

Consistent effects of nitrogen amendments on soil microbial communities and processes across biomes

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Abstract

Ecosystems worldwide are receiving increasing amounts of reactive nitrogen (N) via anthropogenic activities with the added N having potentially important impacts on microbially mediated belowground carbon dynamics. However, a comprehensive understanding of how elevated N availability affects soil microbial processes and community dynamics remains incomplete. The mechanisms responsible for the observed responses are poorly resolved and we do not know if soil microbial communities respond in a similar manner across ecosystems. We collected 28 soils from a broad range of ecosystems in North America, amended soils with inorganic N, and incubated the soils under controlled conditions for 1 year. Consistent across nearly all soils, N addition decreased microbial respiration rates, with an average decrease of 11% over the year-long incubation, and decreased microbial biomass by 35%. High-throughput pyrosequencing showed that N addition consistently altered bacterial community composition, increasing the relative abundance of *Actinobacteria* and *Firmicutes*, and decreasing the relative abundance of *Acidobacteria* and *Verrucomicrobia*. Further, N-amended soils consistently had lower activities in a broad suite of extracellular enzymes and had decreased temperature sensitivity, suggesting a shift to the preferential decomposition of more labile C pools. The observed trends held across strong gradients in climate and soil characteristics, indicating that the soil microbial responses to N addition are likely controlled by similar wide-spread mechanisms. Our results support the hypothesis that N addition depresses soil microbial activity by shifting the metabolic capabilities of soil bacterial communities, yielding communities that are less capable of decomposing more recalcitrant soil carbon pools and leading to a potential increase in soil carbon sequestration rates.

Keywords: anthropogenic change, bacteria, extracellular enzymes, microbial activity, pyrosequencing, soil decomposition, temperature sensitivity

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Introduction

Nitrogen (N) inputs from anthropogenic sources are currently estimated to be 30% to 50% greater than those from natural terrestrial sources and tenfold greater than anthropogenic inputs from 100 years ago (Galloway *et al.*, 2008; Schlesinger, 2009; Canfield *et al.*, 2010). Understanding how this additional N will impact terrestrial ecosystems is becoming increasingly important within the context of the terrestrial carbon (C) budget (Singh *et al.*, 2010; Zaehle *et al.*, 2010). Since N is a limiting nutrient to plants in most terrestrial ecosystems (Vitousek *et al.*, 2002), anthropogenic N enrichment can often have strong effects on aboveground primary productivity and plant community composition (Tilman, 1987; Gough *et al.*, 2000; Vitousek *et al.*, 2002). In contrast, the belowground microbial responses to elevated N inputs are less well understood. Recent

work indicates that N additions can alter the diversity and composition of soil microbial communities (Allison *et al.*, 2008; Campbell *et al.*, 2010; Ramirez *et al.*, 2010b; Bates *et al.*, 2011) though it remains unclear if microbial community shifts are predictable and consistent across ecosystems as few experimental studies have examined responses across a wide range of soil or ecosystem types. Additionally, with field-based studies it is difficult to determine if microbial community shifts are a direct result of the N addition or an indirect result of changes to plant communities and their C inputs to soils (Waldrop *et al.*, 2004; Ramirez *et al.*, 2010b).

Nitrogen enrichments not only influence microbial community composition but also can strongly impact microbial C dynamics – though not in ways that would be predicted from traditional limitation theory (Vitousek & Howarth, 1991) as N enrichments typically decrease microbial activity. Strong reductions in soil microbial activities have been documented in both field and lab-based studies with the magnitude of the reduction in soil respiration and microbial biomass strongly

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related to the duration and amount of N added to an ecosystem (Treseder, 2008; Janssens *et al.*, 2010), with reductions in soil respiration regardless of the form of N added (Ramirez *et al.*, 2010a). Likewise, microbial enzyme activities can be highly sensitive to increases in N, especially those enzymes that degrade complex C compounds (Sinsabaugh *et al.*, 2005; Waldrop & Zak, 2006). Further, Craine *et al.* (2007) found that N additions decrease recalcitrant-C mineralization, causing an overall decline in soil decomposition rates. This commonly observed suppression of microbial activity under N enrichment could be considered somewhat counterintuitive given that plant productivity generally increases with addition of N (Gough *et al.*, 2000), which would likely increase C inputs to soils.

As traditional nutrient limitation theory does not explain declines in soil microbial activity, a number of hypotheses have been proposed to explain this paradox. For example, N additions may have indirect effects on microbial communities by altering plant community composition and/or the amounts and types of plant C inputs to soils, reducing microbial activity levels (Meier & Bowman, 2008). Other hypotheses involve N-induced shifts in microbial physiologies and/or microbial community compositions that are directly associated with increased soil N availability. For example, the *enzyme inhibition* hypothesis proposes that N additions directly inhibit those enzymes needed for the decomposition of recalcitrant C thereby reducing overall microbial activity (Fog, 1988; Gallo *et al.*, 2004). Similarly, the *N-mining* hypothesis suggests that under N enrichment, microbes reduce decomposition of recalcitrant C in response to their lowered N requirements (microbes no longer 'mine' recalcitrant organic matter to obtain N), resulting in a shift towards labile C decomposition and reduced overall microbial activity (Moorhead & Sinsabaugh, 2006; Craine *et al.*, 2007). This shift might occur at the level of the individual microbe and/or be a consequence of shifts in the relative abundances of specific microbial taxa. A similar hypothesis, the *copiotrophic* hypothesis, suggests that N additions decrease microbial activities by directly shifting microbial community composition (Fontaine *et al.*, 2003; Fierer *et al.*, 2007, 2011; Ramirez *et al.*, 2010a,b). Under this hypothesis, increases in N reduce the relative abundance of oligotrophic taxa (those taxa that are adept at catabolizing more recalcitrant C pools) as the alleviation of N limitation allows them to be out-competed by more copiotrophic taxa (those taxa that have higher N demands and are more restricted in their catabolic capabilities, specializing on more labile C pools).

To identify if N enrichment elicits consistent responses in microbial activity and community composition across ecosystems and to determine what

mechanisms may be responsible for the observed responses, soils were collected from 28 sites throughout North America representing a broad range of biomes and soil types. Soils were amended with inorganic N and incubated for 1-year. By incubating the soils under standardized, laboratory conditions we eliminated the indirect effects of N additions on plant C inputs and instead focused only on direct N responses across the soils. We examined effects of N amendments on the soil bacterial community composition, but did not examine effects on the fungal communities, even though such effects may be important, particularly with regard to the process responses examined here (Allison *et al.*, 2008; Edwards *et al.*, 2011; Fontaine, 2011). We tested the validity of the various hypotheses outlined above by assessing N effects on soil respiration, microbial biomass, bacterial community composition, extracellular enzyme activities and soil temperature sensitivity after the 1-year incubation under controlled conditions.

Methods

Site and soil description

Soils were collected from 28 sites across North America, ranging from 17.97 to 68.63 °N and representing a range of climates (mean annual temperatures from -10.3 to 25.7 °C and mean annual precipitation from 17.3 to 300 cm) (Fig. 1, Table 1). The collected soils also represented a broad range of edaphic characteristics (e.g. pH from 4.24 to 8.32, soil C : N from 7.62 to 36.94, and % silt + clay from 5 to 62.5) (Table 1). At each site, soil was collected from the top 10 cm of the A horizon, sieved to 2 mm and stored at 5 °C for 10–60 days until incubations began. Soil C and N concentrations, pH and texture were measured using methods described in Craine *et al.* (2010).

Soil incubation, respiration and microbial biomass

To determine the response of soil microbial communities to N addition, subsamples of soils (20 g dry-equivalent) were weighed out into 50 ml polyethylene centrifuge tubes (Craine *et al.*, 2010). Subsamples were kept at ambient N levels (no N added) or treated with 5 mg N in the form of NH_4NO_3 , which is approximately equivalent to adding 125 g N m^{-2} to a 50-cm soil profile, an amount similar to that applied in many field studies (Treseder, 2008; Ramirez *et al.*, 2010b). All samples were adjusted to 35% water-holding capacity and incubated at 20 °C in a humid environment with soil moisture held constant through the incubation. Soil respiration was measured 15 times over the 365-day incubation period (Craine *et al.*, 2010). Briefly, at each measurement, the two replicates of each soil sample were sealed with a gas-tight cap equipped with a rubber septum and incubated for 18–72 h. At the end of this period, CO_2 concentrations were measured using a Licor 6252 infrared gas analyzer (LiCor, Lincoln, NE, USA) with respiration



Fig. 1 Map of the 28 soil collection sites.

rates determined from the net accumulation of CO₂ over time. Respiration from ambient samples was used as an estimation of available C, where we assumed that in the absence of moisture limitation and at a constant temperature, the rate of CO₂ production corresponds to the amount of C readily available for microbial mineralization (Fierer *et al.*, 2003).

After the 365-day incubation, microbial biomass was measured using the substrate induced respiration (SIR) approach (Wardle & Ghani, 1995; Fierer *et al.*, 2003). Briefly, replicates were placed at 20 °C for 48 h, at which point 400 mg of glucose was added to one replicate of each soil, the tubes capped after 20 min of equilibration, and respiration rates (as described above) were determined after 2–4 h.

Bacterial community composition

To characterize bacterial community composition from each of the 28 soil samples at the end of the 365-day incubation soils were harvested, frozen and stored at –20 °C for 1 month. We then extracted DNA using the MoBio PowerSoil DNA extraction kit following the manufacturer's instructions with modifications described previously (Lauber *et al.*, 2009). A portion of the 16S rRNA gene was amplified in triplicate PCR reactions using the F515 and R805 primers designed to be universal for bacterial and archaeal taxa (Bates *et al.*, 2011; Bergmann *et al.*, 2011). Here, we only report results for the bacterial community as archaea were rare (typically less than 1% of 16S rRNA sequences). Amplicons were pooled and equimolar concentrations pyrosequenced at EnGenCore (University of South Carolina) on a Roche FLX 454 automated pyrosequencer running the Titanium chemistry. Processing of raw bacterial sequence data was performed using QIIME (Caporaso *et al.*, 2010). Briefly, quality sequence reads were grouped by their unique barcode and assigned to phylotypes ($\geq 97\%$ similarity), representative sequences were aligned using PyNAST against the Greengenes core set (DeSantis *et al.*, 2006) and taxonomy was assigned using the Ribosomal Database Project classifier

(Wang *et al.*, 2007). A phylogenetic tree containing the aligned sequences was then produced using FastTree (Price *et al.*, 2009). All downstream analyses were determined after samples were rarified to 200 sequences/sample (to include the maximum number of samples), but the patterns were nearly identical to those observed with 1000 sequences/sample (data not shown). Relatedness of bacterial communities between each pair of samples (ambient vs. N treatment) was determined using the unweighted Unifrac algorithm, hereon referred to as 'pairwise Unifrac' (Lozupone & Knight, 2005). Of the 28 soils, DNA was successfully amplified from 26 soils and used for subsequent analyses (GCE and SJQ were excluded).

Enzyme activity and temperature sensitivity

To understand the response of microbial carbon and nutrient breakdown activity to N addition across soils, we measured potential extracellular enzyme activity (EEA) for eight extracellular enzymes in all 28 soils: β -1,4-glucosidase (BG) and cellobiohydrolase (CBH), β -N-acetylglucosaminidase (NAG), leucine aminopeptidase (LAP), phosphatases (AP) and peroxidase (PER) and phenol oxidase (POX) (Table 3). Following Saiya-Cork *et al.* (2002), soil was suspended in sodium acetate (pH 5.0) or sodium bicarbonate (pH 8.0) buffer and aliquots were dispensed into 96-well microplates with 16 replicate wells per sample per assay. Blank, negative control, and quench wells each had eight replicates. After incubation (20 °C, for up to 4 h depending on substrate), fluorescence was measured using 365 nm excitation and 450 nm emission filters. Phenol oxidase and peroxidase activities were measured spectrophotometrically (450 nm) using L-3,4-dihydroxyphenylalanine (DOPA) (Sinsabaugh *et al.*, 2005). All EEAs were log-transformed to normalize distributions. In addition to comparing the response of individual EEA levels to N addition, we also examined ratios of EEAs to identify relative shifts in nutrient acquisition activities: $\ln(\text{BG}):\ln(\text{NAG} + \text{LAP})$ (C : N

Table 1 Description of the 28 collection sites and the soils used in this study

Site	Abbreviation	Ecosystem	Latitude	Longitude	MAT (°C)	MAP (cm)	Soil pH	WHC	C (mg g ⁻¹)	N (mg g ⁻¹)	C : N	Sand	Silt	Clay
American Prairie	AP	Prairie/Grassland	47.80	-107.95	6.2	27.2	7.29	0.68	22	2.1	10.48	47.5	25	27.5
Andrews LTER	AND	Coniferous Forest	44.20	-122.20	7.8	147.0	5.58	1.02	42.1	2	21.05	82.5	15	2.5
Arctic LTER	ARC	Arctic Tundra	68.63	-149.60	-10.3	17.3	5.47	0.45	22.9	1.5	15.27	40	35	25
Baltimore Ecosystem Study	BES	Urban-temperate	39.61	-76.74	13.6	89.4	5.15	0.92	84.9	4	21.23	77.5	15	7.5
Bonanza Creek LTER	BNZ	Boreal Forest	64.77	-148.27	-3.7	24.4	4.99	0.82	28.9	1.7	17	50	42.5	7.5
Cedar Creek Natural History Area	CDR	Grassland	45.40	-93.20	5.9	60.7	6.22	0.40	9.9	1.3	7.62	87.5	10	2.5
Coweeta LTER	COW	Deciduous Forest	35.03	83.43	11.1	164.2	4.63	0.94	59.1	1.6	36.94	77.5	10	12.5
Florida Coastal Everglades LTER	FCE	Coastal	25.56	-80.39	23.6	119.0	7.52	0.43	21.2	0.7	30.29	85	10	5
Georgia Coastal Ecosystems	GCE	Coastal	31.40	-81.30	19.4	106.0	4.24	0.57	29.9	1.4	21.36	95	2.5	2.5
Guánica	GUA	Dry forest	17.97	-65.50	24.2	162.6	7.81	0.96	68.6	6.9	9.94	65	20	15
Harvard Forest LTER	HFR	Deciduous Forest	42.30	-72.10	8.1	94.1	4.45	1.10	62.1	3.7	16.78	82.5	12.5	5
Hubbard Brook Experimental Forest	HB	Deciduous Forest	43.96	-71.72	5.5	87.0	4.37	1.49	61.8	3.8	16.26	77.5	15	7.5
Itasca State Park	ITA	Deciduous Forest	47.27	-95.33	3.0	65.0	6.95	1.09	59.3	4.7	12.62	75	17.5	7.5
Jepson Prairie	JEP	Prairie-Grassland	38.35	-121.82	15.7	49.7	5.92	0.44	13.9	1	13.9	45	40	15
Jornada Basin LTER	JRN	Desert	32.49	-106.78	14.5	22.9	7.28	0.33	1.8	n/a	n/a	80	12.5	7.5
Kankakee Sands	KAN	Prairie-Grassland	41.05	-87.46	9.9	77.2	6.73	0.59	14.5	1.1	13.18	57.5	32.5	10
Kellogg Biological Station LTER	KBS	Grassland	42.40	-85.40	9.2	76.2	6.51	0.80	35.2	2.8	12.57	70	27.5	2.5
Konza Prairie Biological Station	KNZ	Prairie-Grassland	39.08	-96.57	12.5	72.4	6.93	0.99	42.8	3.5	12.23	42.5	40	17.5
Luquillo	LUQ	Tropical forest	18.31	-65.76	25.7	157.5	5.08	1.36	29.1	2.3	12.65	37.5	42.5	20
Niwot Ridge	NIW	Alpine Tundra	40.09	-105.66	3.9	39.0	5.35	0.70	41.4	1.4	29.57	60	25	15
Olympic National Park	OLY	Coniferous Forest	47.86	-124.04	5.0	300.0	4.63	1.00	65.1	2.8	23.25	80	15	5
Ordway-Swisher	ORD	Wetland	29.68	-82.00	20.5	110.6	5.87	0.32	6.2	0.4	15.5	95	2.5	2.5
San Joaquin Experimental Range	SJQ	Dry Shrubland	37.06	-95.68	14.9	59.7	6.45	0.47	10.7	0.7	15.29	82.5	12.5	5
Santa Rita Experimental Range	SRE	Desert	31.83	-110.85	19.1	27.4	6.01	0.29	2.8	0.1	28	80	15	5
Sevilleta LTER	SEV	Desert	34.35	-106.88	12.9	19.6	8.29	0.32	2.2	0.2	11	82.5	10	7.5
Short Grass Steppe	SGS	Prairie-Grassland	40.81	-104.19	7.9	32.3	5.93	0.48	9.1	0.8	11.38	65	22.5	12.5
Walker Branch	WB	Deciduous-Forest	35.97	84.28	13.1	117.4	6.01	0.78	37.8	1.9	19.89	47.5	37.5	15
Wind River	WR	Coniferous- Forest	45.82	121.95	7.7	179.4	5.6	0.77	32.7	1.6	20.44	82.5	10	7.5

acquisition); In BG:ln(AP) (C : P acquisition); and ln(LAP + NAG): ln(AP) (N : P acquisition) (Sinsabaugh *et al.*, 2009).

To determine responses of temperature sensitivity to N additions, five replicates for each soil were sealed with a cap containing a rubber septum, and then distributed over five temperatures (10, 15, 20, 25 and 30 °C). These soils were incubated for 18–72 h and then respiration rates were determined as described above. This process was repeated 15 times over 365 days. We used the Arrhenius equation to calculate the apparent activation energy of the chemical reactions that contributed to respiration (E_a) where the rate of respiration is relative to total SOC (Laidler, 1984). Specifically, E_a was calculated as the slope of the relationship between $-1/RT$ and the natural logarithm of relative respiration rates to yield an index of the relative recalcitrance of the SOC pools mineralized by microbes with and without added N (Craine *et al.*, 2010).

Statistical analysis

The differences between ambient and treatment pairs in the responses of soil respiration, microbial biomass, the relative abundances of bacterial groups, enzyme activity and temperature sensitivity were assessed with paired *t*-tests. Our goal was to assess responses across the entire soil collection, rather than isolate differences between individual pairs of sites, thus our experimental design did not rely on replicated ambient and treatment pairs of each soil type. To identify significant

changes within the whole bacterial community from ambient to treatment samples, we used a one-sample *t*-test on pairwise Unifrac values. Bivariate relationships were measured with Pearson correlations and regression analysis (temperature sensitivity), with each sample from one of the 28 sites representing an individual data point as our goal was to assess responses of the entire soil collection, rather than isolate differences between individual pairs of sites. All statistical analyses were performed using the program R version 2.13.1 (The R Foundation for Statistical Computing 2011).

Results

Nitrogen effects on soil respiration and microbial biomass

Across the 28 soils, N addition tended to suppress soil respiration and microbial biomass. N addition decreased soil respiration for 75% of the soils and decreased microbial biomass for 89% of the soils (Fig. 2a,b and Table 2, Table S1). On average, the N addition decreased soil respiration by 11.8% and microbial biomass by 35.2% ($P < 0.05$, and < 0.001 respectively) but the magnitude of the N response ranged across the soils (from -44.2% to 30.4% for soil respiration, and -86.7% to 36.8% for microbial biomass) (Fig. 2c). N addition reduced respiration the most for soils with lower soil N content ($r = 0.41$, $P = 0.04$) and higher available C ($r = -0.63$, $P < 0.001$)

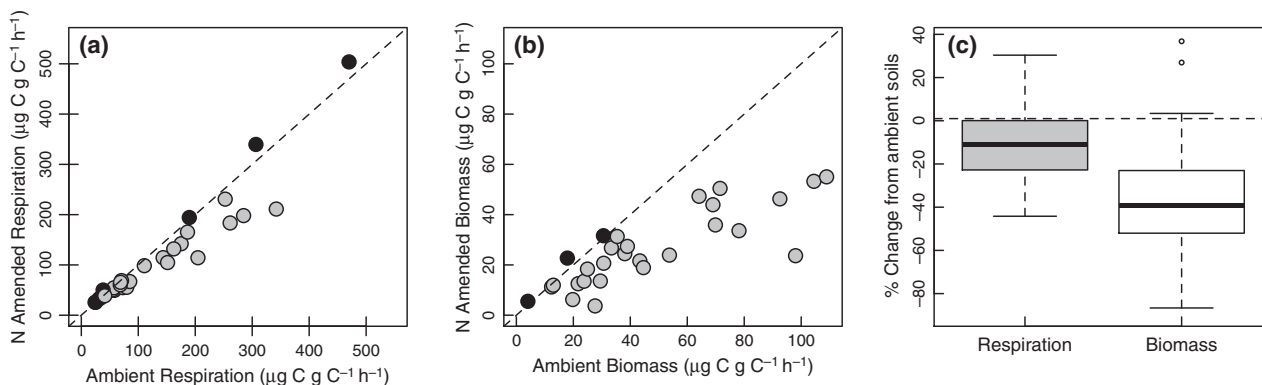


Fig. 2 Ambient (no N added) soil respiration plotted against N amended soil respiration (a) and ambient (no N added) microbial biomass plotted against N amended microbial biomass (b), where points below (gray) the dashed line indicate a decrease with the N amendment. Boxplot summarizing relative change in respiration and biomass with the N amendment (c); the solid black line represents the mean of all soils.

Table 2 Pearson correlation coefficients relating microbial and soil changes to edaphic factors

	N	C	CN	Available C	pH	Latitude	MAT	MAP	Texture
Δ Respiration	0.41*	0.15	-0.34	-0.62**	0.22	-0.09	0.34	0.3	-0.26
Δ Biomass	0.31	0.17	0.01	-0.23	-0.19	0.36	0.05	-0.08	0.1
Pairwise Unifrac	-0.52**	-0.50**	0.08	0.31	0.08	-0.19	-0.17	-0.34	0.1

* $P < 0.05$;

** $P < 0.01$.

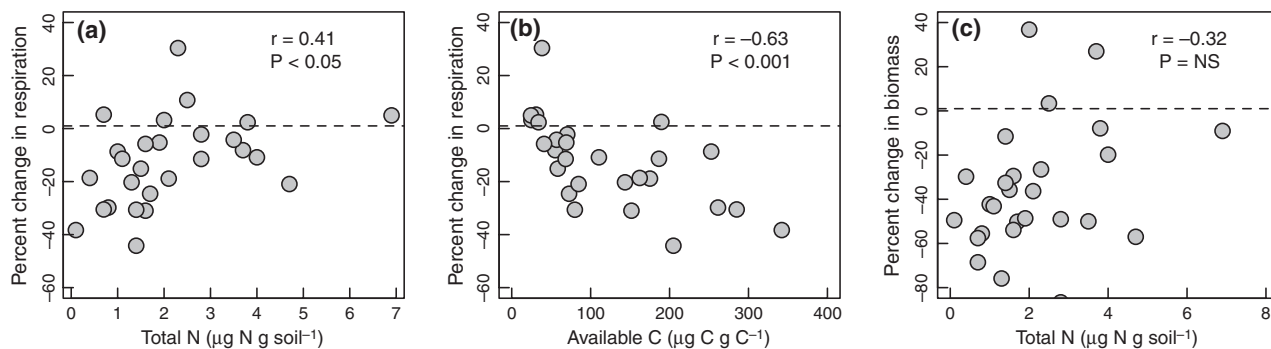


Fig. 3 Percent change in soil respiration rates (panels a and b) and microbial biomass (panel c) against soil N and available C levels. The percent change was calculated as the difference between the ambient (no N added) treatment and the N amendment treatment. Pearson correlation coefficients are reported for each relationship.

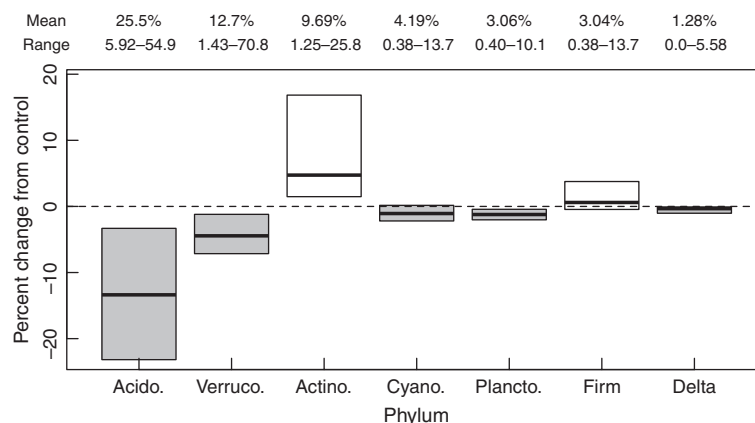


Fig. 4 Bacterial groups (*Acidobacteria*, *Verrucomicrobia*, *Actionobacteria*, *Cyanobacteria*, *Planctomycetes*, *Firmicutes*, and *Deltaproteobacteria*) that demonstrated a significant change from ambient soils to N amended soils. Gray bars indicate a general decrease and white bars are a general increase. Mean percent abundances and ranges for each phylum in the ambient soils are listed along the top.

(Table 2, Fig. 3a,b). Similarly, N addition tended to cause the largest reductions in microbial biomass in soils with low N content ($r = 0.32$, $P = 0.10$) (Table 2, Fig. 3c).

Bacterial community responses to nitrogen amendments

Of the 26 soils that were successfully sequenced, N addition had consistent effects on bacterial community composition regardless of community starting point (Table S2). Overall pairwise Unifrac distances between ambient and N treatment samples indicates that, on average, bacterial communities significantly shifted with the N treatment ($P < 0.001$, one sample t -test). N addition induced the most pronounced shifts in overall bacterial community composition in those soils with lower C and N concentrations (Table 2). More specifically, N addition increased the relative abundance of *Actinobacteria* and *Firmicutes* by an average of 11.8% and 2%, respectively, while decreasing the abundance

of five phyla: *Acidobacteria* (–13.5%), *Verrucomicrobia* (–5%), *Cyanobacteria* (–1%), *Planctomycetes* (–1%) and *Deltaproteobacteria* (–1%) (Fig. 4, Table S3) (all $P < 0.05$). Unlike whole community shifts, the change in relative abundance of individual phyla between ambient and treatment samples could not be predicted by initial soil C or N concentrations ($P > 0.05$ in all comparisons).

Enzyme activities and temperature sensitivity

Similar to the declines in soil respiration and microbial biomass, N addition also decreased the potential activities of four extracellular enzymes: β -1,4-glucosidase (–11.6%), acid phosphatase (–15.1%), leucine amino peptidase (–71.1%) and peroxidase activity (–24.7%) (all $P < 0.001$) (Table 3, Table S4). The other three extracellular enzymes also exhibited N-induced declines in activities, but the changes were not significant. N addition did not alter the ratios of potential extracellular

Table 3 Mean enzyme activities and significance values from paired *t*-tests comparing ambient to samples receiving added N

Enzyme	EC	Mean relative change in activity (%)	<i>P</i>
β -1,4-glucosidase	3.2.1.21	-23.6	<0.001
Cellobiohydrolase	3.2.1.91	-11.4	0.21
β -N-acetylglucosaminidase	3.2.1.14	-0.21	0.98
Leucyl aminopeptidase	3.4.11.1	-64.1	<0.001
Acid phosphatase	3.1.3.2	-36.0	<0.001
Phenol oxidase	1.10.3.2	-15.4	0.06
Peroxidase	1.11.1.7	-24.9	<0.001
BG : AP	-	17.3	0.20
BG:(LAP + NAG)	-	0.91	0.98
(LAP + NAG):AP	-	4.99	0.80

EC, enzyme commission number.

Table 4 Regression results for E_a measured across 15 time points during a 1-year incubation as a function of site identity, N treatment, time into the incubation, and all pairwise interactions

	Sum of squares	<i>P</i>
Site	132196	<0.001
N	1352	0.002
Time	11213	<0.001
Site*N	7760	<0.001
Site*Time	13407	<0.001
N*Time	31	0.64

enzyme activities that would indicate a change in nutrient requirements (Table 3), instead suggesting that the decline in enzyme activities are non-selective and simply associated with the decreases in microbial biomass with N. Unlike soil respiration and microbial biomass, the magnitude of change in enzyme activities was not associated with variation among soils in total N, C or available C ($P > 0.05$ for all comparisons, Table S5) as we observed with soil respiration and microbial biomass.

Across the incubation period, N-amended microbial communities were degrading C with lower biochemical recalcitrance than ambient communities, as determined by the average decrease in activation energy (E_a) from 85.7 to 83.2 kJ mol⁻¹ ($P = 0.002$). The effect of N on the temperature sensitivity of microbial decomposition did not change linearly over time, but did differ between sites (Table 4). Sites with an intermediate pH (5.5) had the lowest decline in E_a with N addition, while sites with extreme pH had the greatest declines ($dEaN = 11.1 - 1.81 * pH - 2.37 * (pH - 5.88)^2$; $r^2 = 0.38$, $P = 0.003$). There were no relationships between the

response of E_a to N addition with microbial biomass, site climate, or soil characteristics ($P > 0.05$ for all comparisons).

Discussion

Across the wide array of soil types, soil respiration and microbial biomass were consistently suppressed under N amendments by an average of 11.8% and 35.2%, respectively. These findings correspond with results obtained in field studies, where decreases in soil respiration ranged between 8% and 15% and biomass decreases ranged between 11% and 20% (Treseder, 2008; Janssens *et al.*, 2010; Liu & Greaver, 2010). However, unlike previous studies, this work demonstrates that belowground responses to N enrichment are not solely related to changes in plant C inputs to soil, as the soils in this study did not receive any C inputs during the course of this incubation. Admittedly, by eliminating plant C inputs, we are choosing to focus on the direct effects of N alone and ignoring indirect N effects that likely contribute to changes in soil communities and processes observed in field experiments. In the field, N amendments generally increase aboveground primary productivity and therefore field studies cannot fully separate the direct effects of N from indirect increases in the quantity or quality of plant C inputs to soil. In contrast, our lab incubation demonstrates that microbial activities, biomass, and community composition can change independently of alterations in litter quality and C inputs, and were most likely a direct response to increased N. Therefore, to better identify the mechanisms driving the suppression of decomposition, we combined the measurements of microbial respiration and biomass with analyses of bacterial community structure, extracellular enzyme activities and temperature sensitivity.

Sequencing analysis revealed that with the N amendment there were consistent phylum-level changes in the bacterial communities across soils. Generally, *Actinobacteria* and *Firmicutes* increased in abundance, and *Acidobacteria* and *Verrucomicrobia* decreased in abundance. These findings are similar to previous field studies, where comparable increases and decreases were observed in the same bacterial groups (Nemergut *et al.*, 2008; Campbell *et al.*, 2010; Ramirez *et al.*, 2010b). Shifts in bacterial phyla may be a function of changes in relative abundances at finer levels of taxonomic resolution, but for this study higher resolution comparisons were not appropriate as all soils had such different starting communities, with little overlap at the order or class levels. Overall, the changes observed here and in other studies, support the *copiotrophic* hypothesis. This hypothesis predicts that under N amendments, microbial groups

that have fast growth rates and rely on more labile C sources are more likely to increase in abundance under nutrient inputs, while other groups that likely thrive under lower nutrient conditions and grow more slowly would decline (Fontaine *et al.*, 2003; Fierer *et al.*, 2007). Indeed, we observed increases in predicted copiotrophic groups (*Actinobacteria* and *Firmicutes*) (Iizuka *et al.*, 1998; Cleveland *et al.*, 2007; Nemergut *et al.*, 2010), and decreases in predicted oligotrophic groups (*Acidobacteria* and *Verrucomicrobia*) (Janssen *et al.*, 1997, 2002; Jones *et al.*, 2009; Bergmann *et al.*, 2011). As the *copiotrophic* hypothesis would suggest, the N-induced shifts in microbial community structure should yield corresponding shifts in the functional and metabolic potentials of the communities, and in this case, resulting in a change in decomposition rates.

In addition to changes in bacterial communities, N enrichment also altered EEA and soil temperature sensitivity, further providing evidence that the physiological capabilities of the communities shifted in response to the N addition. We observed consistent decreases in EEAs that are associated with decomposition of recalcitrant C. Across the 28 soils, the potential activities of BG, LAP, PER, and AP were all reduced with added N. Declines in LAP and PER activity suggest that N acquiring processes and N mining decrease under N amendments (Saiya-Cork *et al.*, 2002; Sinsabaugh *et al.*, 2002). Strong decreases in BG and AP activities, representing enzymes associated with cellulose breakdown and phosphorous acquisition, respectively, may be partially attributed to the strong declines in microbial biomass though also highlight a decline in decomposition processes (Sinsabaugh *et al.*, 2002). The reduction in soil temperature sensitivity with N enrichment suggests that the biochemical recalcitrance of the respired carbon was lower under N enrichment indicating that microbes were respiring more labile C and/or less recalcitrant C (Conant *et al.*, 2008; Craine *et al.*, 2010). The EEA data and temperature sensitivity data highlight physiological changes in the communities consistent with the *enzyme inhibition* and *N-mining* hypotheses, but given that neither measurement correlated well with soil factors or soil process changes, the physiological changes may be better explained by the *copiotrophic* hypothesis.

Quantifying ecosystem responses and identifying a clear mechanism underlying the decreases in decomposition rates can be difficult as each soil has a unique set of starting characteristics (e.g. distinct edaphic characteristics and microbial communities) that may have very different, unquantifiable impacts on soil responses (Zeglin *et al.*, 2007; McCrackin *et al.*, 2008). In this study, soil responses could partially be explained by initial soil N and available C, where lower initial nutri-

ent levels resulted in larger decreases in soil respiration and microbial biomass, similar to results reported by Cusack *et al.* (2010). Indeed N saturation will likely factor into microbial responses as demonstrated in above-ground studies where responses are rarely linear and the driving factors are difficult to pinpoint (Ågren & Bosatta, 1988; Aber *et al.*, 1998). Still, in this work, we observed consistent suppression of decomposition and predictable community shifts supporting our assertion that a wide-spread mechanism, or set of mechanisms, contributes to the observed effects of the N additions. Further, our results complement previous studies suggesting that increases in soil N increase soil C sequestration (Liu & Greaver, 2010).

Quantifying the responses of soils to N inputs has been a focus of soil ecology for over twenty years, especially with respect to decomposition rates (Fog, 1988). The *enzyme inhibition* and *N-mining* hypotheses may explain decreased microbial activity, but the concomitant shifts in bacterial community composition suggest that the N does not simply alter enzyme production by the extant microbial community, but also (directly or indirectly) leads to shifts in community composition (Ramirez *et al.*, 2010b; Fierer *et al.*, 2011). Previous work has hypothesized that functional classifications of the microbial community are appropriate when considering belowground process changes (Fontaine & Barot, 2005; Moorhead & Sinsabaugh, 2006; Strickland *et al.*, 2009). Even broad classifications, such as copiotrophic and oligotrophic can elucidate the ecological roles of different bacterial groups; though admittedly within each taxa there is likely a functional spectra (Pianka, 1970). While our work supports the *copiotrophic* hypothesis, where specific community shifts are responsibly for observed decreases in decomposition, we acknowledge that the mechanisms proposed in the *enzyme inhibition* and *N-mining* hypotheses may also partly account for the process-level shifts observed here or may be acting in conjunction with the *copiotrophic* hypothesis in ways that we could not determine here. Furthermore, we recognize that the soil microbial community does not solely consist of bacteria; additional work is needed to determine if fungal communities exhibit responses to N amendments that parallel the bacterial responses observed here.

Conclusions

Understanding the impacts of increases in anthropogenic N on belowground systems is of specific concern as soils are critical in cycling terrestrial nutrients and hold seemingly incalculable diversity (Pace, 1997; Gans *et al.*, 2005; Falkowski *et al.*, 2008; Canfield *et al.*, 2010). Results presented here provide evidence that, regardless

of the soil or ecosystem type in question, N increases elicit similar responses in bacterial communities and soil processes. Nitrogen enrichments appear to decelerate microbial mineralization of the SOC pool, effectively slowing down decomposition. These changes may lead to enhanced soil C sequestration under N addition (Liu & Greaver, 2010) via mechanisms involving specific shifts in the bacterial community and that are separate from just N-induced changes in plant productivity. This work highlights that microbial community shifts may be important in understanding soil carbon storage and provides an important step towards identifying the likely community shifts associated with N addition.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Soil respiration and microbial biomass measurements from each site for ambient (Amb) and N amended (N) soils.

Table S2. Relative abundance (percentage of sequences) of bacterial groups from each site for ambient (Amb) and N amended (N) soils.

Table S3. Pairwise *t*-test results comparing bacterial abundance between ambient and N amended soils.

Table S4. Mean soil extracellular enzyme activity at each site, log transformed ($n + 1$), units (nmol h⁻¹ g⁻¹) from both ambient (Amb) and N amended (N) soils.

Table S5. Pearson correlation coefficients between change in enzyme concentration and change in measured soil processes (soil respiration and microbial biomass) and soil factors.

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