Oxygen isotope ratios of plant available phosphate in lowland tropical forest soils

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1. Introduction

Nutrient availability limits the productivity of many tropical forests (Vitousek, 1984; Cleveland et al., 2011; Wright et al., 2011). Phosphorus (P) is widely believed to be the primary limiting nutrient on the old, strongly weathered soils that characterize much of the lowland tropics, because many such soils have high P fixation capacities and contain low concentrations of soluble phosphate. Therefore, the maintenance of bioavailable P in sufficient amounts to sustain the productivity of lowland tropical forests depends strongly on the continuous recycling of P within the soils (Vitousek, 1984; Cleveland et al., 2011; Turner et al., 2013). The aim of this project was to derive information on the processes that control phosphate cycling in tropical forest soils, based on natural variation in the isotopic composition of oxygen in soil phosphate (δ18O). This technique has provided valuable information on soil P dynamics in different ecosystems worldwide (Zohar et al., 2010; Angert et al., 2011, 2012; Tamburini et al., 2012; Gross et al., 2013; Jaisi et al., 2014; Gross and Angert, 2015), but has not so far been applied in tropical soils.

We took advantage of two long-term and well-studied field experiments in lowland tropical forest in Panama: an experiment initiated in 1998 involving factorial P, nitrogen (N) and potassium (K) additions (Wright et al., 2011; Yavitt et al., 2011; Sayer et al., 2012; Mirabello et al., 2013; Schreeg et al., 2013; Turner et al., 2013).
Wright, 2014), and a second experiment initiated in 2003 at the same site involving litter manipulation (removal and addition) (Sayer and Tanner, 2010; Vincent et al., 2010; Sayer et al., 2012). These experiments allowed us to isolate the role of nutrients and leaf litter status in the soil P cycle and study the processes that mediate soil P dynamics, while controlling other soil properties such as texture and organic matter content.

The use of δ18O as a tracer for phosphate cycling in soils is based on the stability of the P–O bond, which under natural soil conditions can only be broken by biological reactions (Tuclidge, 1960; Dahms and Boyer, 1973; Kolodny et al., 1983). Thus, the involvement of various reactions in the soil P cycle can be determined from changes in δ18O caused by the incorporation of new oxygen with a different isotopic composition. The major processes that affect the isotopic composition of bioavailable soil phosphate on short time scales are (Jaisi and Blake, 2014; Gross and Angert, 2015):

1) the exchange of phosphate molecules among soil P pools may cause isotopic dilution if their δ18O values are different (Zohar et al., 2010; Angert et al., 2012). Such abiotic exchange reactions do not cleave the P–O bond of the phosphate.
2) the assimilation of phosphate by microorganisms which preferentially take up lighter isotopologues, and therefore cause the remaining phosphate in the soil to be enriched in 18O (Blake et al., 2005).
3) Biological reactions that promote the exchange of oxygen between phosphate and water. These include:
   a) the hydrolysis of organic-P compounds by extracellular phosphatases, which induce strong kinetic effects and produces phosphate depleted in 18O (Liang and Blake, 2006, 2009; Von Sperring et al., 2014).
   b) the microbial turnover of phosphate, which produces phosphate in isotopic equilibrium with water as part of cell metabolism (Blake et al., 2005; Stout et al., 2014). This equilibrium is temperature dependent and is well described by the following equation (Longinelli and Nuti, 1973; Kolodny et al., 1983):

\[
\delta^{18}O_P = \delta^{18}O_{\text{water}} + \left(111.4 - T\right)/4.3 \text{ (where } T \text{ is the temperature in } ^\circ\text{C)}
\]  

where T is the temperature in °C and δ18Owater is the isotopic composition of oxygen in soil water.

We designed a series of controlled incubation experiments with labeled phosphate compounds to evaluate the role of each of these processes on phosphate δ18O values in lowland tropical soils (Gross and Angert, 2015). We focused our measurements on phosphate that can be extracted with anion exchange resin (hereafter “resin-P”), which is considered to approximate the phosphate pool that is available for plant uptake (Cooperband et al., 1999; Qian and Schoenau, 2002). The incubations had three aims. The first aim was to determine whether a biologically mediated cleavage of the P–O bond of the added phosphate compound occurred or simply an exchange of the added phosphate with P from different soil P pools.

The second aim was to determine the role of organic-P mineralization and phosphate uptake by microbes. The third aim was to determine whether changes in the soil-resin-P δ18O values reflect a movement towards isotopic equilibrium with soil water, as was reported for the resin-P pool in a series of natural extra tropical soils (Zohar et al., 2010; Angert et al., 2011, 2012; Tamburini et al., 2012; Gross et al., 2013; Jaisi and Blake, 2014; Gross and Angert, 2015).

To detect whether the resin-P δ18O values we measured at the end of the incubation period can be also found in soils in the field, we determined the δ18O values for the same soils in such field circumstances. We hypothesized that in accordance with reports from other ecosystems, microbial biomass rapidly recycles phosphate added to the soil, altering the δ18O values towards isotopic equilibrium with soil water both in the incubations and in the field. Our second hypothesis was that δ18O responds to the addition of other nutrients (i.e., P, N and K) and litter manipulations. Specifically, we predicted that soil phosphate δ18O values would (1) deviate from isotopic equilibrium in soils that received chronic P additions because of lower microbial demands for P (Turner and Wright, 2014) and (2) reflect the kinetic isotopic fractionation induced during mineralization of organic-P in soils that received double amounts of litter.

2. Methods

2.1. Study site

This study was performed at the Gigante Peninsula (9.063°N; 79.50°W) (Wright et al., 2011), which is part of the Barro Colorado Nature Monument (BCNM), Republic of Panama. The tropical monsoon (Köppen system) climate has a mean annual temperature of 27 °C and mean annual rainfall of 2600 mm (Windsor, 1990). Soils on the upper parts of the landscape 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). Lower parts of the landscape are Inceptisols (Corre et al., 2010) with a shallow water table during the wet season. The soils are moderately acidic (pH 4.8–6.1 (Koehler et al., 2009)) with low readily available phosphate concentrations (<1 mg P kg⁻¹) low nitrate concentrations (~1 µg N g soil⁻¹) and a moderate potassium (K) concentration (~100 µg K g soil⁻¹), which are representative of forest soils in central Panama (Turner et al., 2013).

The fertilization experiment began in 1998 and is described in detail elsewhere (Yavitt et al., 2009; Wright et al., 2011). The experiment is remarkable in its duration and in the range of responses observed in terms of forest productivity, with significant effects involving N, P, and K (Wright et al., 2011). Briefly, fertilizer has been added four times per year at intervals of 45–60 days during the wet season between May and October to thirty two 40 m x 40 m plots which replicates each treatment in a full factorial design four times. Nitrogen is added as urea, P as triple superphosphate (Ca(H2PO4)2), and K as KCl. Annual doses were 125 kg N ha⁻¹, 50 kg P ha⁻¹ and 50 kg K ha⁻¹. To preclude movement of nutrients among plots, and reduce uptake by trees rooted in plots with different treatments, the minimum distance between plots was 40 m. Adjacent to the fertilization experiment, the Gigante Litter Manipulation Project (GLIMP) was established in 2000 (see Sayer and Tanner, 2010 for a detailed description of the experiment). Briefly, the litter was removed from five 45 m x 45 m plots once every three months (L plots) starting in 2003 and immediately spread over five plots (effectively doubling the litter standing crop; L plots). All GLIMP plots were trenched to a depth of 50 cm, lined with plastic and refilled to minimize transport of nutrients and water between treatments by roots and mycorrhizas.

2.2. Soil sampling

We sampled soils during the wet season when 90% of annual rainfall occurs (Windsor, 1990) and maximal biological activity is expected. The sampling was conducted in two campaigns, during August and September 2012, four and eight weeks after the most recent application of fertilizers. Soil samples were collected in each sampling campaign from the same three replicates of the full
N × P × K factorial design (Control, P, NP, KP, NPK, N, NK, K), and from two replicate plots receiving litter manipulations (L− and L+). This generated six samples for each of the factorial N × P × K treatments and four samples for each of the litter manipulation treatments. Rain occurred both nights before soils were sampled and also during the soil sampling in September. We collected rainfall samples on each of the sampling days; we assume the rain water δ18O\text{rain} values are the same as soil water δ18O values (Hsieh et al., 1998).

Soils were taken from the upper soil profile (0–10 cm deep) using a 2.5 cm diameter corer at nine random points in the central 20 × 20 m of each plot. Samples from each plot were composited in the field and returned immediately to the lab, where roots, stones, and large fauna were removed by hand. Soil moisture content was determined gravimetrically by drying a subsample of field-moist soils for 24 h at 105 °C. The incubation experiments were started the following day, using field-moist soils stored overnight at 4 °C to retain their biological activity. A final subsample of each soil sample was oven dried at 60 °C to prevent biological activity from altering phosphate isotopic composition, transported to the Hebrew University labs, ground and sieved to 2 mm.

2.3. Determination of basal concentrations of resin-P in fresh soils and of phosphate released after rewetting of dry soils

To determine the resin-P concentrations in fresh soils, a sub-sample of 1 g field moist soil was shaken on an orbital shaker with anion exchange resin membranes (BDH-55164) in 50 mL of double deionized water for 24 h. These resin membranes, which act as passive ion sinks, are considered analogous to biological uptake (Cooperband et al., 1999; Qian and Schoenau, 2002). To remove the extracted phosphate, the resin membranes were shaken overnight in 5 mL of 0.2 M HNO3 (Weiner et al., 2011; Gross and Angert, 2015).

To determine P concentrations following rewetting of dry soils, 1 g of field moist soil (fresh mass) was first dried at 60 °C for 24 h and stored sealed at 4 °C until analyzed. The dried soil samples were rewetted with 50 mL of double deionized water and shaken on an orbital shaker for 24 h. During the rewetting step, the released phosphate was extracted from the soils with anion exchange resins, as described above for resin-P.

Phosphate concentrations in the extracted solutions of both the fresh and dried/rewetted soils were determined by molybdate colorimetry (Murphy and Riley, 1962). The average difference between duplicate samples was 1.3%. The reported concentrations are the average value for three different plots for each treatment for the N:P:K factorial experiment and for two different plots for the litter manipulation experiment.

2.4. Laboratory incubation experiments

The incubations were carried out with biologically active fresh soils from the different fertilization and litter manipulation treatments, supplemented with a pulse of phosphate compound with known initial δ18O values. 20 g (fresh mass) of field moist soil was supplied with 160 μg P g−1 soil as triple superphosphate fertilizer Ca(H2PO4)2 or KH2PO4 in 1 L of de-ionized water (with known δ18O\text{water} values).

We conducted three incubations with three different combinations of δ18O\text{P}, and δ18O\text{water} values (described below). An isotopic equilibrium (Eq. (1)) can be calculated for each δ18O\text{water} value and the constant incubation temperature of 23 °C. Deviations from this equilibrium provide insight into the processes affecting P recycling (as described below). The amounts of P added in all of the incubations (160 μg P g−1 soil) were large relative to the original soil resin-P concentrations in the control, K, L− and L+ plots (see section 3.1). The high amounts added minimized any artifacts that might result from isotopic dilution between the added P and the original soil P. The amounts of P added were close to the resin-P concentrations in the P and NPK plots (see 3.1). The superphosphate fertilizer added in our incubations was the same as the phosphate fertilizer used in the N:P:K long-term field experiment. This way we prevented artifacts that might result from isotopic dilution between the original and added P. Based on a series of preliminary experiments and on a previous study (Gross and Angert, 2015) we found that incubations that last five days are sufficient to approach steady state soil phosphate δ18O\text{P} values.

The first incubation included P as triple superphosphate fertilizer, with δ18O\text{P} of 21.6 ± 0.3‰ (Hummer International, Earth City, Missouri, USA) and 18O labeled de-ionized water as the incubation media water with δ18O\text{water} of 27.6‰. The expected isotopic equilibrium in this incubation is 48‰. In the first incubation we used control soils only and determined whether oxygen atoms from the labeled media water were incorporated to the phosphate.

The second incubation included the same P compound (triple superphosphate fertilizer, with δ18O\text{P} of 21.6‰) and incubation media water with δ18O\text{water} of −4.6‰. In the second incubation we used soils from the control, K, P, NPK, L− and L+ plots. By using soils from the different treatments, we studied the effect of soil nutrient status and litter amounts on δ18O\text{P}.

The third incubation included P as potassium di-hydrogen phosphate (KH2PO4, Sigma–Aldrich Israel Ltd, Rehovot, Israel), with δ18O\text{P} of 12.5 ± 0.2‰, and the same incubation media water with δ18O\text{water} of −4.6‰. In the third incubation we used control soils only. The δ18O\text{P} values of the two added P compounds were above and below the equilibrium expected with δ18O\text{water} of −4.6‰, which is 16‰. Thus, we could determine whether changes in the soil phosphate δ18O\text{P} values are driven below, above or towards isotopic equilibrium. δ18O\text{P} values that are driven below the expected equilibrium will reflect the mineralization of organic-P compounds whereas δ18O\text{P} values that are driven above equilibrium will reflect microbial uptake.

Each incubation experiment was carried out for 5 days on an orbital shaker at a constant temperature of 23 °C in 1.2 L plastic containers. The containers were sealed to keep the water δ18O\text{water} values constant during the entire experiment and thereby minimize the isotopic enrichment effect associated with evaporation. This was verified by measuring the incubation δ18O\text{P} at the end of the incubation period and comparing final and initial values. We monitored oxygen conditions using standard oxygen indicator tablets and regularly ventilated the containers to enable oxygen from ambient air to enter the containers and preserveoxic conditions. We also monitored pH, which remained constant (−7) throughout the incubations. We performed two replicate incubations for each combination of δ18O\text{P} and δ18O\text{water} and soil (control, K, P, NPK, L− and L+).

Resin-P was extracted from the containers after 5 incubation days with anion exchange resins that were added for an additional 24 h and its δ18O\text{P} values were determined. As the anion exchange resins also retain small organic compounds (Cheesman et al., 2010), we examined whether our measurements included phosphate that was unintentionally released from soil organic compounds during the nitric acid extraction, which might bias our isotopic measurements. For this purpose we used control and isotopically enriched 18O HNO3 solution for the extraction step and measured the δ18O\text{P} values of the extracted phosphate. This test showed no incorporation of 18O atoms in the resin-P, from which we conclude that no organic P was mineralized during the resin extraction steps.

2.5. δ18O\text{water} measurements after rewetting dry field samples

Since resin-P concentrations are extremely low in control soils at our site and in many lowland tropical soils (Quesada et al., 2010;
Turner et al., 2013), it was not possible to measure $\delta^{18}O_P$ values directly. For this reason, we measured the values of phosphate that is released after rewetting of dry soils, which is a good indicator for the P that will be available in the onset of the wet season (Turner and Haygarth, 2001; Mirabello et al., 2013) and thus can be an important source of soluble phosphate especially in seasonal tropical forests that experience strong dry and wet seasons each year (Windsor, 1990). A sub-sample of 100 g of dry soil from plots that received chronic P additions (P, NPK, PK, NP) and from plots that did not receive P additions (N, K, NK, L) and control plots were put in 6 L plastic containers and shaken for 24 h with 5 L unlabeled de-ionized water. The measurements were performed for three and two replicate plots from each fertilizer and litter treatment, respectively. Phosphate concentrations were determined as described in section 2.3. In order to calculate the expected isotopic equilibrium in the soil we assumed that the rain water $\delta^{18}O_{rain}$ values are indicative of the soil water (~2.4‰). It is likely that this assumption was valid because this study was conducted in the rainiest period of the year, when rain occurred almost every day, including on the sampling days, and modeled water evaporation from the soil is very low in high rainfall sites and thus soil water $\delta^{18}O_{soil}$ values similar to rain water $\delta^{18}O_{rain}$ are typical following periods of rain (Hsieh et al., 1998).

2.6. Phosphate extraction and purification for isotopic analysis

The $\delta^{18}O_P$ values of the resin-P at the end of the incubation period and the phosphate that was released after the rewetting of dry soils were determined as described in Weiner et al. (2011). Briefly, the phosphate was first extracted from either the incubated fresh soils or the rewetted dry soils using anion exchange resins as described above. Solubilized phosphate was then eluted from the membranes by shaking them in 0.2 M HNO₃ overnight. To isolate the eluted phosphate from other oxygen containing compounds we performed several additional steps. First, organic matter was removed from the resin extract by shaking overnight with 20 mL of Superlite™ DAX-8 resin (Supelco/Sigma Aldrich). The phosphate was then extracted and purified by the precipitation of cerium phosphate. The precipitate was dissolved in nitric acid, the cerium was then extracted and purified by the precipitation of cerium phosphate. The precipitate was dissolved in nitric acid, the cerium was removed by a cation-exchange resin, and finally the phosphate was precipitated as silver phosphate and dried in a vacuum oven for 24 h at 50 °C.

2.6.1. Isotopic composition determinations

For isotopic composition determinations, three replicates of ~0.3 mg silver phosphate from the same sample were packed in silver capsules and introduced into a high temperature pyrolysis unit (Sercon HT-EA, Sercon Ltd, Crewe UK), where they were converted to CO in the presence of glassy carbon (Venemmann et al., 2002). The HT-EA is interfaced in continuous-flow mode through a gas chromatograph (GC) column to an isotope ratio mass spectrometer (Sercon 20–20, Sercon Ltd, Crewe UK). All isotopic values are given in the delta notation relative to Vienna Standard Mean Ocean Water (VSMOW). The average standard deviation between three replicates of the same sample was 0.3‰. All measurements were performed against our own Ag₃PO₄ laboratory standards, which were pre-calibrated against the following Ag₃PO₄ standards, with their isotopic signatures in parentheses: TU–1 (21.1‰) and TU–2 (5.4‰) (Venemmann et al., 2002), UMCS-1 (32.6‰) and UMCS-2 (19.4‰) (Halai et al., 2011), and against the IAEA-601 benzoic acid standard (23.3‰) (Coplen et al., 2005).

The water used in the incubations and a sample of rain water taken during rainfall in the BCNM were analyzed for $\delta^{18}O_w$ by equilibration with CO₂ and measured on a Thermo Gas Bench coupled in continuous-flow to a Thermo Delta Plus XL mass-spectrometer (Thermo, Bremen, Germany). To detect whether isotopic variations occurred between different rain events during the study period (as reported in tropical forest elsewhere (Goldsmith et al., 2012)), rain samples were taken twice, at the 15th of September and at the 5th of October. No isotopic variations were observed between the two samples.

2.7. Statistical analysis

Comparisons between treatments were made using post-hoc Tukey HSD analysis and significant differences were determined at $p < 0.05$.

3. Results

3.1. Incubation experiments

The $\delta^{18}O_P$ values at the start of the first incubation ($\delta^{18}O_{w}$ of 27.6‰) were driven from the original value of the added P compound ($\delta^{18}O_P$ of 21.6‰) to a value (~±SD) of 40.6‰ (~±0.2) by the end of the incubation (Table 1). These values deviate significantly (by ~8‰) from the expected isotopic equilibrium of ~48‰ ($p < 0.05$). The resin-P concentrations at the end of the incubation showed that 78% of the added phosphate was gradually removed from the resin-P fraction (Table 1).

The $\delta^{18}O_P$ values in the second incubation ($\delta^{18}O_{w}$ of ~4.6‰) were driven from the original value of the added P compound ($\delta^{18}O_P$ of 21.6‰), and ranged from 20.0‰ to 21.0‰ among the six soil treatments (control, K, P, NPK, L, and L−) at the end of the incubation period (Fig. 1, Table 1). These values deviate by ~4‰ from the expected isotopic equilibrium of ~16‰ and are statistically significant for all treatments ($p < 0.05$). Measurements of resin-P concentrations at the end of the incubation period show that the added phosphate fixed during the incubation was significantly lower in soils that received chronic P additions (20 and 28% of the added P was fixed in the NPK and P treatments, respectively) than in soils that did not (59%, 66%, 67% and 75% in the L, L−, K and control plots, respectively (Fig. 1, Table 1; $p < 0.05$)).

The $\delta^{18}O_P$ values in the third incubation ($\delta^{18}O_{w}$ of ~4.6‰) were driven from the original value of the added P compound ($\delta^{18}O_P$ of 12.5‰) to a mean value (~±SD) of 18.8‰ (~±0.4) at the end of the incubation (Fig. 1, Table 1). This value deviates significantly from the expected isotopic equilibrium of 16‰, $p < 0.05$. Measurements of the resin-P concentrations at the end of the incubation period show that 85% of the added phosphate was gradually removed from the resin-P fraction (Fig. 1, Table 1).

3.2. Rewetting dry soils

3.2.1. Soils not receiving P addition

The resin-P concentrations from plots that did not receive P additions (i.e., soil samples from control, K, N, NK and L− plots) ranged from 0.4–0.9 µg P g⁻¹ soil for fresh soil samples and increased significantly to 6.0–7.1 µg P g⁻¹ soil after rewetting dry soil samples (Table 2, $p < 0.05$). The $\delta^{18}O_P$ values of phosphate that was extracted after rewetting dry soils from these plots ranged between 18.4‰ and 19.7‰ (Fig. 2, Table 2, no significant differences between treatments). These values deviate significantly from the expected isotopic equilibrium with the soil water (~17‰, $p < 0.05$), which was calculated based on equation (1), taking into account the average ambient temperature of 27°C and assuming the rain water $\delta^{18}O_w$ value are indicative of the soil water (~2.4‰). The largest deviation from isotopic equilibrium was found in the litter addition plots with a $\delta^{18}O_P$ value of 23.8‰ (~±0.9) after rewetting dry soils (Fig. 2, Table 2, $p < 0.001$).
3.2.2. Soils receiving chronic P addition

In fresh soil samples from plots that received chronic P additions (P, PK, NP and NPK), the resin-P concentrations were significantly higher than in the non-P soils (p < 0.05) and ranged from 45 to 52 μg P g⁻¹ soil eight weeks after the soils were recently fertilized. The concentrations further increased to 89—108 μg P g⁻¹ soil after rewetting dry soils (Table 2). The δ¹⁸O_P values of the phosphate extracted after rewetting dry soils from these plots, ranged from 21.4‰ to 22.2‰, with no significant differences between treatments (Fig. 3). These values deviate significantly from the expected isotopic equilibrium with soil water (~17‰, p < 0.05) and are close to the original value of the added triple superphosphate fertilizer (21.6‰).

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Treatment</th>
<th>δ¹⁸O_P values of the added P compound (%)</th>
<th>δ¹⁸O_w of the media water (%)</th>
<th>Expected isotopic equilibrium with soil water (%)</th>
<th>Measured δ¹⁸O_P values (t = 5 days)</th>
<th>Resin-P concentration (μg P g⁻¹ soil⁻¹) after P addition (t = 0 days)</th>
<th>Resin-P concentration (μg P g⁻¹ soil⁻¹) after P addition (t = 5 days)</th>
<th>P fixation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>Control</td>
<td>21.6</td>
<td>27.6</td>
<td>48</td>
<td>40.6 ± 0.2</td>
<td>160</td>
<td>35</td>
<td>78</td>
</tr>
<tr>
<td>Second</td>
<td>Control</td>
<td>21.6</td>
<td>−4.6</td>
<td>16</td>
<td>20.0 ± 0.2</td>
<td>151</td>
<td>38</td>
<td>75</td>
</tr>
<tr>
<td>Second</td>
<td>K</td>
<td>21.6</td>
<td>−4.6</td>
<td>16</td>
<td>20.1 ± 0.3</td>
<td>149</td>
<td>49</td>
<td>67</td>
</tr>
<tr>
<td>Second</td>
<td>P</td>
<td>21.6</td>
<td>−4.6</td>
<td>16</td>
<td>20.0 ± 0.2</td>
<td>240</td>
<td>172</td>
<td>28</td>
</tr>
<tr>
<td>Second</td>
<td>NPK</td>
<td>21.6</td>
<td>−4.6</td>
<td>16</td>
<td>21.0 ± 0.2</td>
<td>186</td>
<td>149</td>
<td>20</td>
</tr>
<tr>
<td>Second</td>
<td>L⁻</td>
<td>21.6</td>
<td>−4.6</td>
<td>16</td>
<td>20.6 ± 0.1</td>
<td>154</td>
<td>63</td>
<td>59</td>
</tr>
<tr>
<td>Second</td>
<td>L⁺</td>
<td>21.6</td>
<td>−4.6</td>
<td>16</td>
<td>20.3 ± 0.1</td>
<td>144</td>
<td>48</td>
<td>66</td>
</tr>
<tr>
<td>Third</td>
<td>Control</td>
<td>12.5</td>
<td>−4.6</td>
<td>16</td>
<td>18.8 ± 0.4</td>
<td>174</td>
<td>24</td>
<td>86</td>
</tr>
</tbody>
</table>

Table 2

Results for δ¹⁸O_P concentrations and δ¹⁸O_w values after rewetting dry soils. Soils were collected eight weeks after the N, NK, K, P, PK, NP and NPK treatments were fertilized. Values are means (±1 SD) over three forest plots (two for L⁻).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>N</th>
<th>K</th>
<th>NK</th>
<th>P</th>
<th>PK</th>
<th>NP</th>
<th>NPK</th>
<th>L⁻</th>
<th>L⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ¹⁸O_P (%)</td>
<td>18.5 ± 0.9</td>
<td>6.0 ± 0.6</td>
<td>8.0 ± 0.1</td>
<td>6.5 ± 0.7</td>
<td>6.0 ± 0.1</td>
<td>6.0 ± 0.1</td>
<td>6.0 ± 0.1</td>
<td>6.0 ± 0.1</td>
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<td>6.0 ± 0.1</td>
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<tr>
<td>Dry–Wet P (μg P g⁻¹ soil⁻¹)</td>
<td>0.8 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.4</td>
<td>0.4 ± 0.2</td>
<td>0.9 ± 0.4</td>
<td>0.9 ± 0.4</td>
<td>0.9 ± 0.4</td>
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</tr>
<tr>
<td>Resin-P (μg P g⁻¹ soil⁻¹)</td>
<td>0.7 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.2 ± 0.2</td>
<td>0.2 ± 0.2</td>
<td>0.2 ± 0.2</td>
<td>0.2 ± 0.2</td>
<td>0.2 ± 0.2</td>
<td>0.2 ± 0.2</td>
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</tr>
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Fig. 1. Results from the second (a) and third (b) incubation experiments. The initial δ¹⁸O_P values of the added phosphorus (P) compounds are presented at zero percent of P fixed in the soil. Lines connect this initial value with values after five days of incubation. The dashed line in panels (a) and (b) is the incubation steady state value (~19.5‰). The dotted line in panel (b) is the expected isotopic equilibrium range for the constant incubation temperature of 23 °C and the media water δ¹⁸O_P value of ~4.6‰ (after Longinelli and Nuti, 1973; see text eq. 1). Fertilizer plots are shown as squares and dashed lines and include P, potassium (K), and nitrogen (N), P, and K (NPK). Litter plots are shown as triangles and dotted lines and include litter addition (L⁻) and litter removal (L⁺). The control plots (CTL) are shown as circles with solid lines. Note the different scales on the Y and x axes in panels (a) and (b). Error bars are standard error of two replicate analyses.

Fig. 2. Results for δ¹⁸O_P values after rewetting dry soils from control plots and plots that received additions of nitrogen (N), potassium (K), N plus K (NK), and leaf litter (L⁻). The dashed line is the incubation steady state value (~19.5‰). The dotted line is the expected isotopic equilibrium range for the mean forest soil temperature of 27 °C and the δ¹⁸O_P value of rain water (after Longinelli and Nuti, 1973; see text eq. 1). Error bars are standard error of three replicate analyses.
and the is the expected isotopic equilibrium range for the mean forest soil temperature of 27°C approached a steady state between microbial uptake (Fuptake) and microbial biomass. If we assume our experimental system roundings and 2) the uptake of phosphate molecules by the soil re

Fig. 3. Results for $\delta^{18}O_p$ values after rewetting dry soils from plots that received addition of phosphorus (P), nitrogen (N) plus P (NP), potassium (K), N, and P (NPK), or P plus K (PK). The solid line is the $\delta^{18}O_p$ value of the triple superphosphate fertilizer (21.6‰). The dashed line is the incubation steady state value (~19.5‰). The dotted line is the expected isotopic equilibrium range for the mean forest soil temperature of 27°C and the $\delta^{18}O_w$ value of rain water (after Longinelli and Nuti, 1973; see text eq. 1). Error bars are standard error of three replicate analyses.

4. Discussion

4.1. Microbial activity drives above-equilibrium $\delta^{18}O_p$ values in incubation experiments

Results from the first incubation ($\delta^{18}O_p$ of 27.6‰) demonstrate that oxygen from the water was incorporated into the phosphate molecules (Table 1). At temperatures below 60°C, such incorporation occurs only through biological reactions (Kolodny et al., 1983). The observed deviations from isotopic equilibrium found in all of the incubations contrasts with our expectations based on previous studies in extra-tropical soils that reported soil $\delta^{18}O_p$ values in isotopic equilibrium with soil water (Zohar et al., 2010; Angert et al., 2011, 2012; Tamburini et al., 2012; Gross et al., 2013; Gross and Angert, 2015). The authors of these studies hypothesized that the close to equilibrium $\delta^{18}O_p$ values were induced inside the soil microbial cells and the $\delta^{18}O_p$ values they measured in the soil reflected phosphate released from within the microbial biomass.

We suggest the above equilibrium values we found in our study reflect a dynamic balance between two microbial processes: 1) the efflux of cell internal inorganic phosphate back to the soil surroundings and 2) the uptake of phosphate molecules by the microbial biomass. If we assume our experimental system approached a steady state between microbial uptake (Fuptake) and the microbial efflux (Fefflux), we can use the following steady state equation:

$$\delta^{18}O_{\text{mic}} \times F_{\text{efflux}} = (\delta^{18}O_{\text{resin}} + \varepsilon) \times F_{\text{uptake}}$$

where $\delta^{18}O_{\text{mic}}$ and $\delta^{18}O_{\text{resin}}$ are the isotopic values of microbial cell internal phosphate and of the resin-P pool, respectively, and $\varepsilon$ is the isotopic fractionation factor induced by uptake.

Since in steady state $F_{\text{efflux}} = F_{\text{uptake}}$, equation (2) can be rearranged to:

$$\delta^{18}O_{\text{Resin-SS}} = \delta^{18}O_{\text{Mic}} - \varepsilon \quad (3)$$

and can be reorganized to:

$$\delta^{18}O_{\text{Resin-SS}} - \delta^{18}O_{\text{Mic}} = -\varepsilon \quad (4)$$

where $\delta^{18}O_{\text{Resin-SS}}$ is the isotopic value of the soil resin-P, which is in a steady state equilibrium between the microbial uptake of phosphate and its release from within the cells (and thus does not depend on the isotopic composition of the phosphate source). By adopting the calculated isotopic equilibrium between phosphate and cell water based on equation (1) (16‰) as $\delta^{18}O_{\text{Mic}}$ and $\delta^{18}O_{\text{Resin-SS}}$ as the steady state values (19.5‰, Fig. 1), we calculate $\varepsilon$ to be $-3.5$‰, which is similar to the isotopic fractionation factor that was reported during phosphate uptake in laboratory cultures of Escherichia coli (Blake et al., 2005). The isotopic fractionation of $-3$‰ has not been detected previously in extra-tropical soils (Gross and Angert, 2015).

In contrast to the higher than equilibrium values that were measured in the second and third incubations, the $\delta^{18}O_p$ values measured at the end of the first incubation were significantly lower than the expected isotopic equilibrium, by almost 8‰. These differences are related to the relative differences between the $\delta^{18}O_p$ values of the added P compound and the expected isotopic equilibrium in the three incubations (-27‰ in the first incubation and 5.5‰ and 3.5‰ in the second and third incubations, respectively). Based on the larger difference in the first incubation, it seems likely that more incubation time would have been required for the phosphate $\delta^{18}O_p$ values to exceed isotopic equilibrium levels.

4.2. Nutrient status and soil phosphate $\delta^{18}O_p$ values in the incubation experiments

The difference between the incubation steady state value (which reflects an ongoing microbial turnover of the added P) and the measured $\delta^{18}O_p$ value can be used as an indicator for the rate of the soil microbial phosphate turnover. As the activity of the microbial biomass usually depends on the nutrient status of the soil, this indicator can also be used to study the effect of the nutrient status on microbial phosphate turnover (Fig. 1). In soils not receiving P additions (i.e., control, K, litter removal treatment), the large fraction of the added P that was removed from the resin-P pool (Table 2) was probably fixed either chemically by oxidized iron (Fe) and aluminum (Al) oxides or consumed biologically by the end of the incubation period. Even though the isotopic fractionation that accompanies phosphate sorption to iron oxides is negligible (Jaisi et al., 2010), the $\delta^{18}O_p$ values of the resin-P pool changed from the value of the added P compound and deviated only slightly from the microbiologically mediated incubation steady state value at the end of the 5 incubation days (by 0.5–0.8‰, Fig. 1 and Table 1). These measurements show that the microbial biomass responded rapidly to the pulse of added P. This rapid response is presumably related to microbial P limitation in these soils, as was shown in a previous study at the same site (Turner and Wright, 2014).

The significantly lower amounts of P that were fixed in the incubations of soils from the L+ plots (59% compared to 66–75%, p < 0.05) can be attributed to the competition between additional organic molecules derived from the plant litter and the original soil phosphate for sorption sites. This process was shown to affect the quantities and strength of phosphate sorption to the soil in the same site (Schreeg et al., 2013).

Even though no difference was found in the soil microbial C or N in the upper 10 cm of soils from the L+ control, K and L- plots six
years after the start of the experiment (Sayer et al., 2012), we suggest that the microbial phosphate turnover rate (measured by us nine years after the start of the experiment) was significantly slower in the L+ plots, as inferred from the significantly larger difference between the incubation steady state values and the values measured in the incubations of these soils (1.1‰, Fig. 1, p < 0.05). This might indicate a reduced degree of microbial P limitation in these soils, due to the release of bioavailable phosphate from organic P compounds in decomposing additional litter (Vincent et al., 2010).

The significantly lower P fixation amounts in soils that had received chronic P additions (28% and 20% in the P and NPK plots, respectively, Fig. 1) probably reflects the saturation of P sorption sites after 15 years of intensive fertilization (Mirabello et al., 2013; Turner et al., 2013). We predicted that the response of the microbial biomass to the phosphate pulse, added in our incubation experiments, would be smaller in soils that received chronic P additions, due to alleviation of microbial P limitations in these plots (Turner and Wright, 2014). Indeed, we found that the difference between the measured 18O values at the end of incubations of soils from the combined N:P:K treatment and the microbially mediated incubation steady state value was significantly larger than in the non-P soils (1.6‰, p < 0.05). In addition, the measured value deviated only slightly from the value of the added P compound (21.6‰). This shows that the microbial turnover rate of the added phosphate was slower in the plots with added N:P:K than in the non-P plots, probably because the microbial biomass was not limited by P (Turner and Wright, 2014).

In contrast to the N:P:K fertilized soils, soils that received only P addition (i.e. no N or K addition), had resin-P 18O values that were closer to the incubation steady state values (deviation of 0.5‰ at the end of the experiment, similar to control, K, litter removal treatment, Fig. 1) even though most of the added P was retained in the resin-P pool. This indicates that the microbial phosphate turnover was rapid despite high resin-P concentrations. This suggests that the status of N and K in the soil also affects the rate of microbial phosphate turnover, at least in the short term, even though neither of these nutrients affected the microbial biomass or soil phosphate activity in the long term (Turner and Wright, 2014). Our results show that high amounts of N and K when P is in excess slows microbial phosphate turnover. Overall, our results provide more evidence of the importance of multi-element interactions in the Gigante long-term fertilization experiment (Kaspari et al., 2008).

4.3. The 18O values of phosphate extracted after rewetting of dry soils that did not receive P addition is close to the incubation steady state value

Strong biological P demand in lowland tropical forests means that phosphate molecules entering the resin-P pool will be rapidly consumed, and the resin-P concentrations remains exceptionally low (0.4–0.9 µg P g−1 for the soils that did not receive P additions in our study site, Table 2). As a result, the processes that affect soil bioavailable-P 18O values could not be inferred from isotopic analysis of the resin-P fraction alone. In our study site, P that was extracted after rewetting dry soils that did not receive P additions exceeded the resin-P fraction by 5–10 fold (Table 2). Therefore, the processes that affect soil readily available P 18O values can be inferred from isotopic analysis of phosphate that is extracted after rewetting dry soils. This phosphate pool represents P that is released from microbial cells that are lysed by the osmotic shock of rewetting and from other loosely sorbed P fractions in the soil such as the bicarbonate extractable P fraction (Turner and Haygarth, 2003; Turner et al., 2003).

The 18O values of phosphate extracted after rewetting dry soils from the control plots and plots that were treated with chronic N and K additions (N, K, NK) were close to the incubation steady state value of 19.5‰ and were above isotopic equilibrium (~17‰) by 1.5‰–3‰ (Fig 2). These values reflects turnover of the bulk of the soil phosphate by the microbial biomass.

The significantly higher 18O values in the L+ plots (Fig 2, p < 0.05) may reflect the release of P from structural organic P compounds in plant leaves, which were shown to be isotopically enriched relative to isotopic equilibrium, with remarkably high 18O values (up to 68‰) (Pfahler et al., 2013). Such high values were attributed to different isotopic disequilibrium metabolic process that produce isotopically enriched organic phosphates and, to a lesser extent, to the isotopic enrichment of the leaf water in comparison to the water in the soil. In this study we did not measure the plant litter 18O values. However, if we assume that the extremely high 18O values are indicative also of plant litter in our site, it is probable that the phosphate extracted after the rewetting of dry soils from the L+ plots contains a portion of phosphate that was mineralized from isotopically enriched organic P compounds in leaf litter by extracellular phosphatases. Although enzymatic mineralization of organic P usually reduces 18O values (Liang and Blake, 2006; 2009; Gross and Angert, 2015), mineralization of organic P with high 18O value would still yield phosphate with 18O value that is greater than isotopic equilibrium (Pfahler et al., 2013). This is especially true in acid lowland tropical soils, in which acid phosphatase predominates compared to alkaline phosphatase (Turner, 2010), since the isotopic fractionation induced by acid phosphatase is 20–30‰ smaller than the one induced by alkaline phosphatase (Von Sperber et al., 2014).

4.4. The 18O values of phosphate extracted after rewetting dry soils that received chronic P additions remained close to that of the P fertilizer

The high concentrations and the preservation of the 18O values of the P fertilizer in the phosphate extracted after rewetting dry soils that received 15 years of P additions (P, PK, NP and NPK, Fig 3 and Table 2) reflects a reduction in the microbial turnover rate of phosphate in those plots. In the long term, the isotopic effect imparted by microbial turnover is probably masked by the high amount of plant available P and the release of P from less readily available P pools to the more labile P pools in the fertilized soils. This low microbial P turnover is consistent with a previous study at this site that suggested the long-term P addition alleviated microbial P limitation (Turner and Wright, 2014).

In this study we showed that together with the data gained from conventional measurements of soil bioavailable P concentrations, 18O measurements provide novel insight into the biological processes that mediate P availability in lowland tropical soils. From measurements of soil 18O values we infer that the soil bioavailable phosphate is rapidly recycled by the microbial biomass. We found that the rate of the phosphate microbial turnover is influenced by the soil nutrient status, probably by altering the soil microbial P demand.

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