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## RESEARCH ARTICLE

# Nitrogen pulses increase fungal pathogens in Amazonian lowland tropical rain forests

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## Abstract

1. Animals represent an overlooked source of horizontal redistribution of primary production and concentration of elements in ecosystems. For example, the high nutrient concentration of excretions by animals creates a mosaic of short-term nutrient hotspots. However, how this impacts soil microbial communities, especially fungi, and in turn plant species diversity remains little known.
2. This study quantified the temporal dynamics of soil mineral nitrogen (N) availability and its relationship with soil fungal community and functional group composition in simulated high-N patches in an Amazonian rain forest. We hypothesised that (H1) changes in local resource dynamics would increase the abundance of pathotrophs and reduce that of saprotrophs and that, (H2) compared with previously reported bacterial community dynamics, fungi would be more resilient after a pulse disturbance event.
3. A single urea pulse was applied and the relationship between fungal community composition and functional groups and soil N availability were determined before and twice after the urea treatment.
4. An increase in mineral N availability and soil pH two months after the applied urea pulse was found to be associated with significant changes in fungal community composition and the abundance of functional groups. There was a notable decrease in the relative abundance of saprotrophs, accompanied by an increase in plant pathogenic fungi. Five months after the treatment, no differences were detected either in mineral N availability and soil pH or the composition of fungal communities and functional groups between the control and urea treatment.
5. *Synthesis*. By locally favouring the abundance of plant pathogens, temporally short-lived, but frequent high-N patches created by animal excretions could potentially be involved in maintaining spatially and temporally variable soil

[Correction added on 18 June 2022, after first online publication: The Graphical abstract text has been updated and a Portuguese abstract added.]

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microbial diversity and thus contributing to high plant community diversity in tropical rain forests as predicted by the Janzen–Connell hypothesis. The tendency of soil fungal communities in this study to return to their initial composition after 5 months suggests that they are resilient to perturbation by N pulse, and more so than previously observed in bacterial communities.

#### KEYWORDS

Amazonia, functional groups, Janzen–Connell hypothesis, pathogens, soil fungal communities, tropical rain forest, urea-N addition

## 1 | INTRODUCTION

One of the fundamental characteristics of ecosystem dynamics is that disturbances that affect ecosystems vary in magnitude (causal impact), amplitude (extent) and frequency (Gaiser et al., 2020). At different temporal and spatial scales of aggregation, disturbances can be considered as part of the natural functioning of ecosystems under normal conditions, that is, not considering extreme events. Such aggregation results in emergent properties at larger scales, for example, biogeochemical cycles over time at the ecosystem level, that result from disturbance at lesser scales. To understand the functioning of ecosystems, it is important to identify disturbances that affect ecosystems at different spatiotemporal scales and quantify their effects on emergent properties (Gaiser et al., 2020). For example, the high diversity of tree species in tropical rain forests may be caused, as suggested by the Janzen–Connell hypothesis (JCH; Connell, 1971; Janzen, 1970), by 'small-scale disturbances' regulating the regeneration of conspecifics. We define small-scale disturbance, after van der Maarel (1993), as disturbance of small spatial extent, leading to patch dynamics—in our case that of soil microbiota—without changing the prevailing tree community in the short term; in an Amazonian context, urine patches deposited on the forest floor by mammals, or latrines (e.g. by *Tapirus terrestris*; Fragoso, 1997).

The spatially variable availability of soil nutrients has been attributed to multiple causes including topography, hydrology, vegetation patterns and litter quality (Birkenland et al., 2003). Its impact has been shown to be a potentially important factor for biodiversity patterns such as the distribution of plant species (see e.g. Laurance et al., 2010 for central Amazonia) and microbiota (de Oliveira Freitas et al., 2014) and it has also been correlated with genetic differences in understorey plants in temperate forests (Lechowicz & Bell, 1991). The static view of spatially variable nutrient availability needs to be re-examined in view of the temporal dynamics of disturbances that affect ecosystems (Jentsch & White, 2019). Small-scale disturbances are often poorly characterised because they create a mosaic of heterogeneous patches that are often functionally and structurally different from the landscape that surrounds them (Morris et al., 2016).

Dynamic and nonlinear interactions between animals, vegetation and soil properties vary in space and time and are influenced by the heterogeneity of resource distribution, plant-tissue chemistry and movements made by animals, which determine the direction

of the dominant causal effect (Pastor et al., 1998). A relatively neglected aspect in natural ecosystems is that of nutrient patchiness associated with deposition of nutrients by animal excretions. Most research has targeted the role and impacts that ungulates have in grassland and savanna ecosystems (but see e.g. Clay et al., 2015 and Milton & Kaspari, 2007 for urine impacts on tropical forest soil webs), exploring top-down and bottom-up regulation (Leroux & Loreau, 2015; Pastor et al., 1997, 1998; Sitters & Andriuzzi, 2019), with clear implication for ecosystem nutrient dynamics (Schmitz et al., 2010, 2018).

However, tropical rain forests and their animal assemblages differ greatly from those of grassland and savanna ecosystems. First, tropical forests lack herds of herbivores and thus are not affected by the zoogeographical cycles that are characterised by large offtake, soil compaction and erosion, and large input of excreta and carcasses (Sitters & Olde Venterink, 2018; Valdés-Correcher et al., 2019). Second, a considerable proportion of herbivory in tropical forests is undertaken by arboreal mammal species whose density can vary greatly; in Amazonia, it can reach an estimated 953 kg km<sup>-2</sup> (Peres, 2008) and it has been estimated that arboreal mammals consume 1.5–5 times as much plant matter than is consumed by terrestrial herbivores. Third, as most primary production is concentrated in the foliage of the forest canopy and there are physical (body mass) and physiological (energy intake, nutritional quality of the leaves) limits on arboreal life forms, mammalian densities are lower in tropical rain forests than in savannas (see Coley & Barone, 1996 and references therein). Thus, while terrestrial mammals certainly contribute small-scale disturbances in tropical rain forests, for example, tapir latrines have been shown to be important for seed dispersal (O'Farrill et al., 2013) and for better survival and growth of seedlings when compared with those under mother trees (Fragoso, 1997), they have relatively little impact by trampling and offtake.

While recent literature has emphasised the theoretically important role of horizontal redistribution of phosphorus by the extinct megafauna in neotropical savannas and the rain forests in the Amazon river basin (Doughty et al., 2013) and nutrients in general across ecosystems (Subalusky & Post, 2019), very little is known about the redistribution of primary production and its concentration by contemporary animals within forest ecosystems (Leroux, 2021; Seagle, 2003; Villar et al., 2021). The importance of the redistribution and concentration of nutrients in forest ecosystem is associated

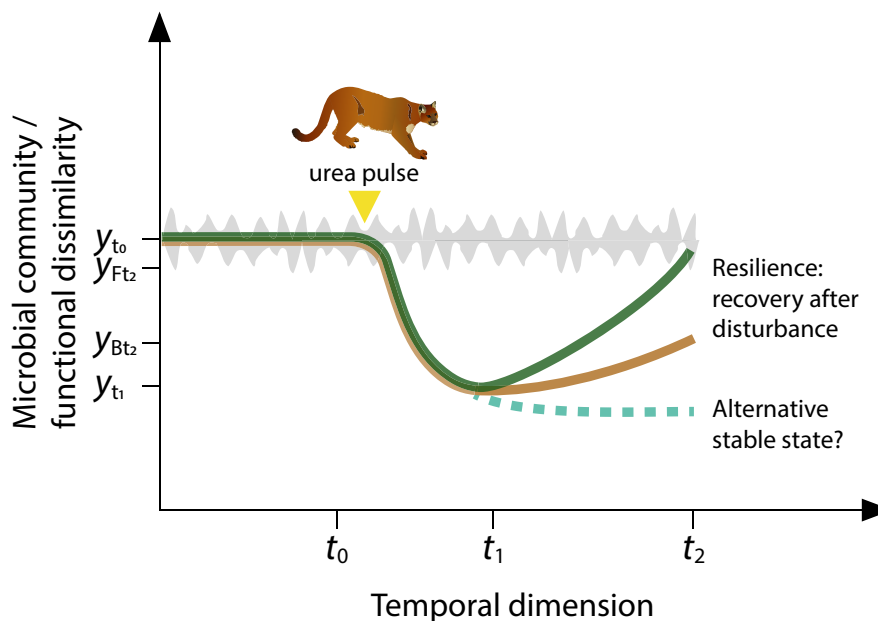
with the high concentration of elements in deposited excreta, it being two to three magnitudes higher than that received by annual litterfall input per area (Buscardo et al., 2016; Buscardo, Geml, Schmidt, Freitas, et al., 2018). Thus, deposition of urine or faeces constitutes a pulse disturbance with high local impact. How the impact of animal excreta manifests in affecting trophic structure and ecosystem structure and function (Subalussy & Post, 2019) depends on the one hand, on the input (type of input, animal size and physiology, population size, life history and use of habitat), and, on the other hand, on the seasonal resource availability at the affected patches and their biota (trophic guilds/functional types, taxonomic identity, life history and mobility). These factors, in turn, are related to the quantity, quality, frequency and duration of inputs and their legacy effects. In a soil context, this disturbance can affect the composition of species and functional types of microbiota and change resource dynamics, which, in turn, locally reconfigures the rate of decomposition and mineralisation processes and the composition and structure of soil organisms.

While the amplitude and frequency of pulse disturbance is dependent on the size of mammals and their population density (see e.g. Clark et al., 2005 for small mammals in grasslands), it is also greatly affected by the behaviour and the ecology of animal species (e.g. solitary vs. gregarious; midden forming; Pastor et al., 1997, 1998). Patch size and the number of patches are generally positively related to body size and population density of mammals, respectively (see Table S1 for the relative densities of terrestrial mammals recorded by camera traps and their mean body mass in central Amazonia). Thus,

pulse disturbance alone or when turns into press type disturbance via recurrence at suitably frequent intervals can lead to maintaining small-scale soil patch diversity and acting as a filter/selective pressure on microbial diversity (cf. the postulates of the theory of pulse dynamics of Jentsch and White (2019)) and it can affect patterns of recruitment of plants.

For a pulse disturbance to have an impact on ecosystem dynamics, it has to affect ecosystem structure or function and have a legacy effect (e.g. Gaiser et al., 2020). Our exploratory model simulations of such legacy effect have shown that there would be a substantial number of soil patches affected by urine input from mammals of different sizes present at any time in a forest plot (L. Nagy et al., unpublished). The impacted patches either return to their original state (over a length of period), or they, in extreme cases (e.g. mid-dens), may assume an alternative stable state (Shade et al., 2012; Figure 1). A pulse disturbance event may directly affect mortality or change in the relative frequency of soil micro-organisms, or indirectly affect relative abundances and turnover rates by changing the environment (Buscardo, Geml, Schmidt, Silva, et al., 2018; Jentsch & White, 2019; Shade et al., 2012). Soil microbial community response to pulse disturbance may occur through shifts in community structure via patch dynamics and species sorting, that is, differential growth or mortality, in combination with dispersal (immigration and emigration; wash-out effect; Wallenstein & Hall, 2012).

While press disturbance type effects on microbial community structure/processes have been receiving increased attention, especially in the context of global changes—for example, increased



**FIGURE 1** Conceptual representation of change in soil microbial community and functional diversity, initiated by a urea pulse and followed by a recovery over time (resilience vs. permanent change, or 'alternative stable state'). We hypothesised that fungal (green) and bacterial (brown) communities have different levels of resilience and follow therefore different temporal recovery dynamics, with fungal communities being more resilient than bacterial communities to a urine-N pulse disturbance.  $t_0$ , pre-treatment state;  $t_1$ , 59 days after urea application;  $t_2$ , 159 days after urea treatment;  $y_{t_0}$ , microbial community/functional dissimilarity baseline and associated natural spatiotemporal fluctuation (grey wavy pattern);  $y_{t_1}$ , impact of pulse disturbance on fungal and bacterial community and functional diversity at  $t_1$ ;  $y_{Ft_2}$  and  $y_{Bt_2}$ , recovery at  $t_2$  by fungal and bacterial community and functional diversity towards initial state

atmospheric nitrogen (N) deposition (Cusack et al., 2011), and climate change (Buscardo et al., 2021)—and studies have indicated that different components of soil microbial communities respond differently to press-type nutrient addition disturbance (Cassman et al., 2016; Gravuer et al., 2020), there is a general paucity of observational and experimental research on the effects of pulse disturbance caused by biological nutrient deposition on the composition and function of soil communities (Shade et al., 2012), especially in tropical rain forests. Singh et al. (2009) have shown a clear shift in soil bacterial T-RFLP community profile, and no response by fungal communities in a temperate pasture 1 month after an experimental pulse disturbance event by urea addition. For tropical rain forest, changes in soil bacterial composition and functional groups associated with the N-cycle have been reported by Buscardo, Geml, Schmidt, Silva, et al. (2018), based on the same experiment reported here. The authors found an increase in the relative abundance of soil bacterial taxa involved in the physiologically 'narrow' processes of nitrification and denitrification and a decrease in N<sub>2</sub>-fixers following urea addition. Furthermore, there was a temporal legacy effect on both bacterial community composition and functional group profile, 2 and 5 months following treatment. In addition, Buscardo et al. (2021) have shown that changes in fungal community structure and function resulting from altered water and nutrient availability affected biotic interactions (pathogens, endophytes) with plants, and nutrient cycling (saprophytes), and thus, they are likely to have a modulating role in the recruitment of forest trees (e.g. Sarmiento et al., 2017).

Microbial functional groups may respond differently to nutrient availability because of the difference in their use of labile versus recalcitrant nutrient sources (Paterson et al., 2008), which is likely to affect ecosystem structure and function. We hypothesised that changes in local resource dynamics resulting from a pulse disturbance would affect differently the abundance of fungal functional groups. Decomposers, characterised by a slow but efficient growth strategy and often associated with the degradation of recalcitrant nutrient sources, represent the dominant trophic guild in tropical forest soils (Brinkmann et al., 2019; Buscardo et al., 2021). Their potential responses to labile N are varied and depend on numerous factors including litter quality, type of soil and source/amount of added N (Cleveland et al., 2006; Fanin et al., 2012; Hobbie et al., 2012; Kaspari et al., 2008; Knorr et al., 2005). However, according to the home-field advantage hypothesis (Gholz et al., 2000) saprotrophs may be specialised towards the litter, they most frequently encounter, and their abundance should therefore be reduced by a labile-N pulse. Previous work has also shown that saprotroph abundance correlates negatively with soil pH (Rousk et al., 2009). Since the transformation of urea results in the production of ammonium that has been found to temporarily raise soil pH (see e.g. Buscardo, Geml, Schmidt, Silva, et al., 2018), we hypothesised that the relative abundance of saprotrophs would (H1a) be locally reduced by the combined direct and indirect effects of added urea.

Nutrient inputs are known to increase pathogen abundance in agroecosystems (Veresoglou et al., 2013) and recent work has shown

that fungal pathogens thrive also in nutrient-rich, mainly N-rich natural ecosystems (Hersh et al., 2012; Revillini et al., 2016; Reynolds et al., 2003). While it is yet unknown whether shifts in fungal functional groups precede, and possibly cause, shifts in plant communities (direct effect of nutrient availability), or if fungal communities respond to changes in plant species/traits (indirect effect), it has recently been reported that pathogens are affected by changes in soil properties and their abundance positively correlates with soil pH (Lekberg et al., 2021). We hypothesised therefore that the increase in labile-N availability combined with the increase in soil pH would cause an increase in the abundance of pathogens (H1b).

Fungi are generally better adapted to obtain nutrients and carbon (C) from recalcitrant sources because of their low nutrient requirement and metabolic activity, while bacteria, which have a smaller C:N ratio and a larger N demand per unit C than fungi, are more efficient in using labile nutrients (Hodge et al., 2000; Moore et al., 2003). A study conducted in tropical rain forests in Puerto Rico, has shown, for example, that N addition increased the abundance of bacteria in the lowlands, facilitating the mineralisation of labile C sources, while an increased fungal growth in upper montane forests was associated with the mineralisation of recalcitrant nutrient sources (Cusack et al., 2011). We hypothesised that (H2) fungal communities would be more resilient than bacterial communities to a urea-N pulse disturbance, that is, they would return to their original compositional/structural state over a shorter length of time than bacteria (Figure 1). To do this, we compared the resilience of fungal communities, characterised in the present study, with that of bacterial communities derived from the same urea addition experiment (Buscardo, Geml, Schmidt, Silva, et al., 2018).

To test our hypotheses, we mimicked urine-N deposition at a rate corresponding to that deposited by a sheep-sized mammal (*Ovis aries*; similar body mass in Amazonia would be represented by, for example, brocket deer *Mazama americana*, or puma *Puma concolor*), by applying a single urea pulse to small permanent plots in a lowland evergreen rain forest in Amazonia. Short-term changes (over a 5-month period) in fungal communities and functional groups were characterised using molecular methods before and twice after the urea treatment and were related to changes in soil mineral N availability. Our experimental design allowed the detection of local impacts of simulated urine input on microbial taxonomic and functional diversity. Allowing for the factors identified by Subalusky and Post (2019) regarding the quantity, quality, frequency and timing and the legacy of the impacts, the results can be used for model input for exploring upscaling to landscape and ecosystem levels.

## 2 | MATERIALS AND METHODS

### 2.1 | Site details

The study was conducted in the Adolpho Ducke Forest Reserve (ADFR), a 10,000-ha block of remaining evergreen tropical 'terra firme' (i.e. land not affected by seasonal flooding) lowland forest on

the outskirts of Manaus, Brazil. The ADFR (03°00'00"–03°08'00"S; 59°52'40"–59°58'00"W) has an equatorial climate with a mean annual temperature of 26°C and a mean annual precipitation of 2550 mm (de Oliveira Freitas et al., 2014). The year of study, 2013, was anomalously wet with a total rainfall of 3385 mm recorded. There are three main soil types closely related to topographic position: plateaux with oxisols, slopes with ultisols and valleys with spodosols (Chauvel et al., 1987). Forest community composition and structure are related to topography and emergent trees, up to c. 45 m are frequent on the plateaux where the canopy reaches 35–40 m (Ribeiro et al., 1999).

Studies of mammals in Amazonia have shown carnivores and ungulates to be the most species-rich orders in protected forest reserve areas surrounded by intact forests, while rodents and Xenarthra (anteaters, armadillos and sloths) may replace ungulates in reserves inserted in an anthropised landscape (Luna et al., 2017 and references therein), the ADFR being one such reserve (Table S1). There have been 42 species of medium- and large-sized mammal species reported from the ADFR (Pontes et al., 2008). Recent observations by camera traps (Gonçalves, 2013) have detected eight species of carnivores (body masses ranging between 3 and 90 kg) and indicated highest frequency for rodents (1.5–7.5 kg) and opossums (1 kg), followed by deer species (14.5–36 kg; Table S1). *Dasyurus novemcinctus* (armadillo), *Myoprocta acouchy* (red acouchi) and *Mazama gouazoubira* (grey brocket deer) appear to have preference for the plateaux areas (Gonçalves, 2013) where the study plots were established, but none were sighted during our study.

## 2.2 | Experimental design and urea application

In all, 20 sampling sites were selected randomly in a plateau area (Buscardo, Geml, Schmidt, Silva, et al., 2018), allowing for potential losses that might occur owing to treefall or other extreme factors such as mass fleshy fruit fall covering the plots during the experiment. A minimum distance of c. 40 m between any two sites was observed to avoid autocorrelation of soil variables. Four permanent plots of 1 m × 1 m in size were established at each site at a distance of 1.5 m from a focal reference tree. The ground cover of plants (herbaceous and tree seedlings) in the plots was sparse, as generally found in primary tropical rain forest (Poulsen & Balslev, 1991). Samples collected from all 80 plots during the first sampling (Time 0, 13 April 2013), before applying the urea treatment on 31 May 2013 were in part used for the characterisation of the spatial structure of bacterial communities and are described in Buscardo, Geml, Schmidt, Freitas, et al. (2018). Two subsequent samplings (Time 1, 28 July 2013; Time 2, 6 November 2013) were made in 20 control and 20 plots treated with urea.

Urea was applied in an aqueous solution by spraying 1 L of solution per each 1-m<sup>2</sup> plot at a rate of 400 kg N ha<sup>-1</sup>, mimicking the N load that an average 'sheep-sized' animal would deposit in 1 L of urine (Hamilton III et al., 1998; Seagle, 2003). In our study site, there are several species of mammals of similar body mass of c. 30–40 kg

(e.g. deer, wild pig, giant anteater, puma; Gonçalves, 2013; Table S1). Control plots were not sprayed with equivalent amounts of water (1 L m<sup>-2</sup> is equivalent to 1 mm rainfall) as an additional 1 mm to the 450 mm rain that fell in May 2013 would have had no impact. Mammal urine may contain organic components other than urea depending on mammal dietary adaptation. The additional labile N sources (e.g. amino acids) could either be immediately immobilised by micro-organisms or easily decomposed. The capacity for the uptake of amino acids by micro-organisms however saturates as N-source concentration increases (Ge et al., 2009). Since our simulated urine input constitutes a temporary labile N saturation and given that the composition of urine is dominated by urea (Bristow et al., 1992), we assumed that urea application alone would sufficiently model urine input applied in situ in a tropical rain forest.

The effects of urea addition on soil properties previously reported by Buscardo, Geml, Schmidt, Silva, et al. (2018) indicated that differences in soil chemistry between control and urea-treated plots were significant at Time 1, that is, 2 months after N addition, for ammonium (NH<sub>4</sub><sup>+</sup>; 17.1 vs. 125.1 μg g<sup>-1</sup>), nitrate (NO<sub>3</sub><sup>-</sup>; 1.9 vs. 29.1 μg g<sup>-1</sup>), pH, (3.9 vs. 4.1), exchangeable acidity (H<sup>+</sup> plus Al<sup>3+</sup>; 194 vs. 137 mmol kg<sup>-1</sup>), soil organic matter (OM; 7.1% vs. 6.3%) and cation exchange capacity (CEC; 201 vs. 145 mmol kg<sup>-1</sup>).

## 2.3 | Soil sampling

Every 1 m × 1 m plot was divided into 15 subplots of 33.3 cm × 20 cm. After removing litter, soil cores were extracted from three subplots, selected in a stratified random manner, to a depth of 5 cm, using a soil corer (ø = 3 cm). A sterile needle was used to sample the three soil cores per plot and the samples for molecular analyses were pooled in a 2.5-ml Eppendorf vial. Samples were kept in a cool box until transporting to the laboratory where they were conserved at -20°C until analyses.

## 2.4 | Molecular analyses

In all, 15 of the 20 sites were included in molecular analyses. Previous experience with soil analyses has shown that this number of samples allowed the detection of statistically significant changes at the level of 30% minimum detectable difference, which is also likely to be ecologically significant. DNA was extracted from a total of 120 samples (Time 0, 15 sites × 4 plots; Time 1, 15 sites × 2 plots; Time 2, 15 sites × 2 plots), using 250 mg of soil with a PowerSoil DNA Isolation Kit (MoBio Laboratories). In all, 90 samples (control: 3 sampling dates × 15 plots; treatment: 1 pre-treatment × 15 plots plus 2 post-treatment × 15 plots) were used in the present study. Amplicon libraries were prepared by PCR amplification of the fungal internal transcribed spacer 2 (ITS2) with the primers fITS7 (Ihrmark et al., 2012) and ITS4 (White et al., 1990) and sequenced by an Ion Torrent Personal Genome Machine (Thermo Fisher Scientific Inc.) at the Naturalis Biodiversity Center,

The Netherlands. Sequencing was successful for a total of 89 of the 90 samples.

## 2.5 | Bioinformatics

Primers were removed and poor-quality ends were trimmed off, based on a probability of error of 0.02 cut-off in Geneious Pro 5.6.1 (BioMatters). In a next step, using USEARCH v.8.0 (Edgar, 2010), all sequences were truncated to 200bp and sequences with an expected error  $>0.5$  were discarded. For each sample, sequences were collapsed into unique sequence types, while preserving their counts. Unique sequences were grouped into OTUs at 97% sequence similarity using USEARCH while excluding putative chimeric sequences. As the sequencing depth of our samples was very high and because the reliability of OTUs with a low number of sequences may be questionable (although see Shade et al. (2012) for the impact it might have had for excluding rare taxa), we followed a conservative approach and removed all OTUs that had less than five reads per sample and did not occur in at least two samples (Lindahl et al., 2013). A total of six OTUs that were present either in the positive or negative control were also eliminated. The number of sequences used in the numerical analyses was thus reduced to 2,287,893, with an average of 25,707 per sample (range 10,243–57,569). These sequences corresponded to a total of 2163 OTUs and were deposited, together with those obtained for the characterisation of spatiotemporal structure of soil fungal communities (5312 OTUs in total) at DDBJ/EMBL/GenBank under the BioProject accession number PRJNA579099. Sequences were assigned to taxonomic groups based on pairwise similarity searches against the UNITE fungal ITS sequence dynamic SH database (Kõljalg et al., 2013), and subsequently they were assigned to functional groups using the FUNGuild database (Nguyen et al., 2016).

## 2.6 | Statistical analyses

To explore the completeness of our sampling, rarefaction accumulation curves were made for each sample. Rarefaction curves, OTU richness (SR), the Shannon–Wiener diversity index ( $H'$ ) and abundance-based coverage estimator (ACE) were computed in VEGAN v.2.3-5 (Oksanen et al., 2016). Temporal differences in the taxonomic composition of the fungal communities between control and treatment were assessed using both presence/absence and relative abundance data (both non-rarefied and rarefied being 10,243 sequences the size of the smallest library) at each sampling time with the ADONIS function in VEGAN implemented with the Bray–Curtis distance and 10,000 permutations. Non-metric multidimensional scaling (NMDS) ordinations based on Bray–Curtis distance matrices were carried out on presence/absence and relative abundance data to visualise differences in OTU-based community composition. After excluding highly correlated variables, the 'envfit' R function was used to fit soil variables onto the NMDS ordinations.

Since there were no substantial differences between rarefied and non-rarefied data, differences in relative abundances of the first 120 most abundant OTUs (75% of the total reads) between control and urea treatment at each sampling time were computed on rarefied data at phylum and at lower taxonomic levels (i.e. genus, family, order and class) and evaluated with the Wilcoxon signed-rank test. Of the 2163 OTUs, 959 were assigned to single (e.g. saprotrophs, pathotrophs, symbiotrophs) or multiple functional groups. Mucoromycotina had an unusually large proportion of sequence reads (c. 18%), with all but one of the OTUs belonging to the Umbelopsidales. Following the FUNGuild database (Nguyen et al., 2016) and Naranjo-Ortiz and Gabaldón (2019), we classified all Umbelopsidales as saprotrophs.

Differences between control and urea-N treatment in rarefied relative abundances of different functional groups were evaluated with the Wilcoxon signed-rank test. Differences in SR and  $H'$  among fungal communities and functional groups between urea-N treatment and control at different sampling dates were evaluated with the paired *t*-test and with the Wilcoxon signed-rank test. The false discovery rate (FDR) method (Benjamini & Hochberg, 1995) was used to correct significances for multiple comparisons.

## 3 | RESULTS

### 3.1 | Fungal community assessment

The number of OTUs (SR) per plot varied between 38 and 274 (mean of 145), while  $H'$  ranged between 1 and 5 (mean of 3.2). ACE predicted identical average, maximum and minimum numbers of OTUs per plot that were identified by sequencing. This indicates that fungal communities were satisfactorily represented in all samples which is also shown by the rarefaction curves, where OTU numbers levelled off in every sample (Figure S1).

According to the UNITE dynamic SH database, 1762 OTUs, corresponding to 81.5% of the total, were classifiable to a taxonomic level, while 959 OTUs (equivalent to 44.3%) were assigned to unique or multiple functional groups by searching against the FUNGuild database. At the taxonomic level, sequence reads were dominated by Ascomycota (55.7%), followed by Mucoromycota (18.3%) and Basidiomycota (17.6%), with Rozellomycota, Chytridiomycota, Mortierellomycota and Entomophthoromycota summing up together less than 2% of the total number of reads. Unidentified OTUs accounted for 8.2% of the total number of reads. Regarding functional groups, fungal communities were dominated by saprotrophs (71.3%), followed by pathotrophs (6.9%) and symbiotrophs (3.9%).

### 3.2 | Impact of urea addition: Fungal community level

Fungal community composition assessed by applying the ADONIS function (rarefied presence/absence; relative abundance of OTUs)

was not significantly different between control and urea plots at Time 0. After urea application, significant differences between control and treatment were detected at Time 1, that is, 2 months after urea treatment (Table 1; for results obtained with non-rarefied data see Table S2).

Similar results were obtained by the NMDS analysis based on relative abundance but not on presence/absence data (Figure 2a; see Figure S2 for results obtained using non-rarefied data), with differences between control and treatment plots exclusively at Time 1. A complete overlap of fungal communities (95% confidence intervals using standard error of the weighted average sample scores per treatment) between control and urea addition was instead observable at Time 0 (i.e. no pre-treatment differences between control and treatment plots) and at Time 2, that is, 5 months after the urea treatment, there were no post-treatment differences between control and treatment.

Differences among communities on a per sample basis were related to soil factors such as  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ ,  $\text{Mg}^{2+}$ , OM,  $\text{PO}_4^{3-}$ , CEC and fine root biomass (Figure 2a; Table S3).

Regarding OTU diversity ( $H'$ ) and OTU richness (or SR), no significant differences were detected for  $H'$ , for all paired comparisons at any of the three sampling dates; while SR was found to be lower in the control than in the urea treatment at Time 0, that is, before the application of urea.

### 3.3 | Impact of urea addition: Taxonomic level

Urea addition caused significant shifts at all taxonomic levels in fungal composition which did not differ between control and pre-treatment at Time 0. The Wilcoxon test indicated 10 taxa to have changed significantly following the urea treatment exclusively at Time 1 (Figures 3–4), while no differences were detected at Time 2. At the phylum level, Mucoromycota in the urea treatment decreased in relative abundance when compared with the control (Figure 3). At a finer taxonomic resolution, 2 classes, 3 orders, 2 families and 2 genera exhibited statistically significant differences in pairwise comparisons between control and urea addition plots at Time 1 (Figure 4). Among these, there was an increase in the relative abundance of taxa belonging to the class *incertae sedis* of Ascomycota. There was also an increase in Pleosporales and *Trichocladium* spp. and a concomitant decrease in Umbelopsidales and *Heterospora* spp. (Figure 4).

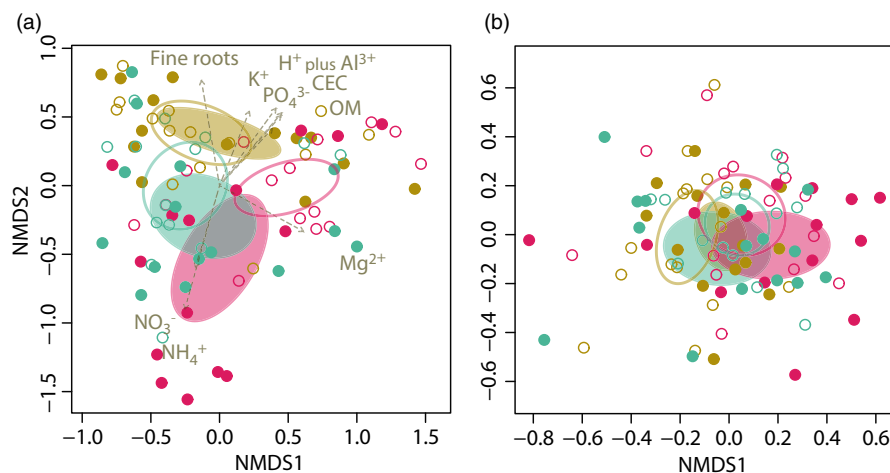
### 3.4 | Impact of urea addition: Functional (trophic) group level

At the functional group level, no differences were detected at any sampling occasion in SR and  $H'$  between control and urea-treated

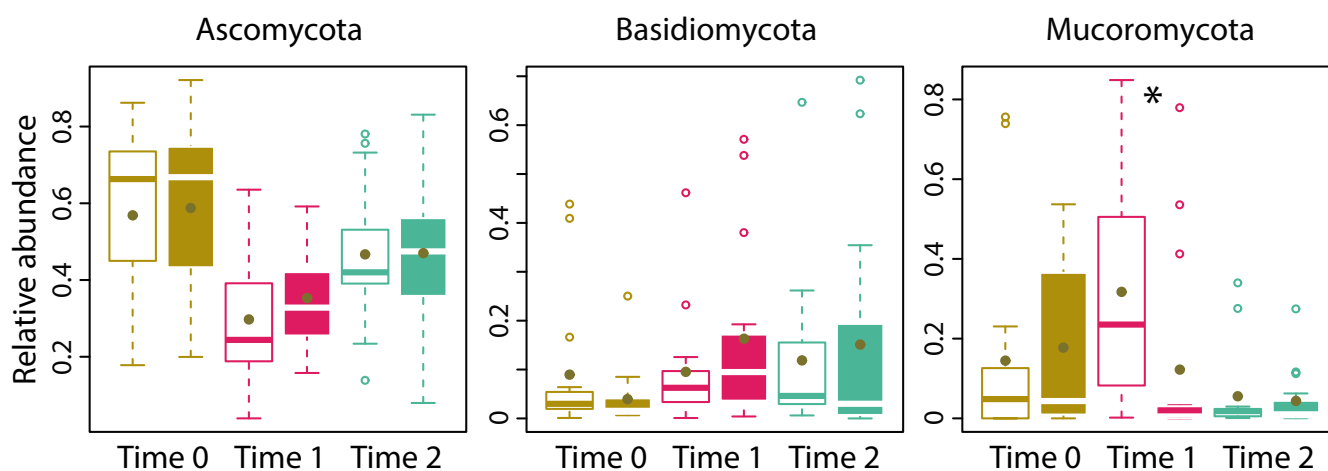
TABLE 1 Overview of differences in soil fungal community composition between control and urea-treated plots in a lowland tropical rain forest in Amazonia, obtained by the ADONIS function. The function was applied to rarefied presence/absence and relative abundance data at each sampling time, using 10,000 permutations

		df	Sum of squares	Mean squares	F model	R <sup>2</sup>	Pr (>F)
Presence/Absence							
Time 0	Urea addition	1	0.2278	0.22785	0.85652	0.03	0.855
	Residuals	28	7.4485	0.26602		0.97	
	Total	29	7.6763				
Time 1	Urea addition	1	0.4319	0.43186	1.6028	0.05	0.004
	Residuals	28	7.5446	0.26945		0.95	
	Total	29	7.9765				
Time 2	Urea addition	1	0.3145	0.31447	1.1784	0.04	0.102
	Residuals	27	7.2054	0.26687		0.96	
	Total	28	7.5198				
Relative abundance							
Time 0	Urea addition	1	0.2274	0.22742	0.76947	0.03	0.694
	Residuals	28	8.2757	0.29556		0.97	
	Total	29	8.5031				
Time 1	Urea addition	1	0.6929	0.69286	2.0167	0.07	0.018
	Residuals	28	9.6165	0.34355		0.93	
	Total	29	10.3124				
Time 2	Urea addition	1	0.3493	0.3493	0.97311	0.03	0.515
	Residuals	27	9.6917	0.35895		0.97	
	Total	28	10.041				





**FIGURE 2** Non-metric multidimensional scaling (NMDS) ordination plots based on rarefied relative abundance (a) and presence/absence data (b) relative to soil fungal communities in response to urea addition in a tropical lowland rain forest in Amazonia. Stress 4D: 0.134 (a); 0.156 (b). Time 0 (pre-treatment), ochre; Time 1 (59 days after urea application), fuchsia; Time 2 (159 days after urea treatment), sea green; empty symbols, control plots; filled symbols, urea-treated plots. Vectors representing environmental variables were fit into the ordination (a) using the 'vegan' envfit function and their significance assessed under 999 permutations (see Figure S2). OM, total soil organic matter;  $\text{PO}_4^{3-}$ , extractable phosphorous; CEC, cation exchange capacity;  $\text{H}^+$  plus  $\text{Al}^{3+}$ , exchangeable acidity; Fine roots, fine root biomass. Ellipses denote a 95% confidence interval using standard error of the weighted average plot scores at each sampling time



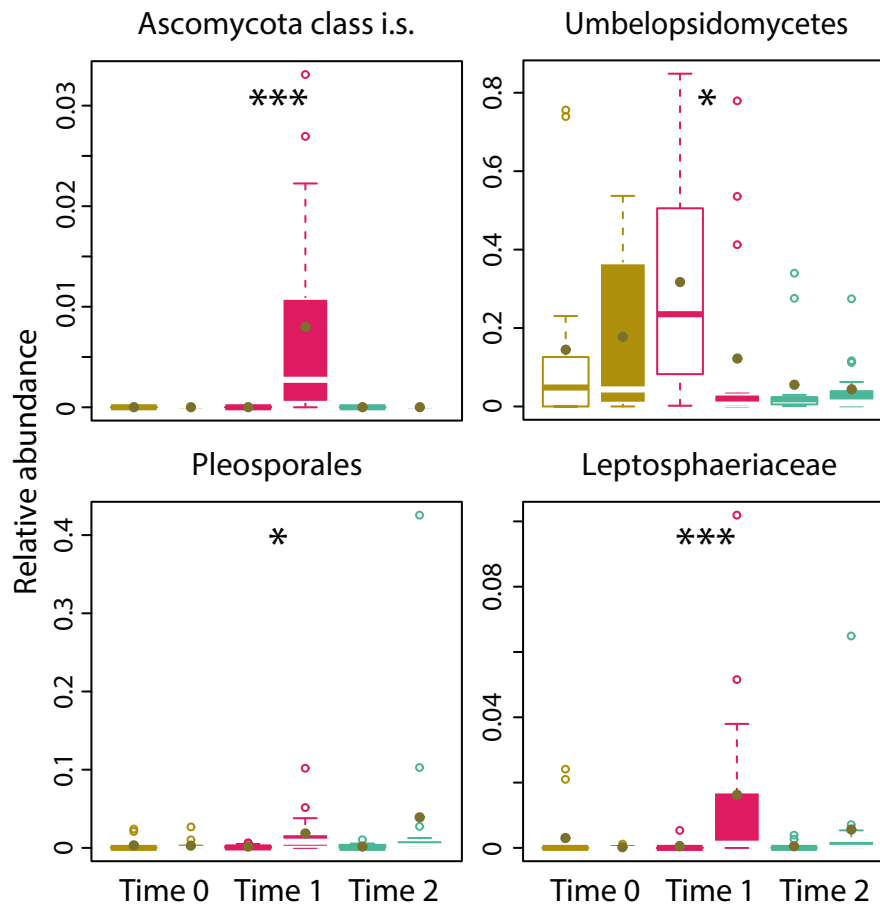
**FIGURE 3** Relative abundances of main soil fungal phyla in response to a pulse of urea in a lowland evergreen rain forest in Amazonia. Time 0, ochre; Time 1, fuchsia; Time 2, sea green; empty box plots, control plots; filled box plots, urea-treated plots. Significant differences between control and treatment at each sampling time as determined by paired *t*-tests or Wilcoxon signed-rank tests are indicated by: \* $p < 0.05$ . Box plots: centre line, median; grey circle, mean; box limits, 25th and 75th percentiles; whiskers, 1.5 times interquartile range; unfilled circles, outliers

plots. Significant differences in functional group relative abundances between control and urea treatment were detected for saprotrophs and plant pathogens at Time 1. Saprotrophs decreased while plant pathogens increased following the urea treatment (Figure 5).

#### 4 | DISCUSSION

This study confirms first the potentially important role played by animals in creating, through their urine deposition, spatiotemporal

patches of nutrient loads which promote spatial heterogeneity of diversity in soil organisms, with potential implications for elemental cycling (Buscardo, Geml, Schmidt, Silva, et al., 2018). Second, it opens the possibility to consider the potential role that animals might have in promoting/maintaining high diversity levels in tropical rain forest plant communities, mediated by their impact on fungal guilds. Large N loads in animal urine could in fact, by creating patches of high plant pathogen abundance, reduce the survival rates of conspecific propagules and contribute, as suggested by the Janzen–Connell Hypothesis (JCH; Connell, 1971; Janzen, 1970), to the maintenance of high plant diversity.



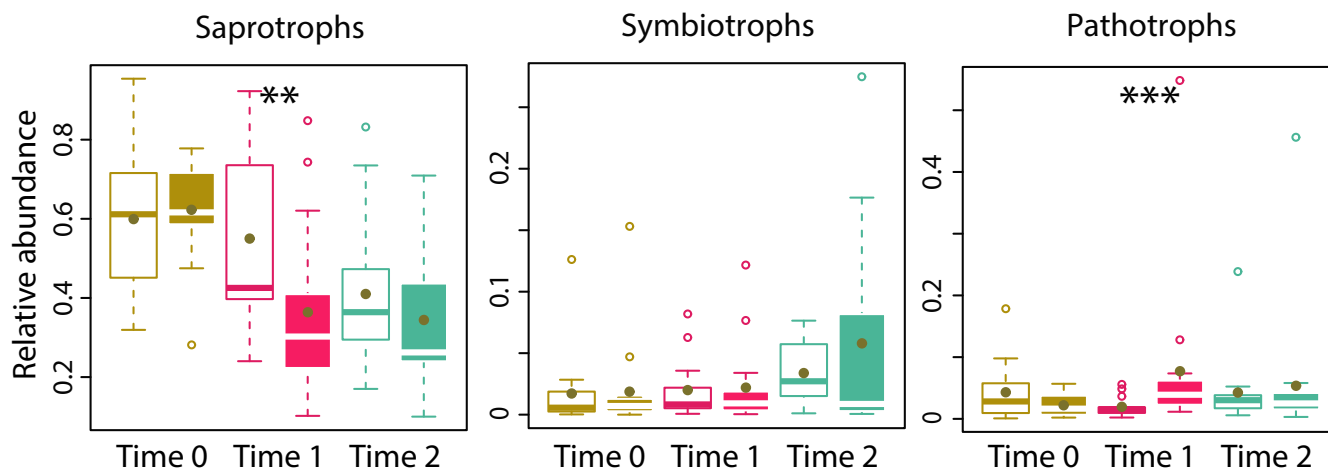
**FIGURE 4** Relative abundances of main soil fungal classes and lower taxonomic levels that significantly changed in response to a pulse of urea in a lowland evergreen rain forest in Amazonia. Time 0, ochre; Time 1, fuchsia; Time 2, sea green; empty box plots, control plots; filled box plots, urea-treated plots. Significant differences between control and treatment at each sampling time as determined by paired t-tests or Wilcoxon signed-rank tests are indicated by: \* $p < 0.05$ ; \*\*\* $p < 0.001$ . For Ascomycota class *incertae sedis* (*i.s.*) identical abundance values were found at order (*i.s.*), family (*i.s.*) and genus (*Trichocladium* spp.) and therefore only the class level figure is shown; for Umbelopsidomycetes identical abundance values were found at order level and therefore only the class level figure is shown; for Leptosphaeriaceae identical abundance values were found at genus level and therefore only the family level figure is shown (*Heterospora* spp.). Box plots: centre line, median; grey circle, mean; box limits, 25th and 75th percentiles; whiskers, 1.5 times interquartile range; unfilled circles, outliers

#### 4.1 | The functional role of fungal groups

Our results confirmed both our hypotheses H1a and H1b and showed that fungal functional groups are differently affected by urea-N pulse: the high nutrient-load patches created in the soil by a single urea pulse had an unbalancing effect on the relative abundance of fungal functional groups at Time 1, that is, 2 months following urea addition, by decreasing the relative abundance of saprotrophs and increasing that of pathotrophs. (Since soil fungal primers used are not designed to amplify arbuscular mycorrhizal fungi—the dominant symbiosis type in these tropical rain forests—symbiotrophs are likely to be underrepresented, therefore we refrained from formulating hypotheses as to their response to urea addition). The reduction in saprotrophs following N addition indicates that this functional group is sensitive to urine-N inputs (H1a) that are c. 1000 times the average daily amount received from litterfall in Amazonian lowland evergreen rain forests (five times higher than annual N inputs; Buscardo, Geml, Schmidt, Silva, et al., 2018). The rapid recovery of

fungal communities suggests however that the N pulse indirectly affects the relative abundance and turnover rate of this functional group by changing the environment (e.g. changes in soil pH; Shade et al., 2012, Jentsch & White, 2019), and give support to the home-field advantage hypothesis (Gholz et al., 2000) according to which saprotrophs are specialists of the substrate they most frequently encounter.

Our work brought to the fore an important issue regarding the functional role of fungal groups, especially in relation to Umbelopsidales, a recently defined order (Spatafora et al., 2016). At the taxonomic level, the urea treatment decreased the relative abundance of Umbelopsidales, classified as saprotrophs, following Nguyen et al. (2016) and Naranjo-Ortiz and Gabaldón (2019). The interpretation of this result requires careful consideration. While little is known about the taxa within this order, some of its members have previously been detected on living roots and isolated as endophytes (Hoff et al., 2004; Qin et al., 2018; Terhonen et al., 2014; Yang et al., 2018). Herre et al. (2007) have described foliar endophytes



**FIGURE 5** Relative abundances of main functional groups in response to a pulse of urea in a lowland evergreen rain forest in Amazonia. Time 0, ochre; Time 1, fuchsia; Time 2, sea green; empty box plots, control plots; filled box plots, urea-treated plots. Significant differences between control and treatment at each sampling time as determined by paired *t*-tests or Wilcoxon signed-rank tests are indicated by: \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . Box plots: centre line, median; grey circle, mean; box limits, 25th and 75th percentiles; whiskers, 1.5 times interquartile range; unfilled circles, outliers

in tropical forests as living as ‘sit-and-wait saprotrophs’ (they apparently spend a long time ‘waiting’ as endophytes before completing their life cycles, essentially as saprotrophs), strongly selected to ‘guard their turf’ from potential usurpers represented by other endophytes and/or by pathogens as well as herbivores. It appears therefore that both endophytic/symbiotic and saprotrophic stages could be part of the life cycle of at least some of the species of Umbelopsidales.

Endophytic fungi can play a potentially important mutualistic role by augmenting the defensive response their host against pathogens either by inducing or increasing the expression of intrinsic host defence mechanisms, or by providing additional sources of defence such as endophyte-based chemical antibiosis (Herre et al., 2007). The role of biotic interactions, including competition and mutualism, in determining response to disturbance and shaping microbial communities has previously been reported (Little et al., 2008; Shade et al., 2012). The response of a community to a disturbance event is more complex than the sum of the traits of individual species—for example, tolerance to disturbance and competitive ability (Hoeksema et al., 2020)—and is closely dependent on interspecific interactions (Violle et al., 2010). The temporary lack of the ‘guard’ role, represented by a decrease in the relative abundance of Umbelopsidales following the N pulse in our study, could therefore have resulted in the significant increase in the abundance of plant pathogens.

## 4.2 | Plant pathogens and tropical forest diversity

The urea pulse favoured pathotrophs, and in particular plant pathogens, confirming our hypothesis H1b. The contribution of animal urine (and other excreta) to the maintenance of locally high pathogen abundance is particularly relevant in the context of forest dynamics. A general increase in the abundance of fungal pathogens

following high doses of urea addition has been documented in tropical forests and agricultural systems (Brinkmann et al., 2019; Kerekes et al., 2013; Paungfoo-Lonhienne et al., 2015). However, the potential ecological implications of creating spatiotemporal nutrient availability patterns, which may have an impact on microbial functional groups in a tropical rain forest ecosystem, have previously not been considered.

The increase in plant pathogen abundance in N-enriched patches offers a hitherto unexplored interpretation/extension of the JCH. This hypothesis proposes that specialist natural enemies may be involved in maintaining high plant diversity by reducing the survival rates of conspecific propagules located close to reproductive adults, and/or in areas of high conspecific density. The role of fungal pathogens in generating density- or distance-dependent mortality of juveniles is increasingly being recognised as a potential key driver of plant species coexistence in tropical forest (Bagchi et al., 2010; Bell et al., 2006; Laliberté et al., 2015; Mangan et al., 2010). Therefore, a significant increase in the abundance of pathogenic fungi after a single N pulse is likely to result in changes in tree seedling recruitment/survival potentially contributing to the maintenance of plant community diversity as predicted by JCH. Spear and Broders (2021) have recently suggested that generalist pathogens eliciting host-specific impacts may contribute to diversity patterns at the local and regional scales. While both N availability and fungal community recover rapidly, the localised pulse perturbation by mammal urine is two to three magnitudes higher than that received by annual litterfall input per area and affects the forest floor daily in tropical rain forests, creating a dynamic mosaic of urea-N enriched patches in various phases of temporal recovery. This results in a spatiotemporal legacy that needs to be taken into consideration when evaluating the importance of mammal urine for forest dynamics and thus, potentially for biogeochemical cycles. The mostly short-lived, but spatially and temporally frequently occurring N-enriched patches are likely to contribute to

high spatiotemporal turnover in soil microbial diversity in tropical lowland forest and thereby they are an important underlying factor in shaping soil microbial diversity. Additionally, by locally and temporally favouring the abundance of plant pathogens, they might also contribute to maintaining the high diversity of plant communities. While the patchiness of nutrient availability caused by N deposited in ecosystems by animal urine has been little studied, further research on the potential impact of this phenomenon on patterns of biodiversity and ecosystem function (e.g. Hobbs, 1996) could help in disentangling ecological questions that aim at explaining the maintenance of tropical rain forest diversity (Laliberté et al., 2015; Levi et al., 2019).

### 4.3 | Resilience to pulse disturbance: Soil fungi versus bacteria

While studies in ecosystems other than tropical forests on the effects of nutrient amendments have reported higher resilience of fungal communities in comparison with bacterial communities to disturbances (Mueller et al., 2015; Singh et al., 2009), a recent study has shown the reduction of spatial heterogeneity in fungal community composition and little change in that of the bacterial community in response to nutrient addition (Gravuer et al., 2020). At our tropical forest site, fungal communities returned to their pre-disturbance composition—defined by Beisner et al. (2003) as stable state or community equilibrium—c. 5 months after the urea application, in line with our hypothesis H2 that fungal communities, by being better adapted to obtain nutrients from recalcitrant sources, would be more resilient than bacterial communities to a pulse disturbance caused by the urine input by animals.

At local scales, dynamic 'hotspots' of high rates of nutrient cycling caused by animal excreta (McClain et al., 2003; McNaughton, 1984) could potentially impact the distribution and trophic interactions of a wide range of micro- (Buscardo, Geml, Schmidt, Silva, et al., 2018; Cline et al., 2018) and macro-organisms (Bengtson et al., 2006; Lechowicz & Bell, 1991) and affect ecosystem processes (Bernhardt et al., 2017). The uptake of labile N primarily by bacteria and a build-up of bacterial population affect, for example, predator-prey interactions by increasing grazing by microbivores (Clark et al., 2005). When feeding on abundant source of food, bacterivorous nematodes return to the soil significant amounts of the total N in a labile form, increasing substrate utilisation and N mineralisation and thus maintaining high rates of N cycling (Anderson et al., 1981). We expected that the assumed uptake of urea-N mainly by bacteria, and the consequent changes in cycling of N with the involvement of the soil microfauna would create a feedback loop (i.e. increase in labile N, used mainly by bacteria) that would delay the return of bacterial communities to their original state when compared to that in fungal communities. The high values of soil mineral N at Time 1, that is, 2 months following urea addition suggest that the existence of this loop is plausible. This is because highly leachable mineral N, if not immobilised, would have been readily lost, even at the transition

between the rainy and dry seasons, with an accumulated precipitation of c. 300 mm following urea application. While it is plausible to assert that trophic interactions between bacteria and microbivores may therefore have allowed the maintenance of the urea pulse legacy in bacterial communities through high rates of N cycling, this aspect will require further targeted research on disentangling complex below-ground–above-ground networks.

Thus, our findings suggest that fungal communities are indeed more resilient to perturbation caused by a N pulse than previously observed in bacterial communities at the study site. By changing the balance in fungal functional groups (i.e. increasing the relative abundance of plant pathogens and decreasing that of putative plant symbionts), the N pulse highlights the important role played by animals in the redistribution of primary production and its local concentration in forest ecosystems. Through their excreta, animals create spatiotemporally variable high-load nutrient patches which promote spatiotemporal heterogeneity and niche differentiation in soil organisms. The results of the present study thus open the possibility to speculate on the potential role that animals play in indirectly promoting and maintaining high levels of diversity in tropical rain forest plant communities. By locally favouring the abundance of plant pathogens, temporally short-lived, but frequent high N patches created by animal urine, could reduce the survival rates of conspecific propagules and thus contribute to high plant community diversity in tropical rain forests as predicted by the Janzen–Connell hypothesis.

### AUTHORS' CONTRIBUTIONS

L.N. planned the research; L.N. and E.B. established the experiment and carried out all the field work; E.B. extracted the DNA and made the statistical analyses; J.G. conducted the bioinformatical analyses; E.B. and L.N. led the writing of the manuscript with input from J.G., S.K.S. and H.F.; H.B.C. contributed new reagents; A.P.S. contributed analytical tools. All authors read and approved the final version of the manuscript.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1111/1365-2745.13904>.

## DATA AVAILABILITY STATEMENT

Representative sequences of all OTUs in this study have been submitted at DDBJ/EMBL/GenBank under the BioProject accession number PRJNA579099.

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