Consistent effects of vegetation loss on abundant and rare soil microbial phylotypes across nitrogen-enrichment levels

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Abstract

Soil harbors highly diverse abundant and rare microbial phylotypes that drive multiple soil functions. Given increasing intensity and frequency of vegetation loss and anthropogenic reactive nitrogen (N) inputs to the soil in the future, we lack a mechanistic understanding of how vegetation loss may influence abundant and rare microbial phylotypes at various N-enrichment levels. In the current study, we assessed the effects of vegetation loss on abundant and rare phylotypes of soil bacteria and fungi across three N-enrichment levels in a semi-arid grassland ecosystem. After six years of experimentation in with and without vegetation plots, the vegetation loss increased the total relative abundance of abundant soil bacterial phylotypes but not that of abundant fungal phylotypes at across N-enrichment levels. It is very likely because the number of abundant bacterial phylotypes with positive than negative responses to vegetation loss was higher; however, the number of abundant fungal phylotypes with positive than negative responses to vegetation loss was similar during this period. Moreover, the vegetation loss did not alter the alpha-diversity of abundant or rare bacterial phylotypes, or, of abundant fungal phylotypes; however, it reduced the alpha-diversity of rare fungal phylotypes at across N-enrichment levels. The vegetation loss, however, altered the beta-diversity of abundant and rare bacterial and fungal phylotypes across N-enrichment levels. We found that, against expectations, the effects of vegetation loss on the diversity of abundant and rare phylotypes of both bacteria and fungi were relatively consistent across N-enrichment levels. Our findings provide, for the first time, the phylotype-based data on how vegetation loss affects abundant and rare phylotypes of soil bacteria and fungi across N-enrichment levels. The results also indicate that the effects of vegetation loss on belowground functions may be relatively insensitive to the differences in the N-deposition rates.

Background

Soils harbor highly diverse microbial communities that contain abundant and rare phylotypes, which are crucial for regulating multiple ecosystem processes [1, 2]. While, a major challenge is to understand how these complex microbial communities respond to anthropogenic stressors, such as abrupt loss of vegetation that is predicted to increase in frequency and intensity in the future [3, 4]. Many previous studies have demonstrated that vegetation loss significantly affects soil microbial communities since these depend on plant-derived resource inputs to meet their nutrient and energy demands in soil [5-7](De Vries and Shade 2013, Kaisermann et al. 2017). The different groups of the microbial community respond differently to anthropogenic stressors; soil fungi, for instance, are generally more resistant to the vegetation loss than soil bacteria [8, 9]. A large number of studies have demonstrated that the effects of anthropogenic stressors on soil microbial communities are context-dependent, and depend on soil, vegetation, and climatic conditions [10, 11]. A number of recent studies, however, have found that the effects of plant functional group loss [12, 13] and N-enrichment [14, 15] on soil microbial functional groups (i.e. bacteria, fungi, and nematodes) were relatively similar in different ecosystems. At present, we do not know whether and how the loss of vegetation affects the abundant and rare microbial phylotypes across a gradient of anthropogenic stressors.

In addition to containing a small number of abundant or dominant phylotypes, the soil microbial communities also show a large number of rare microbial phylotypes [16-18]. The mass ratio hypothesis suggests that dominant than rare species will have greater effects on ecosystem functions [19, 20]. The rare phylotypes may have little effects on species interactions and ecosystem functions in the soil ecosystems that demonstrate phylotype-rich, phylogenetically diverse, and functionally redundant microbial communities [1, 2, 20]. Though the role of rare phylotypes in ecosystem functions has been neglected, recent work predicts that rare biosphere may serve as important reservoirs of genetic diversity and have disproportionate importance in community structure, ecosystem functioning and stability [16, 17]. For instance, *Desulfosporosinus* sp., represents < 0.006% of the microbial community but it has a fundamental role in sulfate reduction in the peatland soils [21]. Therefore, it is important to elucidate the impact of anthropogenic stressors on rare phylotypes and their functions in the context of biodiversity loss.

Because plants are the primary producers and provide resources to the belowground communities, the vegetation loss may alter the composition, structure, and diversity of soil microbial communities by at least three non-mutually exclusive pathways [12, 13, 22]. First, the vegetation loss reduces litter and rhizodeposition, which are major sources of carbon (C), energy, and nutrients for soil microorganisms [7, 22, 23]. Second, the vegetation loss also influences microbial communities by altering the soil nutrient availability and composition [22, 24]. Third, vegetation loss also affects microbial communities by changing the soil abiotic conditions (edaphic properties) and biotic interactions (species interactions) [9, 12]. Although these pathways predict the effects of vegetation loss on the structure of soil microbial communities, we know little about their relative importance across gradients of anthropogenic stressors that can affect both soil biotic interactions and abiotic conditions [25, 26]. The lack of consistency among studies addressing the effects of vegetation loss on microbial communities across different ecosystem types might be due the failure to partition the relative effects of biotic and abiotic variables.

As an important anthropogenic stressor, the N input to terrestrial ecosystems has increased three- to five-fold over the past century [26, 27]. Although a large number of studies have shown that N enrichment to the grassland soil ecosystems can alter plant and microbial communities [14, 25, 26], we lack an experimental evidence of how N-enrichment affects the abundant and rare microbial phylotypes in the context of vegetation loss. To obtain such evidence, we developed a vegetation-free treatment to a long-term multiple-level N-enrichment experiment conducted in a typical semi-arid steppe [9], where the N-deposition rate is expected to increase substantially due to rapid industrialization and urbanization [28]. Before a vegetation-free treatment was added, this experiment showed that N enrichment significantly increased above- and below-ground plant biomass mainly by promoting fast- than slow-growing species [29, 30]. While, the N-enrichment also altered the community structure of soil microbes and nematodes and reduced their taxonomic and functional diversity [9, 31]. Overall, this multiple-level N-enrichment experiment has demonstrated dramatic changes in the plant communities, and soil edaphic properties, while it provides an excellent opportunity to examine the effects of vegetation loss on abundant and rare soil microbial phylotypes under different biotic and abiotic conditions.

We established two subplots (one with vegetation and another without vegetation) in each plot of the multiple-level N-enrichment experiment as described above. Six years after subplots were established, we collected data of soil bacterial and fungi communities (bar-coded pyrosequencing data), plant community parameters, and edaphic properties as described below. We attempted to address three major questions in this manuscript. First, how does vegetation loss affect soil bacteria and fungi at the phylotype level across N-enrichment levels in a semiarid grassland? Second, how does vegetation loss affect the diversity of abundant or rare soil bacterial and fungal phylotypes across N-enrichment levels? Third, how do changes in plant community parameters and soil edaphic properties caused by the vegetation loss affect the diversity of abundant and rare phylotypes of soil bacteria and fungi?

Results

Responses of soil microbial phylotypes to vegetation loss across N-enrichment levels

Among all bacterial phylotypes, about 8% were abundant (227 of 2575) while 92% were rare (2348 of 2575) (Fig. 1 and Fig. 2a). The abundant and rare phylotypes accounted for 52 and 48%, respectively, of the total relative abundance of the bacterial phylotypes. For fungi, about 11% were abundant (81 out of 723) and 89% were rare (642 of 723) (Fig. 1 and Fig. 2a). The abundant and rare phylotypes accounted for 91 and 9%, respectively, of the total relative abundance of the fungal phylotypes. The split-plot ANOVA at the phylotype level showed that the vegetation loss altered about 42% of abundant and 30% of rare bacterial phylotypes but only 26% of abundant and 25% of rare fungal phylotypes (Fig. 1 and Fig. 2b). The N-enrichment altered about 94% of abundant and 82% of rare bacterial phylotypes but only 43% of abundant and 29% of rare fungal phylotypes. Only 9-15% of bacterial and fungal phylotypes were altered by the interaction between vegetation loss and N enrichment (Fig. 1 and Fig. 2b). Across N-enrichment levels, the effects of vegetation loss on abundant or rare phylotypes differed between bacteria and fungi (Fig. 1 and Fig. 2c). From low to high N-enrichment levels, the abundant bacterial phylotypes with significant responses to the vegetation loss increased from 19 to 35%, while the rare phylotypes remained stable (21-30%). Similarly, from low to high N-enrichment levels, the abundant fungal phylotypes with significant responses to vegetation loss increased from 16 to 24%, while the rare phylotypes increased from 35 to 48% (Fig. 1 and Fig. 2c).

The vegetation loss increased the total relative abundance of abundant bacterial phylotypes at the medium and high N-enrichment levels but it did not alter the total relative abundance of abundant fungal phylotypes at any N-enrichment level (Fig. 3a-3d). The N-enrichment greatly decreased the total relative abundance of abundant bacterial and fungal phylotypes; however, it increased the total relative abundance of rare bacterial and fungal phylotypes (Fig. 3a-3d). The interaction between vegetation loss and N enrichment had no effect on the total relative abundance of abundant and rare bacterial or fungal phylotypes (Fig. 3a-3d). The vegetation loss also increased the total relative abundance-based ratio of abundant bacteria to fungi at the low and medium N-enrichment levels but it did not alter the ratio of rare bacteria to fungi at any N-enrichment level (Fig. 3e-3f). At bacterial phylum level, the vegetation loss decreased the relative abundance of dominant *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes* but it

increased the relative abundance of dominant *Acidobacteria*, *Chloroflexi*, *Nitrospirae*, and *Planctomycetes* (Fig. 4a). Regarding comparison at fungal class level, the vegetation loss decreased the relative abundance of dominant *Dothideomycetes* and *Sordariomycetes* (Fig. 4b). Overall, the effects of vegetation loss on microbial phylotypes were more obvious at medium and high N-enrichment levels, while the number of phyla or classes with significant responses to vegetation loss increased from 1 to 8 for bacteria, and from 0 to 4 for fungi across the N-enrichment gradient (Fig. 4).

Responses of soil microbial diversity to vegetation loss across N-enrichment levels

The vegetation loss did not alter the alpha-diversity (as indicated by the Shannon-Wiener index) of all bacterial and fungal phylotypes; however, it reduced the alpha diversity of rare fungal phylotypes at low and medium N-enrichment levels (Fig. 5). The N-enrichment reduced the alpha-diversity of all bacterial phylotypes but it only decreased the alpha diversity of rare fungal phylotypes (Fig. 5). The interaction between vegetation loss and N-enrichment showed no effect on the alpha-diversity of all bacterial and fungal phylotypes (Fig. 5). Regarding phylotype richness, we found the vegetation loss did not alter richness of all, abundant, and rare bacterial or fungal phylotypes (Additional file 1: Fig. S1). The linear regression indicated that the alpha-diversity of all bacterial phylotypes was determined by both abundant and rare phylotypes; however, the alpha-diversity of all fungal phylotypes was influenced only by the abundant fungal phylotypes (Additional file 1: Fig. S2). The SEM analysis showed that the vegetation loss-induced changes in the alpha-diversity of rare fungal phylotypes were mainly explained by the soil edaphic properties such as SOC and TSN at the low-N enrichment level, while these changes were mainly explained by the ANPP at the medium N-enrichment level (Additional file 1: Table S1). These soil variables and ANPP were dramatically decreased by the vegetation loss at these N-enrichment levels (Additional file 1: Fig. S3).

The principal coordinate analysis (PCoA) based on the Bray-Curtis similarity at the phylotype level showed that both vegetation loss and N-enrichment, but not their interaction, altered the beta-diversity (an indicator of community structure) of all bacterial and fungal phylotypes across all N-enrichment levels (Fig. 6). The PERMANOVAs showed that vegetation loss altered the beta diversity of all bacterial phylotypes at each N-enrichment level (Fig. 6). Moreover, it also altered the beta-diversity of all fungal phylotypes. However, vegetation loss did not alter the beta-diversity of abundant fungal phylotypes at all N-enrichment level (Fig. 6). The Mantel test based on the Bray-Curtis similarities showed that the beta-diversity of all microbial phylotypes was mainly explained by the beta-diversity of both abundant and rare bacterial phylotypes, and by the abundant fungal phylotypes (Additional file 1: Fig. S4). The SEM analysis showed that the vegetation loss-induced changes in the beta diversity (as indicated by PCoA1) of all microbial phylotypes showed strong associations with SOC contents at low and medium N-enrichment levels. Moreover, the vegetation loss-induced changes in the beta diversity of microbial phylotypes also showed strong associations with the plant-related variables and soil pH at the high N-enrichment level (Additional file 1: Table S1), because these variables were dramatically decreased by the vegetation loss across all N-enrichment levels (Additional file 1: Fig. S3). In addition, the determinants of vegetation loss-induced changes in the beta diversity differed between abundant and rare phylotypes

(Additional file 1: Table S1). For example, at low N-enrichment level, the beta-diversity of abundant fungal phylotypes was mainly explained by the SOC and TSN contents, but that of rare fungal phylotypes was determined by the ANPP and soil pH (Additional file 1: Table S1).

Discussion

Effect of vegetation loss on abundant and rare microbial phylotypes across N-enrichment levels

We found that vegetation loss increased the total relative abundance of abundant bacterial phylotypes, while a corresponding decrease in the total relative abundance of rare bacterial phylotypes at the medium and high N-enrichment levels. An increase in the total relative abundance of abundant bacterial phylotypes was due to a greater percentage of the abundant phylotypes with positive than negative responses to the vegetation loss (22-31 vs. 4-5%). Our results support the idea that vegetation loss-induced increase in the soil pH at medium or high N-enrichment levels may favor soil bacteria. Substantial data indicate that relatively high soil pH favors the growth and activity of bacteria in various soil ecosystems [9, 31, 32]. However, the vegetation loss did not alter the total relative abundance of abundant or rare fungal phylotypes at any N-enrichment level. This was true because there was no difference in the percentages of abundant fungal phylotypes with positive than negative responses to the vegetation loss, thus implying that most of the abundant fungal phylotypes (77-84%) did not respond to vegetation loss at any N-enrichment levels. The different responses of abundant soil bacterial and fungal phylotypes to vegetation loss were consistent with a previous study of the Mongolian grasslands that also showed positive or no effect of vegetation loss on abundant bacteria and fungi, respectively [13]. While, a higher ratio of the abundant bacteria to abundant fungi caused by vegetation loss in current study was also consistent with other plant diversity experiments [23, 33], which reported that biomass-based ratio of bacteria to fungi increased with a decrease in the input of plant material into soil. The difference in soil bacterial and fungal responses to vegetation loss may be due to their differential sensitivity to the environmental disturbance in the form of vegetation loss, and due to the high requirements of nutrients per unit C for bacteria than fungi [12].

Interestingly, we found that N-enrichment substantially reduced the relative abundance of abundant bacterial and fungal phylotypes but it increased the relative abundance of rare bacterial and fungal phylotypes. However, these trends differ from those exhibited by the plant communities, i.e., the N-enrichment generally increased the biomass of dominant plant species but it reduced the biomass of rare species [9, 31]. The responses of abundant vs. rare microbial phylotypes to the N-enrichment differ probably because the former has evolved strategies to reduce the resource use under water- and nutrient-limited conditions, while these strategies may not be sufficient to deal with the strong environmental disturbances (e.g. low soil pH) at medium or high N-enrichment levels [9, 14]. Our findings are in line with the recent theoretical and empirical predictions [18, 34] that the abundance of rare bacterial phylotypes is usually low but it can increase under suitable conditions. Overall, the vegetation loss had a much stronger effect on the relative abundance of soil bacterial than fungal phylotypes.

Effects of vegetation loss on microbial alpha diversity across N-enrichment levels

Unlike the relative abundance, the alpha-diversity of soil bacterial or fungal phylotypes did not respond to the vegetation loss, which is contrasted with recent reports that bacterial and fungal alpha-diversity increased with plant species richness in the grassland ecosystems at local [35] and regional [36, 37] scales. The lack of response of the alpha-diversity of all phylotypes to the vegetation loss strongly supports the view that plant and soil organisms are largely uncoupled [38-41]. However, a recent research have suggested that soil bacterial than fungal community properties are more closely associated with the plant biomass [42], in addition, we also found that the vegetation loss reduced the alpha-diversity of rare fungal phylotypes at low and medium N-enrichment levels. The stronger responses of alpha-diversity of fungal than bacterial phylotypes to the vegetation loss are partly in line with the recent empirical evidences predicting variable responses of soil microbial groups to the vegetation loss [13, 22, 23]. In the current study, the decline in alpha-diversity of rare fungal phylotypes with vegetation loss were mainly explained by the variables related to soil nutrients and ANPP, which is reasonable, because fungi are the first consumers of belowground plant-derived inputs to the soil [12, 43]. Overall, our results indicate that the alpha-diversity of soil fungal than bacterial phylotypes is more susceptible to the vegetation loss; in terms of relative abundance, however, the fungal than bacterial phylotypes are less sensitive to the vegetation loss.

We also found that the alpha-diversity of all bacterial phylotypes was determined by both abundant and rare bacterial phylotypes, while the abundant fungal phylotypes explained the alpha-diversity of all fungal phylotypes. These results indicate that, for bacteria, the rare phylotypes serve as a reservoir of species and can maintain the alpha-diversity of the bacterial community [16, 17]. We propose that the conflicting results, particularly those, describing the relationships between plant and soil microbial alpha-diversity within single sites [41, 44] can be explained by the differences between the abundant and rare microbial phylotypes, while these differences mainly result from differences in the environmental contexts under investigation. Furthermore, our results revealed that N-enrichment strongly reduced the diversity of both abundant and rare bacterial phylotypes but it decreased the diversity of rare fungal phylotypes, which nevertheless suggests that fungi than bacteria have higher environmental tolerance [9, 45, 46]. These findings differ from results reported for the plant communities, which showed that species richness was more sensitive to the chronic N-enrichment for rare than common species in the grasslands of Asia, America and Europe under arid and non-arid conditions [26, 47]. Overall, our findings provide direct evidence that soil microbial groups differ in their responses to a reduction in plant-derived inputs to soil and that the responses of alpha-diversity depend on the microbial phylotype abundance (rare vs. abundant) and soil nutrient status.

Effects of vegetation loss on microbial beta diversity across N-enrichment levels

The vegetation loss significantly altered the beta diversity of abundant and rare phylotypes of soil bacteria and soil fungi, while these effects were relatively consistent across all N-enrichment levels. However, previous research has reported weaker responses of the bacterial alpha- than beta-diversity to

the vegetation loss [5, 48]. These substantial changes in the bacterial beta-diversity could be due to the substantial percentage of the abundant (19-35%) and rare (21-30%) phylotypes that responded to the vegetation loss across the N-enrichment levels, although previous studies have reported such changes at the phylum level [49]. The large changes in the fungal beta-diversity could be due to the substantial percentage of rare (35-48%) than abundant fungal phylotypes that responded to vegetation loss across N-enrichment levels. While, the changes in the fungal beta-diversity could also be ascribed to the declines in plant-associated classes such as *Dothideomycetes* and *Sordariomycetes* [50]. However, the lack of response of the beta-diversity of abundant fungal phylotypes to the vegetation loss might be due to their non-responsiveness to the vegetation loss across the N-enrichment levels. The SEM analysis further showed that the beta-diversity of all bacterial and fungal phylotypes was mainly explained by the plant-induced changes in the SOC at the low and medium N-enrichment levels [22], and by the plant-related variables [6] and soil pH [32] at the high N-enrichment level. We also found large differences in the determinants of the beta-diversity of abundant vs. rare phylotypes at each N-enrichment level. Previous studies on macro-organisms showed that the occurrence of abundant and rare species is often regulated by different ecological processes [51]. In the case of microbial communities, relatively little is known about how ecological processes regulate the occurrence of dominant and rare phylotypes [18, 34, 52]. Our results, therefore, suggest that vegetation loss-induced changes in the beta-diversity of soil bacteria and fungi at different N levels arises from niche and stochastic processes; however, further research is needed to partition the relative influence of either ecological process.

The Mantel test showed that the beta-diversity of all bacterial phylotypes was explained by both abundant and rare phylotypes but that of all fungal phylotypes was determined by only abundant phylotypes. Our results evidenced that rare phylotypes make substantial contributions to beta-diversity [16, 17]. For instance, Gobet *et al.* [17] showed that, after removing 50% of the rare phylotypes, a significant change in the beta-diversity of soil bacterial community cross coastal sands was disappeared. However, our results indicate that a substantial contribution of rare phylotypes to the community structure was not evident in either bacteria or fungi. Furthermore, our results indicate that the effects of N-enrichment on bacterial and fungal beta-diversity could be explained by the fact that N-enrichment altered 82-94% of bacterial and 29-43% of fungal phylotypes. However, previous studies have mostly described such changes at the phylum or class level [14, 15, 45]. Although strong effects of N-enrichment on the beta-diversity of microbial communities are previously studied, our findings, for the first time, show phylotype level responses of soil bacterial and fungal phylotypes and their beta diversity to the vegetation loss and N enrichment.

Consistent effects of vegetation loss on microbial properties across N-enrichment levels

We did not find a statistically significant interaction between the effects of vegetation loss and N-enrichment levels on microbial community properties at the phylotype level, thus suggesting that the vegetation loss-induced changes in the structure of microbial communities is relatively not obvious across N-enrichment levels, though changes in the N-enrichment levels substantially affected soil fertility and plant productivity (Fig. 7). Our finding is supported by a recent study, which reported that the effects

of vegetation loss on soil microbial functional groups did not differ among 30 island ecosystems differing in the soil fertility and plant productivity [12]. Our results differed, however, from those of several previous studies, which indicated that the effects of vegetation loss on microbial ecosystems can be strongly influenced by environmental contexts [53, 54]. The interaction between vegetation loss and N-enrichment level in the current study was not statistically significant probably because the interaction altered only 13-15% of bacterial and 9-10% of fungal phylotypes. Another possible explanation for the lack of this interaction might be that the soil microbial properties are regulated more by the soil N availability and pH than by vegetation loss under various levels of N-enrichment [9, 30]. There is often some decoupling between the above- and below-ground biota because the soil biota can be driven by abiotic factors independent of vegetation [12, 15]. The current results revealed that the effects of vegetation loss were relatively consistent across N-enrichment levels, i.e., the effects were largely independent of the environmental contexts (Fig. 7). Our findings also suggest that the belowground functions of different ecosystems may have similar negative responses to the vegetation loss irrespective of the large differences in their productivity or fertility.

Conclusions

Global change factors such as reactive N inputs and warming can cause profound changes or even losses in plant vegetation, particularly in fragile ecosystems like arid or semi-arid grasslands. Changes in plant cover are assumed to impact soil microbes, but the resulting effects have rarely been quantitatively assessed. Our results provide the first phylotype-based dataset of vegetation loss effects on soil bacteria and fungi across N-enrichment levels. Vegetation loss increased the relative abundance of abundant bacterial (but not fungal) phylotypes at all three N levels. More abundant bacterial phylotypes positively responded to vegetation loss than these with negative responses, but the opposite was right for abundant fungal phylotypes. Vegetation loss only reduced the alpha diversity of rare fungal phylotypes, but altered the beta diversity of both abundant and rare bacterial and fungal phylotypes. Effects of vegetation loss on the diversity of abundant and rare phylotypes of both bacteria and fungi were consistent across three N levels. The results indicate that the effects of vegetation loss on belowground functions may be relatively insensitive to the differences in the N-deposition rates.

Methods

Experimental design

The long-term N-enrichment experiment is located at the Inner Mongolia Grassland Ecosystem Research Station (43°38'N, 116°42'E) of the Chinese Academy of Sciences. This region is characterized by a semi-arid temperate continental climate with mean annual precipitation of 334 mm, mean annual temperature of 0.9 °C, and elevation of 1200 m (Chen *et al.* 2019). The soil type here at the study site is mainly loamy-sand. The grassland plant community is dominated by perennials, including *Leymus chinensis* (Trin.) Tzvel., *Stipa grandis* P. Smirn., and *Agropyron cristatum* (L.) Gaertn. In 1999, we established an N-

enrichment experiment which had a randomized block design with six levels of N enrichment and three N application times, as described by Bai *et al.* [29].

To understand the influence of vegetation loss on abundant and rare microbial phylotypes across N-enrichment levels, in 2010, we performed vegetation-removal treatment on a subset of the N-enrichment experiment, i.e., in those plots treated three levels of N enrichment (0.00, 5.25, and 17.5 g N m⁻² yr⁻¹) and two N application times (May 1–3 and July 1–5) in first 5 replicate blocks. Our preliminary analysis found no significant effects of the timing of N addition on most microbial parameters, and then we combined the two N application times. In line with previous work [45], we also defined the three N treatments as low, medium, and high N levels. The designation of the two subplots (each 0.5-m × 0.5-m), one with plants and the other without plants, within each plot of the N-enrichment experiment was described by Chen *et al.* [9]. In late August 2016, one composite soil sample including four soil cores (2 cm diameter and 0-15 cm depth) was collected from each with- and without-plant subplot. After passing through a 2-mm sieve, the soils were used for determination of soil moisture, NH₄⁺-N, NO₃⁻-N, microbial community, soil pH, soil organic C (SOC), and total soil N (TSN). We also sampled aboveground plant biomass in a 0.5-m × 0.5-m quadrat in each plot. Consistent with previous work in this N-enrichment experiment [9], N enrichment had increased plant biomass, plant community structure, soil inorganic N, and TSN; it had decreased soil pH but had not altered SOC (Additional file 1: Fig. S1).

Soil DNA extraction, amplification, and sequencing

Microbial DNA was extracted from 0.5-g soil subsamples using the FastDNA® Spin Kit for Soil (MP Biomedical, Solon, OH). A NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA) was used to measure the DNA concentration and purity, and 1% agarose gel electrophoresis was used to check DNA quality. The V3-V4 region of the bacterial 16S rRNA gene was amplified by PCR using primers 338F and 806R, and the fungal ITS sequence of the 18S rRNA gene was amplified using primers ITS1F and ITS2, and a GeneAmp 9700 thermocycler PCR system (ABI, USA). Fungal internal transcribed spacer (ITS) rRNA genes were amplified with PCR primers ITS1F and ITS2. Purified amplicons were sequenced by Shanghai Majorbio Bio-pharm Technology (Shanghai, China) using an Illumina MiSeq platform (San Diego, CA, USA). Raw fastq files were analyzed as the criteria described by Li *et al.* [55]. Gene sequences were grouped into phylotypes (operational taxonomic units) with 97% similarity cutoff using UPARSE (version 7.1, <http://drive5.com/uparse/>), and chimeric sequences were identified and removed using UCHIME. The taxonomy of each gene sequence was analyzed by the RDP Classifier algorithm (<http://rdp.cme.msu.edu/>) against the Silva database (Silva 128) for bacteria and against the Unite database (Unite 7.0) for fungi using a confidence threshold of 70%.

Diversity of abundant and rare phylotypes of soil microorganisms

Relative abundance is widely accepted as a metric of the abundance of phylotypes in their environment, and is therefore useful for identifying abundant or rare phylotypes [1, 2]. We defined the phylotypes as abundant or rare if their frequency was >0.1% or <0.1%, respectively, in plots treated with 0.00 g N m⁻²

yr⁻¹ [56, 57]. All of the pre-processes for determining the alpha and beta diversity of the soil bacterial and fungal communities were conducted by using the script in QIIME (<http://qiime.org/scripts/>). The phylotype table was rarefied by using the single_rarefaction.py script (Additional file 2). Values of the Shannon-Wiener index (an indicator of alpha diversity) of abundant or rare phylotypes were calculated using the alpha_diversity.py script. Values for Bray-Curtis similarity (an indicator of beta diversity) of abundant or rare phylotypes were calculated by cumulative sum scaling transformed phylotype abundances using the normalize_table.py script and beta_diversity.py script. The beta diversity of abundant or rare phylotypes in treatments (+ vegetation removal and N-enrichment levels) were also visualized by principal coordinate analysis (PCoA) using the pcoa function of the ape library in R version 3.5.1 (R Development Core Team 2018).

Statistical analyses

The effects of vegetation loss and N enrichment on each response variable (all, abundant, and rare phylotypes for soil bacteria and fungi; soil nutrients; and soil environments) were examined using split-plot ANOVA, with vegetation loss, N enrichment, and their interactions as fixed factors and N-enrichment level nested within block as an error term. The split-plot ANOVAs were performed using the “aov” function in the base package of R. One-way ANOVAs with Duncan’s multiple-range tests were performed across all response variables to compare the means between vegetation-loss treatments for each N-enrichment level. Four additional statistical analyses were performed. First, we constructed phylogenetic trees of soil bacteria and fungi and visualized their responses to vegetation loss and N enrichment at the phylotype level using the interactive Tree Of Life (iTOL) [58]. Second, we used permutational analyses of variance (PERMANOVA) to test the effects of vegetation loss and N enrichment on the Bray–Curtis distance matrix (beta diversity). Pairwise PERMANOVAs were also used to test for significant effects of vegetation loss at each N-enrichment level. PERMANOVAs were performed using the adonis function in vegan. Third, we used linear regression to assess the relationships of Shannon-Wiener index values between abundant and all phylotypes and between rare and all phylotypes. Mantel tests were used to assess the correspondence in the Bray–Curtis distance matrix based on microbial community composition between abundant and all phylotypes and between rare and all phylotypes. Fourth, we also used structural equation modelling (SEM) to analyze the effects of vegetation loss via biotic and abiotic variables on the diversity of abundant and rare phylotypes at each N-enrichment level; SEM was performed with Amos 21.0 software (IBM SPSS Inc., Chicago, IL, USA). Other statistical analyses were performed using r version 3.5.1 (R Development Core Team 2018).

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets analyzed during the current study are available from their original source (Additional file 2). Additional result files and scripts are available from the corresponding author on reasonable request.

Competing interests

We have no conflicts of interest to disclose.

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Authors' contributions

DC and YB designed the study. DC, YW and BW performed the study. DC and YW compiled and analyzed the data. DC, MS, SH and YB led the writing, with input from all co-authors.

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Figures

small, medium, and large pie charts indicate low, medium, and high N-enrichment levels, respectively. The innermost ring indicates the distribution of dominant and rare phylotypes.

Figure 2

Effects of vegetation loss on abundant and rare soil microbial phylotypes across N-enrichment levels. (a) The percentage of abundant and rare phylotypes and the relative abundance of the abundant and rare phylotypes for soil bacteria and fungi. The numbers of phylotypes number and relative abundances are indicated on the colored bars. The outermost three rings indicate the effects of vegetation removal (R), N level (L), and their interaction (R × L) on each phylotype based on split-plot one-way ANOVAs (non-significant, $P > 0.05$; significant, $P < 0.05$). (b) The percentages of phylotypes significantly affected by vegetation removal, N level, or their interaction. The remaining three middle rings indicate the effects of vegetation loss on each phylotype at different N-enrichment levels based on one-way ANOVAs (decrease, increase, and non-response indicate that vegetation loss decreased, increased, or did not significantly affect the relative abundance of the phylotype at $P < 0.05$). (c) The percentages of phylotypes that were decreased, increased, or not affected by vegetation loss at each N-enrichment level.

Figure 3

Responses of total relative abundance of abundant bacterial and fungal phylotypes (a-b) and of rare bacterial and fungal phylotypes (c-d) and their ratios (e-f) to vegetation loss (+Plant, with plants; -Plant, without plants) at different N-enrichment levels. Values are the means ($\pm$ SE) of 10 replicate subplots. The effects of vegetation removal (R), N level (L), and their interaction (R $\times$ L) on each response variable were assessed using split-plot one-way ANOVAs (ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Asterisks between +Plant and -Plant violin bars indicate significant differences between with-plant and without-plant treatments for each N-enrichment level (one-way ANOVA).

Figure 4

Responses of relative abundance (%) of phyla for bacteria (a) and classes for fungi (b) to vegetation loss (+Plant, with plants; -Plant, without plants) at different N-enrichment levels. The symbols '+' and '-' indicate a significant ($P < 0.05$) increase and decrease, respectively, in relative abundance in response to vegetation loss based on one-way ANOVAs. The effects of vegetation removal (R), N level (L), and their interaction ($R \times L$) on each phylum or class based on split-plot one-way ANOVAs are also indicated in brackets (ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 5

Responses of the Shannon-Wiener index (alpha diversity) of bacteria (a, all phylotypes; b, abundant phylotypes; c, rare phylotypes) and fungi (d, all phylotypes; e, abundant phylotypes; f, rare phylotypes) to vegetation loss (+Plant, with plants; -Plant, without plants) at different N-enrichment levels. Values are the means ($\pm$ SE) of 10 replicate subplots. Statistical comparisons are described in Figure 2.

Figure 6

Responses of community composition (beta diversity) of bacteria (a, all phylotypes; b, abundant phylotypes; c, rare phylotypes) and fungi (d, all phylotypes; e, abundant phylotypes; f, rare phylotypes) to vegetation loss (+Plant, with plants; -Plant, without plants) at different N-enrichment levels. The community composition was assessed by principal coordinates analysis (PCoA) at the phylotype level based on Bray-Curtis similarity distance. Each group is represented by a different color and shape. The effects of vegetation loss (R), N level (L), and their interaction (R × L) on community composition were assessed using permutational analysis of variance (PERMANOVA) (ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). The effects of vegetation loss for each N-enrichment level were also assessed using pairwise PERMANOVAs (r^2 and P values; ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Figure 7

A conceptual diagram showing the responses of community composition of bacteria and fungi to vegetation loss at different N-enrichment levels.

Supplementary Files

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