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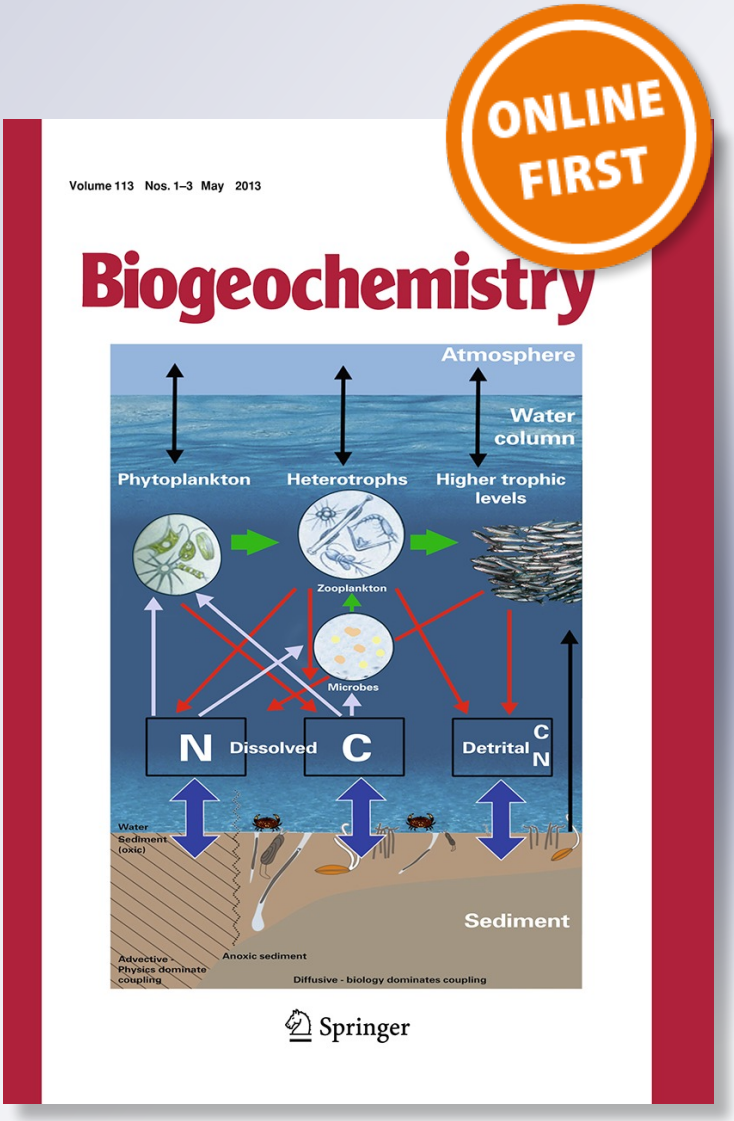

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The response of microbial biomass and hydrolytic enzymes to a decade of nitrogen, phosphorus, and potassium addition in a lowland tropical rain forest

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Abstract Nutrient availability is widely considered to constrain primary productivity in lowland tropical forests, yet there is little comparable information for the soil microbial biomass. We assessed microbial nutrient limitation by quantifying soil microbial biomass and hydrolytic enzyme activities in a long-term nutrient addition experiment in lowland tropical rain forest in central Panama. Multiple measurements were made over an annual cycle in plots that had received a decade of nitrogen, phosphorus, potassium, and micronutrient addition. Phosphorus addition increased soil microbial carbon (13 %), nitrogen (21 %), and phosphorus (49 %), decreased phosphatase activity by ~65 % and *N*-acetyl β-glucosaminidase activity by 24 %, but did not affect β-glucosidase activity. In contrast, addition of nitrogen, potassium, or micronutrients did not significantly affect microbial biomass or the activity of any enzyme. Microbial nutrients and hydrolytic enzyme activities all declined markedly in the dry season, with the change in microbial biomass equivalent to or greater than the annual nutrient flux in fine litter fall. Although multiple nutrients limit tree productivity at this site, we conclude that phosphorus limits microbial biomass in this strongly-weathered lowland tropical forest soil. This finding indicates that efforts to include enzymes in biogeochemical models must account for the disproportionate microbial investment in phosphorus acquisition in strongly-weathered soils.

Keywords Gigante Peninsula · β-glucosidase · Fertilization · Microbial biomass · *N*-acetyl β-glucosaminidase · Panama · Phosphodiesterase · Phosphomonoesterase

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Introduction

Nutrient availability is widely considered to constrain primary productivity in lowland tropical forests (Vitousek 1984; Tanner et al. 1998) and is likely to regulate the response of these ecosystems to increasing atmospheric carbon dioxide concentrations (Cleveland et al. 2011). For example, recent evidence indicates that nutrient availability will modulate the physiological responses of tropical tree seedlings to future changes in climate and atmospheric chemistry (Cernusak et al. 2007, 2009), while nitrogen, phosphorus, and potassium can all affect components of plant productivity in lowland tropical forests (Wright et al. 2011; Santiago et al. 2012).

In contrast, there is little information on the nutrient status of soil microbial communities in tropical forests. It is often assumed that heterotrophic soil microbes are limited by the availability of suitable organic carbon substrate (Wardle 1992). However, nitrogen addition increased soil microbial biomass in

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lower montane tropical forests of Puerto Rico and Panama (Li et al. 2006; Corre et al. 2010; Cusack et al. 2011), while phosphorus appears to regulate microbial processes in lowland tropical forests (Cleveland et al. 2002; Cleveland and Townsend 2006) and plantations (Gnankambary et al. 2008). These findings support the assumption that nitrogen is the limiting nutrient in montane forests and phosphorus the most limiting in lowland forests (Vitousek 1984; Tanner et al. 1998), but do not account for the marked variation in soil properties, including nutrient concentrations, that occur in tropical regions (Baillie 1996; Andersen et al. 2010). The role of potassium, other base cations, and micronutrients in regulating microbial processes has not been addressed.

The nutrient status of soil microbial communities can be inferred from measurements of hydrolytic enzymes, because investment in enzyme synthesis is assumed to reflect biological nutrient demand. For example, soil phosphatase activity provides a sensitive measure of phosphorus demand, being reduced markedly by phosphorus addition (Marklein and Houlton 2012) and responding closely to changes in phosphorus availability along natural gradients in both temperate and tropical rain forests (Olander and Vitousek 2000; Allison et al. 2007). Similarly, enzymes involved in the acquisition of carbon (e.g., β-glucosidase, involved in cellulose degradation) and nitrogen (e.g., *N*-acetyl β-glucosaminidase, involved in chitin degradation) can be used to infer microbial nutrient demand for carbon and nitrogen, respectively (e.g., Sinsabaugh and Moorhead 1994).

It was recently suggested that enzymes involved in carbon, nitrogen, and phosphorus acquisition are synthesized by microbes in a consistent stoichiometric ratio across ecosystems (Sinsabaugh et al. 2009). Although this appears to be the case for freshwater systems, it seems unlikely to occur in tropical soils, given the markedly greater phosphatase activity compared to other enzymes in such systems (Olander and Vitousek 2000; Turner 2010; Turner and Romero 2010; Cusack et al. 2011; Nottingham et al. 2012). There remains, however, a remarkable lack of data on hydrolytic enzyme activities in lowland tropical forests, particularly in terms of the response to experimental nutrient manipulation at the field scale.

Here we report the response of soil microbial biomass and hydrolytic enzyme activities to a decade of fertilization with nitrogen, phosphorus, potassium, and micronutrients in a lowland tropical rain forest in central Panama. We hypothesized that addition of the limiting nutrient would result in (1) an increase in soil microbial biomass, as well as the concentration of the nutrient in the microbial pool, and (2) a reduction in the investment in hydrolytic enzymes associated with the acquisition of the limiting nutrient. Our aim was to use measurements of microbial biomass and hydrolytic enzymes to determine the microbial nutrient status of this lowland tropical forest soil.

Methods

The Gigante fertilization experiment

The study was conducted on the Gigante Peninsula, part of the Barro Colorado Nature Monument, Republic of Panama. The site supports mature moist semi-deciduous rain forest at least 200 years old (Wright et al. 2011). On nearby Barro Colorado Island, the mean annual temperature is 26 °C and annual rainfall averages 2,600 mm, with just 10 % falling during the 4 month dry season between December and April (Windsor 1990). Soils on Gigante Peninsula are clay-rich Oxisols developed on Miocene basalt and are morphologically similar to the Typic Eutrudox (AVA and Marron soil classes) on nearby Barro Colorado Island (Dieter et al. 2010). Soil analyses of control plots indicates very low concentrations of readily available phosphate (<1 mg P kg^{-1}, extracted in Mehlich-3 solution or by anion-exchange membranes; Turner et al. 2013) and extractable inorganic nitrogen (e.g., ammonium and nitrate concentrations of ~ 1 mg N kg^{-1} in samples extracted in the field; B.L. Turner, unpublished). Potassium concentrations (~ 100 mg K kg^{-1}) are moderate for the central Panama region and tropical forests worldwide (Barthold et al. 2008; Yavitt et al. 2011). Foliar nutrient concentrations indicate that the site is relatively rich in phosphorus (Sayer et al. 2012), yet fine litter fall increased by ~ 25 % following phosphorus addition (Wright et al. 2011; see below).

The fertilization experiment began in 1998 and is described in detail elsewhere (Wright et al. 2011; Yavitt et al. 2011; Sayer et al. 2012; Turner et al. 2013). The experiment is remarkable in its duration and in the range of responses observed in terms of forest productivity, with significant effects involving

nitrogen, phosphorus, and potassium (Wright et al. 2011). Briefly, fertilizer has been added four times per year during the wet season to 40 × 40 m plots replicated four times for each treatment in a full factorial design. Nitrogen is added as urea, phosphorus as triple superphosphate, and potassium as potassium chloride. A further treatment (four replicate plots) includes calcium and magnesium (from dolomitic limestone) and micronutrients. However, we assume that calcium, magnesium, and micronutrients do not limit the microbial community at this site, because the dolomite/micronutrient treatment did not significantly affect microbial biomass or enzyme activities ($p > 0.15$; results not shown). Fertilizer was added at the following times during the study period: 13–15 September 2006, 16–18 May 2007, 4–6 July 2007, 22–24 August 2007, and 10–12 October 2007 (Fig. 1).

Summary of changes in response to nutrient addition

Changes in soil nutrients were reported previously (Yavitt et al. 2009; Yavitt et al. 2011; Turner et al. 2013). Phosphorus addition increased available phosphorus markedly from <1 to >30 mg P kg^{-1}. Nitrogen addition increased soil nitrate, had no effect on extractable ammonium, and decreased soil pH (~0.8 units in plots receiving only nitrogen), with a corresponding decline in extractable soil cations. Extractable potassium increased in the potassium treatments but declined in the nitrogen treatments, almost certainly due to the acidification of soils under urea application. There was significant seasonal variation in soil pH and all nutrients measured, although patterns varied among nutrients (Turner et al. 2013).

In addition to the increase in fine litter fall in response to phosphorus addition, tree basal area growth was influenced by a combination of nitrogen and potassium (amelioration of a long-term decline), while fine root growth was reduced by potassium addition (Wright et al. 2011; Yavitt et al. 2011; Santiago et al. 2012). At the seedling stage, potassium and phosphorus appear to play key roles, influencing growth and rates of herbivory (Santiago et al. 2012). There is currently no information on the nutrient status of the soil microbial community, although multiple nutrients

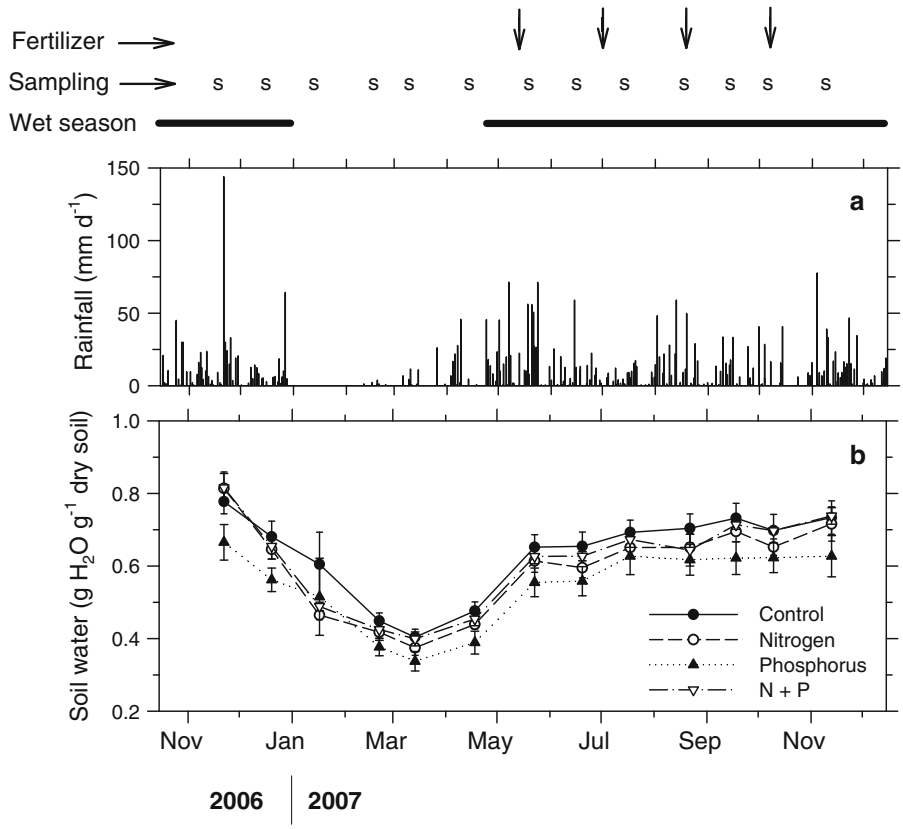

Fig. 1 Daily rainfall for Barro Colorado Island, Panama (**a**), and soil water content in experimental plots on Gigante Peninsula (**b**) between October 2006 and December 2007. The dates of fertilizer addition and sampling, and the approximate extent of the wet season are shown (From Turner et al. 2013). *Error bars* are standard errors of four replicate plots in each treatment

influence litter decomposition (Kaspari et al. 2008) and microbial community composition varies among nutrient treatments (Kaspari et al. 2010). However, determination of microbial carbon and nitrogen after 6 years of nutrient addition yielded no significant differences among treatments (Sayer et al. 2012).

Soil sampling

Soils were sampled monthly between November 2006 and November 2007 (Fig. 1). On the third week of every calendar month, soils were collected from 16 plots in an N × P factorial design (control, +N, +P, +NP). Every 4 months all 36 plots were sampled, including 32 plots in the full N × P × K factorial design plus the four dolomite/micronutrient plots.

Soils were sampled to 10 cm depth using a 2.5 cm diameter corer at nine systematically distributed points in the central 20 × 20 m quadrat of each plot. Samples from each plot were composited in the field and returned immediately to the laboratory, where roots, stones, and mesofauna were removed by hand. Soils were then stored overnight at 4 °C before extraction of microbial biomass nutrients within 24 h of sampling and hydrolytic enzyme activities within 48 h of sampling. A previous study showed no significant effect of cold storage on soil microbial phosphorus or hydrolytic enzyme activities for at least 2 weeks (Turner and Romero 2010).

During the study period, the dry season began at the end of December 2006 and continued until late April 2007, although there were a few days of heavy rain in early April (Fig. 1a; Turner et al. 2013). The maximum daily rainfall was 147 mm in November 2006. Soil moisture was highest ($\sim$0.8 g H_2O g^{-1} soil) in November 2006, when sampling occurred immediately following the highest rainfall event, but was otherwise relatively stable throughout the wet season (0.60–0.75 g H_2O g^{-1} soil) (Fig. 1b). Dry season soil moisture was lowest in March when values ranged between 0.34 and 0.40 g H_2O g^{-1} soil.

Analytical procedures

Microbial biomass

Microbial biomass carbon and nitrogen were determined by chloroform fumigation and extraction in K_2SO_4 by standard procedures (Brookes et al. 1985; Vance et al. 1987). Briefly, fresh samples of fumigated (24 h in a desiccator with ethanol-free chloroform) and unfumigated soil were shaken in a 1:5 soil to solution ratio with 0.5 M K_2SO_4 for one hour and then centrifuged at 8,000×*g* for 10 min. Extracts were then frozen at −35 °C until analysis. Organic carbon and total nitrogen were determined simultaneously on a TOC-V_{CHN} analyzer (Shimadzu, Columbia, MD) after five-fold dilution of the extracts. The difference in organic carbon and total nitrogen in fumigated and unfumigated samples was assumed to originate from microbial biomass. Both microbial carbon and microbial nitrogen concentrations were corrected for unrecovered biomass using a k factor of 0.45 (Jenkinson et al. 2004).

Microbial phosphorus was determined by hexanol fumigation and extraction with anion-exchange membranes based on a method described by Kouno et al. (1995) as modified in Turner and Romero (2010). Briefly, anion exchange membrane strips were prepared by shaking twice in 0.5 M $NaHCO_3$. For each sample, two portions of fresh soil (5 g on a dry-weight basis) were weighed into 120 mL bottles with 80 mL deionized water and five anion-exchange membrane strips. One bottle received 1 mL of hexanol and the samples were shaken for 24 h. The membranes were then removed and rinsed in deionized water and the phosphate recovered by shaking for 1 h in 50 mL of 0.25 M H_2SO_4, with detection by automated molybdate colorimetry at 880 nm using a Lachat Quikchem 8500 (Hach Ltd, Loveland, CO). Microbial phosphorus was calculated as the difference between the fumigated and unfumigated samples and corrected for unrecovered biomass using a k_p factor of 0.40 (Jenkinson et al. 2004). We did not correct for phosphate sorption during the assay, because preliminary studies showed that the majority of the phosphate spike was recovered by the membranes in control soils, but that variable results were obtained for phosphorus addition soils. Ratios of C:N, N:P, and C:P in soil microbial biomass are expressed on a molar basis.

Hydrolytic enzyme assays

The activities of four hydrolytic enzymes were determined using fluorogenic substrates as described previously (Turner 2010; Turner and Romero 2010). The enzymes and substrates were: (i) acid

phosphomonoesterase (Enzyme Commission (EC) number 3.1.3.2) assayed with 4-methylumbelliferyl phosphate; (ii) phosphodiesterase (EC 3.1.4.1) assayed with bis-(4-methylumbelliferyl) phosphate; (iii) β-glucosidase (EC 3.2.1.21) assayed with 4-methylumbelliferyl β-D-glucopyranoside; and (iv) *N*-acetyl β-D-glucosaminidase (EC 3.2.1.52) assayed with 4-methylumbelliferyl *N*-acetyl β-D-glucosaminide.

Substrates were purchased from Glycosynth Ltd (Warrington, UK) and 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 NaN_3 to inhibit microbial activity) by stirring rapidly 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 M NaOH (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 NaN_3 (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 NaN_3 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}.

Statistical analysis

We performed repeated measures analyses of variance (ANOVA) for each response variable to evaluate temporal variation. Between-subject (or between-plot) effects evaluate responses to treatments over the entire experiment. Within-subject (or within-plot) effects evaluate variation among sampling dates and interactions among treatments and sampling dates. Repeated measures ANOVA assumes compound symmetry of the variance–covariance matrix if there are more than two repeated measures. We therefore used the conservative Greenhouse-Geisser correction for violations of the compound symmetry assumption. All analyses were performed with SYSTAT© 11.0 (Richmond, CA).

The NPK factorial design is replicated four times along a 36-m north–south topographic gradient and blocked within each replicate along the perpendicular east–west gradient to control spatial variation in soil properties (Wright et al. 2011). However, the NP design does not account for blocks within replicates, so must overcome additional uncontrolled spatial variation. The quarterly NPK design therefore has greater power to detect treatment effects, while the monthly NP design has greater power to detect seasonal effects. Throughout the results we report treatment effects with respect to the quarterly NPK factorial design and seasonal effects with respect to the monthly NP factorial design. Time × nutrient interactions were evaluated in the monthly NP factorial design except for time × potassium effects, which were evaluated in the quarterly NPK factorial design. A one-way analysis of variance (ANOVA) was used to contrast the micronutrient and control treatments without blocking.

Results

Soil microbial biomass

Microbial carbon was increased significantly by phosphorus addition ($F_{1,18} = 8.0$, $p = 0.011$), but not by nitrogen or potassium addition (Fig. 2a; Table 1, which summarizes significant treatment and seasonal effects for all microbial and enzymatic measurements). Mean microbial carbon concentrations were 972 ± 38 mg C kg^{-1} in no-phosphorus plots and 1,096 ± 36 mg C kg^{-1} in phosphorus addition plots, an increase of 13 % (Fig. 2a). Microbial carbon varied seasonally ($F_{12,108} = 18.6$, $p < 0.001$), with lowest concentrations in the dry season (Fig. 2b). In no-phosphorus plots, concentrations ranged from 751 ± 35 mg C kg^{-1} in the dry season (March 2007)

to 1321 ± 118 in the wet season (September 2007), an increase of 76 % (Fig. 2b).

Microbial nitrogen was increased significantly by phosphorus addition ($F_{1,18} = 7.7$, $p = 0.013$), but not by nitrogen or potassium addition (Fig. 2c). The mean microbial nitrogen concentration was 188 ± 8 mg N kg^{-1} in no-phosphorus plots and 227 ± 15 mg N kg^{-1} in plots receiving phosphorus, an increase of 21 % (Fig. 2c). Microbial nitrogen varied seasonally ($F_{12,108} = 26.2$, $p < 0.001$), with lowest concentrations in the dry season (Fig. 2d). In no-phosphorus plots, microbial nitrogen concentrations ranged from 137 ± 7 mg N kg^{-1} in the dry season (March 2007) to a 255 ± 11 mg N kg^{-1} in the wet season (July 2007), an increase of 86 %. In phosphorus addition plots, concentrations ranged from 173 ± 9 mg N kg^{-1} in the dry season (March 2007) to a 280 ± 22 mg N kg^{-1} in the wet season (July 2007), an increase of 62 % (Fig. 2d).

Microbial phosphorus was increased significantly by phosphorus addition ($F_{1,18} = 14.5$, $p = 0.001$), but not by nitrogen or potassium addition (Fig. 2e). We ignore a significant but obscure N × K × time interaction (not shown). The mean microbial phosphorus concentration was 64.3 ± 3.1 mg P kg^{-1} in no-phosphorus plots and 95.9 ± 7.7 mg P kg^{-1} in plots

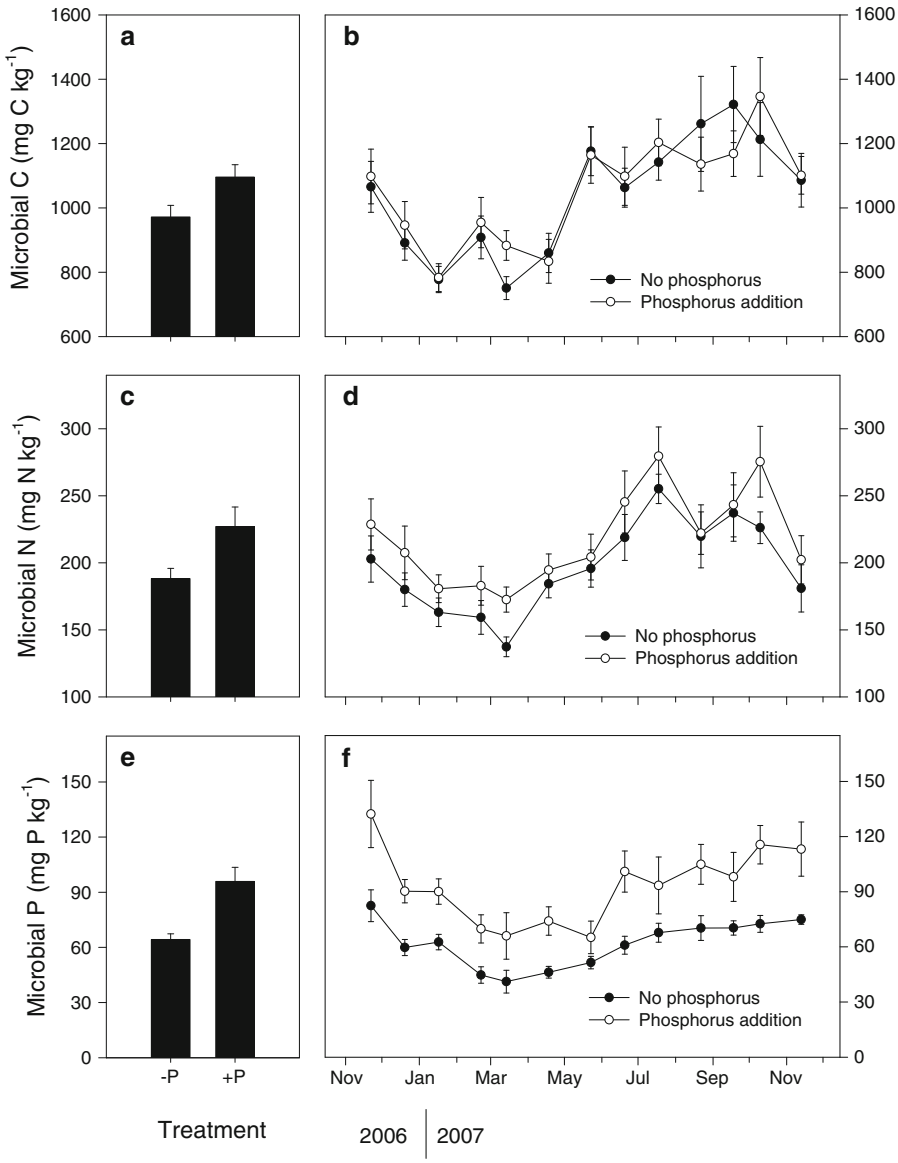

Fig. 2 Microbial biomass carbon (**a**, **b**), nitrogen (**c**, **d**), and phosphorus (**e**, **f**), following a decade of fertilization of a lowland tropical forest on Gigante Peninsula, Panama. Graphs on the left show the significant effect of phosphorus addition in the full NPK factorial design (means and standard errors of 16 plots in each treatment sampled four times between November 2006 and November 2007). Graphs on the right show significant seasonal changes in the NP factorial design (means and standard errors of eight no-phosphorus and eight phosphorus addition plots sampled monthly between November 2006 and November 2007)

Table 1 Summary of seasonal and treatment effects for soil microbial biomass and hydrolytic enzyme activities in the Gigante fertilization experiment

Parameter	Treatment ($F_{1,18}$)	Season ($F_{12,108}$)	Time × nutrient interactions ($F_{12,108}$)
Microbial C	Increased by P ($F = 8.1$*)	Lowest in dry season ($F = 18.6$***)	ns
Microbial N	Increased by P ($F = 7.7$*)	Lowest in dry season ($F = 26.2$***)	ns
Microbial P	Increased by P ($F = 14.5$**)	Lowest in dry season ($F = 13.3$**)	ns
Microbial C:N	Increased by N ($F = 7.4$*)[a]	Variable ($F = 16.2$***)	ns
Microbial C:P	ns	Lowest in late wet season ($F = 9.1$***)	ns
Microbial N:P	ns	Lowest in late wet season ($F = 6.9$**)	ns
Phosphomonoesterase	Reduced by P ($F = 129$***)	Lowest in dry season ($F = 8.4$**)	Time × P interaction ($F = 5.7$**)
Phosphodiesterase	Reduced by P ($F = 169$***)	Lowest in dry season ($F = 6.5$**)	Time × P interaction ($F = 4.1$*)
β-glucosidase	ns	Lowest in dry season ($F = 8.9$***)	ns
N-acetyl β-glucosaminidase	Reduced by P ($F = 4.9$*)	Lowest in dry season ($F = 13.9$***)	ns
Enzymatic C:N (βG:NAG)	Increased by P ($F = 10.4$**)	Highest in late wet season ($F = 3.6$*)	ns
Enzymatic C:P (βG:PME)	Increased by P ($F = 188$***)	Highest in late wet season ($F = 5.1$**)	Time × P interaction ($F = 5.6$**)
Enzymatic N:P (NAG:PME)	Increased by P ($F = 110$***)	Lowest in dry season ($F = 8.5$***)	Time × P interaction ($F = 3.5$*)

Treatment effects were evaluated in the quarterly NPK factorial design, seasonal effects in the monthly NP factorial design, and time × nutrient interactions in the NP design. *F* values are shown in parentheses, with significant effects indicated by *, **, and ***, representing probability at the 5, 1, and 0.1 % levels, respectively. All microbial ratios are on a molar basis

ns not significant ($p > 0.05$), *βG* β-glucosidase, *NAG N*-acetyl β-glucosaminidase *PME* phosphomonoesterase

[a] Marginally insignificant phosphorus effect of decreasing microbial C:N ratio ($F_{1,18} = 4.2$, $p = 0.055$)

receiving phosphorus, an increase of 49 %. Microbial phosphorus varied seasonally ($F_{12,108} = 13.2, p < 0.001$), with lowest concentrations in the dry season (Fig. 2f). In no-phosphorus plots, concentrations ranged from 41.1 ± 6.2 mg P kg^{-1} in the dry season (March 2007) to 82.4 ± 8.6 mg P kg^{-1} in the late wet season (November 2006), a 100 % increase (Fig. 2f). In phosphorus addition plots, concentrations ranged from 65.9 ± 12.6 mg P kg^{-1} in the dry season (March 2007) to 132.3 ± 18.3 mg P kg^{-1} in the wet season (November 2006), an increase of 101 % (Fig. 2f).

Microbial nutrient ratios

The microbial C:N ratio (molar) was increased significantly by nitrogen addition ($F_{1,18} = 3.8$, $p = 0.014$), but was not altered by phosphorus or potassium addition ($p > 0.05$) (Fig. 3a). However, a decrease in the ratio following phosphorus addition was marginally insignificant in the quarterly NPK factorial design ($F_{1,18} = 2.1, p = 0.055$) (not shown). Microbial C:N varied significantly among months ($F_{12,108} = 16.2, p < 0.001$), but there was no seasonal pattern (Fig. 3a). The mean microbial C:N ratio (molar basis) was 5.8 ± 0.2 in no-nitrogen plots and 6.2 ± 0.1 in plots receiving nitrogen.

The microbial C:P ratio (molar) was not affected by addition of any nutrient ($p > 0.05$). The mean microbial C:P ratio (molar basis) across all 32 plots in the NPK factorial design was 40.9 ± 2.2, but this conceals strong seasonal variation ($F_{12,108} = 9.1$, $p < 0.001$), with lowest ratios in the late wet season and early dry season (Fig. 3b). The monthly mean microbial C:P ratio across all plots in the NP factorial design ranged from 28.2 ± 1.6 in the early dry season (January 2007) to 54.9 ± 2.8 in the early wet season (May 2007) (Fig. 3b).

The microbial N:P ratio (molar) was not affected by addition of any nutrient ($p > 0.05$). The mean microbial N:P ratio (molar basis) across all 32 plots in the NPK factorial design was 6.9 ± 0.4, but this conceals strong seasonal variation ($F_{12,108} = 6.9$, $p = 0.001$), with lowest ratios in the late wet season and early dry season (Fig. 3c). The monthly mean

microbial N:P ratio across all plots in the NP factorial design ranged from 4.7 ± 0.2 in the late wet season (November 2007) to 8.6 ± 0.8 in the mid-wet season (July 2007).

Hydrolytic enzyme activities

Phosphomonoesterase activity

Phosphomonoesterase activity was decreased significantly by phosphorus addition ($F_{1,18} = 129, p < 0.001$; Fig. 4a), but was unaffected by nitrogen or potassium addition ($p > 0.05$). However, the effect of nitrogen was marginally insignificant ($F_{1,18} = 3.2$, $p = 0.09$) and activity was always higher where nitrogen was added alone compared to control plots (Fig. 4b). The mean phosphomonoesterase activity in the NPK design was 83.1 ± 3.5 nmol MU g^{-1} min^{-1} in no-phosphorus plots and 27.0 ± 0.9 nmol MU g^{-1} min^{-1} in plots receiving phosphorus, a decline of 68 %.

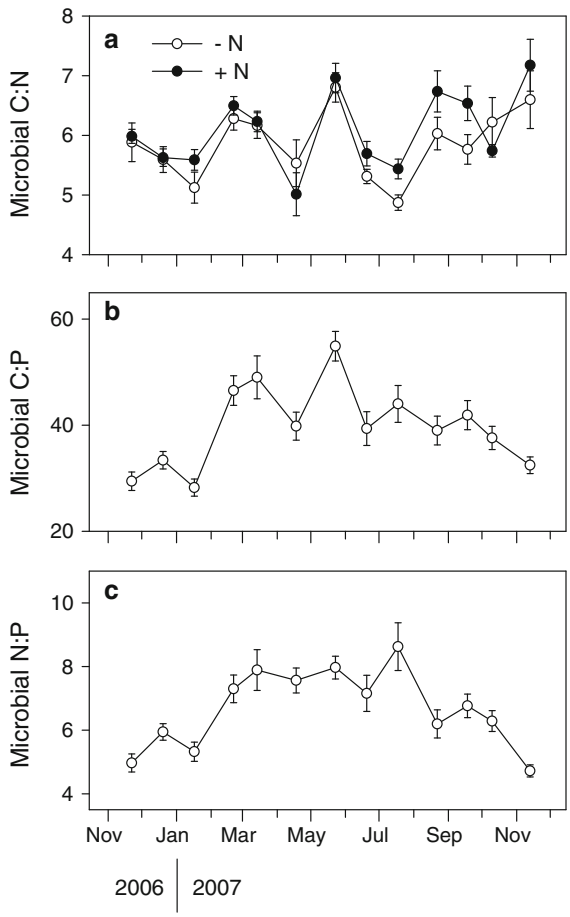

Fig. 3 Seasonal changes in the ratios of microbial carbon to nitrogen (**a**), carbon to phosphorus (**b**), and nitrogen to phosphorus (**c**) following a decade of fertilization of a lowland tropical forest on Gigante Peninsula, Panama. *Graphs* show the significant seasonal variation in all cases, and the significant effect of nitrogen addition on the microbial carbon to nitrogen ratio (panel **a**). Values are monthly means and standard errors in the NP factorial design (i.e. eight plots per treatment in **a**, 16 plots in **b** and **c**)

Phosphomonoesterase activity showed strong seasonal variation ($F_{12,108} = 8.4$, $p = 0.001$), with lowest activity in the dry season (Fig. 4b). For no-phosphorus plots in the monthly NP factorial design, activity ranged from 63.9 ± 4.1 nmol MU g^{-1} min^{-1} in the late dry season (April 2007) to 93.8 ± 7.9 nmol MU g^{-1} min^{-1} in the late wet season (November 2006), an increase of 47 %. For phosphorus addition plots, activity ranged from 23.3 ± 1.5 nmol MU g^{-1} min^{-1} in the dry season (February 2007) to 30.1 ± 2.1 nmol MU g^{-1} min^{-1} in the wet season (July 2007), an increase of 29 %. There was a significant time × phosphorus interaction ($F_{12,108} = 5.7$, $p = 0.005$), because the seasonal change in phosphomonoesterase activity was much smaller where phosphorus was added compared to no-phosphorus plots (Fig. 4b).

Phosphodiesterase activity

Phosphodiesterase activity was decreased significantly by phosphorus addition ($F_{1,18} = 169.2$, $p < 0.001$; Fig. 4c), but was unaffected by nitrogen or potassium addition ($p > 0.05$). The mean phosphodiesterase activity in the NPK design was 11.97 ± 0.41 nmol MU g^{-1} min^{-1} in no-phosphorus plots and 4.14 ± 0.08 nmol MU g^{-1} min^{-1} in plots receiving phosphorus, a decline of 65 %. There was significant seasonal variation in phosphodiesterase ($F_{12,108} = 6.5$, $p = 0.002$), with lowest activity in the dry season (Fig. 4d). There was a significant time × phosphorus interaction ($F_{12,108} = 4.1$; $p = 0.019$), because the seasonal variation in phosphodiesterase activity was much smaller where phosphorus was added compared to no-phosphorus plots (Fig. 4b).

β-Glucosidase activity

β-Glucosidase activity was unaffected by addition of any nutrient ($p > 0.05$; Fig. 5a), but showed strong seasonal variation ($F_{12,108} = 8.9$, $p < 0.001$), with

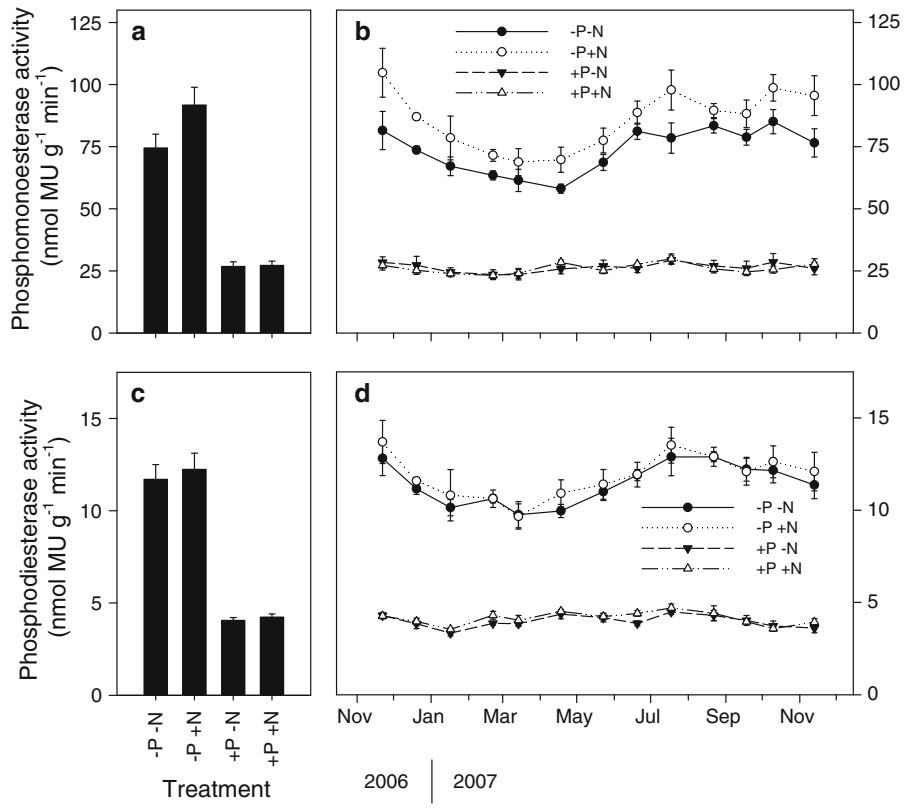

Fig. 4 Phosphomonoesterase activity (**a**, **b**) and phosphodiesterase activity (**c**, **d**) following a decade of fertilization of a lowland tropical forest on Gigante Peninsula, Panama. Graphs on the left show treatment effects in the full NPK factorial design (means and standard errors of eight plots in each treatment sampled four times between November 2006 and November 2007). Graphs on the right show significant seasonal changes in the NP factorial design (means and standard errors of the four replicate plots in each treatment sampled monthly between November 2006 and November 2007). See Table 1 for significant effects. In the units of enzyme activity, MU = methylumbelliferone

lowest activity in the dry season (Fig. 5b). The mean β glucosidase activity varied from 2.43 ± 0.13 nmol MU g^{-1} min^{-1} in the dry season (March 2007) to 3.75 ± 0.16 nmol MU g^{-1} min^{-1} in the wet season (July 2007), an increase of 35 %.

N-acetyl β-glucosaminidase activity

The activity of *N*-acetyl β-glucosaminidase was decreased significantly by phosphorus addition ($F_{1,18} = 4.9$, $p = 0.04$), but was unaffected by nitrogen or potassium addition (Fig. 5c). The mean *N*-acetyl β-glucosaminidase activity in the NPK factorial design was 6.18 ± 0.41 nmol MU g^{-1} min^{-1} in no-phosphorus plots and 4.71 ± 0.20 nmol MU g^{-1} min^{-1} in plots receiving phosphorus (Fig. 5c), a decline of 24 %. There was significant seasonal variation ($F_{12,108} = 13.9$, $p < 0.001$), with lowest activity in the dry season (Fig. 5d). Mean monthly *N*-acetyl β-glucosaminidase activity in no-phosphorus plots ranged from 3.82 ± 0.53 nmol MU g^{-1} min^{-1} in the dry season (March 2007) to 7.41 ± 1.08 nmol MU g^{-1} min^{-1} in the late wet season (November 2006), an approximate two-fold difference. For phosphorus addition plots, activity ranged from 3.29 ± 0.30 nmol MU g^{-1} min^{-1} in the dry season (March 2007) to 5.98 ± 0.64 nmol MU g^{-1} min^{-1} in the wet season (July 2007), an 82 % increase.

Stoichiometric enzyme ratios

The β-glucosidase to *N*-acetyl β-glucosaminidase ratio (C:N) was increased significantly by phosphorus addition ($F_{1,18} = 10.4$, $p = 0.005$), but was not affected by nitrogen or potassium addition (Fig. 6a). The mean β-glucosidase to *N*-acetyl β-glucosaminidase ratio in the

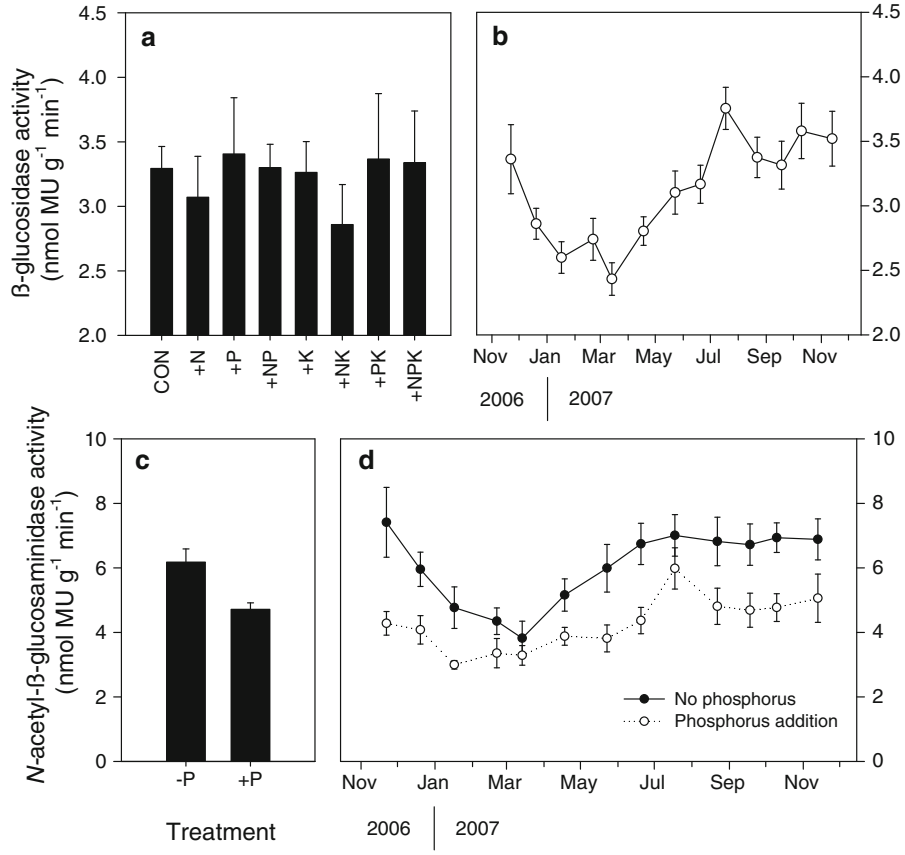

Fig. 5 The activity of β-glucosidase (**a**, **b**) and *N*-acetyl β-glucosaminidase (**c**, **d**) following a decade of fertilization of a lowland tropical forest on Gigante Peninsula, Panama. For β-glucosidase, the figure shows **a** the absence of a significant treatment effect in the full NPK factorial design (means and standard errors of four replicate plots in each treatment sampled four times between November 2006 and November 2007) and **b** the significant seasonal variation in the NP factorial design (means and standard errors of 16 plots per treatment sampled monthly over the same period). For *N*-acetyl β-glucosaminidase, the figure shows **c** the significant effect of phosphorus addition in the full NPK factorial design (means and standard errors of sixteen replicate plots in each treatment sampled four times between November 2006 and November 2007) and **d** the significant seasonal variation in the NP factorial design (means and standard errors of eight no-phosphorus and eight phosphorus addition plots sampled monthly over the same period). In the units of enzyme activity, MU = methylumbelliferone

NPK factorial design was 0.57 ± 0.03 in no-phosphorus plots and 0.75 ± 0.02 in phosphorus addition plots, an increase of 32 % (Fig. 6a). The ratio varied seasonally ($F_{12,108} = 3.6$, $p = 0.01$), being highest in the dry season (Fig. 6b). The seasonal effect appeared strongest for plots that did not receive phosphorus, although the time × phosphorus interaction was not significant ($p = 0.22$).

The β-glucosidase to phosphomonoesterase ratio (C:P) was increased by phosphorus addition ($F_{1,18} = 188$, $p < 0.001$), but was not affected by nitrogen or potassium addition (Fig. 6c). The mean β-glucosidase to phosphomonoesterase ratio in the NPK factorial design was 0.039 ± 0.002 in no-phosphorus plots and 0.126 ± 0.004 in phosphorus addition plots, a greater than three-fold increase (Fig. 6c). The ratio varied seasonally ($F_{12,108} = 8.5$, $p < 0.001$), being lowest in the dry season (Fig. 6d). There was a significant time × phosphorus interaction ($F_{12,108} = 3.5$, $p = 0.010$), because the seasonal variation was greater in the phosphorus addition plots compared to the no-phosphorus plots (Fig. 6d).

The *N*-acetyl β-glucosaminidase to phosphomonoesterase ratio (N:P) was increased by phosphorus addition ($F_{1,18} = 103$, $p < 0.001$), but was not affected by nitrogen or potassium addition (Fig. 6e). The mean *N*-acetyl β-glucosaminidase to phosphomonoesterase ratio in the NPK factorial design was 0.073 ± 0.0053 in no-

phosphorus plots and 0.174 ± 0.005 in phosphorus addition plots, a greater than two-fold increase (Fig. 6e). The ratio varied seasonally ($F_{12,108} = 5.1$, $p = 0.005$), being lowest in the dry season (Fig. 6f). There was a significant time × phosphorus interaction ($F_{12,108} = 5.6$, $p = 0.003$), because the decline in the dry season was greater in the phosphorus addition plots compared to the no-phosphorus plots (Fig. 6f).

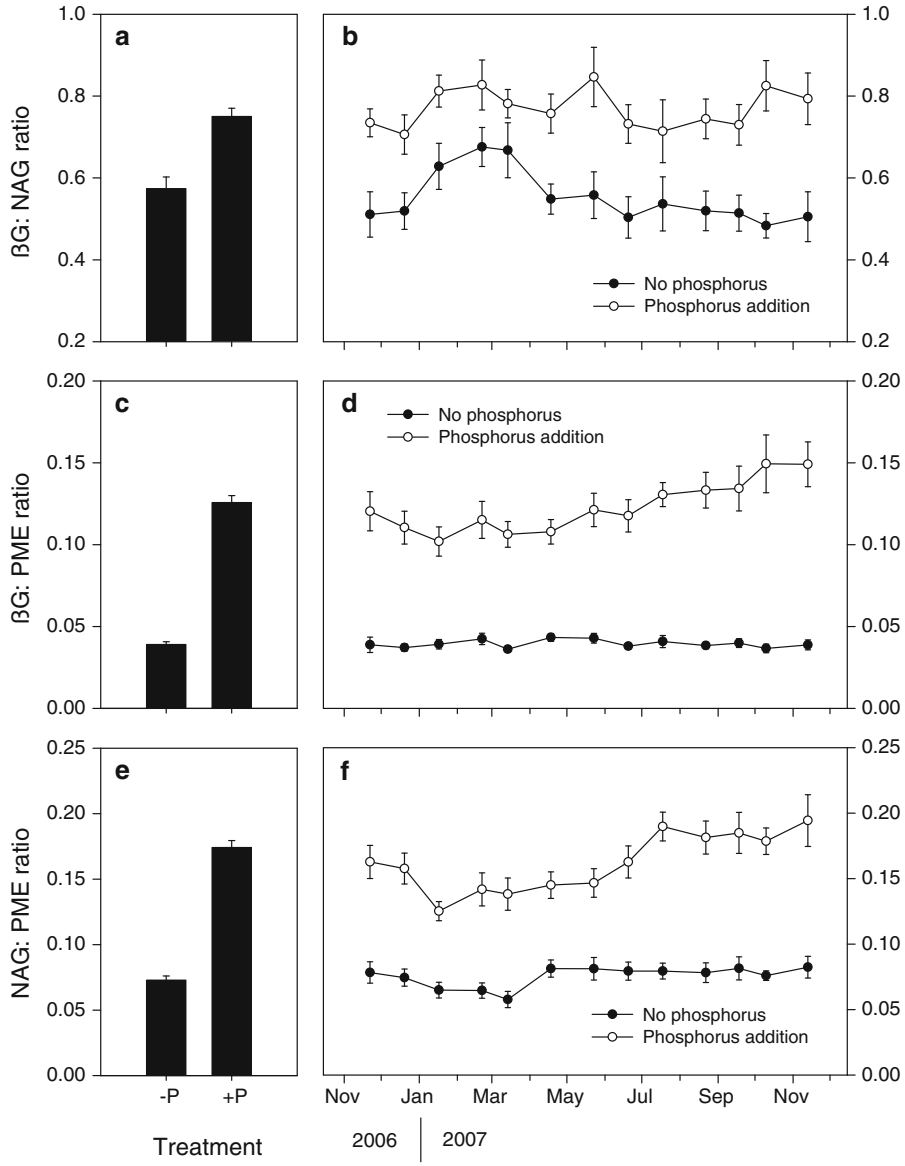

Fig. 6 Stoichiometric enzyme ratios, showing the ratio of carbon to nitrogen enzymes (β-glucosidase to *N*-acetyl β-glucosaminidase) (**a**, **b**), the ratio of carbon to phosphorus enzymes (β-glucosidase to phosphomonoesterase) (**c**, **d**), and the ratio of nitrogen to phosphorus enzymes (*N*-acetyl β-glucosaminidase to phosphomonoesterase) (**e**, **f**). *Graphs* on the *left* (**a**, **c**, **e**) show the significant effect of phosphorus addition in the full NPK factorial design (means and standard errors of 16 replicate plots per treatment sampled four times between November 2006 and November 2007). *Graphs* on the *right* show significant seasonal changes in the NP factorial design (means and standard errors of eight no-phosphorus and eight phosphorus addition plots sampled monthly over the same period). *βG*, β-glucosidase; *NAG*, *N*-acetyl β-glucosaminidase; *PME*, phosphomonoesterase

Discussion

The clear response of the soil microbial biomass and hydrolytic enzyme activities to long-term phosphorus addition demonstrates the significance of phosphorus as the element limiting the microbial community in this lowland tropical forest. Phosphorus addition reduced soil phosphatase activities and increased carbon, nitrogen, and phosphorus concentrations in the microbial biomass, while the addition of nitrogen, potassium, other base cations, and micronutrients elicited no response. The high rates of phosphatase activity compared to enzymes involved in carbon and nitrogen acquisition (i.e., β-glucosidase and *N*-acetyl β-glucosaminidase to phosphomonoesterase ratios <0.1; Fig. 6) further point to the strong microbial phosphorus limitation on Gigante Peninsula. Indeed, the phosphomonoesterase activities reported here are greater than the majority of the values reported in a global compilation (which included only a single lowland tropical forest), while activities of β-glucosidase and *N*-acetyl β-glucosaminidase are within the global range (Sinsabaugh et al. 2008).

These results are consistent with evidence for limitation of litter decomposition by phosphorus and micronutrients at this site (Kaspari et al. 2008), but differ from evidence for nutrient limitation of plant productivity, which responds to nitrogen, phosphorus, and potassium addition (Wright et al. 2011). The increased microbial biomass with phosphorus addition could have been caused indirectly by the approximately 25 % increase in fine litter fall in the phosphorus treatment (Wright et al. 2011), but this seems unlikely given that doubling the litter standing crop in an adjacent experiment did not significantly increase microbial biomass (Sayer et al. 2012). Collectively, results from the Gigante fertilization experiment provide strong evidence that plants and microbes are limited by different nutrients in this lowland tropical forest.

Although soil microbes are usually assumed to be limited by organic carbon availability (Wardle 1992), several previous studies have reported phosphorus limitation of microbial processes in tropical forests (Cleveland et al. 2002; Cleveland & Townsend 2006; Liu et al. 2012) and plantations (Gnankambary et al. 2008) on strongly-weathered soils. For example, 3 years of phosphorus addition promoted an increase in soil respiration during the dry to wet season transition in a lowland forest in Costa Rica (Cleveland and Townsend 2006). In contrast, studies in tropical montane forests have reported increases in microbial biomass in response to nitrogen addition (Li et al. 2006; Corre et al. 2010; Cusack et al. 2011). This supports the assumption that tropical montane forests are limited by nitrogen availability (Tanner et al. 1998), although none of the three montane studies included a phosphorus treatment, and hydrolytic enzyme activities reported by Cusack et al. (2011) indicate a much greater investment in phosphorus acquisition compared to nitrogen acquisition. Given the strong nutrient gradients that can occur in tropical montane forests (e.g., Andersen et al. 2010), it seems likely that phosphorus might also regulate microbial biomass in those environments.

It was recently shown that microbial nutrient ratios are relatively well-constrained across ecosystems worldwide (Cleveland and Liptzin 2007). This is consistent with the results reported here, because despite marked seasonal variation, microbial nutrient ratios were not affected greatly by a decade of nutrient addition, with a significant effect only for microbial C:N in response to nitrogen addition. Mean microbial C:N:P ratios in the fertilization experiment across all plots and sampling dates were 41:6:1, which is reasonably close to the global mean for all soils of 60:7:1 (at least for N:P), but lower than the global mean for forest soils of 74:9:1 (Cleveland and Liptzin 2007). The fact that our ratios are lower than the global mean for forest soils is surprising given that tropical soils are under-represented in the global data set and suggests that temperate forests are more strongly phosphorus limited than commonly assumed. In their meta-analysis, Cleveland and Liptzin (2007) suggested that microbial N:P might provide greater insight into ecosystem nutrient status than foliar N:P in tropical forests given marked variation in foliar N:P among tropical tree species. Here, microbial N:P ratios were lower than the global mean for forest soils and did not vary greatly following long-term phosphorus addition, despite considerable changes in microbial nutrient concentrations. This suggests that microbial nutrient ratios are well constrained for a given soil and therefore provide limited insight into microbial nutrient status.

In a meta-analysis of the response of phosphatase activity to experimental nutrient addition, Marklein and Houlton (2012) found that phosphorus addition

strongly suppressed root and soil phosphatase activity, while nitrogen addition enhanced phosphatase activity, although the increase was slight for soil phosphatase (Fig. 3b in Marklein and Houlton). Results for the lowland tropical forest studied here support the strong suppression of phosphatase by phosphorus addition, but not the enhancement by nitrogen addition. This might be due to the relatively high intrinsic nitrogen availability at the site (Corre et al. 2010; Hietz et al. 2011).

Olander and Vitousek (2000) showed that the activity of *N*-acetyl β-glucosaminidase declined as nitrogen availability increased in tropical montane forests along the Hawaiian Islands soil chronosequence. They also detected a decrease in activity following nitrogen addition in the organic horizon of a young, nitrogen limited site. Our finding that *N*-acetyl β-glucosaminidase decreased with phosphorus addition, but not nitrogen addition, was therefore unexpected. We suggest two possible mechanisms. First, the activity of *N*-acetyl β-glucosaminidase might reflect factors other than nitrogen demand, such as fungal biomass and activity (Miller et al. 1998). Second, reduced investment in nitrogen-rich phosphatase enzymes under phosphorus addition would increase cellular nitrogen and, in turn, allow microbes to reduce investment in nitrogen-acquiring enzymes such as *N*-acetyl β-glucosaminidase. This explanation is consistent with the increase in microbial nitrogen under phosphorus addition.

Despite a decade of phosphorus addition and a marked increase in extractable soil phosphorus, phosphatase activity in the phosphorus addition treatment remained relatively high in comparison to β-glucosidase and *N*-acetyl β-glucosaminidase activities (e.g., a mean β-glucosidase to phosphomonoesterase ratio of 0.13 in phosphorus addition plots). Possible explanations include (i) phosphatase activity in phosphorus addition plots is derived from stabilized extracellular enzymes, rather than actively synthesized enzymes (i.e. the extracellular fraction of the activity is insensitive to phosphorus status), (ii) the phosphatase assay includes constitutive or intracellular enzymes involved in cellular function and not linked to phosphorus acquisition from the ambient environment (Allison and Vitousek 2005), and (iii) phosphatase is involved in processes other than phosphorus acquisition (e.g., carbon acquisition; Heath 2005; Spohn and Kuzyakov 2013). Although a number of soil microbes synthesize phosphatase constitutively, the first possibility seems likely to make the greatest contribution, given that phosphatase is readily stabilized by sorption onto clay surfaces or by complexation with soil organic matter (Quiquampoix 2000; Rao et al. 2000) and can then constitute a permanent potential activity that is independent of the soil microbial biomass (Burns 1982). If this is the case, it indicates that up to one-third of the phosphatase activity in untreated soils is associated with stabilized extracellular enzymes.

There was marked seasonal variation in all measures of microbial biomass and hydrolytic enzymes, with lowest concentrations and activities in the dry season. Similar seasonal variation in microbial biomass has been reported previously for other tropical forest sites (Singh et al. 1989; Ruan et al. 2004; Liu et al. 2012) and is linked to the death of microbial cells following desiccation (Salema et al. 1982; West et al. 1992). Drying also reduces enzyme activity (Speir & Ross 1978), including in tropical forest soils (Turner and Romero 2010). The death of microbial biomass in the dry season can represent a considerable release of nitrogen and phosphorus to the soil (Singh et al. 1989; Srivastava 1997). Here, the dry season declines in microbial nitrogen and phosphorus equate to the release of 118 kg N ha^{-1} and 41 kg P ha^{-1}, calculated for the upper 10 cm of soil and assuming a bulk density of 1.0 g cm^{-3} (Sayer and Tanner 2010). These amounts are similar to those added experimentally as fertilizer. The amount of nitrogen released from microbial biomass is similar to that returned annually in fine litter fall on Gigante Peninsula (143 kg N ha^{-1}), but the amount of phosphorus is several times greater (6 kg P ha^{-1} in annual litter fall) (Sayer and Tanner 2010).

The seasonal changes in microbial nutrients were accompanied by parallel changes in microbial C:P and N:P ratios, which both declined in the late wet season and increased again in the mid-dry season. Stoichiometric enzyme ratios also varied seasonally, with higher β-glucosidase to *N*-acetyl β-glucosaminidase ratios in the dry season and higher β-glucosidase and *N*-acetyl β-glucosaminidase to phosphomonoesterase ratios in the wet season. These patterns suggest either changes in the nutrient content of microbial cells in response to seasonal variation in nutrient availability, or a seasonal shift in microbial community composition (e.g., changing proportions of bacteria and fungi; Eaton et al. 2011). Seasonal changes in the microbial

community seem most likely, because the ratios varied in a similar manner in both control and nutrient addition plots and were therefore not a simple response to fertilizer addition in the wet season.

Microbial carbon and nitrogen were previously determined in the Gigante experiment after 6 years of nutrient addition (a single sampling in the mid-wet season), but no significant differences were detected among treatments (Sayer et al. 2012). This means that either additional years of treatments, or multiple analyses over the annual cycle, were necessary to detect the significant effect of phosphorus addition on microbial biomass in this experiment. This might explain why no significant differences in microbial carbon or nitrogen were detected after 1 year of nitrogen and phosphorus addition to an Oxisol supporting secondary tropical forest (Davidson et al. 2004) and highlights the value of long-term ecological experiments in tropical forests.

Given that previous measurements after 6 years of fertilization in the Gigante experiment showed no change in microbial carbon and nitrogen among treatments (Sayer et al. 2012), and that the results presented here show no effect of nitrogen addition on the microbial biomass, we cannot explain why a previous study during the wet season after 9 years of fertilization detected a 46 % decline in microbial carbon and a 29 % decline in microbial nitrogen in the four plots of the nitrogen only treatment (Corre et al. 2010). The microbial carbon concentration reported for control plots in that study (1.23 g C kg^{-1}) is similar to values reported here for wet season samples, whereas the concentration in the nitrogen addition plots is considerably lower and similar to our values for dry season samples (Fig. 2). As we conducted multiple measurements across the entire experiment (i.e. all 32 plots in the NPK factorial design plus the four dolomite/micronutrient plots), compared to the limited number of samples collected on one occasion in Corre et al. (2010), we disregard the latter result and conclude that microbial biomass did not respond to nitrogen addition in the experiment.

The strong limitation of microbial biomass by phosphorus availability in this lowland tropical forest suggests that efforts to incorporate enzymes into biogeochemical models must account for the disproportionate investment in phosphorus acquisition by microbial communities in strongly-weathered soils. Some recent models of soil organic matter cycling explicitly incorporate nitrogen availability, but do not include phosphorus as a factor regulating microbial processes, including enzyme synthesis (e.g., Schimel and Weintraub 2003). The high rates of phosphatase activity and low ratios of carbon and nitrogen to phosphorus enzymes reported here demonstrate clearly the extent to which below-ground phosphorus limitation can influence microbial communities. Despite this, we caution against the assumption that microbial communities will be limited by phosphorus in all lowland tropical forests. Although vast areas of tropical forest occur on strongly-weathered soils, many tropical forests are supported by fertile soils in which microbial phosphorus limitation is unlikely. In central Panama, for example, soils contain total phosphorus concentrations between 74 and 1,650 mg P kg^{-1} (Turner and Engelbrecht 2011) compared to $\sim$400 mg P kg^{-1} on the Gigante Peninsula (Vincent et al. 2010).

In summary, microbial biomass in a lowland tropical forest soil responded strongly to phosphorus addition, but not to nitrogen or potassium addition. Phosphorus addition increased microbial carbon, nitrogen, and phosphorus, and caused a marked decline in the activity of enzymes involved in the acquisition of phosphorus from organic compounds. Nitrogen addition did not increase investment in the activity of any enzyme, including phosphatases, perhaps due to the relatively high intrinsic nitrogen availability at the site. Microbial biomass and enzyme activities showed strong seasonal variation, with the dry season decline in microbial nitrogen and phosphorus accounting for a similar or greater amount of these nutrients than in annual fine litter fall. Although a combination of nutrients limits plant productivity on Gigante Peninsula, we conclude that microbial biomass is constrained by phosphorus availability in this strongly-weathered tropical forest soil.

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