand to sandy loam, while soils at Nouragues contain more clay and span the range of sandy loam to silty clay.

2.2. Nutrient Addition Experiment

2.2.1. In the Field

The field fertilization experiments at both Paracou and Nouragues were initiated in October 2016 and were ongoing at the time of publication. In each site, three blocks of four 50 × 50 m^2 plots were selected; each block contained four treatments, that is, (1) N addition (+N), (2) P addition (+P), (3) N and P addition (+N+ P), and a (4) control plot where the soil nutrient content remained undisturbed. Fertilizer was applied twice per year by hand and we used commercial urea ($(NH_2)_2CO$; N) and triple superphosphate ($Ca(H_2PO_4)_2$; P) at a rate of 125 kg N ha^{-1} yr^{-1} (+N treatment) or 50 kg P ha^{-1} yr^{-1} (+P treatment), or both 125 kg N ha^{-1} yr^{-1} and 50 kg P ha^{-1} yr^{-1} together (+N+P treatment), as fertilizer. See Verryckt (2021) and Table S4 for a detailed chemical analysis of the fertilizer composition. These fertilizer amounts were chosen to enable future comparison of our results to ongoing fertilization) experiments (e.g., Barro Colorado Nature Monument in Panama (Wright et al., 2011); Amazon Fertilization Experiment (AFEX, Lugli et al., 2021). The Mo-only treatment (+Mo) and the combination of P and Mo treatment (+P+Mo) were realized by adding Mo (1.1 kg Mo ha^{-1} yr^{-1} laboratory-grade sodium molybdate ($MoNa_2O_4$) dissolved into 500 ml of distilled water) to a 2 × 2 m^2 area within each control and each +P plot. The applied amount of Mo is roughly 10 times higher than the frequently applied amount of Mo fertilizer in agricultural studies (Adams, 1997; Razmjoo & Henderlong, 1997), but was chosen as we expected molybdate to leach out of the soil quickly given the acidity of the soil and the high solubility of molybdate under acidic conditions (Reddy & Gloss, 1993). Mo was only added during the first year of nutrient additions.

For the BNF measurements, from each plot, five soil samples were collected using a 2-cm diameter corer to a depth of 5 cm and five litter samples of roughly 10 g wet weight were collected by hand at the same location. Samples were taken according to a five-on-dice sampling pattern, that is, samples were taken in the center and the four corners of the plot. Each sample was assayed separately. Samples were collected and BNF was measured before nutrient additions were initiated (September 2016), after 1 year (September 2017) and after 3 years of nutrient additions (September 2019).

2.2.2. In the Laboratory

For the laboratory nutrient addition experiment conducted in July 2017, we collected leaf litter and soil in Paracou from the control plots that had not received any nutrient amendments during the field fertilization. We did not conduct a laboratory nutrient experiment in Nouragues because it is a remote site from which we could not take the soil and litter to the lab in time. From each of the three control plots, five litter samples were randomly collected by hand and five soil samples to a depth of 5 cm were taken with a shovel. Each sample ($n = 15$) was homogenized and then split into six aliquots of ca. 10 g litter sample (wet weight) or ca. 40 g soil sample (wet weight). Each aliquot received 14 ml of distilled water, in which nutrients were dissolved to obtain the respective concentrations: none (Control), 50 g N L^{-1} (+N), 50 g N and 20 g P L^{-1} together (+N+P), 20 g P L^{-1} (+P), 48 mg Mo L^{-1} (+Mo) and 20 g P and 48 mg Mo L^{-1} together (+P+Mo). For N additions, we used urea (CH_4N_2O), for P we used sodium phosphate (NaH_2PO_4) and for Mo we used sodium molybdate ($MoNa_2O_4$), all laboratory grade and >99% pure. After nutrient addition, samples were left at ambient temperature (~28°C) for 24 h before incubations were initiated. These nutrient additions were chosen based on the methodology used by Wurzburger et al. (2012), thereby maximizing the potential to render both studies comparable.

2.3. Acetylene Reduction Assay

Nitrogen fixation rates were determined using the acetylene reduction assay (Hardy et al., 1968). Litter and soil samples collected in the field or coming from the laboratory experiment were placed in clear 100 ml borosilicate jars. The jars were sealed with rubber septa and 10 ml of air was replaced with 10 ml of acetylene gas (welding grade, Air Liquide) to create a 10% headspace concentration by volume. The samples were then incubated in situ at ambient forest light (no direct sunlight) and temperature for 18 h. Sample moisture was not manipulated in any way. After incubation, a 12 ml subsample from the sample headspace was injected into a pre-evacuated 12 ml borosilicate vial (Labco Limited, Ceredigion, UK) and shipped to the Laboratory of Functional Plant Biology in Ghent, Belgium, for analysis. Ethylene concentrations were measured using laser-based photoacoustic spectroscopy (ETD-300, Sensor Sense) and acetylene reduction activity (ARA, nmol C_2H_4 g^{-1} dry mass d^{-1}) was calculated from the slope of the regression line between ethylene concentration and time. Ten parallel ethylene blanks (no leaf litter or soil) were created to assess background levels of ethylene in the acetylene gas (1.5 ± 0.4 nl ethylene ml^{-1} acetylene gas), which were subtracted from the sample ethylene concentrations. Soil and leaf litter samples that emitted ethylene concentrations below the machine detection limit (0.01 nl ethylene ml^{-1} air) over the total incubation time were recorded as half of this value.

In 2017, we calibrated ARA to BNF using isotopically labeled $^{15}N_2$ gas on 20 litter and 20 soil samples (10 from each forest site) gathered in control plots at the same time as the ARA samples were collected. Each litter and soil sample was divided into two equal parts, one of which received isotopically labeled $^{15}N_2$ gas while the other acted as control. In $^{15}N_2$ treated samples, we replaced the sample headspace with a gas mixture of 80% $^{15}N_2$ (≥98 atom %; Sigma Aldrich, St Louis, USA) and 20% O_2. In control samples, the headspace was replaced with ambient air. All samples were allowed to incubate for 24 h and incubations were terminated by evacuating each jar and drying the samples at 60°C, after which they were ground into powder. ^{15}N abundance and N concentration were determined at the University of Vienna, Austria, in an elemental analyzer (EA 1110; CE Instruments, Milan, Italy) coupled to a Finnigan MAT Delta Plus IRMS (Thermo Fisher Scientific, Waltham, USA). Reference gas (high purity N_2) was calibrated to an atmospheric N_2 standard using IAEA-NO-3, IAEA-N-1, and IAEA-N-2 reference materials (International Atomic Energy Agency, Vienna, Austria). The standard deviation of repeated measurements of laboratory standards was ±0.15‰. By comparing ^{15}N treated litter samples to control litter samples, we calculated a mean ARA:BNF conversion factor of 4.1 ($SD = 1.5$, $SE = 0.3$, $n = 20$). It was not possible to establish a conversion factor for soil due to a combination of low BNF rates and high background N concentrations (Van Langenhove et al., 2019). For soil, we therefore decided to use the theoretical conversion factor of 3 mol ethylene produced per mole of N fixed (Hardy et al., 1968). It bears mentioning that Dabundo et al. (2014) reported on the contamination of $^{15}N_2$ gas with other enriched ^{15}N forms at levels that could potentially impact BNF, and suggested caution when interpreting results derived from incubations with $^{15}N_2$ gas. Furthermore, Saiz et al. (2019) reported that the ARA:BNF conversion factor can vary for the same diazotroph species and at

the same location through time. Hence, BNF rates have to be interpreted with the uncertainty that is inherently linked to the methods used.

2.4. Data Analysis

We used linear mixed-effects regression models (LMER) to assess the differences in BNF rates over time within a single treatment and to compare treatments to control plots. We analyzed the data from different sites and substrates (soil and litter) separately. For models assessing changes over time we used timing as fixed effect and topographic position of the plot as random effect (Table S1). For models assessing changes due to the different treatments, we set fertilization treatment as fixed effect and topographic position of the plot as random effect. The validity of the linear models' assumptions (linearity, normality of residuals, no influential outliers, homoscedasticity) was evaluated with standard functions of R (R Core Team 2018, version 3.5.1), including the Shapiro-Wilk test, Breush-Pagan test, Cook's test, and diagnostic plots. Before analysis, data were log-transformed if their distribution was right-skewed to improve the normality of model residuals. Multiple comparisons within a factor were analyzed using Bonferroni corrected Tukey's post hoc analysis.

Analyses were conducted in R statistical environment, version R.3.5.1 (R Core Team, 2018), using the packages plyr (Wickham, 2011), dplyr (Wickham et al., 2018), MASS (Venables & Ripley, 2010), lmerTest (Kuznetsova et al., 2017), and emmeans (Lenth, 2018) for data analysis and ggplot2 (Wickham, 2016) for visualization.

3. Results

Across the two sites and three time points in the field nutrient addition study, we found that BNF rates in control plots ranged between 0.1 and 1,730.5 ng N g^{-1} d^{-1} in litter and between 0.1 and 55.58 ng N g^{-1} d^{-1} in soil. The litter overall median and mean BNF rates were 153.1 and 226.1 ng N g^{-1} d^{-1}, respectively, while the soil overall median and mean BNF rates were 10.7 and 11.16 ng N g^{-1} d^{-1}, respectively. Litter BNF rates were roughly 20 times higher than soil BNF rates. We refer to the supporting information for a visualization of the data distribution (Figures S1–S8).

3.1. Litter

In both forest sites, the effects of the in situ nutrient additions on leaf litter were comparable (Figure 1), with only a few exceptions. After 1 year, plots that had received only N (+N) showed a significant 50% decrease ($p < 0.01$) in BNF rates compared to pre-fertilization BNF rates and remained ca. 50% lower ($p < 0.01$) even after 3 years of fertilizer additions. The addition of both N and P (+N+P) caused no changes in BNF, except after the third year of nutrient additions in Paracou. There, rates were 50% lower than those of the control plots because control plot litter BNF rates were slightly higher than the years before. In both sites, P additions (+P and +P+Mo) significantly increased BNF rates by over 300% ($p < 0.001$) after 1 and 3 years of nutrient additions, compared to the controls (Figure 1). In Nouragues, the litter BNF rates following P (+P) additions increased even further after 3 years of nutrient additions (500%, $p < 0.001$), while in Paracou, the BNF rates after 1 and 3 years of P additions were similar. Additions of Mo only (+Mo) caused no change in BNF rates compared to control plots in either of the sites.

Similar as in the field experiment, in the laboratory experiment with litter collected from Paracou, there was no change in BNF following N (+N and +NP) additions, whereas P additions (+P and +P+Mo) increased BNF rates by 200% ($p < 0.001$) (Figure 2). Free-living BNF rates did not change in litter fertilized with Mo alone, matching the results observed in the field fertilization experiment.

Exact values of litter BNF rates in response to the various nutrient addition treatments and across the different sampling times can be found in Tables S1 and S3.

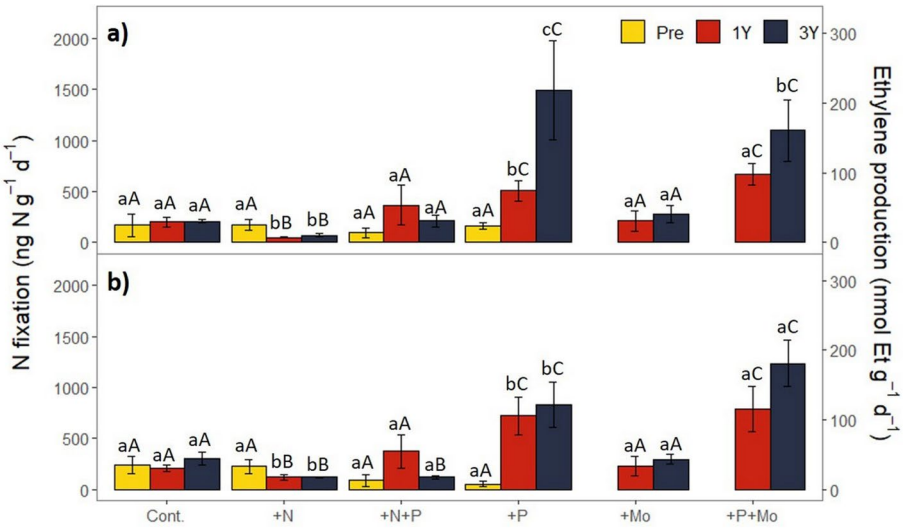

Figure 1. Litter BNF rates and their response to the various nutrient additions in (a) Nouragues and (b) Paracou. Different colors indicate whether samples were collected before (Pre), after 1 year (1Y), or 3 years (3Y) of field nutrient additions. Note that for plots fertilized with +Mo and +P+Mo no pre-treatment data were available. Significant differences within a treatment over the 3-year period are indicated with different lowercase letters while treatments that were different from the control (Cont.) within one sampling period are indicated with uppercase letters. Significant differences are at the $p < 0.05$ level. The different treatments are control (Cont.), N only (+N), both N and P together (+N+P), P only (+P), Mo only (+Mo), and both P and Mo together (+P+Mo). Error bars indicate standard errors (*SE*). For each bar, $N = 15$; five litter samples from three plots.

3.2. Soils

The in situ nutrient addition treatments in Nouragues and Paracou resulted in different responses between the two sites. In Nouragues, after 1 year of fertilizer addition, we observed a significant 200% increase ($p < 0.001$) in the BNF rate of soils in plots where Mo was added and a 300% increase ($p < 0.001$) in soils where both P and Mo were added together, compared to control plots in that same year. No other nutrient

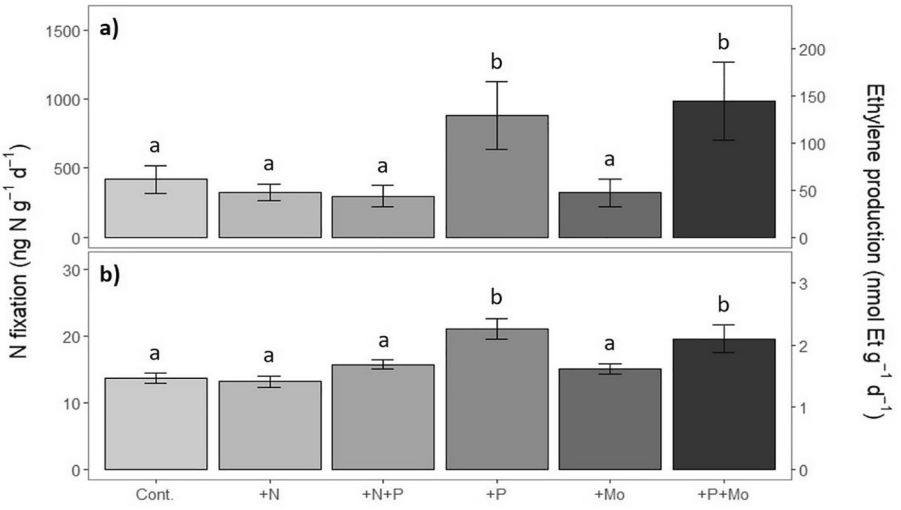

Figure 2. (a) Paracou litter and (b) Paracou soil BNF rates following nutrient additions in the laboratory. Significant differences between treatments are indicated with different lowercase letters ($p < 0.05$). The different treatments are shown in the *x*-axis: control in which only distilled water was added (Cont.), N only (+N), both N and P together (+N+P), P only (+P), Mo only (+Mo), and both P and Mo together (+P+Mo). Error bars indicate standard errors (*SE*). For each bar, $N = 15$; five litter and soil samples from three plots. Note that the scale of both *y*-axes differs between (a) litter and (b) soil.

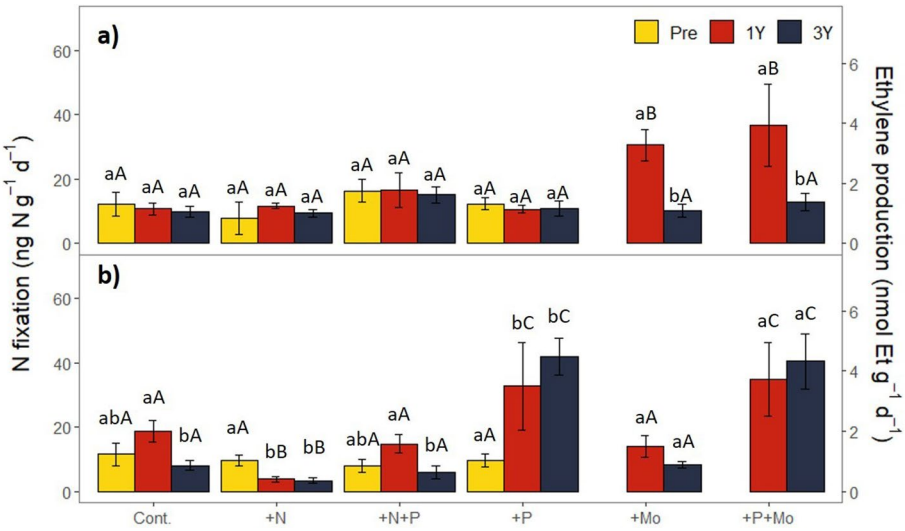

Figure 3. Soil BNF rates and the response to the various nutrient additions in (a) Nouragues and (b) Paracou. Different colors indicate whether samples were collected before (Pre), after 1 year (1Y), or 3 years (3Y) of field nutrient additions. Note that for plots fertilized with +Mo and +P+Mo no pre-treatment data were available. Significant differences within a treatment over the 3-year period are indicated with different lowercase letters while treatments that were ifferent from the control (Cont.) within one sampling period are indicated with uppercase letters. Significant differences are at the $p < 0.05$ level. The different treatments are control (Cont.), N only (+N), both N and P together (+N+P), P only (+P), Mo only (+Mo), and both P and Mo together (+P+Mo). Error bars indicate standard errors (*SE*). For each bar, $N = 15$; five soil samples from three plots.

addition treatments provoked any change in soil BNF rates after a single year of nutrient additions. After 3 years of fertilizer addition, there were no significant differences compared to the control for any of the treatments, including Mo treated plots wherein BNF had decreased back to rates similar to those of the control plots (Figure 3a).

In Paracou, after 1 year of nutrient additions, only the plots receiving N (+N) showed a decrease (60%, $p < 0.01$) compared to pre-fertilization and control plot measurements. Plots that had received P, but not N (+P and +P+Mo), had significantly higher soil BNF rates than their pre-fertilization or control plot counterparts ($p < 0.001$). After 3 years of fertilizer addition, N additions (+N) resulted in a significant 50% decrease ($p < 0.001$) of BNF rates compared to the controls, while both treatments adding P and not N (+P and +P+Mo) resulted in ca. 300% increased BNF rates compared to the controls. Both in the control plots and in the plots receiving N and P together (+N+P) soil BNF rates increased slightly (non-significant) after 1 year of additions and decreased again after 3 years, resulting in no differences with the pre-fertilization treatments but a significantly different BNF rate ($p < 0.05$ for both) from the rates measured after only 1 year of nutrient additions. The addition of Mo alone had no effect on the BNF rates in Paracou.

The laboratory nutrient addition treatments carried out on Paracou soils resulted in a significant 50% increased BNF rate for both the +P and +P+Mo treatment (Figure 2b), while the addition of Mo alone caused no change. Just as in the litter, N additions to the soil (+N and +N+P) in the laboratory nutrient addition experiment did not affect BNF rates (Figure 2b).

Exact values of soil BNF rates in response to the various nutrient addition treatments and across the different sampling times can be found in Tables S2 and S3.

4. Discussion

4.1. The Effect of N

The observed decrease in BNF rates in response to field N additions was in agreement with results from several tropical field studies (Cusack et al., 2009; Fan et al., 2019; Matson et al., 2014; Smercina et al., 2019; Z. Wang et al., 2019) and confirmed that N additions suppressed BNF in litter and soil. The presence of BNF

in the litter layer is linked to the suboptimal litter C:N ratio for microbial consumption, as the capability to obtain additional N from the atmosphere can provide the required N to decompose the litter (Manzoni et al., 2010; Menge et al., 2009). By adding N to the litter, the need to fix N from the atmosphere as nutrient source likely decreases and, under the assumption that diazotrophs are able to dial down their BNF (Menge et al., 2009), BNF decreases as well. Surprisingly, the soil BNF rates in Nouragues remained constant even after 3 years of N additions, which could be related to the N status of the soil at this site. In Nouragues, soils have much higher total and extractable N concentrations than in Paracou and the high N may have led to intrinsically low soil BNF rates that are virtually immune to additional N (Perakis et al., 2017).

The observed decrease of BNF rates following N additions is important at the ecosystem scale, because it suggests that increases in anthropogenic N deposition (R. Wang et al., 2017) could reduce this key microbial process from ecosystems. The complete elimination of free-living BNF in soil and litter due to increased tropical N deposition, as suggested by Dynarski and Houlton (2018), however, seems unlikely as even after 3 years of adding 125 kg N ha^{-1} yr^{-1} BNF remained an active process in both forest sites. Also, in other ecosystems, such as boreal forests (Rousk et al., 2014), the addition of N did not eliminate BNF entirely. While N deposition could potentially replace the loss of N from fixation, the long-term consequences of the suppression of free-living N fixers on terrestrial nutrient cycles and plant productivity remain unknown (Dynarski & Houlton, 2018).

In line with our expectations, the addition of N and P together (+N+P) caused no change to the litter BNF rates. This is similar to results reported for Ecuador (Matson et al., 2014), where N and P added together caused no change in soil BNF rates, but different from results reported for Costa Rica (Reed et al., 2007) and China (Zheng et al., 2016), where adding N and P together still yielded an increase in BNF rates in both litter and soil. In Belize, the addition of N suppressed BNF even when added together with P and/or Mo (Winbourne et al., 2017). As suggested by Matson et al. (2014) and Zheng et al. (2016), it is likely that adding N and P will have different effects on BNF depending on the local nutrient balances and the amount of fertilizer added. For instance, both Reed et al. (2007) and Zheng et al. (2016) added N and P together in equal doses (150 and 100 kg ha^{-1} yr^{-1}, respectively) and found a stimulating effect on BNF, likely because the stimulating effect of P outweighed the downregulating effect of N. Alternatively, Matson et al. (2014) added five times more N than P and found BNF rates that were not different from control rates. Just as seems to be the case in our study, they suggested that the downregulating effect of N was offset by the stimulating effect of P.

4.2. The Effects of P and Mo

In line with our expectations, we found that P additions (+P and +P+Mo) triggered significant increases in BNF rates in litter from both forests and during the laboratory incubation (Figures 1 and 2a). This matches results from both a field (Reed et al., 2007) and laboratory (Reed et al., 2013) nutrient manipulation experiment in Costa Rica. In Hawaii, however, P addition by itself stimulated litter BNF in one forest, while in another forest, both P and micronutrient (all essential plant nutrients except N and P, but including Mo) additions together stimulated litter BNF (Vitousek & Hobbie, 2000). The litter P concentrations in both Nouragues and Paracou were low (Table 1), even for French Guiana and the extended Amazon (Fanin et al., 2012; Hattenschwiler et al., 2008; Vitousek, 1984), and much lower than the litter P concentrations in African, Malaysian or even Neotropical forests (Alvarez-Clare & Mack, 2015; Kaspari et al., 2008; Vitousek, 1984). It is highly likely that the addition of P to the litter layer at least partially relieved the P constraints present in the litter, just as suspected by Vitousek and Hobbie (2000) and Reed et al. (2013) in their respective studies. The global increase in P deposition (R. Wang et al., 2017), already measurable in French Guiana (Barkley et al., 2019; Van Langenhove et al., 2020), is thus likely to cause increases in litter BNF at our forest sites. As for Mo, the P fertilizer we added in the field contained Mo as a hidden trace element (<0.15 mg/kg), shedding doubt on the validity of our results following P additions (Barron et al., 2009). However, the unchanging litter BNF rates following Mo additions alone (Figures 1 and 2a) provide strong evidence that in leaf litter, Mo is not limiting BNF rates. In both forest sites, the litter Mo concentration was high (Table 1) compared to the litter Mo concentration reported for other tropical sites (Barron et al., 2009; Bowell & Ansah, 1993; Reed et al., 2013; Vitousek & Hobbie, 2000). In this framework of litter stoichiometry at our forest sites, it is very likely that P and not Mo are limiting litter BNF.

In soils, we observed two distinct responses to P and Mo additions. First, in Nouragues, Mo (+Mo and +P+Mo), but not P (+P) additions, increased soil BNF rates, indicating that only Mo is limiting soil BNF there (Figure 3a). Second, in Paracou, soil BNF increased following P (+P and +P+Mo) instead of Mo (+Mo) additions. Together, this suggests that nutrient limitation might be site-specific, reminiscent of studies carried out in Panama that found both Mo and P and Mo co-limitation along a soil P and soil Mo gradient (Barron et al., 2009; Wurzburger et al., 2012), but contrast to Central Brazil where no effect of P and Mo addition was found on soil BNF (Wong, Neill, et al., 2020). It is noteworthy that Panamanian and Brazilian soils in these studies had lower (0.3–0.9 and 0.09 ppm, respectively) total soil Mo than the soils here in French Guiana (Table 1), a discrepancy that among other reasons could be linked to the influence of transatlantic atmospheric mineral dust transported from the North African Bodélé Depression (Wong, Mahowald, et al., 2020).

It seems that the balance between P or Mo limitation for soil BNF in our study varied at the landscape scale and was likely determined by the interplay of inputs (from weathering and dust), outputs (mainly leaching), soil stocks, and the pools available for microbial uptake. In the case of Nouragues, this balance was tipped toward Mo limitation, while in the case of Paracou, it was tipped toward P limitation. Previous research in French Guiana, especially in Paracou, indicated that soil P stocks and soil extractable P are low (Allié et al., 2015; Sabatier et al., 1997; Soong et al., 2020). Additionally, a recent study of BNF and its drivers in French Guiana identified P as a regulating driver for soil BNF in Paracou, with higher soil P responsible for higher BNF rates (Van Langenhove et al., 2019). That same study, however, did not identify Mo as regulating driver for soil BNF, even though it clearly caused an increase in Nouragues soil BNF rates here (Figure 3a). A possible explanation for Mo limitation in Nouragues but not in Paracou is that the Mo present in acidic soils (pH < 5.5) is primarily attached to secondary mineral surfaces, particularly iron oxides (Kaiser et al., 2005). In the loamy Nouragues soils, secondary minerals are more abundant than in the sandy Paracou soils (Bongers et al., 2001; Gourlet-Fleury et al., 2004), which leads to more Mo being bound to these soil minerals and less Mo available for microbial uptake. So, although Nouragues has a slightly higher soil total Mo concentration, the available soil Mo concentration is only half of what was found in Paracou (Table 1).

As there are currently no studies investigating the community composition of diazotrophs in either of the sampled forest sites, we cannot rule out the possibility that at least a portion of these diazotrophs could be employing Mo-independent nitrogenases. The latter use V (Hales, 2004) or Fe (Schneider & Müller, 2004) instead of Mo as co-factor and are thus insensitive to changes in Mo concentration in their environment. Traditionally, the majority of BNF is considered to be carried out by diazotrophs using Mo-dependent nitrogenases (Reed et al., 2011; Seefeldt et al., 2009). However, Mo-independent nitrogenases may occur more commonly than previously thought (Bellenger et al., 2014; McRose et al., 2017) and are responsible for up to 55% of the N fixed in some substrates harboring free-living diazotrophs (Zhang et al., 2016). There are no studies, however, that have identified a complete absence of Mo-dependent diazotrophs in the N_2 fixing community. So, even if a high percentage of the diazotrophs in the forests in this study use Mo-independent nitrogenases, we would still expect to detect a change in BNF rates carried out by the Mo-dependent diazotrophs following Mo additions, provided that Mo is indeed limiting. The complete absence of a response to Mo in leaf litter and in Paracou soils indicates that this is not the case there. Finally, in our study, the Mo addition plots were much smaller than the P and N addition plots, and therefore soil and or litter heterogeneity could have had an impact on the results.

4.3. Timing

In contrast to the field experiment, the addition of N to litter and soil in the lab did not change BNF rates (Figure 2). The P additions, however, did lead to increased BNF rates matching results from other studies (Jean et al., 2013; Perakis et al., 2017; Wurzburger et al., 2012) and the responses identified in the field here, although the magnitude of increase was much smaller in the lab than in the field. To our knowledge, this is the first time that a short-term laboratory experiment is combined with a long-term field study. The results unequivocally demonstrate that a short-term laboratory study is not equivalent to a field study carried out over a longer period. There are two distinct options for how N additions could result in decreased BNF rates. First, N in the added urea first needs to be metabolized into ammonia, which is then able to downregulate BNF directly through the inhibition of nitrogenase synthesis by directly downregulating *nifA* gene

transcription (Dixon & Kahn, 2004; Reed et al., 2011). This only occurs if diazotrophs are facultative fixers and able to adjust BNF rates. A second, indirect way through which N additions could lead to decreased BNF rates is through the accumulation of N, which may lead to a change in microbial community composition (Zhong et al., 2015). Following evolutionary theory, the disappearance of the ecological advantage of diazotrophs in a substrate that is no longer N limited may cause them to be outcompeted by other decomposers (Menge et al., 2009). This likely only occurs if the diazotrophs are obligate and incapable of adjusting their BNF rates. In both the direct and indirect way, there is a time lag between N addition and the decrease in BNF rates, which could explain why we observed no change in BNF following the short-term (24-h incubation period) laboratory N additions, in contrast to, for example, Perakis et al. (2017) who found decreases in soil BNF following N additions in the lab but used a slightly longer incubation period (48 h). So far, we do not know whether the decrease in BNF was caused by downregulation of the N_2 fixation pathway itself, or through an ecological response impacting the microbial community structure. Future comparisons of the microbial communities from the fertilized plots may help answer this question. For P, the change in BNF following its addition in the short-term nutrient addition experiment yielded the same clear increase as in the field, but the striking difference in magnitude of change suggests that after only 24 h the BNF has not yet reached its full potential. As a consequence, we recommend that care should be taken when using data obtained through laboratory experiments for the extrapolation to ecosystem effects.

Due to logistical constraints, we were only able to add Mo in the field during the first year. This provided an opportunity in itself as a single Mo addition of 1.1 kg Mo ha^{-1} resulted in increased soil BNF in Nouragues, but this effect was not sustained and after 3 years soil BNF returned to control levels. Since Mo was added to the soil as molybdate, a highly soluble form susceptible to leaching (Reddy & Gloss, 1993; Wedepohl, 1995), 2 years after initial addition, it could have either leached out of the soil, been taken up by plants or microorganisms or bound to mineral surfaces (Wurzburger et al., 2012) and may have no longer been available for N fixers after 3 years.

Previous research has shown that the timing of sampling can have an effect on the observed responses to nutrient additions because of biotic and abiotic changes that occur throughout the year (Reed et al., 2007). Mostly, BNF increases with water availability and is higher during the wet season in tropical forests (Barron et al., 2009; Cusack et al., 2009; Hofmockel & Schlesinger, 2007; Reed et al., 2007), yet higher rates during the dry season have been reported (Matson et al., 2014). In French Guiana, seasonal changes in BNF were only present in the leaf litter but not in the soil (Van Langenhove et al., 2019). Here, we sampled exclusively in September 2016, 2017, and 2019, which is the second month of the yearly dry season; thus, BNF rates measured were likely lower than in the wet season. Additionally, responses to the various nutrient addition treatments could potentially differ if measured at different times throughout the year. However, the only other tropical field fertilization study that we could find which measured BNF at different time points throughout a single year showed that the effects of nutrient fertilization were consistent and stronger in the wet season than during the dry season (Reed et al., 2007). In future studies, measuring BNF at different time points throughout the year will enable a more detailed assessment of seasonal changes combined with nutrient additions.

We expressed the rates of free-living BNF as ng N g^{-1} d^{-1} because this seemed a more intuitive unit than nmoles of ethylene produced per gram of substrate per day (nmol Et g^{-1} d^{-1}). For an accurate conversion from measured ethylene production to nitrogen fixation in litter samples, we used a conversion factor calculated using $^{15}N_2$ calibrations performed in 2017 at the same time, location, and under comparable conditions as the ARA incubations of that year. Such an independently calibrated conversion factor is more accurate than using a theoretical conversion factor (Soper, Taylor, et al., 2021). However, because the ARA:BNF conversion factor can change over time (Saiz et al., 2019), mainly through differences in physical conditions such as, for example, water content (Liengen, 1999), the conversion factors would have been even more accurate when calibrated at each sampling time point, instead of only once. Furthermore, it is possible that the nutrient additions changed the contribution of alternative nitrogenase (V-only or Fe-only based isoenzyme) activity in the substrate, which could have also changed the conversion factor as isoenzymes vary in their BNF efficiency (Bellenger et al., 2020). For soils, we were unable to calibrate a conversion factor (see Section 2), but had to apply the theoretical conversion factor of 3 (Hardy et al., 1968). In a meta-analysis, Soper, Simon, et al. (2021) found that in soils, the reported conversion factors centered on the commonly

applied theoretical value of 3, but the distribution of conversion factors was wide to the extent that the 25th–75th percentile generated a range of BNF rates that varied by a factor up to 2.5. By highlighting some of the caveats regarding the conversion of acetylene production into BNF we aim not to dissuade, but rather to stress that any upscaling of the free-living BNF rates reported here should be done within the framework in which the samples were gathered and should allow for errors inherent to the methodology that was used.

5. Conclusion

In line with our first hypothesis, in the two studied forest sites in French Guiana leaf litter BNF decreased following N additions and increased following P additions, most likely because litter N:P ratios were unfavorable for free-living diazotrophs. The addition of N disturbed the stoichiometric imbalance between N and P even further while the addition of P improved it. Furthermore, we observed that N and P additions may affect BNF rates interactively by canceling each other out leading to similar rates as in control plots. Our second hypothesis was only partly true as in soils, a clear difference in nutrient limitation was visible between both studied sites. Nouragues showed Mo limitation for BNF, while in Paracou the addition of P, but not Mo, caused increased BNF rates, indicative of P limitation. We rejected our third hypothesis, as the addition of P did not cause increased BNF rates in Nouragues while it did in Paracou. So it would seem that even small differences in P availability have the potential to change the response of BNF rates to P additions.

Nevertheless, the severe increase in soil BNF rate following Mo additions is a good indicator that BNF is limited by Mo in Nouragues. From our study, we conclude that BNF in tropical forests is likely not limited by a single nutrient, but rather by multiple nutrients depending on the site-specific nutrient availability. Our study furthermore highlights that, in contrast to what we hypothesized (V), litter and soil BNF rate responses to nutrient addition can differ strongly between long term field studies and short term laboratory incubations, a clear indication that in a controlled laboratory environment and on a short timescale changes brought about by nutrient additions do not necessarily represent the changes found in the field on a longer timescale. We thus suggest caution when extrapolating from laboratory studies to the ecosystem scale, as these results may not be a bona fide representation of what happens in the field.

Data Availability Statement

Data sets for this study are publicly available in Zenodo data repository (Van Langenhove, 2021).

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