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Stability of hydrolytic enzyme activity and microbial phosphorus during storage of tropical rain forest soils

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ABSTRACT

Storage can markedly influence microbial and biochemical properties in soils, yet recommendations for sample storage are based on studies of temperate soils that regularly experience extended cold periods. We assessed the influence of storage conditions on microbial phosphorus and the activity of four hydrolytic enzymes (phosphomonoesterase, phosphodiesterase, β-glucosidase, and *N*-acetyl-β-D-glucosaminidase) in three lowland tropical forest soils from the Republic of Panama that experience a constant warm temperature. The soils spanned a strong rainfall gradient and contained contrasting physical and chemical properties (pH 3.6–5.9; total carbon 26–50 g C kg^{-1}; clay 33–62%; total phosphorus 0.30–0.60 g P kg^{-1}). Storage treatments were: (i) room temperature (22 °C in the dark), (ii) refrigerated (4 °C in the dark), (iii) air-dried (10 d, 22 °C), and (iv) frozen (−35 °C). There were significant changes in enzyme activities and microbial phosphorus during refrigerated and room temperature storage, although changes were relatively small during the first two weeks. An initial marked decline in enzyme activities for one soil analyzed within 2 h of sampling was attributed to a flush of activity caused by sampling and soil preparation (sieving, etc.). For longer-term storage (>2 weeks), ambient laboratory temperature appeared preferable to freezing and cold storage, because one month of storage caused a marked decline in enzyme activities and microbial phosphorus in one soil. Freezing preserved the activities of some enzymes in some soils at rates comparable to cold or room temperature storage, but caused a marked decline in microbial phosphorus in two soils. Air-drying caused a marked decline in microbial phosphorus and the activity of all enzymes. We therefore conclude that enzyme assays and microbial phosphorus should be determined in tropical forest soils after no more than two weeks storage in the dark at ambient laboratory temperature.
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1. Introduction

Measurements of nutrients contained within soil microbes and the activities of enzymes involved in the turnover of nutrients from organic compounds provide important information on biogeochemical cycles in tropical soils (Olander and Vitousek, 2000; Waldrop et al., 2000; Dinesh et al., 2004; Yavitt et al., 2004; Sinsabaugh et al., 2008). Obtaining such information in tropical forests is challenging, however, because microbial and biochemical properties can change rapidly following sampling (e.g., DeForest, 2009), yet research sites are often remote from laboratory facilities and soil samples may require long periods of transport and/or storage before preparation and analysis. Robust information on changes during storage is therefore a prerequisite for detailed studies of microbial and biochemical properties in tropical forest soils.

Numerous studies have assessed the effects of storage conditions on soil microbial and biochemical properties and almost all have recommended refrigerated or frozen storage, because such conditions minimize changes in microbial biomass (e.g., Pancholy and Rice, 1972; Petersen and Klug, 1994; Sternberg et al., 1998) and enzyme activities (e.g., Ross, 1965; Speir and Ross, 1975; Lee et al., 2007; DeForest, 2009) compared to fresh samples. In contrast, storage at room temperature (typically 21–23 °C) has not been recommended, due to poor preservation of both microbial biomass (e.g., Anderson, 1987; Petersen and Klug, 1994) and enzyme activities (e.g., Pancholy and Rice, 1972; Speir and Ross, 1975; Ross et al., 1980). However, all previous studies of storage effects on microbial and biochemical properties have been conducted on temperate soils, which usually experience cold temperatures, including freezing, on an annual basis. It might therefore be expected that microbes in such soils are adapted to withstand extended periods of cold. In contrast, tropical soils typically experience a stable warm temperature, so it might be expected that microbes in tropical soils are not able to withstand cold temperatures. If so, storage at temperatures similar to those

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encountered in the field may be most appropriate to preserve microbial and biochemical properties in tropical forest soils.

To address this, we assessed the influence of storage on microbial phosphorus and five hydrolytic enzymes involved in the cycles of carbon, nitrogen, and phosphorus in three soils under lowland tropical rain forest in Panama. The soils spanned a strong rainfall gradient and contained contrasting chemical and physical properties expected to influence microbial and biochemical properties. Our aim was to determine the most acceptable conditions for soil storage to approximate microbial phosphorus and enzyme activity in field soils.

2. Methods

2.1. Site description

Three soils were sampled from central Panama (Table 1). The soils spanned a strong rainfall gradient (see below) and contained contrasting physical and chemical properties, including pH (3.6–5.9 in deionized water), clay (33–62%), organic matter (26.2–50.1 g C kg^{-1}), and total P (0.297–0.598 g P kg^{-1}). All three sites were under lowland rain forest and were adjacent to a one-hectare forest census plot. Detailed information on the forest communities in two of these plots was reported previously (Fort Sherman, plot code P02; Pipeline Road, plot code P15; Pyke et al., 2001).

Air temperature in the region is stable throughout the year. The long-term mean annual temperature on Barro Colorado Island, a few km from Pipeline Road, is 27 °C and monthly averages vary by <1 °C throughout the year (Windsor, 1990). Mean annual rainfall varies from 1811 mm at Albrook to 3072 mm at Fort Sherman (Table 1). There is a strong dry season from January to April, although the duration varies among the three sites. Mean dry season length, calculated from a network of rainfall stations throughout the area, is 148 d at Albrook, 133 d at Pipeline Road, and 118 d at Fort Sherman (Engelbrecht et al., 2007).

2.2. Soil sampling and storage treatments

Soil sampling and storage treatments were described in detail previously (Turner and Romero, 2009). Briefly, the Albrook soil was sampled on 5 June 2006, while the Pipeline Road and Fort Sherman soils were sampled on 22 August 2007. At each location, approximately 10 kg of soil was taken to 10 cm depth from an area adjacent to the forest census plot and returned immediately to the laboratory (see below). This was completed within 30 min for the Albrook soil, which was then prepared and analyzed on the day of collection. Transportation time was longer for the Pipeline Road (2 h) and Fort Sherman (4 h) soils, so samples were prepared and separated into storage treatments (see below) on the day of collection, but the first analyses were conducted the following morning (within 19 and 22 h of collection for the Pipeline Road and Fort Sherman soils, respectively).

Table 1
Site characteristics and properties of three soils under lowland tropical rain forest in central Panama.

	Albrook	Pipeline Road	Fort Sherman
Latitude	08° 58′ 37″ N	09° 09′ 42″ N	09° 19′ 23″ N
Longitude	79° 33′ 50″ W	79° 44′ 43″ W	79° 57′ 43″ W
Rainfall (mm y^{-1})	1811	2455	3072
Soil pH (H_2O)	3.63	5.89	5.25
Topsoil texture	Clay	Clay loam	Clay
Clay (%)	54	33	62
Total C (g C kg^{-1})	50.1	26.2	30.6
Total N (g N kg^{-1})	3.83	2.43	3.06
Total P (g P kg^{-1})	0.598	0.384	0.297

Upon return to the laboratory, soils were screened (<9 mm mesh) to break up large aggregates and visible stones and roots were removed by hand. The Albrook soil in particular contained many fine roots and small stones. Samples were not further sieved due to the difficulty in sieving field-moist soils that are rich in clay. Each soil was then split into four treatments designed to simulate commonly used storage protocols:

1. Storage in the dark at ambient laboratory temperature and humidity (22 ± 0.5 °C and 55 ± 5%, respectively).
2. Storage in the dark at 4 °C.
3. Storage frozen at −35 °C.
4. Air-dried at ambient laboratory temperature and humidity for 10 d (Pipeline Road and Fort Sherman soils only).

Each treatment was replicated four times for each soil, except for air-dried samples ($n = 3$). Samples were stored in sealed plastic bags, with air-dried samples stored at ambient laboratory temperature. Frozen and air-dried samples contained approximately 200 g of soil per replicate and were analyzed one month after sampling. Samples stored at 4 °C and 22 °C contained approximately 800 g of soil per replicate and were subsampled periodically for analysis. The Albrook soil was analyzed on the day of sampling, then again 1, 3, 7, 15, 35 and 95 d after sampling. The Sherman and Pipeline soils were analyzed on the day following sampling, then again 3, 7, 15, and 28 d after sampling. Frozen soils were thawed overnight at 4 °C prior to analysis the following morning.

Air-dried samples were analyzed for pH and the concentrations of clay and total C, N, and P (methods described in Turner and Romero, 2009). Soil properties are shown in Table 1. Information on short-term changes in inorganic nutrients in the soils was reported previously and indicated that both inorganic nitrogen and phosphorus concentrations changed rapidly (within hours) following sampling for both refrigerated and room temperature storage (Turner and Romero, 2009).

2.3. Enzyme assays

The activities of four hydrolytic enzymes involved in the cycles of carbon, nitrogen, and phosphorus were determined using fluorogenic substrates based on a method described by Marx et al. (2001). The enzymes and substrates were: (i) acid phosphomonoesterase (EC 3.1.3.2) assayed with 4-methylumbelliferyl phosphate; (ii) phosphodiesterase (EC 3.1.4.1) assayed with bis-(4-methylumbelliferyl) phosphate; 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. All substrates were purchased from Glycosynth Ltd (Warrington, UK). Substrates were dissolved in 0.4% methylcellosolve (2-methoxyethanol; 0.1% final concentration in the assay).

For each sample, soil suspensions were prepared in a 1:100 ratio of soil to deionized water (containing 1 mM NaN_3 to prevent microbial activity) by stirring on a magnetic stir-plate for 15 min. Soil suspension (50 μL) was then pipetted into wells on a micro-well plate (16 wells per substrate) containing 100 μL of 200 μM substrate (100 μM final concentration in the assay) and 50 μL of 200 mM sodium acetate–acetic acid buffer (50 mM final concentration in the assay) adjusted to the soil pH. Plates were incubated for 30 min at 26 °C to approximate the daytime soil temperature in lowland forests in central Panama. The reaction was terminated by adding 50 μL of 0.5 M NaOH (final solution pH > 11) and 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 reduction of fluorescence in the presence of soil (quenching). All enzyme activities are expressed as nmol MU g^{-1} soil (dry-weight) min^{-1}.

2.4. Microbial biomass

Microbial phosphorus was determined by hexanol fumigation and extraction with anion-exchange membranes based on a method described by Kouno et al. (1995) and Myers et al. (1999). Anion-exchange membrane strips (1 × 4 cm; manufactured by BDH, Poole, UK; distributed by VWR International, West Chester, PA) were prepared in advance by initially shaking 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 at 880 nm, following online neutralization, by automated molybdate colorimetry using a Lachat Quickchem 8500 (Hach Ltd, Loveland, CO, USA). Microbial phosphorus was calculated as the difference between the fumigated and unfumigated samples, without correction for phosphate sorption or unrecovered microbial phosphorus.

2.5. Statistical analysis

Changes during storage were determined for each soil by repeated measures analysis of variance using time and treatment (4 °C and 22 °C) as factors. Values for fresh samples were compared with those from soils stored for 4 or 5 weeks (room temperature, refrigerated, frozen, and air-dried) by one-way analysis of variance with Tukey's Honestly Significant Difference test for means separation ($P < 0.05$). All statistical analysis was performed using R software (version 2.2.0; www.r-project.org).

3. Results

3.1. Hydrolytic enzymes

Phosphomonoesterase activity in fresh soil ranged from 12.54 ± 1.59 nmol MU g^{-1} min^{-1} in the Pipeline Road soil to 62.3 ± 4.8 nmol MU g^{-1} min^{-1} in the Fort Sherman soil (Table 2). Phosphodiesterase activity was between three and six-fold less, being greatest in the Fort Sherman soil (11.36 ± 0.86 nmol MU g^{-1} min^{-1}). β-glucosidase activity was greatest in the Albrook soil (7.60 ± 0.75 nmol MU g^{-1} min^{-1}), while *N*-acetyl-glucosaminidase activity was greatest in the Fort Sherman soil (3.12 ± 0.32 nmol MU g^{-1} min^{-1}).

For the time series data (storage at 4 °C and 22 °C) there were significant effects of storage time for all enzymes in the Albrook and Fort Sherman soils ($P < 0.001$), although for the Pipeline Road soil only phosphomonoesterase varied significantly during storage (the time effect was marginally not significant for phosphodiesterase and β-glucosidase: $P = 0.06$ and 0.052, respectively). In contrast, there were no significant differences between samples stored at 4 °C or 22 °C for any enzyme in any soil ($P > 0.05$), except for phosphodiesterase activity in the Pipeline Road soil ($P = 0.014$) (Table 2). However, activities in soils stored at 22 °C were generally higher and closer to the original values than were those in refrigerated samples (Fig. 1).

The Albrook soil was assessed over 95 d, but despite the significant time effect during this period there were no significant differences compared to fresh samples after 5 weeks of storage for phosphodiesterase or *N*-acetyl-glucosaminidase stored at either 4 °C and 22 °C, nor for β-glucosidase activity stored at 22 °C (Table 2). For the Pipeline Road and Fort Sherman soils, three of four enzymes (phosphomonoesterase, phosphodiesterase, and β-glucosidase) were relatively stable for the first two weeks of storage, but declined markedly in the Fort Sherman soil after 4 weeks of storage at both 4 °C and 22 °C (Fig. 1). For the Albrook soil the effect of storage time was influenced strongly by a rapid decline from the initial fresh sample, notably for phosphomonoesterase and β-glucosidase. There appeared to be an increase in enzyme activity in all three soils between 1 and 2 weeks of storage (Fig. 1).

Freezing appeared to preserve the activity of all enzymes well compared to 4 or 5 weeks of storage at either 4 °C or 22 °C (Table 2). For the Albrook soil, activities of all enzymes in frozen samples were significantly lower than in the fresh sample, which was analyzed on the day of collection (Table 2). However, for the Pipeline Road and Fort Sherman soils, which were analyzed after overnight storage, values for frozen samples were similar to initial values, particularly for β-glucosidase. Air-drying of the Pipeline Road and Fort Sherman soils caused a marked reduction in activity of all enzymes tested, although it did not eliminate activity for any enzyme (Table 2).

Table 2
Storage effects on the activity of four hydrolytic enzymes in three soils under lowland tropical rain forest in central Panama. Fresh samples were analyzed on the same day as sampling (Albrook) or after overnight storage at 4 °C or 22 °C (Pipeline Road and Fort Sherman), with other treatments analyzed after 4 weeks of storage (5 weeks for the Albrook soil). Values are the mean ± standard deviation of four replicate samples (three for the air-dried treatment), and values within a column for each enzyme with the same letter are not significantly different at the 5% level (Tukey's HSD). nd, not determined.

	Albrook	Pipeline Road	Fort Sherman
Phosphomonoesterase			
Fresh (same day)	26.60 ± 1.26a		
Fresh (22 °C overnight)		12.54 ± 1.59ab	62.30 ± 4.80a
Fresh (4 °C overnight)		13.46 ± 1.05a	59.17 ± 6.04a
Room temperature (22 °C, 4 weeks)	21.05 ± 1.49b	11.51 ± 1.14ab	48.77 ± 1.62b
Refrigerated (4 °C, 4 weeks)	21.02 ± 0.72b	10.73 ± 1.39bc	42.00 ± 1.01b
Frozen (−35 °C, 4 weeks)	20.59 ± 1.82b	10.81 ± 0.65bc	56.12 ± 0.88a
Air-dried	nd	8.41 ± 0.33c	23.01 ± 1.44c
Phosphodiesterase			
Fresh (same day)	8.54 ± 0.80a		
Fresh (22 °C overnight)		2.04 ± 0.34a	11.36 ± 0.86a
Fresh (4 °C overnight)		2.28 ± 0.22a	10.45 ± 0.75ab
Room temperature (22 °C, 4 weeks)	7.12 ± 0.74ab	1.98 ± 0.14a	8.67 ± 0.22c
Refrigerated (4 °C, 4 weeks)	6.91 ± 0.76ab	1.93 ± 0.29a	7.24 ± 0.21d
Frozen (−35 °C, 4 weeks)	6.53 ± 0.88b	2.05 ± 0.11a	9.38 ± 0.12bc
Air-dried	nd	1.05 ± 0.06b	5.13 ± 0.27e
β-Glucosidase			
Fresh (same day)	7.60 ± 0.75a		
Fresh (22 °C overnight)		1.69 ± 0.22a	2.46 ± 0.39a
Fresh (4 °C overnight)		1.73 ± 0.21a	2.22 ± 0.33a
Room temperature (22 °C, 4 weeks)	6.64 ± 0.95ab	1.55 ± 0.24a	1.81 ± 0.12ab
Refrigerated (4 °C, 4 weeks)	5.93 ± 0.56b	1.59 ± 0.37a	1.58 ± 0.23bc
Frozen (−35 °C, 4 weeks)	5.84 ± 0.55b	1.39 ± 0.13a	2.10 ± 0.17ab
Air-dried	nd	0.59 ± 0.03b	1.28 ± 0.17c
N-acetyl-β-D-glucosaminidase			
Fresh (same day)	1.07 ± 0.13a		
Fresh (22 °C overnight)		1.41 ± 0.16a	3.12 ± 0.32a
Fresh (4 °C overnight)		1.48 ± 0.37a	2.89 ± 0.63a
Room temperature (22 °C, 4 weeks)	1.04 ± 0.15ab	1.27 ± 0.33ab	1.93 ± 0.21b
Refrigerated (4 °C, 4 weeks)	0.88 ± 0.10ab	1.07 ± 0.18bc	1.89 ± 0.42b
Frozen (−35 °C, 4 weeks)	0.80 ± 0.07b	0.83 ± 0.13bc	2.18 ± 0.09ab
Air-dried	nd	0.56 ± 0.11c	1.08 ± 0.04c

3.2. Microbial phosphorus

Microbial phosphorus concentrations varied in the three soils in the order: Albrook > Fort Sherman > Pipeline Road. For the time

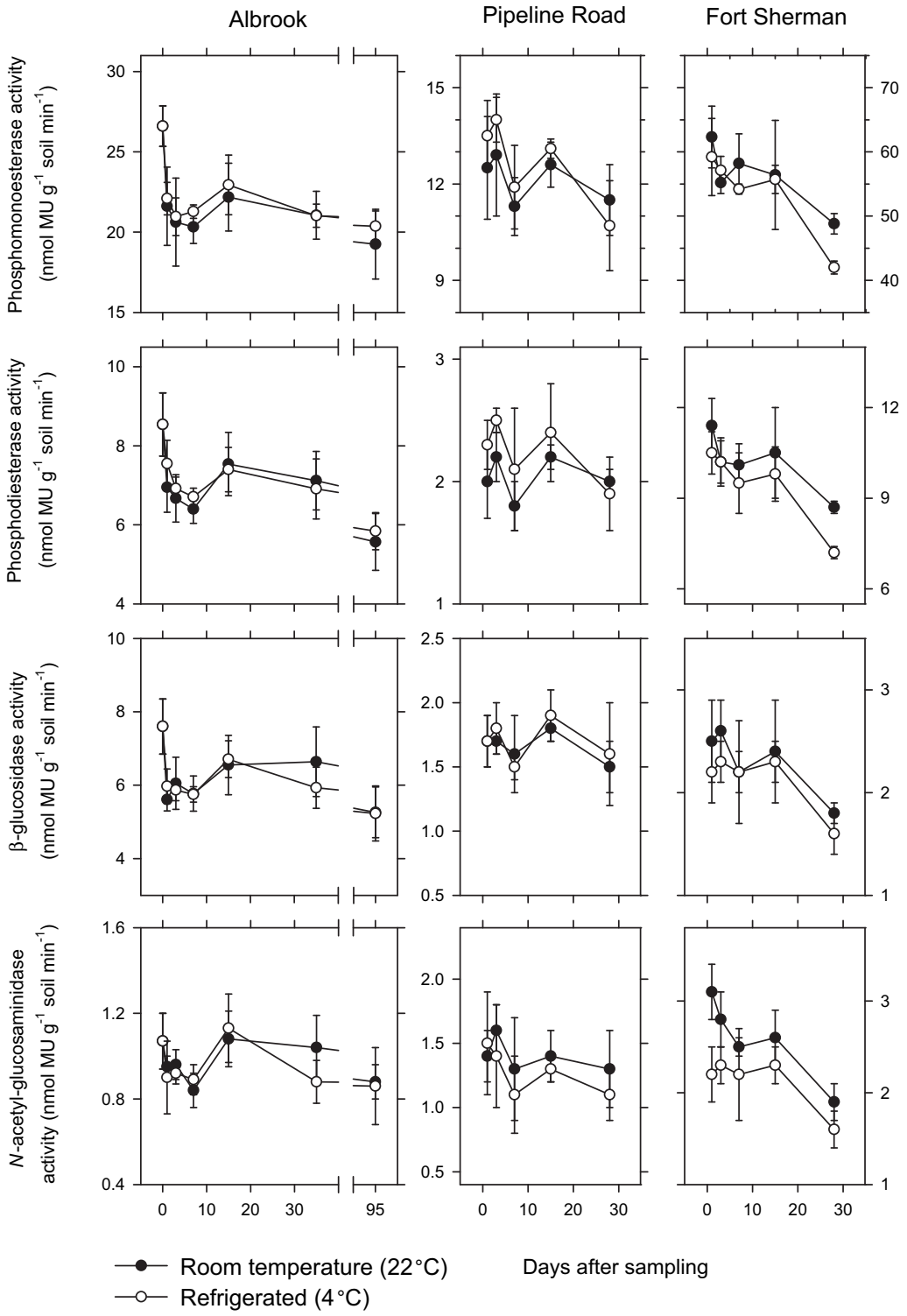

Fig. 1. Temporal changes in the activity of four hydrolytic enzymes in three soils under lowland tropical rain forest in central Panama during storage at 4 °C and 22 °C. Error bars show the standard deviation of four replicate samples. Note the differences in the *Y* axis scales for the three soils.

series data (storage at 4 °C and 22 °C) there was no significant time effect for the Pipeline Road soil ($P > 0.05$), but there were significant changes during storage of the Albrook and Fort Sherman soils ($P < 0.001$), although these were relatively small during the two weeks following sampling (Fig. 2). For the Fort Sherman soil there was a marked decline in microbial phosphorus after 4 weeks of storage at 4 °C, but concentrations in samples stored at 22 °C changed little during the same period (Fig. 2).

Microbial phosphorus in refrigerated samples was almost always lower than in samples stored at room temperature (Fig. 2), although there was a significant treatment effect (storage at 4 °C and 22 °C) only for the Pipeline Road soil ($P = 0.0011$) (Table 3).

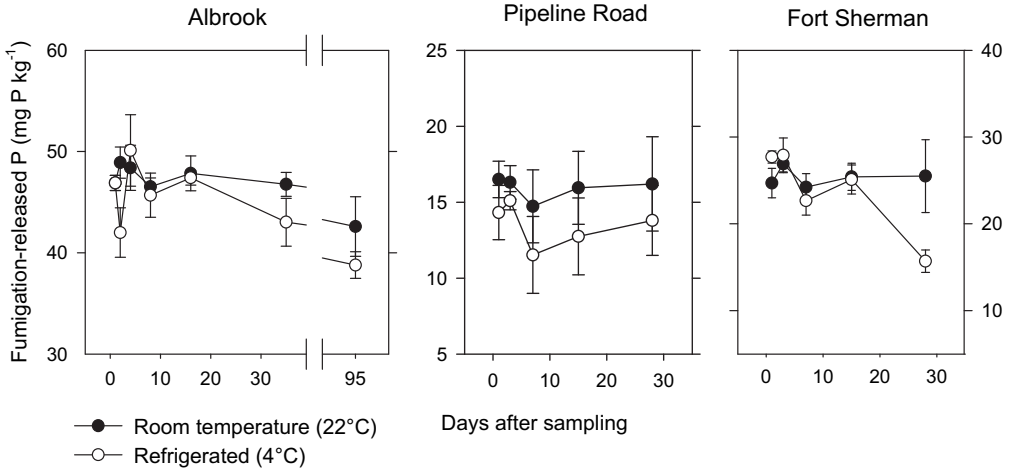

Fig. 2. Temporal changes in microbial phosphorus (fumigation-released P) in three soils under lowland tropical rain forest in central Panama during storage at 4 °C and 22 °C. Error bars show the standard deviation of four replicate samples.

The only treatment × time interaction was for the Fort Sherman soil ($P < 0.001$). There were no significant differences in samples stored overnight at 4 °C and 22 °C for Pipeline Road and Fort Sherman soils (Table 3). Freezing reduced microbial phosphorus concentrations compared to those of fresh samples for all three soils, except for Pipeline Road soil stored overnight at 4 °C.

4. Discussion

Although rapid analysis is usually considered to be the best option for assays of soil enzyme activity (e.g., within 2 h of sampling; DeForest, 2009), this is impractical for virtually all studies, especially those in tropical forests, where sampling sites may be many hours (or days) from suitable laboratory facilities. Here, microbial phosphorus and enzyme activities changed significantly during storage, but values were relatively stable for the first two weeks and it appears that a short period of storage prior to analysis will not greatly influence results. Storage at 22 °C appeared preferable to storage at 4 °C, although differences were small and not significant for most enzymes. In one soil, however, cold storage caused a marked decline in microbial phosphorus and some enzyme activities after 4 weeks. We therefore recommend that both hydrolytic enzymes and microbial phosphorus can be determined on soils stored in sealed plastic bags (to maintain field moisture) stored at either 4 °C or 22 °C for up to two weeks.

The stability of hydrolytic enzyme activities and microbial phosphorus is in marked contrast to the rapid changes in extractable inorganic nutrients in these soils (Turner and Romero, 2009). In particular, both inorganic nitrogen (ammonium and nitrate) and phosphate concentrations changed markedly within hours of sampling when stored at either 4 °C or 22 °C. This means that nutrients must be extracted from soil within 24 h of sampling to ensure that values are representative of those in the field. If it can be demonstrated that enzyme activities reflect nutrient demand at a site, such assays will provide a more robust measure of nutrient status than time-sensitive extractions of inorganic nutrients (Turner and Romero, 2009).

Table 3
Storage effects on fumigation-released (microbial) phosphorus concentrations in three soils under lowland tropical rain forest in central Panama. Fresh samples were analyzed either on the same day as sampling (Albrook) or after overnight storage at 4 °C or 22 °C (Pipeline Road and Fort Sherman). All other treatments are for samples analyzed after 4 weeks of storage (5 weeks for the Albrook soil). Values are the mean ± standard deviation of four replicate samples, and values within a column for each nutrient with the same letter are not significantly different at the 5% level (Tukey's HSD).

	Albrook	Pipeline Road	Fort Sherman
Fresh (same day)	46.9 ± 0.8a		
Fresh (22 °C overnight)		14.3 ± 1.8ab	24.7 ± 1.7a
Fresh (4 °C overnight)		16.5 ± 1.2a	27.7 ± 0.7a
Room temperature (22 °C, 4 weeks)	46.8 ± 1.2ab	16.2 ± 3.1a	25.5 ± 4.2a
Refrigerated (4 °C, 4 weeks)	43.0 ± 2.4b	13.8 ± 2.3ab	15.7 ± 1.3b
Frozen (−35 °C, 4 weeks)	30.7 ± 2.3c	11.1 ± 1.8b	19.7 ± 1.2b

To our knowledge, all previous studies on storage effects on enzymes and microbial biomass have been conducted on temperate soils and most have recommended storage at 4 °C for subsequent analysis of hydrolytic enzymes and microbial biomass (e.g., Ross, 1965; Speir and Ross, 1975; Ross et al., 1980; Lee et al., 2007; DeForest, 2009). For microbial carbon, Ross (1991) reported no significant decline when determined by fumigation–extraction in a silty-clay loam under maize and pasture after 14 months of storage at 4 °C, although there was a 41% decline in the same soil when microbial carbon was determined by fumigation–incubation. Petersen and Klug (1994) measured shifts in microbial community composition by phospholipid fatty acid analysis in a sandy arable soil from southern Michigan (organic C < 1%, pH 5.1). Little change was detected in individual lipids during storage at 4.5 °C for 7 weeks, although samples had been taken from 5 to 15 cm below a frozen topsoil, so it was perhaps not surprising that significant changes were detected during storage at 25 °C. Interestingly, the concentration of the phospholipid 18:2 ω6c, which is of mainly fungal origin, declined significantly during 21 d storage at 4.5, 10, and 25 °C.

For enzymes, Ross (1965) reported that the activities of enzymes hydrolyzing glucose (invertase) and starch (amylase) changed least following storage of pasture soils at 4 °C, while for a Mollisol (sandy loam, organic carbon 0.3%, pH 6.1), Pancholy and Rice (1972) reported no significant changes in urease, invertase (sucrose hydrolysis), or amylase (starch hydrolysis) after 30 d storage at 4 °C. In some cases, storage at 4 °C caused a marked decline in enzyme activities; for example, Speir and Ross (1975) reported variable effects of cold storage on phosphatase in three soils, with a 36% decline during 21 d storage in one soil. Similarly, Lee et al. (2007) reported that although phosphomonoesterase and *N*-acetyl-glucosaminidase activities were not affected by refrigerated storage, the activities of four other enzymes were significantly reduced in at least one soil.

In contrast, storage at room temperature is not generally recommended due to measured changes in microbial biomass

(Anderson, 1987) and enzyme activities (Pancholy and Rice (1972). For example, Anderson (1987) reported a 39% loss of microbial biomass following 70 d storage at 22 °C, compared to an 18% decline for samples stored at 2 °C. However, Speir and Ross (1975) reported that urease activity in three pasture soils (silt loam, organic C 3.8–8.0%, pH 5.8–7.5) was stable at room temperature (18–25 °C) for up to 22 d, and that the smallest changes in phosphatase occurred in soils stored at room temperature, although some of these changes were significant. Also, in one of the few studies to assess changes in microbial phosphorus during storage, West et al. (1986) reported that microbial phosphorus did not change significantly during a 7 d incubation at 25 °C of two pasture soils (silt loams, organic carbon 3.7 and 4.6%, pH 5.2 and 6.0) when they were sampled in the summer. However, a significant increase in microbial phosphorus was detected for one of the soils when sampled in the winter.

Storage at −20 °C was found to best preserve microbial biomass in a series of Swedish soils (Sternberg et al., 1998), presumably due to the acclimation of the microbial community to prolonged periods of annual freezing. Similarly, Tabatabai and Bremner (1970) reported that only freezing, but not storage at room temperature or 5 °C, preserved sulfatase activity over a 3-month period. However, Speir and Ross (1975) reported marked reductions in the activity of several enzymes in pasture soils stored frozen, while Lee et al. (2007) reported that although freezing did not markedly affect microbial properties in a clay-rich soil, many properties, including hydrolytic enzyme activities, were affected significantly in two other soils of coarser texture.

In the current study, frozen storage appeared to preserve hydrolytic enzyme activities at levels close to those in field soils, but caused a marked decline in microbial phosphorus. This suggests that microbial biomass in lowland tropical soils is adversely affected by freezing, whereas enzyme activity is preserved, perhaps due to the dominance of extracellular enzymes stabilized by sorption to clays or organic matter (Skujiņš, 1976; Lee et al., 2007). For microbial biomass, freezing tropical soils might be acceptable if it could be shown that a period of stabilization at room temperature prior to analysis (e.g., one week at room temperature) restores microbial communities to field values, although such a pre-incubation can lead to marked changes in microbial communities and is not recommended for molecular-level studies (Zelles et al., 1991).

Although many soils, including those studied here, experience an annual period of drying, most previous studies have reported strong negative effects of air-drying on microbial biomass and enzyme activities (e.g., Ross, 1966; Ramírez-Martínez and McLaren, 1966; Speir and Ross, 1975; Bandick and Dick, 1999; Parham and Deng, 2000; Lee et al., 2007) and although air-drying was widely used in early studies of soil enzymes (reviewed in Speir and Ross, 1978), it is not usually recommended for such analyses. An exception was Ross (1965), who found no significant decline in the activities of enzymes hydrolyzing glucose (invertase) and starch (amylase) in air-dried samples of naturally arid soils. In addition, Speir and Ross (1975) reported that air-drying best preserved sulfatase activity at values close to those in fresh samples, although Tabatabai and Bremner (1970) reported that air-drying was not suitable for sulfatase assays in a series of thirteen soils from the Midwest USA. All three soils in the current study undergo a period of strong drying for approximately 4 months each year (Engelbrecht et al., 2007), but the marked reduction in all measured properties following air-drying confirms the unsuitability of this treatment for microbial and biochemical analyses of tropical forest soils.

DeForest (2009) reported recently that although enzyme activities in refrigerated and frozen soils did not differ significantly, the effects of storage differed among enzymes. Specifically, storage did not significantly influence the activity of β-glucosidase or xylanase, while phosphomonoesterase and *N*-acetyl-glucosaminidase were significantly affected. Similarly, Lee et al. (2007) reported variable responses of seven hydrolytic enzymes to storage of three soils differing markedly in texture, but with similar pH (5.4–6.1) and (low) organic matter contents. In the current study we did not find major differences in the response of four hydrolytic enzymes to soil storage, although it is possible that differences may be greater for other soils depending on the physical protection offered by the soil matrix, based on properties such as the particle-size or aggregate size distribution (e.g., Marx et al., 2005; Muruganandam et al., 2009).

It was of interest to note the initial rapid decline in enzyme activity for the Albrook soil, after which values were relatively stable. This soil was analyzed within a few hours of sampling, whereas the other two soils, for which this rapid decline was not observed, were analyzed the following morning after sampling. The initial high activity in the Albrook soil may have been caused by the release of intracellular enzymes from roots and fungal hyphae following soil disturbance during sampling and preparation (i.e., sieving) (Petersen and Klug, 1994). Indeed, the Albrook soil contained a large number of very fine roots compared to the other soils. Such intracellular enzymes might be expected to be denatured rapidly, leaving the more stable extracellular component. In this case, very rapid analysis of soils following sampling (i.e., the same day) may overestimate enzyme activities in undisturbed samples.

Differences in phosphatase corresponded to differences in soil phosphorus status, either in terms of total phosphorus (Table 1) or readily-exchangeable phosphate (Turner and Romero, 2009), with lowest phosphorus concentrations corresponding to highest phosphatase activity. Differences in β-glucosidase corresponded to differences in total carbon concentrations (Table 1), but also to microbial biomass concentrations, at least as indicated by microbial phosphorus (Table 2), with higher β-glucosidase activity in soils with high total carbon and microbial biomass. This agrees with the close correlation between β-glucosidase and total and microbial carbon in temperate pasture soils (Turner et al., 2002). Factors regulating the differences in *N*-acetyl-glucosaminidase activity among the three soils are less obvious, but may relate to either microbial demand for nitrogen (Olander and Vitousek, 2000) or the general activity of the fungal community (Miller et al., 1998). Increases in readily-exchangeable phosphate during storage (Turner and Romero, 2009) may have contributed to the slow decline in phosphatase activity measured here, because soluble phosphate reduces phosphatase activity by product inhibition (Speir and Ross, 1978), although the changes were probably too small to have a detectable impact. It is less clear how changes in extractable nutrients might have influenced the other enzymes; for example, inorganic nitrogen concentrations increased markedly in all soils during storage, but *N*-acetyl-glucosaminidase activity is not necessarily depressed by high inorganic nitrogen concentrations (Olander and Vitousek, 2000). Changes in the four enzymes may also have been influenced by the small changes in pH, becoming more acidic, that occurred in all soils during storage (Turner and Romero, 2009).

In summary, significant changes in microbial phosphorus and enzyme activities occurred during storage of three lowland tropical rain forest soils, although changes were relatively small during the first two weeks at either 4 °C or 22 °C. Freezing appeared to be an acceptable form of storage for some enzymes in some soils, but caused a marked decline in microbial phosphorus in two soils after 4 weeks of storage. Air-drying caused a marked reduction in all biological properties measured. We therefore recommend that enzyme activity and microbial phosphorus should be determined in tropical forest soils after no more than two weeks storage in the dark at either 4 °C or 22 °C. For longer-term storage (>2 weeks), room temperature storage appears preferable to refrigeration. Freezing will preserve enzyme activities for future analysis, but will not yield microbial phosphorus values that approximate field conditions.

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