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**Journal**

Applied and Environmental Microbiology, 81(20)

**ISSN**

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**Publication Date**

2015-10-15

**DOI**

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Peer reviewed

# Nitrogen Cycling Potential of a Grassland Litter Microbial Community

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Because microorganisms have different abilities to utilize nitrogen (N) through various assimilatory and dissimilatory pathways, microbial composition and diversity likely influence N cycling in an ecosystem. Terrestrial plant litter decomposition is often limited by N availability; however, little is known about the microorganisms involved in litter N cycling. In this study, we used metagenomics to characterize the potential N utilization of microbial communities in grassland plant litter. The frequencies of sequences associated with eight N cycling pathways differed by several orders of magnitude. Within a pathway, the distributions of these sequences among bacterial orders differed greatly. Many orders within the *Actinobacteria* and *Proteobacteria* appeared to be N cycling generalists, carrying genes from most (five or six) of the pathways. In contrast, orders from the *Bacteroidetes* were more specialized and carried genes for fewer (two or three) pathways. We also investigated how the abundance and composition of microbial N cycling genes differed over time and in response to two global change manipulations (drought and N addition). For many pathways, the abundance and composition of N cycling taxa differed over time, apparently reflecting precipitation patterns. In contrast to temporal variability, simulated global change had minor effects on N cycling potential. Overall, this study provides a blueprint for the genetic potential of N cycle processes in plant litter and a baseline for comparisons to other ecosystems.

Microorganisms play a key role in the decomposition of terrestrial plant litter (1–3), a process that controls the balance of plant carbon (C) released into the atmosphere as $CO_2$ with that stored in the soil. Less often considered is the role that litter microorganisms play in nitrogen (N) cycling. The N available to microorganisms degrading plant litter comes from several sources. One source is organic N bound in plant tissues and microorganisms. Because the average C/N ratio is much higher in plant litter than in microbial decomposers, N availability is thought to limit litter decomposition (4–6). Fungal hyphae can further translocate N from the soil into plant litter (7). And in some ecosystems, atmospheric deposition of inorganic N from human-driven nitrogen oxide ($NO_x$) emissions can also be an important source (8–11).

Microbes can rapidly alter the forms of N in plant litter through a variety of different N cycle pathways, and these changes in N availability can feed back to influence overall ecosystem functioning (12, 13). During decomposition, bacteria utilize N in both assimilatory and dissimilatory pathways. Assimilatory pathways require energy and lead to the conversion of inorganic N to organic N in microbes (e.g., utilizing N for protein, nucleic acid, and cellular component assembly). Dissimilatory pathways use N compounds to provide energy to microbes. Thus, the pathways by which microbes use N affect the fate of N in the ecosystem, specifically whether it is converted into microbial biomass or whether it is converted to new forms and released into the environment. For example, through the ammonia assimilation pathway, organic N in plant litter may be used by microorganisms for growth (14). Through the denitrification pathway, N may be removed from the plant litter-soil system and lost to the atmosphere as $N_2O$ or $N_2$ (15).

Environmental conditions also influence plant litter decomposition and therefore N cycling (2, 16–18). In particular, moisture availability is known to be important to plant litter decomposition

rates (19, 20). Climate models predict decreased precipitation in the southwestern United States in the next century (21), a change that may also alter decomposition indirectly via changes in plant composition and litter quality in grasslands ecosystems (22, 23). In addition, N availability also plays a role in plant litter decomposition (17, 24). N loading from anthropogenic sources (estimated at 20 to 45 kg ha$^{-1}$ year$^{-1}$ in Southern California) is expected to continue to increase (25) and to affect plant communities and ecosystem functioning (26–28).

To investigate the effects of such changes on grasslands, an experiment manipulating nitrogen and precipitation was established in 2007 at Loma Ridge in Irvine, CA (23, 29). Previous work indicates that both drought and added N, as well as seasonal and annual climate variations, affect the microbial composition of litter (30, 31). Moreover, these shifts in microbial composition have functional consequences (32). A reciprocal transplant experiment demonstrated that microbial communities altered by drought had lower rates of plant litter decomposition even under ambient en-

vironmental conditions (22). Further, taxonomic changes in the litter community were correlated with changes in the frequency of glycoside hydrolases, genes responsible for C utilization (31).

Given the intertwined nature of N and C cycling during litter decomposition, we investigated the genetic potential of N utilization in plant litter microbial communities. We analyzed metagenomic samples to identify genes for N cycling in microbial communities from the Loma Ridge experimental plots. We focused our work on prokaryotes, because they are the most abundant organisms on the litter (33), but we also quantified sequences associated with Fungi. Although metagenomic sequences indicate only the functional potential of a community, they provide a holistic description of potential N utilization across many pathways in the N cycle. Specifically, we asked the following questions. (i) How do the abundance, taxonomic composition, and diversity of N cycling genes differ among pathways? (ii) Within a pathway, do these patterns differ over time? (iii) Does N cycling potential respond to global change manipulations (drought and N addition)?

## MATERIALS AND METHODS

**Field experiment and DNA sequencing.** The Loma Ridge global change experiment, Irvine, CA (33°44′N, 117°42′E; elevation, 365 m), was established in 2007 with precipitation and N manipulations (22, 31). The precipitation manipulation reduced the amount of water by 50%, creating drought plots. Surface soil moisture was significantly lower in drought plots than in ambient treatment plots (30). The nitrogen addition plots received 60 kg CaNO₃ ha⁻¹ year⁻¹. Previous studies at the site have shown that litter from nitrogen addition plots contained significantly more nitrogen and lower concentrations of carbon substrates, such as cellulose, hemicellulose, and lignin, than control plots (22). Furthermore, the level of plant-available nitrogen in soil was significantly lower in drought plots than in control or nitrogen addition plots (23). Drought treatment reduced, and nitrogen addition increased, decomposition rates, as measured by mass loss, over those in control plots (22).

The climate in this Southern California grassland ecosystem is semiarid, with mean annual precipitation of 325 mm, most of which occurs between October and April. Beginning in 2010, plant litter samples were taken seasonally for 2 years in control, drought, and N addition plots. Sampling dates were 14 April, 20 August, and 17 December 2010; 29 February, 10 June, 21 September, and 14 December 2011; and 12 March 2012.

For each of the three treatments (ambient, drought, and N addition), eight plots were sampled at 8 time points (for a total of 192 samples). To balance replication with sequencing costs, we pooled equal concentrations of DNA extracts from four plots undergoing the same treatment; extracts from the same plots were pooled on all dates (31). Thus, six metagenomic libraries (two replicate libraries per treatment) were sequenced at 8 time points, for a total of 48 libraries. Although two replicates per treatment is not ideal and limits our statistical power to test for treatment effects, pooling four independent plots into two composite "replicates" provides improved mean estimates (such as the mean abundance of a particular gene) over those obtained by sampling only two plots (34). Further, sampling over 8 time points also gives us additional statistical power to test for treatment effects by reducing the error variance (35).

Metagenomic libraries were prepared using a TruSeq library kit (part 15026484, revision C, July 2012; Illumina), were sequenced with an Illumina HiSeq 2000 system (100-bp paired ends), and yielded 107.4 Gbp (which passed quality control [QC]) (31). In total, 46 libraries were analyzed, of which 2 libraries were excluded due to low sequence counts (April 2010, reduced precipitation [MG-RAST accession no. 4511045]; August 2010, increased N deposition [MG-RAST accession no. 4511064] [see Table S1 in the supplemental material]). Sequences were uploaded onto the MG-RAST server, where 53% of the sequences were annotated (31).
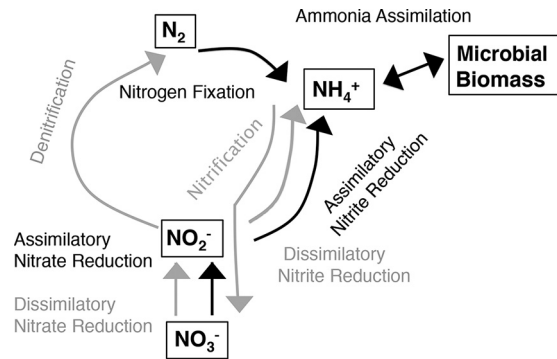


FIG 1 Nitrogen cycling pathways considered in this study. Pathways in gray are categorized as dissimilatory, and pathways in black are categorized as assimilatory.

Taxonomic assignment for the metagenomic libraries was performed by MG-RAST (36) using the KEGG database (37), and annotations were downloaded using the MG-RAST API, version 3.2 (36). Taxonomic annotation was considered for sequences with an E value of $\leq 10^{-5}$ (31). Each sequence was assigned to the closest related species in the database; however, in order to be conservative in our taxonomic assignment, we report bacterial taxonomy at the corresponding order level.

**N cycle pathway identification.** Eight N cycling pathways were defined for this analysis: nitrification (number of genes targeted [$n$], 2), N fixation ($n = 20$), denitrification ($n = 20$), dissimilatory nitrate reduction to nitrite [DNRA($NO_3^- \rightarrow NO_2^-$)] ($n = 9$), dissimilatory nitrite reduction to ammonia [DNRA($NO_2^- \rightarrow NH_3$)] ($n = 4$), assimilatory nitrate reduction to nitrite [ANR($NO_3^- \rightarrow NO_2^-$)] ($n = 2$), assimilatory nitrite reduction to ammonia [ANR($NO_2^- \rightarrow NH_3$)] ($n = 2$), and ammonia assimilation ($n = 10$) (Fig. 1; see also Table S2 in the supplemental material). Although nitrification includes both ammonia oxidation and nitrite oxidation, we combined them here because of their low level of representation in the samples. Finally, we excluded the anammox pathway from our analyses. The functional genes for this pathway are poorly represented in genome databases (and currently are not defined in the KEGG or SEED database). After some analysis, we were not confident in our ability to distinguish between genes in the annamox pathway and related non-annamox genes.

To detect genes in the eight pathways, we first identified the corresponding genes in the KEGG (37) and SEED (38) databases. For the KEGG database, KEGG Orthology (KO) numbers (37) were obtained from the Functional Ontology Assignments for Metagenomes (FOAM) database (39). For the SEED database, FIGfam numbers were obtained for the N fixation and denitrification pathways. Next, MD5 identifiers (IDs) for each KO and FIGfam number sequence were retrieved from the nonredundant M5nr database (40). Finally, we searched for the MD5 IDs in our samples annotated by the MG-RAST server. For each pathway, we checked the functional assignments of a subset of sequences using the BLAST algorithm against the MicrobesOnline database. This allowed us to compare the annotations and genome contexts of those hits in fully sequenced genomes (41).

**Data standardization and statistical analyses.** We first compared the relative abundances of prokaryotic and fungal reads across the different N cycle pathways. To do this, we first took the average number of sequences associated with each pathway across all 46 samples and then divided it by the number of different genes searched in the pathway. While we recognize that it is common to standardize by gene length as well (see, e.g., reference 42), the variation in copy number and gene length for all 80 genes across a wide range of microbial taxa makes this infeasible. Conveniently, our results show that the average abundances of microbial taxa differ between most pathways by orders of magnitude, while gene lengths usually differ to a lesser degree. This suggests that the relative differences observed would likely persist even with such standardization.
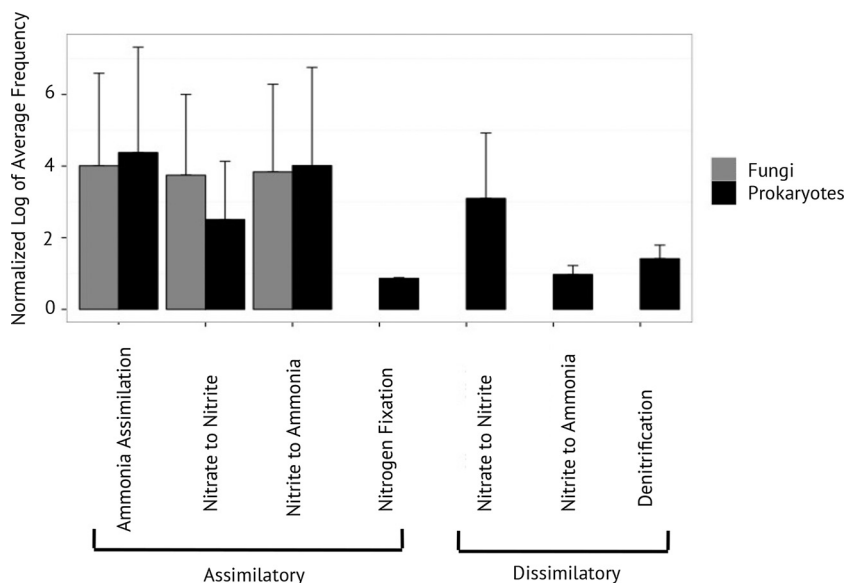
FIG 2 Log abundances of prokaryotic and fungal reads for each N cycle pathway. Abundance was calculated as the average across all samples ($n = 46$), standardized by the number of genes targeted in the pathway (see Table S2 in the supplemental material).

All statistical analyses were performed using the "nlme" and "vegan" packages in the R software environment (43, 44). To test for differences in the abundances of bacterial communities across treatments and sampling dates, we used one-way repeated-measures analysis of variance (ANOVA) with "plot" included as an error term. For this analysis, we standardized the number of reads associated with each pathway by the total number of annotated bacterial reads in that library. We standardized fungal read abundances for each sample by the total fungal reads in each library.

The taxonomic diversity of the genes associated with each pathway was quantified using the Shannon evenness index and the observed richness of the number of orders. To test whether evenness changed over time, we performed one-way repeated-measures ANOVA as described above.

To assess the effects of treatment and time on bacterial community composition, we performed a permutational multivariate analysis of variance (PERMANOVA) (45) including treatment and sampling date as fixed effects (45, 46). Taxa were first standardized by their relative frequencies within each pathway; then the analysis was run for each pathway. The analyses were run using partial sums of squares on 999 permutations of residuals under a reduced model. If the model returned nonsignificant variables, the variables were removed, and the model was tested again. This procedure does not alter the significance of the remaining variables but reduces the effect of spurious relationships between variables (47). To identify the taxa contributing to significant compositional differences, we used similarity percentage (SIMPER) analysis (48). Specifically, we tested which taxa accounted for differences between the samples from rainy (winter/spring) and dry (summer/fall) seasons. Lastly, Pearson's correlations were used to test whether the number of sequences attributed to an N cycling pathway was distributed among bacterial orders in proportion to the total abundances of those orders in the metagenomes.

**Extracellular enzyme activity.** To assay the functional potential of the litter microbial community, we measured the potential activities of nine extracellular enzymes—α-glucosidase (AG), acid phosphatase (AP), β-glucosidase (BG), β-xylosidase (BX), cellobiohydrolase (CBH), leucine aminopeptidase (LAP), $N$-acetyl-β-$D$-glucosaminidase (NAG), polyphenol oxidase (PPO), and peroxidase (PER)—on litter from all treatments on seven sample dates between September 2011 and March 2013. Fluorimetric and oxidative enzyme assays were conducted as described in reference 33, and the initial results from these analyses are reported in references 2 and 30. For this study, we used the same data to calculate the ratio of N-acquiring enzymes to C-acquiring enzymes (NAG/$C_{enz}$ ratio) (49). The ratio of these two metrics has been proposed for the estimation of relative allocation to nutrient acquisition versus energy (49, 50). The extracellular enzyme NAG measures potential chitinase activity, a proxy for the conversion of organic N to ammonium for assimilation. $C_{enz}$ is defined as the sum of four extracellular enzymes involved in C cycling (AG, BG, CBH, BX). We used a repeated-measures ANOVA to test for differences in NAG/$C_{enz}$ ratios across all treatments and enzyme sampling dates.

To test for correlations between potential enzyme activities and genomic potential, we examined data from three sampling dates when both types of data were collected (September 2011, December 2011, March 2012). Specifically, we tested whether the NAG/$C_{enz}$ ratio could be predicted by the ratio of the abundance of assimilatory N cycling genes to that of dissimilatory N cycling genes (A/D ratio), as a genomic index for the allocation of nutrient acquisition versus energy. We used analysis of covariance (ANCOVA) with NAG/$C_{enz}$ activity as the independent variable, the A/D ratio as the dependent variable, and time as a covariate. (We excluded treatment as a covariate, because treatment did not significantly affect the NAG/$C_{enz}$ ratio in the test described above.)

## RESULTS

Across all the plant litter metagenomic libraries, 59% of the annotated sequences were bacterial (294,674,419 reads). Of the annotated sequences, 0.31% were associated with an N cycling pathway, and of these, 896,943, 197,944, and 3,278 were assigned to *Bacteria*, Fungi, and *Archaea*, respectively. The vast majority of these sequences were associated with ammonia assimilation (84%, 75%, and 98%, from *Bacteria*, Fungi, and *Archaea*, respectively). All N cycling fungal sequences were associated with two phyla, Ascomycota (94.5%) and Basidiomycota (5.5%).

**Abundance.** The total abundances of prokaryotic reads related to different N cycling pathways differed by several orders of magnitude (Fig. 2). Broadly, assimilatory pathways were much more prevalent (96.5%) than dissimilatory pathways (3.5%). After ammonia assimilation, ANR($NO_3^- \rightarrow NH_3$) and DNRA($NO_3^- \rightarrow NO_2^-$) were the next most frequently detected pathways, while

TABLE 1 Effects of treatment, sample date, and their interaction on the abundance of sequences from each N cycle pathway for prokaryotes and Fungi

| N cycle pathway | Significance ($P$ value)[a] of effect of: | | | Total no. of sequences detected |
|---|---|---|---|---|
| | Treatment | Time[b] | Interaction of treatment and time | |
| Prokaryotes | | | | |
| Dissimilatory nitrate to nitrite | NS | <0.0001 | 0.07 | 35,920 |
| Dissimilatory nitrite to ammonia | NS | NS | NS | 130 |
| Denitrification | NS | <0.01 | NS | 1,846 |
| Nitrification | — | — | — | 17 |
| Assimilatory nitrate to nitrite | NS | NS | NS | 2,009 |
| Assimilatory nitrite to ammonia | NS | <0.0001 | NS | 102,360 |
| Ammonia assimilation | NS | <0.0001 | 0.05 | 757,470 |
| Nitrogen fixation | NS | <0.01 | 0.02 | 413 |
| Fungi | | | | |
| Assimilatory nitrite to ammonia | NS | <0.01 | 0.05 | 32,172 |
| Assimilatory nitrate to nitrite | NS | NS | 0.06 | 17,169 |
| Ammonia assimilation | NS | NS | NS | 148,602 |

[a] Based on repeated-measures ANOVA. NS, not significant; —, not tested.
[b] Sample date.

the nitrification pathway was the least frequently detected (Table 1). All fungal N cycling sequences were associated with assimilatory pathways (Fig. 2).

The frequency of prokaryotic genes in each pathway differed significantly over time for 5 of the 8 pathways: the ammonia assimilation, ANR($NO_2^-$ → $NH_3$), DNRA($NO_3^-$ → $NO_2^-$), N fixation, and denitrification pathways (by repeated-measures ANOVA) (Table 1; Fig. 3a). Gene abundances in these pathways tended to covary over time and were lowest in August 2010 and June 2011. This pattern correlated with cumulative precipitation at the site in the 2 weeks prior to sampling (Fig. 3b). The only

fungal pathway in which the frequency of genes differed significantly over time was that associated with ANR($NO_2^-$ → $NH_3$). In contrast to prokaryotic sequences from this pathway, the frequency of fungal sequences was highest in August 2010 and June 2011 (Fig. 3a).

**Composition.** The distribution of N cycling potential among prokaryotic taxa differed distinctly by pathway (Fig. 4). Genes involved in ammonia assimilation were generally detected in proportion to their abundances in each bacterial order ($R^2 = 0.877$; $P < 0.0001$), as were genes involved in ANR($NO_2^-$ → $NH_3$) and DNRA ($NO_3^-$ → $NO_2^-$) ($R^2$, 0.714 and 0.657; $P < 0.0001$).
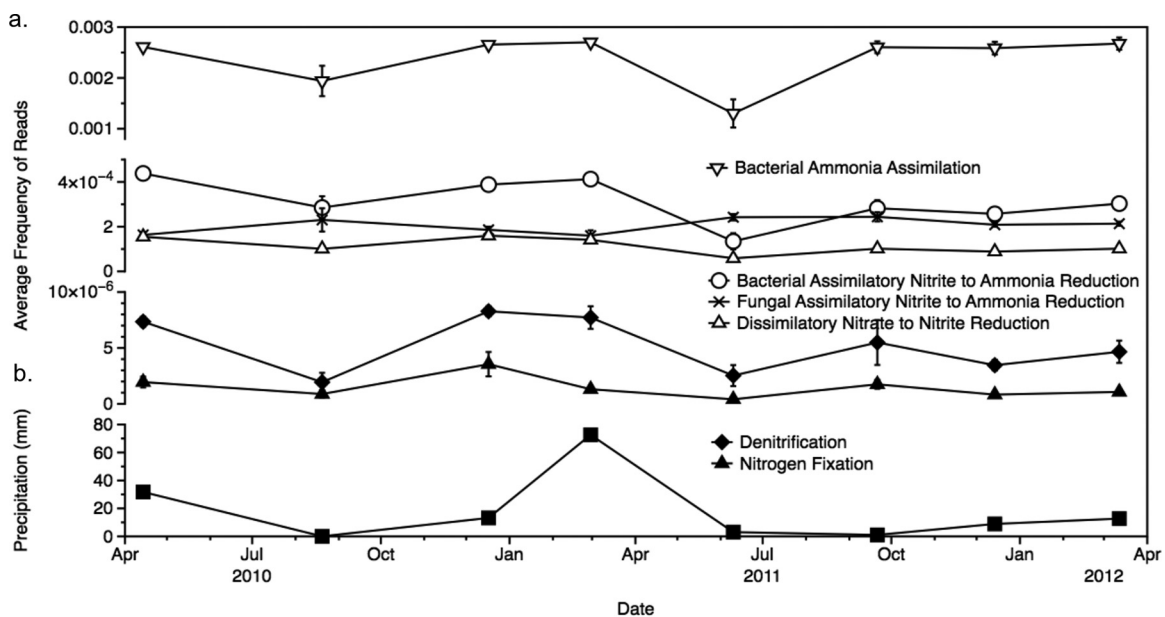


FIG 3 (a) Average frequencies of sequences by sample date for those pathways in which they differed significantly over time, including the denitrification, nitrogen fixation, dissimilatory nitrate reduction to nitrite, bacterial assimilatory nitrite reduction to ammonia, fungal assimilatory nitrite reduction to ammonia, and bacterial ammonia assimilation pathways. Error bars, ±1 standard error. (b) Cumulative precipitation (in millimeters) in the 2 weeks prior to sampling, shown by sampling date.
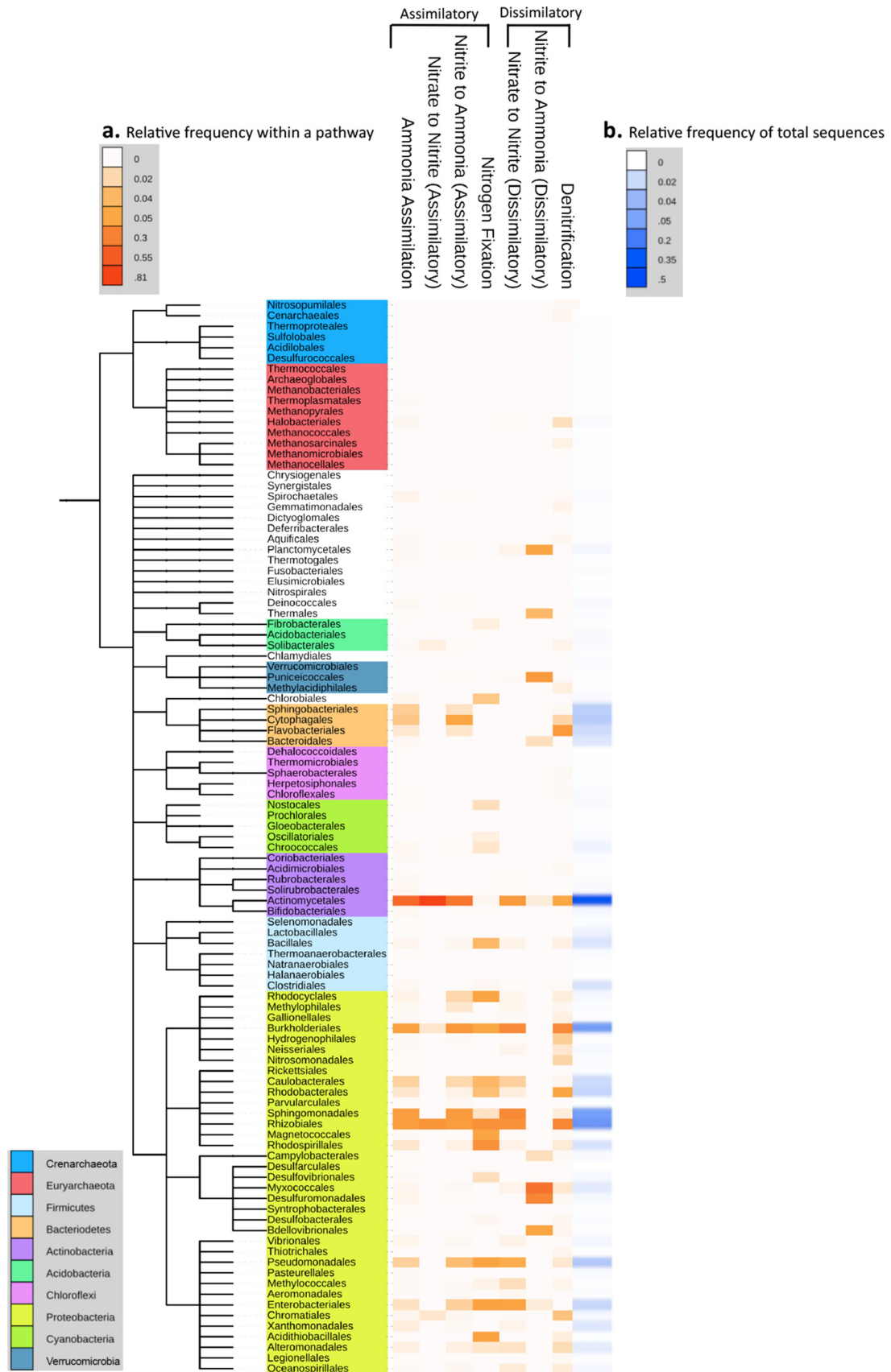
FIG 4 Bacterial and archaeal composition at the order level by N pathway. Data are combined across all sampling dates. A coarse phylogeny is shown on the left to highlight the major phyla (Interactive Tree of Life [iToL]) (94). (a) The orange heat map represents the relative distribution of sequence reads by order for each pathway. (b) For comparison, the blue heat map shows the relative abundances of all bacterial and archaeal sequences (all predicted proteins and rRNA genes) by order. (Relative abundance was calculated with all bacterial/archaeal taxa; however, only orders with predicted N cycle reads [101/130] are included in this figure.)
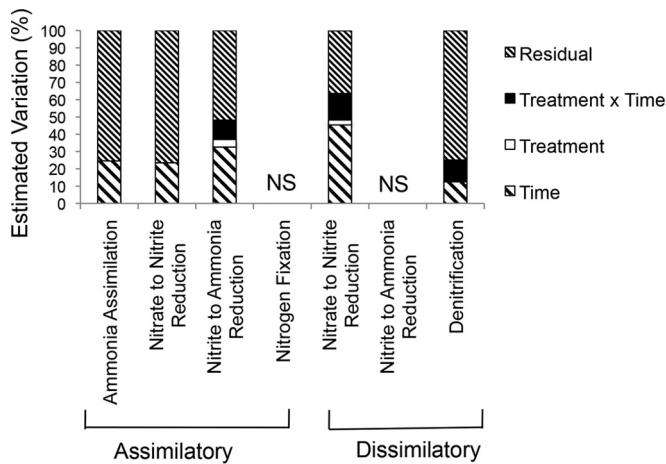
FIG 5 Percentages of the estimated variation in prokaryotic community composition explained by time, treatment, and their interaction for each N cycle pathway. Estimates were derived from PERMANOVA models, where NS means the test result was not significant ($P$, ≥0.1).

$ANR(NO_3^- \rightarrow NO_2^-)$ was the most taxonomically restricted pathway. Even though genes for this process were relatively abundant in the metagenomic libraries (Fig. 2), they were detected for only five bacterial orders and thus were not well correlated with the total abundance in each order ($R^2 = 0.459$; $P < 0.0001$). Finally, genes for $DNRA(NO_2^- \rightarrow NH_3)$, were rare among abundant orders but common among some rare orders and hence were poorly correlated with overall abundance ($R^2 = 0.084$; $P > 0.05$) (Fig. 4).

In an intermediate case, denitrification genes were common among many abundant bacterial orders (e.g., *Rhizobiales*, *Burkholderiales*, and *Actinomycetales*) but were also found in other, less abundant orders, including orders of *Archaea* (*Halobacteriales*, *Methanosarcinales*, and *Cenarchaeales*) and known ammonia oxidizers (*Nitrosomonadales* and *Nitrosopumilales*) ($R^2 = 0.539$; $P < 0.0001$) (Fig. 4). This was also true for N fixation ($R^2 = 0.497$; $P < 0.0001$), which was common in some the most abundant taxa (e.g., *Rhizobiales* and *Burkholderiales*) but absent in the most abundant order (*Actinomycetales*).

Distinct bacterial taxa appeared to have different N cycling potentials in these plant litter communities (Fig. 4). The most abundant taxa (e.g., *Actinomycetales*, *Rhizobiales*, *Burkholderiales*, and *Sphingomonadales*) appeared to be N cycling generalists in that they carried genes from most (five to six) of the seven pathways. Other taxa seemed to be more specialized. Notably, the orders from the phylum *Bacteroidetes* (*Bacteroidales*, *Cytophagales*, *Flavobacteriales*, and *Sphingobacteriales*) carried genes for only a couple of pathways (two to three), even though the taxa were relatively abundant (Fig. 4).

Beyond these average trends, the composition of potential N cycling litter prokaryotes differed among litter samples. Much of this variation could be attributed to temporal variability (see Fig. S1 in the supplemental material). In 5 of the 8 pathways examined, time explained 12% to 45% of the compositional variation among samples (Fig. 5; see also Table S3 in the supplemental material). These trends were driven largely by seasonal differences in a few abundant bacterial orders. For instance, across all N pathways, the relative abundances of *Burkholderiales* and *Sphingomonadales*

were higher, and that of *Enterobacteriales* was lower, in the rainy (winter/spring) than in the dry (summer/fall) seasons (by SIMPER analysis; see Table S4 in the supplemental material). However, the seasonal abundances of at least two orders, *Actinomycetales* and *Rhizobiales*, depended on the N pathway examined. For example, for *Actinomycetales*, $ANR(NO_2^- \rightarrow NH_3)$ genes were relatively abundant in winter/spring, but *Actinomycetales* $ANR(NO_3^- \rightarrow NO_2^-)$, $DNRA(NO_3^- \rightarrow NO_2^-)$, and ammonia assimilation genes were higher in summer/fall (see Table S4).

In contrast to time, treatment (drought or N addition) had minor effects on the composition of N cycling prokaryotes, explaining only a small percentage of variation in the $DNRA(NO_3^- \rightarrow NO_2^-)$ (2.9%) and $ANR(NO_2^- \rightarrow NH_3)$ (4.4%) pathways (Fig. 5). This result was similar to that for analyses of community composition using 16S rRNA, where treatment explained ~3% of estimated variation (30). Treatment also interacted with sampling date to account for 11% to 15% of compositional variation in the denitrification, $DNRA(NO_3^- \rightarrow NO_2^-)$, and $ANR(NO_2^- \rightarrow NH_3)$ pathways (Fig. 5). For instance, N addition altered the composition of bacterial orders carrying $ANR(NO_2^- \rightarrow NH_3)$ pathway genes during the dry season, but not during the rainy season (see Fig. S1 in the supplemental material).

**Diversity.** Prokaryotic evenness at the order level was relatively constant over time for all pathways examined (Fig. 6). The richness and evenness of prokaryotic orders associated with each N pathway were highly correlated; pathways encoded by a higher number of orders also tended to be more evenly distributed across those orders. Generally, evenness was low for the communities with the potential for N fixation, $ANR(NO_3^- \rightarrow NO_2)$, and $DNRA(NO_2^- \rightarrow NH_3)$ (Fig. 6). In the case of N fixation and $DNRA(NO_2^- \rightarrow NH_3)$, this reduced diversity may be due to undersampling, since the total number of sequences detected was in the hundreds (Table 1). As mentioned above, however, only five bacterial orders appeared to carry genes for $ANR(NO_3^- \rightarrow NO_2^-)$, even though thousands of sequences were sampled. Most (81%) of these genes were attributed to one order, the *Actinomycetales*.

**Extracellular enzymes.** Like the abundances of N cycling genes, the potential activities of nine extracellular enzymes differed over time, but not by treatment, in this plant litter system (30). In particular, the ratio of N- to C-acquiring enzymes (NAG/$C_{enz}$ ratio) differed by month ($P$, <0.0001 by repeated-measures ANOVA), with the lowest NAG/$C_{enz}$ ratios in the dry fall months (September 2012 and 2013). Further, the A/D gene abundance ratio explained almost half of the variation in the NAG/$C_{enz}$ ratio ($P$, 0.045 by ANCOVA; adjusted $R^2$, 0.47) (see Fig. S2 in the supplemental material).

## DISCUSSION

Our analysis provides a blueprint for the genetic potential of N cycle processes in plant litter without the biases associated with targeting individual genes or a subset of microbial communities (47, 48). Of course, there are a number of caveats to interpreting metagenomic data (51). Most importantly, for many pathways, gene abundance in a community has not been found to correlate consistently with environmental process rates, which limits our ability to draw conclusions about activity (52–54). Still, a powerful feature of metagenomics data is the ability to make comparisons across many functional pathways and taxa at the same
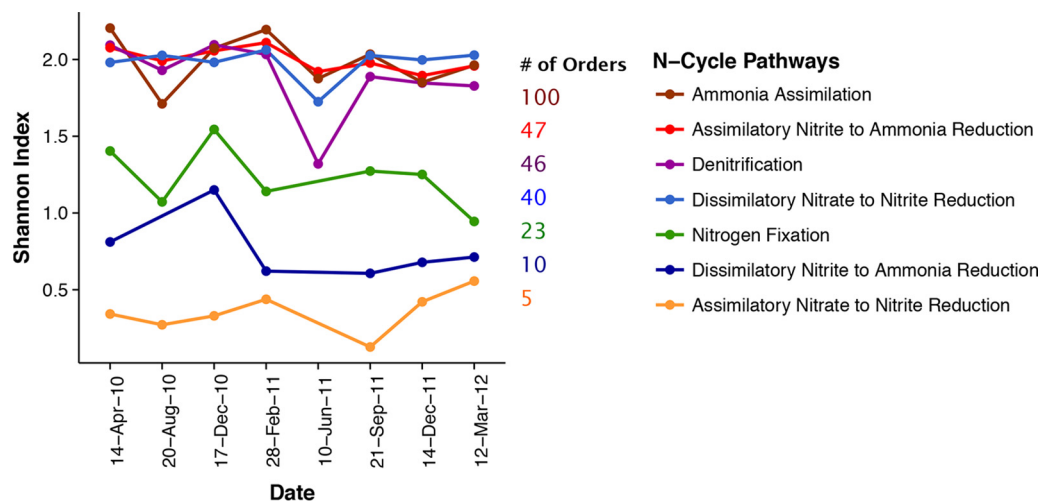
FIG 6 Diversity of orders (calculated by the Shannon index) for each N cycling pathway by collection date. The total number of orders for each pathway is also noted.

time. Indeed, we found that the abundance, composition, and diversity of N cycling genes differed greatly among the eight targeted pathways. By aggregating the data across pathways, prokaryotes and fungi appear to play equally important roles in N assimilation in this system. Assimilatory pathways were much more prevalent than dissimilatory pathways. And orders within the *Actinobacteria* and *Proteobacteria* appeared to be N cycling generalists, carrying genes from most pathways, in contrast to those from the *Bacteroidetes*, which were also relatively abundant in the litter.

The metagenomic picture of N cycling potential was further correlated with the functional potential of the community, as assayed by extracellular enzyme activities. Specifically, the ratio of assimilatory to dissimilatory gene abundance explained almost half of the variation in the ratio of N- to C-acquiring enzymes (NAG/$C_{enz}$), an index of the relative allocation to nutrient acquisition versus energy (49, 50). This relationship indicates that the metagenomic variation observed may have direct functional relevance for the plant litter community.

**Comparisons across N cycling pathways.** The three most abundant N cycling pathways [i.e., ammonia assimilation, ANR($NO_2^- \rightarrow NH_3$), and DNRA($NO_3^- \rightarrow NO_2^-$)] were associated with prokaryotes in proportion to their total abundances, indicating that the pathways are broadly distributed across the prokaryotes. For instance, ammonia assimilation is an ability shared by virtually all microorganisms, and sequences in this pathway made up the majority of N cycling sequences, a finding similar to those in other metagenomic studies across various environments (55–57).

The high abundance of ANR pathways suggests that $NO_3^-$ and $NO_2^-$ may be important sources of N on plant litter. This availability may be due in part to the high rates of atmospheric N deposition that occur in the Southern California region (28), although microbial ANR appears to be an important process in a wide range of terrestrial systems, including undisturbed soils and agricultural fields (58–60). In terrestrial systems, heterotrophic bacteria seem to prefer ammonium ($NH_4^+$) over $NO_3^-$ or $NO_2^-$ due to energetic costs (61, 62), unless they are limited by $NH_4^+$, in which case they may also use $NO_3^-$ or $NO_2^-$ (58, 63). Thus, the

ability to assimilate $NO_3^-$ and $NO_2^-$ may provide an advantage in an N-limited ecosystem (64).

The DNRA($NO_3^- \rightarrow NO_2^-$) pathway was also abundant in the plant litter. Relatively little is known about DNRA in terrestrial systems (65). While the DNRA pathway in soil bacteria was discovered in the 1930s (66), until recently many studies considered denitrification the only dissimilatory reduction process in soil (67). Indeed, DNRA and denitrification are competitive processes, which occur primarily under anaerobic conditions (65). DNRA is now recognized as a key process in wetlands and has been observed in moist tropical soils (68). Modeling studies suggest that DNRA may be important in temperate grassland soils (69, 70), but its general significance in aerobic upland soils remains unclear (71).

There are several reasons why the plant litter environment may be conducive to DNRA. First, while the process is most likely to occur under anaerobic conditions, some studies have shown that DNRA is less sensitive to variable $O_2$ and redox conditions than denitrification (72, 73). Second, DNRA is thought to be favored in high-C/N-ratio environments (65), like that of plant litter. Finally, oxygen gradients that range from 100 to 0% saturation within a few micrometers have been measured in plant litter layers (74, 75). Thus, we speculate that plant litter could be suitable for DNRA, particularly after rains, when the wet, matted-down litter may contain anaerobic pockets. Indeed, the highest abundances of DNRA($NO_3^- \rightarrow NO_2^-$) sequences in the litter samples were correlated with increased precipitation at the site. In the future, it would be useful to verify the activity of the pathway at the site by transcriptomics.

While less abundant, denitrification and N fixation genes were also present in the plant litter metagenomes. Denitrification is known to be an important process in terrestrial grasslands, where soils are a major source of $N_2O$ emissions to the atmosphere (15, 76). Denitrification in soils occurs primarily after precipitation events (77, 78). Like DNRA, the denitrification pathway was detected across a wide range of bacterial orders, but it was most common in orders from the phylum *Proteobacteria*.

N fixation appears to be one of the rarest of the pathways targeted in the litter community; only nitrification is less frequent.

Although N fixation does occur during litter decomposition, estimated rates in the litter/soil layer are low, between <0.5 and 20 kg N ha$^{-1}$ year$^{-1}$ (79)—much lower than the symbiotic N fixation rates in various agricultural crops (80). Like that for DNRA($NO_3^- \rightarrow NO_2^-$), the genetic potential for N fixation was distributed across a distinct group of bacterial orders, many of which were present at low abundances in the community. It is often assumed that *Rhizobiales* carry out most N fixation in soils (81, 82); while 18% of all N fixation genes were classified as *Rhizobiales*, 82% were from other orders, including *Pseudomonadales*, *Enterobacteriales*, and *Burkholderiales*.

**Temporal variation and sensitivity to global change manipulations.** In addition to the broad patterns of prokaryotic diversity supporting N cycling in plant litter, we investigated how these communities differed across the seasons and in response to drought and N addition treatments. Within pathways, gene abundance and taxonomic composition differed over time, but they differed little in response to the global change manipulations. Specifically, the manipulations did not impact N cycle pathway abundance and altered composition only within the DNRA($NO_3^- \rightarrow NO_2$) and ANR($NO_2^- \rightarrow NH_3$) pathways. This limited response of the N cycling pathways is somewhat surprising considering that the percentage of N in the plant litter increased significantly in the nitrogen addition plots (22). However, we may have missed some responses because of low statistical power and/or our focus on primarily inorganic pathways.

Previous metagenomic studies have detected changes in potential N cycling in response to environmental perturbations such as N addition (see, e.g., references 83 and 84); however, many of these studies consider the N cycle quite broadly, making direct comparisons difficult. A few studies highlight mixed responses by pathway (85–87); for instance, burning tended to increase the relative abundance of dissimilatory processes and decrease that of assimilatory processes (88).

In contrast to the response to global change manipulations, N cycling pathway abundance showed significant temporal variability. Other global change experiments have also revealed this pattern of strong temporal versus treatment effects on microbial communities (89–92). Such a result is perhaps not unexpected; annual mean precipitation at the Loma Ridge site is 30 cm, and almost all of this precipitation falls between November and April (29). As a result, microbial biomass on plant litter is reduced to less than 25% of peak levels during the summer dry season (22). Indeed, the abundance of some N cycling pathways followed this broad trend. In these cases, the frequency of the pathway (relative to all prokaryotes) was stable over time (no significant time effect), indicating that there was no differential selection for the pathway across seasons above and beyond the fluctuations in total prokaryotic abundance.

However, for the majority of the N pathways, gene abundance differed significantly over time, generally increasing during the wetter months. Corresponding to this trend, the NAG/$C_{enz}$ activity ratio was lowest during the driest months. Thus, under wet conditions, selection may favor traits allowing for rapid N assimilation, whereas dry conditions may select for drought tolerance traits associated with different taxa (93).

Like the overall taxonomic composition of the litter community (30), the diversity and composition of bacterial lineages involved in most N cycling pathways differed across seasons. Compositional differences were driven by changes in the relative abundances of the most abundant taxa in the system. For example, some potential $NO_2^-$ assimilators (e.g., *Actinomycetales*, *Enterobacteriales*, and *Burkholderiales*) displayed a seasonal signal, whereas others (e.g., *Cytophagales* and *Rhodocyclales*) were less affected by seasons. This pattern suggests that this pathway itself may not be selected for by season but instead may be linked to other traits that have a distinct distribution in bacterial lineages.

**Conclusions.** This study provides an overview of microbial N cycling potential in a plant litter system and points to several directions for future research. In particular, the high abundance of DNRA pathway genes is intriguing and suggests that further work on this process in grassland ecosystems is warranted. We also observed that the degree of N pathway specialization among bacterial orders tended to decrease with their increase in abundance in the plant litter, suggesting that N cycling generalists may have an advantage in plant litter. However, it is not clear whether this pattern is specific to this environment or whether it may be a general feature of N-limited environments. Indeed, the results described here will be most useful in direct comparison to other ecosystems.

## ACKNOWLEDGMENTS

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