**Biogeochemical responses of a lowland tropical wet forest to 12 years of nutrient addition**

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# Abstract

Large-scale, long-term forest fertilization experiments in tropical forests have been used for almost thirty years to investigate the role of nutrients on ecosystem function. Paradoxically, despite large additions of nitrogen (N) and phosphorus (P), vegetation responses are highly variable. The Earth Forest Fertilization Experiment (EFFEX), located in the humid lowlands of Costa Rica, started in 2007 and has been continuously fertilized for 12 years. Here we provide a detailed analysis of chemical properties of EFFEX soils, in order to gain insight into possible causes for the lack of response of the vegetation. Additionally, we tested nutrient limitation to soil microorganisms in terms of microbial biomass carbon (C), N and P; soil enzyme activity and soil respiration. Nitrogen addition decreased pH by 0.2 units, while P addition doubled Ca concentration (the fertilizer contains Ca). Total carbon (C), N and P were unchanged by nutrient addition. Base cations at this site are some of the lowest reported for the Neotropics, and (other than Ca) were unchanged by nutrient addition. Phosphorus addition increased labile inorganic phosphate concentration by 15-fold, and doubled soil P saturation and soil microbial P. Fertilization had no significant effects on microbial biomass C and N, nor on long-term soil respiration. Fertilization had no significant effect on P-acquiring enzymes, but plots that received +N had higher soil sulfatase activity, and lower cellobiohydrolase and xylanase activity, perhaps due to the lower pH observed in +N plots. Overall, we show a modest biogeochemical response to nutrient addition despite large changes in soil phosphorus concentrations, perhaps related to base cation limitation or the duration of the experiment.

# Introduction

Nutrient limitation to terrestrial ecosystems is common (Elser et al. 2007). Because plants are the basis of ecosystems, many studies have focused on nutrient limitation to net primary productivity (NPP). The highest NPP on Earth is found in lowland, species rich tropical forests (LSRTF), thus studying the drivers of NPP in LSRTFs is important for understanding the global carbon and water cycles in a changing world. Fertilization experiments are recognized as the most direct way to evaluate nutrient limitation to NPP, but there are currently only nine fertilization studies in old growth LSRTFs (Wright et al. 2018, Luigli et al. 2021), and they mostly show weak fertilization effects on NPP (Wright et al. 2018). A weak response could be related with native tree adaptations to low soil fertility, or an increase in herbivory with fertilization (Wright et al. 2018). However, it could also be related with a high backdrop variability in soil chemistry (Townsend et al. 2008) which adds “noise” to the fertilization treatments, precluding clear ecological responses. Additionally, the extent to which fertilizer applications induce a corresponding change in soil chemistry is not always clear, for instance due to high denitrification rates in N addition plots, characteristic of hot and humid climates (refs).

Furthermore, it is possible that other organisms are more nutrient-limited than plants in some contexts, such as soil microbes (Camezind et al. 2017). “Phosphorus limitation appears to transcend different trophic levels and affects plants as well as soil microorganisms.”camezind. Here we provide detailed information of the effects of 12 years of fertilization with N and phosphorus (P) on soil chemical and microbial variables, in order to (1) understand possible experimental artefacts behind a weak plant response, (2) expand our understanding of forest nutrient limitation beyond autotrophs, to also include soil heterotrophs.

Although microbes are traditionally considered to be mainly limited by C (Demoling et al. 2007, Kamble and Baath 2014), a recent meta-analysis of 42 studies from 34 tropical forest sites points to generalized P limitation (Camezind et al. 2018 UPDATE). This limitation would arise due to the much narrower C:N:P ratios in microbial than in plant biomass (Cleveland and Liptzin 2007, Manzoni et al. 2010, Mooshammer et al. 2014), which means that microbes have higher N and P requirements per unit C than what the plant substrate provides. Camenzind et al. (2018) found a consistent positive response of P fertilization on microbial biomass and several microbial process rates such as litter decomposition, soil microbial respiration, free-living N-fixation, N mineralization, and P immobilization. On the other hand, responses to N addition were either positive (N mineralization, net nitrification) or negative (e.g. soil microbial respiration, free-living N-fixation) depending on the specific process considered, and hence resulted in an overall neutral N fertilization effect (Camezind et al. 2018). The study did not consider the response of hydrolytic enzymes released by soil microbes due to lack of data, but recognises that the stoichiometry of enzyme activity represents a useful indicator for relative microbial limitation (Camezind et al. 2018, Sinsabaugh et al. 2008, Waring et al. 2014). Camezind´s response may not be universal to all old growth tropical forests, however. Of the 20 fertilization experiments included in the meta-analysis, only eight are field fertilization experiments in tropical lowland forests (e.g. Liu et al. 2013, Cusack et al. 2010, Cleveland et al. 2006, 2013; Turner and Wright 2014, Fanin et al. 2015, McGroddy et al. 2004, Fisher et al. 2013), and only two were conducted for ≥ 5 years (Cusack et al. 2010, Turner et al. 2014). As such, there is a lack of data on the microbial response of old growth lowland tropical forests to long-term differences in nutrient supply. This information has practical applications, for example predicting ecosystem function in the face of atmospheric N pollution from tropical cities (Matson, Hietz, etc.).

**L ong-term fertilization experiments** “limitation by a nutrient is shown if the Rate of an ecosystem process is increased by addition of That nutrient, and strictly speaking it can only be deter- mined experimentally” (Tanneretal.1998, Sayerand Banin 2016). CAMEZIND. For example in a long-term fertilization experiment in old growth LSRTF in Limón, Costa Rica, fertilization had no significant effect on trees > 100 mm DBH at the community level after three years of fertilization (Alvarez-Clare et al. 2013), and the same was true after 12 years (Alvarez-Clare et al. *unpublished*).

“Additionally, the fate of N is very different from that of P once added to the soil, since N may be rapidly lost through leaching and gaseous emissions (Hall and Matson 1999, Corre et al. 2010, Velescu et al. 2016), whereas P will be less available by geochemical sorption, though kept in the system for longer periods (Olander and Vitousek 2004). These dif- ferential effects must be considered in the interpretation of nutrient manipulation experiments, also in light of shifts in stoichiometric ratios by the addition of multiple elements (Cleveland and Liptzin 2007, Kaspari 2012).” Camezind.

Question the paradigm of all lowland tropical soils being super nutrient poor. Talk about how EFFEX falls in place cf other tropical fertilization experiments (Kelly). Mention Kelly´s new nature paper of P effects on AFEX.

Biogeochemical theory predicts that phosphorus (P) limitation, rather than nitrogen (N), should be widespread in humid tropical lowlands (Walker and Syers, 1976). There is at present insufficient evidence to show that P limitation to plants is stronger and more frequent than N limitation, but also a paucity of fertilization studies, and most of them younger than 5 years, which may be insufficient to elicit a response in trees (Wright et al. 2018).

Paragraph 5. Hypotheses.

H1 +N and +P increase soil N and P

H2 Microbial biomass is P limited, hence microbial C should increase, microbial N not, microbial P yes (immobilization). N and P addition should inhibit N and P hydrolases, respectively. Nutrient addition should stimulate soil microbial activity and hence soil CO2 flux, unless it reduces root growth and autotrophic respiration, leading to no change in total soil respiration.

# Methods

## Study site

The Earth Forest Fertilization Experiment (EFFEX) is located in a 900-ha forest reserve located on the campus of EARTH University in Limón, Costa Rica (10° 11’ N and 84° 40’ W), at approximately 30 m.a.s.l. (Fig. 1). The site consists of mature and regenerating rainforest and wetlands. Mean annual temperature is 25.1°C and mean annual precipitation (MAP) is 3,464 mm, with less rainfall in March, April and September (Fig. 2). The study region is located on the distal section of a coalescence of alluvial fans (Madrigal and Rojas, 1980). The general topography of the region is flat to slightly undulating. Geologically, the region is formed by fine lahars, andesitic lava, volcanic tuffs, and pyroclastic materials, and in low-lying areas close to rivers, alluvial sediments have accumulated over these materials (Madrigal y Rojas, 1980). Soils in the area have been classified as Inceptisols and Ultisols of volcanic origin, high in clay, with poor drainage, although there are as yet no detailed classification for soils at the EFFEX sites specifically. Total N and P, and macronutrient concentrations are relatively high in comparison to other lowland tropical forests (Alvarez-Clare and Mack, 2015). Moreover, tree growth, litter productivity, and root growth index in this forest are high, suggesting that this is a relatively fertile tropical lowland wet forest ecosystem (Alvarez-Clare and Mack, 2015). Soils, climate, floristic composition and tree density at the EARTH Forest Reserve are similar to those from the alluvial soils at La Selva Biological Station, a well-studied forest in Costa Rica (McDade et al. 1994). Published soil nutrient analyses for EFFEX exist for samples collected two years after the start of fertilization, but this is the first comprehensive soil study published since.

Forests at EARTH, in a similar fashion to La Selva, are dominated by the legume tree *Pentaclethra macroloba*, which represents approximately 30% of the total tree basal area within our study plots. Our study sites also have a high palm density, which is characteristic of forests in the Caribbean lowlands of Costa Rica (Hartshorn 1983, Hartshorn and Hammel, 1994). At EARTH Forest, the second most important species after *P. macroloba* is *Socratea exorrhiza*, a single-stem canopy or subcanopy palm with a high frequency and density at our study plots.

## Experimental design

The experiment consists of 24 plots 30 × 30 m in size, established in 2007 within mature, non-flooding areas of the reserve. They were randomly assigned to three fertilizer treatments or a control, in a complete block design (n = 6). All plots were separated from each other by at least 100 m. Fertilizer is broadcast by hand twice a year on the surface of the 900 m2 plots to yield the following treatments: +P (47 kg ha-1 yr-1 of P as super triple phosphate from 2008 to 2014, and as rock phosphate from 2014 to the present, which consists of 30% P2O5, 40% CaO (“cal viva” in Spanish) and 10% SiO2), +N (100 kg ha-1 yr-1 of N applied as ammonium nitrate, NH4NO3, and urea, NH2CONH2), and +NP (N and P added together in the same amounts as in the +N and +P plots). The Ca in the fertilizer is applied at a rate equivalent to 110 kg Ca ha-1 yr-1. All measurements are confined to the internal 400 m2 of each plot (20 × 20 m) to reduce edge effects. Published data exists for properties of soils collected at the onset of the experiment and 1 and 2 years after fertilization (Alvarez-Clare & Mack, 2015), but there are no published soil data for the next 10 years.

## Soil sampling

Soils were sampled on 5 and 6th August 2018 at EFFEX sites. Eight cores were taken per plot at randomly selected locations within the 500 m2 buffer zone, in order to minimize damage to the central 400 m2 of each plot, using a 2.5 mm diameter punch corer and to 0−10 cm depth. The eight cores were pooled to yield one composite sample per plot. Field moist soils were shipped on 8th August to the Smithsonian Tropical Research Institute in Panama and were kept refrigerated until the start of analyses on August 14th, 2018.

## Soil pH, labile inorganic nutrients, base cations and oxalate-extractable nutrients

Total soil C and N were determined by combustion on an elemental analyzer (brand), and total P by ignition (1 h, 550°C) and acid extraction (1.0 M H2SO4) followed by automated molybdate colorimetry on a Lachat Quickchem 8500 spectrophotometer (Hach Ltd, Loveland, CO). Soil pH was determined in a 1:2 soil to solution ratio in both deionized water and 10 mM CaCl2 with a glass electrode. Extractable phosphate (i.e., exchangeable phosphate in competitive equilibrium with the soil exchange complex) was extracted by anion exchange membranes (AEM-P) (BDH # 55164 2S, BDH laboratory supplies, Poole, England) in bicarbonate form, elution of the phosphate in 0.25 M H2SO4, and detection by automated molybdate colorimetry (Turner and Romero, 2009). Inorganic N was extracted in 0.5 M K2SO4, with ammonium and nitrate determined by automated colorimetry on a Lachat Quikchem 8500 (Hach Ltd, Loveland, CO). Exchangeable cations (Al, Ca, Fe, K, Mg, Mg, Mn, Na) and effective cation exchange capacity (ECEC) were determined with a 0.1 M barium chloride extraction (BaCl2) followed by inductively-coupled plasma optical-emission spectrometry (ICP-OES) on an Optima 7300 V (PerkinElmer Inc., Shelton, CT). Oxalate-extractable Al, Fe, Mn, Si and P were determined by extraction with a 0.2M ammonium oxalate solution followed by ICP-OES (Optima 7300 V Perkin Elmer) (Schoumans 2000, Courchesne and Turmel 2008). Total elements (Al, B, Ca, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Zn) were determined with a digestion with 70% nitric acid followed by ICP-OES (Optima 7300 V Perkin Elmer).

We compared selected variables measured on soils sampled in 2018 with soils sampled in 2007, immediately before fertilization treatments started. Total C and N in 2007 were determined with combustion with an elemental analyzer (marca) and total P with (poner protocolo y marca de equipo). Labile inorganic phosphate was determined with a Mehlich-II extraction followed by molybdate colorimetry. Soil pH was determined in water with a x:x soil to solution ratio. Total Ca was measured with a double nitric acid extraction (dar más detalle). All 2007 analysis were conducted at the University of Florida.

## Microbial C, N, and P

Chloroform-labile C and N (hereafter referred to as microbial C and N) were determined using the CHCl3 fumigation-extraction procedure (Brookes et al. 1985; Vance et al. 1987). Fumigated (24 h) and corresponding unfumigated soil subsamples of each soil were extracted with 0.5 M K2SO4 and centrifuged for 15 min at 8000 *g*, and extracts analysed by automated combustion (Shimadzu TOC analyser, Tokyo, Japan). Hexanol-labile P (hereafter referred to as microbial P) for each soil was determined using a simultaneous hexanol fumigation and extraction method with anion exchange resins (BDH # 55164 2S, BDH laboratory supplies, Poole, England) in bicarbonate form for 16 h (Kuono et al. 1995), using hexanol instead of chloroform as a fumigant (McLaughlin et al. 1986). Resins were eluted with 0.25M H2SO4. Phosphorus in the extracts was measured by molybdate colorimetry after a 5-fold dilution for unfumigated samples or a 15-fold dilution for fumigated samples. Microbial C, N, and P for each soil were each estimated as the difference in the concentration of a given element in fumigated and non-fumigated subsamples. Both microbial carbon and microbial nitrogen concentrations were corrected for unrecovered biomass using a k factor of 0.45, and microbial phosphorus using a k factor of 0.40 (Jenkinson et al. 2004). We did not use extractability factors to convert microbial C, N and P into actual biomass values, because these factors are known to be soil-specific and therefore vary considerably among different soils (e.g. Oberson et al. 1997; Turner et al. 2002). Instead we present them as relative figures, i.e. the difference between the fumigated and unfumigated fractions.

## Hydrolytic enzyme activity

We measured the activity of nine hydrolytic soil enzymes using fluorogenic substrates as described in Turner (2010) and Turner and Romero (2010) (Table 1). The enzymes assayed were β-glucosidase (BG), leucine aminopeptidase (LAP), monoesterase (MUP), diesterase (BIS), N-acetyl glucosaminidase (NAG), sulfatase (S), α-glucosidase (AG), xylanase (XYL), and cellobiohydrolase (CEL) (Table 1). β-glucosidase carries out the hydrolysis of glycosidic bonds to terminal non-reducing residues in β-D-glucosides and oligosaccharides, with release of β-D glucose. It hydrolyses glucose chain fragments to glucose (Paul, 2007). NAG degrades peptidoglycan, a major component of chitin. Chitin is a long-chain polymer of N-acetylglucosamine, and a derivative of glucose. It is a primary component of cell walls in fungi and the exoskeletons of arthropods (ref). Sulfatase degrades organic sulfate esters into inorganic sulfate and play important roles in the cycling of sulfur in the environment, in the degradation of sulfated glycosaminoglycans and glycolipids in the lysosome, and in remodelling sulfated glycosaminoglycans in the extracellular space. Together with sulfotransferases, sulfatases form the major catalytic machinery for the synthesis and breakage of sulfate esters (Wikipedia). AG breaks down starch and disaccharides to glucose (wikipedia). Xylanase breaks down hemicellulose, major components of plant cell walls (wikipedia). Cellulase is any of several enzymes produced chiefly by fungi, bacteria, and protozoans that catalyze cellulolysis, the decomposition of cellulose and of some related polysaccharides. Wikipedia.

Briefly, enzymes were assayed using a microplate fluorimetric assay based on Marx, Wood and Jarvis (2001) and adjusted to tropical lowland soils by Turner and Romero (2007) and Turner (2010). The substrates used were conjugates of the highly fluorescent compounds 4-methylumbelliferone (MUB) and 7-amino-methyl-coumarin (AMC). All substrates were purchased from Glycosynth Ltd (Warrington, UK). Substrates were dissolved in 0.4% methylcellosolve (2-methoxyethanol; 0.1% final concentration in the assay). For each sample, soil suspensions were prepared in a 1:100 soil to water ratio (containing 1 mM NaN3 to inhibit microbial activity) by stirring on a magnetic stir-plate for 15 min. Soil suspension (50 μL) was then pipetted into wells on a micro-well plate (16 wells per substrate) containing 100 μL of 200 μM substrate dissolved in deionized water and 50 μL of 200 mM sodium acetate–acetic acid buffer (pH 5.0). Final concentrations in the assay mixture were therefore 100 μM substrate and 50 mM buffer. Plates were incubated for 30 min at 26 °C to approximate the daytime temperature in the upper 10 cm of soil in lowland forests in central Panama (Marthews et al. 2008). Incubation times were based on preliminary assays to assess the linearity of the reaction over time. The reaction was terminated by adding 50 μL of 0.5 MNaOH (final solution pH>11) and the fluorescence determined immediately on a FLUOstar Optima multi-detection plate reader (BMG Labtech, Offenburg, Germany), with excitation at 360 nm and emission at 460 nm. Control wells were prepared for each substrate and contained substrate, buffer, and 1 mM NaN3 (no soil suspension). Blank wells contained soil suspension and buffer only (no substrate). Standard wells contained buffer, 1 nmol methylumbelliferone (MU), and either soil suspension or 1 mM NaN3 to account for the reduction of fluorescence in the presence of soil (quenching). Standard curves showed that fluorescence was linear to at least 2 nmol MU under these assay conditions. All enzyme activities are expressed as nmol MU g-1 soil (dry weight) min-1. Soil enzyme C:N ratios were calculated as β-glucosidase (BG)/ N-acetyl glucosaminidase (NAG), C:P as BG/MUP5, N:P as NAG/MUP5, C:S is BG/S, N:S is NAG/S, P:S is MUP/S.

## Soil CO2 fluxes

Soil CO2 flux measurements were conducted on three locations per each of 22 fertilization plots using an infrared gas analyzer (LI-8100, Li-Cor Biosciences, Lincoln, Nebraska). Measurements were made on three PVC rings (20 cm ∅, 5 cm tall) per plot, inserted in the soil at least two weeks before measurements. Volumetric water content was measured with time-domain reflectometry (TDR) (GS1 soil moisture sensor, Decagon devices), and soil temperature to 5 cm depth, for each CO2 flux reading. Three consecutive readings were taken per collar and averaged. We took a first set of measurements on April 23-24, 2017, six months after the last fertilization (“pre-fertilization measurements”). On April 25th we applied one of the bi-yearly fertilization doses, and re-measured soil CO2 fluxes 24 h, 72 h, 9 d and 3.6 months after this fertilization event. Overall, we made ~200 soil CO2 flux readings per each of the five measurement campaigns, for a total of 1000 soil CO2 flux measurements. A set of soil samples were collected (0-10 cm) for the determination of nitrate and ammonium concentrations with KCl extraction and spectrophotometry, and for instantaneously bioavailable phosphate with Mehlich-III.

## Statistical analyses

In order to test for fertilization effects on soil chemical variables and soil respiration, we used one-way analysis of variance (ANOVA), after checking that the data met normality and homogeneity of variances assumptions, using Shapiro-Wilk and Levene’s tests respectively, together with visual examination of plots of the residuals (q-q plots) (Table 2). In the case of soil respiration, we did separate ANOVAs for each of the five measurement periods. Where significant effects of fertilization were found (at p= 0.05), differences between treatment levels and the control were tested with Dunnet’s tests. There was a high within-treatment variation in most of the variables measured, so to test for fertilization effects on biological response variables, such as microbial biomass and enzyme activity, we used two-way ANOVAs which included main effects for N and P addition and their interaction (Winer 1971, Wright et al. 2011, Wurzburger et al. 2015). Normality and homogeneity of variances assumptions were checked by visual inspection of plots of the residuals. Although this type of analysis precludes determining differences between a treatment level and the control, in the case of biological response variables we were more interested in looking at possible main effects of the fertilization treatments. Homogeneity of variances assumptions were checked with xyz. Data were analysed with the program R (r-project.org).

# Results

## Soil pH, base cations, labile inorganic and oxalate-extractable nutrients

Eleven years of fertilization has not had a significant effect on total soil C, N, and P; nor on C:N, C:P and N:P ratios (Table 2, Fig. 3). The same is true for the concentration of soil nitrate (NO3-) (Table 2, Fig. 3). However, 11 years of fertilization with +N significantly decreased pH by 0.2 units relative to the control (F(3,18)=5.72, p=0.006; Table 2, Figure 3), and fertilization with +P and +NP increased extractable phosphate by 15-fold (1500%) relative to control plots (F(3,17)=22.1, p<0.001; Table 2, Figure 3). Consequently, the degree of P saturation (DPS, %) also increased in +P and +NP treatments (F(3,17)=7.18, p=0.003; Table 2). Of the exchangeable cations measured, the concentration of Ca doubled (F(3,18)=6.88, p=0.003; Table 2, Fig. 3), and total exchangeable bases (TEB) (F(3,17)=4.00, p=0.024) and base saturation (BS) (F(3,17)=6.60, p=0.003) increased significantly, in +P plots relative to controls, whereas Al saturation decreased significantly in +P plots (F(3,17)=5.83, p=0.006; Table 2). This is likely because the rock phosphate fertilizer we use contains CaO. The concentration of the other base cations measured (Al, Fe, K, Mg, Mn, Na) remained unchanged with nutrient addition, as was the case for oxalate-extractable Al and Fe (Table 2).

## Microbial C, N and P

Microbial biomass C values ranged from 990−1800 mg C g-1 soil, microbial N from 200−350 mg N g-1, and microbial P from 14 to 200 mg P g-1 soil (Fig. 4). Fertilization treatment had no significant effects on the concentration of microbial C or N, either when expressed per g of soil (Fig. 4) or per g soil organic C (*data not shown*). However, plots that received +P (+P, +NP) had double the concentration of microbial P than plots that did not (Control, +N) (F(1,18)=6.06, p=0.024; Fig. 4). Microbial molar C:N values were in the range of 6.1−6.2, microbial N:P 8.5−9.9 and microbial C:P 49.7−61.5, but fertilization did not significantly influence those ratios (Table 2).

Both microbial C and N were strongly and positively correlated with Total C and N (Table 3). Microbial P was strongly positively correlated with labile phosphate, but not with Total P (Table 3). Microbial C and N were moderately significantly correlated with the base cations Mg and Na, and all three microbial C, N and P were moderately positively correlated with Ca, TEB and ECEC (Table 3).

## Hydrolytic enzyme activity

Of the nine hydrolytic soil enzymes tested, only three responded significantly to fertilization, specifically to N addition (+N, +NP). Sulfatase was significantly higher in plots that received N than in those that did not (F(1,18)=12.5, p=0.002; Fig. 5) whereas cellobiohydrolase (F(1,18)=5.03, p=0.038; Fig. 5) and xylanase (F(1,18)=3.38, p=0.083; Fig. 5) decreased with N addition (+N, +NP), although results for xylanase were only marginally significant. Following this, stoichiometric enzyme ratios involving sulfatase (S), such as C:S, N:S and P:S, were all significantly influenced by N addition (Table S1). No enzyme responded significantly to P addition. Of all the soil variables measured, soil pH seemed to have the strongest effect on soil enzyme activity, through being moderately positively related with LAP and S (Table 3). Other moderately strong and positive correlations were observed between CEL with Fe and Al, between XYL and total C and N (Table 3). A weak negative correlation was observed between AG and NO3- (Table 3).

## Soil CO2 fluxes

Overall, we found no evidence of a long-term fertilization effect on soil CO2 fluxes, given that treatments had no significant effect on soil CO2 flux measured 1 day before fertilization (equivalent to 6 months after the last fertilization) (F3, 205=1.57, p=0.204) (Fig. 6) nor 124 days (3.6 months) after fertilization (F3, 193=0.21, p=0.889). We did find an up to four-fold increase in soil CO2 fluxes in +N plots 24 h (F3, 201=48.0, p<0.001) and 72 h (F3, 198=13.2, p<0.001) after fertilization, but the effect was transient and differences had disappeared 9 days after fertilization (F3, 197=2.24, p<0.085).

# Discussion

## Fertilization effects on soil nutrients

We found no effects of 12 years of fertilization on total C, N and P. This is consistent with findings from a large-scale, long-term fertilization experiment in lowland humid forest in Panama, where total soil C and N AND SOIL P? were unaffected by 10 years of nutrient addition (Turner et al. 2015) and Puerto Rico, where seven years of fertilization did not have a significant influence on total C (N was not measured) (Li et al. 2006). In EFFEX, Panama and Puerto Rico, soil pH decreased with +N fertilization. Nitrogen addition decreased pH by 0.2 units in EFFEX, and by ca. 0.8 units in Panama (Turner et al. 2013). However in Panama the decline in pH caused a corresponding decline in extractable base cations (Ca and K) and increased extractable Al, a pattern we did not observe in EFFEX. Soil pH also decreased with simultaneous fertilization with NPK+ micronutrients in Puerto Rico, although the authors do not discuss the role of N addition in this pattern (Li et al. 2006). In terms of easily extractable (“labile”) N and P, we saw no effect of +N addition on soil NO3-, but in Panama it led to a 47% increase in soil NO3- concentration (Wright et al. 2011). In contrast, we saw a ~1200% increase in labile PO4- (Mehlich P) concentration, but in Panama Bray P only increased by 34%. PERHAPS COMPARE SILVIAS NITRIFICATION RATES TO CORRE DATA AND ESTIMATE?)

In terms of cations, our +P and +NP treatments increased the concentration of exchangeable Ca, total exchangeable bases (TEB) and base saturation (BS), whereas Al saturation decreased significantly in +P plots. This is likely because the rock phosphate fertilizer we use contains CaO. The experiment was fertilized with triple superphosphate during its first six years (2008-2014) but we had to switch to rock phosphate due to a ban on triple superphosphate imports into Costa Rica. The concentration of the other base cations measured (Al, Fe, K, Mg, Mn, Na) remained unchanged with nutrient addition, as was the case for oxalate-extractable Al and Fe. In general, the natural concentration of soil cations in our experiment is low. For example, EFFEX K, Ca and Mg concentrations are in the middle of the range of what is reported across the Amazon basin (Quesada et al. 2012) which is often considered to be poor in cations. LOOK UP FOR MORE PAPERS

Fyllas 2009 also has something on Amazonian nutrients. Look at new Nature paper on Amazon fertilization experiment.

Barthold et al. 2008 have a Mesoamerican comparison of exchangeable K values.

Turner and yavitt 2013 gigante inorganic nutrients: their pH decreased by 0.8 units in +N plots. The decline in pH caused a corresponding decline in extractable base cations (Ca and K) and increased extractable Al, highlighting an important but poorly understood consequence of long-term atmospheric N deposition onto tropical forests.

Interestingly, soil organic P was significantly increased by P addition in Panama, by 46% (Turner et al. 2015). Soil C:N ratios also were unaffected by fertilization, while the C to organic P ratio did decrease significantly after P addition

Also in Puerto Rico, Fertilization had little effect on soil moisture and soil bulk density in both the 0–10 and the 10–25 cm soil layers.

Here compare results with Silvia´s measurements

Cellulose is the most abundant organic polymer in nature, chitin is the second most abundant.

In terms of extractable Al and base cations,

* Resultados de las mismas variables que medimos nosotros pero para otros experimentos de fertilización en bosque tropical lluvioso de bajura. Buscar cationes
* Comparar los valores de todas las variables con los de otros experimentos tropicales (yo le paso la lista) y hacer una tabla.

## Microbial biomass C, N and P

Our microbial C, N and P values were in the range of those reported for a fertilization experiment in Panama (Turner and Wright 2013). In EFFEX, fertilization had no significant effect on microbial C and N, but plots that received P had In Gigante, P addition significantly increased microbial C by 13%, but N and K addition did not. Microbial nitrogen increased significantly with P addition, but not with N or K addition (Turner et al. 2013). Microbial P increased by 49% in +P plots relative to no P plots

The microbial molar C:N ratios observed here were on the lower end of the range reported in Panama, as was the case for microbial N:P ratios. However, the microbial C:P ratios in our plots tended to be lower than in Panama, although in that study they followed changes at several points during the year and our data only represent one sampling point in July.

Rosinger et al. 2019 Because microbial biomass C:N:P ratios are relatively invariant across ecosystems (Cleveland and Liptzin, 2007), differences in microbial efforts to acquisition C, N and P should be expected between ecosystems that differ in nutrient quantity as well as quality in order to maintain microbial homeostasis (Sinsabaugh et al., 2014)

* Microbial ratios in this study varied A LOT. See if they varied so much in Panama or other studies mentioned in camezind
* Microbial homeostasis…. In CNP ratios?

Rosinger 2019 Thus, high enzyme activities targeting a particular resource are thought to indicate nutrient limitation by that resource (Sinsabaugh et al., 1993), and ratios of N-acquiring (e.g. chitinase, urease, peptidase), C-acquiring (e.g. cellulase, hemicellulase, glucosidase) and P-acquiring (e.g. phosphatase) enzymes, to the activity of enzymes targeting other resources, should reflect relative nutrient availability and thus limiting factors for microorganisms (Sinsabaugh and Moorhead, 1994).

The evidential basis for the enzymatic method to assess microbial resource limitation is correlations between soil physicochemical stoichiometry and the enzymatic stoichiometry (Moorhead and Sinsabaugh, 2006; Sinsabaugh et al., 2008, 2009).

That is, soils poor in N have a high activity of N-acquiring enzymes relative to other enzymes, soils poor in P have high activities of P-acquiring enzymes relative to the activity of other enzymes, while soils rich in all nutrients show relatively high activity of C-acquiring enzymes in relation to the activity of other enzymes (Moorhead and Sinsabaugh, 2006; Sinsabaugh et al., 2008, 2009). However, the elemental composition of soil is known to poorly reflect microbial availabilities (Hart et al., 1994; Demoling et al., 2007; Månsson et al., 2009; Xu et al., 2013). To validate

the power and precision of enzymatic assessments to determine



## Enzymes

Es posible que los niveles de fósforo en el suelo sean naturalmente tan bajos que se necesite un aumento aún mayor en las concentraciones de fosfato en suelo para lograr inhibir la mineralización del fósforo orgánico. También es posible que la actividad enzimática del suelo, al reflejar la actividad no solo de enzimas recientemente producidas sino también enzimas estabilizadas por años, no sea un buen reflejo de la demanda actual de fósforo por parte de la biota. En todo caso, estos hallazgos sugieren que la relación entre la disponibilidad de fosfato inorgánico biodisponible, la demanda biológica por P y la mineralización del P orgánico es más compleja que lo que se reconoce actualmente.

# Conclusions

Lack of P limitation to trees but indeed limitation to microbial biomass… what does this mean.

Why do we see P limitation to microbes (immobilization) when there is so much P around

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