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Metagenomic reconstruction of nitrogen cycling pathways in a CO_2-enriched grassland ecosystem

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ABSTRACT

The nitrogen (N) cycle is a collection of important biogeochemical pathways mediated by microbial communities and is an important constraint in response to elevated CO_2 in many terrestrial ecosystems. Previous studies attempting to relate soil N cycling to microbial genetic data mainly focused on a few gene families by PCR, protein assays and functional gene arrays, leaving the taxonomic and functional composition of soil microorganisms involved in the whole N cycle less understood. In this study, 24 soil samples were collected from the long-term experimental site, BioCON, in 2009. A shotgun metagenome sequencing approach was employed to survey the microbial gene families involved in soil N cycle in the grassland that had been exposed to elevated CO_2 (eCO_2) for >12 years. In addition to evaluating the responses of major N cycling gene families to long-term eCO_2, we also aimed to characterize the taxonomic and functional composition of these gene families involved in soil N transformations. At the taxonomic level, organic N metabolism and nitrate reduction had the most diverse microbial species involved. The distinct taxonomic composition of different N cycling processes suggested that the complex N cycle in natural ecosystems was a result of multiple processes by many different microorganisms. Belowground microbial communities that mediate N cycling responded to eCO_2 in several different ways, including through stimulated abundances of the gene families related with organic decomposition, dissimilatory nitrate reduction, and N_2 fixation, and suppressed abundances of the gene families in glutamine synthesis and anammox. This study provides a genetic basis of the microorganisms involved in key processes in the N cycle in complex ecosystems, and shows that long-term eCO_2 selectively affects N cycling pathways instead of tuning up every process.

1. Introduction

The nitrogen (N) cycle, by which different forms of N are transformed, is a collection of important biogeochemical pathways mediated by microbial communities (Galloway, 1998; Galloway et al., 2004; Gruber and Galloway, 2008), including N mineralization, immobilization, and various oxidation-reduction reactions that transform different forms of N. The N cycle is a complex biogeochemical cycle with multiple steps observed and concluded from cultured microbes and *in situ* experiments. In addition to absorbing NO_3^- and NH_4^+ from the environment, the plant root also uptakes amino acids as N sources (Nelson et al., 2016; Schimel and Chapin, 1996). Previous genetic studies of the N cycle focused on specific N cycling gene families such as *nifH* (nitrogenase, key

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enzyme for N_2 fixation) (Collavino et al., 2014; Tu et al., 2016a, 2016b), *amoA* (ammonium monooxygenase, key enzyme for nitrification) (Bru et al., 2011; Leininger et al., 2006; Pester et al., 2012; Sintes et al., 2013), *narG* (nitrate reductase) (Bru et al., 2011; Kandeler et al., 2006), *nirK*/*nirS* (nitrite reductase) (Braker et al., 2000; Bru et al., 2011; Kandeler et al., 2006), and *nosZ* (nitrous oxide reductase) (Bru et al., 2011; Henry et al., 2006; Kandeler et al., 2006), and were carried out by amplification-based approaches. The results have provided novel insights, especially highlighting the importance of uncultured organisms in carrying out these processes in many ecosystems.

Recent efforts have focused on characterizing functional genes involved in multiple N cycling processes using genetic approaches and relating genetic information to ecosystem functioning. For example, Petersen et al. (2012) analyzed nitrification and denitrification processes across a vegetation gradient in Alaska by qPCR amplification of gene families including *amoA*, *nirK/S*, and *nosZ*, and suggested that the abundance of these functional genes can be used as good predictors for biogeochemical process rates. However, comprehensive surveys of genes involved in all N cycling processes in complex ecosystems have rarely been carried out, because of the lack of working primers for many of the gene families involved. With high throughput microbial ecological microarray technologies such as GeoChip (He et al., 2012a; Tu et al., 2014), several studies were previously carried out to analyze N cycling processes in different ecosystems (He et al., 2012c), such as grassland ecosystems (He et al., 2010; Xu et al., 2013; Zhou et al., 2012), river sediments (Xu et al., 2014), and tundra (Xue et al., 2016). However, microarray technologies are insufficient to estimate novel genes and highly divergent gene variants in the environment (Zhou et al., 2015). Because the abundance and composition of gene families are at the core in linking microbial communities to N cycling processes (i.e. who are doing what in the environment), there remains a need to comprehensively quantify N cycling processes, especially for those gene families not targeted by microbial ecological microarrays and/or without working primers.

Under elevated CO_2 (eCO_2), several inter-related processes greatly affect N cycling pathways mediated by belowground microbial communities. Stimulated plant photosynthesis and plant growth (both aboveground and belowground) (Luo et al., 2006b; Norby et al., 2005; Reich and Hobbie, 2013; Reich et al., 2001) provide more carbon (C) to the belowground microbial communities, and at the meanwhile demand more biologically available N in the soil. As a result, microbial N_2 fixation is expected to increase under eCO_2, leading to a larger N-input to the soil ecosystem (Hungate et al., 1999; Soussana and Hartwig, 1995), though some studies also show declined N_2 fixation (Hungate et al., 2004). Microbial decomposition and N mineralization processes can be affected by increased C:N ratios in the plant biomass as well as by increased availability of labile C that can fuel processes such as N_2 fixation, denitrification and priming of soil organic matter decomposition (Luo et al., 2006b; Norby et al., 2005; Reich and Hobbie, 2013; Reich et al., 2001). Previous studies (Carney et al., 2007; Hu et al., 2001) also suggested that the effects of eCO_2 on microbial decomposition is related with litter quality (BALL, 1997) and C:N ratio in plant litter (McLaren and Cameron, 1996). In addition, increased plant growth may strengthen the degree of N limitation (Finzi et al., 2006; Hu et al., 2001; Luo et al., 2004; Norby et al., 2010; Reich and Hobbie, 2013; Reich et al., 2006), increasing plant demand for N and plant N uptake. Progressive N limitation may ultimately constrain plant responses to eCO_2 (Hu et al., 2001) and may as well affect belowground microbial community composition, structure, and functional potentials (Deng et al., 2012; Drigo et al., 2008; He et al., 2010, 2012b; Tu et al., 2015; Tu et al., 2016b; Xu et al., 2013). Although much work has been carried out to study the microbial responses to eCO_2 at the BioCON and other sites, a linkage between microbial community composition and N cycling pathways remains largely elusive.

In this study, we aimed to survey the major N cycling gene families, and their responses to eCO_2 at the BioCON (Biodiversity, CO_2 and Nitrogen) experimental site located at the Cedar Creek Ecosystem Science Reserve in Minnesota. A total of 24 soil samples were collected from the BioCON experimental site in 2009. A shotgun metagenome sequencing approach was implemented here to survey important N cycling gene families in an unbiased manner. We hypothesized that multiple N cycling processes, especially those related to organic matter decomposition and N_2 fixation, would be stimulated due to increased C input and N limitation in the ecosystem as a result of long-term eCO_2. In addition to characterizing the responses of microbial N cycling pathways to long-term eCO_2, this study also focused on the actual relative abundance of each key gene family involved in N cycling processes as well as their taxonomic composition. According to our discoveries, we also present a schema to illustrate the N cycling process in the BioCON grassland experimental site, as well as its responses to long-term eCO_2.

2. Materials and methods

2.1. Site description and sample collection

The study was conducted at the BioCON experimental site located at the Cedar Creek Ecosystem Science Reserve in Minnesota, USA (Lat. 45N, Long. 93W). The long-term experiment was started in 1997 in a secondary successional grassland on a sandy outwash soil after removing the previous vegetation (Reich et al., 2001). The main BioCON field experiment has 296 (of a total of 371) evenly distributed plots (2 × 2 m) in six 20-m diameter FACE (free air CO_2 enrichment) rings, three with ambient CO_2 (aCO_2) concentrations, and three with CO_2 concentrations elevated by 180 μmol/mol (Lewin et al., 1994). In this study, 24 plots (12 from aCO_2, 12 from eCO_2, all with 16-species and without additional N supply) were used.

All of the 16 plant species used in this study are native or naturalized to the Cedar Creek Ecosystem Science Reserve, and can be classified into four functional groups: (i) four C_3 grasses (*Agropyron repens*, *Bromus inermis*, *Koeleria cristata*, *Poa pratensis*), (ii) four C_4 grasses (*Andropogon gerardii*, *Bouteloua gracilis*, *Schizachyrium scoparium*, *Sorghastrum nutans*), (iii) four N_2-fixing legumes (*Amorpha canescens*, *Lespedeza capitata*, *Lupinus perennis*, *Petalostemum villosum*), and (iv) four non N_2-fixing herbaceous species (*Achillea millefolium*, *Anemone cylindrica*, *Asclepias tuberosa*, *Solidago rigida*). Plots were regularly manually weeded to remove unwanted species, although the 16 species plots used in this study require minimal weeding.

Bulk soil samples were taken in July, 2009 from plots (four per FACE ring) planted with 16 species (four species from each of four functional groups, C_4 grasses, C_3 grasses, legumes, and non-legume forbs) under aCO_2 and eCO_2 conditions for microbial community analysis. Each sample was composited from five soil cores at a depth of 0–15 cm. All samples were immediately transported to the laboratory, frozen and stored at −80 °C for DNA extraction, PCR amplification, and 454 pyrosequencing. Fine roots were carefully examined and removed from the soils.

2.2. Plant biomass and soil nitrogen properties

The aboveground and belowground (0–20 cm) biomass were measured as previously described (Reich et al., 2001, 2006). In August, 2009, a 10 × 100 cm strip was clipped at just above the soil

surface, and all plant material was collected, sorted to live material and senesced litter, dried and weighed. Roots were sampled at 0–20 cm depth using three 5-cm diameter cores in the area used for the aboveground biomass clipping. Roots were washed, sorted into fine (<1 mm diameter) and coarse classes and crowns, dried and weighed. A composite sample was taken from aboveground and belowground biomass from each plot from the August harvest, ground and analyzed for N using a Costech ECS 4010 element analyzer (Costech Analytical Technologies, Inc., Valencia, CA).

Soil pH and volumetric soil moisture were measured in a KCl slurry and with permanently placed TRIME Time Domain Reflectometry (TDR) probes (Mesa Systems Co., Medfield MA), respectively. Net ammonification and nitrification were measured concurrently in each plot for one-month *in situ* incubations with a semi-open core method from 0 to 20 cm depth during midsummer of each year (Reich et al., 2001, 2006). Incubated soil cores (2 mm), as well as soil cores taken at the start of each incubation, were sieved and extracted with 1 M KCl. Extracts were analyzed for NO_3^- and NH_4^+ on an Alpkem auto-analyzer (Alpkem, Perstorp Analytical Company, Wilsonville, OR). Net ammonification was calculated by the difference between the final and initial NH_4^+-N pool sizes of the one-month *in-situ* incubation. Net nitrification was calculated by the difference between the final and initial NO_3^--N pool sizes of the one-month *in-situ* incubation.

2.3. DNA extraction, purification and quantification

Soil DNA was extracted by freeze-grinding mechanical SDS-based lysis as described previously (Zhou et al., 1996), and was purified using a low-melting agarose gel followed by phenol extraction for all 24 soil samples collected. DNA quality was assessed by the ratios of 260/280 nm, and 260/230 nm using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE), and final soil DNA concentrations were quantified with PicoGreen (Ahn et al., 1996) using a FLUOstar Optima (BMG Labtech, Jena, Germany).

2.4. Shotgun metagenome sequencing, processing and data analysis

All 24 samples were subjected to shotgun metagenome sequencing by Roche 454 pyrosequencing approaches. For each sample, 500 ng DNA was used for library construction using the GS FLX Titanium Rapid Library Preparation Kit (454 Life Sciences, Branford, CT). Library construction and sequencing were carried out by Los Alamos National Lab (New Mexico, USA) using standard shotgun protocols. A total of 18,890,805 raw reads (385,097 to 1,385,378 reads per sample, average length 339bp) were obtained. Quality control for 454 shotgun sequences was carried out by the LUCY program (Chou and Holmes, 2001) with minimum quality score of 21 and maximum error rate of 0.01, resulting in 17,096,024 high quality sequences. Gene prediction was carried out by FragGeneScan (Rho et al., 2010), which predicts high quality gene fragments from short, error-prone reads and overcomes homopolymer errors. A total of 17,578,392 genes were predicted by FragGeneScan. Functional and taxonomic assignments of the predicted genes were carried out by BLAST searching protein sequences against eggNOG (Muller et al., 2010) and NCBI nr database, respectively. For functional assignment, the best hit with eggNOG database was used. For taxonomic assignment, lowest common ancestors were assigned based on the best hits within 1/10 top e-value using the MEGAN program (Huson et al., 2007). A random resampling effort to the minimum number of sequences in the samples was carried out. The total number of sequences was then normalized to 1 million per sample for further analysis. The response ratio analysis was used to measure the statistical differences of gene family relative abundances between aCO_2 and eCO_2 sites. All metagenomic data generated in this study was deposited in the NCBI database and can be found under accession number SRP034704.

3. Results

3.1. eCO_2 effects on plant and soil properties

Both aboveground and belowground plant growth were significantly stimulated by eCO_2, by 66 and 105 g/m^2, respectively ($P = 0.01$, Fig. 1A). Soil ammonium concentrations measured in June (years 2005–2009) increased ($P = 0.08$) under eCO_2, while nitrate concentration did not change significantly (Fig. 1B), which was consistent with past studies that analyzed June ammonium and nitrate concentrations over a longer time period (Mueller et al., 2013). C:N ratio in both aboveground and belowground plant biomass increased under eCO_2 ($P = 0.008$ and $P = 0.09$, respectively) (Fig. 1C), suggesting a progressive N limitation as a result of eCO_2. Soil pH, however, did not change significantly ($P = 0.45$) (Fig. 1D) under eCO_2.

3.2. An overall schema of nitrogen cycling pathways in the soil

We first proposed a schema to illustrate the whole N cycle in the BioCON experimental site according to our results (Fig. 2). A total of seven pathways were presented and analyzed, including nitrification, denitrification, dissimilatory nitrate reduction to ammonium, assimilatory nitrate reduction, anammox, N_2 fixation, and organic N metabolism. Among all of the gene families, *nxrAB*, *GS*, *nao*, *nasAB*, *nirBD*, *ureC*, and *gdh* were the most abundant gene families in the ecosystem. And among all these pathways, organic N metabolism and glutamine synthesis were the most diverse at the taxonomic level. Regarding their responses to long-term eCO_2, significant changes in the abundance of gene families involved in dissimilatory nitrate reduction, organic N metabolism, N_2 fixation, and anammox were found. Abundance of gene families involved in nitrification, assimilatory nitrate reduction, and denitrification of NO_2^- to N_2, however, did not change under eCO_2.

3.3. Nitrification and denitrification

Nitrification is the biological oxidation process that converts ammonia to nitrite, and then to nitrate. Four gene families are mainly responsible for microbial biological nitrification, including *amoA* encoding ammonia monooxygenase, *hao* encoding hydroxylamine dehydrogenase, and *nxrAB* encoding nitrite oxidoreductase. Among them, *amoA* is responsible for oxidizing ammonia to hydroxylamine, *hao* is responsible for oxidizing hydroxylamine to nitrite, and *nxrAB* subunits are responsible for oxidizing nitrite to nitrate. Metagenome sequencing showed *nxrA* and *nxrB* subunits were the most abundant gene families involved in nitrification, with ~235 and ~400 normalized reads detected, respectively (Fig. 3). Low relative abundances were found for both *amoA* and *hao* gene families, with only about 30 and 3 normalized reads, respectively (Fig. 3). No significant differences were found for any gene family involved in nitrification between CO_2 treatments (Fig. 3).

In contrast, denitrification is a biological reductive process that converts NO_3^- to NO_2^-, NO, N_2O, and finally to N_2. Gene families including *narGHIJ* and *napAB* are responsible for nitrate reduction to NO_2^-. The abundances of *narHJ* and *napB* increased significantly under eCO_2 (Fig. 3), which could be a coincidence with increased abundance of gene families involved in dissimilatory nitrate reduction. Gene families including *nirS/K* (nitrite reductase), *norBC* (nitric oxide reductase) and *nosZ* (nitrous-oxide reductase) are

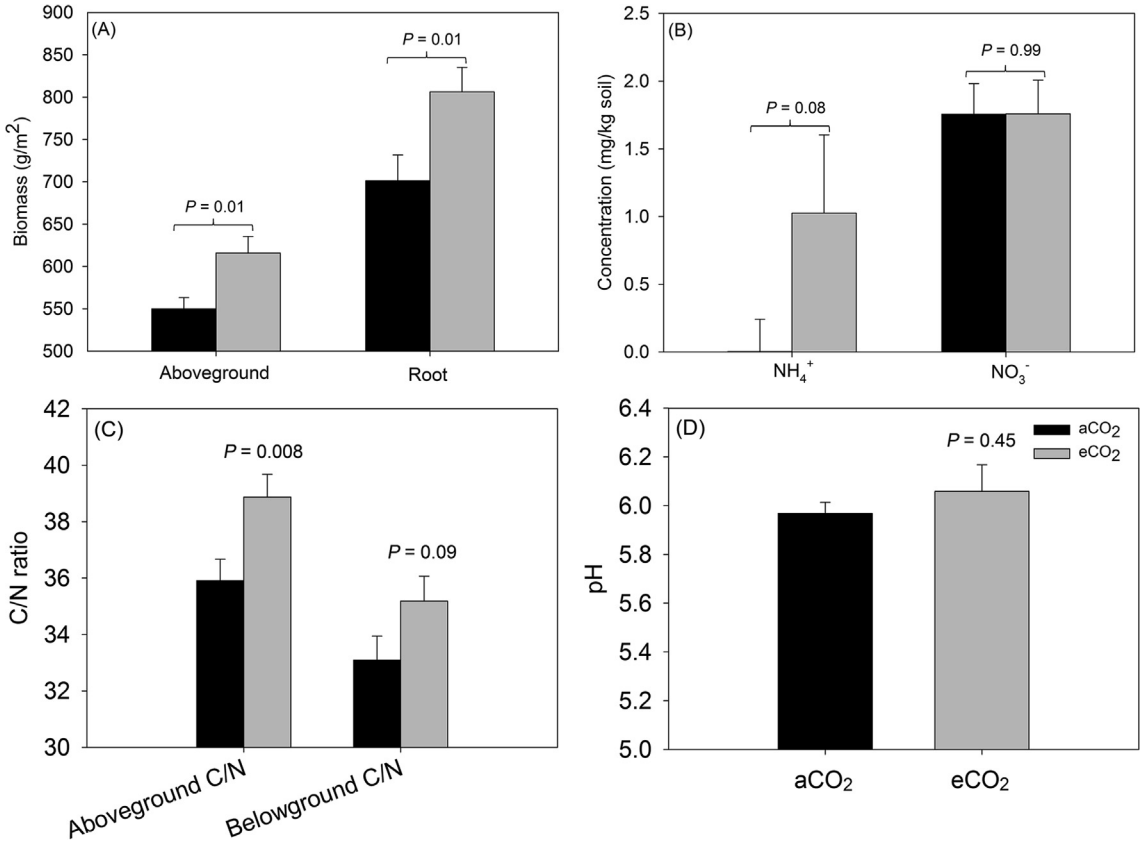

Fig. 1. Elevated CO_2 effects on plant biomass (A) and soil nitrogen (NH_4^+ and NO_3^-) (B). Both aboveground and root biomass were significantly stimulated under eCO_2 treatment. Long-term eCO_2 also significantly stimulated ammonification in the soil, but not nitrification. Data from year 2005–2009 were collected and analyzed by the Student's *t*-test.

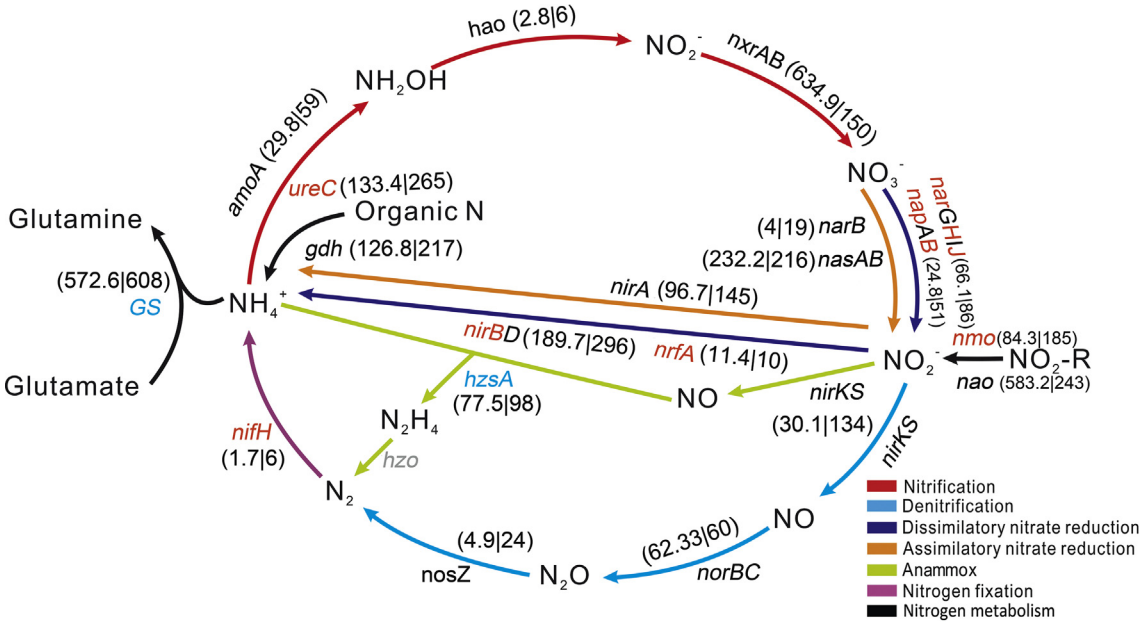

Fig. 2. An overall schema illustrating the nitrogen cycling processes in the grassland ecosystem as well as their responses to long-term eCO_2. Under eCO_2, the gene families marked in red indicate a significantly increased abundance, the gene families in light blue indicate a significantly decreased abundance, and the gene families in black indicate no significant changes. The numbers in the brackets beside each gene represent the normalized abundance (left) and observed number of microbial species (right) for the gene family. Different colors represented different functional processes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

responsible for denitrification of NO_2^- to N_2. Low abundances were detected for all these gene families, with less than 40 normalized reads in each sample (Fig. 3). Again, no significant differences of abundance were found for these gene families between CO_2

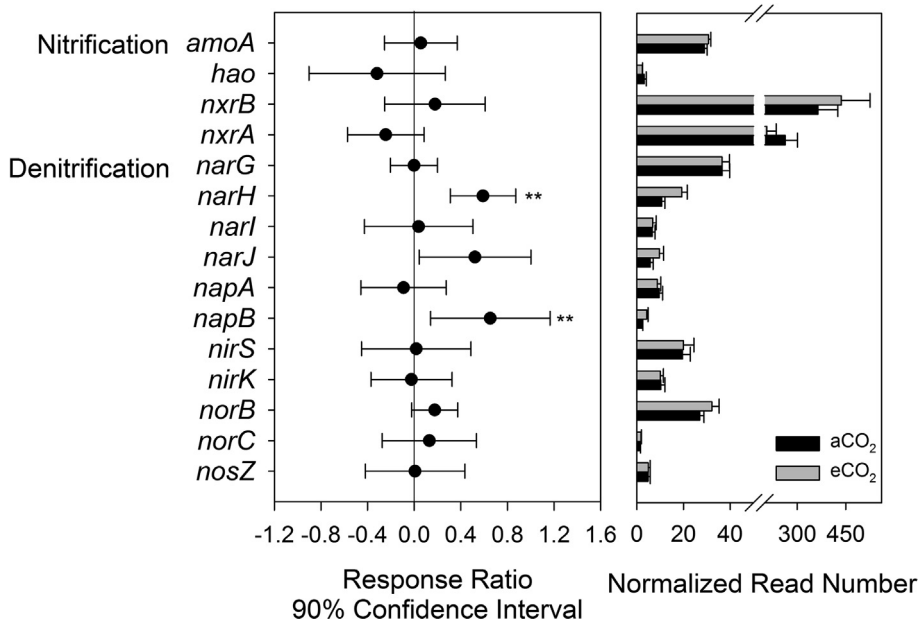

Fig. 3. Response ratio analysis (90% confidence interval) and the normalized abundance of gene families involved in nitrification and denitrification processes. The total number of reads in each sample was normalized to one million. ** represents significant changes at 95% confidence interval.

treatments.

3.4. Assimilatory and dissimilatory nitrate reduction

Based on the final fate of produced ammonium, there are two pathways for nitrate reduction to ammonium in the environment—dissimilatory and assimilatory nitrate reduction to ammonium. Ammonium produced by dissimilatory nitrate reduction (dissimilatory nitrate reduction to ammonium, DNRA) is released to the environment when the reduction of nitrate to ammonium is coupled to the oxidation of organic C to yield energy; the ammonium can subsequently be absorbed by plants and microorganisms. Assimilatory nitrate reduction is an energy-consuming process whereby nitrate is reduced to ammonium for use in biosynthesis of microbial biomass (Sias et al., 1980). Different gene families are involved in these two pathways of nitrate reduction. The assimilatory process is catalyzed by enzymes encoded by *narB* (nitrate reductase), *nasAB* (nitrate reductase) and *nirA* (nitrite reductase) gene families, while the dissimilatory process is catalyzed by enzymes encoded by *narGHIJ* (nitrate reductase), *napAB* (nitrate reductase), *nirBD* (nitrite reductase), and *nrfA* (nitrite reductase). Interestingly, abundances for gene families related with assimilatory nitrate reduction did not change significantly under eCO_2, though relatively high copies of sequences were detected for *nasA* and *nirA* (Fig. 4). In contrast, relative abundance for several dissimilatory gene families increased ($P \leq 0.1$) under eCO_2, including *narHJ*, *napB*, *nirB*, and *nrfA* (Fig. 4). Among these, *nirB* was the most abundant gene family involved in nitrate reduction, with more than 200 normalized reads in each sample, indicating that dissimilatory reduction of nitrite into ammonium might be an important process in these soils.

3.5. Anammox, organic N decomposition and N_2 fixation

Anammox is the process that converts NO and ammonium into N_2H_4 and then N_2. Enzymes encoded by gene families *hzsA* (hydrazine synthase) and *hzo* (hydrazine oxidoreductase) are responsible for this process. The gene family *hzo* was rarely detected in either aCO_2 or eCO_2 metagenomes. An average of 90.6 ± 6.8 and 64.4 ± 8.7 normalized reads were detected in aCO_2 and eCO_2 metagenomes, respectively (Fig. 5). Relative abundance of gene family *hzsA* decreased significantly ($P < =0.05$) under eCO_2, suggesting a decreased conversion of ammonium into N_2H_4 under eCO_2.

Many microbial gene families are responsible for organic N decomposition, metabolism, and biosynthesis in soil. Here, five gene families directly related with N cycling processes were extracted and analyzed, including *nao* (nitroalkane oxidase), *nmo* (nitronate monooxygenase), *gdh* (glutamate dehydrogenase), *ureC* (urease) and *GS* (glutamine synthetase). These gene families were clearly dominant in the soil ecosystem, with a range of ~77.1 ± 4.1 to ~645.8 ± 84.7 normalized reads in the metagenome (Fig. 5). The abundances for gene families *nao* and *gdh* did not change under eCO_2, but increased significantly for *nmo* and *ureC*, and decreased for *GS* (Fig. 5). This indicated that eCO_2 stimulated organic N metabolism, but suppressed biosynthesis of glutamine from ammonium.

N_2 fixation in soil is mainly carried out by nitrogenase encoded by *nifH* gene family, and is considered as an important source of ammonium. However, *nifH* genes were rarely detected in shotgun metagenomes, with only 1–2 copies per sample. This is possibly due to relatively low abundance of *nifH* genes in the immense soil microbial communities and/or in the bulk soil, as well as random sampling issues in metagenomic studies. Nevertheless, although *nifH* was detected with low abundance, *nifH* gene abundance increased significantly ($P < 0.05$), indicating possibly increased N_2 fixation as a result of eCO_2.

3.6. Taxonomic profiles of microorganisms involved in soil N cycling

In order to identify the taxonomic compositions that are mainly responsible for N cycling processes, the five most abundant microbial orders and/or those with >3% contribution to the processes were extracted and analyzed (Fig. 6). Taxonomically, the N cycling processes were mainly carried out by *Actinomycetales* and *Rhizobiales* species, except denitrification and anammox. Substantial

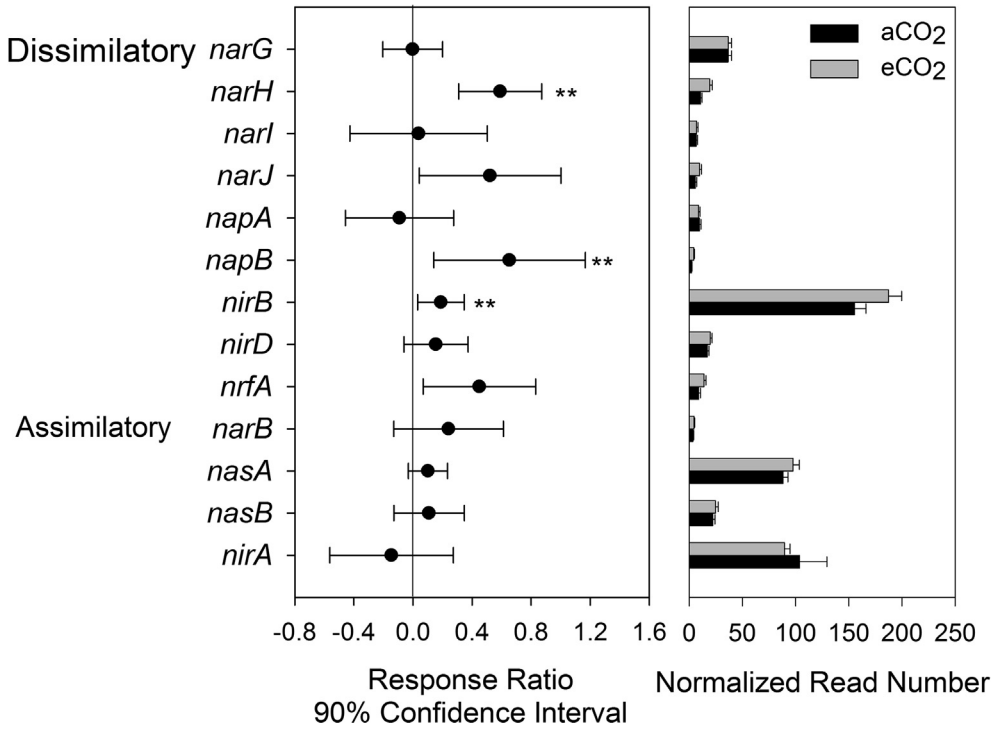

Fig. 4. Response ratio analysis (90% confidence interval) and the normalized abundance of gene families involved in dissimilatory and assimilatory nitrate reduction processes. Significantly increased abundances of gene families for dissimilatory nitrate reduction were detected, but not for assimilatory nitrate reduction. The total number of reads in each sample was normalized to one million. ** represents significant changes at 95% confidence interval.

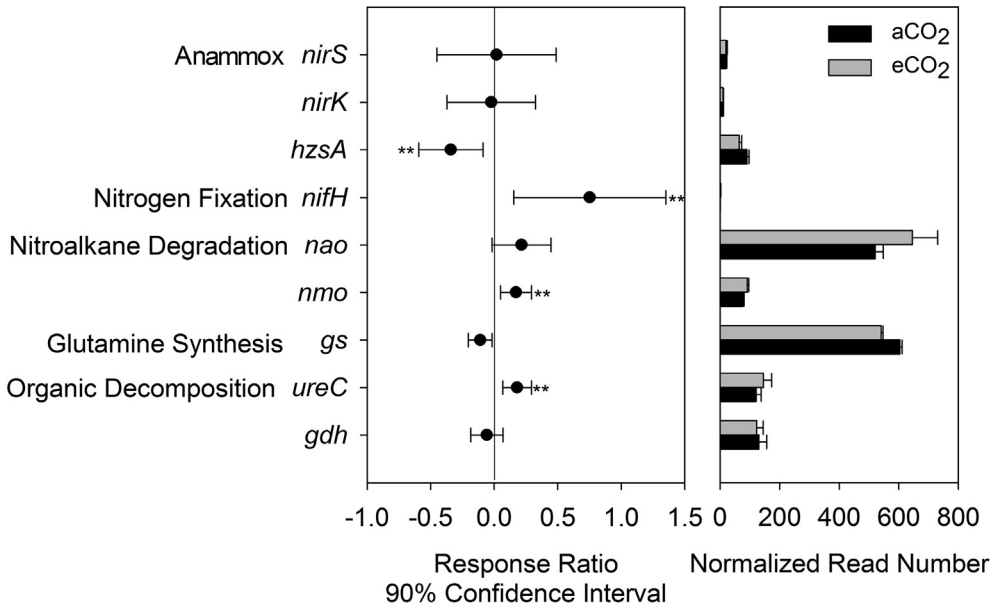

Fig. 5. Response ratio analysis (90% confidence interval) and the normalized abundance of gene families involved in anammox, N_2 fixation, organic N metabolism, and glutamine synthesis processes. Significantly changed abundances of gene families for anammox, N_2 fixation, and organic N metabolism were detected. The total number of reads in each sample was normalized to one million. ** represents significant changes at 95% confidence interval.

differences in taxonomic profiles could be observed for less abundant taxonomic groups among different N cycling processes. For example, *Nitrospirales* species contributed to ~6.9% of the total detected genes in nitrification; *Solirubrobacterales* contributed to ~11.9% of the genes in organic N metabolism; *Planctomycetales* species contributed to ~4.4% in assimilatory nitrate reduction. Among these, organic N metabolism was the most diverse process in the ecosystem, with only 4 families having >3% relative abundance in the taxonomic profile but with a total of 758 microbial species detected. No significant changes could be observed for these dominant microbial orders, except *Actinomycetales* and *Nitrospirales* that respectively showed marginally significant increase ($P = 0.1$) and decrease ($P = 0.08$) in abundance.

Compared to other N cycling processes, denitrification and

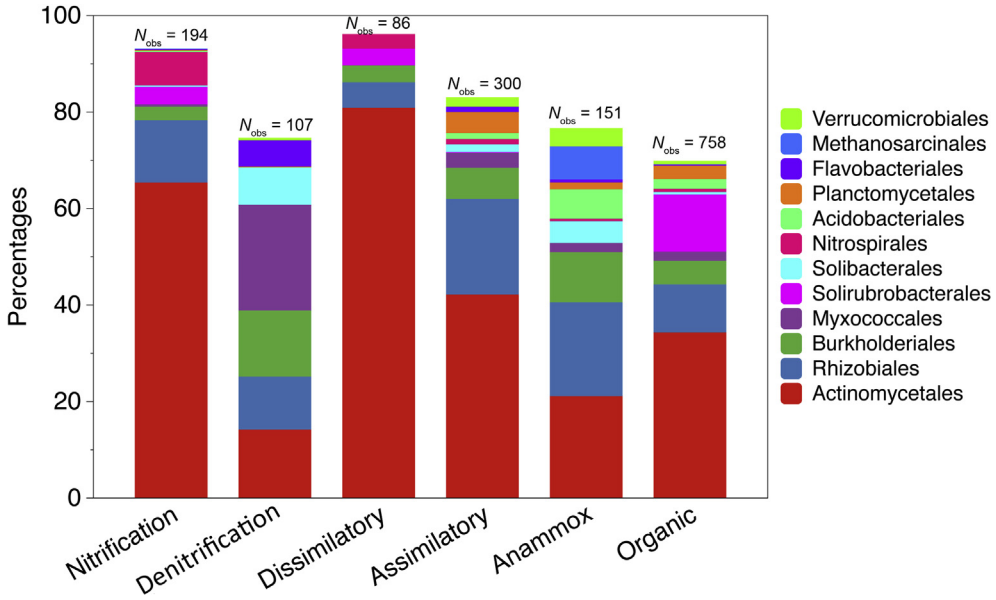

Fig. 6. Taxonomic groups involved in major nitrogen cycling processes. Only the top five most abundant microbial orders and the ones with >3% relative abundance are shown in the color legends. The numbers above each bar represent the observed number of microbial species in their corresponding processes.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

anammox were the processes with the most even distribution among different microbial orders, with six and seven microbial orders contributing to more than 3% of genes involved in each process, respectively. The taxonomic profiles of involved microorganisms were substantially different from the other processes. For example, *Myxococcales* species, with less than 3% contribution in other processes, were the major player in denitrification processes with 21.9% contributions. *Acidobacteriales* species contributed 6.1% of the genes in the anammox process, but were rarely detected in other processes. In addition, microbial species belonging to *Burkholderiales* and *Solibacterales* played important roles in both denitrification and anammox. Notably, about 7%–35% of the genes involved with each process could not be assigned to any known microbial order.

At the family and gene levels, a mosaic distribution of microbial families was observed across the investigated N cycling gene families (Table S1). Among them, only a few microbial families contributed to more than 20% of the sequences of some gene families. For example, *Bradyrhizobiaceae* contributed 25%, 36%, 48%, 65%, and 25% to the taxonomic composition of *nirK*, *amoA*, *napA*, *napB*, and *nifH* gene families, respectively. *Nocardioidaceae* contributed 37% to the *nasB* gene composition. *Burkholderiaceae* contributed 31% to the *nirK* gene composition. These results suggest that a diverse microbial community is responsible for N cycling in the soil ecosystem.

4. Discussion

Although many studies have been carried out in the past to analyze N cycling pathways in various ecosystems, the abundance of corresponding gene families and their taxonomic composition are not easy to characterize. By taking advantage of shotgun metagenome sequencing approach, this study surveyed the gene families responsible for N cycling processes in a CO_2-enriched grassland ecosystem. Although only one time-point samples were analyzed, a balance among the ecosystem, plant and microbial communities was expected to be reached after more than 12-year eCO_2 exposure. Changes of microbial communities should be representative for the response to long-term eCO_2, especially that samples were taken during peak growth season when plant communities have the highest productivity. Therefore, the information learned should provide some insights into understanding of N cycling pathways in a grassland ecosystem, as well as the potential responses of different N cycling pathways to long-term eCO_2.

Shotgun metagenome sequencing was performed in this study to characterize the taxonomic and functional composition of nitrogen cycling processes in the grassland soil. Compared to microarrays and PCR amplification methods, this approach is expected to provide relatively unbiased observations (Condron et al., 2010) for the gene families involved in the N cycle. However, issues have also been noticed regarding usable information gained for low abundant gene families, such as *nifH*, *hao*, *nosZ*, and *hzo*, which play important roles but often have very low abundances in the environment. Although this could be explained by the fact that *nifH* or *nosZ* occur only in a very small portion of the whole microbial community in natural ecosystems (Henry et al., 2006; Kandeler et al., 2006; Wang et al., 2015) as well as the random sampling issues in metagenomic studies (Zhou et al., 2013), previous GeoChip based analysis (He et al., 2010; Xu et al., 2013) and amplicon sequencing of *nifH* genes (Tu et al., 2016b) at the same experimental site have suggested a much higher diversity of N_2 fixation community in the ecosystem than we detected here. Thus, from the point of usable data and information obtainable by different technologies, application of shotgun metagenome sequencing to analyze gene families of low abundance should be interpreted carefully (Zhou et al., 2015). To gain more usable information for low abundant gene families, a higher sequencing depth is needed. When necessary, it is recommended to use shotgun metagenome sequencing to quantify the (relative) abundance, while using functional gene arrays and/or amplicon sequencing technologies to analyze the community structure and diversity for low abundant gene families.

The N cycle (Francis et al., 2007; Zehr and Kudela, 2011; Zehr and Ward, 2002) is far more complex processes than we have presented here. New knowledge is still gained in recent years, such as anaerobic ammonia oxidation (anammox) (Francis et al., 2007;

Strous et al., 1999), ammonia oxidation by Crenarchaea (Francis et al., 2007; Konneke et al., 2005), and a complete denitrification pathway in foraminifera (Risgaard-Petersen et al., 2006). This study mainly focused on the known major N cycling processes mediated by microbial communities as well as some recently characterized steps, including anammox (Francis et al., 2007; Strous et al., 1999). Consistent with a recent global metagenomic survey that showed a high diverse phylogenetic distribution of N cycling processes in soil (Nelson et al., 2016), this study also suggested that each process in the N cycle was carried out by a series of highly diverse microbial species. A recent study have suggested that such high microbial diversity is required to maintain robust N cycling processes in soil ecosystems due to a potentially limited functional redundancy of the soil microbial community (Philippot et al., 2013). Of the seven major processes we analyzed, organic N metabolism and nitrate reduction (both assimilatory and dissimilatory) had the highest gene abundance and the most diverse microbial species involved. This is probably because organic N metabolism and nitrate reduction are two major processes from which microorganisms gain energy and nutrition (Condron et al., 2010; Moreno-Vivián et al., 1999), as well as the increased C:N ratio in plant biomass at the BioCON site. At the taxonomic level, *Actinomycetales* was the major taxonomic group responsible for several processes, especially for nitrification, to which *Actinomycetales* contributed more than 60% of the detected genes. Notably, only a few copies of genes were detected for the gene family *hao*, which serves as the key gene in transforming NH_2OH to NO_2^- in the nitrification process (Zehr and Kudela, 2011). However, a high number of *amoA* and *nxrAB* genes were found. This suggested a potential existence of not yet known alternative genes that play a similar role as *hao*. In addition, the distinct taxonomic profiles among different components also suggested that the complex N cycling was a result of collective actions of many different microbial groups.

Under eCO_2, increased organic C input to the soil and intensified N limitation are two major ecological impacts affecting belowground microbial communities (Hu et al., 2001; Luo et al., 2004, 2006b; Norby et al., 2010; Reich and Hobbie, 2013; Reich et al., 2006). The relationship between increased C and N limitation seems unclear as revealed by previous studies (Luo et al., 2006a). Different studies have reported contrasting results, including constrained microbial decomposition by decreased N availability (Hu et al., 2001), increased N cycling as a result of increased C input (Luo et al., 2006b), and no effects on any microbial N cycling pool or process (Zak et al., 2003). In the BioCON experimental site analyzed in this study, N limitation of ecosystem response to eCO_2 has been observed (Reich and Hobbie, 2013; Reich et al., 2006). To sustain the stimulated plant growth under eCO_2, the belowground microbial communities are expected to provide more N for plant growth. It remains blurry whether microbial decomposition is constrained by N limitation. Interestingly, the current study suggested that relative abundance of several key genes related with organic decomposition to ammonium increased under eCO_2. Consistent with the current study, previous GeoChip-based analysis also suggested increased C degradation and N_2 fixation genes of belowground microbial communities (He et al., 2010; Xu et al., 2013).

Because the extent of N availability in the ecosystem is mainly determined by soil N_2 fixation rate, organic decomposition, and the N uptake by the plants increased under eCO_2, we hypothesized that multiple N cycling processes would be stimulated as a result of increased plant biomass, C input to soils, and N limitation. The degree of N limitation of plant responses to eCO_2 depends in part on how eCO_2 influences soil N cycling and availability (Hu et al., 2001; Luo et al., 2004; Reich and Hobbie, 2013; Williams et al., 2000). Previous studies have suggested contradictory results for responses of N cycling processes to eCO_2. For example, enhanced N_2 fixation in the ecosystem as a result of eCO_2 have been observed in several studies (Hungate et al., 1999, 2003; Levitan et al., 2007), while declined N_2 fixation has also been reported (Hungate et al., 2004). Microbial decomposition, another most attended N cycling process, has also been found with contradictory results in different studies (Carney et al., 2007; Cheng et al., 2012; Hu et al., 2001). Such different observations could be due to different types of ecosystems, extent of N limitation in the ecosystem, length of CO_2 treatment, climate, time of sampling, soil pH, and litter quality (Nelson et al., 2016).

In our results, genes related to NH_4^+ cycling were most sensitive to eCO_2. First, genes encoding urease and nitronate monooxygenase increased in abundance with eCO_2. Of these two gene families, *ureC* produces NH_4^+ from organic decomposition, and *nmo* can generate more NO_2^- for nitrate reduction. Second, abundances of dissimilatory nitrate reduction gene families encoding enzymes that convert NO_3^- into NH_4^+ were stimulated, providing more NH_4^+ from NO_3^-. Among them, abundances of *nmo* and *hzsA* genes were significantly correlated with soil NH_4^+ concentrations. Both findings were consistent with our metadata that the soil ammonification rate and ammonium concentration were significantly higher under eCO_2 than under aCO_2. However, assimilatory nitrate reduction that produces NH_4^+ for microbial organic synthesis did not change. Third, consistent with our previous analyses at this site (He et al., 2010; Tu et al., 2016b; Xu et al., 2013), relative abundance of the N_2 fixation gene family *nifH* increased. Finally, relative abundances for gene families that utilize NH_4^+ for glutamine synthesis and anammox decreased under eCO_2, indicating that microbial communities not only accelerated NH_4^+ production by stimulating corresponding gene families, but also suppressed gene families that consume NH_4^+. Compared to previous studies (He et al., 2010; Xu et al., 2013) that showed that only N_2 fixing genes significantly increased under eCO_2, the current study, using shotgun metagenome sequencing, showed that several other aspects of microbial N cycling were sensitive to long-term elevated CO_2.

Dissimilatory and assimilatory nitrate reduction responded distinctly to eCO_2 at the BioCON experimental site. Although both processes reduce nitrate to ammonium, the roles they play in the ecosystem are fundamentally different in that assimilatory nitrate reduction utilizes nitrate as a nitrogen source for growth, while dissimilatory nitrate reduction dissipates excess reducing power for redox balancing through the process (Moreno-Vivián et al., 1999). Under eCO_2, the soil ecosystem is expected to provide more biologically available nitrogen to sustain stimulated plant growth due to increased photosynthesis rate (Luo et al., 2006b; Norby et al., 2005; Reich and Hobbie, 2013; Reich et al., 2001). Since soil microbial communities underpin the soil N cycle, the increased microbial DNRA gene families could be a result of microbial feedback to eCO_2, especially the progressive nitrogen limitation in the ecosystem. This suggests that long-term eCO_2 selectively stimulates DNRA pathways that help relieve N limitation in the ecosystem, rather than tunes up every process in the N cycle.

In summary, this study employed shotgun metagenome sequencing to survey the taxonomic and functional composition of N cycling gene families in a grassland ecosystem exposed to eCO_2 for >12 years. Abundances for certain gene families involved in ammonium production pathways increased under long-term eCO_2, including N_2 fixation, organic N metabolism, and DNRA, while abundances for some gene families involved in ammonium consumption processes such as glutamate synthesis and anammox decreased under eCO_2. The results also showed that technologies like shotgun metagenome sequencing should be applied carefully when focusing on low abundant gene families, such as *nifH* and *nosZ*. The study provides insights into how ongoing global change affects belowground microbial communities.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2016.12.017.

Conflict of interest

None declared.

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