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### **Fire affects asymbiotic nitrogen fixation in southern Amazon forests**

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#### **Key Points:**

- Asymbiotic N<sub>2</sub> fixation (ANF) was quantified for the first time in fire-affected seasonally flooded Amazon forests.
- ANF rates were, on average, 24% lower in burned compared to unburned surface soils (0–10 cm).
- Soil carbon:nitrogen ratio and available phosphorus explained 58% of ANF variance in burned and unburned soils.

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## Abstract

In this study, we investigate the biogeochemical consequences of fire in seasonally flooded Amazon forests, where recent declines in forest cover have been linked to increases in fire frequency and severity. Previous studies have hypothesized that a quasi-permanent state-shift transition from typical Amazon forests to open savannas can occur when fire results in further depletion of already impoverished soil nutrient pools. Asymbiotic  $N_2$  fixation (ANF) is an essential pathway for fire-affected forests to acquire nitrogen (N) after disturbance, but ANF response to fire has yet to be quantified in Amazonia. Here, we quantify ANF through field sampling and laboratory incubations using  $^{15}N$ -labeled dinitrogen ( $^{15}N_2$ ) and measurement of 14 biogeochemical parameters in surface [0–10 cm] and subsurface [10–30 cm] soils. Our data represent burned and unburned replicated sampling sites, across five stands, spanning a gradient from infrequent (once in 13 years) to frequent (five times in 13 years) fire occurrences. ANF did not vary with fire frequency but was, on average, 24% lower in burned than in unburned surface soils across all stands. Burned and unburned subsurface soils had similar ANF rates. About 58% of ANF variance was explained by the joint effect of carbon(C):N ratio and available phosphorus (P) in burned and unburned soils. ANF increased linearly with C:N and P availability in unburned soils, but a highly non-linear relationship was observed in burned soils. Our findings show that fire alters soil C-to-nutrient stoichiometry, which resulted in lower N inputs via ANF into burned relative to unburned tropical forest soils.

**Keywords:** Climate change, disturbance, drought, free-living diazotrophs, nitrogen-15, stable isotopes, stoichiometry, tropical soils, wildfire.

## Plain Language Summary

The impact of fire on biological  $N_2$  fixation in tropical forests is poorly understood. In Amazon forests, most of the N is recycled through the litter layer, which is mostly consumed during a fire event. Therefore, asymbiotic biological  $N_2$  fixation (ANF) in the soil—the conversion of atmospheric  $N_2$  into plant-available N by free-living soil bacteria or archaea—is expected to be a significant source of N for vegetation growth after fire events. We measured rates of soil ANF as well as soil C and P, which are

known to affect N cycling in tropical forests. Rates of ANF were 24% lower in burned compared to unburned surface soils (0–10 cm), while no difference was detected in subsurface soils (10–30 cm). At both soil depths, ANF was expected to increase linearly with C:N ratio and P availability, but non-linear relationships between soil variables make predictions more challenging in burned soils regardless of fire frequency. Our findings suggest that decreased N inputs due to post-fire ANF suppression could help accelerate the transition from Amazon forests to open savannas.

## 1 Introduction

Approximately 41% of total global forest loss between 1990 and 2015 occurred in the Brazilian Amazon–Cerrado transition zone (Bonini et al., 2018; FAO, 2016; Hansen et al., 2008), the largest transitional area on the planet. In that region, observed biodiversity loss and disruption of biogeochemical cycles are related to recent changes in fire regimes (i.e., increase in fire frequency and severity), rampant forest-to-monocrop conversion, and intensification of the management of agricultural and livestock areas (Aragão et al., 2018; Brando et al., 2014, 2019; Chen et al., 2013; Fearnside, 2018; Silva et al., 2018; Silverio et al., 2013). That transition zone is fire-prone due to a seasonal climate (~5- to 6-month dry season) and mosaic-type distribution of open savannas characterized by a discontinuous cover of fire-adapted woody species with thick bark in a continuous grass understory, and fire-sensitive forests dominated by trees with thin bark and lacking grass understory (Hart et al. 2005; Hoffmann et al., 2009; Ratnam et al., 2011). Recent increases in dry season length (Agudelo et al. 2018; Fu et al., 2013) and intensity (Barkhordarian et al., 2018a, 2018b) can escalate the likelihood of fires throughout the season in that region (Barlow et al., 2019). As a result, without proper fire management, positive feedbacks between fire frequency, dry season length, land-use change and management (i.e., the use of fire on pastoral and agricultural land) are expected to facilitate shifts from fire-sensitive to fire-adapted vegetation (Brando et al., 2014; Cano-Crespo et al., 2015; Dantas et al., 2015; Flores et al., 2017; Hansen et al., 2008; Hoffmann et al., 2012; Lovejoy & Nobre, 2018; Silverio et al., 2013).

Seasonally flooded forests in the Amazon–Cerrado transition are examples of fire-sensitive forests affected by recent changes in fire regimes. Those forests are sensitive to fire because trees

adapted to humid tropical forests are generally not fire-adapted (Franco et al., 2014); thus, high tree mortality can occur as a result of fire (Hoffmann et al., 2012). Ongoing long-term (>12 years) ecological research has indicated above-average tree mortality and diversity loss in seasonally flooded forests due to fire (da Silva et al., 2018; Maracahipes et al., 2014). Fire disturbance can disrupt biological processes and biogeochemical cycles, notably those concerning N (Pellegrini et al., 2018), which can influence natural succession in dystrophic, nutrient-poor soils (de Oliveira et al., 2017; Nardoto et al., 2014; Taylor et al., 2019). For example, the effect of fire includes transformations of the soil organic and inorganic pools with subsequent loss through volatilization or via export of particulate matter (Certini, 2005; Crutzen & Andreae, 2016; Dionizio et al., 2018; Mataix-Solera et al., 2011; Schlesinger et al., 2016). Fires can lead to significant losses of carbon (C) and N through combustion and volatilization (Pellegrini et al., 2014; Silva et al., 2015), even though mineral elements, such as phosphorus (P), can become readily available following fire-induced mineralization of soil organic matter (Butler et al., 2018). Therefore, during the early stages of post-fire succession characterized by low N and high P, biological N<sub>2</sub> fixation (BNF, i.e., the biological conversion of N<sub>2</sub> gas into bioavailable forms) is expected to be a relevant source of N to the vegetation.

Inputs of new N into tropical forests via symbiotic BNF depend on the presence of thin-barked fire-sensitive trees (i.e., members of the Fabaceae family and their endosymbiotic N<sub>2</sub>-fixing bacteria), which, like other tropical trees, show high mortality after fire events (da Silva et al., 2018; Maracahipes et al., 2014). However, BNF also includes asymbiotic N<sub>2</sub> fixation (ANF), which is not well documented in Amazonia. In the soil, ANF alleviates N limitation via heterotrophic or autotrophic soil bacteria (or archaea) that exist independently of C transfer (Reed et al., 2011), which has the potential to replenish part of the lost N due to fire. This expectation is supported by previous studies in tropical forests showing correlations between low N availability and high ANF (Dynarski & Houlton, 2017; Reed et al., 2011); this is attributed to the suppression of nitrogenase activity—the enzyme that catalyzes BNF—under energetically favorable soil inorganic N uptake relative to atmospheric N<sub>2</sub> fixation (Norman & Friesen, 2017). In some cases, ANF may respond negatively to fire-induced shifts in critical regulators such as soil C:N ratio and P availability (Bomfim et al., 2019; Dynarski & Houlton, 2017; Mirza et al., 2014; Reed et al., 2011).

In this study, we quantify soil ANF rates in fire-affected seasonally flooded Amazon forests to examine patterns and biogeochemical controls with explicit consideration of fire frequency and presence. Specifically, we focus on the following questions: i) Does fire presence affect soil ANF? ii) Does fire frequency affect soil ANF? iii) Which biogeochemical variables best explain ANF variance in fire-affected soils? We hypothesize that: i) soil ANF increases with N depletion due to fire presence; ii) soil ANF increases with fire frequency due to further depletion of N pools in frequently burned areas, and; iii) soil C:N:P stoichiometry correlates with ANF activity across burned and unburned areas. We developed these hypotheses on the basis that ANF may increase as a result of fire-induced N losses and P gains via organic matter mineralization. However, soil C volatilization and the biomass removal caused by fire may negatively impact ANF. Based on field observations and laboratory measurements, we report: i) the first measurements of ANF in fire-affected seasonally flooded soils in southern Amazon with explicit consideration of fire frequency, fire presence, and soil depth, and; ii) statistical relationships between soil biogeochemical properties and ANF considering fire presence.

## **2 Materials and Methods**

### **2.1 Site description and sample collection**

During the austral winter (July) of 2016, we collected soil samples in semideciduous seasonal alluvial forests in Araguaia State Park, one of the most extensive continuous seasonally flooded areas in South America where tropical forests and savannas co-exist (Figure 1). The park covers >200 thousand hectares of southern Amazon forest in Mato Grosso State, Brazil (Marimon et al., 2014). Climate is classified as Köppen's Aw (Kottek et al., 2006)— 26°C mean annual temperature and 2,000 mm rainfall—with a dry season between April and September and a rainy season between October and March (INMET, 2017). Elevation ranges from 210 to 240 m above sea level.

Araguaia State Park has a well-documented spatiotemporal fire frequency record between 2000 and 2013, which represents the number of fire events detected during that period using burned area data from the Moderate Resolution Imaging Spectroradiometer (MODIS) MOD45 product (Neves et al., 2018). This fire frequency gradient, among other factors, depends on differences in flooding regimes

across the landscape. We assessed the effects of fire frequency (from one to five events), and fire presence (burned vs. unburned) on soil ANF and its biogeochemical controls by selecting five seasonally flooded (seasonal semideciduous alluvial) forest stands in the Park to establish a fire frequency gradient ranging from one to five fire events between 2000 and 2013 (Figure 1; Table 1). In all five stands, a permanent one-hectare monitoring plot is systematically surveyed since 2007/2008 as part of the long-term ecological research of the RAINFOR network (<http://www.rainfor.org/>). For our fire frequency gradient, we selected one stand affected once by fire (F1) to serve as a control for comparisons with the other forests affected twice (F2 and F2b), four times (F4) and five times (F5).

Between 2007 and 2016 (the most recent survey), aboveground biomass loss (Nogueira et al., 2019), increased floristic similarity, and higher plant mortality than recruitment (da Silva et al., 2018; Maracahipes et al., 2014) were reported for the same stands included in this study (Table 1). The most recent survey data [2014 and 2016], including trees with a diameter at breast height-DBH  $\geq 10$  cm in each stand, are presented in Tables S1 (species abundance), S2 (beta diversity matrix), and S3 (density, basal area and relative basal area of trees belonging to Fabaceae). While stem density and basal area in F1 (control) increased between 2007 and 2014, both parameters decreased by nearly half in F5 between 2008 and 2016 (Table 1). During the same periods, tree species richness did not change in F1 but declined from 56 to 26 species per hectare in F5. Based on the most recent surveys (2014 for F1, F2, and F2b, and 2016 for F4 and F5), all five stands have very similar Shannon diversity indices (between 2.2 and 2.7), but tree species richness ranged from 21 in F1 to 33 species per hectare in F4. Floristic similarity (based on species abundance) is higher among F4 and F5, F1, and F2b, while F2 is less floristically similar to the other stands (Figure S1; Table S2). Stand basal area range from 15.3 m<sup>2</sup> per hectare in F5 to 33.5 m<sup>2</sup> per hectare in F1. All these changes in the vegetation have been attributed to longer dry seasons, and higher frequency of fire events by da Silva et al. (2018), Maracahipes et al. (2014), and Nogueira et al. (2019), but not to a particular fire event detected between 2007 (first survey year) and 2013 (last year of the fire frequency record).

We employed a systematic cluster scheme (Wang et al., 2012) to sample multiple soil cores in each stand and account for the spatial heterogeneity of fire, which we only detected *in situ* because of the spatial resolution of the MOD45 product (30 m). We established two sampling clusters within each

forest: one in an area unaffected by fire (unburned) and another in an area affected by fire (burned). We did not observe fire scars on the standing trees surrounding the unburned sampling points, nor did we find charcoal pieces in the unburned soil samples. Additionally, we did not explicitly consider burn severity when selecting our burn clusters. Each 100 m<sup>2</sup> cluster consisted of 2 N–S and 2 E–W samples as well as one center point, which we did not sample, totaling four samples per cluster. At each sampling point, we collected soil cores (0–10 and 10–30 cm depths) for the determination of ANF rates and physicochemical properties (listed in section 2.3).

Seasonally flooded forests are natural fragments occurring on depressions where organic material has accumulated over time to form, in most cases, organic soils (i.e., soil organic C  $\geq$  12 to 18%; Buol et al., 2011; see section 2.3). In all studied forests, soils fall under the Brazilian Classification of “*Gleissolo Melânico*” (EMBRAPA, 2006), which is equivalent to Typic Haplosaprists (Histosols) or Aquepts (Entisols) in the USDA system (Staff, 2014). The studied forests are naturally surrounded by grasslands/herbaceous fields (Marimon et al., 2012), which favors fire ingress into the forest during the dry season (~May to September) when the grass is parched and flammable (Maracahipes et al., 2014).

## 2.2 Quantification of ANF rates

We collected soil samples (0–10 and 10–30 cm depths) at each sampling point and placed them into sterile bags, mixed, and stored at 4°C until all sampling was completed (7 days). Then, the samples were transported to the University of California-Davis, where ANF was immediately quantified as in Bomfim et al. (2019) (see more details in Text S1). Briefly, three replicates of each sample were incubated in 12-ml sealed gas vials (Exetainer, Labco, UK) containing 1 g (dry weight) of sample and 2 ml of <sup>15</sup>N-labeled dinitrogen gas (98 atom% <sup>15</sup>N, catalog number NLM-363-PK, Cambridge Isotope Laboratories). At the same time, two corresponding controls were incubated in 12 ml vials containing the same sample (~1g) but not receiving any isotopically labeled gas. Vials were incubated in a dark incubator (at 25°C) for 48 hours, after which the vials were opened, samples were removed, and ANF rates were calculated according to standard procedures (Hsu & Buckley, 2009). All samples were oven-



dried at 40°C and ground in a sterile ceramic mortar. Their <sup>15</sup>N content was determined using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). The amount of N fixed (ng N g dry weight<sup>-1</sup> h<sup>-1</sup>) was calculated in comparison with background (control) levels. A total of 400 samples were analyzed for <sup>15</sup>N content representing a total of 80 forest soil samples collected in Araguaia State Park (Table S4).

### **2.3 Soil physical and chemical characterization**

Soil samples were air-dried, sieved to 2 mm, manually ground using a ceramic mortar, and physically and chemically characterized in the laboratory at the University of California-Davis. Precisely, 14 known controls on ANF were quantified (Reed et al., 2011) and are listed for each sampled depth in Table S5. Gravimetric moisture content (weight of water divided by dry soil weight) was determined by oven drying ~5 g of soil at 100°C for 48 h; soil pH was determined in H<sub>2</sub>O (2:1) and KCl (1 M); total C and N were measured using a Costech Elemental Combustion System 4010; potentially mineralizable nitrogen (PMN, as NH<sub>4</sub>-N) by anaerobic incubation (Waring & Bremner, 1964); total P and Fe content by sulfuric acid digestion (Claessen et al., 1997) followed by colorimetric determination (Murphy & Riley, 1962; Stookey, 1970), and; available P (M3-P) and Fe (M3-Fe) by Mehlich-3 extraction (Claessen et al., 1997; Mehlich, 1984; Tran et al., 1990) followed by colorimetric determination (Dominik & Kaupenjohann, 2000; Murphy & Riley, 1962).

### **2.4 Statistical analysis**

To assess the effects of fire on soil biogeochemical parameters and ANF, we performed all statistical tests using mass-based average ( $n = 3$ ) rates of ANF (ng N g dry weight<sup>-1</sup> h<sup>-1</sup>). We used the Shapiro-Wilk test to verify data normality, and several log transformations were necessary ( $p \geq 0.05$  or  $W \geq 0.90$ ). First, we used linear mixed-effects models to test the fixed effects of fire frequency (1, 2, 4, and 5 years), fire presence (burned and unburned), forest stand (F1, F2, F2b, F4, and F5), soil depth (0–10 and 10–30 cm)—with interactions—on soil ANF (response variable). We included cluster replicates

(four in each cluster) as a random effect using the *lmer* function (residual maximum likelihood method) in the *lme4* R package (Bates et al., 2013).

Because the estimate of the variance explained by the random effect was not significant at the 95% confidence level, we fit generalized additive models (GAMs) to test the effects of the same categorical variables on soil ANF and the best model was chosen based on AIC (Akaike Information Criterion), with the lowest value indicating the best model. In case any category showed significant differences, least-squares means at the 95% significance level, adjusted for Tukey's Honestly Significant Difference (HSD), was used for comparisons of means.

We used factor-smooth GAMs (Bomfim et al., 2019; Wood, 2006) to evaluate the effects of fire frequency and fire presence on soil physicochemistry and ANF, including the effects of stand, for both 0–10 and 10–30 cm soil depths combined (details in Text S2). Gaussian error distribution was used for GAMs because normality was achieved after log transformations. The Generalized Cross-Validation (GCV) was used as the prediction error criteria to find the appropriate degree of smoothness (Wood, 2006). Variance explained and AIC values—here too the lowest AIC value indicates the best model—and the adjusted coefficient of determination (adjusted  $R^2$ ) were considered in the GAM selection. Concurvity, the non-linear form of collinearity among explanatory variables, was assessed in the best-fitted GAM using function *concurvity* in *mgcv* R package (Wood, 2008). We use Spearman correlation to examine relationships among soil variables (Figure S4). We used R version 3.6.1 (R Core Team, 2019) for all statistical analyses.

### 3 Results

#### 3.1 Asymbiotic nitrogen fixation rates across stands, fire frequency, and fire presence

ANF activity was detected in 76% of the 80 soil samples, and rates ranged from 0.026 to 3.31 ng N g dry weight<sup>-1</sup> h<sup>-1</sup> in F1 and F2b burned soil samples, respectively (Figures 2 and S2). Fire presence negatively affected ANF in surface (0–10 cm) soils (Tukey's test,  $p = 0.05$ ), while no significant effect ( $p = 0.9$ ) was detected in the subsurface layer (10–30 cm). The average ANF rate was  $0.97 \pm 0.72$  ng N g dry weight<sup>-1</sup> h<sup>-1</sup> in burned surface soils and  $1.27 \pm 0.56$  ng N g dry weight<sup>-1</sup> h<sup>-1</sup> in unburned surface

soils, a significant 24% decrease. The categorical variable fire presence ( $F = 5.34$ ,  $p = 0.03$ ), the interaction between fire presence and soil depth ( $F = 3.96$ ,  $p = 0.05$ ), and the interaction between fire presence and fire frequency ( $F = 2.94$ ,  $p = 0.06$ ) significantly affected ANF across the studied soils (Table S6). There was no significant effect of stand on ANF ( $p > 0.1$ ), and no significant effect of soil depth or fire frequency apart from that in the interactions (Figure S2).

### 3.2 Soil biogeochemical controls on ANF

Based on the best GAM, total C:N ratio and available P, interacting with fire presence, explained 58% of ANF variance (Figure 3; Table 2). Their joint effect on ANF was significant in both burned and unburned soils (0–10 and 10–30 cm depths) across all stands. Although ANF increased linearly with C:N and P availability in unburned soils, a highly non-linear relationship was observed in burned soils. ANF in unburned soils was highest when soil C:N was greater than 40 and log available P higher than  $1.5 \mu\text{g g}^{-1}$ . ANF in burned soils was highest when C:N was  $\sim 10$  and log available P greater than  $1.5 \mu\text{g g}^{-1}$ , or when C:N was  $\sim 20$  and log available P less than  $0.5 \mu\text{g g}^{-1}$ .

Total soil C:N and available P were significantly correlated ( $r = 0.45$ ), yet the nature of their relationship varied with fire presence (Table 3; Figures S3 and S4). On average, total C:N was significantly higher in unburned than in burned surface soils, but subsurface soils did not differ. P availability was  $\sim 20\%$  higher in unburned than in burned surface soils, but burned and unburned subsurface soils did not differ in P availability. Total soil C was higher in unburned surface soils, while total N was higher in burned surface soils, though not significantly (Figure S5).

In addition to total C:N and available P, few parameters varied with fire presence in surface and subsurface soils (Tables 3 and S5). For instance, potentially mineralizable nitrogen (PMN, as  $\text{NH}_4\text{-N}$ ) was higher in burned than in unburned surface soils ( $p = 0.2$ ), but lower in burned than in unburned subsurface soils ( $p = 0.1$ ). PMN was also significantly positively correlated with total soil C ( $r = 0.7$ ) and N ( $r = 0.6$ ), but not with C:N ratio or available P (Figure S4). Available Fe was higher in unburned than in unburned surface and subsurface soils, though not significantly. Available Fe was also

significantly positively correlated with total C ( $r = 0.25$ ) and N ( $r = 0.3$ ), in addition to available P ( $r = 0.4$ ) and PMN ( $r = 0.35$ ).

### 3.3 Soil parameters across stands and fire presence

In addition to total soil C, N, C:N, and available P, the following soil parameters (0–30 cm) varied with fire presence across stands: available Fe, available Fe:P ratio, total P, and total Fe:P (Table S5). Total N in burned soils did not vary significantly across stands but was significantly higher in F2 ( $9.0 \pm 4.2$  g kg<sup>-1</sup>) than in unburned soils in F4 ( $2.8 \pm 2.1$  g kg<sup>-1</sup>;  $p < 0.01$ ). On the other hand, total C in burned soils was significantly higher in F2 ( $227.1 \pm 121.2$  g kg<sup>-1</sup>) than in F2b ( $119.0 \pm 71.8$  g kg<sup>-1</sup>;  $p = 0.07$ ), in F4 ( $122.5 \pm 40.4$  g kg<sup>-1</sup>;  $p = 0.08$ ) and in F5 ( $51.7 \pm 40.4$  g kg<sup>-1</sup>;  $p < 0.01$ ). Within F5, burned soils had significantly lower total C ( $51.7 \pm 40.4$  g C kg<sup>-1</sup>;  $p = 0.04$ ) than unburned soils ( $138.0 \pm 67.2$  g C kg<sup>-1</sup>). Among unburned soils, total C only varied significantly between F2 ( $209.7 \pm 121.2$  g C kg<sup>-1</sup>) and F4 ( $76.4 \pm 56.1$  g C kg<sup>-1</sup>;  $p = 0.02$ ). Total C:N in unburned soils did not vary across stands. Among burned soils, C:N was significantly higher in F1 ( $32.7 \pm 6.3$ ) than in F5 ( $13.2 \pm 6.1$ ;  $p < 0.01$ ).

## 4 Discussion

### 4.1 Fire reduces ANF in Amazon forest soils

The frequent fires in southern Amazonia have led to aboveground biomass loss in the studied forests (da Silva et al., 2018; Maracahipes et al., 2014; Nogueira et al., 2019) with, as of yet, unquantified consequences for microbially-mediated soil processes. Fire-induced aboveground biomass loss may influence ANF through, among other factors, reduced C inputs from photosynthesis into the soil (Perakis et al., 2017), and changes in litterfall mass, decomposition, and resulting shifts in forest N and P cycling (Butler et al., 2017; Mataix-Solera et al., 2009; Nardoto et al., 2014; Reed et al., 2011; Sayer & Tanner, 2010). Here, we focused on fire-induced changes in the soil and tested the hypothesis that fire frequency and fire presence alter ANF in southern Amazon forests.

Because the general lack of mineral elements (e.g., P) in tropical soils limits ANF (Bomfim et al., 2019; Dynarski & Houlton, 2017), we expected ANF rates to increase in response to fire-induced

N volatilization and increase in P availability. Because P has a high ( $>700^{\circ}\text{C}$ ) volatilization temperature (Butler et al., 2018; Glaser & Lehr, 2019), it is enriched in ash, which is incorporated into the soil through recycling of combusted plant material. Surprisingly, we found that regardless of fire frequency, ANF was ~24% lower in burned surface (0–10 cm) soils (Figure 2). On the other hand, fire did not affect ANF in the subsurface layer (10–30 cm) probably because of the lower influence of fire temperature and soil combustion in depths below 10 cm (Certini, 2005), which may also explain the very few significant changes in soil physicochemistry at the 10–30 cm depth (Tables 3 and S5). The dominant trend that emerged is that fire presence had a direct negative effect on ANF, which is an essential microbially-mediated biogeochemical process that alleviates N limitation in impoverished tropical soils (Reed et al., 2011).

The observation that fire frequency did not directly affect ANF contradicts our hypothesis of greater ANF in more frequently burned soils. Given our study provides an initial step toward understanding fire-induced changes on ANF in seasonally flooded tropical forests, comparisons with other studies within the same ecosystem are limited. However, our results corroborate previous findings that fire frequency does not change soil N in tropical savannas (Aranibar et al., 2003), or some temperate forests and grasslands after frequent prescribed fires (Alcañiz et al., 2018). ANF has decreased after a fire in temperate grassland soils as a result of greater soil inorganic N (Hobbs & Schimel, 1984), the uptake of which is energetically favorable compared to atmospheric  $\text{N}_2$  fixation (Norman & Friesen, 2017). Given that fire events can cause different impacts on different ecosystems (i.e., severity; Hoffmann et al., 2019), future studies in the tropics might further test this fire frequency hypothesis in controlled experimental settings.

Another important aspect related to fire effects on ANF regards the capacity of diazotrophs to recolonize the soil after repeated fires. Diazotroph communities are affected by a high temperature such that  $\text{N}_2$  fixation falls off beyond  $40^{\circ}\text{C}$  (Mittal et al., 2011; Sylvia et al., 2005). However, fire generally affects the first centimeters of the soil, as observed in this study, and recolonization from deeper soil or adjacent areas should happen promptly (Neary et al., 1999). Therefore, our observed lower diazotroph activity in burned surface soils, six years after the last documented fire event, is probably more related

to the observed changes in soil C:N:P (discussed in section 4.2) than to the lower abundance of soil diazotrophs. However, it is possible that fire-induced shifts in diazotroph community composition also affected rates of  $N_2$  fixation in the soil (Andersson et al., 2004).

#### **4.2 Fire alters C:N:P stoichiometry and ANF**

Our data reveal that fire presence can induce changes in soil C, N, and P stoichiometric ratios, which were strong predictors of ANF (Figure 3). Variation in ANF mirrored changes in C:N, with both decreasing significantly in surface soils exposed to fire. An initial decrease in C:N following a fire event may occur due to the formation and accumulation of new recalcitrant heterocyclic N compounds and more rapid volatilization of C compounds, as found in temperate systems (Rodríguez et al., 2018). In general, however, the short- and long-term impacts of fire on C and N may be different, even though both have relatively low volatilization temperatures (Butler et al., 2018; Nave et al., 2011; Novak et al., 2009). We observed that soil C decreased, and N increased in burned surface soils, though these trends were not significant (Table 3).

While total C:N and available P correlated linearly and positively with ANF in unburned soils, a highly non-linear relationship emerged in burned soils. The observed nonlinearity in fire-disturbed soils is opposite to the trend detected in soils affected by land-use change in the Brazilian Atlantic Forest (Bomfim et al., 2019). In that study, non-linearity was verified in undisturbed soils under native vegetation, and disturbance (i.e., forest conversion to monocropping systems) homogenized the stoichiometric relationships between biogeochemical controls and ANF. Possibly, land-use change served as a homogenizing force on ANF (Rodrigues et al., 2013), while fire disturbance created patchiness (i.e., heterogeneity) in the studied forests, evinced by increased variance and non-linearity in the relationship between ANF, C:N and available P observed only in burned soils (Figure 3). This finding supports the assumption that fire influences the overall balance of elements within forest ecosystems (Butler et al., 2017).

Soil organic matter (SOM) quality can influence ANF (i.e., lower C:N was associated with lower ANF in the burned surface soils) because diazotrophs can acquire energy from SOM decomposition (Perakis et al., 2017; Reed et al., 2011). As diazotrophs decompose SOM, they can

decrease the availability of P (Perakis et al., 2017) and molybdenum-Mo (not measured here but see Barron et al., 2008), which are also essential in regulating ANF (Dynarski & Houlton, 2017; Perakis et al., 2017; Reed et al., 2011; Wurzbürger et al., 2012). Rapid mineralization of SOM via burning and ash production can temporarily offset this effect by increasing P availability in burned soils (Butler et al., 2018). Typically, P does not easily volatilize, and so P concentrations tend to increase or remain constant following fires (Alcañiz et al., 2018; Butler et al., 2018), which is also reflected the ratio of inorganic to organic P pools (Butler et al., 2018; Santín et al., 2018). Once in the ash component, P loss from the system can happen by water and wind erosion (Santín et al., 2018). In fact, we found lower soil P availability in burned surface soils, suggesting that fire can aggravate P limitation in tropical soils. Therefore, fire-induced changes in soil biogeochemistry can vary by soil type even though fire inevitably disturbs element ratios within ecosystems and, consequently, alters biological processes such as ANF.

#### **4.3 ANF as a potential N source after fire**

Predicting changes in N biogeochemistry after fire events is a challenge. Soil N can increase, decrease, or remain unchanged after wildfire (Wan, 2001; Wang, 2012) and prescribed fires (Alcañiz et al., 2018; Aranibar et al., 2003). Our data suggest that about six years after the last detected fire event (Table 1), burned tropical forest soils present a diminished capacity to provide N for vegetation recovery via ANF compared to unburned soils. Considering the regional changes in fire regimes (Agudelo et al., 2018; Brando et al., 2019; Chen et al., 2013), and state-shifts from forest to open savanna (Hoffmann et al., 2012; Silverio et al., 2013), ANF response to future fires remains uncertain.

Previous studies showed that N cycling following tropical pasture abandonment (Taylor et al., 2019) and selective logging (Winbourne et al., 2018) recovers in less than 20 years. This recovery may be related to disturbance type and suppression during secondary succession. Considering that most of the N recycling in the Amazon region comes from litter decomposition (Nardoto et al., 2014) and that the litter layer is both burned and negatively affected by fire-induced tree mortality, soil ANF fire response deserves more considerable attention and further investigation. In tropical savannas, for instance, legume N<sub>2</sub> fixation appears not to be the mechanism that balances N losses (Aranibar et al.,

2003), suggesting that other biological N<sub>2</sub> fixation pathways may be critical in those frequently-burned systems. Future work should quantify ANF (in different substrates) and symbiotic N<sub>2</sub> fixation simultaneously, in addition to their interrelationships with the vegetation and soil microbial communities during secondary succession to assess the contribution of plant and microbial inputs to the successional N dynamics of regenerating tropical forests.

## 5 Conclusions

To understand how tropical forest soil biogeochemistry in seasonally flooded landscapes of southern Amazon respond to fire, we combined field observations and laboratory measurements of ANF rates and 14 biogeochemical parameters in surface (0–10 cm) and subsurface (10–30 cm) soils from burned and unburned areas. We selected forest stands to represent a fire disturbance gradient from low (once 13 years) to high (five times in 13 years) fire frequencies. Across all stands, we found that ANF was ~24% lower in burned relative to unburned surface soils, but burned and unburned subsurface soils did not differ. The joint effect of total soil C:N ratio and available P in burned and unburned soils, at both 0–10 cm and 10–30 cm depths, explained 58% of ANF variance. As explained above, ANF increased linearly with C:N and P availability in unburned soils, but a non-linear relationship was observed in burned soils as fire history influenced soil C-to-nutrient ratios, which regulate inputs of N into tropical forest soils via ANF. Coupled with frequent fires and decreased litterfall due to tree mortality after the fire, lower ANF rates in the soil could have long-term consequences for secondary forest succession trajectories in southern Amazonia, given that most of the N recycling occurs through the litter layer, which is mostly combusted during a fire event. We suspect that the effects of fire presence may be related to changes in the vegetation and that fire frequency further affects ANF via plant-induced feedbacks with the soil microbial community. Future studies stand to gain valuable information by investigating linkages between above- and belowground composition, structure, diversity, productivity, and C, N, and P biogeochemistry in tropical ecosystems affected by fire disturbance. Such studies would benefit from applying our GAM model, under controlled experimental settings, and with explicit consideration of burn severity, since our study, while taking advantage of real variation in wildfire



occurrence, was subject to natural irregularities among sampling sites under the uneven frequency of fires.

### **Acknowledgments, Samples, and Data**

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**Table 1.** Fire frequency (number of fire events between 2000 and 2013) and structural and diversity parameters of the studied stands in Araguaia State Park, Mato Grosso State, Brazil.

Stand ID <sup>a</sup>	Fire frequency	Years of fire events	Census year	Tree richness (number of species ha <sup>-1</sup> )	Shannon diversity index	Basal area (m <sup>2</sup> ha <sup>-1</sup> )	Tree density (stems ha <sup>-1</sup> ) <sup>e</sup>
F1	Control <sup>b</sup>	2010	2007 <sup>c</sup>	21	-	31.9	1467
			2014	21	2.36	33.5	1623
F2	2	2007, 2010	2007 <sup>c</sup>	27	-	19.9	646
			2014	29	2.55	17.8	656
F2b	2	2007, 2010	2007 <sup>c</sup>	35	-	29.5	950
			2014	30	2.21	21.2	664
F4	4	2001, 2006, 2007, 2010	2008 <sup>d</sup>	48	-	27.6	966
			2016 <sup>d</sup>	33	2.71	28.7	917
			2008 <sup>d</sup>	56	-	31.6	922
F5	5	2006, 2007, 2010	2016 <sup>d</sup>	26	2.71	15.3	411

*Note.* <sup>a</sup> All stands are semideciduous seasonal alluvial forests. <sup>b</sup> This stand was burned once between 2000 and 2013 and, based on long-term inventory data showing low tree mortality and floristic composition change, is used as the control in this study. <sup>c</sup> Data included trees with a diameter at breast height (DBH, i.e., the stem diameter at 1.3 m above ground level)  $\geq 10$  cm (da Silva et al., 2018). <sup>d</sup> Unpublished data. <sup>e</sup> Number of live individuals ha<sup>-1</sup>.

**Table 2.** Summary of the best GAM fitted for ANF as a function of the joint effect of total soil C:N and log available P (M3-P) interacting with fire presence (0–10 and 10–30 cm soil depths).

Parametric coefficients	Estimate	Standard error	t-value <sup>a</sup>	<i>p</i> (> t ) <sup>b</sup>
Intercept	-0.2228	0.05	-4.98	<0.0001****
Fire presence - Unburned	0.1470	0.06	2.54	0.014**
Factor-smooth interaction terms				
	Edf <sup>c</sup>	Ref df <sup>d</sup>	F	<i>p</i> (>F) <sup>e</sup>
<i>s</i> (C:N, log M3-P) * Burned	13.52	16.63	3.35	<0.001***
<i>s</i> (C:N, log M3-P) * Unburned	2.00	2.00	4.16	0.020**

Significance levels: 0 \*\*\*\*\*, 0.001 \*\*\*, 0.01 \*\*, 0.05 \*, 0.1

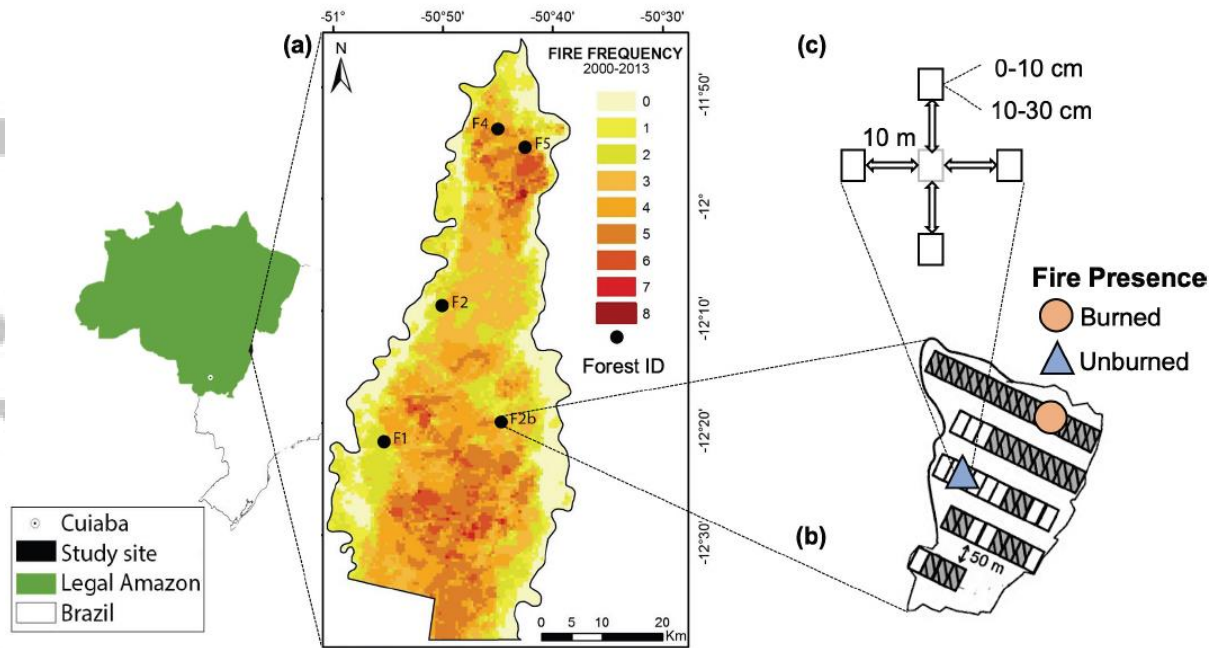
*Note.* <sup>a</sup> The t-value is a t-statistic for testing whether the corresponding regression coefficient is different from 0. <sup>b</sup> Indicates the probability of the change in slope from the reference level to the indicated category level in the same factor. <sup>c</sup> Effective degrees of freedom = the amount of the complexity used in the smooth—higher Edf values ( $\geq 8$ ) indicate that the curve is non-linear, Edf = 1 is ~ a straight line see (Wood, 2006). <sup>d</sup> Effective residual degrees of freedom. <sup>e</sup> Associated with Wald tests that the whole function *s*( $\cdot$ ) = 0. Low *p* indicates a low likelihood that the splines that make up the function are jointly zero.

**Table 3.** Biogeochemical parameters (means and standard deviations) of burned (B) and unburned (UB) soils across five forest stands in Araguaia State Park, Mato Grosso State, Brazil.

Fire presence Soil depth (cm)	UB	B	UB	B
	0–10		10–30	
ANF (ng g dry weight <sup>-1</sup> h <sup>-1</sup> )	1.27 (0.6) a	0.97 (0.7) b	0.75 (0.3) b	0.81 (0.5) b
Total C:N	29.6 (5.3) a	23.8 (6.1) b	19.5 (5.3) b	20.3 (8.1) b
Available P (μg g <sup>-1</sup> )	25.7 (15.6) a	22.3 (13.4) b	10.4 (8.8) b	14.0 (21.8) b
Total C (g kg <sup>-1</sup> )	205.0 (80.9) a	195.9 (92.3) a	74.9 (40.4) b	74.1 (47.6) b
Total N (g kg <sup>-1</sup> )	7.3 (3.2) a	8.3 (3.1) a	4.0 (2.0) b	3.7 (2.0) b
PMN (μg NH <sub>4</sub> -N g <sup>-1</sup> )	69.1 (54.7) a	90.7(56.0) a	42.5 (62.1) ab	15.2 (10.0) b
Total P (g kg <sup>-1</sup> )	1.1 (0.6)	1.3 (0.6)	1.0 (0.6)	1.1 (0.8)
Total N:P	8.4 (7.0) a	8.2 (6.1) a	5.9 (4.9) ab	5.2 (3.7) b
Total Fe (g kg <sup>-1</sup> )	9.2 (3.0)	9.5 (2.4)	10.6 (4.5)	10.9 (2.9)
Total Fe:P	12.6 (14.6)	9.5 (5.4)	21.4 (33.1)	23.4 (28.8)
Available Fe (μg g <sup>-1</sup> )	127.4 (114.9) a	93.9 (67.8) a	36.9 (53.6) b	31.1 (29.3) b
Available Fe:P	6.3 (8.0)	6.3 (7.4)	4.7 (6.8)	7.7 (7.1)
pH (H <sub>2</sub> O)	5.3 (0.5)	5.4 (0.5)	5.5 (0.5)	5.4 (0.3)
pH (KCl)	4.1 (0.4)	4.1 (0.3)	4.3 (0.3)	4.2 (0.2)
Total C:P	247.2 (190.8) a	170.5 (85.8) a	102.4 (74.8) b	88.5 (52.6) b

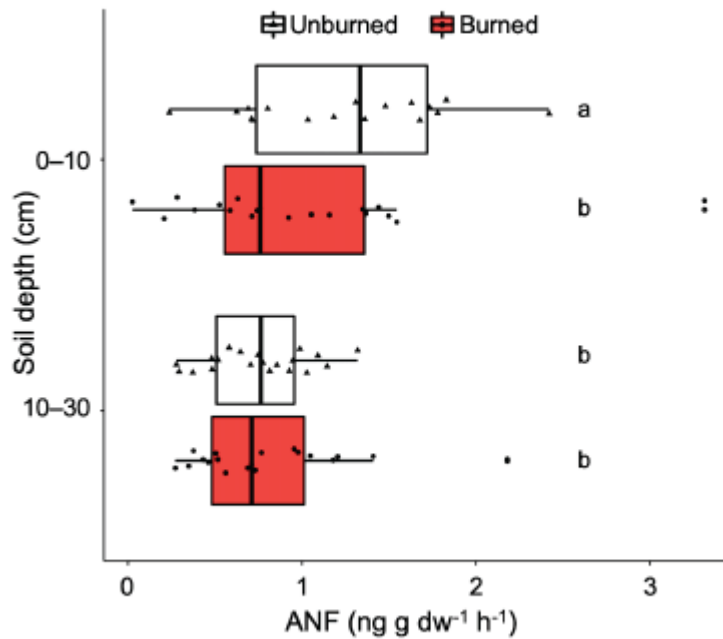
*Note.* Means across each row, followed by different letters, differ at the 95% confidence level (least-squares means adjusted for Tukey's test). Means without letters across each row do not statistically differ. Supplementary Table S5 includes the full list of soil parameters

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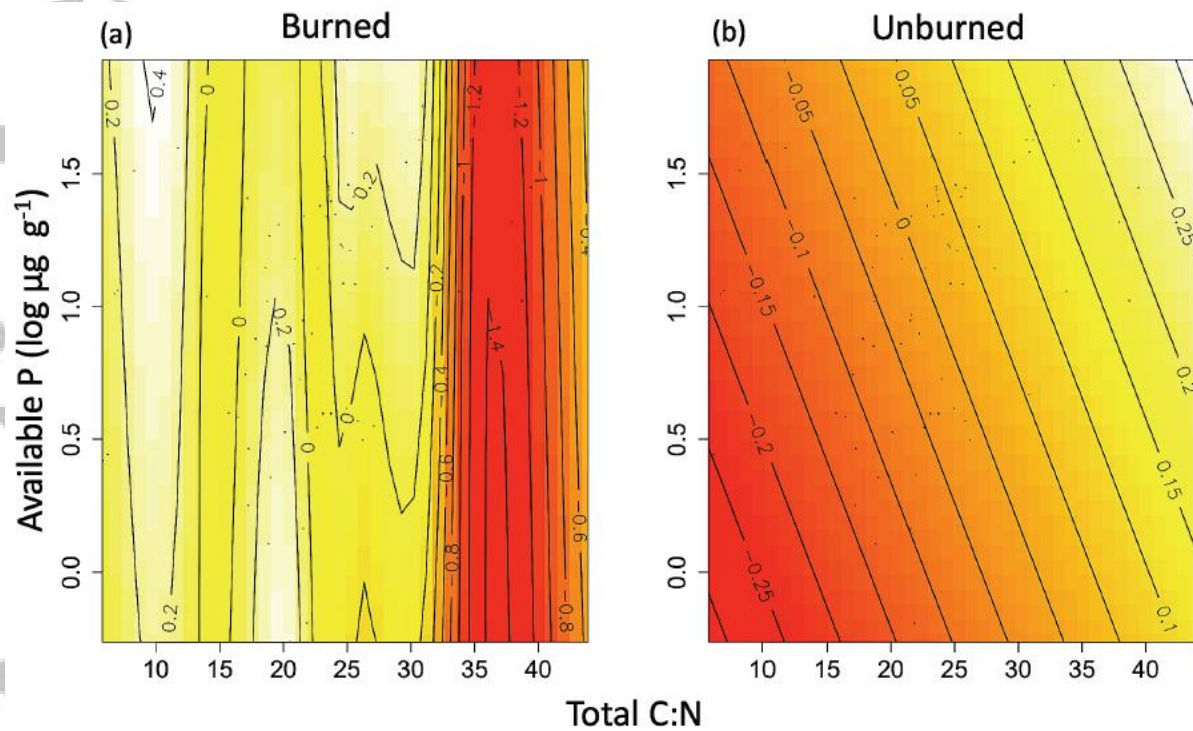


**Figure 1.** Schematic map showing the location of Araguaia State Park, Mato Grosso State, Brazil, and our sampling design based on (a) the spatial interpolation of fire frequency (i.e., the number of wildfire events between 2000 and 2013) increasing from light-yellow to red. F1, F2, F2b, F4 and F5 are the sampled stands, where the labels match the number of fire events over the 13 years (e.g., F1 burned once, F2 and F2b twice, F4 four times and F5 five times between 2000 and 2013; Neves et al., 2018). The gray squares in (b) represent the burned subplots (20 m × 10 m) within the permanent 1-ha plot, and the white squares represent the unburned subplots. This fine-scale spatial heterogeneity of fire was detected *in situ* during sample collection and not by MOD45 product. We systematically established one cluster (pink circle) in a burned and another (blue triangle) in an unburned area within the 1-ha plot to account for fire presence. (c) Each burned and unburned cluster consisted of four sampling points (each point is 10 m apart from the center point), where soil samples were collected at two depths (0–10 and 10–30 cm).





**Figure 2.** Asymbiotic nitrogen fixation rates (ANF, ng N g dry weight<sup>-1</sup> h<sup>-1</sup>) measured in soil samples (0–10 and 10–30 cm) collected in burned (B; red) and unburned (UB; white) areas within five seasonally flooded forest stands studied at Araguaia State Park, Mato Grosso, Brazil. In each box, the middle band is the median ANF rate, and the top and bottom of the box are the first and third quartiles, respectively. Whiskers indicate the maximum and minimum values. Different letters (right of each box) indicate significant differences at the 95% significance level (least-squares means adjusted for Tukey’s test).



**Figure 3.** Fitted generalized additive model (GAM) predictions of asymbiotic nitrogen fixation (ANF, in  $\log \text{ ng N g dry weight}^{-1} \text{ h}^{-1}$ ), including both soil depths (0–10 and 10–30 cm) and all sampled forest stands, as a function of total soil C:N (x-axes) and available P, in  $\log \mu\text{g P g}^{-1}$  (y-axes) in each fire presence level: (a) Burned and (b) Unburned. Each panel is a heatmap with overlaid contours of the 2-dimensional smooth functions [ $s(\text{C:N}, \log \text{M3-P}, \text{by}=\text{Fire presence})$ ] from the best GAM, which fitted soil ANF as a function of total soil C:N, available P, and the categorical variable fire presence. Dark contour lines represent ANF rates, where points along the same contour line have the same value; numbers shown on contour lines are the response variable ANF ( $\log \text{ ng N g dry weight}^{-1} \text{ h}^{-1}$ ); the red color indicates where ANF is lowest and light-yellow indicates where ANF is highest.