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ORIGINAL ARTICLE

Comparative metagenomic, phylogenetic and physiological analyses of soil microbial communities across nitrogen gradients

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Terrestrial ecosystems are receiving elevated inputs of nitrogen (N) from anthropogenic sources and understanding how these increases in N availability affect soil microbial communities is critical for predicting the associated effects on belowground ecosystems. We used a suite of approaches to analyze the structure and functional characteristics of soil microbial communities from replicated plots in two long-term N fertilization experiments located in contrasting systems. Pyrosequencing-based analyses of 16S rRNA genes revealed no significant effects of N fertilization on bacterial diversity, but significant effects on community composition at both sites; copiotrophic taxa (including members of the *Proteobacteria* and *Bacteroidetes* phyla) typically increased in relative abundance in the high N plots, with oligotrophic taxa (mainly *Acidobacteria*) exhibiting the opposite pattern. Consistent with the phylogenetic shifts under N fertilization, shotgun metagenomic sequencing revealed increases in the relative abundances of genes associated with DNA/RNA replication, electron transport and protein metabolism, increases that could be resolved even with the shallow shotgun metagenomic sequencing conducted here (average of 75 000 reads per sample). We also observed shifts in the catabolic capabilities of the communities across the N gradients that were significantly correlated with the phylogenetic and metagenomic responses, indicating possible linkages between the structure and functioning of soil microbial communities. Overall, our results suggest that N fertilization may, directly or indirectly, induce a shift in the predominant microbial life-history strategies, favoring a more active, copiotrophic microbial community, a pattern that parallels the often observed replacement of *K*-selected with *r*-selected plant species with elevated N.
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Introduction

Ecosystems across the globe are receiving elevated levels of nitrogen (N): anthropogenic activities have doubled rates of N deposition and fixation compared with pre-industrial levels (Galloway *et al.*, 2004). Rates of N deposition already exceed 10 kg N $ha^{-1} yr^{-1}$ in regions of western Europe, southeast Asia and North America (Dentener *et al.*, 2006), and the average global rate of N deposition to terrestrial ecosystems is likely to increase by a factor of 2.5 over the next century (Lamarque *et al.*, 2005). Just as important, a large proportion of the global land surface is used for crop production where significant quantities of N (often >100 kg N $ha^{-1} yr^{-1}$) are applied directly to soils as fertilizer.

As a key factor impacting terrestrial ecosystems worldwide, the effects of elevated N inputs on biota and biogeochemical cycles have been the focus of many studies. In most systems, elevating N inputs increases plant productivity (LeBauer and Treseder, 2008), with corresponding changes in plant community composition and, in most cases, decreases in local plant species diversity (Clark *et al.*, 2007; Cleland and Harpole, 2010). It is also

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well-established that microbial taxa associated with specific components of the soil N cycle (for example, nitrifiers) often change in relative abundance when soils are amended with N. We know far less about how elevated N inputs will influence the structure, and associated functioning, of the entire belowground microbial community.

Recent studies have demonstrated N effects across a range of sites on both bacterial (for example, Campbell *et al.*, 2010; Wessen *et al.*, 2010) and fungal communities (for example, Frey *et al.*, 2004; Egerton-Warburton *et al.*, 2007). These shifts in the composition of soil microbial communities with N amendments are likely associated with shifts in the functional capabilities of these communities. Recent lab-based studies (for example, Strickland *et al.*, 2009; Fukami *et al.*, 2010) support this possibility by demonstrating that microbial communities are neither functionally redundant nor similar, with changes in their structure influencing rates of ecosystem-level processes. However, beyond the well-established direct effects of elevated N inputs on specific N cycling processes (Lu *et al.*, 2011), it remains to be tested whether changes in the abundances of microbial taxa with N fertilization translate to changes in community functioning. To test this possibility, one promising approach is to pair phylogenetic analyses with shotgun metagenomics, the direct sequencing of whole-community DNA to characterize the genomic attributes of microbial communities. However, shotgun metagenomic analyses are rarely applied to replicated, experimental studies of soil microbial communities because it is implicitly assumed that deep sequencing is required for comparative analyses of highly diverse communities.

We used a suite of complementary approaches to determine how the structure and functional capabilities of soil microbial communities respond to long-term N amendments. We analyzed soils from two experimental N gradients that both received N fertilizer as NH_4NO_3, but have contrasting soil and vegetation types. The phylogenetic structure of the soil bacterial communities was determined by barcoded pyrosequencing (at least 1000 16S rRNA gene sequences per sample), and the functional potentials of the communities were characterized using catabolic assays and shotgun metagenomic profiling (with $\approx$75 000 sequences per sample). We used this unique data set to test the overarching hypothesis that prolonged increases in N availability shift the composition of soil microbial communities, their catabolic capabilities and their metagenomes. Such shifts may be, directly or indirectly, related to the changes in microbial carbon dynamics that are typically observed in soils receiving experimental N additions (Treseder, 2008; Janssens *et al.*, 2010; Liu and Greaver, 2010), but the mechanisms underlying these changes in carbon dynamics remain largely undetermined.

Materials and methods

Sample collection

Soils were collected in July 2008 from two long-term N addition experiments: one located at the Cedar Creek Ecosystem Science Reserve (CC; Bethel, MN, USA) and one at the Kellogg Biological Station (KBS; Hickory Corners, MI, USA). The CC site was established in 1982 on successional grasslands. The KBS experiment was established in 2001 and is located in an agricultural field with a yearly rotating cropping system that is tilled at the end of each growing season. Both sites receive N fertilizer as NH_4NO_3, with the fertilizer applied either twice (CC) or once (KBS) per year. Detailed information on these two experiments is available online (http://www.cedarcreek.umn.edu and http://www.lter.kbs.msu.edu). Sample collection and processing methods have been described previously in Ramirez *et al.* (2010). For this study, we selected a subset of the soils included in that study for more detailed re-analysis. Specifically, we analyzed bulk soils from three N amendment levels per site (0, 34 and 272 kg N ha^{-1} yr^{-1} at CC; 0, 101 and 291 kg N ha^{-1} yr^{-1} at KBS), with soils collected from three replicate plots per N level (collecting and compositing six soil cores from randomly selected locations within each plot). Although no N fertilizer was applied to the control plots directly, these soils do receive N from atmospheric deposition (likely 10–40 kg N ha^{-1} yr^{-1}, Dentener *et al.*, 2006). Thus, we refer to these three N treatments as 'low', 'medium' and 'high' N levels. These levels were selected to represent current N deposition rates (the 'low' level), elevated atmospheric N deposition rates (the 'medium' level) and N amendment rates in intensively managed agricultural soils receiving direct fertilizer applications (the 'high' level). In total, 18 samples were included in this study (3 N levels $\times$ 2 sites $\times$ 3 replicate plots per N level per site). Characteristics of these sites and the collected soils are detailed in Supplementary Table S1, with soil properties and the effects of N on these properties discussed in detail in Ramirez *et al.* (2010). Plant biomass and composition at these sites have been described in Clark and Tilman (2008) and McSwiney and Robertson (2005).

Barcoded pyrosequencing of the 16S rRNA gene

We chose to focus solely on the composition and diversity of bacterial communities, because bacteria dominated the metagenomes in all 18 soils (see below) and archaea were rare in these soils ($<$3% of the 16S rRNA sequences; Bates *et al.*, 2011). To analyze the composition of the bacterial communities, we used the procedure described in detail in Bates *et al.* (2011) and Lauber *et al.* (2009). Briefly, we used the MoBio Powersoil kit to extract DNA from 0.25 g of each of the 18 soil samples, with the soils carefully homogenized before subsampling. Amplifications were conducted in triplicate using

the primer set 515f/806r that targets a region of the 16S rRNA gene that yields accurate phylogenetic information (Liu *et al.*, 2007) and should have few biases against any bacterial taxa (Bates *et al.*, 2011; Bergmann *et al.*, 2011). The forward primer also included a 454 adapter, and the reverse primer contained both a 454 adapter and a 12-bp error-correcting barcode sequence. All amplicons were cleaned and pooled in equimolar concentrations into a single tube before sequencing on a Roche 454 GS FLX using the Titanium chemistry. The full length of the targeted 16S region was sequenced (approximately 300 bp reads, including the barcode) and, after quality filtering, all samples were rarefied to 1000 sequences per sample before conducting downstream analyses. All sequence analyses were conducted using the QIIME pipeline (Caporaso *et al.*, 2010). Phylotypes were selected at the 97% sequence similarity level and the taxonomic identity was determined using the RDP scheme. Pairwise distances between communities were determined using unweighted Unifrac, a metric that measures the phylogenetic relatedness of whole communities and is well suited for comparing beta-diversity patterns between complex bacterial communities (Lozupone *et al.*, 2006, 2011).

Shotgun metagenomic analyses

To obtain sufficient DNA from each of the 18 soil samples for metagenomic analyses (that is, analyses of whole community DNA via random sequencing, not just sequencing of specific, targeted genes), DNA was extracted from the soils (six replicate extractions per soil) using the MoBio PowerSoil kit as described above with modifications to the elution step. Instead of using separate aliquots of solution C6 to elute the DNA from each spin filter, the eluate collected from one spin filter served as the eluent for the next spin filter. Eluent collected from the first spin filter (100 µl) was pipetted onto the second filter and incubated again for 5 min at room temperature before centrifugation. These steps were carried out until all spin filters for a given sample had been eluted. Using this approach, DNA yields were between 1.0 and 2.5 µg per soil, as quantified using the PicoGreen dsDNA kit. The 18 DNA samples (1.0 µg per sample) were prepared for sequencing by nebulization, followed by tagging with GS-FLX-Titanium Rapid Library MID Adapters Kit (454 Life Sciences, Branford, CT, USA) and sequenced with the Titanium LR70 chemistry on a Roche 454 Gene Sequencer. The run yielded 518 Mbp of data, 1.35 million reads in total, with an average read length of 384 bp.

Sequences were assigned to subsystem categories using the MG-RAST web-server (Meyer *et al.*, 2008), with a BLAST threshold of 1e-10 and a minimum read length of 50 bp. Only those reads that matched at least one subsystem category were included in downstream analyses. To estimate pairwise distances between metagenomes, the MG-RAST sequence assignments were first converted to percentages (% of assigned sequences per sample assigned to each category) with pairwise Euclidean distances calculated between each of the 18 samples.

Catabolic profiling

To compare the relative catabolic abilities of the microbial communities in each of the 18 soils, we used a catabolic profiling assay adapted from Degens and Vojvodic-Vukovic (1999). Briefly, 22 different organic carbon substrates (see list in Supplementary Table S4) were added individually to 4 g (dry wt. equivalent) of each soil. Net CO_2 accumulation was measured on an infrared gas analyzer after an incubation period at 20 °C of 5 or 24 h (for the more recalcitrant substrates). The 22 substrates were chosen to represent a range of substrates potentially available to soil microorganisms, and included more recalcitrant substrates (chitin, cellulose, humic acid and lignin) as well as those that are more labile (amino acids, proteins, carboxylic acids and sugars). Substrates were added at concentrations ranging from 15 to 100 mM in 8 ml of solution with concentrations chosen following Degens and Vojvodic-Vukovic (1999). Assays were conducted in duplicate, yielding 828 assays in total (18 soils × 22 substrates plus the 'water control' × 2 analytical repeats per substrate per soil). Substrate catabolic rates were calculated as the mean difference from the 'water controls' (those samples that only received water) for each substrate per soil combination. As with the metagenomic data, pairwise distances in the catabolic potentials of the 18 soils were calculated using the Euclidean distance metric after expressing catabolic rates as percentages (CO_2 production for a given substrate as a percentage of CO_2 production for all substrates combined).

Statistical analyses

Relationships between samples were visualized using principal coordinate analyses (PCoA) from pairwise distances (Euclidean distances for the metagenomic and catabolic profiling data, unweighted Unifrac distances for the 16S data). Using the PRIMER software package (PRIMER-E, Plymouth, UK), the significance levels for differences between sample categories were determined using analysis of similarity tests, with correlations between pairwise distances from different assays determined using Mantel tests (Clarke and Gorley, 2006). Taxonomic and catabolic diversity levels per sample were estimated using the Shannon index (H') as calculated from either phylotype relative abundances or percentages of CO_2 production from each substrate.

Results and discussion

Composition of the bacterial communities across the N gradients

We analyzed soils from three N amendment levels per site, with soils collected from three replicate

field plots per N level for a total of 18 samples. We obtained up to 3000 high-quality 16S rRNA gene sequences per sample, but all samples were rarefied to 1000 sequences per sample and thus one sample (CC23) was not included in any analyses of bacterial community structure due to insufficient sequence coverage. The soils from the Cedar Creek

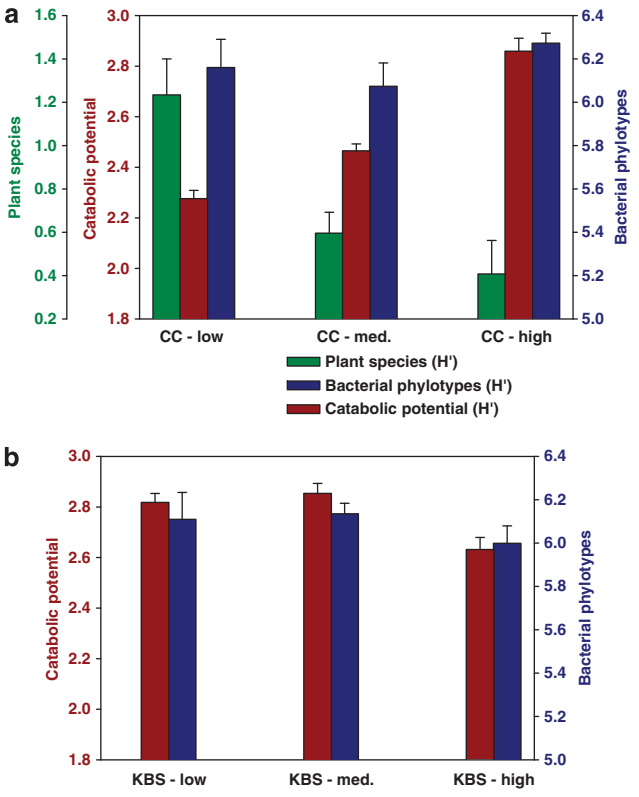

Figure 1 Comparison of taxonomic diversity (both bacteria and plants) and catabolic diversity for the plots studied at CC (**a**) and KBS (**b**). In all cases, diversity was calculated using Shannon's index (H'). KBS is a crop monoculture and thus plant species diversity is held constant across all nitrogen levels. Bars indicate 1 s.d.

successional grassland site (CC) had slightly higher levels of bacterial taxonomic diversity (average $H' = 6.18$, s.e.m. $= 0.04$) than the agricultural site at the KBS (average $H' = 6.05$, s.e.m. $= 0.03$) (Figure 1), but there was no significant correlation between N addition level and phylotype diversity at either site (Spearman's $r < 0.2$, $P > 0.2$ in both cases; Figure 1). In contrast, Campbell *et al.* (2010) found a decrease in bacterial diversity with N additions, suggesting that the effects of N amendments on bacterial diversity levels are variable and likely site-dependent.

The absence of any significant changes in bacterial diversity across the N gradient at CC contrasts with the plant community responses at this site, where plant diversity strongly decreased with increasing N additions (a pattern examined in detail in Clark and Tilman (2008) and references therein). This qualitative contrast between bacterial and plant diversity patterns across the N gradient suggests that the controls on plant diversity and bacterial diversity may not be the same and that one should not necessarily expect higher levels of bacterial diversity in soils with more plant species (Fierer and Jackson, 2006).

At both sites, the bacterial communities in the plots receiving the highest levels of N addition were significantly different from the communities receiving intermediate levels or no added N (Figure 2a). These high-N bacterial communities differed from those receiving lower amounts of N in having higher relative abundances of Proteobacteria (alpha-Proteobacteria at KBS and gamma-Proteobacteria at CC), Bacteroidetes and Actinobacteria, with lower relative abundances of Acidobacteria (Figure 3). These taxon-specific patterns were fairly consistent across the two sites, even though the sites are distinct with respect to site, soil and vegetation characteristics, a finding discussed in more detail in Ramirez *et al.* (2010). Bacteroidetes and many of the dominant actinobacterial and proteobacterial groups that

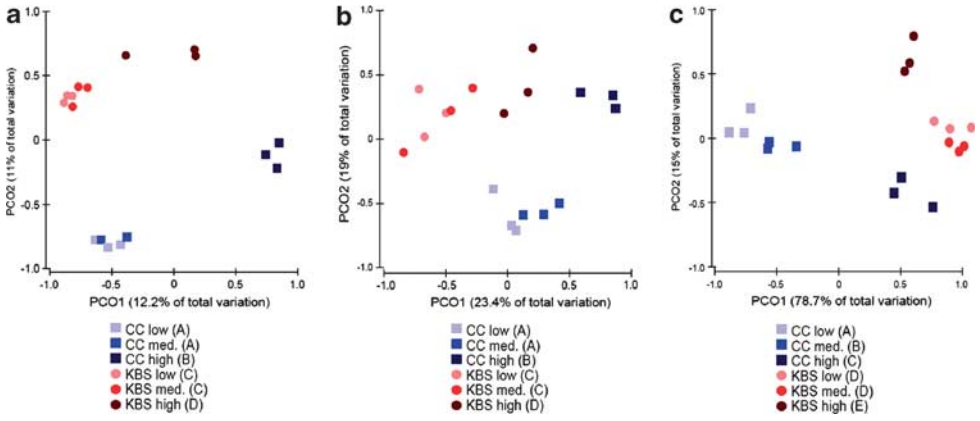

Figure 2 PCoA plots illustrating distances between microbial communities in individual samples. (**a**) Bacterial unweighted Unifrac distances (a measure of phylogenetic dissimilarity between communities). (**b**) Euclidean distances between metagenomes and (**c**) Euclidean distances between catabolic profiles. Letters within parentheses in the legends indicate groups of samples that were significantly different ($P < 0.01$).

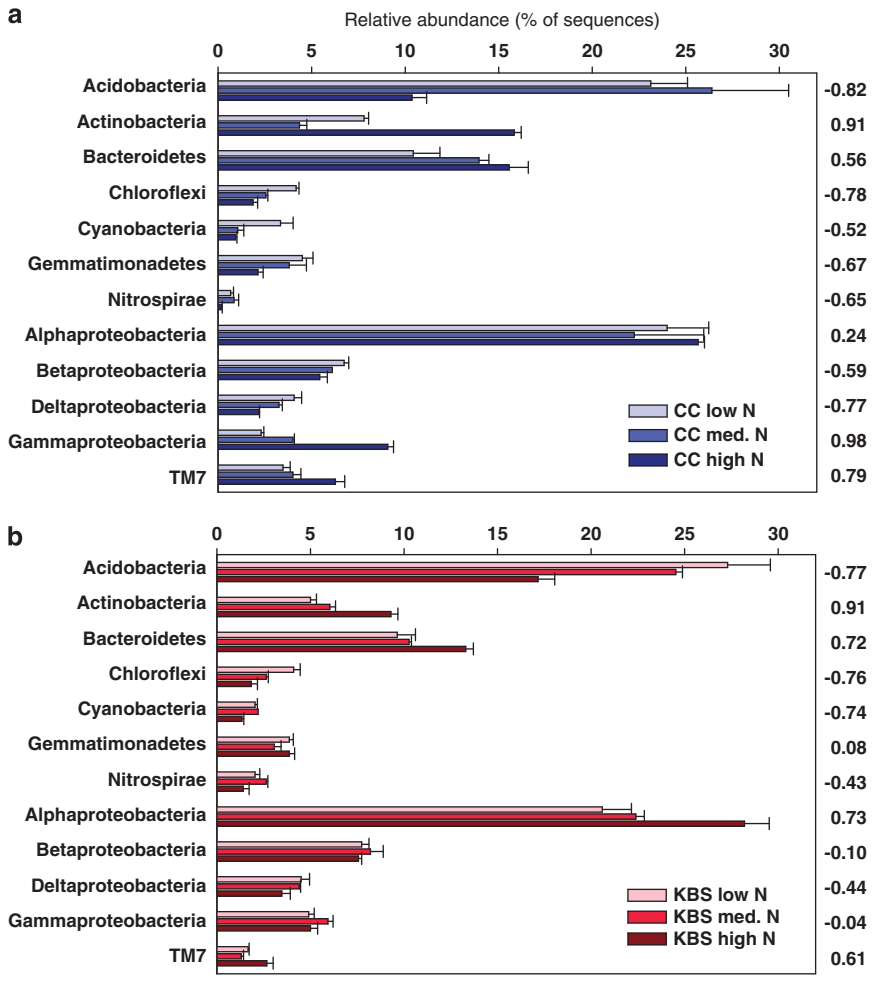

Figure 3 Changes in the relative abundances of bacterial taxa across the N gradients at the CC (**a**) and KBS sites (**b**). Only shown are those taxa with average relative abundances >1% at either of the two sites. The numbers on the right indicate the correlation (Spearman's ρ values) between N input rates and relative abundances calculated across all samples for that site. Bars indicate 1 s.d. For more details on the relative abundances of taxa in these soils, see Supplementary Table S2.

increased in relative abundance across the gradient (Figure 3) have been putatively identified as being copiotrophic taxa (those taxa that thrive in conditions of elevated C availability and exhibit relatively rapid growth rates; Fierer *et al.*, 2007; Eilers *et al.*, 2010). In contrast, there was a decline in the abundance of Acidobacteria, a group that is often considered to be oligotrophic (Fierer *et al.*, 2007; Davis *et al.*, 2011) with slower growth rates and, in all likelihood, the ability to metabolize nutrient-poor and recalcitrant C substrates. Although these copiotrophic–oligotrophic categories are clearly over-simplifications of the broad range of ecological attributes and life-history strategies exhibited by soil microbes, previous work does allow us to identify bacterial taxa that likely fall into these general categories and our results are consistent with community-level copiotroph–oligotroph shifts across the N gradients. This presumed shift in the dominant life-history strategies may be a direct result of the increase in N availability as we would expect copiotrophic taxa to have lower biomass C:N ratios and higher N demands than more oligotrophic taxa (Fontaine and Barot, 2005; Fierer *et al.*, 2007). Alternatively, the taxon-specific responses might be an indirect N effect arising from the increase in organic carbon availability associated with the increase in plant productivity in soils amended with N.

Similar taxon shifts to those we observed have been found in other studies examining bacterial community responses to N addition. For example, Campbell *et al.* (2010) noted that Acidobacteria were relatively less abundant and Proteobacteria (particularly gamma-Proteobacteria) were more abundant in fertilized plots, a pattern also observed in Wessen *et al.* (2010). This apparent consistency suggests that very high levels of N addition may have similar effects on the composition of bacterial communities across sites, but more comprehensive cross-site studies are required to test this hypothesis.

Characteristics of the metagenomes across the N gradients

Of the approximately 75 000 shotgun metagenomic sequences obtained per sample, only 25–35% of these sequences could be assigned to functional categories, percentages that are similar to those reported in other metagenomic surveys of environmental samples (Dinsdale *et al.*, 2008; Mou *et al.*, 2008). Although the metagenomic libraries were generated from bulk DNA extracted directly from the soil, the metagenomes are most likely reflective of the collective bacterial genomes as >98% of the ssu rRNA gene sequences identified from the metagenomic data were bacterial with, at most, 1–2 fungal ssu rRNA gene sequences detected compared to 70–130 bacterial ssu rRNA gene sequences per sample. Although the fungi can represent a significant proportion of belowground biomass (Strickland and Rousk, 2010), their minimal representation in the metagenome was likely a product of bacteria having higher rRNA gene concentrations per unit biomass.

The level of sequencing depth we used clearly resolved differences in metagenome composition; just as the soils from the two sites harbored distinct bacterial communities, they also differed in their metagenomic characteristics (Figure 2b). Also, within each site the characteristics of the metagenomes were significantly affected by N addition, with the soils receiving the highest levels of N having metagenomes that were significantly different from those receiving lower or no N inputs (Figure 2b). Although the metagenomes from the soils at the two sites were distinct, a subset of the specific gene categories that changed across the N gradients were similar between the two sites, suggesting that some of the soil metagenomic responses to N addition are similar across sites. The shifts in the relative abundances of specific gene categories observed across each of the N gradients (Figure 4) are consistent with the bacterial taxon responses, suggesting that the communities become more copiotrophic as N inputs increase. Indeed, we found that genes associated with DNA/RNA replication, electron transport and protein metabolism increased in relative abundance across the N gradients (Figure 4). We would expect all of these gene categories to be more common in copiotrophic than in oligotrophic bacteria (Fierer *et al.*, 2007), but more detailed genomic analyses of bacteria from across the oligotroph–copiotroph continuum is required to confirm this hypothesis. At both sites, genes associated with urea decomposition are relatively less abundant in the plots receiving the highest levels of N, suggesting that the communities at both sites become less reliant on organic forms of N as additional fertilizer is applied. With the relatively shallow sequencing effort conducted here, many possible genes or gene categories were not sufficiently abundant in the data set to determine accurately changes in their relative abundances across the experiments. This includes genes associated with specific processes likely to be of critical importance to carbon and nitrogen cycling in these systems (including genes associated with N_2 fixation, lignin degradation and ammonia oxidation). Deeper metagenomic sequencing will be required to capture these genes and to build a more comprehensive understanding of how the functional capabilities of the soil microbial communities are impacted by N fertilization.

Catabolic diversity across the N gradients

The catabolic profiling assays provide a very different perspective on the functional capabilities of these communities across the N gradients as we measured the potential for these communities to catabolize a range of organic carbon substrates. As expected, all substrates were catabolized in each of the 18 soils examined. However, the soils were not equivalent in their relative catabolic capabilities. At CC, the soils receiving the highest levels of N had greater catabolic diversity than the soils receiving less or no added N inputs, a pattern that was reversed at KBS (Figure 1). Nitrogen addition level also clearly influenced the catabolic capabilities of the microbial communities (Figure 2c). Just as with the phylogenetic and metagenomic patterns (Figures2a and b, respectively), soils receiving the highest levels of N had significantly distinct catabolic capabilities from the unamended soils (Figure 2c), with minimal variability between replicate plots. However, the specific substrates that were favored at high N levels versus lower N levels were not the same at the two sites, that is, the shifts in the relative rates at which individual substrates were catabolized were not consistent across the two gradients (Figure 5). This may partly reflect phylogenetic or physiological responses of the microbial communities to differences in the amounts and/or types of organic C substrates available at each site across the N gradients (Degens *et al.*, 2000; Frey *et al.*, 2004). Alternatively, some of the catabolic shifts described in Figures 2c and 5 could be related to shifts in substrate use efficiencies or shifts in the fungal communities, which were poorly represented in the metagenomes, but could represent a significant portion of the active biomass pools (Strickland and Rousk, 2010). Advances in understanding links between microbial community structure and function in soils clearly relies on the development of methods to independently assess the functioning of bacterial and fungal assemblages in intact microbial communities or molecular tools that permit simultaneous characterization of the whole community at resolutions proportional to bacterial and fungal activities.

Methodological considerations

As demonstrated in this and other studies, metagenomics provides a powerful tool for studying the

a

Subsystem Hierarchy 1	Subsystem Name	Subsystem #	Low N			Med. N			High N			Rho	Mean %
			A22	A45	A54	D8	D23	D38	H17	H40	H52		
Respiration	Ubiquinone-cytochrome reductase complexes	155	-0.3	-0.4	-0.4	-0.8	-0.5	-1.3	1.0	1.6	1.2	0.91	1.2
Respiration	F0F1-type ATP synthase	154	-0.1	-0.4	-1.2	-0.4	-0.8	-0.8	1.3	0.8	1.6	0.91	0.3
Cofactors, Vitamins	Lipoic acid metabolism	96	-0.9	-0.5	-1.1	-0.7	-0.4	-0.2	1.2	1.6	1.1	0.97	0.1
Protein Metabolism	tRNA aminoacylation	139	-0.9	-0.7	0.1	-0.6	-0.9	-0.7	0.7	1.4	1.6	0.90	4.3
Protein Metabolism	General Secretion Pathway	143	-0.9	-1.2	-0.3	-0.1	0.4	-1.0	1.9	0.4	0.8	0.81	0.5
DNA Metabolism	DNA structural proteins	106	-0.3	-0.8	-1.1	-0.4	0.1	-1.0	0.6	1.4	1.6	0.90	0.4
DNA Metabolism	DNA-replication	104	-0.9	-1.4	-0.7	-0.8	0.0	0.6	1.5	1.2	0.3	0.80	1.4
RNA Metabolism	ATP-dependent RNA helicases	145	-0.7	-0.1	-0.1	-0.3	-1.0	-0.4	1.9	1.5	-0.8	0.83	1.5
Nucleosides/Nucleotides	Purine conversions	128	-1.4	-0.9	-0.8	0.3	0.3	-0.6	0.6	0.7	1.8	0.62	1.1
Amino Acids and Derivatives	Urea decomposition	2	2.0	0.5	-0.1	-0.8	0.5	0.6	-0.8	-0.9	-1.0	-0.70	1.2
Motility and Chemotaxis	Bacterial Chemotaxis	125	1.1	-0.4	0.6	1.4	0.5	-0.6	-1.7	-0.1	-0.9	-0.67	1.3
Motility and Chemotaxis	Bacterial motility:Gliding	124	-0.1	0.3	1.1	1.7	-0.7	0.6	-0.7	-0.8	-1.3	-0.70	0.6
Aromatic Cmpd. Metabolism	Phenylpropanoid compound degradation	120	0.2	2.0	-0.4	-0.2	0.7	0.7	-1.1	-1.1	-0.6	-0.73	0.6
Cofactors, Vitamins	Fe-S cluster assembly	93	1.6	1.1	0.0	0.7	0.0	-0.1	-0.8	-1.1	-1.3	-0.84	0.1
Regulation and Cell Signaling	cAMP signaling	153	1.1	1.1	1.1	-0.1	0.2	0.1	-1.4	-1.4	-0.6	-0.91	2.5
Clustering-based Subsystems	Tricarboxylate transporter	80	0.4	1.0	0.6	0.5	0.4	0.8	-1.9	-1.1	-0.8	-0.94	0.5

b

Subsystem Hierarchy 1	Subsystem Name	Subsystem#	Low N			Med. N			High N			Rho	Mean %
			201	301	401	204	304	404	209	309	409		
Respiration	Ubiquinone-cytochrome reductase complexes	155	-1.3	-0.1	-0.5	-0.4	-0.6	-0.2	-0.2	1.3	2.0	0.76	1.2
Cofactors, Vitamins	Folate Biosynthesis	94	-0.8	0.0	-1.5	0.1	-1.1	-0.1	0.9	1.2	1.3	0.86	1.5
Protein Metabolism	Lipoprotein Biosynthesis	138	-1.7	-0.3	0.2	-0.2	-1.0	-0.1	1.2	1.6	0.3	0.74	0.1
RNA Metabolism	ATP-dependent RNA helicases	145	-1.0	-0.6	0.2	-1.2	-0.5	0.6	-0.1	2.0	0.7	0.62	1.6
Cell Wall and Capsule	Cellulosome	31	-0.7	-1.1	-0.4	-0.2	-0.3	-0.5	-0.1	1.7	1.7	0.82	0.1
Potassium metabolism	Potassium-efflux system	135	-0.9	-0.1	-1.0	-0.8	0.4	-0.5	0.9	0.0	2.1	0.74	0.9
Clustering-based subsystems	Pyrimidine biosynthesis	62	-1.7	0.3	-1.5	-0.2	0.2	0.5	0.8	0.2	1.4	0.74	0.1
Membrane Transport	Fructose and Mannose Inducible PTS	115	-0.9	-0.7	-0.2	0.2	0.1	-1.5	0.0	1.2	1.7	0.71	0.1
Carbohydrates	Chitin and N-acetylglucosamine utilization	11	-0.2	-0.2	-0.4	-1.0	-1.1	-0.3	-0.1	1.7	1.7	0.67	0.2
Amino Acids and Derivatives	Urea decomposition	2	1.9	-0.7	0.7	0.2	-0.3	0.7	-1.3	0.1	-1.1	-0.61	1.3
Secondary Metabolism	Phytoalexin biosynthesis	163	1.5	1.0	1.0	-0.7	-0.4	-0.6	-1.5	0.2	-0.5	-0.69	0.1
Nucleosides and Nucleotides	Pyrimidine utilization	129	1.0	0.4	0.7	-0.2	0.3	0.7	-2.2	0.3	-0.9	-0.73	0.4
Clustering-based subsystems	Tricarboxylate transporter	80	-0.4	1.1	0.3	0.9	0.5	1.1	-1.2	-1.4	-0.9	-0.74	0.7
Cell Wall and Capsule	Lipid A-Ara4N pathway (Gram negative)	29	0.9	1.6	0.2	-0.1	0.7	-0.4	-0.6	-1.7	-0.7	-0.83	1.2
Cell Wall and Capsule	Rhamnose containing glycans	27	1.4	1.0	1.2	-0.6	0.4	-0.6	-0.4	-1.0	-1.3	-0.85	1.2
Virulence	Resistance to fluoroquinolones	189	1.9	0.4	0.8	0.2	-0.4	0.2	-0.8	-0.9	-1.4	-0.88	3.2

Figure 4 Selected gene categories that changed in relative abundance across the N gradient, as estimated from the metagenomic data. For a full list of all gene categories and their relative abundances in each sample, see Supplementary Table S3. Data are expressed as *z*-scores, with red colors indicating higher *z*-scores (higher relative abundance compared with the mean for that gene category). (**a**, **b**) Results from the CC and KBS sites, respectively, with the header row indicating the specific plot numbers. Only those gene categories that were well correlated with nitrogen addition rates (ρ values >0.6 or <-0.6) are shown here. ρ Values for each gene category (relating relative gene abundances to N addition rates) are reported to the right of the heatmap along with the details on the mean percentage of assignable sequences in each gene category.

a		Low N			Med. N			High N				
	Substrate	A22	A45	A54	D8	D23	D38	H17	H50	H52	Rho	Mean %
	D-Glucose	-1.0	-0.8	-0.9	-0.5	-0.3	-0.4	1.4	1.3	1.2	0.99	2.39
	L-Glutamic acid	-0.7	-0.8	-0.8	-0.5	-0.5	-0.5	1.3	1.6	1.0	0.99	2.57
	L-Glutamine	-0.7	-0.7	-1.0	-0.4	-0.3	-0.6	1.7	1.3	0.9	0.97	1.94
	D-Mannose	-0.9	-0.7	-1.1	-0.1	-0.5	-0.6	1.2	1.6	0.9	0.97	2.44
	L-Glycine	-0.8	-0.5	-1.1	-0.7	-0.2	-0.5	0.7	1.8	1.2	0.94	0.99
	Sucrose	-0.9	-0.8	-0.8	-0.5	-0.4	-0.3	0.4	1.7	1.6	0.93	2.67
	D-Fructose	-0.9	-0.8	-1.0	-0.2	-0.2	-0.5	0.6	2.0	1.0	0.92	2.88
	Palmitic acid	-0.2	-0.5	-1.5	-0.2	-0.3	-0.9	1.5	0.8	1.3	0.91	0.80
	Cellulose	-1.3	-1.0	-0.8	-0.1	-0.8	0.7	1.2	1.1	1.0	0.85	1.21
	Oxalate	-0.6	-0.6	-0.6	-0.6	-0.3	0.2	-0.5	2.3	0.8	0.69	0.30
	L-Lysine	-0.6	-0.5	-1.5	-0.4	0.5	0.0	1.7	1.3	-0.5	0.65	1.24
	Lignin	-0.9	-1.5	-0.9	-0.1	0.7	0.4	-0.1	1.7	0.8	0.64	1.47
	Acetate	1.0	1.3	-0.5	0.2	0.6	0.3	0.0	-1.2	-1.8	-0.73	7.79
	DL-Malic acid	0.4	-0.2	1.4	0.4	0.5	0.8	-0.9	-1.9	-0.4	-0.82	9.90
	L-Histidine	1.3	0.6	0.6	0.7	0.2	0.3	-1.7	-1.3	-0.6	-0.93	5.73
	Citrate	1.0	0.8	0.9	0.5	0.5	0.1	-1.1	-1.6	-1.1	-0.97	26.90
	Oleic acid	1.0	1.0	0.5	0.6	0.4	0.4	-1.3	-1.3	-1.3	-0.99	6.03
b		Low N			Med. N			High N				
		201	301	401	204	304	404	209	309	409	Rho	Mean %
	Oxalate	-0.1	-1.1	-0.7	-0.8	-0.2	-0.8	0.8	1.6	1.3	0.88	1.91
	Citrate	0.0	-0.6	-1.0	-0.6	-0.8	-0.8	1.3	1.1	1.3	0.87	16.90
	Chitin	-0.7	-0.2	0.2	-0.7	-1.1	-0.8	0.6	2.1	0.5	0.68	9.80
	D-Mannose	0.5	-0.4	0.3	0.8	1.3	0.8	-1.1	-1.7	-0.6	-0.67	3.40
	L-Glycine	0.9	0.1	0.7	-0.3	1.1	0.7	-0.2	-1.8	-1.2	-0.76	1.17
	L-Glutamine	0.0	0.6	0.6	0.1	1.2	1.1	-0.8	-1.8	-0.9	-0.77	2.47
	Oleic acid	0.2	1.3	1.1	-0.2	0.2	0.1	-2.1	-0.1	-0.6	-0.78	3.69
	Acetate	0.1	1.0	0.2	1.2	0.3	0.9	-1.5	-0.9	-1.2	-0.80	1.03
	L-Glutamic acid	1.0	0.3	0.7	-0.1	0.5	0.9	-1.2	-2.0	-0.1	-0.81	3.68
	Lactate	0.0	1.3	0.3	1.3	0.6	0.0	-1.3	-1.2	-1.0	-0.81	2.71
	L-Histidine	1.2	0.4	0.3	1.0	0.6	0.0	-1.7	-1.3	-0.5	-0.85	2.12
	Autolysed yeast	1.0	1.1	0.3	0.7	-0.1	0.5	-0.8	-1.7	-1.2	-0.92	9.59

Figure 5 Selected substrates for which the relative catabolic rates changes across the N gradients, as estimated from the catabolic profiling data. For a full list of all substrates and their relative catabolism in each sample, see Supplementary Table S4. Data are expressed as *z*-scores, with red colors indicating higher *z*-scores (higher relative abundance compared with the mean for that gene category). (**a**, **b**) Results from the CC and KBS sites, respectively, with the header row indicating the specific plot numbers. Only those substrates with catabolic rates that were well correlated with N addition rates (ρ values >0.6 or <-0.6) are shown here. ρ Values for each substrate (relating catabolic rates to N addition rates) are reported to the right of the heatmap along with the mean relative catabolic rate (expressed as a percentage of the catabolic rate for all substrates combined for each sample).

functional capacities of individual microbial communities. However, the wider application of metagenomic tools to compare microbial community attributes requires sufficient statistical replication to confirm the validity of any observed patterns. Without replicated metagenomic data, many of the observed differences in metagenome characteristics across sample categories or experimental treatments are merely anecdotal (Prosser, 2010). Field replication is missing from nearly all published metagenomic studies owing, in part, to the assumption that deep sequencing of complex microbial communities is required for robust comparisons of metagenomes. Here we show that this is not the case. Even at a relatively shallow sequencing depth of $<100\,000$ reads per sample, we were able to differentiate the metagenomes across sites and experimental treatments, identifying key gene categories that change in relative abundance across the N gradients. For studies where there is interest in genes that are relatively rare (for example, studies looking for specific genes involved in ammonia

oxidation or lignin breakdown) or interest in the detailed phylogenetic structure of the communities, the metagenomic approach used here would be nearly useless as far deeper sequencing would be required to document changes in the relative abundances of relatively rare genes or taxa across samples. However, as has been demonstrated previously for 16S rRNA-based surveys (Lauber *et al.*, 2009), conducting shallower metagenomic analyses on more samples allows us to detect community-level ecological patterns and confirm their significance. Indeed, our data suggest that advances in the understanding of whole microbial communities, especially with regards to broadly dispersed physiological functions (such as those involved in general C metabolism), are feasible with relatively shallow and replicated metagenomic assessments.

Concordance between phylogenetic, metagenomic and functional changes in the communities

All three assays showed significant changes in microbial community characteristics across both the KBS and CC N gradients (Figure 2). Interestingly, the phylogenetic, metagenomic and catabolic responses were significantly correlated with one another in nearly all cases (Table 1), suggesting some degree of correspondence between these very different metrics of microbial community characteristics in how they are impacted by N amendments. However, it is important to note that the phylogenetic and metagenomic shifts were most pronounced at the highest N levels, with the intermediate N addition levels at both sites yielding communities that were not significantly different from the control plots. This observation is surprising because even the intermediate N levels (34 and 101 kg N ha^{-1} yr^{-1} at CC and KBS, respectively) are higher than most soils likely receive from chronic N deposition in all but the most heavily polluted regions (Dentener *et al.*, 2006). In addition, these intermediate levels of N did lead to pronounced increases in plant productivity at both sites and changes in plant community composition at CC (McSwiney and Robertson, 2005; Clark and Tilman, 2008), suggesting that, in general, plant communities may be more sensitive to N additions than soil bacterial communities. It is possible that the direct effects of N fertilization on soil bacterial communities are subtle in all but those soils (like agricultural soils) that receive large, direct inputs of N fertilizer. The meta-analysis of field and laboratory N amendment studies conducted by Treseder, 2008 supports this hypothesis, as the largest effects of N addition on microbial biomass and activity were observed at the highest fertilization rates.

Table 1 Spearman's correlation (ρ values), determined via Mantel tests, relating pairwise Unifrac distances (phylogeny) to metagenomic distances to catabolic profile distances

	Cedar Creek	*KBS*	*Both sites combined*
Phylogeny, metagenome	0.84*	0.48**	0.63*
Catabolic profile, phylogeny	0.73**	0.76**	0.39**
Catabolic profile, metagenome	0.78*	0.4***	0.35**

All correlations were significant, *$P<0.001$, **$P<0.01$, ***$P<0.05$.

Long-term environmental manipulations cannot be used to tease out causation from correlation in microbial composition and functioning. However, such manipulations do provide observations that inform the development and testing of theory and, in this regard, the apparent predictability of the relationships between the phylogenetic and functional community properties found here is intriguing. Indeed, we found that phylogeny is correlated with metagenomic characteristics, suggesting that both phylogenetic and metagenomic information could be used to predict relative shifts in the catabolic characteristics of soil communities. These results add to the growing body of evidence that accurate prediction of how some ecosystem processes will respond to disturbance requires the explicit incorporation of soil microbes into ecosystem models (Allison and Martiny, 2008). That is, soil microbial communities cannot be assumed to be functionally redundant nor similar. Future work is required to investigate whether relationships between microbial community phylogeny, metagenome and catabolic functioning are generally observed at local scales in response to environmental perturbation and whether these shifts influence ecosystem processes in a manner not predictable from abiotic environmental parameters alone.

As evident from cross-biome (Dinsdale *et al.*, 2008) and cross-taxon (Muegge *et al.*, 2011) studies, shifts in the relative abundances of specific lineages generally lead to corresponding shifts in metagenome characteristics. We show that this same pattern holds at local scales in response to environmental perturbation. Our data are consistent with observations that major bacterial groups show coherent ecological and genomic characteristics (Philippot *et al.*, 2010), and the often strong agreement between 16S rRNA distance and gene content conservation (Konstantinidis and Tiedje, 2005) or similarity in functional pathways (Chaffron *et al.*, 2010). There will obviously be many gene categories for which shifts in relative abundances are not predictable from phylogenetic information (for example, those genes readily transferred horizontally) and we lack basic information on the genomic properties of many soil taxa. However, because metagenomic analyses are not trivial, future studies clearly have to ascertain whether (or in what circumstances) metagenomic sequencing provides 'added value', or if metagenomic shifts could largely be predicted from phylogenetic information. For example, large deviations of measured metagenomes from the baseline prediction generated by 16S

rRNA/reference genome analysis (after appropriate corrections for genome size and phylogenetic distance; Konstantinidis and Tiedje, 2005; Chaffron *et al.*, 2010) might imply ecologically important features of local habitat adaptation.

Possible mechanisms underlying the observed patterns
Both the phylogenetic and metagenomic data suggest that the N additions may result in a shift from a more oligotrophic bacterial community to one that is more copiotrophic, just as fertilizer often increases the abundance of *r*-selected 'weedy' plant species over longer-lived, slower-growing *K*-selected plant species (Suding *et al.*, 2005; Cleland and Harpole, 2010). This overall shift in life-history strategies, with copiotrophic microbes increasing in relative abundance in heavily fertilized soils, may explain the widely observed decrease in soil microbial respiration rates and biomass with added N (Treseder, 2008; Janssens *et al.*, 2010; Liu and Greaver, 2010), even though N additions typically increase both plant productivity and the quality of plant litter inputs to soil (Chapin *et al.*, 1986; Suding *et al.*, 2005). Copiotrophic taxa may be expected to have higher rates of activity per unit biomass, higher turnover rates and higher substrate use efficiencies yielding a smaller standing microbial biomass pool with faster growth rates. However, copiotrophic taxa should also be less likely to access more recalcitrant pools of C (Fontaine *et al.*, 2004; Fierer *et al.*, 2007; Miki *et al.*, 2010), reducing overall rates of heterotrophic respiration as the communities preferentially consume more labile substrates over the more recalcitrant organic C substrates that make up the bulk of the soil organic C pool (akin to eating dessert and ignoring the other meal courses) (Fontaine and Barot, 2005). This oligotroph–copiotroph switch may complement other hypotheses posed to explain the inhibition of microbial activities with N fertilization, namely the N mining hypothesis (Craine *et al.*, 2007) and the extracellular inhibition hypothesis (Fog, 1988; Sinsabaugh, 2010). Of course, the validity of our hypothesis remains undetermined and further studies are required to determine if this presumed oligotroph–copiotroph switch is a more universal response to N additions and if the hypothesis does indeed explain the soil microbial responses to N amendments.

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