

The response of heterotrophic activity and carbon cycling to nitrogen additions and warming in two tropical soils

DANIELA F. CUSACK*, MARGARET S. TORN†, WILLIAM H. McDOWELL‡ and WHENDEE L. SILVER*

*Department of Environmental Science, Policy and Management, University of California – Berkeley, 137 Mulford Hall #3114, Berkeley, CA 94720, USA, †Lawrence Berkeley National Lab, 1 Cyclotron Road, Berkeley, CA 94720, USA, ‡Department of Natural Resources and the Environment, University of New Hampshire, 38 College Road, Durham, NH 03824, USA

Abstract

Nitrogen (N) deposition is projected to increase significantly in tropical regions in the coming decades, where changes in climate are also expected. Additional N and warming each have the potential to alter soil carbon (C) storage via changes in microbial activity and decomposition, but little is known about the combined effects of these global change factors in tropical ecosystems. In this study, we used controlled laboratory incubations of soils from a long-term N fertilization experiment to explore the sensitivity of soil C to increased N in two N-rich tropical forests. We found that fertilization corresponded to significant increases in bulk soil C concentrations, and decreases in C loss via heterotrophic respiration ($P < 0.05$). The increase in soil C was not uniform among C pools, however. The active soil C pool decomposed faster with fertilization, while slowly cycling C pools had longer turnover times. These changes in soil C cycling with N additions corresponded to the responses of two groups of microbial extracellular enzymes. Smaller active C pools corresponded to increased hydrolytic enzyme activities; longer turnover times of the slowly cycling C pool corresponded to reduced activity of oxidative enzymes, which degrade more complex C compounds, in fertilized soils. Warming increased soil respiration overall, and N fertilization significantly increased the temperature sensitivity of slowly cycling C pools in both forests. In the lower elevation forest, respired CO_2 from fertilized cores had significantly higher $\Delta^{14}\text{C}$ values than control soils, indicating losses of relatively older soil C. These results indicate that soil C storage is sensitive to both N deposition and warming in N-rich tropical soils, with interacting effects of these two global change factors. N deposition has the potential to increase total soil C stocks in tropical forests, but the long-term stability of this added C will likely depend on future changes in temperature.

Keywords: C turnover, microbial enzymes, oxidative activity, Q_{10} , radiocarbon, roots, soil respiration

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Introduction

There has been considerable recent interest in the effects of global change factors, such as elevated temperature, precipitation and nitrogen (N) deposition, on carbon dioxide (CO_2) fluxes from soils (Luo *et al.*, 2004; Norby & Luo, 2004; Pendall *et al.*, 2004; Henry *et al.*, 2005). Ecosystem respiration is one of the largest annual emissions of CO_2 to the atmosphere (Prentice *et al.*, 2001), with soil respiration contributing approximately 50% of this flux across ecosystems (Schlesinger, 1997). Humid tropical forests play an important role in the

global carbon (C) budget because of high net primary productivity (NPP), high rates of respiration, and substantial C storage (Melillo *et al.*, 1993). Because humid tropical forests have some of the largest repositories of soil C globally (Post *et al.*, 1982; Jobbagy & Jackson, 2000), even small changes in soil respiration rates with global change could represent large net changes in ecosystem CO_2 emissions. Heterotrophic respiration (i.e., microbial) can account for the majority of soil CO_2 emissions in tropical forests, with autotrophic respiration (i.e., root) often contributing $\leq 50\%$ (Silver *et al.*, 2005). The response of heterotrophic respiration to multiple and interacting factors of global change is poorly understood for tropical forests, but will likely be a key factor determining the direction of soil feedbacks to climate change (Bardgett *et al.*, 2008).

Correspondence: Present address: Daniela F. Cusack, Geography Department, University of California—Santa Barbara, 1832 Ellison Hall, Santa Barbara, CA 93106, USA, e-mail: dcusack@geog.ucsb.edu

Combined changes in N deposition and temperature have considerable potential to affect soil CO₂ fluxes and C storage in tropical regions. N deposition and warming can each alter the activity of microbial decomposers, influencing the quantity of C lost from soils via respiration, the chemical quality of C retained in soils as decomposition byproducts, and the transport of C from the surface into soils as dissolved organic C (DOC) (Guggenberger, 1994; Guggenberger & Zech, 1994; Kalbitz *et al.*, 2000, 2005; Fenner *et al.*, 2007). N deposition in the tropics is increasing rapidly because of increased industrialization (Galloway *et al.*, 1994, 2004), with ecosystem responses likely to diverge from those observed in temperate zones (Matson *et al.*, 1999).

Unlike most temperate and boreal ecosystems, humid tropical forests on highly weathered soils are typically characterized by high soil N availability, rapid rates of N cycling, and the lack of N limitation to NPP (Vitousek & Sanford, 1986; Martinelli *et al.*, 1999). Thus, above-ground NPP is unlikely to increase with N deposition in these forests (Harrington *et al.*, 2001), unlike in temperate forests (Aber & Magill, 2004; Nadelhoffer *et al.*, 2004). Heterotrophic activity, in contrast, is likely to be sensitive to N deposition, both because microbial decomposers are sensitive to changes in the C:N ratios of organic material even in N-rich tropical forests (Hobbie & Vitousek, 2000; Cusack *et al.*, 2009a), and because of potential shifts in microbial community composition with added N (Wallenstein *et al.*, 2006). The extent and direction of change in soil C storage in tropical forests with N deposition is thus likely to depend on heterotrophic responses.

Results from N-addition experiments have led to apparently contradictory hypotheses regarding the direction of change in soil C storage with N deposition. Labile C pools, such as those present during early stages of decomposition, typically respond to added N with increased decomposition rates (Berg & Matzner, 1997; Neff *et al.*, 2002), implying that N deposition may lead to soil C losses. In contrast, N deposition has also been found to decrease decomposition of poorer quality litter, and suppress respiration during later stages of decomposition (Carreiro *et al.*, 2000; Swanston *et al.*, 2004; Waldrop & Firestone, 2004; Knorr *et al.*, 2005; Olsson *et al.*, 2005). N fertilization can also increase fluxes of C into soils from the surface via DOC (Guggenberger, 1994; Pregitzer *et al.*, 2004; Waldrop & Zak, 2006), which can be important for delivery of C into the soil profile and subsequent storage (McDowell & Likens, 1988). Thus, results indicate both increased and decreased soil C losses, and the potential for changes in soil C sequestration with N deposition. Understanding the processes that predominate changes

in different soil C pools will help us reconcile these apparently contradictory results.

While it is important to track changes in bulk soil C stocks, different C pools vary greatly in their importance for long-term soil C storage. Some soil C is readily available for biological degradation and has turnover times on the scale of decades (the active C pool) (Trumbore *et al.*, 1995; Trumbore, 2000), while other soil C is chemically or physically protected and stored for longer time periods (slowly cycling C) (Krull *et al.*, 2003). The turnover of C in these two pools is mediated, in part, by the activity of microbial decomposition enzymes. Soil microbial enzymes can be roughly divided into two classes: (1) Oxidative enzymes, which are primarily produced by fungi and promote cometabolic degradation of complex compounds and structural plant tissues; (2) hydrolytic enzymes, which directly acquire nutrients from relatively labile organic matter and support primary metabolism (Sylvia *et al.*, 2004). Because oxidative enzymes are produced primarily by fungi for nutrient scavenging, these enzymes are often negatively correlated to nutrient supply (Fog, 1988). In contrast, hydrolytic enzymes, which are produced by a broad suite of microbial groups, can respond positively to increased N (Carreiro *et al.*, 2000; Saiya-Cork *et al.*, 2002). Thus, the effects of global change factors on soil C storage in tropical forests may depend on the relative importance of the active and slowly cycling C pools, and the responses of different groups of microbial enzymes.

Warming is an important global change factor in tropical forests that also has the potential to affect the active and slowly cycling C pools differently. Climate change and the C cycle are closely coupled (Cox *et al.*, 2000), and significant changes in C uptake and storage in tropical forests have been predicted under a variety of climate change scenarios (Cox *et al.*, 2004; Friedlingstein *et al.*, 2006), due to both warming and changes in precipitation (Harris *et al.*, 2008). Soil respiration in particular is highly sensitive to temperature change, and is predicted to increase with warming (Raich & Schlesinger, 1992; Raich *et al.*, 2002), likely driving losses of soil C. The often applied Q₁₀ factor of two implies that a 10 °C increase in temperature roughly doubles soil respiration (Raich & Schlesinger, 1992), but the chemical form of soil C within and among C pools may influence the temperature sensitivity of heterotrophic respiration. Chemically complex C compounds are hypothesized to have greater intrinsic temperature sensitivity than chemically simple compounds, because of the higher activation energy needed to decompose them (Davidson & Janssens, 2006; Conant *et al.*, 2008a,b). For example, an increase in the relative complexity of soil C compounds, which could result from

reduced oxidative enzyme activity, may increase the temperature sensitivity of soil respiration. Thus, changes in the activities of the two groups of microbial enzymes mentioned above, resulting in a change in the chemical quality of soil C, may lead to changes in the temperature sensitivity of soil respiration from different C pools.

This study used soils from an on-going N fertilization experiment in two tropical forests to explore the separate and combined effects of N deposition and warming on soil C dynamics under controlled laboratory conditions. Field studies from the research site showed that soil C stocks increased with N fertilization, but above-ground stem growth and litter productivity did not (Cusack *et al.*, 2009). The main objective of this study was to determine whether declines in microbial respiration (i.e., reduced losses of soil C) could account for the observed increases in soil C stocks. We used soils from fertilized and control plots, plus a warming treatment, to address the following hypotheses: (1) long-term N fertilization suppresses heterotrophic respiration, resulting in increased bulk soil C pools; (2) N addition suppresses decomposition of chemically complex C compounds, leading to increased temperature sensitivity of soil respiration; and (3) warming interacts positively with N addition to stimulate losses of more labile C compounds from soils. While we predicted that total losses of C via heterotrophic respiration would decline with fertilization, we expected that the integrated radio-carbon-age of respiration from fertilized soils would be older. This could occur as the results of reduced contributions of labile soil C to respiration because of smaller active C pools after prolonged fertilization.

Materials and methods

Site description

This study was conducted using soils from the Luquillo Experimental Forest (LEF), an NSF-sponsored Long Term Ecological Research (LTER) site in Puerto Rico (Lat. +18.3°N, Long. -65.8°W). Background rates of wet N deposition are relatively low in Puerto Rico ($\sim 2.1 \text{ kg N ha}^{-1} \text{ yr}^{-1}$), but have more than doubled in the last two decades (NADP/NTN, 2009). Soils were collected from two distinct forest types at a lower and upper elevation to examine the effects of N additions in forests that experience different rainfall and temperature environments. The lower elevation site is a wet tropical rainforest (Bruijnzeel, 2001) in the Bisley Experimental Watersheds (Scatena *et al.*, 1993) in Tabonuco-type forest. Long-term mean annual rainfall in the watersheds is 3537 mm yr^{-1} (Garcia-Montino *et al.*, 1996), and the plots were located at 260 m asl. The upper

elevation site is a lower montane forest characterized by abundant epiphytes and cloud influence (Bruijnzeel, 2001) in the Icacos watershed (McDowell *et al.*, 1992) in Colorado-type forest. Mean annual rainfall at the site is 4300 mm yr^{-1} (McDowell & Asbury, 1994; Heartsill-Scalley *et al.*, 2007), and plots were located at 640 m asl. The average daily temperature is 23 °C in the lower elevation forest, and 21 °C in the upper elevation forests (W.L. Silver, unpublished data). The LEF experiences little temporal variability in monthly rainfall and mean daily temperature (McDowell *et al.*, 2010).

The two forest types differ in important ecological factors. The soils in both forests are primarily deep, clay-rich, highly weathered Ultisols lacking an organic horizon, with Inceptisols on steep slopes (Beinroth, 1982; Huffaker, 2002), but the upper elevation forest has lower soil redox potential than the lower elevation forest (Silver *et al.*, 1999) and poorer drainage. Soil C, N and P content are higher in the upper elevation forest, and 1 M HCl extractable P increases with elevation in the LEF (McGroddy & Silver, 2000). The lower elevation forest has significantly higher background soil N concentrations than the upper elevation forest (Cusack *et al.*, 2009b). The two forests also differ in tree species composition and structure (Brown *et al.*, 1983). Average canopy height is 21 m for the lower elevation forest, and 10 m for the upper forest (Brokaw & Grear, 1991). The dominant tree species in the lower elevation forest is *Dacryodes excelsa* Vahl, while four species were codominant in the upper elevation plots: *Cyrilla racemiflora* L., *Micropholis chrysophylloides* Pierre, *Micropholis garcinii-folia* Pierre, and *Clusia krugiana* Urban. Trees of the *Clusia* genus are widespread throughout the Neotropics, and commonly express crassulacean acid metabolism (CAM) which can affect foliar isotopic composition (Ball *et al.*, 1991; Holtum *et al.*, 2004; Luttge, 2008), and potentially the isotopic composition of soil respiration.

N-addition plots in each forest type were established in 2000 at sites described by McDowell *et al.* (1992), and fertilization began in January 2002. Three 20 m $\times$ 20 m fertilized plots were paired with control plots of the same size in each forest type, for a total of 12 plots. The buffers between plots were at least 10 m, and fertilized plots were located so as to avoid runoff into control plots. Soil C concentrations to 30 cm depth, DOC and total dissolved nitrogen in soil solution were all measured before fertilization in 2001, and showed no differences among plots (Macy, 2004). Starting in 2002, $50 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ were added using a hand-held broadcaster, applied in two annual doses of NH_4NO_3 . Fifty $\text{kg N ha}^{-1} \text{ yr}^{-1}$ is approximately twice the average projected rates in Central America for the year 2050 (Galloway *et al.*, 2004). Soils for this experiment were collected in August 2007.

Soil incubation

Soil cores were collected from 0 to 10 cm depth using 7.6 cm diameter PVC tubing with beveled edges. Newly fallen litter was not included in the cores, but more highly decomposed litter attached to surface soil was not removed. Three pairs of cores were collected from each of the 12 plots, for a total of 72 cores. Paired cores were subsequently assigned to separate temperature treatments. After collection, soils were maintained at ambient temperature and shipped to Lawrence Berkeley National Laboratory, where each core was placed in a 1-L leaching vessel fitted with a 0.45 µm glass fiber filter (Millipore Stericup, Billerica, MA, USA). Cores were kept intact in the PVC tubing for the duration of the incubation to maintain soil structure, covered with perforated cellophane to allow gas exchange while maintaining high soil moisture, and stored in the dark. After a 2-week preincubation period, paired cores were split into ambient (21 °C) and warming (31 °C) treatments. Soil moisture was maintained at field capacity by watering with deionized water two times per week. Cores from fertilized plots received aqueous NH_4NO_3 monthly, equivalent to $50 \text{ kg N ha}^{-1} \text{ yr}^{-1}$. Soils were incubated for 245 days.

Soil carbon pools and fluxes

CO_2 fluxes were measured for each soil core every 6–14 days. For gas collection, leaching vessels were sealed with rubber end-caps that were fitted with black butyl rubber gas-impermeable Geo-Microbial Technologies septa (GMT, Ochelata, OK, USA). CO_2 production was tested for linearity, and found to be linear up to 5 h. Ambient headspace was collected at the start of each incubation period and at 2.5 h using a plastic syringe. Samples were stored in 20-mL pre-evacuated glass Wheaton vials fitted with Teflon septa (National Scientific, Rockwood, TN, USA). CO_2 reference standards were prepared similarly during each sampling period. Gas samples were analyzed within 24 h using a thermal conductivity detector on a Shimadzu GC14 gas chromatograph (Shimadzu Corporation, Columbia, MD, USA). Reference standard recovery for all data presented was >95%. Total C respired over the course of the incubation was calculated using linear interpolation between sampling dates, and is presented as a percent of total soil C.

Turnover times for two soil C pools were calculated using respiration data as in Townsend *et al.*, (1997) and Torn *et al.*, (2005). Briefly, an active pool of C was defined as the initial large flux of respiration, which is typically followed by a lower baseline rate of respiration. This steady baseline of respiration reflects decom-

position from the slower C pool (Townsend *et al.*, 1997). For this incubation we used 75 days as the cut-off for the active C pool because this was the period of the large initial flux of respiration, after which respiration was relatively stable. We recognize that the active C pool actually declines gradually, with some active C remaining after the cut-off. However, this initial flux represents the majority of this pool (Townsend *et al.*, 1997; Torn *et al.*, 2005). It should also be noted that soil disturbance likely increases initial respiration rates in soil incubations, potentially inflating active C pool sizes calculated using this method. Nonetheless, comparison between treatments should still be robust. Many studies separate the slower C pool into two pools to account for passive C. We did not do that here since the goal was to compare treatments, not calculate precise turnover times *per se*. Because of the single slowly cycling C pool used here, our turnover times for slow C are biased toward longer time scales (Trumbore, 2000).

Soil C pool sizes and turnover times were calculated as in Torn *et al.*, (2005). The active pool of C was operationally defined as:

$$C_a = \sum_{t=0}^{t=75} R_{\text{OM}}^t - R_s^t,$$

where C_a is the C (g) in the active pool, R the soil respiration ($\text{g C g}^{-1} \text{ soil C day}^{-1}$), t the time (days) and subscripts OM the total soil organic matter (SOM), S the slowly cycling C pool. Respiration for the slow pool (R_s) was calculated as the average respiration rate after the initial flux of C (i.e., >75 days). The size of the slow C pool was calculated as

$$C_s = C_t - C_a,$$

where C_t is the total soil C (g) as measured on bulk soils. Finally, turnover times (τ) were calculated for the active and slow C pools using

$$R = C/\tau,$$

where R is the average soil respiration for R_s or R_a , and C is the the pool size of the slow or fast pool as calculated above.

DOC production was measured as the concentration of C in leachate from soil cores at six time points during the incubation. To collect leachate, 100 mL of deionized water was added to each core. Leaching vessels were then sealed and placed on vacuum to draw leachate through the filter. Samples plus blanks (deionized water drawn through an empty vessel) were frozen and shipped to the University of New Hampshire where DOC was measured as nonpurgeable organic carbon using a Shimadzu TOC 5000 or TOC V (Shimadzu Corporation, Columbia, MD, USA). Total dissolved N, including organic and mineral N, was measured using

the Shimadzu carbon analyzers and a NO_x analyzer (Merriam *et al.*, 1996).

Additional analyses were conducted at the end of the incubation. Each core was removed from PVC tubing, homogenized, and subsamples were collected for chemical and enzyme analyses. Air-dried samples were ground using a mortar and pestle, and C and N concentrations were measured in duplicate for each core on a CE Elantec Elemental analyzer (CE Instruments, Lakewood, NJ, USA) using alanine as a standard. Additional soil measurements included root biomass, pH, and soil moisture. Roots remaining in the cores at the end of the incubation were hand-picked out and oven dried at 60 °C until weight stabilized. Gravimetric soil moisture for each core was measured at 105 °C. Soil pH was measured on soils at the end of the incubation in a 1:2 suspension of 1 M KCl. Enzyme analyses are described below.

Temperature sensitivity (Q_{10})

As a simple measure of temperature sensitivity, the Q_{10} of soil respiration was calculated as the plot-averaged respiration rate for 31 °C divided by the plot-averaged respiration rate for 21 °C. Measuring the temperature sensitivity of more slowly cycling C pools is difficult in the presence of labile C compounds. Because of the separation in time of respiration from the active vs. slower pools (as described above), soil incubations are useful for examining the temperature response of different C pools (Townsend *et al.*, 1997). For example, *Ca* dominates CO_2 fluxes initially until the active C pool is depleted. Values for Q_{10} were calculated for each time point for fertilized and control soils after the preincubation period. Calculating Q_{10} based on the quantity of C respired from soils, rather than from instantaneous measurements of respiration at the same time point, can be useful to standardize for pools and quality of C being compared (Conant *et al.*, 2008a). Therefore, additional Q_{10} values were calculated using respiration rates at cumulative fluxes of 500, 1000, 5000, 10 000, 15 000 and 19 000 μmol of C respired, averaging by plot.

Microbial enzyme analyses

Microbial enzyme activities were measured on fresh soils within 24 h of the end of the incubation. The oxidative enzymes phenol oxidase and peroxidase, which degrade lignin and other complex C compounds, were measured using colorimetric assays. Hydrolytic enzymes that acquire C, N and phosphorous (P) by degrading more simple compounds included cellulose-degrading β -glucosidase and cellobiohydrolase, hemicellulose-degrading β -xylosidase, and carbohydrate-degrading

α -glucosidase, which acquire C; chitin-degrading N-acetylglucosaminidase (NAG) and leucine aminopeptidase which acquire N; and phosphatase which acquires P. All hydrolytic enzymes were measured using fluorescent assays (Saiya-Cork *et al.*, 2002; Caldwell, 2005).

The general method in Sinsabaugh *et al.*, (2003) was followed. Briefly, 2.5 g of homogenized fresh soil (approximately 1.3 g dry-weight equivalent) from each core was slurried in 100 mL of 50 mM, pH 5 acetate buffer on a stir plate for 2 min. For fluorometric assays, aliquots of 200 μL were put onto 96-well plates, with eight analytical replicates per soil. Two hundred microliters of 200 μM fluorescing substrate (listed above) was added to each assay. Background fluorescence of soils and substrates was measured. Standard curves of 4-methylumbelliferone plus quench standards of soil with standard 4-methylumbelliferone were used. The reaction was stopped using 10 μL of 1 N NaOH. Plates were read on a Jobin Yvon Horiba SPEX DataMax with a Micromax microwell plate reader (Horiba Scientific, Longjumeau, France) at 365 nm excitation, 450 nm emission.

For colorimetric assays, 0.75 mL of soil slurry was pipetted into 2 mL deep-well plates, and 0.75 mL of 2500 μM L-3,4-dihydroxy-phenylalanine (DOPA) substrate was added. For peroxidase, 10 μL of 0.3% H_2O_2 was added. Absorbance was read on a Molecular Devices SpectraMax Plus with a microplate spectrophotometer (MDS Analytical Technologies, Toronto, Canada) at 450 nm. Background absorbance of DOPA was measured, and an extinction coefficient was calculated using a standard curve of DOPA degraded with mushroom tyrosinase. Assays were incubated at 27 °C for 0.5 to 8 h for fluorescent assays, and up to 24 h for colorimetric assays. Incubation times were based on initial tests.

Radiocarbon ($\Delta^{14}\text{C}$) and $\delta^{13}\text{C}$ measurements

The radiocarbon content of CO_2 respired during incubations was used as an integrated measure of the relative age of substrates utilized by heterotrophic microbes (Trumbore, 2000). Respiration for radiocarbon analysis was collected from one to two cores from each plot at 90 and 230 days. Individual intact cores were transferred to glass reaction vessels fitted with rubber o-rings, metal clamps, and Swagelok fittings. Cores were incubated at treatment temperatures for enough time to evolve at least 2 mg of C as CO_2 . The vessels were then evacuated on a glass manifold, and CO_2 was cryogenically purified, split into two samples, and stored in Pyrex tubing. Radiocarbon content of CO_2 was measured at the Keck Accelerator Mass

Spectrometry (AMS) facility at the University of California, Irvine, following sample graphitization (Vogel *et al.*, 1984). Radiocarbon results are reported as $\Delta^{14}\text{C}$, the per mil deviation from a standard normalized for ^{13}C (Stuiver & Polach, 1977). AMS analytical precision was reported as 1.4–3.4‰ for these samples. Split samples were analyzed for $\delta^{13}\text{C}$ on an isotope ratio mass spectrometer at the University of California, Irvine, with $\pm 0.15\text{‰}$ analytical precision.

Statistical analyses

The effects of fertilization and warming on soil respiration and Q_{10} values over time were tested for each forest type using a repeated measures multivariate analysis of variance (MANOVA), including forest type as a factor. A subsequent analysis was run separating Q_{10} values for the active C pool (defined as above, <75 days), from the slower pool (>75 days), since these fluxes likely represent the utilization of different C pools (Townsend *et al.*, 1997; Torn *et al.*, 2005). Results from Wilk's lambda tests are reported for multivariate analyses (NumDF = numerator degrees of freedom, DenDF = denominator degrees of freedom), as well as significance levels for individual factors. Fertilization treatment and warming were also tested as predictors of soil C concentrations, total CO_2 evolved, and average DOC production in each forest type using analysis of variance (ANOVA). A fertilization effect was calculated as the simple difference between fertilized and control paired plots for response factors where this measure shows a meaningful change in C fluxes with fertilization. This calculation takes advantage of the experimental paired plot design to look specifically at the effect of fertilization on fluxes, and minimizes noise from landscape-scale variability.

Total oxidative and hydrolytic enzyme activities were tested as predictors of C fluxes. For microbial enzyme activities, a fertilization effect size was calculated rather than a simple difference. Because enzyme assays give potential activities, and units are not directly translatable to field rates, we calculated the fertilization effect size as the natural log of the response ratio (fertilized : control) for each set of paired plots (Knorr *et al.*, 2005). Using the natural log standardized fertilization effects to zero, such that average ratios >0 show a positive effect of fertilization, and ratios <0 show a negative effect of fertilization (e.g., a value of 0.7 represents a 100% increase). Response ratios for each enzyme were tested for significant difference from 0. In addition to reporting total enzyme activities per gram of soil, we also report specific enzyme activities per gram of soil C. Enzyme activities tend to respond strongly to the quantity of soil organic matter (Sinsabaugh *et al.*, 2008), so normalizing activities to soil C content provides a

measure of direct responses to added N, rather than responses to changes in soil C.

The $\Delta^{14}\text{C}$ and the $\delta^{13}\text{C}(\text{‰})$ of CO_2 were compared among fertilization and temperature treatments, and time points for each forest type using ANCOVA. The presence of the CAM tree genus (*Clusia*) in the upper elevation forest had the potential to enrich leaf litter and SOM in $\delta^{13}\text{C}$ (Holtum *et al.*, 2004). Likely because of this genus, the $\delta^{13}\text{C}$ of respiration in the upper elevation had higher variability than in the lower elevation. General environmental drivers of $\Delta^{14}\text{C}(\text{‰})$ of CO_2 were also of interest. All environmental factors (oxidative and hydrolytic enzyme activities, root biomass, pH, soil moisture, % C and % N) were initially included in a multiple regression to predict $\Delta^{14}\text{C}$ and removed sequentially if not significant. Predictors of $\delta^{13}\text{C}$ were tested similarly. Data were log transformed where necessary to meet the assumptions for ANOVA (root biomass and DOC). Analyses were performed using JMP 7.0.2 software (SAS institute). For all ANOVAs, data were averaged at the plot-scale ($n = 3$ per forest type), and means are reported $\pm 1\text{S.E.}$ unless otherwise noted. Statistical significance was determined as $P < 0.05$ for models and model factors unless otherwise noted.

Results

Soil carbon pools and fluxes

Bulk soil C concentrations were significantly higher in fertilized soils for both forest types, and losses of C via microbial respiration were lower from fertilized soils (Table 1, Figs 1 and 2). Soil C concentrations increased from $3.7 \pm 0.3\%$ in control cores to $4.2 \pm 0.2\%$ in fertilized cores for the lower elevation forest, and from $3.5 \pm 0.3\%$ in control cores to $6.7 \pm 1.8\%$ in fertilized cores in the upper elevation forest.

Forest type, fertilization, and warming were all significant factors in the analysis of respiration ($\text{CO}_2 \text{ g}^{-1} \text{ soil C h}^{-1}$) over time, with an interaction between time and temperature (Fig. 1, NumDF = 48, DenDF = 16, $P < 0.01$). Comparing among treatments, temperature had a strong positive effect on C losses via respiration. Respiration was significantly higher from upper elevation forest soils than from lower elevation soils (Fig. 1). In both forest types, N fertilization suppressed CO_2 respired per g of soil C (Fig. 1). Comparing total C lost via respiration over the course of the incubation, fertilized plots lost significantly less C as a percent of total soil C than did control plots, and warming increased C losses overall (Fig. 2).

Although total soil C concentrations increased, added N had a different effect on the active C pool vs. the slowly cycling C pool. As a proportion of total soil C, the

Table 1 Soil Characteristics

Forest	Temp. (°C)	Trt.	pH§*	Soil (% C)†	Soil (% N)†	Roots (mg g ⁻¹) soil	DOC (mg ⁻¹ C L ⁻¹)§*,¶	TDN (mg N L ⁻¹)†,‡
Lower	21	C	4.0 ± 0.03	3.7 ± 0.3	0.27 ± 0.03	6.6 ± 2.3	1.0 ± 0.2	0.7 ± 0.1
Lower	21	N	4.1 ± 0.08	4.2 ± 0.2	0.33 ± 0.02	2.8 ± 1.5	1.2 ± 0.1	6.0 ± 3.5
Lower	31	C	4.0 ± 0.07	3.3 ± 0.2	0.25 ± 0.02	2.4 ± 0.7	1.4 ± 0.3	2.7 ± 1.4
Lower	31	N	4.1 ± 0.15	3.9 ± 0.3	0.31 ± 0.02	2.1 ± 0.8	1.0 ± 0.1	7.1 ± 4.9
Upper	21	C	4.1 ± 0.09	3.5 ± 0.3	0.18 ± 0.02	6.2 ± 1.3	3.4 ± 0.4	1.8 ± 0.4
Upper	21	N	3.9 ± 0.11	6.7 ± 1.8	0.31 ± 0.07	4.3 ± 1.5	4.1 ± 0.6	6.8 ± 0.5
Upper	31	C	4.2 ± 0.27	3.5 ± 0.2	0.18 ± 0.02	3.1 ± 1.2	5.6 ± 1.8	3.6 ± 0.6
Upper	31	N	3.8 ± 0.10	6.7 ± 1.9	0.32 ± 0.08	4.4 ± 0.7	10.9 ± 4.0	10.5 ± 1.7

Soil chemistry (pH, % C and % N), root biomass, dissolved organic C (DOC), and total dissolved N (TDN) are shown for two tropical forest types (lower and upper elevation), with two temperature (Temp.) treatments, and a fertilization treatment (Trt.). Averages ± one standard error are shown for soils from Control (C) and N-fertilized (N) plots ($n = 3$).

*Denotes $P < 0.1$, otherwise $P < 0.05$.

†Significant effect of N for both forest types.

‡Significant effect of temperature for both forest types.

§Significant effect of N for upper elevation forest.

¶Significant effect of temperature for upper elevation forest.

active C pool was smaller in fertilized vs. control soils (Table 2). The active C pool comprised <0.3% of total soil C, but it contributed significantly to soil respiration in the beginning stages of the incubation. Fertilization affected the slowly cycling C pool differently. In fertilized soils, the slowly cycling pool had significantly slower turnover (Table 2), indicating that increased C retention in this pool accounted for the observed increases in bulk soil C. Turnover times for the slower pool ranged from 76 to 195 years (Table 2).

Fertilization increased DOC production and decreased pH in the upper elevation forest only (Table 1). There was also a significant positive effect of warming on DOC production in the upper elevation forest. Soil pH decreased slightly but significantly at both temperatures in the upper elevation forest (Table 1). Soil pH, oxidative enzymes, temperature and fertilization treatment were all significant factors in the analysis of DOC concentrations for the upper elevation forest.

There were no significant differences in root biomass among treatments at the end of the incubation (Table 1), and there were no significant correlations between root biomass and soil C pools. In the lower elevation forest only, root biomass was weakly positively correlated with total C respired over the course of the incubation across treatments ($R^2 = 0.18$, $n = 36$, $P < 0.05$).

Q_{10} values

Initial Q_{10} values ranged from 1.4 to 2.1 (Fig. 3), and were not different among treatments for the initial stage of the incubation. In the later stages of the incubation, N fertilization increased the temperature sensitivity of

SOM in the slowly cycling C pool in both forests. Using Q_{10} values calculated for each time point, fertilized cores had significantly higher Q_{10} values after the first 75 days in the lower elevation forest (NumDF = 1, DenDF = 4, Fig. 2). Using Q_{10} values calculated for given quantities of C respired, there was a trend toward higher temperature sensitivity of fertilized soils for both forests after the initial 1000 μmol of CO_2 respired (NumDF = 2, DenDF = 9, $P = 0.1$, Fig. 3). When Q_{10} values were based on the amount of C respired, rather than the day of the incubation, variability decreased and clearer patterns emerged (Fig. 3). Time was a significant factor for each set of Q_{10} values, with values significantly lower for the later time period (>75 days) in both forest types. When Q_{10} values were based on the amount of C respired, the upper elevation forest had significantly higher overall Q_{10} values than the lower elevation forest (NumDF = 1, DenDF = 10).

Microbial enzyme activities

Microbial enzyme activities responded to fertilization in both forest types. Looking first at total enzyme activities per gram soil, fertilization had a significant positive effect on hydrolytic enzyme activities in both forest types with fertilization. In contrast, fertilization had a negative effect on total oxidative enzyme activities in the lower elevation forest (Fig. 4, Table 3). Across all treatments and forest types, soil C concentration was the strongest single predictor of total hydrolytic enzyme activities ($R^2 = 0.56$, $n = 72$, $P < 0.05$). Total oxidative enzyme activities were also correlated to soil C concentrations, although the relationship was weak ($R^2 = 0.09$,

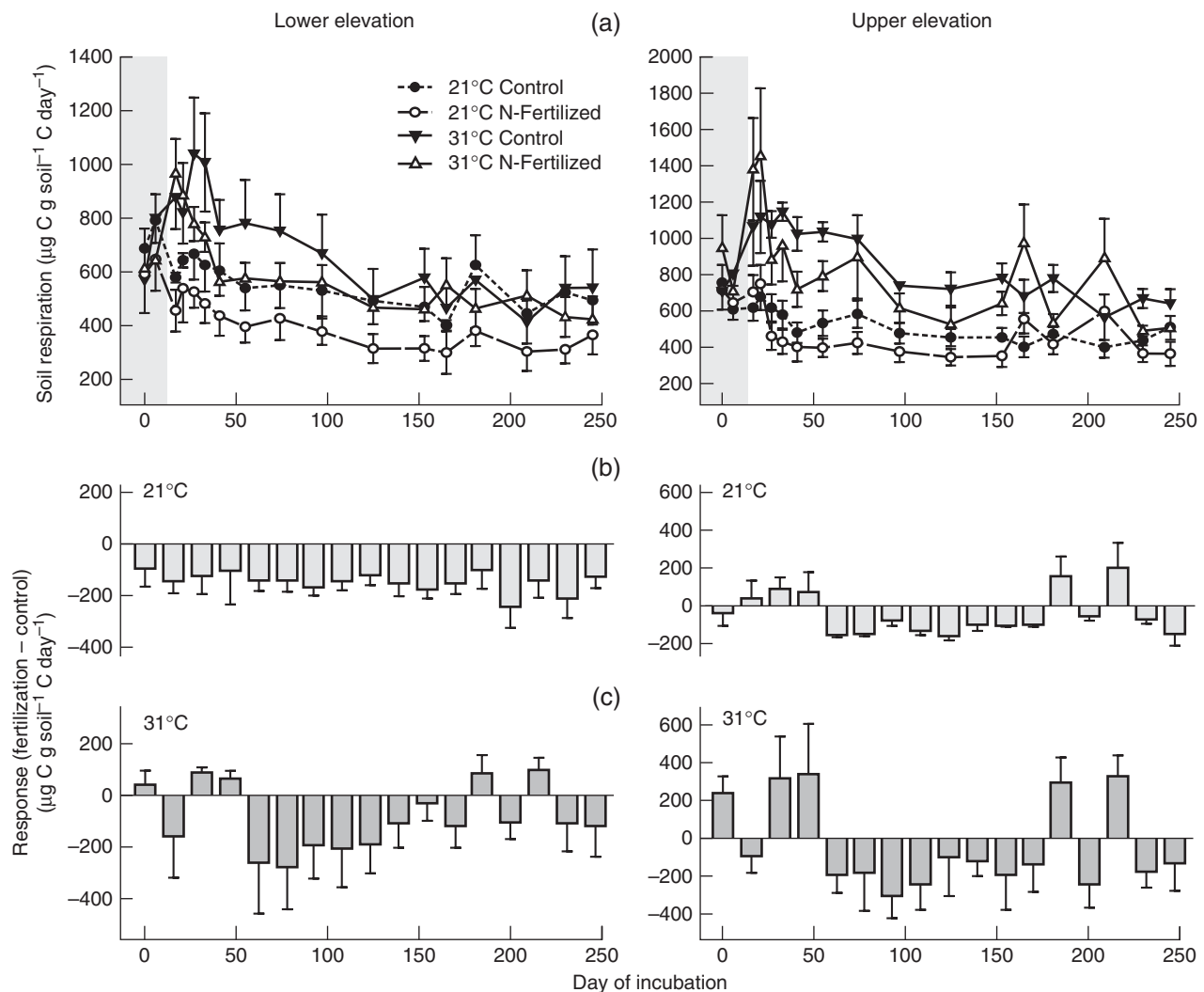


Fig. 1 (a) Soil Respiration per gram of soil carbon (C) is shown over a 245-day incubation. Dark symbols are for control soils, and open symbols are Nitrogen (N)-fertilized soils. Ambient temperature respiration (21°C) is shown in circles, warmed (31°C) is shown in triangles. The first two sampling dates which are shaded in gray, show respiration data for a pre-incubation period (no temperature treatment). (b) The difference in C respired between fertilized and control soils for each set of paired plots is shown for ambient (21°C) soils ($n=3$). (c) The difference in C respired between fertilized and control soils for each set of paired plots is shown for warmed (31°C) soils ($n=3$). Repeated measures MANCOVA showed a trend of lower respiration from N-fertilized cores over time in both forest types ($P=0.1$), a significant effect of temperature ($P<0.05$), and an interaction between time and temperature. For simplicity, significant differences for individual dates are not shown on the figure.

$n = 72$, $P<0.05$). Temperature did not affect the magnitude of the fertilization effect size for hydrolytic enzymes (i.e., paired-plot ratios of fertilized:control), but total hydrolytic enzyme activities were significantly lower in warmed vs. ambient temperature cores at the end of the experiment. Temperature did not affect oxidative enzyme activities (Table 3).

The fertilization effect on specific enzyme activities (i.e., normalized to soil C content) helped explain the accumulation of soil C. Per gram of soil C, fertilized soils had lower oxidative enzyme activities than control soils in both forests, with suppression of some hydro-

lytic specific enzyme activities as well (Fig. 4). This indicates that a lower proportion of the total soil C was likely to be decomposed by enzymes in fertilized plots, potentially indicating a direct response of oxidative enzymes to added N (i.e., not purely a response to soil C changes).

Across all treatments and sites, oxidative enzyme activity was the strongest single predictor of DOC ($\ln \text{DOC mg C L}^{-1} = 0.02 + 0.03(\Sigma \text{ oxidative enzyme activity mmol g}^{-1} \text{ soil h}^{-1})$; $R^2 = 0.61$, $n = 72$, $P<0.05$), whereas hydrolytic enzymes were weaker but also significant predictors of DOC ($R^2 = 0.31$, $n = 72$,

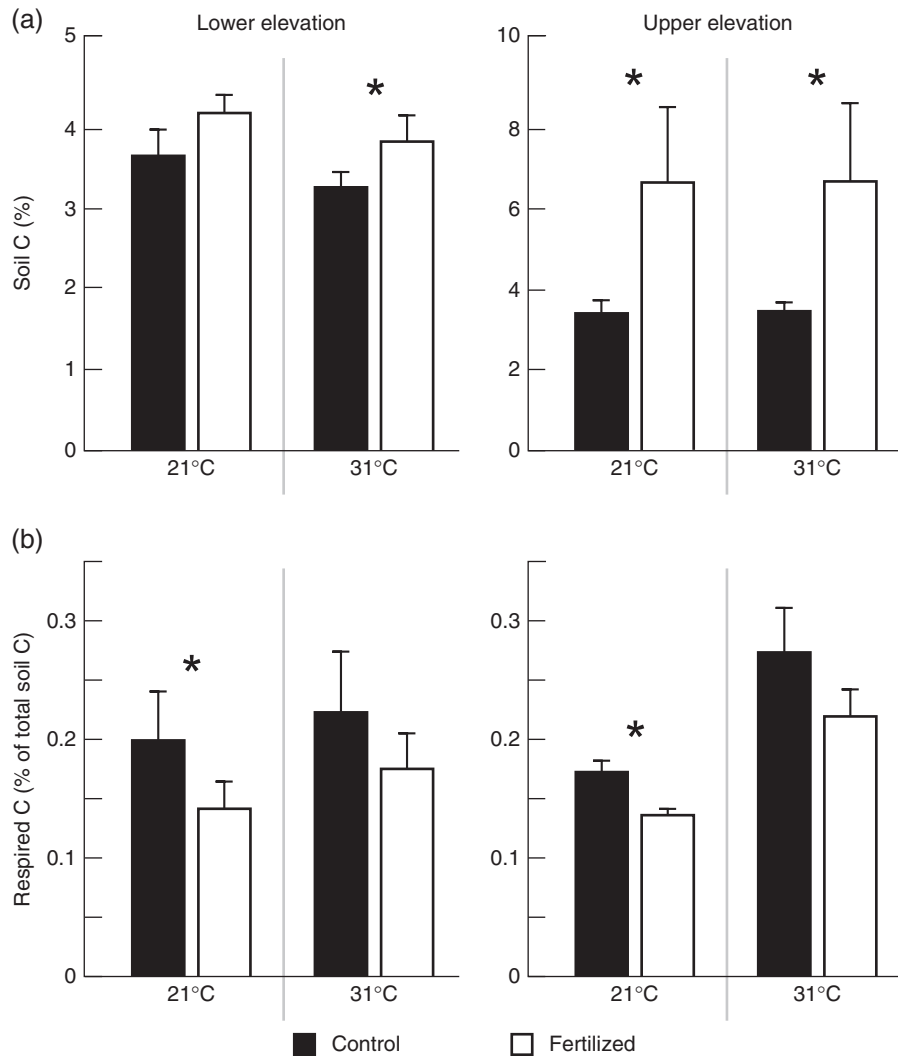


Fig. 2 (a) Soil carbon (C) concentrations, and (b) respired C as a % of total soil C, are shown for soils from control plots (dark bars), and Nitrogen (N)-fertilized plots (open bars), at two temperatures. Averages $\pm$ 1 SE are shown ($n=3$). N-fertilized soils generally had significantly higher soil C concentrations and lower respiration rates of C. Temperature significantly increased respired C for the upper elevation forest soils (not indicated on figure, $P < 0.05$). For significant differences between control and fertilized plots, * $P < 0.1$, ** $P < 0.05$.

$P < 0.05$). There were strong positive correlations of enzyme activities with soil C:N ($R^2 = 0.58$ for hydrolytic enzymes, $R^2 = 0.46$ for oxidative enzymes, $n = 72$ each, $P < 0.05$), while enzyme activities were not significantly correlated with root biomass. Total enzyme activities were significantly but weakly positively correlated with the cumulative C lost via respiration from each core ($R^2 = 0.18$ for hydrolytic enzymes, $R^2 = 0.25$ for oxidative enzymes, $n = 72$ each, $P < 0.05$).

Radiocarbon ($\Delta^{14}\text{C}$) and $\delta^{13}\text{C}$ in respired CO_2

In the lower elevation forest, N fertilization and time point (90 vs. 230 days) significantly affected the radio-

carbon content of respired CO_2 (Table 2). Temperature was not a significant factor in the analysis. Carbon respired from fertilized soil cores had significantly higher $\Delta^{14}\text{C}$ than C respired from control cores (Fig. 5, Table 2). If the radiocarbon signal primarily reflects relatively recent C (i.e., post the bomb spike in the 1950s), then higher $\Delta^{14}\text{C}$ correspond to relatively older C. Across treatments, average $\Delta^{14}\text{C}$ of respired C in the lower elevation forest increased with time from an average of $96 \pm 5\%$ at 90 days to $107 \pm 5\%$ at 230 days ($n = 12$, $P = 0.06$). The $\delta^{13}\text{C}$ of CO_2 was significantly higher for fertilized soils versus control soils in the lower elevation forest (Table 2). Temperature and time point were not significant predictors of $\delta^{13}\text{C}$.

Table 2 Soil Carbon Pools and Fluxes

Forest	Temp. (°C)	Trt.	Active Pool	Active Pool	Slow/ Passive Pool	$\Delta^{14}\text{C}$ (‰)	$\Delta^{14}\text{C}$ (‰)	$\delta^{13}\text{C}$ (‰)	$\delta^{13}\text{C}$ (‰)
			% of total SOM ^{†,‡}	C Turnover (years) ^{¶, ,**}	C Turnover (years) ^{†,‡}	3 months ^{§,¶}	8 months ^{§*}	3 months [§]	8 months ^{§*}
Lower	21	C	0.12 ± 0.02	11.8 ± 0.2	136 ± 34	89.7 ± 9.7	96.8 ± 8.1	-27.3 ± 0.5	-27.7 ± 0.6
Lower	21	N	0.09 ± 0.01	11.6 ± 0.5	195 ± 44	98.9 ± 10.0	103.1 ± 11.9	-27.0 ± 0.3	-26.6 ± 0.5
Lower	31	C	0.18 ± .03	12.9 ± 0.6	110 ± 34	87.4 ± 7.7	109.6 ± 4.0	-28.0 ± 0.4	-26.7 ± 0.3
Lower	31	N	0.13 ± 0.02	12.1 ± 0.2	134 ± 32	109.7 ± 10.7	117.2 ± 12.1	-27.0 ± 0.1	-26.3 ± 0.6
Upper	21	C	0.12 ± 0.02	10.5 ± 0.5	142 ± 32	102.5 ± 7.8	105.0 ± 8.6	-27.4 ± 0.3	-25.9 ± 1.0
Upper	21	N	0.08 ± 0.01	12.2 ± 0.6	179 ± 34	99.4 ± 9.3	98.6 ± 9.3	-27.6 ± 0.2	-27.1 ± 0.7
Upper	31	C	0.22 ± 0.01	12.6 ± 0.3	76 ± 4	113.0 ± 12.0	112.9 ± 14.1	-26.7 ± 0.6	-26.1 ± 0.9
Upper	31	N	0.17 ± 0.03	13.7 ± 0.5	124 ± 22	100.5 ± 2.1	110.3 ± 10.3	-24.7 ± 2.0	-24.4 ± 2.2

Soil C pools, turnover times and the isotopic content of CO₂ are shown for two tropical forest types (lower and upper elevation), with two temperature (Temp.) treatments, and a fertilization treatment (Trt.). Averages ± one standard error are shown for soils from Control (C) and N-fertilized (N) plots (*n* = 3).

*Denotes *P* < 0.1, otherwise *P* < 0.05.

†Significant effect of N for both forest types.

‡Significant effect of temperature for both forest types.

§Significant effect of N for lower elevation forest.

¶Significant effect of temperature for lower elevation forest.

||Significant effect of N for upper elevation forest.

**Significant effect of temperature for upper elevation forest.

In the upper elevation forest the isotopic content of respired CO₂ did not show clear trends with fertilization or temperature. None of the main factors in this analysis (fertilization, temperature, or time point) were significant predictors of the $\Delta^{14}\text{C}$ of respired CO₂ in the upper elevation forest.

There were several significant environmental predictors for time-averaged $\Delta^{14}\text{C}$ of CO₂ across forest types. Oxidative enzyme activity and root biomass were the strongest predictors of $\Delta^{14}\text{C}$ (Fig. 6). Oxidative enzyme activity had a significant interaction with fertilization treatment, and there was a significant positive correlation between oxidative enzyme activities and $\Delta^{14}\text{C}$ of CO₂ in control soils only. Root biomass was significantly negatively correlated with the $\Delta^{14}\text{C}$ of CO₂ across treatments and forest types. Thus, higher oxidative enzyme activity corresponded to higher $\Delta^{14}\text{C}$, while increased root biomass corresponded to lower $\Delta^{14}\text{C}$ values.

There were some interesting differences in the $\delta^{13}\text{C}$ of CO₂ between the two forest types. First, the $\delta^{13}\text{C}$ of CO₂ was less negative in the upper elevation forest, averaging $-26.0 \pm 0.6\text{‰}$ vs. $-27.1 \pm 0.2\text{‰}$ in the lower elevation forest (*n* = 6, *P* = 0.1). In the upper elevation, five cores consistently gave $\delta^{13}\text{C}$ values of $-21 \pm 0.6\text{‰}$ at both time points. This difference may reflect the influence of the CAM tree in the upper elevation. For time-averaged $\delta^{13}\text{C}$ of CO₂, oxidative enzyme activity was significantly positively correlated in the upper

elevation forest ($R^2 = 0.45$, *n* = 17, *P* < 0.05), while no environmental factors were significantly correlated with $\delta^{13}\text{C}$ in the lower elevation.

Discussion

N fertilization effects on soil carbon

In support of our first hypothesis, we found that N fertilization slowed soil C losses via heterotrophic respiration. This may explain our observation that fertilized soils had higher soil C concentrations than control soils. Carbon concentrations were ~30% higher in fertilized vs. control soils for the lower elevation forest, and ~100% higher in fertilized vs. control soils in the upper elevation forest. Declines in heterotrophic respiration have also been observed with N fertilization in some temperate ecosystems (Burton *et al.*, 2004; Swanston *et al.*, 2004; Olsson *et al.*, 2005), and a temperate grassland study found slower turnover rates of organic matter with high N inputs (Loiseau & Soussana, 1999). In a Hawaiian study, N fertilization also slowed turnover times of slowly cycling C pools, but only for a young soil where NPP was N-limited (Torn *et al.*, 2005). Our results show similar trends for two tropical forests on highly weathered soils, with N fertilization slowing the turnover of the slowly cycling C pool. Since these two forests did not have increased C inputs to soils from ANPP with N fertilization (Cusack *et al.*, 2009), it seems

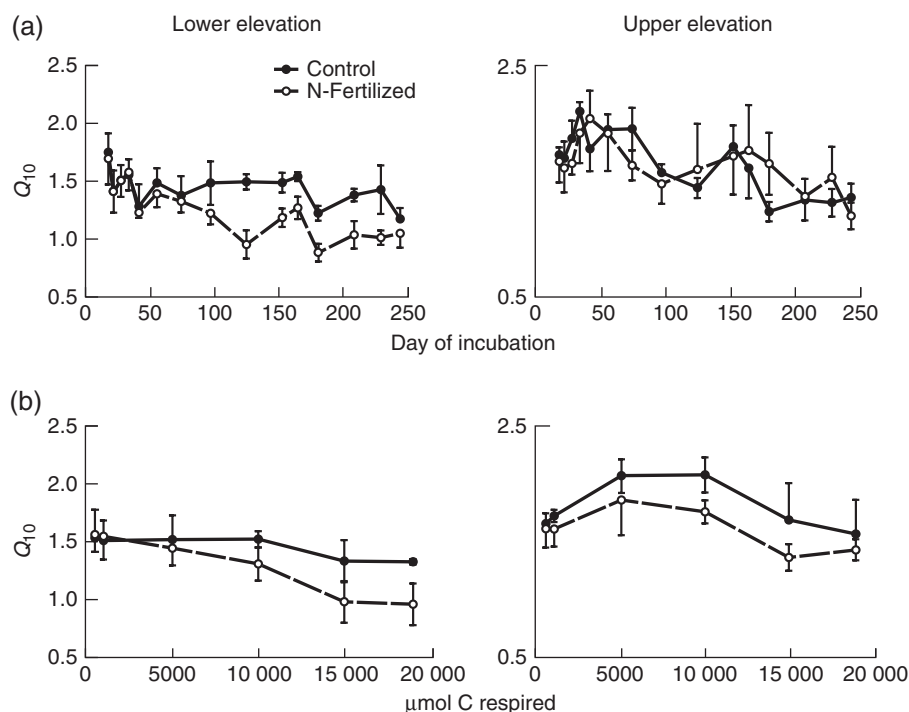


Fig. 3 Q_{10} values are shown for fertilized versus control soils (mean ± 1 SE, $n=3$ for each point). Q_{10} values are compared by (a) time point, and (b) amount of carbon (C) evolved in respiration. Dark symbols are for control soils, and open symbols are Nitrogen-fertilized soils. Fertilized-soil Q_{10} values were higher than control soils after the first 75 days at the lower elevation ($P<0.05$), and after the first 1000 μmol of C respired for both forests ($P=0.1$).

that slower C loss via suppressed heterotrophic respiration is the primary reason for the increased bulk soil C stocks.

Changes in microbial processing of soil carbon

Tissue chemistry appears to play a central role in how heterotrophic decomposition responds to N-addition across ecosystems (Knorr *et al.*, 2005), with implications for changes in soil C storage. Specifically, forests and grasslands with higher lignin content in plant tissues have been shown to increase soil carbon stocks with N fertilization, whereas low-lignin sites have lower or no response (Dijkstra *et al.*, 2004; Waldrop *et al.*, 2004). In contrast to our results, an increase in oxidative enzyme activity with added N was observed in an agricultural tropical soil (Waldrop & Firestone, 2004). In our study, suppressed oxidative enzyme activities with N fertilization corresponded to lower cumulative losses of C via respiration. The litter quality of the tropical forests in this study is poor, with high C:N and high lignin content, especially in the upper elevation forest where the greatest increases in soil C were observed (McGroddy & Silver, 2000; Cusack *et al.*, 2009b). Although plant lignin as a compound does not appear to be retained over the long term in SOM (Gleixner & Poirier, 2001;

Gleixner *et al.*, 2002), there is some evidence that N additions can promote the movement of lignin (or lignin biomarkers) into mineral-associated soil fractions (Neff *et al.*, 2002), and promote the transformation of plant residues into chemically recalcitrant soil C fractions via condensation reactions (Sollins *et al.*, 1996; Moran *et al.*, 2005). Our results indicate that N deposition can lead to the reduced activity of oxidative enzymes in some tropical forests, creating the potential for increased soil C storage.

While oxidative enzyme activity declined, total hydrolytic enzyme activity increased with added N. Similar increases in hydrolytic activity have been observed in temperate sites (Carreiro *et al.*, 2000; Saiya-Cork *et al.*, 2002), where soil N availability is commonly low and limiting to NPP (Chapin, 1980; Aber, 1992). Hydrolytic enzymes typically degrade more chemically simple forms of C (Caldwell, 2005), so the increase in their activity may be related to the long-term declines in active C pool sizes observed here. In this incubation, the active C pool accounted for a very small fraction of total soil C (0.1–0.2%), less than in Hawaiian soils (0.7–5%) (Townsend *et al.*, 1997). Possibly because of their small contribution to total soil C, the decline in active C pools was not a significant contributor to changes in bulk C with fertilization. Thus, the sizes of the active C

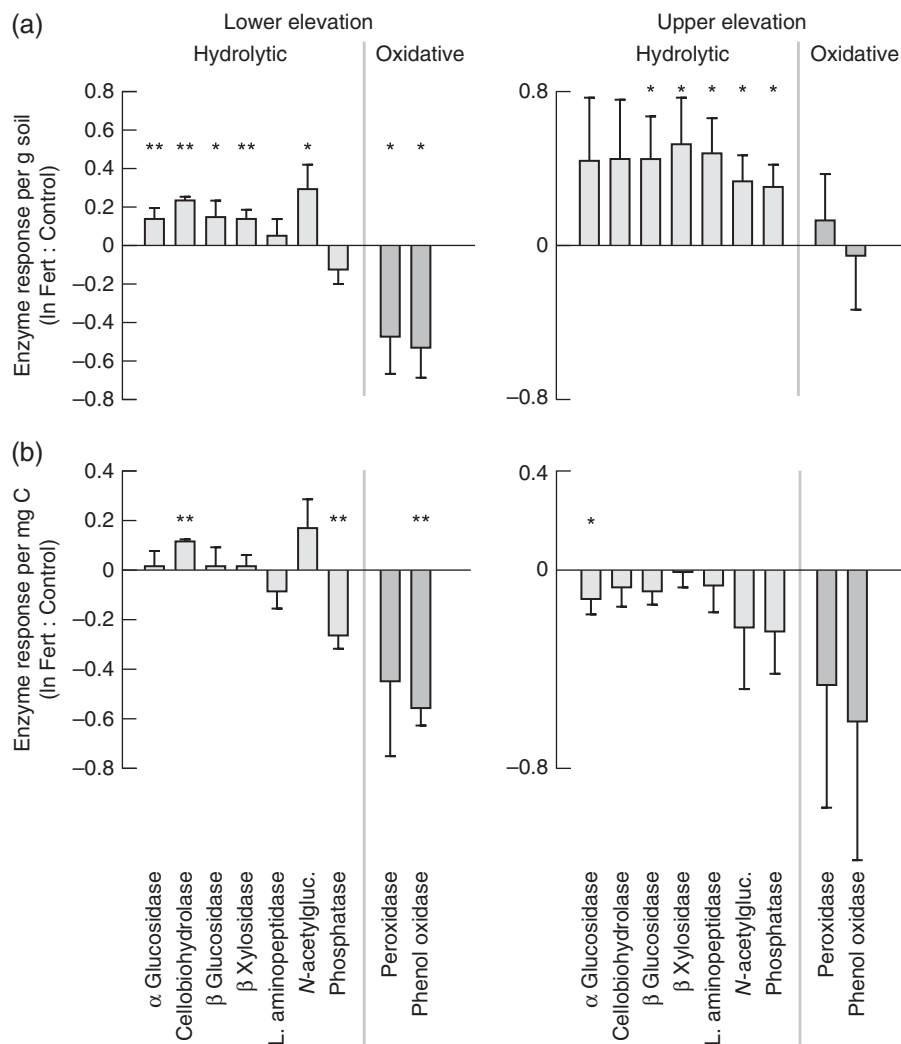


Fig. 4 The fertilization effect size on microbial enzyme activities is shown as the natural log of the ratio of fertilized: control soils, comparing paired plots. (a) The top panel shows the fertilization effect on total enzyme activity (per g dry soil). (b) The bottom panel shows the fertilization effect on specific enzyme activity (per mg soil C). There was no effect of temperature on the effect size, so average ratios from the two temperature treatments for each plot were used (mean $\pm$ 1 SE, $n=3$ for each forest type). (a) Fertilization significantly increased total hydrolytic enzyme activity per gram of soil, and (b) decreased specific oxidative enzyme activity in both forests. For significant difference from zero, * $P < 0.1$, ** $P < 0.05$.

pool and the slowly cycling C pool responded to N fertilization in opposite directions at these sites, but because the slowly cycling C pool was so much larger, the change in this pool dominated the net change in bulk soil C stocks.

The increased DOC production in the upper elevation forest with N fertilization, which may have contributed to the large increases in soil C concentrations in that forest, could also be related to changes in heterotrophic decomposition. The production of DOC at the soil surface and its subsequent downward movement and adsorption to mineral soil is an important mechanism of C delivery and storage for both temperate and

tropical soils (McDowell & Likens, 1988; McDowell, 1998). Altered production of DOC is commonly linked to changes in heterotrophic activity, and/or leaching of humus and litter (Kalbitz *et al.*, 2000). Increased DOC with N additions has been observed in some temperate sites (Guggenberger, 1994; Pregitzer *et al.*, 2004), and may be inversely related to changes in oxidative enzyme activities with N additions (Waldrop & Zak, 2006). That is, as oxidative enzyme activity decreases, a larger proportion of complex organic compounds may be left behind in the ecosystem as DOC, rather than being lost as respiration. We observed this relationship only for the upper elevation forest, where oxidative

Table 3 Microbial Enzyme Activities for Nitrogen-Fertilized and Warmed Soils

Forest	Temp. (°C)	Trt.	α D Glucosid. ($\mu\text{mol g}^{-1} \text{h}^{-1}$) [†] ₁	β D Cellobio. ($\mu\text{mol g}^{-1} \text{h}^{-1}$) [†] ₁	β D Glucosid. ($\mu\text{mol g}^{-1} \text{h}^{-1}$) [†] ₁	β D Xylos. ($\mu\text{mol g}^{-1} \text{h}^{-1}$) [†] ₁	Leucine ($\mu\text{mol g}^{-1} \text{h}^{-1}$) [†] ₁	NAG ($\mu\text{mol g}^{-1} \text{h}^{-1}$) [†] ₁	Phosphatase ($\mu\text{mol g}^{-1} \text{h}^{-1}$) [†] ₁	Peroxidase ($\text{nmol g}^{-1} \text{h}^{-1}$) [†] ₁	Phenol Oxidase ($\text{nmol g}^{-1} \text{h}^{-1}$) [†] ₁
Lower	21	C	68 ± 9	253 ± 82	825 ± 151	697 ± 185	106 ± 9	444 ± 114	10591 ± 1926	5685 ± 3114	346 ± 439
Lower	21	N	76 ± 13	329 ± 106	976 ± 212	835 ± 209	109 ± 3	443 ± 114	9061 ± 712	2975 ± 959	222 ± 151
Lower	31	C	41 ± 5	189 ± 42	691 ± 120	601 ± 96	52 ± 5	227 ± 47	7876 ± 919	7695 ± 5806	1397 ± 323
Lower	31	N	51 ± 9	231 ± 45	798 ± 147	659 ± 85	57 ± 7	405 ± 48	7046 ± 1141	5975 ± 1982	903 ± 538
Upper	21	C	43 ± 1	144 ± 5	518 ± 18	465 ± 36	68 ± 12	267 ± 45	19118 ± 1455	43922 ± 913	4464 ± 741
Upper	21	N	85 ± 29	242 ± 83	817 ± 213	725 ± 203	116 ± 36	401 ± 35	27797 ± 4785	44774 ± 2016	3577 ± 772
Upper	31	C	50 ± 7	84 ± 18	346 ± 79	338 ± 56	44 ± 9	752 ± 114	15771 ± 1914	40144 ± 15496	4250 ± 2299
Upper	31	N	73 ± 17	154 ± 47	634 ± 196	726 ± 196	80 ± 25	930 ± 39	20877 ± 3381	43603 ± 4355	3778 ± 876

Microbial enzyme activities are shown for two tropical forest types (lower and upper elevation), with two temperature (Temp.) treatments, and a fertilization treatment (Trt.). Averages ± one standard error are shown for soils from Control (C) and N-fertilized (N) plots ($n = 3$). The enzymes presented are α D-glucosidase, β D-glucosidase, β D-cellobiohydrolase, β D-xylosidase, leucine aminopeptidase, leucine aminopeptidase (NAG), phosphatase, peroxidase, and phenol oxidase.

*Denotes $P < 0.1$, otherwise $P < 0.05$.

[†]Significant effect of N for both forest types.

[‡]Significant effect of temperature for both forest types.

[§]Significant effect of N for lower elevation forest.

[¶]Significant effect of temperature for lower elevation forest.

^{||}Significant effect of N for upper elevation forest.

activity declined with fertilization and DOC increased. Soil pH also declined in the upper elevation forest, and changes in soil pH with N deposition may have important indirect effects on DOC production and subsequent adsorption to mineral surfaces via changes in soil charge balance and organic matter solubility (Evans *et al.*, 2008). Thus, an increase in DOC production with N fertilization may depend on both reduced oxidative enzyme activities and decreased pH.

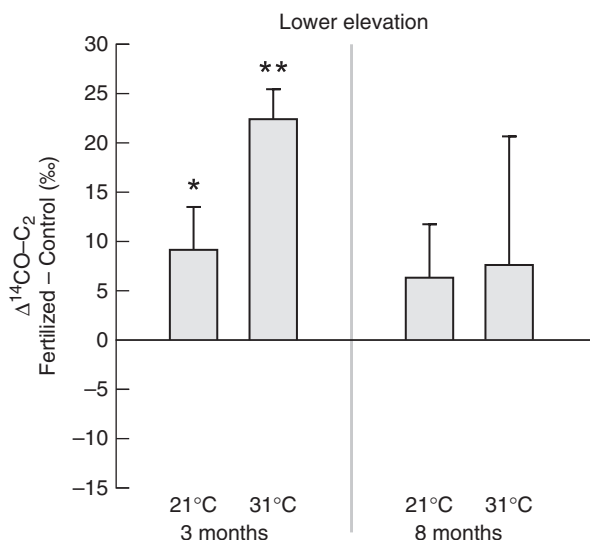


Fig. 5 The effect of fertilization on the radiocarbon content of CO_2 respired at two temperatures is shown for the lower elevation forest at 3 and 8 months of incubation. The effect of fertilization on the $\Delta^{14}\text{C}$ (‰) of CO_2 is shown as the difference between fertilized and control soils for each set of paired plots ($n=3$). For significant difference from zero, $*P<0.1$, $**P<0.05$.

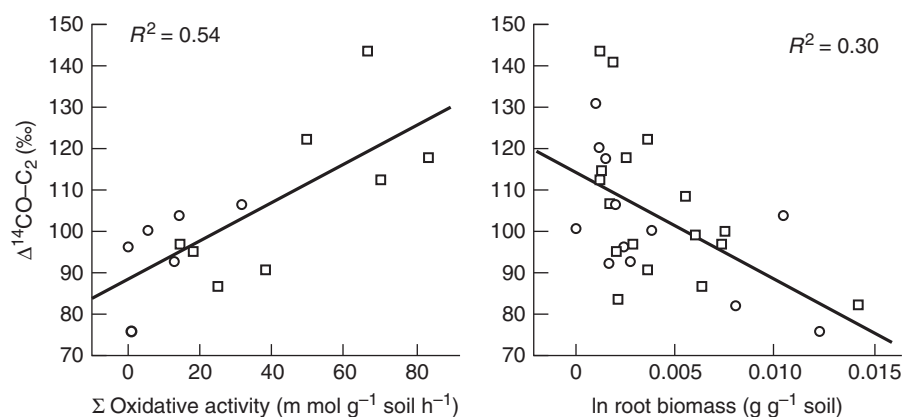


Fig. 6 The best environmental predictors of average $\Delta^{14}\text{C}$ of CO_2 across forest types are shown. Oxidative enzymes are the sum of phenol oxidase and peroxidase, and were significant predictors in control soils only. Root biomass data were transformed using the natural log(biomass + 1). Circles = lower elevation soils, squares = upper elevation soils. The equations for the regressions are: $\Delta^{14}\text{C}$ of $\text{CO}_2 = 94.2 + 0.3$ (oxidative enzyme activity) ($R^2 = 0.54$, $n = 14$, $P < 0.05$). $\Delta^{14}\text{C}$ of $\text{CO}_2 = 114.3 - 2566 [\ln (\text{root biomass} + 1)]$ ($R^2 = 0.30$, $n = 29$, $P < 0.05$).

Temperature sensitivity of soil respiration

N fertilization corresponded to increased temperature sensitivity of soil respiration in both forest types, with the greatest effect in the upper elevation forest. The higher Q_{10} values in fertilized vs. control soils provide support for our second hypothesis, that N additions increase temperature sensitivity of some soil C pools. We further hypothesize that the observed increases in temperature sensitivity for fertilized soils later in the soil incubation are related to a shift in the chemical composition of C in the slowly cycling pool. The observed suppression of oxidative enzyme activity suggests that chemically complex C compounds were retained in larger quantities in fertilized vs. control soils. Lignin has been shown to be more sensitive to warming than many other plant-derived compounds in soils (Feng *et al.*, 2008), likely because of its chemical complexity and the higher activation energy required to decompose it (Davidson & Janssens, 2006).

While the slowly cycling C pool showed increased temperature sensitivity with N fertilization, initial Q_{10} values for the active C pool were even higher than Q_{10} values later in the incubation. There is an apparent contradiction in this, because we attribute greater temperature sensitivity in fertilized soils to higher content of complex C compounds, yet the highest overall Q_{10} values were observed in the beginning of the experiment, when labile, simpler C compounds were most available. This apparent disparity can be explained by a shift in the factor driving Q_{10} values, from a limitation by enzymatic potential in the beginning, to limitation by substrate availability later in the incubation (Nicolardot

et al., 1994; Kirschbaum, 2006; Gershenson *et al.*, 2009). Initially, substrate availability was not limiting to respiration and maximum enzyme potential was the limiting factor, such that the Q_{10} depended primarily on the enzymatic temperature response. Later in the incubation, the availability of C substrate became more limiting, and likely influenced the capacity of soil respiration to respond to warming. As the relatively labile C in the active pool disappeared, we suggest that differences in substrate chemistry of the slowly cycling pool became apparent.

Radiocarbon age of soil carbon losses

N fertilization led to smaller active C pools, and proportionally higher losses of relatively older soil C in the lower elevation forest, supporting our third hypothesis. The $\Delta^{14}\text{C}$ of heterotrophic respiration is an integrated measure of substrate age in the ecosystem. Because the spike in atmospheric $^{14}\text{CO}_2$ from nuclear bomb testing has declined steadily since 1963, the radiocarbon content of plant tissue, decadal-centennial cycling SOM, and microbial respiration, provides an indication of when C in those categories was photosynthetically fixed into an ecosystem. Assuming that the majority of C being respired by heterotrophic microbes in our incubation was fixed since the bomb spike (i.e., it is 'modern' C), higher radiocarbon numbers indicate a shift toward increases in the proportional loss of older soil C (Trumbore, 2000). Thus, the increase in the $\Delta^{14}\text{C}$ of CO_2 with warming in the lower elevation forest indicates relatively greater losses of older soil C from warmer vs. ambient soils. This could be related to a shift in microbial community composition with warming (Andrews *et al.*, 2000), or other factors that increase the availability of older soil C in warmer soils.

N fertilization also increased the $\Delta^{14}\text{C}$ of CO_2 in the lower elevation forest. This presents an apparent contradiction: the longer turnover time of the slowly cycling C pool with N fertilization discussed above suggests decreased losses of older soil C, but the higher $\Delta^{14}\text{C}$ of CO_2 values from fertilized soils suggests larger losses of older C to respiration. There are several potential explanations for this trend. Note that the $\Delta^{14}\text{C}$ unit is a concentration, and not an absolute flux. Therefore, these data likely indicate that as total CO_2 fluxes declined with fertilization, there was a lower flux from the (older) slow-cycling C pool (leading to longer turnover times), and there was an even lower relative flux of C from the active C pool (leading to a lower relative contribution of younger C to $\Delta^{14}\text{CO}_2$). Because fertilization corresponded to smaller active C pools, the youngest C in these soils was depleted faster than in control soils, so there was less young C contributing to

the radiocarbon signal. A second explanation for the higher $\Delta^{14}\text{C}$ of CO_2 from fertilized soils is desorption and subsequent decomposition of older C from mineral surfaces in response to soil chemical changes with N fertilization. The soils in the tropical forests in this study have variable charge clays, and even small changes in soil pH can alter their net charge, changing adsorption and desorption of charged organic matter (Chorover & Sposito, 1995). A change in surface charge could result in a shuffle of older C off of mineral surfaces (potentially to be replaced by newer C of a different charge), exposing older C that was previously physically protected to decomposition. Such a change in adsorption and desorption could also help explain the increases in soil C concentrations observed with N fertilization in these soils, because the new C was found primarily in the mineral-associated soil fraction, and significant increases in pH with fertilization were detected in field measurements (Cusack *et al.*, 2009). A third explanation from the literature, which we rejected for this dataset, is that a shift in substrate utilization by decomposers in N-fertilized plots led to losses of older soil C. A study in a tropical agricultural soil found increased respiration of older soil C with N fertilization, linked to an increase in oxidative enzyme activities (Waldrop & Firestone, 2004). In this study, we did observe a positive correlation between oxidative enzyme activity and the $\Delta^{14}\text{C}$ of CO_2 in control soils. However, this trend did not hold in fertilized soils, which had lower oxidative enzyme activity but higher $\Delta^{14}\text{C}$ of CO_2 , contradicting this explanation for our observed pattern. It seems most likely that a combination of (1) relative declines in the contribution of active-pool C to respiration, and/or (2) desorption from mineral surfaces and subsequent decomposition of older soil C, are responsible for the older $\Delta^{14}\text{C}$ signal of CO_2 from N-fertilized soils.

A general pattern of interest was the relationship between root biomass and the radiocarbon content of soil respiration. Root biomass was negatively correlated with $\Delta^{14}\text{C}$ of CO_2 across all treatments and forest types, indicating that roots provided a more recent source of C relative to other SOM in these soils. Roots are an important precursor to SOM (Bird & Torn, 2006; Bird *et al.*, 2008), and thus should be younger than bulk SOM. While some roots have long life spans, an Amazonian study found that the $\Delta^{14}\text{C}$ of new root biomass corresponded to the atmospheric signal in the year of growth (Trumbore *et al.*, 2006). Interestingly, root biomass was not a significant predictor of total CO_2 fluxes across treatments and sites, and was not correlated with extracellular enzyme activities, indicating that root necromass was not a large factor driving net heterotrophic activity in this experiment.

Conclusion

Our results provide strong evidence that N additions cause changes in heterotrophic respiration and processing of SOM in highly weathered tropical forest soils, and can lead to overall increases in soil C stocks. Suppressed heterotrophic respiration with N fertilization, and decreased activity of oxidative enzymes that degrade chemically complex macromolecules, appear to explain the increases in soil C observed here. Although bulk soil C concentrations increased, the active and slowly cycling soil C pools responded to N fertilization differently, with the response in the slowly cycling C pool dominating the net increase in soil C storage. The temperature sensitivity of the slowly cycling soil C pool in N-fertilized soils increased. This, together with relatively higher losses of older soil C (i.e. higher radiocarbon content) in respiration from N-fertilized soils, may indicate a shift in the chemical character of soil C pool after long-term N additions. The increased temperature sensitivity of soil C in N-fertilized soils means that the larger soil C stocks in these soils are likely to be more vulnerable to future warming. More generally, these results suggest that in tropical soils, the stability of soil C is uncertain across forest types in the context of interacting global change factors, including N deposition and warming.

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