

Article

A Gap in the Prairie Nitrogen Cycle: Nitrogen Fixation Is Low, Despite Presence of Diverse Nitrogen Fixing Bacteria

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Abstract

Nitrogen is integral to all living systems, but its diverse forms are interconverted dynamically through reductions and oxidations that constitute the nitrogen cycle. Ecosystems gain combined nitrogen by bacterial and archaeal reduction in atmospheric N₂, while combined nitrogen is lost to the atmosphere as nitrogenous gases through denitrification. We sought to characterize the diversity and activity of free-living nitrogen-fixing bacteria or diazotrophs in natural prairie grasslands and the activity of the other steps of the nitrogen cycle through metatranscriptomics. DNA and mRNA were obtained from prairie sites that did not contain any leguminous plants to avoid symbiotic nitrogen fixation. Both 16S rRNA gene and *nifH* amplicon pools were sequenced to characterize the diversity of diazotrophs, and the expression levels of N-cycle genes were quantified by RNAseq. Nitrogen fixation without and with added carbohydrates was quantified by measuring the incorporation of ¹⁵N₂. The soil samples contained a diversity of diazotrophs, as reflected both by 16S rRNA gene and *nifH* gene sequences. Carbohydrate amendment of soil samples led to substantial ¹⁵N₂ incorporation, showing that members of the resident microbiota were able to fix nitrogen. However, we did not detect ¹⁵N₂ incorporation in unamended soil samples, pointing to a lack of in situ fixation. The very low levels of nitrogenase gene transcripts supported this finding. In contrast, transcripts for nitrification and denitrification genes were expressed, pointing to a gap in the nitrogen cycle. The prevalence of diazotrophs with undetectable nitrogen fixing activity suggested the absence of available carbohydrates to provide the energy needed. The apparent imbalance in the nitrogen cycle would not be sustainable, so other possible mechanisms of acquiring combined nitrogen should be explored.

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This article is an open access article distributed under the terms and conditions of the [Creative Commons Attribution \(CC BY\)](https://creativecommons.org/licenses/by/4.0/) license.**Keywords:** nitrogen; diazotrophs; natural prairie; nitrogenase; soil microbiome; soil transcriptome

1. Introduction

Soil is home to diverse microbial communities [1], including bacteria catalyzing the conversion of nitrogenous compounds [2]. Nitrogen (N) is critical for all life forms, and available N limits primary productivity in the biosphere [3]. The combined N pool in soils is decreased through the combined action of nitrifiers and denitrifiers, which leads to the return of N₂ and other nitrogenous gases to the atmosphere [2]. Conversely, the available N pool in soils is enhanced by decomposition of soil organic matter, but this is

derived from organisms that have died after growing using soil N reserves. Arbuscular mycorrhizal fungi (AMF) play a key role in making combined nitrogen available to plants. This includes supporting bacteria that mineralize organic N-substrates or convert inorganic N forms further away from roots [4]. AMF also take up inorganic N forms through their widely distributed network of hyphae, and transport this to the plant root [5]. As the pool of organically bound nitrogen in soil is finite, the primary natural source of N introduced into the ecosystem is biological nitrogen fixation by bacteria called diazotrophs [6,7]. In the absence of N input by nitrogen fixation, the biosphere would undergo a net decrease in combined N. Thus, diazotrophs are essential for biological nitrogen fixation [8] and play a vital role in the introduction of combined nitrogen into the ecosystem [9]. Diazotrophs can be either free-living or symbiotic [10]. Symbiotic diazotrophs need plant hosts such as legumes for nodulation and nitrogen fixation [11]. Prairies are dominated by grasses, with legumes occurring unevenly and in low proportions [12,13]. Therefore, the free-living diazotrophic community is expected to play an important role in nitrogen fixation for sustaining the prairie ecosystem where legumes are absent [14].

Free-living diazotrophs in soil are spread across multiple bacterial phyla [7] and display diverse phenotypes and growth requirements. This makes culture-based study of diazotrophs in soils challenging. While the prokaryotic community of the soil is not entirely culturable yet [2], next-generation sequencing has revolutionized the study of microbial communities through culture-independent approaches [15,16]. The 16S rRNA gene is now widely used for taxonomic categorization and has emerged as a marker gene for bacterial classification and diversity analysis [17]. Similarly, for the study of ecological or functional potential, specific functional genes are sequenced and analyzed [18]. Nitrogen fixation function was explored using the *nifH* gene as a marker [19]. It encodes an iron complex of the nitrogenase enzyme system. The combination of these two marker genes can be used to understand the true bacterial community composition and identify *nifH* gene-bearing members [20]. Although much can be deciphered by characterizing the overall diazotroph community composition using next-generation DNA sequencing, additional techniques can be employed for a better picture of ecological functioning. Bacteria are known for tight control over the expression of their many genes. Thus, the first indication of metabolic potential is transcription, evidenced by the presence of specific mRNA transcripts. Quantifying gene transcripts can, therefore, indicate relative activities of the diverse reactions of the N cycle [2]. The horizontal mobility of several N-cycling genes has added to the complexity of characterization of the processes with microbial taxa [21]. In addition, some microbial taxa seem to be either N-cycling specialists or generalists, as previously reported in whole-genome-based phylogenetic analysis of soils across the globe [22]. Traditional methods such as the use of isotopic nitrogen ($^{15}\text{N}_2$) and acetylene reduction assays for the quantification of nitrogen fixation are predominantly used by researchers working with diazotrophs [23,24]. The incorporation of isotopic nitrogen from an enriched atmosphere by the bacteria to the soil system or within cells signifies successful fixation. This adds to the value of gene presence data with confirmation of active fixation. Therefore, along with molecular tools, the use of isotopic nitrogen incorporation helps confirm nitrogen fixation activity by either a single bacterium in monoculture or full consortia in soil [25]. Thus, the study of nitrogen cycling pathways using RNA sequencing and ^{15}N incorporation would help to establish nitrogen fixation, hence adding to the understanding of the nitrogen dynamics and productivity of natural grassland ecosystems.

Little is known about the diversity and activity of free-living diazotrophs in prairie ecosystems where legumes are sparse. Inspired by the isolation of potential diazotrophs from prairie soil onto nitrogen-free medium, diverse free-living bacteria with nitrogen fixation ability were expected to be present in this soil [26]. We hypothesized that (a) prairie

soil contains a diversity of free-living diazotrophs and (b) that these diazotrophs would replenish nitrogen lost through nitrification and denitrification by nitrogen fixation. The goal of our work was to (a) determine the proportion of diazotrophic bacteria using culture-independent approaches, (b) quantify N fixation by incorporation of $^{15}\text{N}_2$, and (c) determine the expression of the various genes contributing to steps in the nitrogen cycle by RNA-seq. We found that diazotrophs were diverse and prevalent, both using 16S rRNA gene signatures and *nifH* amplicon sequencing.

2. Materials and Methods

2.1. Sample Sites

Samples were taken for a prior project at the Sioux Prairie Preserve near Colman, South Dakota, USA, and analyzed for chemical composition as reported previously [12]. Briefly, samples were taken 20 m apart from each other along a randomly obtained transect between 44° 208.500' N, 96° 4705.200' W and 44° 205.600' N, 96° 4706.900' W. Three soil cores were taken at each site to a depth of 5–7 cm using a soil corer of 2.5 cm diameter when the ground was thawed and then monthly until October (six time points). Samples were taken to the laboratory for processing a few hours later. Soil composition, including pH, nitrate, ammonia, total nitrogen by Kjeldahl method [27], potassium, and extractable phosphorous by Olsen method [28], were determined by the South Dakota State University (SDSU) Soil Testing Laboratory using standard protocols, and reported previously as Figure S2.

2.2. Identification of Bacterial Genera from 16S rRNA Data

The bacterial community shifts were previously studied using 16S rRNA gene amplicon pools [12]. The literature for each bacterial genus was explored for evidence of their nitrogen-fixing ability. All the bacterial genera known to fix atmospheric nitrogen were filtered out to make comparisons across the sites and time points. In addition, nitrogen-fixing bacterial community proportions were estimated for each time point. All comparisons were made using the nonparametric Wilcoxon test at the 95% significance level, unless stated differently in cases where another statistical measure was used.

2.3. *nifH* Gene Sequencing

The total genomic DNA previously extracted for overall soil bacterial community analysis was used for *nifH* gene sequencing [12]. Since the bacterial diversity across the time points did not show marked differences and Sites 5 and 6 had legumes, we chose to select the DNA from Sites 1 to 4 collected during October 2018 for characterization of the free-living *nifH*-derived prokaryotic community. The DNA quantities in the extracts were quantified using Nanodrop (Fisher Scientific, Pittsburgh, PA, USA). The DNA samples were sent for analysis to the Novogene Corporation Inc. (Sacramento, CA, USA) sequencing facility. Sequencing was performed on an Illumina platform (NovaSeq 6000 PE250, San Diego, CA, USA) using the PolF (TGC GAY CCS AAR GCB GAC TC) and PolR (ATS GCC ATC ATY TCR CCG GA) degenerate primer sets. After their initial quality filtering process, compressed raw reads were obtained and used for downstream analysis.

2.4. *nifH* Sequence Data Analysis and Visualizations

The sequence reads obtained were denoised using the DADA2 pipeline [29] on QIIME2 [30]. The forward and reverse reads were truncated to 245 bases and merged during the denoising process. The representative sequences were further analyzed on high-performance cluster computers, and taxonomic groups were assigned using Kaiju [31] using a newly created broad *nif* gene database [7]. The taxonomic data on *nifH* sequence diversity were analyzed for statistical comparison and visualizations on RStudio (Team, 2020)

and R (Team, 2021). The following packages were used. Data sorting was performed using the “dplyr” package [32] and then plotted using the package “ggplot2” [32]. Statistical analyses were performed using “agricolae” [33], “DescTools” [1], “psych” [2], “ggpubr” [3], “vegan” [4], and “tidyverse” [32].

2.5. Incorporation of ^{15}N Isotopes

Two soil cores were selected from each of the six study sites (divided into two sub-samples). From each core, 1 g of soil was carefully placed in a 10 mL syringe. To mimic the addition of carbon supplied through root exudates, 1 mL of carbohydrate mixture (2 g per L each of glucose, mannitol, arabinose, and malic acid) was added, termed “supplemented”. Next, the plunger was set at the 10 mL mark. The plunger was pressed to release air and clear 1 mL of headspace. One milliliter of isotopic $^{15}\text{N}_2$ gas (>98% ^{15}N -labeled, Cambridge Isotope Laboratories, Inc., Andover, MA, USA) was added. The control syringes had only unenriched atmospheric air ($^{15}\text{N}_2$ not added). All syringes were incubated at room temperature in the dark for either 14 d (unsupplemented) or 21 d (supplemented). The soil samples were dried for 3 d at 55 °C and crushed, and approximately 10 mg of soil was wrapped in 5 × 9 mm tin capsules (Costech Analytical Technologies, Valencia, CA, USA). The capsulated soil samples were sent to the UC Davis Stable Isotope Facility, Davis, CA, USA, for mass spectrometric analysis. The data obtained were analyzed using R.

2.6. RNA Sequencing and N-Cycling Pathways

The total RNA samples were extracted from the soil (Sites 1–4) using Qiagen Kits using the recommended methods [12]. The samples were sent to Novogene Corporation Inc. for RNA-Seq. After initial quality checks, cDNA was synthesized from purified mRNA and sequenced on an Illumina NovaSeq 6000 PE150. The filtered and high-quality sequences obtained from the sequencing facility were analyzed using a previously described pipeline [7]. It included initial quality checking using FastQC [34], followed by taxonomic classification of the reads by Kaiju [31] with reference to the prokaryotic reference database [35]. The reads were then assembled using Trinity [36] and classified using Kaiju. Counts for each transcript were generated using Salmon [37]. Blastx, HMMER v1 [38], and BLASTP were used for the characterization of the assembled transcripts and later compiled using the SQLite database with the help of the Trinity boilerplate template that has precompiled GO (Table S1), KEGG and taxonomy databases.

3. Results

3.1. Most Nitrogen-Fixing Bacteria Differ by Site and Not Across Months

To obtain an overview of the bacterial taxa with nitrogen fixation potential, we used previously sequenced bacterial 16S rRNA gene pools from sites free from legumes [12] and searched for specific genera with reported nitrogen-fixing isolates. Between 1.5 and 4% of the reads in each sample belonged to members of nitrogen-fixing genera (Figure 1A). *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, *Devosia* and other *Bradyrhizobiaceae* were the most abundant nitrogen-fixing genera at all sites. These genera are known to fix nitrogen as symbionts of legume nodules, but these sites did not contain legumes. The other predominant genera are known as free-living fixers (Figure 1B). Only three bacterial genera were significantly different across the sampling time points: *Paenibacillus*, *Arthrobacter* (Figure S2C,D, Wilcoxon test; $p < 0.05$) and *Rhizobium*. *Paenibacillus* and *Arthrobacter* peaked immediately after May, when they were significantly low in the community. In contrast, *Rhizobium* was significantly low during the month of September (Wilcoxon test; $p < 0.05$). These well-known nitrogen-fixing genera tended to show a seasonal pattern (Figure 1B). Overall, the nitrogen-fixing community was different by sampling site but not by time

points, as indicated by principal component analysis (Figure S1). Many diazotrophic bacterial genera were significantly different across at least one sampling site (Figure S2).

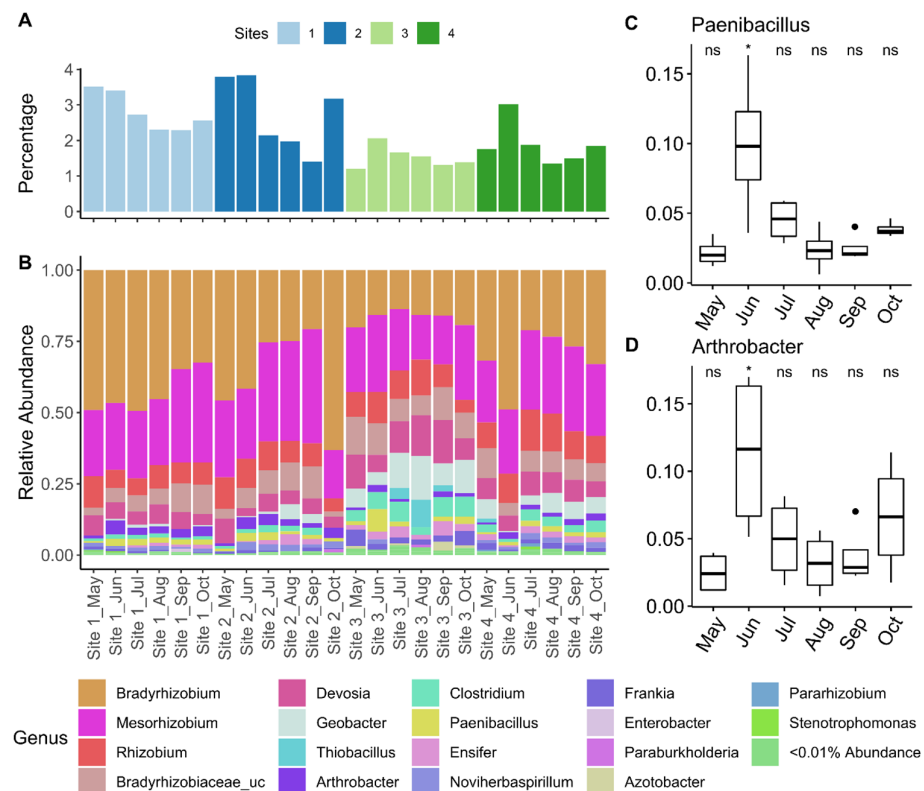


Figure 1. Nitrogen-fixing bacterial genera derived from 16S rRNA sequence data and genera with significantly different proportions across time points. Total N-fixing percentage of bacterial OTUs (A), distribution of N-fixing genera at each time point across sampling sites given as relative abundance (B), and proportions of the bacterial community across time-points for *Paenibacillus* (C) and *Arthrobacter* (D). *p* Values of <0.05 are indicated by *, and >0.05 as ns (not significant).

3.2. Diazotroph Diversity of the Bacterial Community Determined by *nifH* Sequences

To understand the diversity of the nitrogen-fixing bacterial community, we sequenced the marker gene for nitrogenase, *nifH*, using a set of highly efficient degenerate primers, PolF & PolR [39]. The samples passed the initial quality control process and were successfully amplified. A summary of the data quality is given in Table S2. On average, 161,486 reads were clean, with 89.28% of bases having a quality score of 30 or above. The average GC content was 61.29%. The reads had a good quality score and an error rate of only 0.03%. After quality filtering, 0.323 Gb clean raw data were obtained and used for further bioinformatic analysis for nitrogen-fixing bacterial community profiling.

To understand the community structure of the bacteria with an amplifiable *nifH* gene, the clean reads obtained from Illumina sequencing were analyzed on QIIME2 and R. The total number of features identified was 7712, with a mean frequency of 13.42 per feature. On average, 67.41% ($\pm 2.74\%$) of the *nifH* sequences could not be assigned to any bacterial taxa. Therefore, the comparisons of the *nifH*-harboring bacterial community were performed using sequences known to be of bacterial origin. Classification was performed using the recently created *nif* database using available whole-genome sequences [7]. *Proteobacteria* dominated the diazotrophs in all four sample sites with an average relative abundance of 62.35% ($\pm 2.28\%$), followed by *Aquificae* and *Verrucomicrobia* (Figure 2). Among the *Proteobacteria*, the relative abundance of the bacteria from the class Alpha-proteobacteria was highest and over a quarter of the total bacterial community. Some other classes of bacteria known for their nitrogen-fixing ability were *Cyanobacteria*, *Bacteroidetes*, and *Firmicutes*. At

the lower taxon level, all four sample sites had the highest proportion of *Cystobacterineae* of *Deltaproteobacteria*, followed by *Desulfurobacterium* and *Geobacteraceae*. Furthermore, cumulatively, almost a quarter of bacterial taxa were less than 1% in abundance. The distribution of these genera is presented in Figure S3.

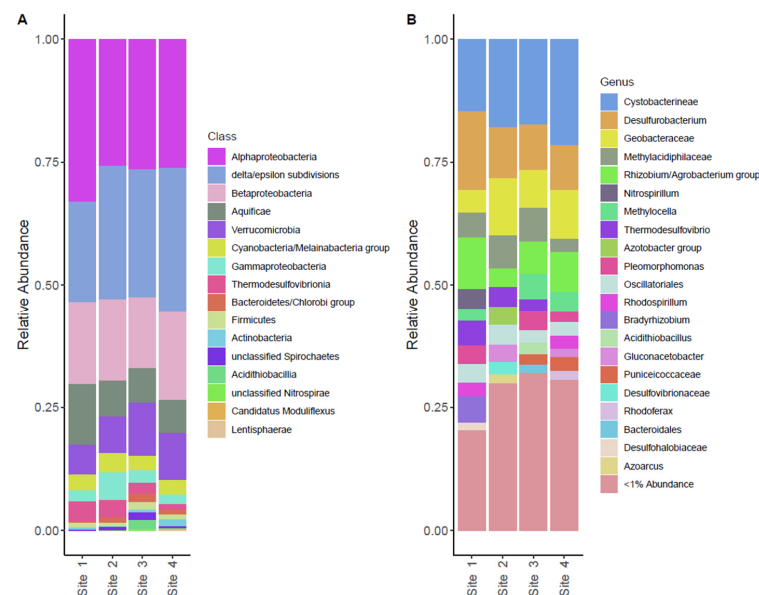


Figure 2. Stacked bar plots showing the bacterial diversity of *nifH*-encoding taxa. Distribution by taxonomic classes (A) and genera (B). The *nifH* region was amplified using PolF-R primers.

3.3. Nitrogen-Fixation Potential in Prairie Soil Using $^{15}\text{N}_2$ Incorporation Assay

To quantify the nitrogen-fixing activity versus potential of the microbiota in these soils, samples were incubated with $^{15}\text{N}_2$ with and without supplemented arabinose, mannitol, glucose, and malic acid for 21 d, dried, and analyzed by mass spectrometry. Isotopic incorporation was calculated using $\delta^{15}\text{N}$. ^{15}N incorporation in unsupplemented soils was very low (Figure 3). In contrast, the addition of sugars yielded substantial incorporation, with a maximum of 2389.0 (Site 4) and a minimum of 194.4 $\mu\text{g}/\text{kg}/\text{d}$ (Site 3) (Figure 3). This indicated that in situ fixation was limited but that the resident diazotroph community had the potential to fix nitrogen, provided an energy source was available. When assuming a nitrogen-fixing period of three months and a 5 cm zone of active fixation, the average in situ incorporation was below the detection limit, equating to 0 kg N per Ha per season, and the potential N incorporation rate was 1307 $\mu\text{g}/\text{kg}/\text{d}$ or 58.8 kg N per Ha per season.

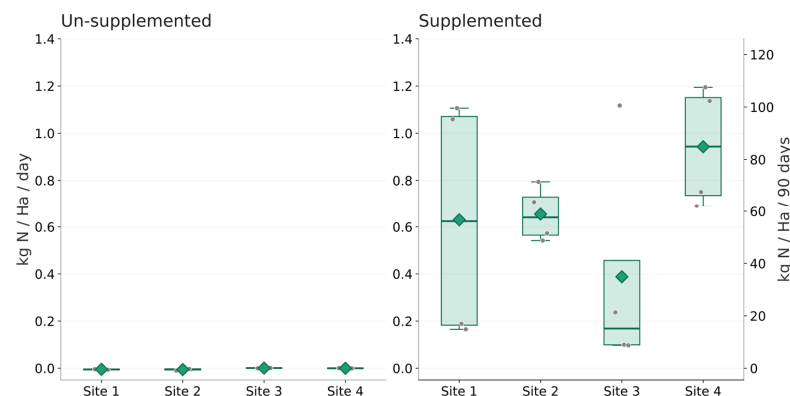


Figure 3. ^{15}N incorporation of soil microbiota in unsupplemented (in situ fixation) versus carbohydrate-supplemented (potential fixation) prairie soil. Nitrogen incorporation per day (left Y axis) was converted to a 90 d period (right Y axis) to reflect contributions during one growing season. Boxes represent Q1–Q3 and green lines represent the mean.

3.4. N-Cycling Pathway Gene Expression

To understand microbial N cycling in natural prairie, mRNA sequencing was performed. The data obtained had 212.4 million paired-end mRNA sequences. On average, reads were 150 bp, with a Phred quality score of above 30 for $95.49\% \pm 1.5\%$ of the reads and an average GC content of 62.48%. After assembly of sequences on Trinity, a total of 6,198,722 transcripts and 5,263,238 genes were identified. Furthermore, the average length of mRNA was 341.92 bases. Using BLASTX, 54.38% of the transcripts were identified in the NCBI nr protein database. Whereas 34.9% of these genes were identified using HMMER search for protein domains on Pfam, approximately 50% of the reads could not be classified. Among the classifications, the largest proportion was bacterial (49%), and the remaining 1% was archaea and viruses (Figure S4A). After assembly, more of the unclassified reads were identified to be of bacterial origin, increasing the proportion to 67.5%. The proportions of Archaea and viruses remained the same (1%). The analysis showed that the most abundant phylum by gene expression activity was *Proteobacteria* (15–22%), followed by *Actinobacteria* (9–18%), *Acidobacteria* (4.6–7%), and *Chloroflexi* (1–2%) (Figure S4B).

Only 24 nitrogenase-related transcripts encoding NifDKHENB or Vnf/Anf were detected. The distribution was as follows: *nifH* (10 transcripts), *nifDK* (5 transcripts each), and 2 each for *nifE* and *nifN*. *nifB* was not found (Figure 4A). The transcripts were from *Proteobacteria*, *Cyanobacteria*, *Chloroflexi*, *Chlorobi*, and the archaeal phylum *Euryarchaeota*. *Alphaproteobacteria* were most common and represented by *Bradyrhizobium*, *Azospirillum*, *Mesorhizobium*, and *Zymomonas*. Gammaproteobacteria were represented by *Azotobacter*, *Teredinibacter*, and *Klebsiella*. *Herbaspirillum* was the only Beta-proteobacteria expressing *nif* genes. The very low number of *nifHDKENB* transcripts detected indicated that nitrogen fixing activity was very low (Figure 4B).

The expression levels of various pathways involved in nitrogen cycling were evaluated by selecting a marker gene for each pathway (Figure 5A). Expression of the marker genes was detected in all the pathways except nitrite oxidation (a part of nitrification) and hydrazine dehydrogenase (a part of anammox). Ammonia monooxygenase was the least expressed pathway, and nitrogen fixation was the second least expressed pathway. The most highly expressed pathway was ammonia assimilation, followed by mineralization. All the other internal cycling pathways showed intermediate expression (Figure 5A). Nitrite oxidation and hydrazine dehydrogenase were not detected. The distribution of overall gene expression by site showed an inconsistent distribution across the sampling sites (Figure 5B).

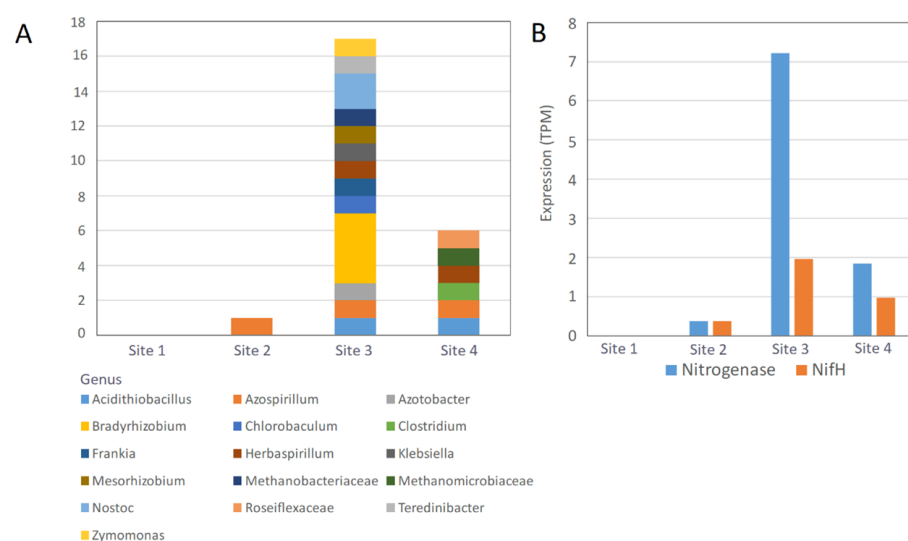


Figure 4. Distribution of bacteria with nitrogenase transcripts across study sites. Diversity (A) and expression (B) of total *nif* transcripts and *nifH* (marker for nitrogenase activity).

Genes expressing ammonia monooxygenase and hydroxylamine dehydrogenase were detected from the single genus *Nitrosomonas*. The expression of AmoA was in the range of 0.2 to 1.4 transcripts per kilobase million (TPM), with Site 1 showing the highest expression. The expression of Hao ranged from 0.06 to 3.1 TPM, with Site 3 showing the highest expression. These expression levels were lower than the expression of nitrogenase genes (Figure 5). Both eukaryotic and prokaryotic organisms were found to be capable of glutamine synthesis (assimilation) and deaminase to ammonia (mineralization) while being more active for assimilation. Assimilation was dominated by *Actinobacteria*, *Proteobacteria*, and *Cyanobacteria* (Figure S5A). *Actinobacteria* producing *glnA* were *Streptomyces*, *Frankia*, and *Mycobacterium*. Assimilating bacteria were *Alpha-proteobacteria*: *Azospirillum*, *Azorhizobium*, *Bradyrhizobium*, *Rhizobium*, *Sinorhizobium*, and *Rhodobacter*; *Betaproteobacteria*: *Cupriavidus*, *Neisseria*, and *Methyloversatilis*; *Gammaproteobacteria*: *Pseudomonas*, *Acinetobacter*, *Azotobacter*, and *Methylococcus*; *Cyanobacteria*: *Nostoc* and *Synechococcus*; and *Firmicutes* were represented by *Bacillus*, *Lactobacillus*, *Staphylococcus*, and *Clostridium*. On the other hand, deamination was dominated by *Euryarchaeota*, *Proteobacteria*, and fungi from the *Eukarya* (Figure S5B). They were distributed as follows: *Euryarchaeota*: *Pyrococcus* and *Saccharolobus*; *Proteobacteria*: *Gamma* (*Pseudomonas*, *Escherichia*, *Salmonella*, *Shigella*, *Haemophilus*) and *Epsilonproteobacteria* (*Helicobacter*). The ammonia-assimilating and -mineralizing organisms were uniformly distributed across the four sites. The expression of *glnA* was much higher, with an average expression of 271.2 TPM and deaminase of only 61.175 TPM (Figure S6). This indicates that ammonia was being used by the organisms at a much higher rate than it was being cycled back to the soil.

Nitrate assimilation marker genes were identified and included NR, NarB, NasA, NarG, and NapA. The distribution of expression levels was abundant for eukaryotes (algae, fungi, and phytoplankton) (Figure S7). NapA was expressed by the most diverse group of taxa, including *Symbiobacterium* (*Firmicutes*), followed by *Proteobacteria* (*Bradyrhizobium*, *Cupriavidus*, *Dechloromonas*, *Desulfovibrio*, *Rhodopseudomonas*, *Rhizobium*, and others). NarB was expressed by *Archaea* (*Haloferax*) and *Cyanobacteria* (*Synechococcus* and *Synechocystis*). NasA was expressed by *Klebsiella* and *Haloferax*. NarG was expressed by the genera *Mycobacterium* (*Actinobacteria*), *Escherichia* (*Proteobacteria*), *Bacillus* (*Firmicutes*), and *Haloferax* (*Archaea*). ANRA is catalyzed by NirA (expressed by *Haloferax* and *Phormidium*) and NIT-6 (expressed by *Bacillus* and some eukaryotes). Dissimilatory nitrate reduction is catalyzed by NirB (expressed by *Escherichia* and *Klebsiella*).

For denitrification, Hzs was the most highly expressed transcript and aligned to the single bacterium *Candidatus Kuenenia* (*Planctomycetes*). NosZ was the next most expressed, observed to be from *Bradyrhizobium*, *Pseudomonas*, *Brucella*, *Sinorhizobium*, *Cupriavidus*, and *Achromobacter*. It was followed by NirK, which was also expressed mainly by *Proteobacteria* (*Alkaligenes*, *Pseudomonas*, *Rhizobium*, *Rhodobacter*, *Sinorhizobium*). It was followed by NorB (*Pseudomonas*) and NrfH (*Wolinella*). The expression of denitrifying genes was also correlated with the number of transcripts recorded, with some variations among different sites (Figure S8).

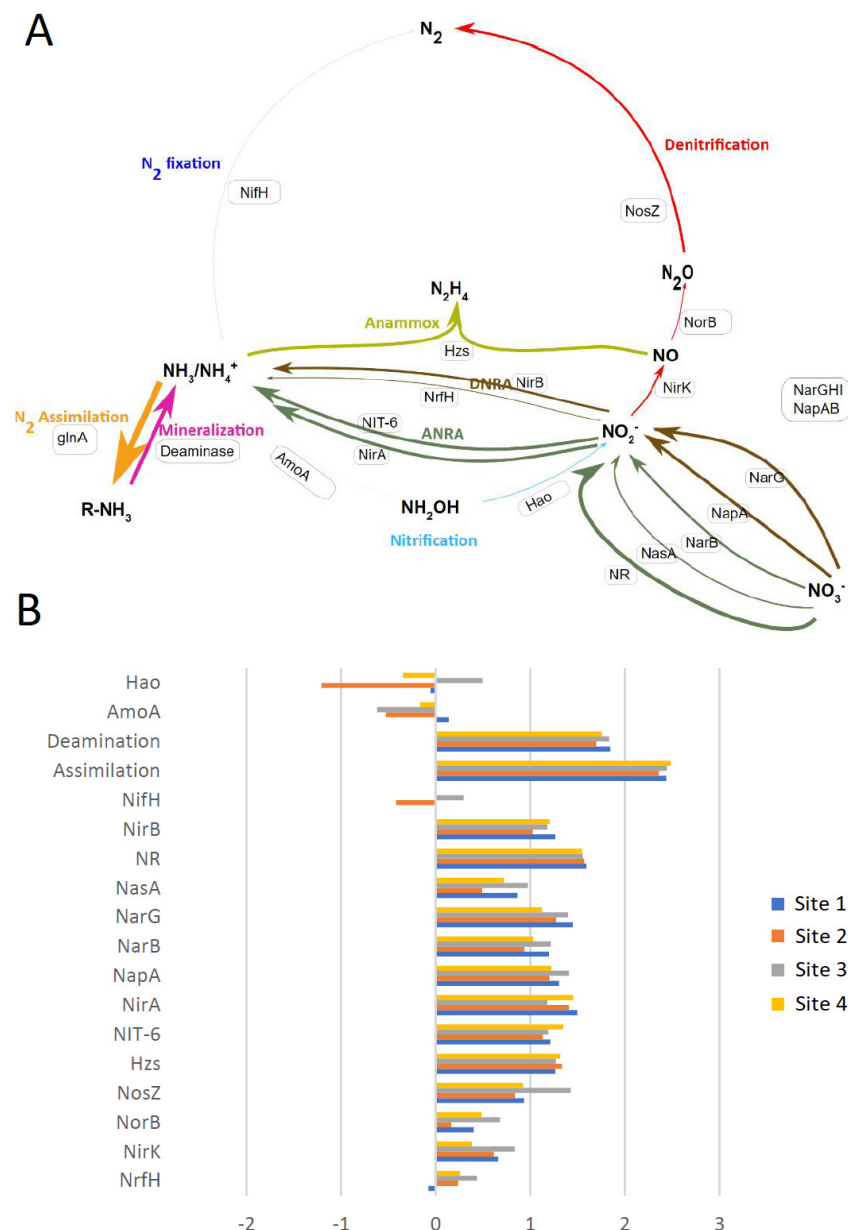


Figure 5. Nitrogen cycle with log₂ expression of marker genes, with thickness of connecting lines indicating expression levels (A), and comparison of N-cycling marker genes as log₁₀ of average expression of genes (B).

4. Discussion

We found that diazotrophs were diverse and prevalent, both using 16S rRNA gene signatures and *nifH* amplicon sequencing. However, we did not find evidence for nitrogen fixation through either ¹⁵N₂ incorporation or mRNA transcripts of core nitrogenase genes. In contrast, transcripts for nitrification and denitrification genes were expressed, pointing to a gap in the nitrogen cycle.

4.1. Prairie Soil Was Rich in Bacteria with Nitrogen Fixing Potential

The prairie soil contained a diverse community of genera known to contain diazotrophs. The community proportion determined by 16S rRNA gene homology varied by site. Proteobacteria constituted greater than one percent and were dominated by *Proteobacteria*. *nifH* amplicon pools also reflected a diversity of nitrogen fixers predominated by *Proteobacteria*. The use of *nifH* sequences for the study of diazotrophic community and

nitrogenase enzyme activity is well established [40,41]. *nifH* gene diversity in Tibetan grasslands [42] and pine plantations [43] was also predominated by *Proteobacteria*. The relative abundances of *nifH*-derived bacterial communities also show that a few bacterial taxa occupy large proportions of the community. This predominance of selected taxa is very common in soil environments [44]. The diversity of diazotrophs falling under 1% abundance fluctuated across the sites (Figure S3).

Many of the sequences amplified using the PolF & PolR primers could not be assigned to specific taxa. One possible reason for this could be the presence of uncharacterized bacterial communities in the prairie soil, warranting a more systematic characterization recently developed for human microbiomes [45]. Furthermore, horizontal gene transfer of *nifH* would enable the presence of the gene in bacteria that did not contain the additional core nitrogenase-encoding genes and would not provide an ability to fix atmospheric nitrogen [46,47]. More importantly, some diazotrophs could have gone undetected by *nifH* PCR because there are no primer sets shown to amplify the full diversity of the gene pool [48]. All published *nifH* primer sets are highly degenerate, leading to both nonspecific amplification and nonamplification of target genes. The majority of predicted diazotrophs fell into rhizobial genera known to fix nitrogen only when inside the nodules of legumes [49]. These sites did not contain legumes. The most prevalent bacteria at all sites and time points were *Bradyrhizobium*, *Mesorhizobium*, and *Rhizobium* (Figure 1B). These are well known for associative nitrogen fixation in legumes by forming nodules [49]. Conversely, some *Bradyrhizobium* fix nitrogen in association with sugarcane, i.e., outside nodules [50], and some nitrogen-fixing species are sole members of genera not known to otherwise fix nitrogen, e.g., *Propionibacterium* or *Spirochaeta* [7]. In addition, a few rhizobia have been reported to fix nitrogen while in free-living mode [51–53]. *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Devosia* likely rely on traits other than nitrogen fixation for fitness under local conditions, as some members are free-living [53,54]. Taken together, some of the taxa found through 16S rRNA gene and *nifH* sequencing were possibly not diazotrophs. However, the data support a substantial diversity of diazotrophs in the prairie soils.

4.2. Nitrogenase Activity in Prairie Soil Was Very Low

Culture-independent evidence pointed to a substantial diazotrophic community in prairie soils where between one and four percent of bacteria were predicted diazotrophs (Figure 1A). However, biochemical quantification by isotopic $^{15}\text{N}_2$ incorporation measurement pointed to fixation activity below the detection limit (Figure 3). The isotopic nitrogen incorporation assay is the gold standard technique to quantify nitrogen fixation [24]. The biochemical evidence was supported by the very low numbers of *nifH* (0.83 TPM) and other nitrogenase gene transcripts (Figure 4). No evidence of alternative nitrogenases *vnf* and *anf* was detected. This indicates a very low rate of nitrogen fixation occurring in the prairie soil. This result is congruent with the result from our previous NiCE chip analysis from the same soil [12]. A similar low expression of *nifH* was observed in another meta-transcriptomic study in saline soil [55]. To help reconcile the discrepancy between the presence and apparent inactivity of diazotrophs, we determined the nitrogen fixation potential by adding a carbohydrate cocktail [56]. The stimulation of fixation by the provision of carbohydrates yielded very high nitrogen fixation (Figure 3), indicating that the resident bacterial community was able to fix nitrogen. Importantly, nitrogen fixation by free-living bacteria is energy-intensive on multiple counts [57]. This includes the reduction in 16 ATP per molecule of N_2 and various bacterial activities to protect the cytoplasmic nitrogen complex from oxygen damage [57]. The strict gene regulation controls on nitrogen fixation are often overlooked [58]. The average carbon-stimulated fixation of 1307 $\mu\text{g/kg/d}$

is higher than the average incorporation rate of 300 µg/kg/d reported for a field plot under continuous maize cultivation for over 30 years [59] and is substantial. The apparent lack of fixation in unamended soil indicated that resident diazotrophs lacked carbohydrates for power fixation. Collectively, the data indicate that diazotrophs were present in the prairie soil but did not fix nitrogen, likely due to a lack of available carbon sources for energy.

4.3. The Nitrogen Cycle in Prairie Soil Appeared to Be Active

While the metagenomic approach informs the presence of specific genes, the meta-transcriptomic approach provides insights into gene expression, which is related to the metabolic functions of interest. Recently, several other studies have also employed HTS of complete transcriptomes to study N-cycling genes [8,9]. Among the nitrogen cycling pathways, nitrogen fixation and nitrification genes were expressed at very low levels. Nitrogenase-encoding genes were expressed only in *Proteobacteria*, *Cyanobacteria*, *Chloroflexi*, *Chlorobi*, and the archaeal phylum *Euryarchaeota*. Although the structural components of nitrogenase were expressed in very low numbers, a significantly higher number of nitrogen fixation regulatory transcripts (16,270) were expressed in this study, indicating the tight regulation of energy-expensive nitrogenase. Nitrification was another least expressed N-cycling pathway (6.1 times per million sequences [22]) and expressed by a very limited number of microorganisms. *amoA* was expressed at only 0.648 TPM, and *hao* had 1.127 TPM. Recently, various Archaea and proteobacteria were shown to be capable of nitrification [9,60], but in this study, only *Nitrosomonas* was found to be able to perform nitrification.

The expression levels of all N-cycling genes except nitrification and nitrogen fixation were found to be high and comparable to reports from other studies [8,12]. The nitrogen-metabolism network proposed by Kuypers et al. [2] suggests that they should be involved in nitrogen homeostasis. While most of the nitrogen is cycled within the ecosystem, some is lost back to dinitrogen through denitrification. This loss should be balanced out by replenishment through nitrogen fixation. Without the replenishment of combined nitrogen loss, the ecosystem would lose biomass over time. Nitrogen fixation could occur in hotspots not specifically sampled here, for example, in the rhizosphere, where roots provide carbon through exudation [61,62]. While nitrogenase gene expression was very low, there could be other ways in which soil bacteria acquire combined nitrogen. Interestingly, ammonia assimilation was the most highly expressed of all genes of the N-cycle pathway. Ammonia uptake genes are widespread in bacteria [63]. The most abundant organisms expressing ammonia assimilation were *Actinobacteria*, such as *Streptomyces*, *Frankia*, and *Mycobacterium*. These *Actinobacteria* genera were also common among bacteria isolated on nitrogen-free medium, yet there was no evidence for nitrogenase [26]. This high expression of ammonia assimilation genes such as *glnA* could assist in scavenging residual combined nitrogen.

5. Conclusions

The data presented here indicate that the natural prairie contains a prolific proportion of nitrogen-fixing taxa, making up 1.5–4% of the community. The nitrogen-fixing community was diverse but contained a high proportion of symbiotic fixers such as *Bradyrhizobium* not known to fix under free-living conditions. While the nitrogen-fixing potential of the bacterial community was high, in situ ¹⁵N₂ incorporation was under the detection limit. This low level of fixation was supported by the very low level of *nifH* mRNA detected. The comparatively high levels of expression of all other genes of the nitrogen cycle suggests an imbalance in the nitrogen cycle, with net loss of combined nitrogen over time. The net loss of combined nitrogen would lead to a decrease in overall biomass that has, however, not

been observed to date. This apparent contradiction requires further study, including the search for potential N-fixing hot-spots such as the rhizosphere and AMF hyphosphere.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/nitrogen7030079/s1>. Table S1. GO terms used to identify the transcripts involved in nitrogen cycling pathways. Table S2. Sequence data summary; Figure S1. Principal component analysis of the prevalence of OTUs aligning with nitrogen-fixing genera shown by sampling site (A) and time obtained from prairie (month) (B). Figure S2. Relative abundances of nitrogen-fixing genera showing a significant difference for at least one sampling site in the natural prairie ecosystem across the study period. Figure S3. The distribution of bacterial taxa occupying less than 1% of the relative *nifH*-bearing abundance across the sampling sites. Figure S4. Prokaryote distribution patterns from assembled RNA reads. Prokaryotic superkingdoms (A) and prokaryotic phyla after excluding unclassified reads (B). Figure S5. Diversity of ammonia-assimilating and -mineralizing organisms across the four sites: glutamine synthase (GlnA) (A), deaminase (B). Figure S6. Expression of assimilation (glnA) and biomineralization (deaminase) across the four sites. Figure S7. Diversity of genes involved in multiple pathways of nitrate assimilation. *napA* was found to be expressed by most diverse microorganisms. Figure S8. Expression of marker genes involved in the denitrification process.

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Data Availability Statement: The raw sequence data analyzed in this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1158199 (BioProject ID: 1158199). The dataset includes RNA-Seq data from four sampling sites (BioSamples SAMN43530905–SAMN43530908) and *nifH* amplicon sequencing data from the same four sites (BioSamples SAMN43530909–SAMN43530912).

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