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Experimental removal and addition of leaf litter inputs reduces nitrate production and loss in a lowland tropical forest

William R. Wieder · Cory C. Cleveland · Philip G. Taylor · Diana R. Nemergut · Eve-Lyn Hinckley · Laurent Philippot · David Bru · Samantha R. Weintraub · Mysti Martin · Alan R. Townsend

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Abstract Environmental perturbations such as changes in land use, climate, and atmospheric carbon dioxide concentrations may alter organic matter inputs to surface soils. While the carbon (C) cycle response to such perturbations has received considerable attention, potential responses of the soil nitrogen (N) cycle to changing organic matter inputs have been less well characterized. Changing litter inputs to surface to soils may alter the soil N cycle directly, by controlling N substrate availability, or indirectly, via interactions with soil C biogeochemistry. We investigated soil N-cycling responses to a leaf litter manipulation in a lowland tropical forest using isotopic and molecular techniques. Both removing

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W. R. Wieder · P. G. Taylor · D. R. Nemergut · E.-L. Hinckley · S. R. Weintraub · M. Martin · A. R. Townsend
Institute for Arctic and Alpine Research; Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, CO 80309, USA
e-mail: philip.taylor@colorado.edu

D. R. Nemergut
e-mail: diana.nemergut@colorado.edu

E.-L. Hinckley
e-mail: ehinckley@neoninc.org

S. R. Weintraub
e-mail: samantha.weintraub@colorado.edu

M. Martin
e-mail: mysti.martin@colorado.edu

A. R. Townsend
e-mail: alan.townsend@colorado.edu

Present Address:
W. R. Wieder (✉)
National Center for Atmospheric Research, TSS, CGD/NCAR, PO Box 3000, Boulder, CO 80307, USA
e-mail: wwieder@ucar.edu

C. C. Cleveland
Department of Ecosystems and Conservation Science, University of Montana, Missoula, MT 59812, USA
e-mail: cory.cleveland@umontana.edu

E.-L. Hinckley
National Ecological Observatory Network, Boulder, CO 80301, USA

L. Philippot · D. Bru
Department of Soil and Environmental Microbiology, INRA, UMR 1229, 21000 Dijon, France
e-mail: laurent.philippot@dijon.inra.fr

D. Bru
e-mail: david.bru@dijon.inra.fr

L. Philippot · D. Bru
Department of Soil and Environmental Microbiology, University of Burgundy, UMR 1229, 21000 Dijon, France

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and doubling leaf litter inputs decreased the size of the soil nitrate pool, gross nitrification rates, and the relative abundance of ammonia-oxidizing microorganisms. Gross nitrification rates were correlated with the relative abundance of ammonia-oxidizing archaea, and shifts in the N-cycling microbial community composition correlated with concurrent changes in edaphic properties, notably pH and C:N ratios. These results highlight the importance of understanding coupled biogeochemical cycles in global change scenarios and suggest that environmental perturbations that alter organic matter inputs in tropical forests could reduce inorganic N losses to surface waters and the atmosphere by limiting nitrate production.

Keywords Nitrogen cycle · Tropical forests · Nitrification · ^{15}N pool dilution · Microbial community composition

Introduction

Coupled biogeochemical cycles result from the metabolic demands and stoichiometric constraints of biota (Falkowski et al. 2008; Schlesinger et al. 2011), and understanding the interactions between biogeochemical cycles is critical for predicting ecosystem responses to environmental change (Hungate et al. 2003; Luo et al. 2004; Rastetter 2011). Work in multiple ecosystems shows that nutrient availability—especially nitrogen (N)—regulates the carbon (C) cycle. For example, N availability often limits rates of aboveground net primary productivity (NPP; Falkowski et al. 1998; Lebauer and Treseder 2008; Vitousek and Howarth 1991). Meanwhile, N additions can have positive, negative, or neutral effects on rates of organic matter degradation, litter decomposition, and soil respiration (Fog 1988; Janssens et al. 2010; Knorr et al. 2005). The effects of N on C cycling have been well documented both conceptually and empirically in previous work (Butterbach-Bahl et al. 2011; Liu and Greaver 2010; Nave et al. 2009). However, comparatively little attention has been given to understanding how changes in C availability may modulate N cycling in terrestrial ecosystems, especially in tropical regions (but see Mo et al. 2003, Sayer and Tanner 2010; Wieder et al. 2011).

Multiple lines of evidence suggest that human-driven environmental perturbations will alter the productivity of tropical ecosystems (Clark et al. 2003; Lloyd and Farquhar 2008)—changes which will likely alter C inputs to surface soils. For example, soil C availability may decrease following major disturbances (e.g., deforestation), increase with elevated atmospheric carbon dioxide (CO_2) concentrations, or shift in either direction in response to climate change. Unfortunately, while the effects of land use change are reasonably well studied (e.g. Davidson et al. 2007; Neill et al. 1997; Neill et al. 1999), manipulative experiments that can simulate other global change drivers such as shifts in climate or rising atmospheric CO_2 are exceptionally challenging and thus rare-to-absent in tropical forests (Clark et al. 2003; Clark 2004; Cleveland et al. 2011). In the absence of such experiments, litter manipulations may represent viable surrogate approaches for gauging possible tropical forest responses to environmental changes that alter plant productivity (Sayer et al. 2011; Sayer et al. 2007; Sayer and Tanner 2010; Vasconcelos et al. 2004; Wood et al. 2009).

To date, most such work has focused on understanding changes in C cycling following litter manipulations (Leff et al. 2012; Sayer et al. 2007; Sayer et al. 2011; but also see Sayer et al. 2012). However, changing soil C resources may concurrently alter the N status of an ecosystem by affecting how N is input, lost, and/or retained in soils. For example, increases in labile C availability may stimulate N inputs through N fixation (Vitousek and Hobbie 2000; Vitousek et al. 2002), increase N retention by increasing biological N demand (Luo et al. 2004), reduce nitrate (NO_3^-) accumulation (Taylor and Townsend 2010), and/or accelerate gaseous N losses through denitrification (Garcia-Montiel et al. 2003; Nobre et al. 2001; Wieder et al. 2011). Because microorganisms catalyze all of these processes, simultaneously assessing changes in process rates and soil microbial community structure (through a functional group lens) in response to changes in C inputs offers the opportunity to explore how the N cycle responds to altered C cycling, and if those responses are better understood with the inclusion of microbial community structure information.

In particular, the production and fate of NO_3^- is crucial for assessing an ecosystem's capacity for N retention versus loss from soils (Likens et al. 1969; Schlesinger 1997; Templer et al. 2008). To investigate how changing soil C resources may affect soil N cycling we used a leaf litter manipulation and paired

soil biogeochemical (using ^{15}N pool dilution techniques) with qPCR-based analyses of the microbial community; specifically focusing on the production and fate of NO_3^-. We hypothesized that eliminating leaf litter inputs would decrease substrate availability to the microbial community, slow rates of N transformations by lowering available organic N stocks for N mineralization, and coincide with a reduction in the relative abundance of N-cycling microbial guilds. Since N mineralization is the precursor for nitrification, lower rates of N mineralization would decrease ammonium (NH_4^+) availability and thereby reduce NO_3^- production and loss (e.g. Davidson et al. 2007). By contrast, since the majority of ecosystem N demand is met via internal recycling through litter decomposition, mineralization, and assimilation (Likens and Bormann 1995), we hypothesized that doubling leaf litter inputs would increase N availability and associated rates of soil N transformations. In particular, lowland tropical forests are widely assumed to cycle N in excess of biological demand (Vitousek 1984). Thus, we expected that increasing N availability by doubling litter inputs would also increase the abundance of ammonia-oxidizing archaea and bacteria (AOA and AOB, respectively), accelerate NO_3^- production, and drive higher rates of denitrification.

Methods

Site description and experimental manipulation

The study was conducted in a lowland tropical forest located on the Osa Peninsula, Costa Rica (8°43′N, 83°37′W). Mean annual soil temperature is 25 °C, rainfall averages ~5,000 mm year^{-1}, and soils at the site are Ultisols (Bern et al. 2005; Berrange and Thorpe 1988) with high clay content (>75 %; Cleveland et al. 2006). Further details on the site can be found in Table 1 (but also see Cleveland and Townsend 2006; Wieder et al. 2009).

We began manipulating litter inputs to surface soils in ten blocks of three 3 × 3 m plots in April 2007. Initially, standing litter stocks were removed from each block, homogenized, and weighed. We redistributed litter so that two-thirds of the standing litter mass was placed on randomly assigned double litter input plots (2×), one-third of the standing litter mass was placed on control plots (1×), and litter removal plots (0×) received no litter inputs ($n = 10$ per treatment). Subsequently, leaf litter was removed monthly from 0× plots, weighed, pooled, and evenly distributed on the 2× plots. We estimate that litter removal decreased fine litter inputs to 0× plots ~450 g C m^{-2} year^{-1} and 10.4 g N m^{-2} year^{-1}, which augmented leaf

Table 1 Mean mineral soil characteristics (±1 SE, 0–10 cm) measured during the second year of the litter manipulation (2008–2009)

	Units	Treatment		
		0×	1×	2×
Soil C[c]	kg C m^{-2}	2.6 (±0.1)[a]	3.0 (±0.2)[b]	3.5 (±0.3)[b]
Soil N[c]	kg N m^{-2}	0.22 (±0.02)[a]	0.26 (±0.01)[b]	0.27 (±0.02)[b]
Soil C: N[c]		11.5 (±0.12)[a]	11.8 (±0.30)[a]	14.1 (±0.43)[b]
Bulk density[d]	g cm^{-3}	0.65 (±0.01)[a]	0.58 (±0.02)[ab]	0.52 (±0.02)[b]
NH_4^+ [e]	μg N g^{-1}	5.35 (±0.58)[a]	6.32 (±0.62)[a]	11.4 (±0.90)[b]
NO_3^- [e]	μg N g^{-1}	1.86 (±0.25)[a]	5.46 (±1.06)[b]	1.56 (±0.27)[a]
Microbial C[c]	μg C g^{-1}	897 (±0.02)[a]	1,206 (±167)[b]	1,397 (±304)[b]
Soil pH[f]		5.00 (±0.06)[a]	5.44 (±0.04)[b]	5.15 (±0.10)[a]

Different superscripts indicate significantly different mean values between treatments for each variable ($P < 0.05$). These data are from previously published work and meant to summarize observed changes in soil resources driven by the litter manipulation

[c] Data from Leff et al. (2012); $n = 10$ treatment^{-1}

[d] Data from Leff et al. (2012); $n = 5$ treatment^{-1}

[e] Data from Wieder et al. (2011); $n = 10$ treatment^{-1}

[f] Data from plots measured in this study, collected Oct 2009

litter inputs to the 2× plots. After 2 years, this litter manipulation significantly altered the soil chemical environment, notably changing mineral soil C and N pools, microbial biomass C and N pools and inorganic N availability (Table 1; previously described and reported in Leff et al. 2012; Wieder et al. 2011). Leaf litter decomposition at the site is rapid (Cleveland et al. 2006; Wieder et al. 2009), which typically prevents litter accumulation and organic layer formation. However, 2× plots did begin to develop a distinct organic horizon; thus we constrain our analysis to soil chemical and physical characteristic in the mineral soil (0–10 cm). Still, we observed significant changes in soil bulk density (Table 1) and fine root biomass (Leff et al. 2012) among litter treatments, which indicates concurrent (although unexplored) changes in soil macrofauna activity and plant uptake with this small-scale litter manipulation.

^{15}N pool dilutions

We conducted a ^{15}N pool-dilution experiment to examine patterns of N-cycling in situ in October 2009, following 2.5 years of litter manipulation. We randomly selected 8 replicate control plots and 5 each of the 0× and 2× plots to receive ^{15}N tracer additions. A tracer experiment was also conducted in 8 control plots in May 2009, but to simplify data presentation, data from these plots are only used in regression and correlation analyses (see below). In each plot we installed seven uncapped, 5 cm (diameter) × 12 cm (length) PVC tubes into the top 10 cm of mineral soil. In each plot three cores were injected with $(^{15}NH_4)_2SO_4$ solution and three cores were injected with a $K^{15}NO_3$ solution. The remaining core was used for natural abundance ^{15}N measurements and soil pH (measured in a 1:2 soil slurry with deionized water). In labeled cores we intended to enrich background inorganic N pools to 15 at.%; thus, $^{15}NH_4^+$ and $^{15}NO_3^-$ labeled cores received six 1-mL injections (Davidson et al. 1991) of 44.1 or 29.2 μg ^{15}N mL^{-1} at 98 at.% enrichment, respectively. In some systems this technique can increase soil moisture and change process rates, however, we observed no change in soil moisture resulting from ^{15}N label addition. Isotopically-labeled cores were incubated in situ and harvested at ~ 0.5 h, 4 h, and 24 h (T_0, T_4, and T_{24}, respectively). Prior to harvesting T_4 and T_{24} soils, we measured $^{15}N_2O$ efflux out of $^{15}NH_4^+$ and $^{15}NO_3^-$ labeled cores (Silver et al. 2005). Unvented, static chambers (13.25 cm tall, 300 ml total volume) were placed over soil cores, and 20 ml of headspace gas was sampled every 10 min for 30 min. Gas samples were stored in pre-evacuated 12 ml Exetainer vials (Labco, Buckinghamshire, UK).

Immediately after sampling, all soil cores were extruded into plastic bags and transported to a nearby field station. Soils were homogenized and two subsamples were placed into sterile whirl–pak bags and frozen (−20 °C for 5 days, transported on ice to the University of Colorado and stored at −80 °C) for subsequent qPCR analysis and determination of gravimetric soil moisture (oven dried at 105 °C for 5 days). Additionally, three 30 g (dry weight equivalent) subsamples from each core were added to preweighed 120 ml specimen cups containing 100 ml of either a 2 M KCl, for inorganic N (one per core); or 0.5 M K_2SO_4 solution, for microbial biomass N (two per core). We added 1 ml of ethanol-free chloroform directly to one of the K_2SO_4 aliquots (Fierer and Schimel 2003) to serve as the fumigated sample for microbial biomass N determination. Soil solutions were capped and vigorously shaken by hand for one minute, and thereafter every 15-minutes for 4 h. Soil solutions settled for an additional hour, were syringed filtered, and frozen.

Soil inorganic N concentrations were determined colorimetrically (OI Analytical, College Station, USA; BioTek U.S. Winooski, USA). To test for possible iron interference with our NO_3^- determination (Colman et al. 2007), we separately analyzed a subsample of KCl extracts (Yang et al. 2012) and found no evidence for iron interference of NO_3^- (S. Weintraub, unpublished data). Microbial biomass N samples were oxidized with a persulfate digestion (Cabrera and Beare 1993), and microbial biomass N was calculated as the difference of N between fumigated and un-fumigated samples, with no correction for extraction efficiency (Silver et al. 2005).

Soil extracts were prepared for ^{15}N isotope analysis using diffusion techniques, including blank correction of ^{15}N enrichment (Stark and Hart 1996). N isotope ratios from diffusions were measured at the Stable Isotope Facility at the University of California Davis using an elemental analyzer interfaced to a PDZ Europa 20-20 IRMS (Sercon Ltd., Cheshire, UK). Facilities at UC Davis also determined N_2O concentration and $^{15}N_2O$ isotope ratios from labeled gas

samples using a trace gas concentration system interfaced with a ThermoScientific Delta V Plus IRMS (Bremen, Germany). For T_4 and T_{24} we calculated rates of gross mineralization, gross nitrification, microbial N immobilization, dissimilatory nitrate reduction to NH_4^+ (DNRA), and $^{15}N_2O$ efflux (Davidson et al. 1991; Kirkham and Bartholomew 1954; Silver et al. 2005; Silver et al. 2001).

Quantitative PCR (qPCR) of genes involved in the N cycle

Soil DNA was extracted with a DNA extraction kit (Powersoil, Mo Bio Laboratories Inc., Carlsbad, USA), but we could not recover DNA from one 0× soil sample. The total bacterial and crenarchaeal communities were quantified using 16S rRNA primer-based qPCR assays (Lopez-Gutierrez et al. 2004; Ochsenreiter et al. 2003). The relative abundances of the N-cycling microbial guilds were estimated by qPCR of the genes encoding catalytic enzymes of N-fixation (*nifH*), ammonia oxidation (bacterial and crenarchaeal *amoA*), nitrate reduction (*narG* and *napA*) and denitrification (*nirK*, *nirS*, and *nosZ*) as molecular markers (primers and PCR conditions are described in Bru et al. 2011; Leininger et al. 2006; Poly et al. 2001; Tourna et al. 2008). Reactions were carried out in an ABI prism 7900 Sequence Detection System (Applied Biosystems, Foster City, USA), with quantification based on the increasing fluorescence intensity of the SYBR Green dye during amplification. The qPCR assay was carried out in a 15 µl reaction volume containing the Absolute QPCR SYBR Green ROX Mix (ABgene, Cedex, France), 1 or 2 µM of each primer, 250 ng of T4 gene 32 (QBiogene, Cedex, France) and 2 ng of DNA. Two independent qPCR assays were performed for each gene. Standard curves were obtained using serial dilutions of linearized plasmids containing the targeted genes. Two to three no-template DNA controls were run for each qPCR assay. Tests for the potential presence of PCR inhibitors in DNA extracted from soil were performed by spiking soil DNA extracts with a known amount of plasmid DNA prior to qPCR. In all cases, no inhibition was detected. PCR efficiency for the different assays ranged between 77 and 96 %. To estimate the relative abundance of different functional communities we calculated ratios between functional gene copy number and the total bacterial 16S rRNA gene copy number, and between AOA and crenarchaeal 16S rRNA gene copy number.

Statistical analyses

We determined treatment effects on soil N pools, fluxes, and the relative abundance of N-cycling microbial guilds using a linear mixed effects (lme) model with plot as the random effect and allowing the intercept to vary by time using the R statistical platform and the nlme package (Pinheiro et al. 2009; R Development Core Team 2011). Using litter treatment and/or sampling time, plus their interaction, as fixed effects we selected the best-fit lme model using Akaikes Information Criterion (AIC, Bolker 2008). For all lme analyses data were ln transformed if necessary, quantile–quantile plots were used to assess the normality of residuals, and data were tested for homogeneity of variance with fitted versus residual plots. Means and standard errors (measured at T_4, unless otherwise noted) were reported as measures of central tendency and dispersion, respectively, of model outputs.

To explore patterns between N-cycling microbial guilds, environmental variables, and gross rates of N-cycling, we used Spearman's rank correlation coefficients with data from individual plots across all treatments (including samples collected in June 2009). Our aim with the correlation tests was to present relationships of gene relative abundance with environmental variables and rates of process in the most straightforward way possible. Results from correlation tests were not corrected for multiple comparisons. We subsequently focus our analysis and discussion on the strongest of these relationships, especially related to rates of nitrification and the relative abundance of ammonia-oxidizing microbes, using linear regressions. We tested if the litter manipulation resulted in shifts in microbial community structure using permutational multivariate analyses of variance using a Bray–Curtis distance matrix generated from ln transformed relative gene abundance data with the adonis function. To visualize differences in microbial community structure between experimental treatments we used Nonmetric Multidimensional Scaling (NMDS) using the metaMDS function with a Bray–Curtis distance matrix of relative gene abundance data. Adonis function and NMDS calculations were performed with the R statistical platform using the vegan,

and MASS packages (Oksanen et al. 2010; R Development Core Team 2011; Venables and Ripley 2002).

Results

Soil NH_4^+ pools in field fresh soils were largest in the 2× plots, but were statistically indistinguishable from 1× plots. Soil NH_4^+ pools in the 0× plots were significantly lower than 1× and 2× plots (Fig. 1). Soil NO_3^- pools were largest in control plots, but not significantly different across litter treatments. Soil NO_3^-: NH_4^+ ratios were consistently <1 in all treatments, and although highest in 1× plots (0.21 ± 0.04) compared to 0× or 2× plots (0.15 ± 0.04 and 0.11 ± 0.01, respectively) this difference was not statistically significant. Inorganic N extracted in the field (Fig. 1) was lower than in previous assays (Table 1), likely reflecting differences in sample handling (Turner and Romero 2009). Gravimetric soil moisture at the time of sampling in 1× and 2× plots (0.44 ± 0.01 g H_2O g $soil^{-1}$) was significantly higher than soil moisture in 0× plots (0.40 ± 0.02, $P = 0.03$). Finally, soil pH was significantly higher in 1× plots than either 0× or 2× plots (Table 1).

We observed large, statistically significant differences in rates of N-cycling among leaf litter treatments. Rates of gross soil N mineralization were significantly higher in the 1× and 2× plots than in 0× plots (Fig. 1), and positively correlated with soil moisture ($r = 0.73$, $P = 0.02$, $n = 18$) and total soil N ($r = 0.5$, $P = 0.04$, $n = 18$). Rates of gross soil

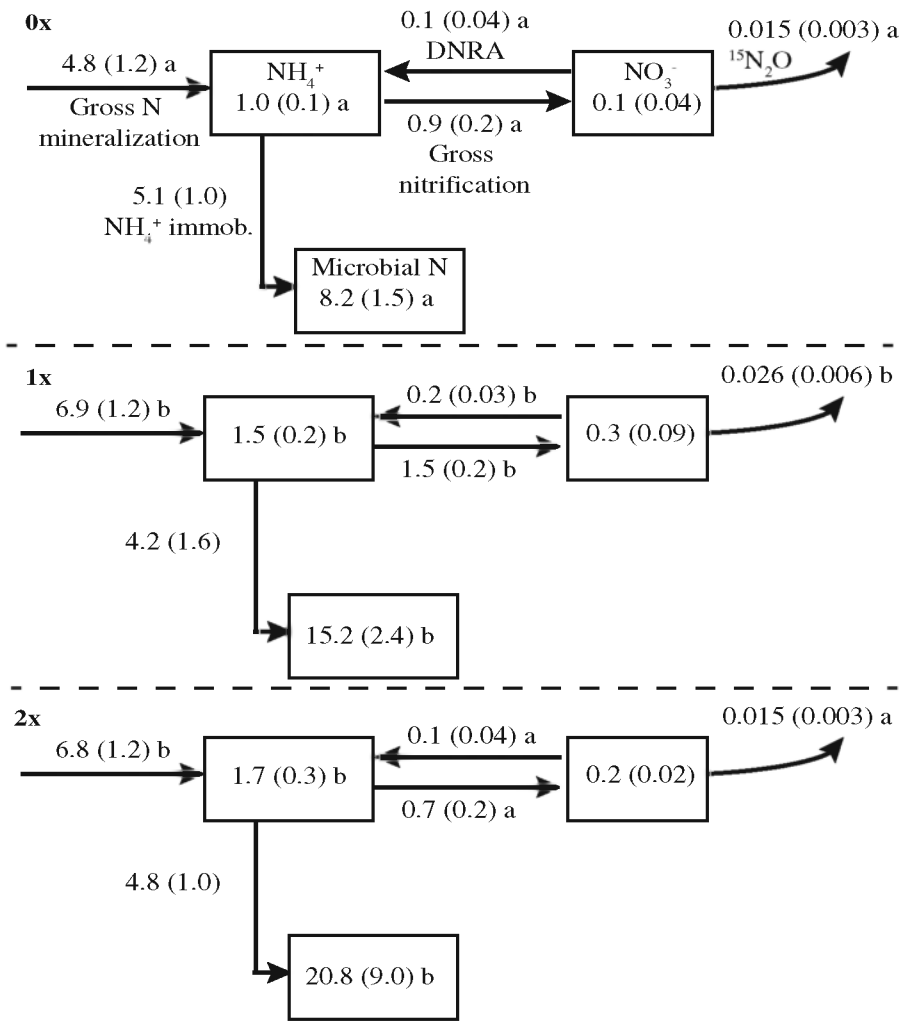

Fig. 1 Mineral soil N pools (0–10 cm) and rates of gross soil N transformations from ^{15}N pool dilutions. Soil N pools (μg N g $soil^{-1}$) were measured from unlabeled (natural abundance) soil cores from litter removal (0×, $n = 5$), control (1×, $n = 8$), and double litter plots (2×, $n = 5$) taken at T_0 and extracted in the field. Rates of soil N transformations (μg N g $soil^{-1}$ day^{-1}) from ^{15}N labeled cores at T_4. Values represent means ± 1 SE and significant differences ($P < 0.05$) between treatments are denoted with different *superscripts*

nitrification were significantly higher in the 1× plots than in either the 0× or the 2× litter treatment plots. Gross nitrification also accounted for a larger proportion of gross N mineralization in 1× plots (27 ± 5.0 %) than in either the 0× or the 2× litter treatments (13 ± 3.8 and 9.8 ± 3.8 %, respectively, $P < 0.003$). Across treatments, gross nitrification rates were positively correlated with gross N mineralization ($r = 0.51$, $P = 0.04$, $n = 18$), and soil pH ($r = 0.71$, $P = 0.001$, $n = 18$).

Microbial biomass N pools were significantly smaller in 0× plots (Fig. 1). In soil cores receiving $^{15}NH_4^+$ label, microbial biomass ^{15}N was slightly enriched (0.391 ± 0.038 at.%, $P = 0.14$) relative to T_0 values (0.284 ± 0.089 at.%), suggesting microbial immobilization of the $^{15}NH_4^+$ label. Mean rates of NH_4^+ immobilization were highly variable, and similar across experimental treatments (4.7 ± 1.0 μg N g^{-1} day^{-1}, Fig. 1). Based on these observations, we estimate that 61 ± 32 % of the mineralized NH_4^+ was immobilized into microbial biomass in 1× plots, whereas a greater proportion of mineralized NH_4^+ tended to be immobilized in 0× and 2× plots (86 ± 27 and 73 ± 27 %, respectively), although these differences are not statistically significant. We observed no enrichment of microbial biomass ^{15}N following $^{15}NO_3^-$ additions, suggesting little or no microbial immobilization of the $^{15}NO_3^-$ label.

Soil cores receiving $^{15}NO_3^-$ label showed enrichment of $^{15}NH_4^+$ pools (0.95 ± 0.08 at.%, $P = 0.02$), relative to mean $^{15}NH_4^+$ values at T_0 (0.67 ± 0.08 at.%), indicating that DNRA occurred in all treatments. Mean DNRA rates were significantly higher in 1× plots than in either the 0× or 2× treatments (Fig. 1); although DNRA represented a smaller percentage of gross nitrification in control plots (11 ± 1.4 %) than in either 0× or the 2× litter treatments (15 ± 3.0 %, $P = 0.02$; and 15 ± 6.6 %, respectively) the difference was not statistically significant. Across treatments, rates of DNRA were positively correlated with gross nitrification rates ($r = 0.73$, $P < 0.001$), gross N mineralization rates ($r = 0.65$, $P = 0.004$), and soil pH ($r = 0.52$, $P = 0.03$; $n = 18$ in all cases).

Efflux of $^{15}N_2O$ was significantly higher out of soil cores receiving $^{15}NO_3^-$ label than cores receiving $^{15}NH_4^+$ label ($t = 2.15$, $P = 0.04$). However, $^{15}N_2O$ emissions from $^{15}NO_3^-$ and $^{15}NH_4^+$ labeled cores were strongly auto-correlated with each other ($r = 0.79$, $P < 0.001$, $n = 18$), so here we report $^{15}N_2O$ production from cores receiving $^{15}NO_3^-$ label. Rates of $^{15}N_2O$ emissions from control plots (5.4 ± 1.3 ng $^{15}N_2O$-N cm^{-2} h^{-1}) were significantly higher than $^{15}N_2O$ emissions from 0× or 2× plots (< 4.3 ng $^{15}N_2O$-N cm^{-2} h^{-1}; $P < 0.002$, Fig. 1). Among all treatments, rates of $^{15}N_2O$ emissions were most strongly correlated with rates of gross nitrification ($r = 0.72$, $P < 0.001$, $n = 18$) and soil pH ($r = 0.69$, $P = 0.002$, $n = 18$). We compared $^{15}N_2O$ emissions to other soil N transformations (sensu Silver et al. 2005), and found that gaseous $^{15}N_2O$ losses from $^{15}NO_3^-$ labeled cores were significantly lower than rates of DNRA (Fig. 1). Estimated rates of $^{15}N_2O$ production were 10 ± 1 % of DNRA rates in 1× plots, and significantly lower in 0× and 2× treatments (<2 ± 2 %, $P < 0.001$).

Abundance of N-cycling microbial guilds

Corroborating observations of microbial biomass C (Table 1), bacterial abundance in 1× and 2× plots was 20 % higher than in 0× plots (Appendix A), while crenarchaeal abundance was statistically indistinguishable among treatments. We also observed significant differences in the relative abundance of N-cycling microbial guilds between litter treatments (Appendix A). Notably, the mean relative abundance of *napA*, *nirS,* AOA, and AOB were significantly higher in 1× plots than either 0× or 2× treatments (Fig. 2a). Concurrently, the mean relative abundances of *nifH*, *narG*, *nirK*, and *nosZ* were significantly higher in 0× and 2× plots, compared to 1× plots. Multivariate statistical analyses showed patterns in N-cycling microbial guilds that demonstrate consistent treatment effects. A permutational analysis of variance using Bray–Curtis distance matrices showed that litter treatment alone explained 65 % of the observed variation in N-cycling microbial community structure (adonis $F = 20.4$, $P = 0.01$). Similarly, NMDS demonstrated strong clustering of microbial communities based on litter manipulation treatment (Fig. 2b).

Soil pH and/or soil C:N were significantly correlated with the relative abundance of N-cycling microbial guilds (Appendix B). The relative abundance of AOA, in turn, explained a significant amount of variation in measured rates of gross nitrification (Fig. 2c).

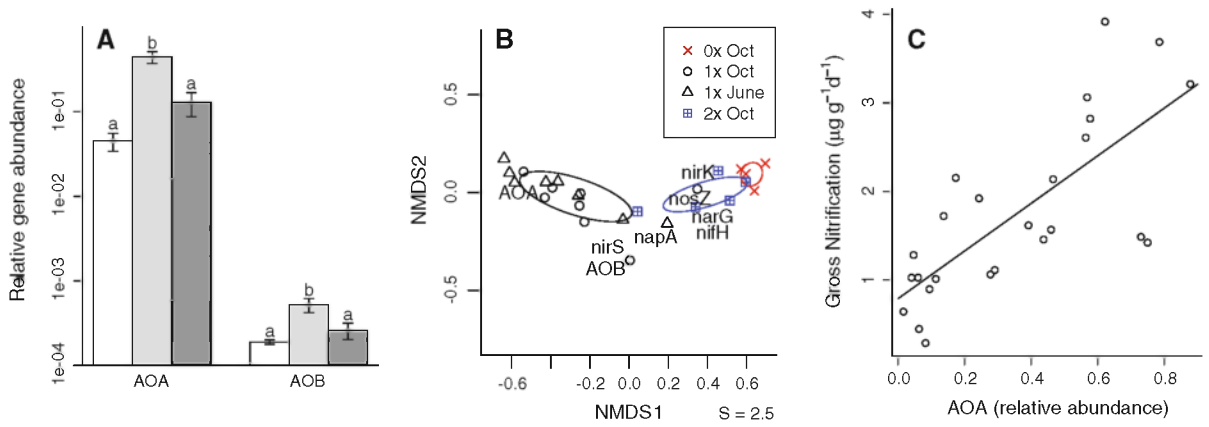

Fig. 2 Selected microbial community responses to leaf litter manipulation showing: **a** the relative abundance of AOA and AOB (mean ± 1 SE) from litter removal (*white bars*), control (*grey bars*), and double litter plots (*dark bars*). Significant differences between litter treatments for each microbial guild are denoted with different *superscripts* ($P < 0.05$). **b** NMDS analysis of relative abundance of N-cycling microbial communities from all litter treatments with the (including samples collected from control plots in June 2009). The relative positions in the NMDS of different functional genes targeted are indicated. *Ellipses* indicate 95 % confidence intervals for leaf litter treatments. Stress value (S) = 2.5. **c** Linear regression between rates of gross nitrification and the relative abundance of AOA ($R^2 = 0.53, P < 0.001, n = 25$, includes data collected in June 2009 from control plots)

Discussion

Experimentally manipulating leaf litter inputs to surface soils profoundly altered the soil N cycle. Unexpectedly, both litter removal and addition decreased rates of NO_3^- production (Fig. 1). Moreover, the changes observed in gross nitrification rates were mirrored by shifts in the relative abundance of ammonia-oxidizing microbes (Fig. 2). The similarity of this response to opposing leaf litter manipulations suggests different mechanisms are at play in 0× and 2× plots. Removing leaf litter inputs reduced soil N availability (Table 1) and depressed rates of N cycling relative to control plots (Fig. 1). By contrast, doubling leaf litter inputs modestly increased soil N pools, but in spite of this greater soil N availability we observed lower rates of gross nitrification—contradicting our own hypotheses and observations of increased NO_3^- availability from another leaf litter manipulation in Panama (Sayer and Tanner 2010; Sayer et al. 2012). Thus, while reducing organic N inputs appeared to directly control the soil N cycle response in 0× plots, increasing soil C pools modulated the soil N cycle response in 2× plots (Fig. 3).

The importance of leaf litter inputs to the N cycle of forest ecosystems is well documented (Attiwill and Adams 1993; Sayer and Tanner 2010; Vitousek 1982). Since our litter removal treatment essentially simulated some aspects of a biomass harvest, we were not surprised to observe soil N responses in the 0× plots that were similar to patterns reported in tropical pastures and secondary forests that commonly exhibit N limitation (Davidson et al. 2007; Mo et al. 2003; Neill et al. 1997, 1999). Removing leaf litter inputs constrained the soil N cycle by decreasing the amount of organic N available for N mineralization. Decreased gross N mineralization rates also reduced downstream soil N transformations (e.g., nitrification and/or denitrification; Fig. 1), essentially down-regulating the entire soil N cycle (Fig. 3). These data are consistent with other studies of land-use change in tropical systems, where losses of organic matter (and soil compaction) often lead to smaller soil NO_3^- pools, lower NO_3^-: NH_4^+ ratios, and lower soil N_2O fluxes (Corre et al. 2006; Davidson et al. 2007; Silver et al. 2005). Removing leaf litter inputs to surface soils reduced stocks of organic C and N, thus constraining rates of soil N transformations in 0× plots.

By contrast, results from the 2× plots offer new insight into how the tropical N cycle may change with increases in litterfall, perhaps including those induced by rising atmospheric CO_2. Despite similar rates of gross mineralization in the 1× and 2× plots, low rates of gross nitrification in 2× plots (Fig. 1) may result

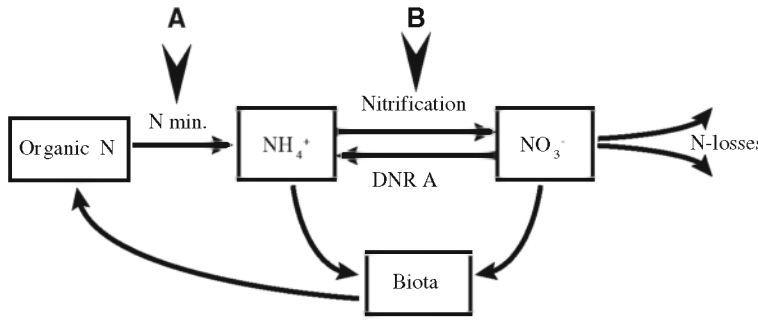

Fig. 3 Conceptual diagram of changes in soil N cycling following leaf litter manipulation. Removing organic N inputs in the 0× plots reduced rates of gross N mineralization (*arrow A*), constraining subsequent rates of N transformations—especially rates of gross nitrification. In contrast, augmenting organic N inputs in the 2× plots resulted in high rates of gross N mineralization, but concurrent changes in the soil chemical environment reduced the relative abundance of ammonia-oxidizers and reduced gross nitrification rates (*arrow B*). Thus, manipulating leaf litter inputs to surface soils reduced soil NO_3^- pools, although via different mechanisms

from increasing C inputs to surface soils. Augmenting litter inputs to 2× plots exacerbated the stoichiometric imbalance between microbes (C:N = 5) and their resources (i.e. litter C:N > 40). The proportional enrichment of C relative to N is reflected by in larger pools of microbial biomass C, higher soil C, and wider soil C:N ratios (Table 1). Increasing soil C availability concurrently increased biologic demand for N in 2× plots from both plants (Leff et al. 2012) and microbes (Weintraub et al. unpublished data). Ammonia-oxidizing bacteria are generally poor competitors for NH_4^+ compared to plants and heterotrophic microbes (Gerards et al. 1998), and the responses we observed here are consistent with a conceptual model in which C rich environments can limit NO_3^- accumulation (Taylor and Townsend 2010; Fig. 3).

Although these findings contradict our initial hypotheses, they build on decades of evidence showing that increasing C availability can drive N immobilization into organic forms. For example, leaf litter provides a relatively large input of C, relative to N, thus N immobilization characterizes the initial stages of leaf litter decomposition (Aber and Melillo 1982; Aerts 1997; Parton et al. 2007). Similarly, increasing ecosystem C availability via elevated CO_2 generates greater biotic N demand and long-term N sequestration in biomass and soil organic matter, resulting in progressive nitrogen limitation (PNL, Luo et al. 2004). This conceptual model was largely developed in temperate forests, and the similar findings we present from a lowland tropical forest are somewhat unexpected given previous work by Sayer and Tanner (2010), and the broad assumption that tropical forests generally cycle N in excess of biological demand (Hedin et al. 2009; Vitousek 1984). Despite the small size of our plots and the drastic manipulation of litter inputs, there may still be good reason to believe the long term response of temperate and tropical forests to elevated C inputs (relative to N) may be fundamentally different. All things being equal, tropical forests growing under elevated CO_2 may find stoichiometric equilibration with greater N inputs via biological N fixation (Cleveland et al. 1999; Vitousek et al. 2002), potentially avoiding, or at least delaying, PNL. The timescale and magnitude of such a response are not well known in the tropics, and depend on more than C availability alone (Houlton et al. 2008; Vitousek et al. 2010).

For example, some evidence suggests that relative N limitation in tropical forests may increase under wetter conditions (Houlton et al. 2006; Nardoto et al. 2008), and past work in our sites—which receive ~5 m of rainfall per year—indicates the potential for stronger N constraints than seen in many drier lowland forests. Specifically, we have observed that N additions increase fine root biomass (Cleveland and Townsend 2006), annual N_2O emissions are comparatively low for a lowland tropical forest (Wieder et al. 2011), and hydrologic losses of N are dominated by organic forms (P. Taylor, unpublished data). Moreover, soil and foliar ^{15}N values (A. Townsend, unpublished data) are also far lower than the mean

tropical forest values reported in Martinelli et al. (1999), in keeping with the negative relationship between foliar ^{15}N values and precipitation reported for sites across Amazonia by Nardoto et al. (2008). Thus, the greater relative N immobilization we observed under 2× litter additions may reflect a response to baseline conditions where N is already under high internal demand.

Supporting this conceptual model, we found that the microbial community functional group responses to litter manipulation (Fig. 2) paralleled changes in soil N transformations. Nitrification regulates NO_3^- production and is fundamentally linked to the activity and/or abundance of ammonia-oxidizing organisms (Carney et al. 2004; Francis et al. 2007; Leininger et al. 2006; Nicol and Schleper 2006). Not only did 1× plots exhibit the highest rates of gross nitrification (Fig. 1), but they also had the highest relative abundance of AOA (Fig. 2a). Furthermore, across all plots, the relative abundance of AOA was positively related to gross nitrification rates (Fig. 2c). AOA expression is unique to archaea, supporting the notion that archaeal, rather than bacterial nitrifiers dominate NO_3^- production at this site. The relative abundance of ammonia-oxidizers was positively related to both soil C:N and pH across all plots (Appendix B). This covariation in response to two potentially important environmental drivers makes it difficult to determine the relative importance of each mechanism since both edaphic properties appear to strongly influence microbial community structure and abundance (Bates et al. 2011; Bru et al. 2011; De Boer and Kowalchuk 2001; Fierer and Jackson 2006; Fierer et al. 2009) and deserves further discussion.

Edaphic properties, like C:N and pH, strongly influence microbial community structure and abundance. Shifting soil C:N ratios may limit NO_3^- production through a stoichiometrically-driven increase in biologic N demand, which disfavors ammonia-oxidizers' competitiveness for available NH_4^+ (Taylor and Townsend 2010). Concurrently, the pH sensitivity of ammonia-oxidizing communities is well documented (De Boer and Kowalchuk 2001, Erguder et al. 2009) but far from resolved (Bru et al. 2011, Nicol et al. 2008). The pH of soil from 0× and 2× plots was lower in this study (Table 1). In 2× plots we attribute lower soil pH to higher inputs of organic acids from root exudates and increased rates of soil respiration (Leff et al. 2012). In 0× plots decreased pH may have been caused by the decline in base cation inputs from fine litterfall (Sayer and Tanner 2010). As such, these changes in C availability and/or pH may have played a role in the observed changes in the relative abundance of particular microbial guilds may help explain some of the biogeochemical responses to our leaf litter manipulation; changing litter inputs altered the soil environment in ways that decreased the relative abundance of ammonia-oxidizers. That decreased abundance mirrors declines in gross nitrification, and suggests the potential for reduced NO_3^- losses following shifting litter inputs may be driven by microbial community—as well as process—based changes that directly affect NO_3^- production and consumption.

The nitrate pool size also reflects consumption processes that include plant (not measured here) and microbial uptake, DNRA, denitrification, and leaching (also not measured here, although small across the region P. Taylor unpublished data). Previous work at this site shows fine roots responds strongly to inorganic N additions (Cleveland and Townsend 2006), and we observed greater fine root biomass in 2× plots (Leff et al. 2012). We suspect that our 2× plots created nutrient hotspots that facilitated greater plant N uptake and translocation out of our small plots. Accordingly, stimulated N uptake by plants could consume the products of nitrification (Stark and Hart 1997) or limit the amount of N available to ammonia-oxidizing microbes, decreasing rates of gross nitrification. We suspect the later, given low relative AOA abundance observed in 2× plots (Fig. 2, Appendix B). Thus, increased biotic demand for N from both plants and decomposer communities in 2× plots likely explains low rates of gross nitrification in 2× plots.

In addition to lower NO_3^- production rates in 0× and 2× plots, a greater proportion of the available NO_3^- was reduced to NH_4^+ via DNRA than was lost to denitrification. DNRA is more energetically favorable than denitrification in environments with high soil C availability, or high C:NO_3^- ratios (Fazzolari et al. 1998; Silver et al. 2005; Tiedje 1988). This suggests that the combination of lower nitrification rates and greater NO_3^- conservation through DNRA constrained the size of the NO_3^- pool in litter-manipulated plots, potentially reducing NO_3^- losses as gases or by leaching. Indeed, observations of lower $^{15}N_2O$ emissions from the 0× and 2× plots (Fig. 1) suggest that external N losses via denitrification may decline

as litterfall inputs change. These same plots, however, also had a higher relative abundance of *nosZ*, which encodes for nitrous oxide reductase (Appendix A). This indicates a greater proportion of denitrifiers capable of reducing N_2O to N_2, which could explain the lower $^{15}N_2O$ efflux out of 0× and 2× plots (Philippot et al. 2011). Thus, while we cannot conclude that manipulating litter inputs decreased gaseous N losses, data from this study do show lower soil emissions of the greenhouse gas N_2O with either litter additions or removal (Fig. 1), a pattern that is consistent with the multiple other lines of evidence that suggest greater relative N conservation following resource manipulation.

Conclusions

Both increases and decreases in organic matter inputs decreased rates of gross nitrification, facilitated greater NO_3^- retention through DNRA, and reduced gaseous N_2O losses. These concerted changes to the N cycle suggest that changing C inputs to tropical forests may reduce N losses to surface waters and the atmosphere by limiting the production of NO_3^- by ammonia-oxidizing microbes, especially archaea. While the magnitude of our litterfall manipulation likely exceeded predicted changes in tropical forests litterfall, our results suggest that changing organic inputs to surface soils may decrease both the production and loss of NO_3^- from tropical forest soils. The degree to which similar observations may occur in larger-scale global change manipulations (especially those involving multiple perturbations) deserves further investigation; especially given the substantial biogeochemical heterogeneity that resides in the tropical biome (Hedin et al. 2009; Townsend et al. 2008). Results from this study highlight the importance of considering changes in resource stoichiometry in evaluating ecosystem responses to perturbations of biogeochemical cycles.

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