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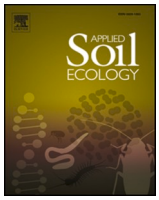

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ABSTRACT

Although involved in key functions of the terrestrial ecosystems, the activity and the diversity of soil microorganisms can be severely limited by energy and nutrients in weathered tropical soils. To optimize nutrient cycling for crop nutrition, assessing which nutrients limit the activity of the microbial food web is thus essential. This was our aim in this study carried out on a tropical ferrallitic soil from a natural grassland in Madagascar. For this purpose, we employed an innovative nutrient-omission approach in microcosms to test the distinct effects of omitting C, six nutrients (N, P, K, S, Ca, Mg) and a cocktail of micronutrients (B, Mn, Cu, Na and Mo) on the composition, biomass and activities of the microbial community and on the abundance and biomass of their nematode grazers. C and P were identified as primary limitations, but other nutrients (N, Mg and S) played significant roles as co-limiting factors. Some bacterial and fungal taxa were significantly associated to specific nutrient deficiencies and can act as biological indicators. Additionally, the abundance and biomass of microbial-feeding nematodes provided valuable insights into the responses of the soil microbial community to nutrient deficiencies. These findings contribute to our understanding of nutrient dynamics and microbial community growth in tropical grassland ecosystems and have implications for sustainable crop fertility management and ecosystem ecology in similar environments.

1. Introduction

The soil micro-food web is a complex, size-structured network consisting of bacterial and fungal cells, along with their grazers, such as protists and nematodes (Geisen et al., 2015; Potapov et al., 2021). It plays a major role in terrestrial ecosystems by transforming and processing carbon and nutrients (Ferris, 2010; Bardgett and van der Putten, 2014; Kou et al., 2018; Ranoarisoa et al., 2020). The breakdown of organic compounds, nutrient mining from mineral soil, nutrient transformation and mineralization, and greenhouse gas emissions greatly depend on the activities of soil microorganisms and their associated grazers (van der Heijden et al., 2008; Trap et al., 2016; van den Hoogen et al., 2019; Wilschut and Geisen, 2020). Better quantifying the energetic and nutritional limitations of the soil micro-food web is a major issue in ecosystem ecology and in the perspective of relevant and sustainable crop fertility management.

Following the "law of minimum" von Liebig framework (Liebig, 1846), carbon (C) is often considered as the primary element limiting the activity of heterotrophic microorganisms in bulk soils (Cheng et al., 1996; Demoling et al., 2007; Soong et al., 2020). However, under tropical climates, most soils are deeply weathered and depleted in available phosphorus (P) (Walker and Syers, 1976), which strongly limits microbial activity (Xiao et al., 2020). The main reason is that mineral available P (phosphates) is adsorbed on insoluble aluminum and iron complexes (Gérard, 2016; Ramaroson et al., 2017). Tropical soils are thus considered severe P-depleted ecosystems (Dubus and Becquer, 2001; Tiessen, 2005), leading to strong P limitation for soil microbial communities (Manzoni et al., 2010). Additionally, nitrogen (N) has also been identified as a limiting element worldwide and in tropical ecosystems (Fanin et al., 2016), but in a lesser extent (Vitousek et al., 2010;

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Camenzind et al., 2018; Du et al., 2020), resulting in lower soil microbial N:P ratio in comparison to higher latitudes (Cleveland and Liptzin, 2007; Li et al., 2014).

As stated by Camenzind et al. (2018), few studies have addressed the importance of mineral nutrients other than N or P for soil micro-food web activity. Potassium (K), sulphur (S), calcium (Ca) and magnesium (Mg) are poorly investigated as possible limiting nutrients, while the amounts of these nutrients decrease within ongoing rock weathering and soil development (Vitousek and Sanford Jr, 1986; Austin and Vitousek, 1998). We suppose that one of the reasons for the limited literature on the subject is methodological. The usual cross-fertilization method commonly used in the literature to test nutrient limitation hypotheses results in a rapid increase in the number of treatments as the number of investigated nutrients grows following a 2^n power law, where 'n' represents in the number of nutrients. When examining the 7 nutrients mentioned earlier (C, N, P, K, S, Mg and Ca) alongside a negative control, it would require a total of 128 treatments. Alternatively, the omission-trial approach presents a viable option. This method involves the systematic removal of one nutrient from a complete fertilization solution, allowing for the investigation of multiple nutrients without the burden of an overly complex experimental design. Widely used in agronomy to assess crop nutrient limitations (Nziguheba et al., 2009; Raminoarison et al., 2020), this approach holds promises for application in soil microbial ecology as well.

In this study, in order to optimize nutrient cycling for crop nutrition, we aimed at assessing which nutrients among C, N, P, K, S, Ca, Mg and micronutrients limit the activity of the microbial food web of a tropical ferrallitic soil (Ferralsol). For this purpose, we employed an innovative nutrient-omission approach in microcosms to test the distinct effects of omitting C, six nutrients (N, P, K, S, Ca, Mg) and a cocktail of micronutrients (B, Mn, Cu, Na and Mo) on the composition, biomass and activities of the microbial community and on the abundance and biomass of their nematode grazers in a Ferralsol from a natural grassland in Madagascar. As previously mentioned, Ferralsols are known for their nutrient scarcity for crops (Raminoarison et al., 2020). We hypothesized that C and P will be the first elements limiting the soil micro-food web.

2. Materials & methods

2.1. Soil sampling and preparation

We collected 10 soil samples from the top 20 cm layer of a Ferralsol soil in the natural savanna of Imerintsiatosika in the Itasy region near Antananarivo (1400 m altitude, 19°05′40"S; 47°25′65″E, Madagascar) in June 2018. The samples were taken from a 10 m^2 plot and combined to create a composite sample. The site had been under grass fallow vegetation, predominantly dominated by *Aristida* sp. grasses, for at least 100 years. The average annual precipitation at the site was 1500 mm, and mean annual temperature was 20 °C. The soil is a Ferralsol with a sandy-clay texture with 28.9 % clay, 42.4 % fine silt, 15.3 % coarse silt and 13.4 % sand, a pH_{H20} of 4.7, a total organic C content of 20.8 g kg^{-1}, a total N content of 1.3 g kg^{-1}, a C:N of 16, a total P content of 380 mg kg^{-1}, an Olsen P content of 7.12 mg kg^{-1}, an exchangeable K, Ca and Mg contents of 30.7, 120.7 and 28.3 mg kg^{-1}, respectively. The cationic exchange capacity was 1.34 $cmol^{+}$ kg^{-1} and the water-holding capacity (WHC) was 0.49 g g^{-1} dry soil. For a detailed description of the soil analyses, please refer to Raminoarison et al. (2020). The day after sampling, the soil was sieved through a 2 mm mesh, and coarse roots were removed. The soil was then air-dried for 7 days and stored overnight in the dark before use.

2.2. Microcosm set up

Microcosm setup consisted of two batches. In the first batch, 20 g of 2-mm sieved dry soil was placed in 150-mL glass microcosms. The soil water content was adjusted to 60 % of the water-holding capacity by adding sterile deionized water. The microcosms were then hermetically sealed and pre-incubated for 7 days in the dark at 26 °C, with daily ventilation. After the 7-day pre-incubation period, the microcosms were opened, and fertilizers were added to each microcosm to reach 80 % of the water-holding capacity. Subsequently, the microcosms were incubated for 28 days in the dark at 26 °C.

In the second batch, 1 L hermetic glass microcosms containing 120 g of 2-mm sieved dry soil were set up in parallel under the same conditions. Both types of microcosms were handled in the same manner. The first batch was used for determining microbial respiration, DNA analyses, biomass carbon and phosphorus contents, and enzyme activities. The second batch was used for nematode extraction requiring larger soil volumes.

2.3. Experimental design: the nutrient-omission fertilization

The experimental design included ten treatments: (1) a positive control coded "PC" with C and all nutrients added; (2) a negative control coded "NC" without addition of C nor nutrients; (3) all added but C omitted coded "-C"; (4) all added but N omitted coded "-N"; (5) all added but P omitted coded "-P"; (6) all added but K omitted coded "-K"; (7) all added but S omitted coded "-S"; (8) all added but Ca omitted coded "-Ca"; (9) all added but Mg omitted coded "-Mg"; (10) all added but a cocktail of micronutrients (B, Mn, Cu, Na and Mo) omitted coded "-mnut". Each nutrient treatment was repeated three times. A total of 30 microcosms were set up for each batch experiment.

2.4. Fertilization

The fertilizers were composed of mass half glucose and half cellulose for C, NH_4NO_3 for N, NaH2PO4 for P, KCl for K, K2SO4 for S, CaCl2 for Ca, MgO for Mg, H3BO3 for B, $MnCl_2$ for Mn, copper standard solution 100 mg L^{-1} for Cu, and Na2MoO4 for Mo. $MgSO_4$ was also used to compensate the loss of accompanying ions. We provided 1 g kg^{-1} of C as glucose (0.5 g kg^{-1}) and cellulose (0.5 g kg^{-1}) corresponding to 3.4 % of soil total C content. The macronutrients N, P and K were applied at the following rates: 300 mg-N/kg, 200 mg-P kg^{-1}, 200 mg-K kg^{-1} corresponding to agronomic recommendations (Raminoarison et al., 2020). The remaining nutrients S, Ca, Mg, B, Mn, Cu, Co, Na and Mo were applied at 65 mg-S kg^{-1}, 150 mg-Ca kg^{-1}, 50 mg-Mg kg^{-1}, 32.5 mg kg^{-1} for B, Mn, Cu, Co, Na and Mo. When providing the nutrients in solution, the soil water content was adjusted to 80 % of its water-holding capacity with sterile water and the microcosms were closed.

2.5. Soil sampling and abiotic measurements at the end of the experiment

After 28 days of incubation, the soil was sampled from each microcosm to analyze soil pH and nutrient contents. Soil pH was measured by stirring a soil-mixture with a ratio of 1:2.5 for 30 min in distilled water. Mineral N (nitrate and ammonium) content was determined from fresh samples (0.5 g) after KCl 1 M extraction by colorimetry according to the Berthelot reaction (ammonium) and the Griess reaction (nitrate) (Mulvaney, 1996) using an automated continuous flow analyzer San^{++} (Skalar analytique, France). A subsample was air-dried during 7 days and total C and N contents were determined by dry combustion in a Flash 2000 CHN analyzer (PerkinElmer Inc., Waltham, MA, USA). Inorganic P was extracted with an ion-exchange resin filled with carbonate 0.5 M (Amer et al., 1955); then, total P concentration in the solution was analyzed in HCl/NaCl 0.1 M solution by colorimetry with malachite green (Rao et al., 1997). The K, Ca and Mg contents were determined by suspending soil in cobaltihexamine chloride solution (100 mg L; 1:10 ratio) at soil pH and measured using an atomic absorption spectrophotometer (iCE 30,000 Series AA Spectrometer, Belgium) with lantana reagent (Ciesielski and Sterckeman, 1997).

2.6. Soil microbial measurements

2.6.1. Microbial respiration

Measurements of CO_2 emissions were performed on days 0, 1, 3, 7, 14, 21 and 28 of the incubation. The atmospheric CO_2 concentration was measured in all microcosms using a micro-CPG (CP-4900, Varian, Middelburg, The Netherlands). After gas sampling, the microcosms were flushed with air to renew the atmosphere, the soil water content was adjusted to 80 % of its water-holding capacity with sterile water and the microcosms were hermetically sealed.

2.6.2. Soil microbial biomass C and P and metabolic quotient

Microbial biomass C (MBC) was quantified using the chloroform-fumigation method from 10 g of equivalent dry soil exposed to chloroform vapor for 24 h. Dissolved soil C was then extracted with 40 mL of 0.025 M K_2SO_4, shaken for 45 min, centrifuged (10 min at 2683 *g*) and then filtered through 0.22-µm PTFE filters. Organic C concentrations in the extracts were measured by a TOC/TON analyzer (OI-Analytical, Aurora 1030, College Station, USA). Microbial biomass P (MBP) was determined from 20 g of fresh soil using anion exchange resin after a hexanol fumigation-extraction procedure (Kouno et al., 1995) adapted from Amer et al. (1955). Organic C concentrations in the extracts were measured by a TOC/TN analyzer (OI-Analytical, Aurora 1030, College Station, USA). MBC and MBP contents were calculated from the difference between the chloroform-fumigated and non-fumigated samples. We applied a conversion factor of 0.45 for MBC and 0.40 for MBP (Jenkinson et al., 2004). Total C of non-fumigated samples was used to quantify dissolved organic C (DOC). The metabolic quotient (qCO_2), defined as the C respired per unit of MBC, was calculated by dividing microbial respiration by MBC.

2.6.3. Soil DNA extraction and analyses

Soil DNA has been extracted from 0.5 g of fresh frozen soil and following the DNA part of the DNA/RNA coextraction protocol described in Tournier et al. (2015). Extracted DNA was quantified by fluorometry using the Quant-iT™ Pico Green DNA Assay Kit (Molecular Probes, Carlsbad, New Mexico) in accordance with the manufacturer's instructions. The purity and integrity of the DNA recovered was also verified after migration on a 1 % agarose gel. DNA extracted from the 30 soil samples were sent to the ADNid company (Qualtech groupe, Montpellier, France) for further NGS analysis on 16S and 18S genes.

Amplicon libraries were constructed according to our dual indexing strategy. Forward R1 consisted of the appropriate Illumina adapter, an 8-nt index sequence, an Illumina overhang adapter, a 3to6-random-nt linker and the specific forward primer. Reverse R2 consisted of the appropriate Illumina adapter, an 8-nt index sequence, an Illumina overhang adapter, a 6 random-nt linker and then the specific reverse primer. For bacteria, primers F479 (5′-CAGCMGCYGCNGTAANAC-3′) and R888 (5′-CCGYCAATTCMTTTRAGT-3′) targeting the V4 hypervariable regions of the 16S rRNA gene were used (Terrat et al., 2017). For fungi, primers FR1 (5'-AICCATTCAATCGGTAIT-3′) and FF390 (5′-CGATAACGAACGAGACCT-3′) targeting the 18S gene was chosen according to (Chemidlin Prévost-Bouré et al., 2011). Amplicons were generated using the Hot start DNA polymerase (Type-it QIAGEN). PCR was conducted on 10 ng of template DNA into 15 µL total mix, submitted to an initial denaturation of 5 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 1 min at 55 °C and 30 s at 72 °C. A final extension of 30 min at 60 °C was also included to complete the reaction. Amplicons were verified on 1.5 % agarose gel, purified using Agencourt® AMPure® XP kit (Beckman Coulter, Italy, Milano), and quantified by fluorimetry (Quant-iT™ Pico Green DNA Assay Kit). PCR products concentration was adjusted to 10 ng/µL. A second PCR step was performed using specific Illumina adapter, the index and the Illumina overhang adapter primers into 2× KAPA Hifi Hotstart Ready Mix (Roche Molecular Systems, Inc). A volume of 5 µL of PCR products previously prepared was used into a 50 µL final volume of PCR mix. A 3mn at 95 °C denaturation step was performed followed by 12 cycles of 30s at 95 °C, 30s at 55 °C, 30s at 72 °C before a 5mn at 72 °C final extension step. PCR2 products were purified and pooled together at 15 µg/µL in a final library. The library quality was controlled using a fragment analyzer. Sequencing runs, generating 2 × 250 bp, reads were performed on an Illumina MiSeq using V2 chemistry.

Samples sequencing data were automatically separated by the sequencer using the tag in our constructs. Samples paired end.fastq.gz files are the all processed together using an CLI (Command Line Interface) pipeline based on FROGS 2.0 (http://frogs.toulouse.inra.fr/ (Escudié et al., 2018). Our two amplicons followed the same pathway: 1 – first, forward and reverse reads were merged together using the common region, 2 – obtained sequences were the clustered using default swarm (Mahé et al., 2014, 2015) distance parameter (1), 3 – chimera were then removed, 4 – poorly represented clustered were removed (Abundance <5e-05), 5 – OTU were generated, using rdp classifier and blastn databases in.biom format (SILVA 132 provided with FROGS for 16S and 18S). Additional affiliation statistics and OTU.tsv files were also produced in further steps. Finally, python script was used to assign samples reads to each taxonomic levels based on the blast assignation made by FROGS, resulting in 7 files, one of each taxonomic level.

2.6.4. Soil potential enzymatic assays

We measured the potential activity of six hydrolytic soil enzymes (Bell et al., 2013), i.e. β-1,4-glucosidase 'GLU', β-D-cellobiosidase 'CBH', α-1,4-glucosidase 'AG', β-xylosidase 'XYL', β-1,4-*N*-acetyl-glucosaminidase 'NAG', L-leucine aminopeptidase 'LAP', and alkaline phosphatase 'AP'. These enzymes were selected to represent the degradation of C (GLU, CBH, AG, XYL), N (NAG, LAP) and P (AP). Briefly, the enzymatic assays involved homogenizing 2.75 g of fresh soil suspended in 91 mL 50 mM TRIS buffer in a blender for 1 min. The soil slurries were then added to a 96-well microplate (400 µl) using an eight-channel repeater pipet. Additional quench control replicates of the soil slurry and 4-methylumbelliferone (MUB) or 7-amino-4-methylcoumarin (MUC) standard curves (0–100, 1 M concentrations) were included with each sample. The soil slurries were incubated with fluorometric substrates for 3 h at 25 °C. After incubation, the plates were centrifuged for 5 min at 3000 rpm, after which 250 µl of supernatant was transferred from each well into a black flat-bottomed 96-well plate and then scanned on a microplate reader (SpectraMax Gemini, Molecular Devices, CA, USA) using excitation at 365 nm and emission at 450 nm.

2.7. Soil nematode measurements

Soil nematodes were extracted from 100 g of fresh soil using a large Petri dish-modified Baermann funnel method (Hooper et al., 2005). Fresh soil was placed in a circular mesh covered by a paper tissue partly submerged in sterile water to avoid anaerobic conditions. After 48 h, we collected the nematode suspension in 50 mL Falcon tubes and nematodes were count in 5 mL subsamples using a stereomicroscope (x40). The counting was repeated three times. Nematodes were fixed in a 4 % formaldehyde solution. Then, 120 specimens per sample were randomly selected on mass slides and identified to genus or family with a microscope (Olympus, France). Nematodes were assigned to the trophic groups as described by Yeates et al. (1993). The length (L, in µm) and maximal diameter (D, in µm) at mi-body of each individual were measured under the microscope. The biomass of nematode assemblages was calculated as the sum of the weights of the individual nematode in each assemblage. The fresh weight (W_{fresh}) of individual nematodes was calculated using the following Eq. 1:

$$W_{fresh} = LD^2/1.6 \times 10^6 \quad (1)$$

The nematode biomass carbon was expressed in µg-C assuming a dry weight of nematodes of 20 % of fresh weight and a proportion of C in the body of 52 % of dry weight (van den Hoogen et al., 2019).

2.8. Statistical analyses

Statistical significance was set at $P < 0.05$. Means and standard deviations (SD) were computed per treatments for all variables (three replications). We performed one-way ANOVA models with 'nutrient treatments' as the main factor using the "aov" functions from "ade4" package. The normality of the data and the homogeneity of variance were checked using Shapiro and Levene's tests, respectively. Principal Component Analyses (PCA) followed by analysis of similarities (ANOSIM) were used on bacterial and fungal taxonomic composition to test statistically whether there is a significant difference between nutrient treatments for each set of communities. For bacteria, the PCA was carried out at the family level. The OTUs for which family affiliation was not possible were labelled as "unknown". In the case of fungi, due to numerous absences of classification at the different lower taxonomic levels (class, order or families), the PCA was carried out on all taxonomic

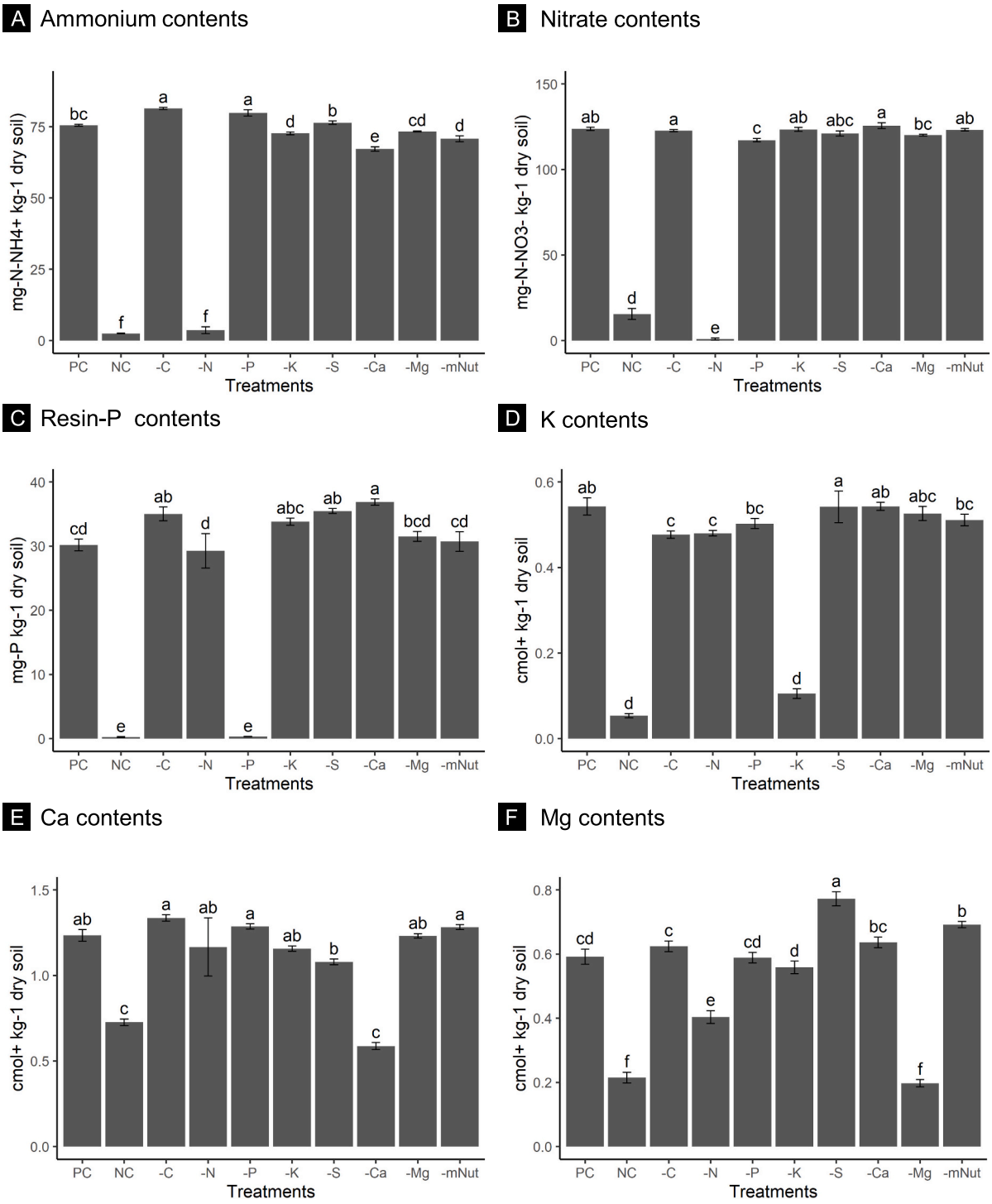

Fig. 1. Soil nutrient contents according to nutrient-omission treatments at the end of the experiment. (A) ammonium, (B) nitrates, (C) resin-phosphorus, (D) potassium, (E) calcium and (F) Mg contents. Whisker corresponds to standard deviation (n = 3). Letters (a, b, c, d, e and f) indicates significant differences according to one-way ANOVA and post-hoc Tukey HSD test at P-value <0.05. "PC" positive control; "NC" negative control; "-C" carbon omitted; "-N" nitrogen omitted; "-P" phosphorus omitted; "-K" potassium omitted; "-S" sulphur omitted; "-Ca" calcium omitted; "-Mg" magnesium omitted; "-mNut" micronutrient omitted.

levels, including the phylum, in order to take into account as much precision in taxonomic affiliation as possible. We calculated alpha-diversity metrics for bacterial and fungal communities through hill numbers using the hillR::hill_taxa function (Chao et al., 2014). Finally, using the Indicspecies::multipatt function, we computed the significant associations between bacterial and fungal taxa patterns and the nutrient treatments (Dufrêne and Legendre, 1997; De Cáceres et al., 2010). This function creates combinations of the nutrient treatments and compares each combination with the species in the input matrix (De Cáceres et al., 2010). The correlation index was used. It returns the pattern that creates a highest inside/outside difference (De Cáceres et al., 2010). We first restricted the combinations by considering single site-groups only. We then computed the analyses for all treatment combinations. All tests were done with the R (version 4.1.2) software (Team, 2016).

3. Results

3.1. Soil nutrient contents at the end of the experiment

For all nutrients, their contents in the soil solution were very low in the negative control (NC) and when they were omitted (Fig. 1). The ammonium contents were significantly higher when C (-C) and P were omitted (-P) in comparison to the positive control (PC), and significantly lower when K, Ca and the micronutrients were omitted (ANOVA F-value 3430, P-value <0.001) (Fig. 1.A). The nitrate levels were only lower in the -P treatment in comparison to PC (ANOVA F-value 2267, P-value <0.001) (Fig. 1.B). Resin-P contents were very low in NC and when P was omitted (-P), i.e. ~0.2 mg-P kg^{-1} soil in average (ANOVA F-value 304, P-value <0.001) (Fig. 1.C). The other treatments exhibited higher values between 25.7 and 37.4 mg-P kg^{-1} soil. Similarly, the soil K contents were low in -C and -N in comparison to PC (ANOVA F-value 333.3, P-value <0.001). The other treatments exhibited higher K values (Fig. 1.D). Ca follows the same general trend with low values under the NC and the Ca omission (-Ca) treatments, ~0.7 $cmol^{+}$ kg^{-1} dry soil, and 1.8-times higher in the others treatments, ~1.3 $cmol^{+}$ kg^{-1} dry soil (ANOVA F-value 39.26, P-value <0.001) (Fig. 1.E). Soil Mg contents were low in the -N treatment and higher in the -S and -mNut treatments in comparison to PC (ANOVA F-value 237.5, P-value <0.001) (Fig. 1.F). The other treatments had higher values, around 0.6 $cmol^{+}$ per dry kg of soil.

3.2. Soil microbial activity and biomass under nutrient deficiencies

The cumulated CO_2 release was very low in NC and when C was omitted, i.e. ~370 µg-C g^{-1} dry soil (Fig. 2.A). It was significantly higher in -P, but still significantly lower than the other treatments (ANOVA F-value 255.2, P-value <0.001). The omission of N and S induced significant higher values than NC, -C and -P, followed by the omission of K and Mg. The other treatments had higher values but not significantly different from the positive control (PC). The microbial biomass C (MBC) showed lower variation, still significant (ANOVA F-value 6.36, P-value <0.001), than microbial respiration with comparable values (~380 mg-C kg^{-1} dry soil) among the treatments (Fig. 2.B). Microbial biomass P (MBP) exhibited more variation, the lowest values were found in NC and -K while the highest ones were observed in -N (ANOVA F-value 17.68, P-value <0.001) (Fig. 2.C). The treatments PC, -P, -Ca, -Mg and -mNut had intermediate values. The lowest microbial metabolic quotient (qCO_2) values were found for NC and -C (ANOVA F-value 16.7, P-value <0.001) (Fig. 2.D).

3.3. Soil microbial enzymatic activities under nutrient deficiencies

We did not find significant variation in β-glucosidase, xylanase and leucine aminopeptidase (Fig. 3.A, B and C). Also, β-D-cellobiosidase exhibited low variation, except slight but significant lower values in NC in comparison to -N, -P, -S, -Ca, -Mg and -mNut (Fig. 3.D). When P, S, Mg and micronutrients were omitted, the β-*N*-acetylglucosaminidase was significantly lower in comparison to PC treatment. Acid phosphatase

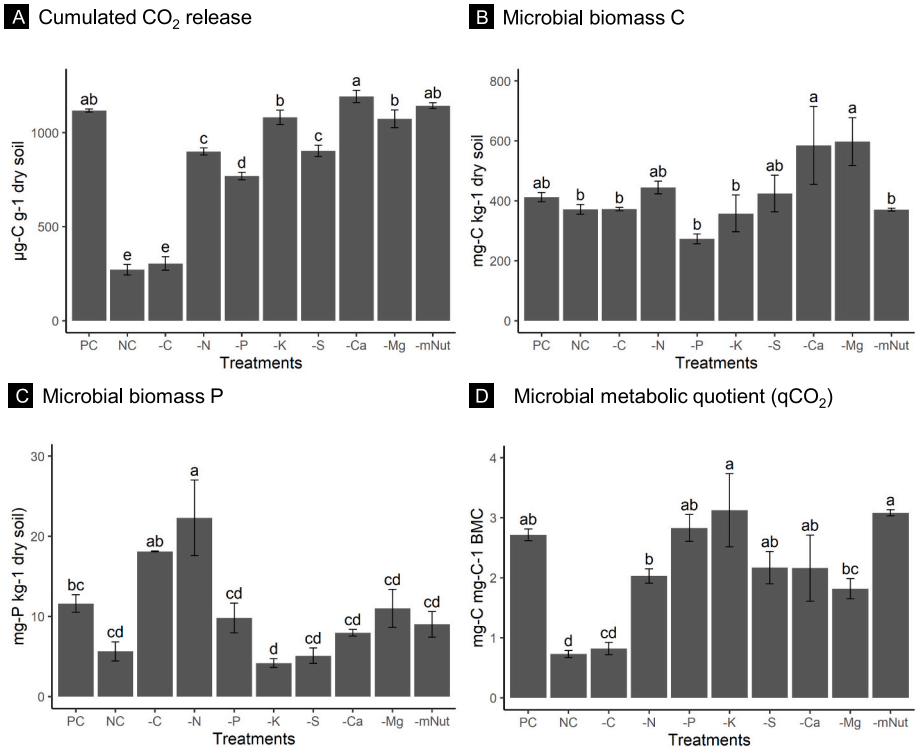

Fig. 2. Soil microbial activity and biomass according to nutrient-omission treatments at the end of the experimental incubation. (A) cumulated CO2 release, (B) microbial biomass C, (C) microbial biomass P and (D) microbial metabolic quotient. Whisker corresponds to standard deviation (n = 3). Letters (a, b, c and d) indicates significant differences according to one-way ANOVA and post-hoc Tukey HSD test at P-value <0.05. See legend to Fig. 1 for treatment codes.

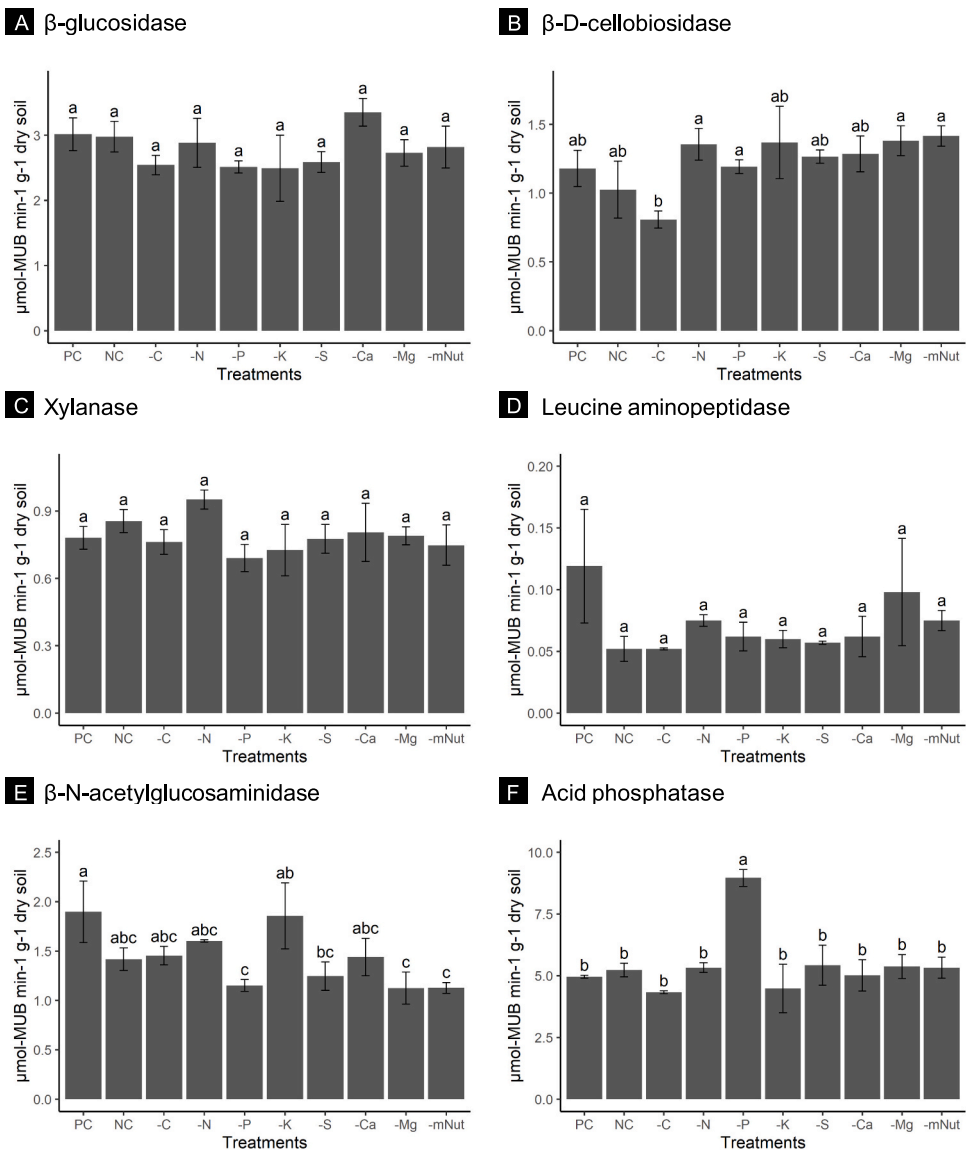

Fig. 3. Soil microbial enzymatic activities according to nutrient-omission treatments at the end of the experimental incubation indicated by (A) β-glucosidase, (B) β-D-cellobiosidase, (C) Xylanase, (D) Leucine aminopeptidase, (E) β-*N*-acetylglucosaminidase, (F) Acid phosphatase. Whisker corresponds to standard deviation (n = 3). Letters (a, b and c) indicates significant differences according to one-way ANOVA and post-hoc Tukey HSD test at P-value <0.05. "PC" positive control; "NC" negative control; "-C" carbon omitted; "-N" nitrogen omitted; "-P" phosphorus omitted; "-K" potassium omitted; "-S" sulphur omitted; "-Ca" calcium omitted; "-Mg" magnesium omitted; "-mNut" micronutrient omitted.

sharply increased when P was omitted in comparison to the other treatments (ANOVA F-value 12.47, P-value <0.001) (Fig. 3.F).

3.4. Soil microbial community composition under nutrient deficiencies

The nutrient omission treatments induced significant and pronounced changes in bacterial composition (Fig. 4.A). The first two PCA axes performed on bacterial taxa explained 48.5 % of total inertia with a significant clustering for treatments (ANOSIM R^2 0.82; P-value <0.01). The first axis opposed NC, -C and -P with positive scores against -K, -Ca, PC and -Mg (no significant differences among these nutrients) with negative scores (Fig. 4.A). The main taxa involved in the first axis were the Gemmatales, Rhizobiales, Myxococcales, Acidobacteriia subgroup 2, Acetobateriales, Solirubrobacteriales and Ktedonobacteriales for the positive scores, and the Betaproeobacteriales, Cytophagales and Shingobacteriales for the negatives scores (Fig. 4.B). The second axis was mainly explained by Bacillales (positive scores). We observed higher bacterial Shannon diversity (hill number order of 1) for PC and when N, S, Ca, Mg or micronutrients were omitted (Table 1). The bacterial Shannon diversity was significantly lower for NC, -C, and to a lesser extent, for -P and -K. The bacterial Simpson diversity (hill number order of 2) had the same pattern.

The multi-level pattern analysis showed significant relationships between C omission and the following taxa: Solirubrobacterales (phi 0.74, P-value <0.01), Bacillales (phi 0.67, P-value <0.01), Ktedonobacterales (phi 0.58, P-value <0.05), among others (Table 2). The Oligoflexales were highly associated with N deficiency (phi 0.89, P-value <0.01, respectively) while the Solirubrobacterales (phi 0.56, P-value <0.01) were associated with P deficiency. We also found strong relationships between the Cytophagales and K limitation, the Betaproteobacteriales and Ca limitation, the Catenulisporales, Legionellales and Xanthomonadales with Mg deficiency. Several bacterial taxa were associated with the negative control, especially the Myxococcales (phi 0.84, P-value <0.01).

The nutrient omission treatments induced significant but less pronounced changes in fungal composition in comparison to the bacterial

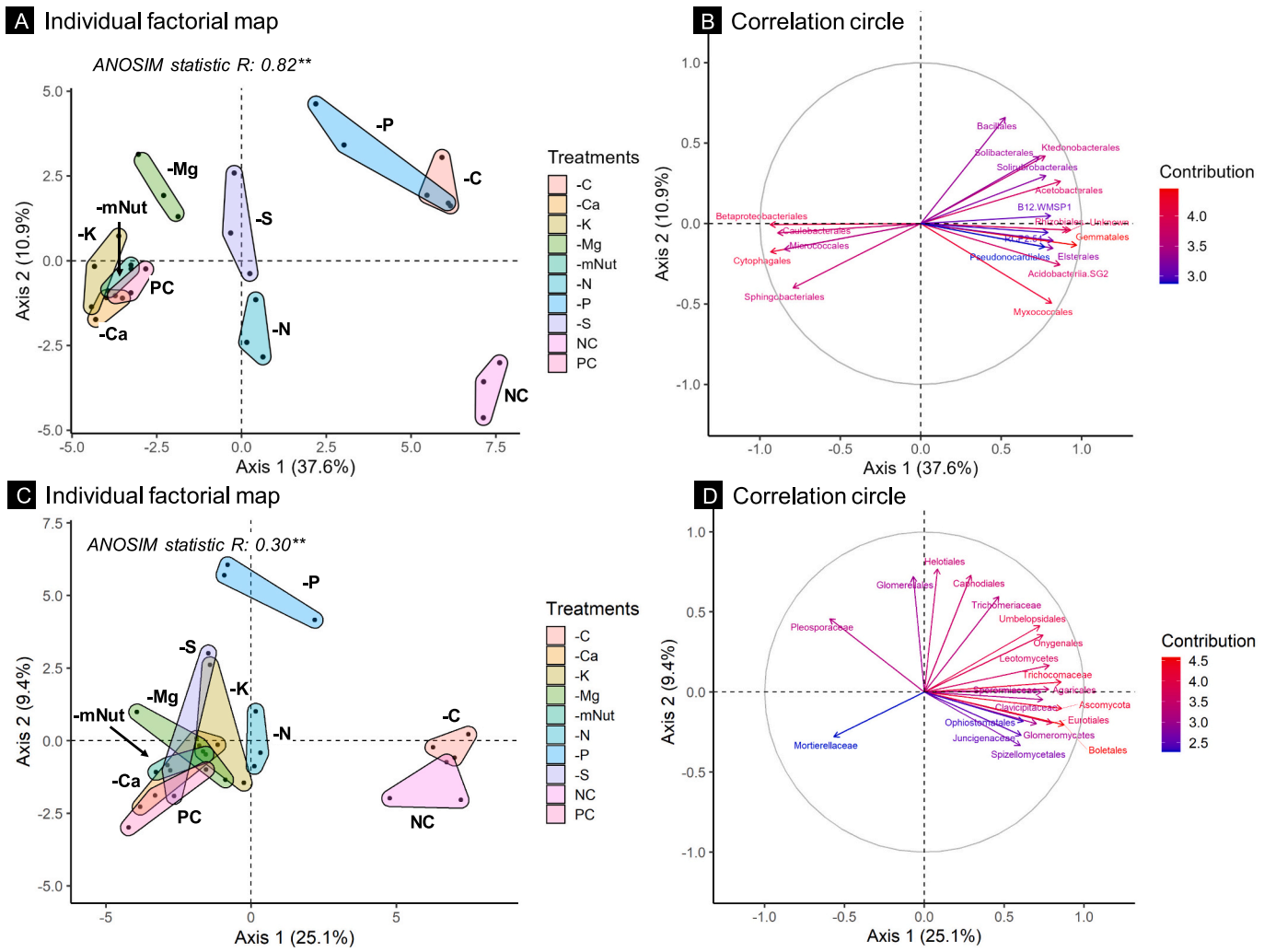

Fig. 4. Principal Component Analyses performed on bacterial and fungal communities and their associated ANOSIM statistics. (A) Individual factorial map based on the bacterial taxa. (B) Correlation circle for bacterial taxa. (C) Individual factorial map based on the fungal taxa. (D) Correlation circle for fungal taxa. "PC" positive control; "NC" negative control; "-C" carbon omitted; "-N" nitrogen omitted; "-P" phosphorus omitted; "-K" potassium omitted; "-S" sulphur omitted; "-Ca" calcium omitted; "-Mg" magnesium omitted; "-mNut" micronutrient omitted.

Table 1
Taxonomic diversity of bacterial and fungal communities using the Hill diversity numbers, Shannon entropy (q = 1) and inverse Simpson index (q = 2) according to treatments. Letters (a, b, c, d and e) indicates significant differences according to one-way ANOVA and post-hoc Tukey HSD test at P-value <0.05. "PC" positive control; "NC" negative control; "-C" carbon omitted; "-N" nitrogen omitted; "-P" phosphorus omitted; "-K" potassium omitted; "-S" sulphur omitted; "-Ca" calcium omitted; "-Mg" magnesium omitted; "-mNut" micronutrient omitted.

Treatments	Bacterial communities						Fungal communities					
Order of diversity	*q = 1*			*q = 2*			*q = 1*			*q = 2*		
PC	23.7	(0.8)	abcd	15.3	(1.0)	abc	15.3	(3.4)	ab	8.2	(2.3)	b
NC	19.0	(0.2)	e	12.2	(0.1)	d	24.7	(0.8)	ab	17.7	(1.3)	a
-C	21.1	(0.3)	de	13.0	(0.3)	cd	25.4	(2.8)	a	16.5	(4.2)	ab
-N	26.1	(0.4)	a	17.4	(0.3)	a	22.6	(0.5)	ab	15.0	(0.8)	ab
	23.1	(1.2)	bcd	14.5	(0.6)	abcd	18.1	(3.2)	ab	11.5	(2.9)	ab
-K	22.7	(1.1)	cd	13.6	(1.3)	bcd	19.2	(0.8)	ab	11.2	(1.3)	ab
-S	24.6	(0.1)	abc	16.6	(0.5)	ab	15.4	(4.4)	ab	9.0	(3.8)	ab
-Ca	24.2	(0.6)	abc	15.8	(0.5)	abc	15.8	(4.8)	ab	8.1	(3.3)	b
-Mg	25.5	(0.6)	ab	16.7	(1.0)	a	15.9	(2.3)	ab	8.9	(1.5)	ab
-mNut	24.1	(1.3)	abc	15.3	(1.4)	abc	14.9	(2.6)	b	8.3	(2.5)	ab

community (Fig. 4.C and D). The first two PCA axes performed on fungal taxa explained 34.5 % of total inertia with a significant clustering for treatments (ANOSIM R^2 0.30; P-value <0.01). The first axis opposed NC and -C (positive scores) against the other treatments (Fig. 4.C). The second axis opposed the -P treatment (positive score) to the others. The main taxa involved in the first axis were Pleosporaceae and Mortierellaceae for the negatives scores and Agaricales, Eurotiales, Glomeromycetes and for the positive scores. The main taxa involved in the second axis (associated to P deficiency) were Helotiales, Glomeralales and Capnodiales (positives scores). The fungal Shannon diversity was

Table 2
Significant (P-value <0.05) associations (phi coefficient) between bacteria taxa and nutrient deficiencies. "All": all nutrients omitted referring to NC.

Bacterial taxa	Nutrient deficiencies						
	C	N	P	K	Ca	Mg	All
Acetobacterales	0.53**						
Acidobacteriales	0.55*						
Acidobacteriia-SG13							
Acidobacteriia-SG2							0.61**
B12-WMSP1							
Bacillales	0.67**						
Betaproteobacteriales					0.33*		
Catenulisporales						0.54*	
Caulobacterales							
Chitinophagales							
Corynebacteriales							
Cytophagales				0.44*			
Diplorickettsiales							
Elsterales							0.64*
Frankiales							0.54*
Gemmatales							0.69*
Isosphaerales							
KF-JG30-C25	0.50*						
Ktedonobacterales	0.58*						
Legionellales						0.56**	
Micrococcales							
Micromonosporales							
Micropepsales							
Myxococcales							0.84**
Oligoflexales		0.89**					
Unknown							0.48*
Paracaedibacterales							
Pseudonocardiales							0.52*
RCP2–54							0.52**
Reyranellales							
Rhizobiales							0.60*
Rhodospirillales							
Solibacterales							
Solirubrobacterales	0.74**						
			0.56**				
Sphingobacteriales							
Streptomycetales							
Streptosporangiales							
Xanthomonadales						0.54*	

*P-value <0.05; **P-value <0.01; ***P-value <0.001.

high for NC, -C and -P but very low for PC and -mNut (Table 1). The fungal Simpson diversity (hill number order of 2) had the same pattern. The multi-level pattern analysis (Table 3) showed significant relationships between C omission and the following taxa: Sporormiaceae (phi 0.85, P-value <0.01), Nucleariidae & Fonticula group (phi 0.79, P-value <0.01), Trichocomaceae (phi 0.72, P-value <0.05) and Ascomycota (phi 0.70, P-value <0.01). The omission of N was only associated to the Chytridiales (phi 0.89, P-value <0.01) while the omission of P was only associated to the Glomerellales (phi 0.80, P-value 0.05). We also found significant association between the Boletales, Endogonales, Eurotiales, Juncigenaceae and Saccharomycetes and the omission of all nutrients (NC).

3.5. Soil nematode abundance and biomass under nutrient deficiencies

We found only two dominant nematode taxa, i.e. *Acrobeloides* (a bacterial-feeding taxa) and *Aphelenchoides* (a fungal-feeding taxa). The negative control (NC) had the lowest nematode abundance with around 10 individuals per g of dry soil (ANOVA F-value 27.24, P-value <0.001) (Fig. 5.A). Similarly, the omission of C, P, Mg and micronutrients induced low nematode abundances with an average of 40 individuals per g of dry soil. Nematode abundance when N, S and Ca were omitted was around 80 individuals per g of dry soil. Finally, the treatments PC and -K have the highest nematode abundances with around 130–160 individuals per g of dry soil. We observed similar pattern for nematode biomass carbon (ANOVA F-value 24.68, P-value <0.001) (Fig. 5.B).

Table 3
Significant (P-value <0.05) association (phi coefficient) between fungal taxa and nutrient deficiencies. "All": all nutrients omitted referring to NC.

Fungal taxa	Nutrient deficiencies			
	C	N	P	All
Agaricales				
Ascomycota	0.70**			
Boletales				0.61*
Chytridiales				
		0.89**		
Clavicipitaceae				
Endogonales				0.66*
Eurotiales				0.57*
Glomerellales				
			0.80*	
Glomeromycetes	0.55*			
Juncigenaceae				0.62*
Leotomycetes				
Microascales				
Mucocomycota				
NFG[$]	0.79**			
Saccharomycetes				0.68*
Sporormiaceae	0.85**			
Tremellomycetes				
Trichocomaceae	0.72*			
Umbelopsidales				

*P-value <0.05; **P-value <0.01; ***P-value <0.001.
[$]Nucleariidae & Fonticula group.

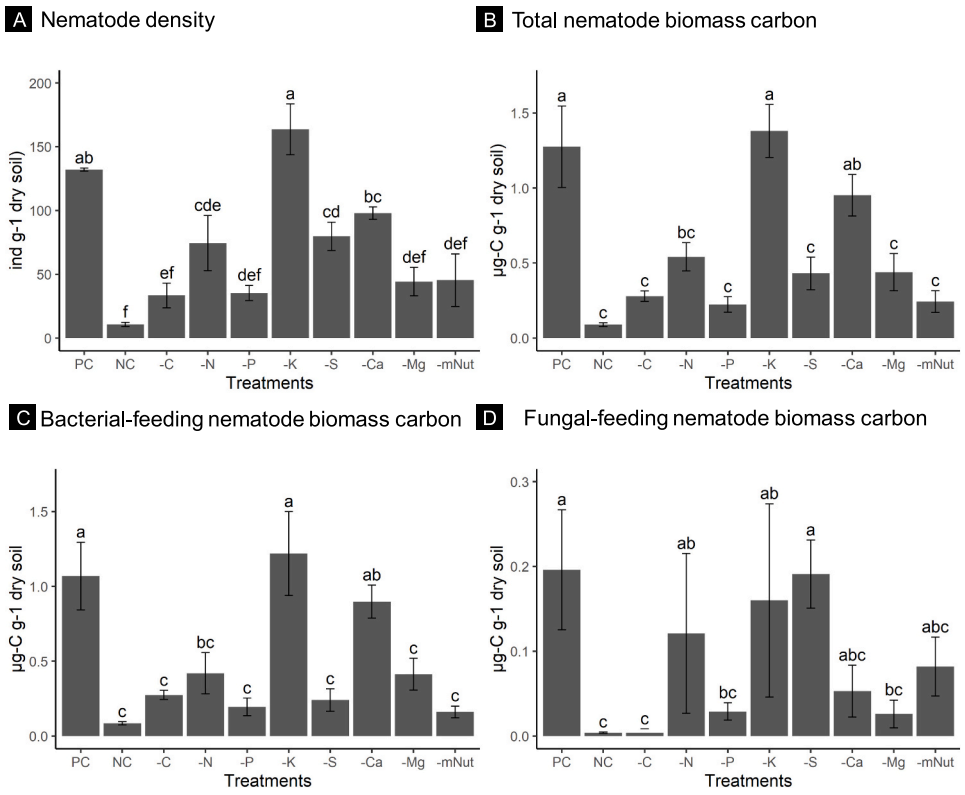

Fig. 5. Soil nematode density and biomass according to nutrient-omission treatments at the end of the experimental incubation. (A) total nematode density, (B) total nematode biomass, (C) bacterial-feeding nematode biomass and (D) fungal-feeding nematode biomass. Whisker corresponds to standard deviation (n = 3). Letters (a, b, c, d, e and f) indicates significant differences according to one-way ANOVA and post-hoc Tukey HSD test at P-value <0.05. "PC" positive control; "NC" negative control; "-C" carbon omitted; "-N" nitrogen omitted; "-P" phosphorus omitted; "-K" potassium omitted; "-S" sulphur omitted; "-Ca" calcium omitted; "-Mg" magnesium omitted; "-mNut" micronutrient omitted.

More limitation was found for bacterial-feeding nematode biomass carbon with the highest values found in PC and -K (ANOVA F-value 18.34, P-value <0.001) (Fig. 5.C) and the lowest ones for NC, -C, -N, -P, -S, -Mg and -mNut. The fungal-feeding nematode biomass carbon was very low for NC and -C, followed by -P, -Mg, -Ca and -mNut. The highest values were found for PC, -N, -K and -S (ANOVA F-value 3.68, P-value <0.007) (Fig. 5.D).

4. Discussion

4.1. Final soil nutrient contents

We verified that the soil nutrient contents at the end of the experiment match with the initial amount of nutrient added in the soil, i.e. suggesting no pollution or manipulation mistake. However, concerning N, a negative balance of ca. 100 mg-N per kg was observed in the treatments that received N. We first hypothesized N loss by denitrification, but the soil was never flooded. Still, we cannot exclude probable N loss through denitrification in anaerobic micro-niches. A similar but more pronounced pattern was found for P. Around 170 mg-P per kg disappeared in the treatments that received P. This P loss is ascribed to rapid P-sorption on the solid phase (Dubus and Becquer, 2001; Perez et al., 2014; Gérard, 2016). The final soil K, Ca and Mg contents were similar to the initial added amount of the beginning of the experiment, suggesting no or low K, Ca and Mg loss from the microcosms.

4.2. C and P co-limitation of the soil micro-food web

Our nutrient-omission approach revealed that when C was omitted from the soil, various variables, including microbial respiration and biomass carbon, exhibited values similar to the negative control and lower ones compared to the positive control. Microbial structure was also deeply affected by C limitation. This finding aligns with the widely accepted fact that C is considered the primary limiting element for heterotrophic microorganisms in bulk soils (Allison and Vitousek, 2005; Curiel Yuste et al., 2007; Krashevska et al., 2010; Fanin et al., 2013; Farrell et al., 2014). The omission of C mainly induces a drop in the C:P ratio of the microbial biomass in comparison to the negative control. The absence of C but the presence of P probably promoted the growth of microbial populations capable of accumulating P in reserve within polyphosphates in such energy-limited situations (Khan and Joergensen, 2019; Asada et al., 2022). This hypothesis is supported by the significant association found between C deficiency and bacteria from the Bacillales, known for their ability to accumulate P in their biomass (Hong et al., 2016; Correa Deza et al., 2023).

P, when omitted, also had a significant impact on microbial activity and diversity, resulting in lower microbial respiration and microbial biomass P (MBP) in this treatment compared to the positive control. Notably, acid phosphatase activity was the only enzymatic activity exhibiting large variation, i.e. a sharp increase when P was omitted. The limitation of P in tropical soils is well-documented (Hattenschwiler et al., 2011; Fanin et al., 2013; Randriamanantsoa et al., 2015; Mori et al., 2018), due to strong adsorption of available P onto insoluble aluminum and iron complexes, making it less accessible to microorganisms (Dubus and Becquer, 2001; Antoniadis et al., 2016). Our results also allow us to address the hypothesis formulated by Mori et al. (2018) on the misinterpretation of P limitation assessed by microbial respiration confounding by a substantial desorption of C after P fertilization. We observed a pronounced P deficiency even with an excessive C input. Therefore, the misinterpretation hypothesis appears unlikely.

Rather, our results support the hypothesis of a C-P co-limitation of the microbial food web in this tropical soil. This co-limitation was

significantly associated to the Solirubrobacterales encompassing keystone taxa involved in C metabolism of hexosephosphate (Zhu et al., 2022); soil multifunctionality (Qiu et al., 2021) and taxa such as *Conexibacter* capable of survival in harsh conditions. This microbial C-P co-limitation is likely a generic case for ferrallitic soils, but there can be differences in microbial nutrient limitations depending on the degree of rock weathering (Raminoarison et al., 2020). Thus, even though fertilization strategies in this tropical region should imperatively meet the C and P requirements of microorganisms, it is essential not to overlook other elements, especially N, which is severely deficient and known to be related to microbial C functions (Razanamalala et al., 2018).

4.3. N, S and Mg co-limitation of the soil micro-food web

Indeed, in addition to C and P co-limitation, we observed the significance of N, S, and Mg as co-limiting nutrients for the soil micro-food web in this tropical Ferralsol under investigation. Many microbial variables exhibited lower values compared to the positive control when these elements were missing. N is known to be a limiting nutrient in various ecosystems (Ågren et al., 2012; Du et al., 2020), including in the Highlands of Madagascar (Raminoarison et al., 2020). Its availability can influence microbial growth and nutrient cycling (Schimel and Bennett, 2004). Sulphur is involved in various enzymatic processes and is essential for the synthesis of amino acids and coenzymes while Magnesium is a crucial cofactor for many enzymes and plays a role in nucleic acid and protein synthesis (Senbayram et al., 2016; Narayan et al., 2022). Although studies have reported the effects of N addition on specific microbial taxa involved in N cycling (Chaudhry et al., 2012; Kuypers et al., 2018), research on the effects of S and Mg is less extensive (Chapman, 1997; Kopáček et al., 2013; Camenzind et al., 2018; Heinze et al., 2021). Here, we showed that the Oligoflexales, and the Chytridiales, known to were highly associated with N deficiency. Fu et al. (2022) analyzed the microbial communities of rhizosphere and bulk soils under different N application levels in boreal forests of China for 8 years and found that Oligoflexales could be used as bacterial biomarkers of the rhizosphere under the low N treatment. Thus, in conditions where C is not limited, this taxon may serve as promising bio-indicators of N limitation in these tropical soils. Similarly, the omission of S and Mg was associated with several bacterial taxa (but not fungi), i.e. the Cytophagales, Catenulisporales, Legionellales but also Xanthomonadales. These associations lack substantial support in the existing literature, emphasizing the need for further studies to validate the potential of these taxa as biological indicators for specific nutrient deficiencies and to comprehend the underlying determinants. Still, the co-limitation of N, S and Mg highlights the complex and intricate interplay of multiple nutrient factors that influence soil microbial communities in highly weathered soils.

4.4. Microbivorous nematodes as proxy of microbial growth

Because measuring microbial growth rate is highly challenging (Rousk and Bååth, 2011), nutrient limitation of the soil micro-food web often relies on measuring microbial respiration and biomass, or using enzymatic activities (Fanin et al., 2013). Despite a huge variation in microbial respiration, the microbial biomass (MBC or MBP) exhibit very low variation. This raises questions regarding the relevance of using this metric to comprehend the nutritional constraints of soil microorganisms (Smith and Paul, 2017). To assess microbial growth rate, methods such as thymidine and leucine incorporation (Bååth, 1998) or, for instance, using isotopes (Baath, 2001; Spohn and Kuzyakov, 2013), are available. Though, these techniques can be complex to implement, particularly in regions with limited access to certain technologies. Interestingly, our study showed that the biomass of microbivorous nematodes, i.e. corresponding to all nematode taxa feeding on bacteria and fungi, responds very significantly to nutrient deficiencies, and relates with certain microbial metrics such as respiration. The strong relationship between soil bacteria and nematode populations has been observed in various settings, including microcosms, fields and modeling studies (Hunt et al., 1989; Bloem et al., 1994; Bouwman et al., 1994; Yeates et al., 1999; Zelenev et al., 2004; Zelenev et al., 2006). Because soil microbivorous nematodes play key roles in nutrient cycling and in regulating the turnover of microbial communities, changes in their abundance can serve as valuable alternative indicators of soil nutrient limitation for the microbial food web.

5. Conclusion

In conclusion, our study highlights the multiple deficiencies faced by microorganisms in this tropical soil. Besides C and P, which strongly limit the activity and diversity of the microbial community, other nutrients are also significantly lacking, especially N, S, and Mg. These findings underscore the need to design fertilization methods that address the full spectrum of nutritional deficiencies in microorganisms, aiming to feed the soil and enhance soil functions sustainably. We have identified bacterial and fungal taxa significantly associated with certain deficiencies, which could potentially serve as indicators of specific nutritional deficiencies in this soil based on their frequency in the future. We cannot exclude variations in responses of microbial communities to nutritional deficiencies in situations where the initial composition of the soil micro-food web differs significantly from our case study. In this regard, additional studies appear necessary. Lastly, our study suggests that characterizing microbivorous nematodes could serve as a rapid indicator to assess nutritional deficiencies in the soil micro-food web, offering a simple implementation, particularly in Southern countries where resources may be limited at times.

CRediT authorship contribution statement

Jean Trap: Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Manoa Raminoarison:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Aurélie Cébron:** Writing – review & editing. **Kanto Razanamalala:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Tantely Razafimbelo:** Writing – review & editing. **Thierry Becquer:** Writing – review & editing. **Claude Plassard:** Writing – review & editing. **Eric Blanchart:** Writing – review & editing, Funding acquisition. **Laetitia Bernard:** Writing – review & editing, Methodology, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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