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Abstract

- Biological nitrogen fixation (BNF) is a fundamental part of nitrogen cycling in tropical forests, yet little is known about the contribution made by free-living nitrogen fixers inhabiting the often-extensive forest canopy.
- We used the acetylene reduction assay, calibrated with $^{15}N_2$, to measure free-living BNF on forest canopy leaves, vascular epiphytes, bryophytes and canopy soil, as well as on the forest floor in leaf litter and soil. We used a combination of calculated and published component densities to upscale free-living BNF rates to the forest level.
- We found that bryophytes and leaves situated in the canopy in particular displayed high mass-based rates of free-living BNF. Additionally, we calculated that nearly 2 kg of nitrogen enters

the forest ecosystem through free-living BNF every year, 40% of which was fixed by the various canopy components.

- Our results reveal that in the studied tropical lowland forest a large part of the nitrogen input through free-living BNF stems from the canopy, but also that the total nitrogen inputs by free-living BNF are lower than previously thought and comparable to the inputs of reactive nitrogen by atmospheric deposition.

Keywords

canopy soil, epiphytes, leaves, litter, bryophytes, $^{15}N_2$, terrestrial LIDAR

Introduction

Except for areas with high atmospheric nitrogen (N) deposition, biological N fixation (BNF) is the most important pathway for introducing 'new' N into unfertilized terrestrial ecosystems (Cleveland *et al.*, 1999; Vitousek *et al.*, 2013). Inert dinitrogen (N_2) gas is reduced to ammonia during fixation by symbiotic or free-living N fixers (also called diazotrophs). Symbiotic diazotrophs, generally found in root nodules, exchange fixed N for carbon with their host plants, whereas hetero- or autotrophic bacteria or archaea freely inhabit and fix N in substrates such as water, soil, rocks, leaves, leaf litter and bryophytes (Dynarski & Houlton, 2018). The contribution of both these life strategies to the N cycle in tropical forests is thought to be substantial, estimated to range between 5.5 – 16 kg N ha^{-1} y^{-1} for symbiotic BNF, and between 0.1 – 60 kg N ha^{-1} y^{-1} for free-living BNF (Reed *et al.*, 2011). Although many tropical forests have a high abundance of leguminous trees (Losos & Leigh, 2004), typically associated with symbiotic N fixers present in root nodules, the contribution of symbiotic BNF to the total BNF in tropical forests has been questioned because mature tropical forests are generally considered N rich compared to other nutrients, removing the need for trees to obtain N through symbiotic interactions with diazotrophs (Hedin *et al.*, 2009). Based on mass-balance approaches and modelling Cleveland *et al.* (2010) showed that, after accounting for free-living N fixation and atmospheric N deposition, only modest inputs of N via symbiotic fixation were necessary to balance the N budget of a mature tropical forest in Amazonia. There is also increasing evidence that root nodulation and symbiotic N fixation is facultative and may decline to near zero in mature tropical forests (Menge *et al.*, 2009; Barron *et al.*, 2011; Batterman *et al.*, 2013; Sullivan *et al.*, 2014; Bauters *et al.*, 2016), and therefore attention has been shifting towards the role of free-living BNF in the N cycle of tropical forests (Reed *et al.*, 2011).

The availability of N, phosphorus (P) and even molybdenum - a necessary co-factor of many nitrogenases (Barron *et al.*, 2009) -, in addition to humidity, have been shown to play an important role in determining free-living BNF rates (Reed *et al.*, 2011; Wurzburger *et al.*, 2012; Camenzind *et al.*, 2018; Dynarski & Houlton, 2018; Van Langenhove *et al.*, 2019). High rates of free-living BNF seem paradoxical (Hedin *et al.*, 2009) in the face of the generally assumed N-rich and P-poor nature of mature tropical forests (Turner & Condron, 2013). However, because free-living BNF generally occurs in substrates decoupled from N conditions in deeper soils, such as the litter layer which is rich in C relative to N compared to decomposers (Menge *et al.*, 2009), N inputs through free-living BNF can still be substantial in mature tropical forests (Hedin *et al.*, 2009; Dynarski & Houlton, 2018). High rates of free-living BNF in tropical forest floor soil and litter have been reported in both Central (Reed *et al.*, 2007; Barron *et al.*, 2009; Černá *et al.*, 2009; Cusack *et al.*, 2009; Wurzburger *et al.*, 2012) and South

America (Matson *et al.*, 2014), although markedly lower rates of free-living BNF were encountered in tropical forests in Mato Grosso State, Brazil (Wong, 2019), and in French Guiana (Van Langenhove *et al.*, 2019), possibly related to the extremely low P availabilities there.

Beyond the forest floor, tropical rainforests possess extensive canopies, generally exceeding 30 m and regularly 45 m in height (Tao *et al.*, 2016), representing a complex matrix of tree leaves and branches colonized by a diverse suite of animals and plants, such as bryophytes (including mosses, liverworts and hornworts), algae, lichens, fungi and vascular epiphytes (Nadkarni, 1994; Sillett & Antoine, 2004; Enloe *et al.*, 2006; Nakamura *et al.*, 2017). An additional component is canopy soil: accumulations of organic matter consisting of decomposing epiphytes, leaf litter, invertebrates, fungi and microorganisms found on branches and in tree junctions (Hietz *et al.*, 2002; Nadkarni *et al.*, 2002). Canopy soils display many similarities to tropical forest floor litter and soil (Vance & Nadkarni, 1990; Nadkarni *et al.*, 2002; Cardelús *et al.*, 2009). Microbial communities associated with these different canopy components do not have access to the soil N and may therefore fix N_2 to meet their N requirements. Indeed, BNF by free-living diazotrophs has been found to occur on tropical leaf surfaces (Fürnkranz *et al.*, 2008; Reed *et al.*, 2013), on tropical bryophytes (Cusack *et al.*, 2009) and in tropical canopy soils (Matson *et al.*, 2014). One study even found that free-living BNF measured in canopy soils was higher than free-living BNF on the forest floor when comparisons were mass-based (Matson *et al.*, 2014). Studies describing the role of vascular epiphytes in harbouring free-living BNF have reported variable results (Sengupta *et al.*, 1981; Dighe *et al.*, 1986; Bermudes & Benzing, 1991; Brighigna *et al.*, 1992). Yet, to date, no study has aimed to quantify free-living BNF within the different canopy components simultaneously, nor attempted to estimate ecosystem-wide BNF including the canopy components.

Therefore, the aims of the present study were (1) to quantify free-living BNF rates of different canopy components (i.e. leaves, bryophytes, vascular epiphytes, canopy soil) and of forest floor (i.e. soil and leaf litter), and (2) to upscale these rates to the forest level to evaluate the relative importance of each component to the total amount of N fixed in an old-growth tropical lowland forest in French Guiana. By nature of the measurement technique, rates of free-living BNF are typically expressed on a per mass of substrate or a per area of substrate basis. However, to obtain ecosystem-wide (per hectare of forest) estimates of free-living BNF for the various canopy and soil components it is necessary to apply an appropriate scalar (Vitousek *et al.*, 2013; Sullivan *et al.*, 2014). In some instances this scalar is easily identified, as with forest floor soil for example. There, a measurement of BNF expressed on a per mass basis multiplied with the soil bulk density, corrected for the depth to which soil samples were taken, will yield an amount of N fixed per area of forest over a certain time period (see e.g. Matson *et al.,*

2014). For other components, however, finding the appropriate scalar is not so straightforward and we here applied a combination of measured scalars for forest floor soil, leaf litter, canopy leaves and bryophytes while for canopy soils and epiphytes we applied scalars derived from a literature survey.

We hypothesized that because the canopy complexity creates niches for many free-living diazotrophs, free-living BNF occurring in the canopy contributes substantially to the total amount of N_2 fixed and could make an important contribution to the N input at the ecosystem scale. We expect that because vascular epiphytes, bryophytes and canopy leaves make up a large part of the forest canopy they will each contribute more to the overall amount of N fixed through free-living BNF than canopy soils, which are much less prevalent.

Methods

Study area

The study was conducted at the Nouragues Nature Reserve, a primary rainforest site in French Guiana, situated 100 km inland from the Atlantic coast and south of the capital city Cayenne (4°2' N, 52°40' W). The site is located between 25 and 40 m above sea level, mean annual air temperature is 26 °C, and mean annual rainfall is 3000 mm (Bongers *et al.*, 2001). The 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 with a short dry period in March and a long dry period, typically characterized by less than 100 mm rainfall $month^{-1}$, from August to November (Aguilos *et al.*, 2019). Soils at the Nouragues site are derived from the Bonidoro series and parent material consists mainly of Caraib gneiss (Bongers *et al.*, 2001). Soils are classified as nutrient poor Acrisols (FAO, 1998). Samples were collected within a fully inventoried 1.5 hectare plot at the Pararé Research Station, where canopy height ranges between 35 to 55 m (Ho Tong Minh *et al.*, 2016).

Sample Collection

Samples were collected in September 2017 from fourteen adult trees. These particular trees were chosen because they possessed extensive, multilayer canopies with several vascular epiphytes visible from the forest floor. Additionally, because trees were climbed using single rope access techniques, another important requirement was safety of access, which required at least one open section in the canopy to allow the positioning of the access line, an absence of large dead branches and no obvious signs of wasp, bee, and/or termite nests near the trunk. The fourteen sampled trees belonged to nine different species and their diameter at breast height (DBH) ranged between 47 and 97 cm (Table 1). From these trees, samples of canopy leaves, vascular epiphytes, trunk and canopy bryophytes and canopy soils were collected by hand, for safety reasons often from within the interior crown. To

account for variation in sunlight exposure per tree, a branch from the upper canopy (sunlit) and another from the lower canopy (shaded) was removed with a handsaw and three leaves from each branch were collected. To avoid confounding BNF rates from leaves with the potential BNF rates of algae, lichens or fungi colonizing leaf surfaces we only selected leaves that bore no visual signs of abundant colonisation. Per host tree we aimed to collect a maximum of three individual vascular epiphytes that each had at least 15 g of canopy soil attached, totalling 30 vascular epiphyte individuals of 17 different species (Table S1). From each vascular epiphyte three 4 cm^2 pieces were cut from three leaves and 15 g of canopy soil was collected from its base. Although several trees had deposits of canopy soil in bifurcations or on top of larger branches, only seven of the fourteen trees had deposits of sufficient size (>15 g). Preliminary analysis showed no difference in free-living BNF rate between the canopy soil that was attached to vascular epiphytes and canopy soil derived from branch deposits, these components were therefore pooled and further analysed as one single canopy component. From each of the sampled trees, bryophytes were collected by scraping off three pieces (> 4 cm^2) from the tree trunk (trunk bryophytes), between 2 and 5 m above the ground. Canopy bryophytes were collected by scraping three pieces (> 4 cm^2) from three different branches within the canopy. For the purposes of this study we did not differentiate between the three divisions of non-vascular plants contained in the bryophyte group (the mosses, liverworts and hornworts). There were very few bryophytes present on the forest floor and these were therefore ignored during sampling. Lastly, five samples of forest floor leaf litter and soil were collected between one and two meters away from the tree trunk in a radial pattern. This was done for only five out of the fourteen trees because earlier sampling at the same site showed that more measurements of soil and litter free-living BNF were superfluous to obtain a robust average BNF rate for these components in this homogenous forest plot. Approximately 5 g of leaf litter were collected by hand from the soil surface and soil samples (around 15 g) were collected with a 2-cm diameter corer to a depth of 5 cm after removing all litter from the surface. All samples were transported in plastic bags to the field station where sample processing was initiated within 2 h after collection.

Acetylene Reduction Assay

BNF rates were determined using the acetylene reduction assay (ARA) as a proxy for BNF, wherein the production of ethylene after acetylene addition to a sample is measured (Hardy *et al.*, 1968). All collected samples 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, Kourou, French Guiana) to create a 10% headspace concentration by volume. The samples were then incubated *in situ* under ambient forest light (no direct sunlight) and temperature for 18 hours.

After incubation, a 12 ml gas subsample from the sample headspace was injected into a pre-evacuated 12 ml borosilicate vial (Labco Limited, Ceredigion, UK) and shipped to Ghent University (Belgium) for analysis. Ethylene concentrations were measured using laser-based photo-acoustic spectroscopy (ETD-300, Sensor Sense, Nijmegen, The Netherlands). Parallel acetylene blanks (no sample) and sample blanks (no acetylene gas) were created in the field to assess background levels of ethylene in the acetylene gas (1.5 ± 0.4 nl ethylene ml^{-1} acetylene gas) and background ethylene production in the samples (0.2 ± 0.1 nl ethylene ml-1). These rates were subtracted from the sample ethylene concentrations.

We reported the rates of ethylene production expressed as nmol ethylene produced per gram of substrate per hour (nmol g^{-1} h^{-1}) and, specifically for canopy leaves, vascular epiphyte leaves, bryophytes and leaf litter only, as nmol ethylene produced per area of substrate per hour (nmol m^{-2} h^{-1}), which was necessary for up scaling (see below). We used an LI-3100C Area Meter (LI-COR, Lincoln, Nebraska USA) to measure the area of each canopy and epiphyte leaf sample. Since measuring with the LI-3100C would require flattening the bryophyte samples, which would erroneously increase the area, we instead photographed each bryophyte sample on a standardized white background with a known scale and used ImageJ software (Schneider *et al.*, 2012) to determine the actual 2D projected area, which was important for enabling the up scaling of the measured rates to the forest level (see further). Mass-based leaf litter ethylene production was converted into area-based ethylene production using the measurements for litter density reported by Van Langenhove et al. (2019) for the same study site (601 ± 44 g leaf litter m^{-2}).

Upscaling of free-living BNF to forest stand level

In order to upscale free-living BNF rates for each sampled component to the forest canopy and entire forest level beyond, three additional steps were required. First, measured ethylene production rates were converted into BNF rates. Second, different canopy component densities were either calculated from *in situ* observations or obtained from the literature (Table 2) and, three, these rates and canopy component densities were combined to scale up to forest-wide rates of free-living BNF.

We calibrated ARA to BNF using $^{15}N_2$ gas, conducting paired simultaneous assays with acetylene and $^{15}N_2$ on all components in the field station. 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 and allowed to incubate for 24 h. Incubations were terminated by evacuating each jar and freezing the samples at -20 °C. Once returned to the lab, samples were dried at 60 °C, ground and analysed for $^{15}N/^{14}N$ and percentage of N at the University of Vienna, Austria by an elemental analyser (EA 1110; CE Instruments, Milan, Italy) coupled to a Finnigan MAT Delta Plus IRMS (Thermo Fisher Scientific, Waltham, USA). The conversion factor of ARA to BNF was calculated as nmol of ethylene produced per hour per gram dry mass (or area) divided

by nmol N incorporated per hour per gram dry mass (or area). We found meaningful conversion factors for canopy leaves, bryophytes and leaf litter (Table 2). It was, however, not possible to establish conversion factors for canopy soil, epiphyte leaves and forest floor soil due to a combination of low BNF rates and high background N concentrations. For these components we decided to use the theoretical conversion factor of 3 mol ethylene produced per mole of N fixed (Hardy et al., 1968). For the calculations we assumed that the standard error on the theoretical conversion factor was equal to the mean standard error identified for the conversion factors of canopy leaves, bryophytes and leaf litter.

Upscaling the canopy leaf area-based free-living BNF rate into a forest wide canopy BNF rate was done by multiplying the leaf area-based BNF rate with a leaf area index (LAI) of 6.5 ± 0.5. This LAI value was based on previous studies conducted in the study area (Cournac *et al.*, 2002; Emmons *et al.*, 2006), which is slightly higher than the 4.2 ± 2 overall tropical forest evergreen broadleaf average (Asner *et al.*, 2003), but within the higher range (between 2.7 and 6.8) of LAI values reported for protected tropical forests in the Americas (Pfeifer *et al.*, 2018). We used the average area-based BNF rates of both sunlit and shaded leaves together because there was no difference in ethylene production rates between these two types of leaves (Fig. 1).

Estimates of vascular plant epiphytic densities or canopy soil loads in the literature are scarce, and almost exclusively represent Central American montane tropical forests (Table S2), which have been shown to have significantly higher loads than lowland tropical forests (Freiberg & Freiberg, 2000). Based on the information contained in table S2 we estimated canopy soil loads at 1 ± 0.5 Mg ha^{-1} and vascular epiphytic loads at 1.5 ± 0.75 Mg ha^{-1}. We refer to the supplementary information for a more detailed explanation for this assumption.

Given the limited information in the literature regarding bryophyte loads in lowland tropical forest canopies (Table S2), a calculation of both canopy and trunk bryophyte density in our plot was made. First, we visually estimated woody surface area covered by bryophytes by assigning each sampled tree to a class (0 – 1%, 1 – 25%, 25 – 50%, 50 – 75% and 75 – 100% of trunk or branch surface covered by bryophytes). Because our 14 trees belonged to the 15% largest trees of the plot, we also estimated the woody surface area covered by bryophytes on tree trunks of 35 smaller trees (DBH between 12 and 27 cm), to test whether our 14 chosen trees were representative for smaller trees (Table S3). Average bryophyte coverage on the smaller trees was similar to bryophyte coverage on the larger trees, indicating that bryophyte coverage was not related to tree size (Li *et al.*, 2015). Second, we quantified the surface area of the woody surfaces (tree trunk and branch surface area separately) of a select number of trees and extrapolated this to the entire plot. For this, we used terrestrial laser scanning

(TLS), an active remote sensing technique able to measure various structural parameters with high spatial accuracy. We collected TLS data from eight of the fourteen climbed trees in a radial pattern around each tree using a RIEGL VZ1000 terrestrial laser scanner (RIEGL, Horn, Austria) mounted on a tripod approximately 1.3 m from the ground. We refer to the supplementary information for more details on the extrapolation and the TLS protocol. We then multiplied the average relative bryophyte coverage for both trunk and branch surface with the estimated trunk and branch surface area to obtain an estimate of bryophyte density in the plot (in m^2 ha^{-1}).

Lastly, for upscaling mass-based BNF rates in leaf litter and forest floor soil we multiplied them with litter and soil densities as reported by Van Langenhove et al. (2019). Table 2 gives an overview of the densities we used for each substrate.

Data analysis

Across the fourteen sampled trees we conducted a total of 455 ARA measurements (including the 45 blanks) to calculate ethylene production rates, comprising forest canopy leaves, vascular epiphytes, bryophytes and canopy soil along with forest floor leaf litter and soil.

We used linear mixed effects regression models (LMER) to assess the differences in ethylene production rates between canopy components located in different abiotic conditions: sunlit canopy leaves versus shaded canopy leaves, and canopy bryophytes versus trunk bryophytes. In each of the models we used sample type as fixed factor and tree number as random factor. The validity of the linear models' assumptions (linearity, normality of residuals, no influential outliers, homoscedasticity) were evaluated with standard functions of R (R core team 2018, version 3.5.1), including diagnostic plots. Prior to analysis ethylene production rates were log transformed if their distribution was right-skewed to improve normality of model residuals.

We used LMERs to assess differences in ethylene production rate between the different soil (forest floor soil and canopy soil) and vegetation (canopy leaves, vascular epiphyte leaves, canopy bryophytes, trunk bryophytes and leaf litter) components. Sample type was used as a fixed factor and tree number as random factor. The validity of the linear models' assumptions was checked with standard functions of R. Variance of homogeneity was checked using the Bartlett test. Multiple comparisons within a factor were analysed using Tukey post hoc tests. We used the same approach to investigate differences in ethylene production between the various species of vascular epiphyte, wherein epiphyte species was used as a fixed factor and, because we measured ethylene production on three pieces of epiphyte leaf from each individual epiphyte, epiphyte identity as random factor. As we sampled no multiple

epiphyte individuals belonging to the same species from a single tree it was unnecessary to include host tree as random effect. Multiple comparisons within a factor were analysed using Tukey post hoc tests.

The errors on the upscaled free-living BNF rates were calculated by propagating the standard errors of the acetylene production rates of each component with the standard errors on the measured conversion factors (canopy leaves, bryophytes and leaf litter) or theoretical conversion factors (canopy soil, epiphyte leaves, forest floor soil), and with the standard errors of the component densities (Table 2).

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.

Results

Free-living BNF rates were highly variable, both within and among ecosystem components (Table 3). Across all ecosystem components, the lowest average ethylene production rate, as proxy for BNF, was 0.022 ± 0.009 (SE) nmol g^{-1} h^{-1}, observed in soil, and the highest was 1.26 ± 0.41 nmol g^{-1} h^{-1}, observed in canopy bryophytes (Table 3).

While there was no significant difference in either mass or area-based ethylene production rates between shaded and sunlit leaves, canopy bryophyte ethylene production rates were almost four times higher than trunk bryophyte ethylene production rates, both on a mass basis ($P < 0.001$, $F_{1,48} = 16.0$) and an area basis ($P < 0.001$, $F_{1,48} = 12.9$) (Fig. 1). For the remainder of the analyses, shaded and sunlit canopy leaves are grouped together and treated as one canopy component, i.e. canopy leaves, whereas canopy and trunk bryophyte are treated separately.

The mass-based ethylene production rates of canopy soil were about eight times higher than the production rate of forest floor soil ($P < 0.001$, $F_{1,147} = 21.6$) (Table 3 and Fig. 2a). The variation in canopy soil ethylene production was much larger than in forest floor soil (Table 3) and after accounting for sample size resulted in the much larger SE (Fig. 2a).

Among vegetation components canopy bryophytes had the highest overall mass- and area-based ethylene production rates (Table 3, Fig. 2). Canopy bryophyte mass-based ethylene production rates were significantly higher than those of the canopy leaves ($P < 0.001$, $F_{1,88.6} = 96.7$), but they were not

different from the rates found in the leaf litter (Fig. 2a). Leaf litter mass-based rates, in turn, were also not different from either canopy leaves or trunk bryophyte rates, although rates were higher on average (Table 3, Fig. 2a). Vascular epiphytic leaf ethylene production rates were significantly (P < 0.001 for all) lower than all other vegetation canopy components.

In contrast, area-based rates of leaf litter ethylene production were roughly three times and eight times higher than both trunk bryophytes (P < 0.05, $F_{1,45}.8 = 4.0$) and canopy leaves (P < 0.001, $F_{1,89.4}$ = 34.3), respectively (Fig. 2b). Trunk bryophyte rates were also twice as high as canopy leaf rates (P < 0.001, $F_{1,92.8}$ = 14.8) (Fig. 2b). These differences in area-based ethylene production rates compared to the mass-based ethylene production rates were caused by differences in area density between the various components. Just as with the mass-based rates, the area-based ethylene production rates of vascular epiphytic leaves were lower than all other vegetation canopy components (P < 0.001 for all) (Fig. 2b).

We found a significant effect of vascular epiphyte species on foliar area-based ethylene production rates (P < 0.01, $F_{16,13}$ = 4.3), which ranged from 0 nmol m^{-2} h^{-1} (*Anthurium sp.2*, *Asplenium sp.2* or *Clusia sp.1*) to 100 nmol m^{-2} h^{-1} and above for *Araceae sp.1* and *Philodendron sp.2*. For six out of the 17 identified species at least two individuals were both accessible and large enough to be sampled (Fig. 3). Considering only the six replicated vascular epiphyte species, we again identified a significant effect of species identity on ethylene production rate (P < 0.05, $F_{5,13}$ = 4.7), and only one of these six species (Pteridophyta sp. 1) showed significant intra species variation (P < 0.01, $F_{2,1}$ = 17.8).

Scaling to the ecosystem

Ethylene production rates were converted into BNF rates and subsequently scaled up to the ecosystem level using estimates and measurements of ecosystem-wide mass or area densities of the respective forest floor and canopy components (Table 2). The upper five cm of soil showed a free-living BNF rate of 810 ± 350 g N ha^{-1} y^{-1}, whereas leaf litter on the forest floor contributed much less to the total BNF, namely 250 ± 90 g N fixed ha^{-1} y^{-1} (Fig. 4). Here and following, all mentioned errors are standard errors.

In the canopy, canopy soil free-living BNF contributed only 15 ± 13 g N ha^{-1} y^{-1} based on an assumed canopy soil density of 1 ± 0.5 Mg ha^{-1} (Table S2). We also estimated the vascular epiphyte density at our site based on previously published values (Table S2) and assumed a density of 1.5 ± 0.75 Mg ha^{-1}. This led to a free-living BNF rate of 15 ± 10 g N fixed ha^{-1} y^{-1}.

Based on the 2015 tree census of the sampling plot (Chave et al., pers. comm.) and data on forest structure provided by terrestrial laser scanning inventory, we calculated an average tree trunk surface area of 8740 ± 210 m^2 ha^{-1} and a branch surface area of 13100 ± 1250 m^2 ha^{-1}. Together with an average trunk bryophyte coverage of 37.5% and an average canopy bryophyte coverage of 62.5%, this led to

an estimated 390 ± 180 g N fixed ha^{-1} y^{-1} for canopy bryophytes and 42 ± 19 g N fixed ha^{-1} y^{-1} for trunk bryophytes (Fig. 4).

Lastly, canopy leaf BNF rates were lower than those of the canopy bryophytes, but higher than those of the trunk bryophytes , amounting to 250 ± 70 g N fixed ha^{-1} y^{-1}, using an LAI of 6.5 ± 0.5 as scalar (Table 1).

When summed across all measured components, free-living BNF amounted to 1.76 ± 0.47 kg N fixed ha^{-1} y^{-1} at this tropical forest site. The soil components (soil and leaf litter) contributed 60%, or 1050 ± 420 g N ha^{-1} y^{-1}, to the total free-living BNF, while the canopy components (canopy leaves, trunk and canopy bryophytes , canopy soil and vascular epiphytes) contributed 40%, or 710 ± 200 g N ha^{-1} y^{-1}.

Discussion

Our results demonstrated that in this mature lowland tropical forest ethylene production following acetylene addition, as a proxy for BNF, was an active process in canopy soil, on tree- and vascular epiphytic leaves, in bryophytes, in forest floor leaf litter and in topsoil. We found that 40% of the total ecosystem free-living BNF was carried out aboveground on tree trunks and within the canopy (Fig. 4), implying that aboveground free-living BNF constitutes a non-negligible contribution to this tropical forest's N cycle. In our study, canopy BNF amounted to 710 ± 200 g N ha^{-1} y^{-1}, which is much lower than proposed by early work estimating the canopy contribution at >60 kg N ha^{-1} y^{-1} (Edmisten, 1970). More recently, studies have estimated canopy free-living BNF inputs in tropical forests between 0.02 and 8 kg N ha^{-1} y^{-1} (Forman, 1975; Carpenter, 1992; Freiberg, 1998; Matzek & Vitousek, 2003; Benner *et al.*, 2007; Fürnkranz *et al.*, 2008; Reed *et al.*, 2008; Cusack *et al.*, 2009; Matson *et al.*, 2014), but still the rates at our study site fall towards the lower end of this range. Total ecosystem free-living BNF (canopy plus forest floor BNF; 1.8 kg N ha^{-1} y^{-1}) also falls in the lower end of the 0.1 – 60 kg N ha^{-1} y^{-1} range reported for free-living BNF in tropical forests, being more similar to rates reported for boreal and temperate forests (Reed *et al.*, 2011).

A recent study estimated inorganic N deposition in French Guiana to range between 1 and 2 kg N ha^{-1} y^{-1} (Wang *et al.*, 2017), a value that is very similar to the yearly deposition of reactive N measured at a coastal lowland tropical forest site in French Guiana (Van Langenhove et al., 2020). To our knowledge, in old growth French Guianese forests symbiotic BNF has not yet been quantified, but Roggy and Prevost (1999) found that 67% of species belonging to potentially nodulating taxa were nodulated in a primary forest. Assuming that symbiotic BNF in this primary lowland tropical forest is similar to the 2 to 4 kg N fixed ha^{-1} y^{-1} found in old growth forests in Eastern Brazil (Winbourne *et al.*, 2018), Costa Rica (Taylor *et al.*, 2019) or Panama (Batterman *et al.*, 2013), this would mean that between 25 and 33% of

the input of 'new' N stems from free-living BNF on the forest floor and in the canopy, highlighting the importance of free-living BNF for the ecosystem's N cycle. Taken together, this shows that in this forest N is introduced into the ecosystem by free-living BNF at a rate that equals the rate of inorganic N deposition, yet this input is lower than previously thought (see e.g. Reed et al., 2011) and likely less important for sustaining the ecosystem N budget than the N that is recycled yearly through litterfall (Chave *et al.*, 2010).

It is important to note that the amount of forest-wide BNF measured in this forest is closely dependent on various characteristics of this particular forest. It is humid (~3000 mm rainfall per year), has tall trees with extensive canopies, leading to favourable conditions for bryophyte growth and an LAI that is higher than average (Pfeifer *et al.*, 2018). Contributions of canopy components to total forest free-living BNF may be different in other forests, such as montane tropical forests that typically have much higher loads of canopy soil and epiphytes (Freiberg & Freiberg, 2000), but are lower in stature leading to lower LAIs (Moser *et al.*, 2007). Canopy free-living BNF in dry lowland tropical forests is likely much lower as humidity plays an important role in determining free-living BNF (Dynarski & Houlton, 2018; Rousk *et al.*, 2018). Finally, lowland tropical forests situated in regions that are subject to higher amounts of (anthropogenic) P deposition could potentially possess higher canopy BNF rates as, besides litterfall, deposition is the main source of P for the canopy dwelling diazotrophs (Stanton *et al.*, 2019).

Free living BNF activity of different ecosystem components

Several studies have assessed free-living BNF in forest floor soil and/or litter in tropical forests (e.g. Vitousek and Hobbie, 2000; Reed et al., 2007; Wurzburger et al., 2012; Reed et al., 2013; Barron et al., 2009; Cusack et al., 2009) and overall found higher rates of ethylene production than in our study. In contrast, only one other study measured BNF in canopy soil from the neotropics and reported ethylene production rates of nearly 0.7 nmol g^{-1} h^{-1} (Matson *et al.*, 2014), more than three-fold higher than the rates in our study. This discrepancy is unlikely to be associated with the nutrient contents of the respective canopy soils since these are very similar between both studies (Table S4), indicating that these rates can strongly vary between forests. Matson *et al.* (2014) found similar mass based BNF rates for soil and canopy soil across their altitudinal gradient and reasoned this was unsurprising because these two types of soil were both organic. In contrast, in our study we found that soil nutrients (Table S4) and mass based BNF rates differed strongly between the mineral forest floor soil and the organic canopy soil (Fig 2). Likely, the higher availability of carbon relative to nitrogen in the canopy soil induced higher BNF rates so heterotrophic fixers could offset the unfavourable C:N stoichiometry of their substrate (Hedin et al., 2009; Menge et al., 2009). On the other hand, the higher N:P ratio in the mineral soil might be expected to favour BNF as compared to the canopy soil. However, total P is a

poor indicator of biologically available P. In tropical mineral soils the majority of the P fraction is occluded by clay minerals and metal-oxides, rendering P inaccessible for microbes (Vitousek et al., 2010; Fink et al., 2016). As such, there probably was much less P available in the mineral soil than in the canopy soil, contributing to the large difference in mass-based BNF rates.

Free-living BNF rates on the surfaces of rainforest plants have been studied more often and highlighted as a potential source of N for the plant (Ruinen, 1961; Bentley, 1987; Fritz-Sheridan & Portecop, 1987; Carpenter, 1992; Freiberg, 1998). Compared to canopy leaf fixation rates in Costa Rica (between 0.003 and 0.070 nmol g^{-1} h^{-1}; Reed et al., 2008) our identified rates, averaging at nearly 0.5 nmol g^{-1} h^{-1} (Fig. 2A), were more than six times higher. Because we selected leaves that were free of any visual signs of colonisation by fungi, lichens, algae or other organisms, it is possible that the main agent responsible for the observed high rates of leaf BNF were endophytic diazotrophs (Moyes *et al.*, 2016; Puri *et al.*, 2020). However, the identification of possible endophytic diazotroph associations in tree leaves was beyond the scope of the present study.

In 40% of vascular epiphyte leaf samples there was no ethylene production and in the remainder of samples ethylene production was low (Table 3), resulting in the lowest overall ethylene production rate of all vegetal substrates (0.12 nmol g^{-1} h^{-1}). Nevertheless, average ethylene production was still higher than reported by the only other study carried out on tropical epiphytes (Brighigna *et al.*, 1992). The low rates of BNF suggest that vascular epiphytes derive their N from alternate sources, e.g. mineralization of intercepted organic material, wet or dry atmospheric deposition or even animal interactions (Leroy *et al.*, 2009). We also detected differences in ethylene production rates between different vascular epiphyte species (Fig 3). In particular leaves of the bromeliad *Achmea aquilega* showed high rates (ca. 12 nmol g^{-1} h^{-1}), even when individuals were collected from different trees. This suggests that the microbial community, or at least the taxa responsible for BNF, present on the leaves is related more to the epiphyte than to the host tree, possibly because of various functional traits of the epiphyte (Kembel *et al.*, 2014).

In our study canopy bryophytes showed the highest mass-based rates of ethylene production and BNF activity compared to all other measured components (Fig 2A). Free-living BNF in mosses has been identified as an important source of N in boreal forests (DeLuca *et al.*, 2002; Lagerström *et al.*, 2007), but studies on tropical forest bryophyte BNF are rare. The ethylene production rates identified in our French Guianase forest site are lower than those found in mosses situated on tree trunks in Puerto Rico (Cusack *et al.*, 2009), but similar to rates identified on forest floor moss in Hawaii (Vitousek, 1994; Matzek & Vitousek, 2003). The differences in ethylene production between trunk and canopy

bryophytes (Fig 1 and 2) may have several explanations. Differences in abiotic conditions, such as humidity (Cusack *et al.*, 2009), is one possible explanation. Because we sampled during the second month of the dry season, during which relative air humidity in the canopy is still substantially higher than in the understorey (Stahl *et al.*, 2010; Gehrig-Downie *et al.*, 2011), the canopy bryophytes were significantly wetter than trunk bryophytes (Fig S5), potentially affecting diazotroph activity. Though not studied in tropical systems, in arctic systems moss moisture was previously found to be the most important factor for BNF, followed by temperature (Rousk *et al.*, 2018). Another possibility is that bryophyte (Leppänen *et al.*, 2015) or diazotroph (Warshan *et al.*, 2016) species composition may have at least been partly responsible for the difference in ethylene production rates between trunk and canopy bryophytes. However, we did not characterize the different bryophyte species occurring in the canopy and on the tree trunks, nor did we identify the diazotroph community. Thus, we cannot rule out that differences in species composition caused the differences in ethylene production rates between canopy and trunk bryophytes.

The large discrepancies between mass-based BNF rates and forest-wide rates for any specific ecosystem component are primarily due to the employed scalars. For instance, the mass-based BNF rate of soil was by far the lowest (Fig 2), while at the scale of the ecosystem the contribution of soil was the highest, representing roughly 45% of the N fixed across all measured ecosystem components (Fig 4), all because the soil density was orders of magnitude higher than the densities of the other components (Table 2). While canopy bryophytes and tree leaves had over 70 times lower component densities than soil, their high mass-based BNF rates relative to soils results in upscaling to just over 35% of the forest-wide free-living BNF and over 90% of the canopy-derived free-living BNF. This demonstrates that in spite of their lower relative abundances, these components are pivotal for forest-level N cycles. Furthermore, bryophyte BNF rates in particular are more susceptible to adverse conditions in, e.g., temperature, humidity or atmospheric deposition than soil because these conditions affect both the bryophyte density in the forest and their associated mass-based BNF rates (Zotz & Bader, 2009; Mendieta-Leiva et al., 2020). Soil, however, has an unchanging density on short timescales and very low BNF rates that, if reduced further, would yield fairly minor changes in soil BNF. In the framework of a changing environment the combination of a high density and high BNF rate could cause bryophytes to be disproportionately affected, leading to changes in forest-wide BNF rates.

Uncertainties of determining free living BNF activity

The free-living BNF rates reported here are the result of careful point measurements that were up scaled to the ecosystem level using estimated and measured densities of the relevant scalars, and are accompanied by a number of uncertainties. First, the conversion of the ethylene production rate to

468 BNF rates requires converting the number of moles of ethylene produced into a number of moles of N
469 fixed (Hardy *et al.*, 1968). This conversion factor is empirically determined, and mostly resembles the
470 theoretical conversion factor of 3:1 (Vitousek, 1994; Vitousek & Hobbie, 2000; DeLuca et al., 2002;
471 Leppänen et al., 2013; Rousk et al., 2017). For canopy leaves, bryophytes and leaf litter we were able
472 to empirically determine the conversion factor, which closely resembled the theoretical ratio (Table
473 2), and use this in our up scaling. However, likely due to low mass based BNF rates and relatively high
474 background N concentrations (Menge & Hedin, 2009; Matson et al., 2014; Van Langenhove et al.,
475 2019), it was not possible to determine conversion factors for vascular epiphyte leaves, canopy soil
476 and forest floor soil. Instead, we used the theoretical 3:1 conversion factor, which is commonly used
477 in BNF studies where $^{15}N_2$ incubations were not possible or not carried out at all (Benner et al., 2007;
478 Reed et al., 2007; Cusack et al., 2009; Matson et al., 2014; Brookshire et al., 2019). To our knowledge,
479 no empirical conversion factors have ever been determined for canopy soil or vascular epiphytic
480 leaves. Regardless of the accuracy of the theoretical conversion factor, because of the low mass based
481 ethylene production rates in these two components any deviations from it would lead to only minor
482 changes in their respective BNF rates. However, the accuracy of the forest floor soil conversion factor,
483 which in Swedish soils was shown to range between 0.8 and 3.6 (Nohrstedt, 1983), has a larger impact
484 on the final result, as soils represented the largest fraction of free-living BNF. Depending on the actual
485 conversion factor the BNF rates in soils could range from 0.7 to 3.0 kg N ha^{-1} y^{-1}.

486 A second source of uncertainty were the applied scaling factors. This is especially true for the amount
487 of canopy soil and density of vascular epiphytes in our forest, as they were not quantified, but
488 estimated based on findings from other tropical forests (Table S2). However, the mass-based ethylene
489 production rates of the canopy soil and vascular epiphytes were comparably low, so much so that even
490 if we assumed unrealistically high canopy soil load and vascular epiphytic density (10,000 and 22,000
491 kg ha^{-1}; respectively), fixed N from both sources combined would still amount to only 0.3 kg N ha^{-1} y^{-1}.
492 Because we measured bryophyte coverage and used quantified values of canopy leaf area, leaf litter
493 abundance and soil density specifically for our site, the errors associated with these scalars were much
494 smaller (Table 2).

495 A third source of uncertainty is the very high spatial variability in BNF rates within forest floor or canopy
496 components, which led to the large errors associated with the ethylene production rates (Table 3). The
497 commonly accepted explanation for this high natural variability is that the free-living N fixer
498 community, as well as nutrient availability, can differ profoundly over very small distances (Reed *et al.*,
499 2011; Dynarski & Houlton, 2018). While different microbial species may be responsible for the BNF in
500 different components, the physiology of all free-living N fixers is affected by the properties and

requirements of the nitrogenase enzyme (Gutschick, 1981). BNF is energy- and nutrient-intensive, and it is often suppressed when N availability is high (Hedin *et al.*, 2009; Menge *et al.*, 2009) and stimulated when phosphorus availability increases (Camenzind *et al.*, 2018; Dynarski & Houlton, 2018), thus differences in substrate N:P ratio even on a small spatial scale may be responsible for varying rates.

An important caveat of the scaling up approach employed here is that we assume constant rates of BNF throughout the year. Free-living BNF rates are variable in time even when measured at the exact same location (e.g. Reed *et al.,* 2007; Matson *et al.,* 2014; Van Langenhove *et al.,* 2019), which makes an upscaling of point measurements of BNF to yearly rates challenging (Stanton *et al.*, 2019). In forest floor soil and leaf litter, changing BNF rates are often related to changing biotic and abiotic conditions, mainly driven by changing moisture impacting the microbial community and decomposition rates, which in turn leads to changes in nutrient availability (Reed *et al.*, 2011). We can imagine similar limitations on canopy BNF, as seasonally changing rainfall and associated air humidity (Gehrig-Downie *et al.*, 2011) could impact microbial communities there and seasonal trends in atmospheric deposition (Eklund *et al.*, 1997) could potentially impact nutrient inputs and thus also BNF. It is possible that the yearly rates of BNF discussed here are lower than the actual rates, given that they were measured in the beginning of the dry season and extrapolated to an entire year. Free-living BNF occurring on arctic mosses, for example, was previously shown to be impacted strongly by changes in moisture (Rousk *et al.*, 2018) and this could very well be the case here too. On the other hand has previous research in French Guiana shown that seasonal differences, at least for leaf litter and soil, are rather small (Van Langenhove *et al.*, 2019). Measuring free-living BNF in canopy compartments two or even four times within one year would have likely provided a better overview of yearly rates and the temporal variability of these rates.

Conclusion

Overall, rates of free-living BNF were low in our tropical forest site. Even so, 40% was carried out in the canopy and of all components both canopy bryophytes and canopy leaves showed the highest mass-based BNF rates and contributed most to total canopy BNF. The contribution made by vascular epiphytes and canopy soils was much smaller, at least in this forest. According to these results, future studies attempting to quantify ongoing BNF in lowland forest canopies will likely benefit from focussing their efforts on bryophytes and canopy leaves. However, in montane forests where canopy soil and epiphyte loads are likely larger their contribution to the total amount of N fixed could still be substantial. Thus far, efforts to construct biome wide rates of BNF have included symbiotic and free-living forest floor BNF, but ignored canopy BNF, often for a variety of reasons including a lack of data.

For future tropical budgeting studies it will be worthwhile to also include estimates of canopy BNF as they can represent a substantial part of the total fixed N.

Acknowledgement

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Tables

Table 1: List of trees sampled during the study. The World Flora Online database (2019) was consulted to validate scientific names as well as to confirm author names. The diameter at breast height (DBH) is listed, along with the number of samples gathered from each tree for each of the different components. Bryo. is an abbreviation for bryophytes

Species	Family	DBH (cm)	Forest floor soil	Canopy Soil	Litter	Canopy Leaves	Vascular Epiphyte Leaves	Canopy Bryo.	Trunk Bryo.
Aspidosperma sprucaneaum Benth. Ex Müll.Arg.	Apocynacea	97.1	-	6	-	6	3	-	3
Tetragastris altissima (Aubl.) Swart	Burseraceae	95.5	-	15	-	6	9	3	3
Tetragastris sp.	Burseraceae	47.4	5	-	5	6	6	3	-
Poraqueiba guianensis Aubl.	Icacinaceae	83.4	-	12	-	3	9	3	3
Couratari oblongifolia Ducke & R. Knuth	Lecythidaceae	79.1	-	18	-	-	6	3	3
Eschweilera coriaceae (DC.) S.A.Mori	Lecythidaceae	53.0	5	3	5	6	-	3	3
Eschweilera coriaceae (DC.) S.A.Mori	Lecythidaceae	53.6	-	9	-	9	9	3	3
Eschweilera coriaceae (DC.) S.A.Mori	Lecythidaceae	53.2	-	-	-	6	6	-	-
Eschweilera coriaceae (DC.) S.A.Mori	Lecythidaceae	48.7	-	3	-	6	3	3	3
Eschweilera coriaceae (DC.) S.A.Mori	Lecythidaceae	54.7	5	12	5	3	9	3	3
Lecythis persistens Sagot	Lecythidaceae	47.4	-	18	-	6	9	3	3
Eperua falcata Aubl.	Leguminosae	81.8	5	15	5	6	9	-	3
Eperua falcata Aubl.	Leguminosae	73.5	-	9	-	3	6	3	3
Micropholis sp.	Sapotaceae	69.6	5	15	5	6	6	-	-
Total samples incubated			25	135	25	72	90	30	33

Table 2: List of conversion factors and component densities used to upscale each substrate for which ethylene production was measured. Indicated errors are standard errors. Standard errors of theoretical conversion factors were assumed to be similar to the mean of the relative standard error of the calculated conversion factors (see Methods).

	Component conversion		Component Density		
Component	**Conversion factor**	**Source**	**Measurement**	**Value**	**Reference**
Forest floor soil	3.0 ± 0.8	Theoretical	Soil bulk density to 5 cm depth	44 ± 1 kg m^{-2}	Van Langenhove et al., 2019
Canopy soil	3.0 ± 0.8	Theoretical	Canopy soil load per hectare of forest	1000 ± 500 kg ha^{-1}	Listed in Table S2
Canopy bryophytes	2.4 ± 0.9	Calculated (*n* = 10)	Branch bryophyte surface area per hectare of forest	8190 ± 780 m^2 ha^{-1}	Calculated
Trunk bryophytes	2.4 ± 0.9	Calculated (*n* = 10)	Trunk bryophyte surface area per hectare of forest	3280 ± 284 m^2 ha^{-1}	Calculated
Canopy leaves	3.5 ± 0.4	Calculated (*n* = 10)	Leaf area index	6.5 ± 0.5 m^2 m^{-2}	Cournac et al., 2002; Emmons et al., 2006
Forest floor litter	4.1 ± 0.6	Calculated (*n* = 10)	Litter density on forest floor	601 ± 44 g m^{-2}	Van Langenhove et al., 2019
Vascular epiphytes	3.0 ± 0.8	Theoretical	Vascular epiphyte mass per hectare	1500 ± 750 kg ha^{-1}	Listed in Table S2

Table 3: Overview of the mass-based ethylene production rates identified in each component. The sampling size (n), overall mean, geometric mean, SE, SD, minimum, maximum and median values are listed, along with the percentage of measurements per components wherein no ethylene production was present (% zero). Values are expressed as nmol ethylene produced g^{-1} h^{-1}.

	n	Mean	Geometric mean	SE	SD	Min	Max	Median	% zero
Forest floor soil	25	0.022	0.012	0.009	0.049	0.003	0.253	0.010	0
Canopy soil	133	0.189	0.037	0.135	0.897	0.001	5.986	0.031	2
Canopy bryophytes	31	1.264	0.300	0.407	2.268	0.006	9.731	0.373	0
Trunk bryophytes	29	0.273	0.080	0.093	0.498	0.000	2.559	0.130	4
Canopy leaves	67	0.479	0.420	0.111	0.905	0.000	5.435	0.066	34
Leaf litter	25	0.691	0.242	0.211	1.056	0.029	4.453	0.234	0
Epiphyte leaves	88	0.119	0.222	0.052	0.286	0.000	1.486	0.031	40

Figures

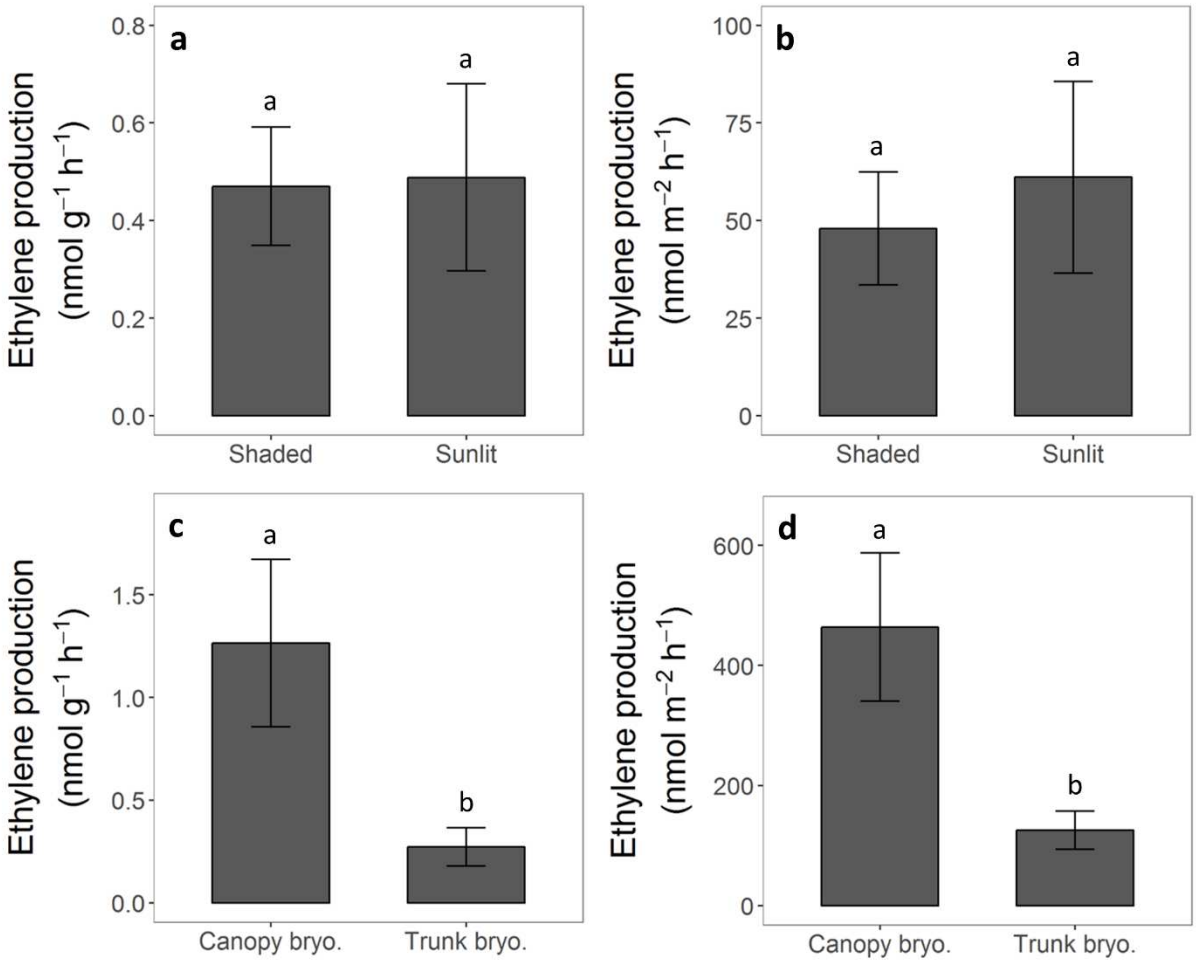


Figure 1: Comparison of mass-based (a, c) and area-based (b, d) rates of ethylene production for shaded versus sunlit leaves (a, b) and canopy bryophytes versus trunk bryophytes (c, d). Error bars represent standard errors. Different lowercase letters represent significant differences ($P < 0.001$) between canopy components. Sample sizes: Shaded leaves n = 35, Sunlit leaves n = 32, Canopy bryophyte N = 31, Trunk bryophyte N = 29 Bryo. is an abbreviation for bryophytes.

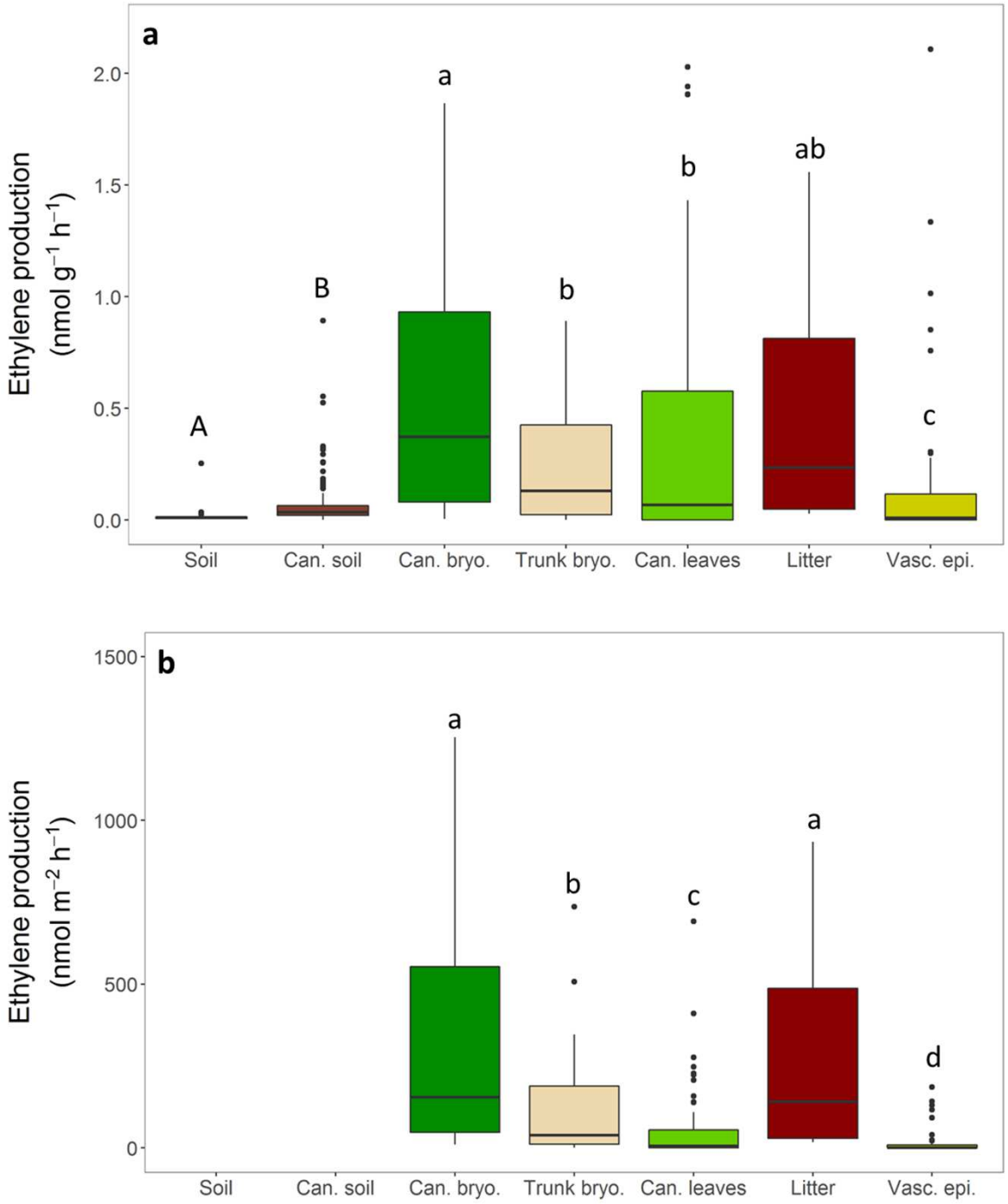
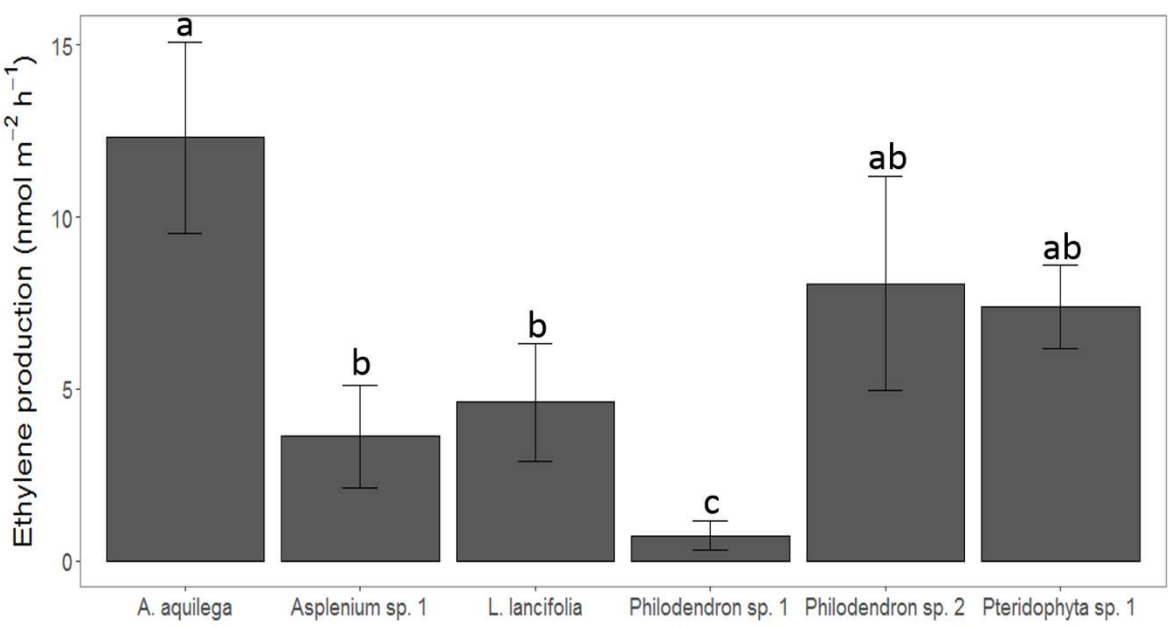


Figure 2: Average mass-based (a) and area-based (b) ethylene production rates for each of the measured components. It was not possible to directly measure area-based rates for forest floor soil and canopy soils. Error bars indicate standard errors. Lowercase letters indicate significant differences at the $P < 0.05$ level for components derived from vegetation; uppercase letters indicate significant differences at the $P < 0.05$ level between soil and canopy soil. Abbreviations: Soil = forest floor soil, Litter = leaf litter, Can. Soil = Canopy soil, Trunk bryo. = bryophytes gathered from the tree trunks, Can. bryo. = bryophytes gathered from the tree canopy, Can. Leaves = canopy leaves, including both sunlit and shaded leaves, and Vasc. Epi. = vascular epiphyte leaves collected from the canopy. Respective sample sizes can be found in Table 3.



580

581 **Figure 3:** Area-based ethylene production rates of leaves of vascular epiphyte species. For each
582 species at least two individuals were sampled. Error bars indicate standard errors. Different
583 lowercase letters indicate significant differences at the $P < 0.05$ level. Species are ordered
584 alphabetically. We sampled two individual Achmea aquilega plants, three Asplenium sp. 1 plants,
585 five Ludovia lancifolia plants, four Philodendron sp. 1 plants, two Philodendron sp. 2 plants and
586 three Pteridophyta sp. 1 plants.

587

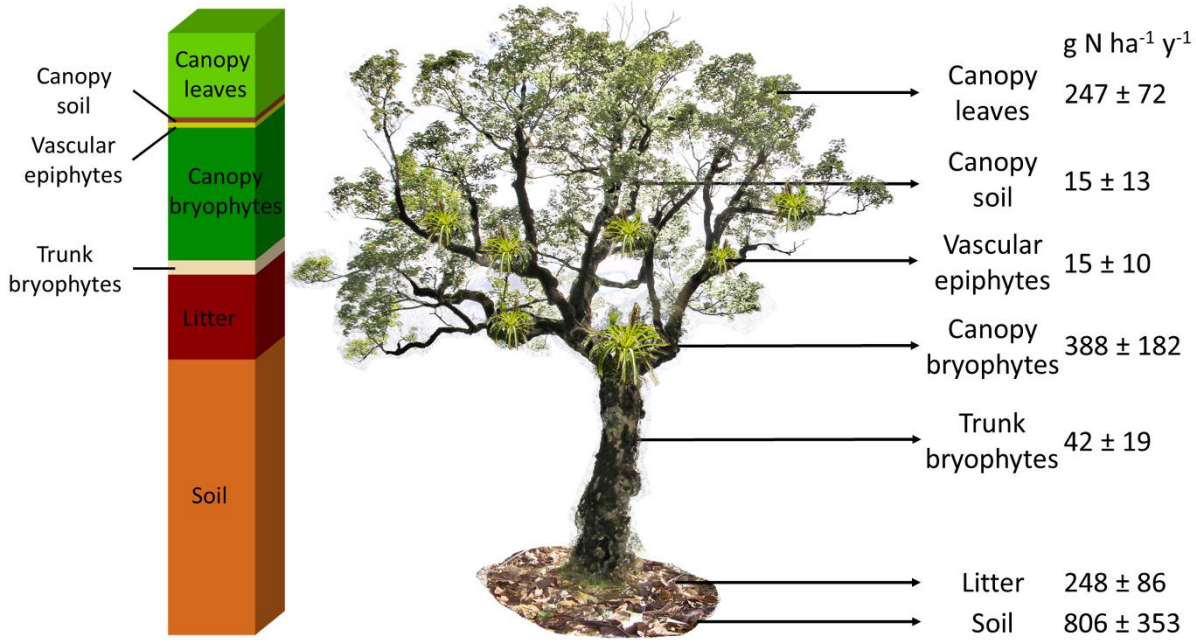


Figure 4: Diagram of calculated amounts of free-living BNF per ecosystem component both on the forest floor and within the canopy in a tropical lowland forest in French Guiana. Free-living BNF rates are expressed in g N fixed ha^{-1} y^{-1} and include standard errors.

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