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RESEARCH ARTICLE

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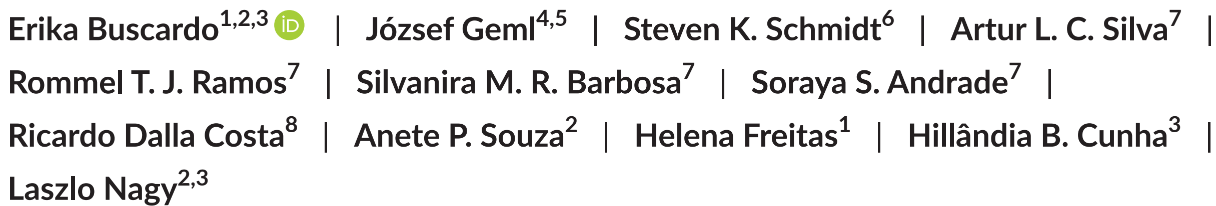
# Of mammals and bacteria in a rainforest: Temporal dynamics of soil bacteria in response to simulated N pulse from mammalian urine



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## Abstract

1. Pulse-type perturbation through excreta by animals creates a mosaic of short-term high nutrient-load patches in the soil. How this affects microbial community composition and how long these impacts last are important for microbial community dynamics and nutrient cycling.
2. Our study focused on the short-term responses to N by bacterial communities and 'functional groups' associated with the N cycle in a lowland evergreen tropical rainforest. We applied a single urea pulse, equivalent to urine-N deposition by medium-sized mammals to simulate N enrichment and changes in soil N availability, and analysed soil bacterial communities using molecular methods, before and after urea application.
3. Urea addition increased mineral N availability and changed bacterial community composition, from phylum to operational taxonomic unit levels, however, taxon richness and diversity were unaffected. Taxa involved in the physiologically "narrow" processes of nitrification (e.g. *Nitrosospira*) and denitrification (e.g. *Phyllobacteriaceae*, *Xanthomonadaceae* and *Comamonadaceae*) increased their relative abundance, while $N_2$-fixers (e.g. *Rhodospirillales*, and *Rhizobiales*) decreased after treatment. While a temporal legacy on both community composition and functional group profile was observable 58 and 159 days after treatment, at the latter date bacterial communities were already tending towards pre-treatment composition.
4. We suggest that pulse-type perturbation by mammal urine that occurs on a daily basis has strong short-term effects on patch dynamics of soil microbiota and N availability. Such a spatio-temporally dynamic soil environment enhances overall microbial richness and diversity, and contributes to the apparent temporal resilience of community composition.



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**KEYWORDS**
Amazonia, pulse-type perturbation, rainforest, soil bacteria, urea

## 1 | INTRODUCTION

In ecosystems, there are three principal sources of input of N to soil that are derived from annual litterfall, atmosphere (N deposition and biological $N_2$-fixation, BNF), and from largely unquantified amounts returned by herbivores. Litter represents internal cycling of N in an ecosystem; animal input through urine is largely part of internal N cycling; however in mixed landscapes, it may also contribute to exchange (input/loss) between ecosystems (Turner & Gardner, 2015); and atmospheric deposition and BNF represent external input. In lowland evergreen rainforests in Amazonia litterfall returns *c.* 80 (range 50–120) kg N $ha^{-1}$ $year^{-1}$, BNF 4–7 kg N $ha^{-1}$ $year^{-1}$ and atmospheric deposition *c.* 12–14 kg N $ha^{-1}$ $year^{-1}$ (Buscardo et al., 2016). The annual total N return per area to soil by animals is yet to be quantified, and probably less than the above values, but is an important and unexplored factor for local N enrichment and soil resource patchiness. The N deposited at high concentrations by animals affects variable-size patches throughout the year. While both litterfall and atmosphere-derived N represent regular/cyclic inputs to soil and litterfall-derived quantities of N are spatially rather homogeneous, input by animals is patchy and likely to reflect density, body size, range use, behaviour and resource availability (see e.g. Hobbs (1996) for ungulates in a temperate forest).

Nutrient addition experiments have been carried out in various ecosystems, including tropical forests (Alvarez-Clare, Mack, & Brooks, 2013; Mirmanto, Proctor, Green, & Nagy, 1999; Vitousek, Walker, Whiteaker, & Matson, 1993; Wright et al., 2011), principally with the objective of demonstrating nutrient limitation to aboveground productivity (mostly with reference to the C cycle/C sequestration), while recently the modelling/evaluation of atmospheric N deposition has been receiving increasing attention (Bowman, Nemergut, McKnight, Miller, & Williams, 2015; Cusack, Silver, Torn, Burton, & Firestone, 2011). These experiments, with the exception of those that simulated atmospheric N deposition, have used arbitrarily chosen nutrient addition rates and application frequencies. On the contrary, a neglected area has been addressing the potential impact on patterns of biodiversity and ecosystem function caused by N deposited in ecosystems by mammals (e.g. Hobbs, 1996). The lack of such studies is not surprising in the apparent absence of formally acknowledging the existence/importance of this phenomenon for creating of spatio-temporal resource availability patterns. While the existence of micro-scale nutrient availability for forest understorey niche delimitation for plant growth and population genetics/diversification has long been known (e.g. Bengtson, Falkengren-Grerup, & Bengtsson, 2006; Lechowicz & Bell, 1991), no information is available on how patchiness of nutrient availability impacts soil microbiota. This is especially true in relation to the deposition of urea-N in animal urine. Shade et al. (2012) have recently identified gaps in understanding microbial responses to pulse disturbances—all application of mineral fertilizers is a pulse disturbance, with the exception where carefully chosen dose and application frequency turn the treatment into a press-type perturbation (Pearce & Van der Wal, 2008). The deposition of urea-N in urine, a general phenomenon in ecosystems, results in patches with instantaneous N inputs that are five times higher than annual N inputs (and are *c.* 1,000 times the daily amount received with litterfall in Amazonian lowland evergreen rainforest) in areas that do not receive urine-N inputs, leading to microbial niche differentiation. We used sources from the literature (Hamilton III, Giovannini, Moses, Coleman, & McNaughton, 1998; Seagle, 2003) for determining the N loading (N concentration of urine) that could simulate urine-N load for generating patchiness for studying microbial functional type responses. We simulated a single pulse-application of urea, applied to small permanent plots in a tropical lowland evergreen rainforest (*terra firme*) in Amazonia and we monitored these patches for soil bacterial community composition and available mineral soil N. We estimated, based on mammal density surveys (*c.* 430 individuals per $km^2$; Terborgh et al., 2008), that animals play an important role in creating a dynamic spatio-temporal mosaic with regard to nutrient availability and that these changes would affect microbial community richness/composition.

We characterized bacterial community composition by molecular methods before and after N treatment and hypothesized firstly that pulse-type perturbation by high-dose N addition would result in a sharp decrease in the taxonomic richness of soil bacteria by mortality/inactivation of disturbance-sensitive taxa and competitive exclusion (Schimel, Balser, & Wallenstein, 2007). This we based on the extension of the "intermediate disturbance" hypothesis (IDH) of Connell (1978). We tested if a high pulse of N reduced bacterial "species richness (SR)", represented by operational taxonomic unit (OTU) richness (Figure 1). According to the IDH, both the lack of disturbance and high disturbance (in this case the pulse-type perturbation by a single high-dose N addition) would lead to reduced (species) diversity, in relation to an intermediate level of disturbance that maximizes diversity. We considered that SR is determined by the spatio-temporal interaction of a series of abiotic and biotic variables. The structure and composition of microbial communities in forests are influenced by both spatial heterogeneity (e.g. lowland evergreen rainforests are highly diverse and the variation in their plant attributes is reflected in diversified resource niches for microbial communities) and temporal patterns (e.g. dynamics of litterfall inputs, fine root turnover and nutrient pulses that are linked to precipitation patterns). A hypothetical example of spatio-temporal variation in bacterial SR is illustrated in Figure 1a. We considered this variation in diversity as the range determined by natural average disturbance (excluding extreme events—the last major drought at the site

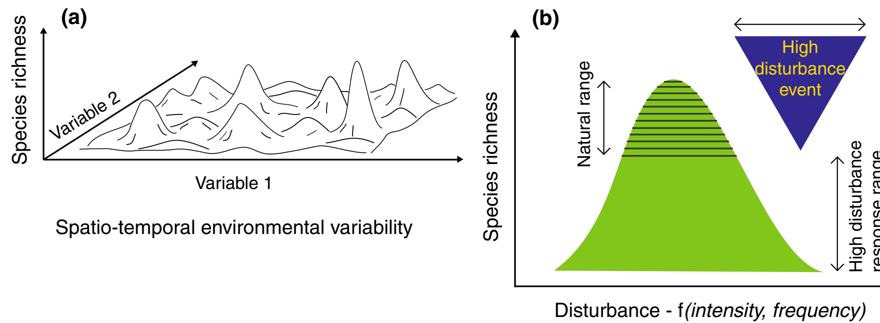


**FIGURE 1** Species richness (SR) is related to the spatio-temporal variability of the environment: (a) The hypothetical spatio-temporal variability of SR, attributable to natural disturbances, excluding extreme events; this range of SR is represented in (b) by the top hatched portion that represents the higher SR than the portion that lies to the right that represents higher than average disturbance event(s) and associated lower SR

occurred in 2009 with 5 months with negative hydrological balance). This natural average disturbance can be taken as intermediate level disturbance within the context of IDH and reflects the range of SR at intermediate levels of disturbance (Figure 1b).

Secondly, we focused on short-term responses by bacterial communities and microbial "functional groups" associated with the N cycle and we hypothesized that an increase in the substrate available for nitrifying and denitrifying bacteria would induce an increase in their relative sequence abundance and a related decrease in the relative abundance of $N_2$-fixing bacteria. Thirdly, we hypothesized that urea addition would leave a temporal legacy that would increase spatial heterogeneity (niche differentiation) for bacterial community composition and functional group profiles.

## 2 | MATERIALS AND METHODS

### 2.1 | Study area

The study was conducted in the "Reserva Florestal Adolpho Ducke" (RFAD), a 10,000-ha reserve, 26 km northeast of Manaus (03°00′00″–03°08′00″S; 59°52′40″–59°58′00″W), Brazil. A detailed description of the area can be found in Oliveira Freitas et al. (2014). Briefly, the region is characterized by an equatorial climate (type Af in the Köppen system), with a mean annual temperature of 26°C and mean annual precipitation of 2,550 mm for the period 1966–2014, measured at the Reserve's meteorological station. The year of study, 2013, was anomalously wet, with 3,385 mm. A precipitation seasonality is characteristic with a drop between June and November, often referred to as "dry season"; however, true negative hydrologic balance (rainfall <100 mm per month) rarely exceeds 2 months. The landscape is undulating, with a clay-rich plateau at about 110–140 m a.s.l., dissected by drainage channels (*c*. 40–50 m a.s.l.). Soil types vary with topography: oxisols on the plateau, ultisols on the slopes and hydromorphic spodosols in the valleys (Chauvel, Lucas, & Boulet, 1987). The water-table is deep on the plateau, while the valley bottoms are usually waterlogged during the rainy season. The dominant vegetation type is lowland evergreen rainforest that is not affected by flooding (*terra firme*); the forest composition changes with topography (Ribeiro et al., 1999). Emergent trees (*c*. 45 m) are abundant on the plateau where the closed canopy reaches 35–40 m (Ribeiro et al., 1999).

### 2.2 | Sampling design and experimental treatment

Twenty sampling areas were chosen in a plateau area, using random points generated by the "Sample" module in IDRISI Selva v.17 software (Eastman, 2012). A minimum distance of *c*. 40 m between any two areas was observed to avoid autocorrelation in soil variables. At each area, four 1 m × 1 m permanent plots were established, at about 1.5 m from a focal reference tree, in a manner to maximize the separation distance among plots (see Figure S1 in supporting information). This resulted in 80 plots in total. Soil was sampled from all plots at the first sampling (13 April 2013, Time 0), before applying the urea treatment on 31 May 2013 (rainy season). Samples from the first sampling were in part used for the characterization of the spatial structure of bacterial and fungal communities, and are reported elsewhere. Two subsequent samplings on 28 July (Time 1, transition between rainy and dry season) and 6 November 2013 (Time 2, "dry season") were made from 40 plots (20 control plus 20 urea treatment) for the evaluation of changes in microbial community after urea addition. The choice of the sampling dates was based on the knowledge that bacterial communities in lowland tropical rainforest soils are subject to patterns of seasonality in rainfall (dry vs. rainy season) and related peaks and troughs in litterfall and fine root turnover (with potential changes in rhizodeposition), and consequently on the availability of carbon and nutrients. Thus, while evaluating the effects of urea addition, we considered the potential seasonal variation in soil bacterial communities.

We applied urea at a rate equivalent to 400 kg N $ha^{-1}$ in an aqueous solution, by spraying 1 L (1 mm rainfall) of solution per plot. The dose (concentration of N) of the urea used represents the N load that an average ungulate would deposit in 1 L of urine (Hamilton III et al., 1998; Seagle, 2003). The principal consideration was to reproduce dose to a large enough area that could then be repeatedly sampled, and adequately replicate it in space to capture spatial variation. A partial Mantel analysis that considered spatial patterns from

within-plot to the full extent of the experimental area has shown that there is a high spatio-temporal autocorrelation among the samples (both on taxonomic and phylogenetic data) within a 1 m × 1 m plot (E. Buscardo et al. unpublished manuscript). This additional information confirms that the experimental design and sampling scheme used are adequate and the results are robust. Control plots were not sprayed with equivalent amounts of water as the precipitation in the month of application was 450 mm.

## 2.3 | Soil sampling

Each plot was divided into 15 subplots and at every sampling, in a stratified random manner, three subplots were selected for sampling. After removing litter, soil cores were extracted to a depth of 5 cm, using a metal cylinder of 3 cm diameter. The cores were subsampled by using a sterile needle, pooled for every plot in a 2.5-ml Eppendorf vial and kept in a cool box until transported to the laboratory, where they were conserved at −20°C for molecular analyses. The remaining soil was pooled per plot, kept in polythene bags and transported to the laboratory for soil chemical analyses and determination of root biomass.

## 2.4 | Molecular analyses

Of the 20 areas, 15 were included in molecular analyses for the characterization of the bacterial communities, resulting in a total of 90 samples (control: 3 sampling dates × 15 plots; treatment: 1 pre-treatment × 15 plots plus 2 post-treatment × 15 plots). DNA extractions were made using 250 mg of soil with a PowerSoil DNA Isolation Kit (MoBio Laboratories). The primers 27F (Amann et al., 1990) and 338R (Lane, 1991) were used for amplification of bacterial 16S rDNA. The forward primer was linked through a barcode adaptor with the Ion-Xpress barcode and the Ion adaptor "A" sequence, while the reverse primer was linked with the P1 sequence (Table S1). Details on PCR settings and library preparation for the emulsion PCR are in the supporting information (Appendix S1). Amplicon libraries were sequenced using Ion 318 Chips (24 barcodes per chip) by an Ion Torrent Personal Genome Machine (Thermo Fisher Scientific Inc.), at the Federal University of Pará, Belém, Brazil.

## 2.5 | Sequence processing

The total number of raw sequences per sample was between 195,000 and 375,000. The primers were removed and poor quality ends were trimmed off, based on .02 error probability limit in Geneious Pro 5.6.1 (BioMatters). Subsequently, sequences were filtered, using USEARCH v.8.0 (Edgar, 2010), based on the following settings: all sequences were truncated to 200 bp and sequences with 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 simultaneously excluding putative chimeric sequences. After excluding singletons, and discarding OTUs that were also present in any of the five negative samples, the dataset contained 1,249,244 sequences in 11,094 OTUs. As the sequencing depth of our samples was very high and because the reliability of OTUs with a low number of sequences may be questioned, we choose to follow the conservative approach suggested by Lindahl et al. (2013) and removed all OTUs that had less than six occurrences across all samples. For the final analyses, the total number of sequences was thus reduced to 1,234,308 (5,467 OTUs; Table S2). Sequences were assigned to taxonomic groups by RDP Naïve Bayesian Classifier Version 2.10 (Wang, Garrity, Tiedje, & Cole, 2007).

## 2.6 | Soil analyses

Fresh moist samples were used for the determination of mineral N concentration (Soil Laboratory, INPA). Soil $NH_4^+$ (Mackereth, Heron, & Talling, 1978) and $NO_3^-$ (Allen, 1989) were quantified by spectrophotometry, after extraction in 0.5 M $K_2SO_4$ and filtration (Whatman No. 44), using flow injection analysis (UV-1800 Shimadzu). Soil moisture content was determined by the gravimetric method by drying at 105°C to constant mass (Allen, 1989). Soil organic matter, pH, phosphorous ($PO_4^{3-}$), potassium ($K^+$), magnesium ($Mg^{2+}$) and exchangeable acidity ($H^+$ plus $Al^{2+}$) were determined following van Raij, Andrade, Cantarella, and Quaggio (2001). Root biomass was determined by oven drying at 65°C to constant mass after separating fine (≤2 mm) and coarse roots in each soil sample.

## 2.7 | Data analyses

All analyses of molecular data were made on both non-rarefied data and on data rarefied to 5,222 sequences (the size of the smallest library). All phylogenetic information was derived from a phylogenetic tree built with an approximate maximum-likelihood approach in FastTree v.2.1 (Price, Dehal, & Arkin, 2010), using the – DUSE_DOUBLE parameter. The phylogeny was midpoint rooted using the Archaeopteryx interface (Han & Zmasek, 2009). All subsequent analyses were made in the R environment (R Development Core Team, 2016). The generalized UniFrac procedure (Chen et al., 2012) was implemented in "GUniFrac" v.1 to compute UniFrac distances (Lozupone & Knight, 2005). Rarefaction curves, alpha-diversity measures, Shannon index and abundance-based coverage estimator (ACE) were computed in "vegan" v.2.3-5 (Oksanen et al., 2016). Phylogenetic diversity was calculated as Faith's phylogenetic diversity (PD) in "picante" v.1.6-2 (Kembel et al., 2010). Changes within the bacterial phylogenetic community composition between control and treatment samples were assessed on presence/absence data at each sampling time with the ADONIS function in "vegan" using 10,000 permutations. Non-metric multidimensional scaling (NMDS) ordinations based on Bray–Curtis and generalized UniFrac (GUniFrac) distance matrices were carried out on presence/absence data to visualize differences in OTU-based community composition. The GUniFrac distance matrix used in both ADONIS and NMDS was made by setting alpha, which controls the weight on lineages with

common taxa, to 0.5 to provide the best overall power (Chen et al., 2012). To visually compare bacterial relative sequence abundances (treatment vs. control), a heat map based on the 300 most abundant OTUs and phylogenetic data was made using NMDS-based ordering (Bray–Curtis distances) in "PHYLOSEQ" v.1.6.1 (McMurdie & Holmes, 2013). Differences between treatments in rarefied relative abundances of the first 300 most abundant OTUs (75% of the total abundance) were computed on data at phylum level and at lower taxonomic levels (i.e. genus, family, order and class depending on the taxonomic resolution available) and evaluated with the Wilcoxon signed-rank test. Significances were corrected for multiple comparisons using the false discovery rate (FDR) method (Benjamini & Hochberg, 1995). In addition, we carried out an indicator species analysis by combining groups (treatment × time) in "INDICSPECIES" v.1.7-6 (Anderson, 2001). Differences in diversity indices among bacterial communities and in soil chemical properties and root biomass (urea treatment vs. control) at different sampling dates were evaluated with the paired *t* test and with the Wilcoxon signed-rank test.

## 3 | RESULTS

### 3.1 | Bacterial community assessment

Sequence read numbers after quality control and removal of all OTUs that had less than six occurrences across all samples averaged 14,521 (range 5,222–50,390) per sample and resulted in a total of 5,467 OTUs (Table S2). OTUs were representative of the total bacterial community as shown by the rarefaction curves that tended to saturation for almost every sample (Figure 2). The abundance-based coverage estimator (ACE) predicted maximum numbers of OTUs per plot ranging from 495 to 1,635 (mean, 978; Table S3). Species richness (SR) per plot varied between 462 and 973 (mean, 724), and Shannon's diversity (H') ranged between 4.1 and 6 (mean, 5.3), while Faith's phylogenetic diversity (PD) ranged between 126 and 208 (mean, 166). No significant differences were found for SR, H' and PD, for all paired comparisons at the three sampling dates (for SR see Figure S2).

There were no significant differences in bacterial communities between control and urea plots before applying the urea treatment (Time 0, Table S4). After urea application, differences between treatment and control on rarefied bacterial communities were significant both at Time 1, 2 months after urea treatment ($\text{ADONIS}_{\text{Time 1}}$: $F = 6.01$; $R^2 = 0.182$; $p < .001$) and Time 2, 5 months after the treatment ($\text{ADONIS}_{\text{Time 2}}$: $F = 4.412$; $R^2 = 0.15$; $p < .001$; see Table S4 for differences obtained on non-rarefied data). The samples partitioned into three different clusters by the two NMDS analyses based on taxonomic and phylogenetic composition (Figure 3; see Figure S3 for the two NMDS obtained on non-rarefied data). All pre-treatment communities at Time 0 clustered together with those of control plots at Times 1 and 2 (Figure 3). In contrast, communities sampled following urea addition formed two partially overlapping clusters, representing Times 1 and 2, and distinctly separated from that at Time 0.

A total of 89.9% of the sequences were classifiable to a taxonomic level. Communities were dominated by *Proteobacteria*, with an average relative abundance across samples of 28.4%, followed by *Firmicutes* (19.7%), *Acidobacteria* (18.6%) and *Actinobacteria* (15.6%).

### 3.2 | Major shifts in bacterial diversity following urea treatment

Urea addition caused significant shifts in bacterial community composition at all taxonomic levels (Figure 4). The heat map showed an evident increase of a group of OTUs and a discernible decrease in the relative sequence abundance of several OTUs in the urea-treated plots (Figure S4). The Wilcoxon test indicated 132 taxa to have changed significantly following the urea treatment (Figure 4; Table S5). At the phylum level, *Acidobacteria* decreased in relative sequence abundance, while *Bacteroidetes* and *Gemmatimonadetes* increased when compared with the control both at Times 1 and 2. At a finer taxonomic resolution, 10 orders, 9 classes, 17 families and 20 genera (Figure 4) and 73 of the

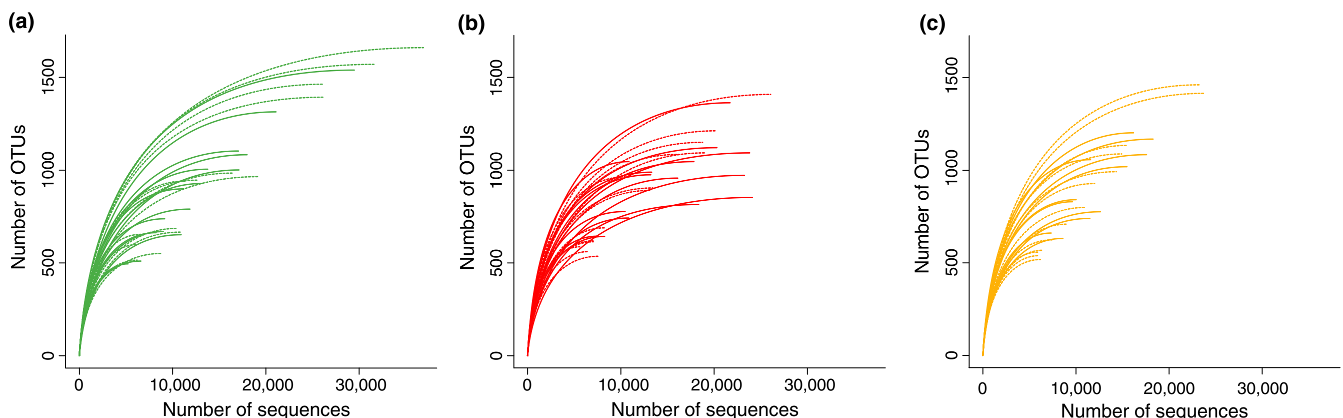


**FIGURE 2** Rarefaction curves representing the relation between the number of sequences (obtained by Ion Torrent sequencing of bacterial 16S rDNA amplicon libraries generated from DNA extracted from soil samples in a lowland Amazonian rainforest affected by N pulse-type perturbation), and the number of operational taxonomic units (OTUs), assigned at 97% sequence similarity. Time 0 (pre-treatment), green (a); Time 1 (59 days after urea application), red (b); Time 2 (159 days after urea treatment), yellow (c); dashed line, control plots; continuous line, urea-treated plots

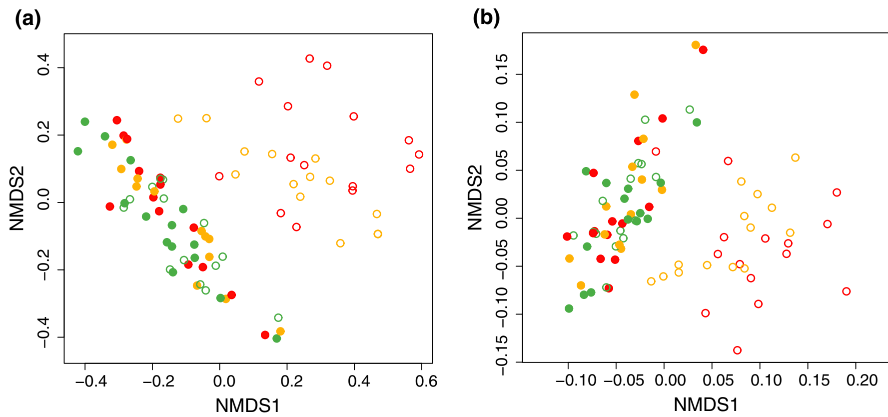


**FIGURE 3** Non-metric multidimensional scaling (NMDS) ordination plots based on taxonomic (a) and phylogenetic rarefied data (b) of soil bacterial communities in response to urea addition in a tropical lowland rainforest. Stress 4D: (a), 0.101; (b), 0.109; Time 0, green; Time 1, red; Time 2, yellow; filled symbols, control plots; hollow symbols, urea-treated plots

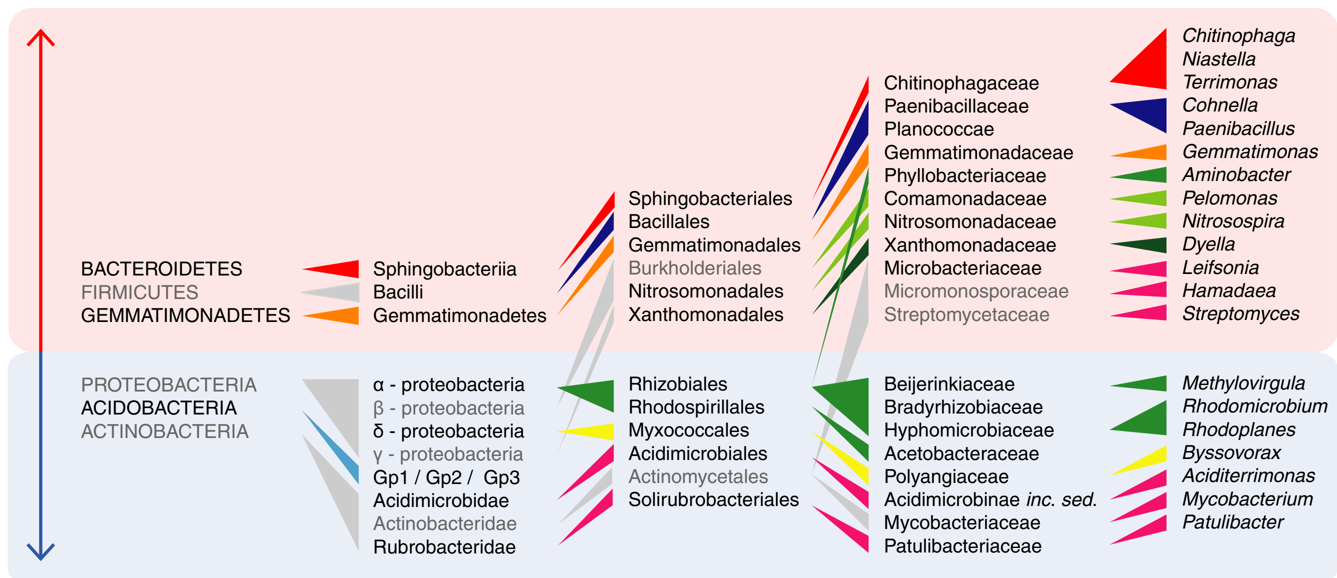


**FIGURE 4** Significant changes in relative abundances of soil bacteria (phylum to genus levels) in response to urea addition in a tropical lowland rainforest. Red arrow, increased post-treatment abundance; blue arrow, decreased post-treatment abundance; grey, taxa affected by urea addition exclusively at lower taxonomic levels. Different colours represent taxa belonging to different phyla: red, *Bacteroidetes*; blue, *Firmicutes*; orange, *Gemmatimonadetes*; medium green, *Alphaproteobacteria*; light green, *Betaproteobacteria*; yellow, *Deltaproteobacteria*; dark green, *Gammaproteobacteria*; light blue, *Acidobacteria*; fuchsia, *Actinobacteria*

300 OTUs (Table S5) exhibited statistically significant differences in pair-wise comparisons between control and urea-addition plots over the three sampling times. Among these, there were taxa known to be involved in the nitrogen cycle whose relative abundances were related to soil mineral $NH_4^+$ plus $NO_3^-$, and are shown in Figure 5 (see Figure S5 for relationship between relative abundances and $NH_4^+$). None of the taxa differed between control and pre-treatment at Time 0. Seventy-one OTUs were significantly different between urea and control plots at Time 1 and 27 OTUs at Time 2. Twenty-five of these differences were observed both at Times 1 and 2, all of them maintaining the same direction of change (Table S5). The indicator species analysis showed large differences between control and urea-treated plots, with 51 OTUs highlighted as indicator species in control plots and another 28 OTUs in urea plots. There were only two common species indicated for control and urea treatment (Time 1); this increased to 13 by Time 2 (Table S6).

### 3.3 | Soil analyses

Differences between control and urea plots were significant at Time 1, 2 months after the urea application, for $NH_4^+$ (17.1 vs. 125.1 μg/g), $NO_3^-$ (1.9 vs. 29.1 μg/g), pH, (3.9 vs. 4.1), exchangeable acidity (194 vs. 137 mmolc/kg), soil organic matter (7.1% vs. 6.3%) and cation exchange capacity (201 vs. 145 mmolc/kg), but not at Time 2, 5 months after the treatment (Table S7).

## 4 | DISCUSSION

The limited number of studies on soil microbiology in *terra firme* forests (e.g. Barberán et al., 2015; Kim, Sparovek, Longo, De Melo, & Crowley, 2007; McGuire, Fierer, Bateman, Treseder, & Turner, 2012; Tripathi et al., 2012) calls for more basic work, particularly on

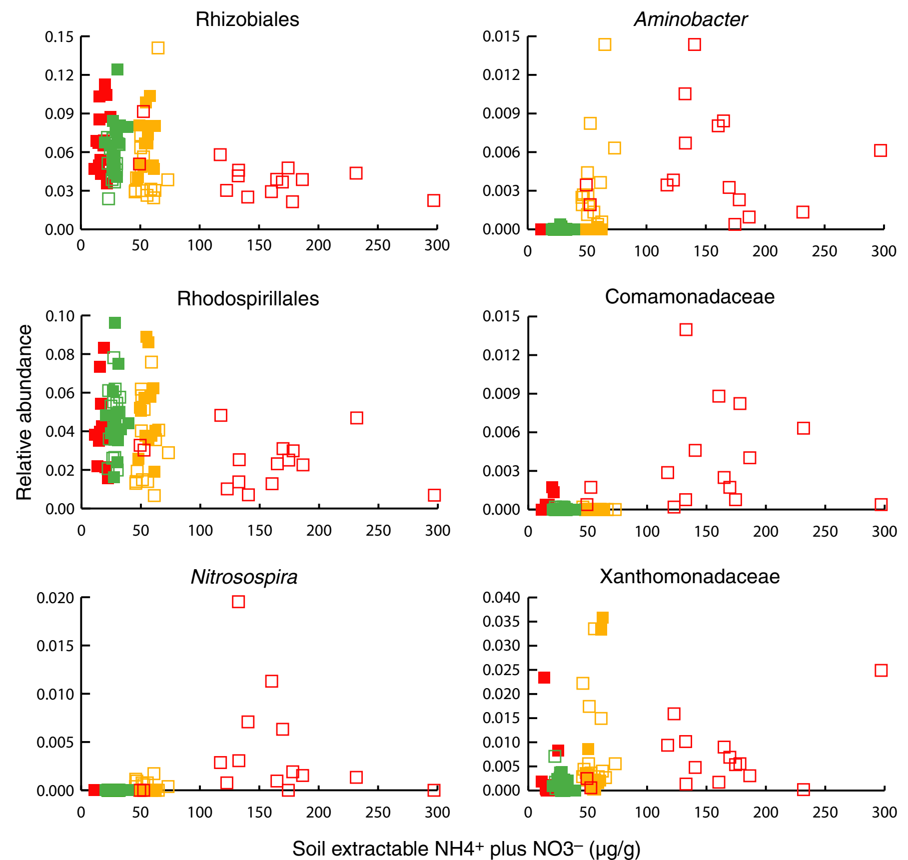


**FIGURE 5** Relationship between the relative abundance of bacterial taxa known to be involved in the nitrogen cycle and soil mineral N. The figure includes taxa whose relative abundance significantly increased or decreased (Table S5), in response to urea addition in a tropical lowland rainforest. Time 0 (pre-treatment), green; Time 1 (59 days after urea application), red; Time 2 (159 days after urea treatment), yellow; filled symbols, control plots; hollow symbols, urea-treated plots

establishing a link between community composition, taxon abundance and function, and rates and controls of biogeochemical transformations, which is particularly challenging in tropical forest soils (Pajares & Bohannan, 2016).

Our experiment represents the first study to simulate the patch dynamics of soil processes attributable to concentrated local deposition of mammalian urine-N and its effects on soil bacterial community composition in tropical forests. The present study highlights an area often overlooked in ecology, namely the importance of the role of animals in creating spatio-temporal resource availability patterns, thus contributing to patch dynamics and niche differentiation in soil organisms and to the spatial heterogeneity of elemental cycling in ecosystems. In our study, the short-term responses of bacterial communities to simulated mammalian urine-N pulse-type perturbation showed significant changes in composition and relative abundance of taxa involved in physiologically "narrow" processes as defined by Schimel, Bennett, and Fierer (2005). We discuss below these changes following our three main hypotheses.

## 4.1 | No change in taxon richness after high-N pulse

Bacterial community change to N pulse could have various component processes at play over time. Some members of the community could simply suffer toxicity and die; others may respond to the pulse by forming resistant structures and remain inactive until environmental condition return to be within their tolerance (Schimel et al., 2007). In a broader community sense, one may evoke a reduction of diversity by competitive exclusion whereby the high-N pulse would favour some bacteria to enter the community and colonize niche space made available by mortality/inactivity of disturbance-sensitive taxa. This mechanism appears to operate in a temporally dynamic manner after N pulse and subsequent patch recovery, during which the recovery from dormancy in situ is likely to result in competitive exclusion of the taxa favoured by high-N pulses.

According to the intermediate disturbance hypothesis (IDH), we expected that a pulse-type disturbance by urea addition would reduce the taxonomic richness of soil bacteria. We did not find evidence for this as urea addition did not alter the species richness (SR) and diversity of bacterial OTUs. The IDH has been tested amply in relation to vascular plant and animal SR (see examples in Cain, Bowman, & Hacker, 2014; Tokeshi, 1999), but not in relation to microbiota. In addition, there are no reports from tropical forests on soil microbial richness responses to N addition. Therefore, results from the literature are difficult to compare with ours (type of ecosystem and organisms studied, doses of N applied and experiment duration). A recent short-term experiment ($NH_4NO_3$, 10 kg ha$^{-1}$ year$^{-1}$, 6-weeks, Campbell, Polson, Hanson, Mack, & Schuur, 2010) in arctic tundra, one of the most N-limited ecosystems, did not show any effect on bacterial diversity. The N dose applied by Campbell et al. is considered to be around the critical annual N load for tundra. However, in comparison with our application dose, it is very low and probably does not represent an acute pulse-type perturbation. In general, long-term N fertilization

experiments (press-type disturbance) in several natural ecosystems, including grassland ($NH_4NO_3$, 10 kg ha$^{-1}$ year$^{-1}$, 20 years, Coolon, Jones, Todd, Blair, & Herman, 2013), Mongolian steppe (urea, 50–150 kg ha$^{-1}$ year$^{-1}$, 8 years, Li et al., 2016) and arctic tundra ($NH_4NO_3$, 10 kg ha$^{-1}$ year$^{-1}$, 24 years, Campbell et al., 2010) have shown a decrease in microbial diversity. The response time of organisms to disturbance differs and depends on the organism in question (Eviner, Chapin, & Vaughn, 2000), especially their generation time. We consider that the length of our experiment was suitable to characterize adequately the temporal development of richness and community composition of soil bacteria.

Although urea addition did not reduce taxon richness and diversity, bacterial community composition significantly changed, from phylum to OTU levels. These changes were still pronounced 2 months after the urea application, while another 3 months later, there was an evident tendency of the bacterial communities becoming similar to their pre-treatment compositions. Changes in bacterial communities were accompanied by corresponding changes in mineralized soil N. Significant increases in nitrate and ammonium concentrations were observed 2 months after urea addition, but mineral N concentrations 159 days after the urea addition were not different from those in the control plots. This may be related to several processes, including increased nitrification and denitrification, immobilization and/or nitrate leaching. N addition generally stimulates the processes of nitrification and denitrification (Compton, Watrud, Arlene Porteous, & DeGrood, 2004; Kolb & Martin, 1988; Prosser, 2005) and increases N leaching (Magill et al., 1997), especially on kaolinitic clays, with poor nutrient-holding capacity as in our study area (Chauvel et al., 1987).

## 4.2 | Increased availability of mineral N leads to an increased abundance of nitrifiers/denitrifiers and to a decreased abundance of $N_2$-fixing bacteria

The increase in soil $NH_4^+$ concentration in response to urea addition may be linked to the increased relative abundance of ammonia-oxidizing bacteria (AOB) belonging to *Nitrosospira*, as reported here (exclusively observed in urea-treated plots) and in previous studies (Fierer, Carney, Horner-Devine, & Megonigal, 2009; Prosser, 2005). The increase in the abundance of nitrifiers following N fertilization has also been indicated by an increase in both cell counts and nitrification potential (Prosser, 2005) and by an almost exclusive presence of the amoA gene in long-term $NH_4NO_3$-treated soils when compared with controls (Compton et al., 2004). These indicate that substrate availability may be an important limiting factor for nitrification in lowland evergreen tropical forests. The observation of a single OTU with low abundance representing a *Nitrosospira* species possibly suggests a relatively low contribution of AOB to nitrification. It has been demonstrated that different AOB and archaeal ammonia-oxidizers (AOA, not studied here) are selected according to soil acidity and that the differences in community structure and abundance are reflected in different contributions to ammonia-oxidizer activity (Nicol, Leininger, Schleper, & Prosser, 2008). The results of several studies have shown that low soil pH favours the niche specialization of AOA (Prosser & Nicol, 2012) and could therefore explain the low AOB presence in our soils.

As distantly related micro-organisms can have denitrifying functions and closely related strains can exhibit different respiratory pathways, their ability to denitrify cannot be predicted simply from taxonomic affiliation (Philippot, Hallin, & Schloter, 2007). Among the phylogenetically diverse group of denitrifiers, several bacteria are also involved in other steps of the N cycle, such as nitrification or biological $N_2$-fixation (BNF, Philippot et al., 2007). Many $N_2$-fixing rhizobia are known for their denitrifying abilities (Shapleigh, 2013): *Phyllobacteriaceae* (e.g. *Aminobacter*), for instance, belong to Rhizobiales and integrate many species that take part in the physiologically "narrow" process of denitrification (Labbé, Parent, & Villemur, 2004). The relative abundance of *Phyllobacteriaceae* at our site increased after urea treatment. This trend was also observed for *Xanthomonadaceae* and *Comamonadaceae*, which also include members with denitrifying capacities (Green et al., 2012; Khan, Horiba, Yamamoto, & Hiraishi, 2002; Xu et al., 2015). The increase in denitrifiers could have been caused by an increased availability of $NO_3^-$ in the urea-treated plots. While this assumption is based exclusively on semi-quantitative taxonomic information, it is supported by complementary studies conducted within the same reserve and in other tropical lowland forests that have shown enhanced denitrification activities and simultaneous increases in N-oxide emissions following N addition (Corre, Sueta, & Veldkamp, 2014; Keller, Kaplan, Wofsy, & Da Costa, 1988; Koehler, Corre, Veldkamp, Wullaert, & Wright, 2009).

Free-living organisms can account for a significant proportion of total BNF in tropical forests (Gehring, Vlek, de Souza, & Denich, 2005), although most of their communities remain uncharacterized, and their role in regulating BNF rates is unknown (Reed, Townsend, Cleveland, & Nemergut, 2010). *Rhizobiales* and *Rhodospirillales*, known for hosting members involved in BNF (Hoppe et al., 2014; Jones et al., 2014) decreased following the urea treatment. This finding is in agreement with a decrease in BNF in tropical forests after application of $NH_4NO_3$ (Cusack, Silver, & McDowell, 2009) and urea (Barron et al., 2009). Kolb and Martin (1988) have shown that the decrease in BNF following the application of $NH_4NO_3$ was accompanied by a decrease in both soil free-living $N_2$-fixers and in the proportion of diazotrophs among heterotrophs, likely by competitive suppression of diazotrophs by non-diazotrophs. A reduced amplification of the nifH gene that detects free-living $N_2$-fixers was observed by Compton et al. (2004) in a long-term study, after $NH_4NO_3$ addition (50–150 kg ha$^{-1}$ year$^{-1}$) to temperate pine forest soils. The authors suggested that chronic N additions suppressed the potential for BNF, indicating a negative feedback between N availability and BNF by free-living microbes. In the present study, Rhizobiales appeared to be very sensitive to urea addition, being the only order among those negatively affected whose abundance did not return to pre-treatment levels 5 months after the application. These results highlight how perturbation can completely displace unique components of $N_2$-fixing bacteria (Shaffer, Widmer, Porteous, & Seidler, 2000), possibly altering links between soil microbial community structure and function that have already been demonstrated for denitrification (Cavigelli

& Robertson, 2000) and nitrification (Carney, Matson, & Bohannan, 2004). Increasing N inputs can select for groups of microbes with distinct physiologies (Schimel et al., 2005). According to the results obtained in the present study, we can accept our second hypothesis and affirm that an increased availability of mineral N led to an increase of the relative abundance of nitrifiers and denitrifiers and to a decrease in the relative abundance of $N_2$-fixing bacteria.

### 4.3 | Temporal legacy of N pulse on community composition and functional group profile

The general trend after N addition across ecosystems, including temperate and lowland tropical forests, has been a reduction in both microbial biomass and respiration and decomposition rates (Compton et al., 2004; Ramirez, Craine, & Fierer, 2012). Fanin, Hättenschwiler, Schimann, and Fromin (2015) have attributed non-significant or negative effects of urea addition on microbial biomass and decomposition rates to high soil N availability compared to C and P that could have allowed sufficient N uptake by soil micro-organisms with no further stimulation by additional N. In their experiment in French Guyana over a 2-year period (130 kg N $ha^{-1}$ $year^{-1}$—*c.* one-third of that applied in the present study), they applied two equal doses of urea per year, and collected soil samples 3–4 months after urea application. It is unlikely that the highly weathered and leached tropical soil was more C or P limited in the study by Fanin et al. than in our study. Although the two studies are not directly comparable, we suggest that the length of time since last N application, the dose and seasonality may determine to a large extent effects/responses. In our study, the composition of soil bacterial communities differed significantly from the pre-treatment communities 58 days and 159 days after urea addition; however, community composition at the latter time tended to be closer to the original, indicating that microbial communities may be more resilient after pulse disturbances than after press disturbances (Shade et al., 2012). The bacterial communities responses in the present study appear to have had a slight lag in following changes in soil mineral N. This tendency was observable at different taxonomic resolutions and also in relation to taxa known to be involved in decomposition, nitrification, denitrification and BNF. However, as there were observable differences in community composition and functional group profile 159 days after urea application, our third hypothesis of a transient temporal legacy of urea addition on community composition and functional group profile can be accepted (Figure 6).

## 5 | CONCLUSIONS

A pulse-type perturbation caused by urea addition in a lowland evergreen tropical forest in central Brazilian Amazonia increased the availability of mineral N and altered significantly the composition of bacterial communities and the relative abundance of taxa involved in physiologically "narrow" processes. Taxa known for their nitrifying and denitrifying capacities responded to short-term urea addition with

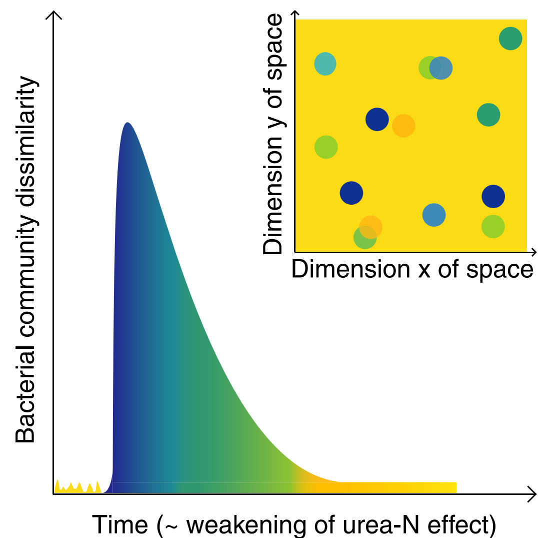


**FIGURE 6** Conceptual model of how nitrogen (N) pulse-type perturbation affects the spatio-temporal dynamics of soil bacterial communities. Mammal urine input causes a mosaic of urea-N-enriched soil patches, which, with time return to the baseline N level, accompanied by shifts in community composition (decreasing dissimilarity from matrix). The main graph represents bacterial community dissimilarity from the matrix in function of urea-N loading in a single patch. The inset shows a hypothetical spatial pattern of urea-N enriched patches in various phases of temporal recovery to baseline. Recovery or community similarity is indicated by colour similarities. The colour gradient represents the dissimilarity in bacterial community composition, induced by N pulses (dark blue) and the gradual recovery of the original community over time (yellow)

an increase in their relative abundance while $N_2$-fixing bacteria abundance decreased after treatment.

While there was a general tendency of bacterial communities to approach the pre-treatment state with time, the pulse-type perturbation left a temporal legacy on both community composition and functional group profile.

All pulse-type nutrient additions act as disturbance and thus need to be interpreted carefully. In our study, we described a specific case, where we characterized the impact of naturally occurring N pulses in a tropical forest ecosystem. We suggest that high-N pulse-type input that occurs on a daily basis creates a spatio-temporally highly patchy soil environment which contributes to the maintenance of the observed high richness and diversity of soil microbiota. The apparent temporal resilience of community composition to N pulse may well reflect this inherent spatio-temporal dynamic.

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## AUTHORS' CONTRIBUTIONS

L.N. conceived the research; E.B. and L.N. established the experiment, applied the urea treatment and carried out the soil sampling; E.B. extracted the DNA, carried out the PCRs and prepared the samples for the Ion Torrent runs; M.S.R.B. and S.S.A. performed the Ion Torrent runs; R.D.C. gave technical support; J.G. conducted the bioinformatics; E.B. made the statistical 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.L.C.S., R.T.J.R. and A.P.S. contributed analytical tools. All authors read and approved the final version of the manuscript.

## DATA ACCESSIBILITY

Representative sequences of all OTUs in this study have been submitted to Genbank (KY542280-KY547746).

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## SUPPORTING INFORMATION

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