1 **Metal ions steer the duality in microbial community recovery from nitrogen**

2 **enrichment by shaping functional groups**

3 Running title: microbe recovery from nitrogen enrichment

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Abstract

Atmospheric nitrogen (N) deposition has been substantially reduced due to reductions in the reactive N emission in major regions of the world. Much less is unknown, however, on how soil microbial communities respond to the declines in N deposition, especially the recovery patterns of functional groups and key drivers modulating their responses. Here, we examined the effects of N addition and cessation of N addition on above and belowground communities through a 17-year field experiment in a temperate grassland. We found that soil bacterial and fungal diversity showed partial recovery after 3-year cessation of N addition, moreover, higher levels of N addition in the past resulted in greater resilience of their communities. Fungal communities exhibited lower resilience than bacteria because they were more dependent on changes in plant communities. However, both bacterial and fungal diversity did not fully recover to the initial stage, suggesting the co-existence of resistance and resilience in their responses to cessation of N enrichment. These two sides of their recovery were mainly steered by toxic metal ions differentially regulating on functional taxa. Specifically, beneficial symbiotic microbes including N-fixing bacteria and arbuscular mycorrhizal fungi (AMF) experienced detrimental effects from metal ions, hindering their recovery from previous N enrichment. Bacterial functional groups involved in N respiration and carbon (C) decomposition, and ericoid mycorrhizal and saprotrophic fungi, were positively influenced by soil metals, and were readily recovered. These findings could advance our mechanistic understanding of belowground community dynamics under

39   ongoing global changes, which also guide management practices to mitigate the adverse

40   effects of N enrichment on soil function.

41   **KEYWORDS**

42   nitrogen deposition, microbial community, community recovery, diversity, community

43   composition, microbial functional groups, soil chemistry, soil function

44

1. INTRODUCTION

Atmospheric deposition of reactive N caused by human activities leads to soil biodiversity loss and simplification of soil community composition (Johnson et al., 1998; Ramirez et al., 2012; Chen et al., 2018b; Zhang et al., 2018). These negative effects induced by N enrichment may impair multiple ecosystem functions, such as decomposition, nutrient retention and nutrient cycling (Liang & Balser, 2012; Wagg et al., 2014; Zhou et al., 2020). Microbial diversity reduction and community composition changes may result from direct effects of increased N availability and indirect modulation by other soil properties (Wang et al., 2018; Zhou et al., 2020; Liu et al., 2021). In addition to increases in availability of N (Pilkington et al., 2005; Clark et al., 2009; Phoenix et al., 2012), the common impacts of N enrichment on soil chemistry are also associated with depletion of nutrient base cations (Bowman et al., 2008; Lu et al., 2014) and reduction in soil pH (Horswill et al., 2008; Tian & Niu, 2015) and consequent impacts on metal availability (Bowman et al., 2008; Tian et al., 2020). Furthermore, N enrichment-induced alternations in plant community composition (Midolo et al., 2019) also contribute to soil microbial community dynamics (Leff et al., 2015; Chen et al., 2018b).

A decline in atmospheric N deposition has occurred in some regions across the globe due to the implementation of emission policies (Payne et al., 2017; Schmitz et al., 2019), This prompts studies focusing on the recovery of natural or semi-natural ecosystems, especially the aboveground community, from cessation of N enrichment (Boxman et al., 1998; Armitage et al., 2011; Britton et al., 2019; Seabloom et al., 2020;

Ladouceur et al., 2023). It has been suggested that N input-induced changes in plant communities may not readily recover after cessation of N inputs (Clark et al., 2009; Clark & Tilman, 2010; Street et al., 2015; Stevens et al., 2012; Stevens, 2016; Tilman & Isbell, 2015). For example, low plant diversity driven by N addition remained 20 years after the cessation of N addition in a successional grassland (Isbell et al., 2013). In contrast, several other studies found that plant diversity and species composition were recovered following the decline of N inputs (Edmondson et al., 2013; Shi et al., 2014; Storkery et al., 2015). These inconsistent findings may be due to differences in the past N-input levels and soil traits (Strengbom & Nordin, 2008; Edmondson et al., 2013).

Compared to the plant community, soil chemical variables seem to recover relatively quickly in response to cessation of enhanced N input (O'Sullivan et al., 2011; Verstraeten et al., 2012; Stevens, 2016). For example, in the North American alpine grasslands, soil inorganic N contents decreased markedly after cessation of N addition (Clark et al., 2009; Isbell et al., 2013). Similar results were also reported in European acid grasslands (Stevens et al., 2012). Soil pH and exchangeable ions were also observed to recover eight years after N addition ceased (Power et al., 2006). The effects of cessation of N input on soil biotic properties, such as microbial biomass, abundance and diversity, have been reported (Högberg et al., 2014; Bowman et al., 2018; Yang et al., 2023). However, these studies have mainly focused on description of microbial response patterns, and few studies have specifically explored the mechanisms underlying microbial recovery from past N enrichment.

Microbial community changes are typically expressed by community α diversity and β diversity, which are respectively considered as species richness and compositional differences between local communities. In addition, community β diversity is more associated with the reordering of species abundances or turnover in species composition (Jones et al., 2017). Therefore, community β diversity usually indicates a more rapid or greater fluctuation in response to environmental changes than community α diversity (Pierik et al., 2011; Dornelas et al., 2014; Diekmann et al., 2014). Comparable sensitivities of individual species or functional groups to environmental variables ultimately determine the variation in community diversity by affecting species growth, reordering, and/or turnover (Lilleskov et al., 2011; Andersen et al., 2013; Pallett et al., 2016). For instance, N enrichment usually inhibits symbiotic bacteria and fungi, i.e. N-fixing bacteria (Berthrong et al., 2014) and arbuscular mycorrhizal fungi (AMF, Chen et al., 2017), but increases the abundance of pathogenic and saprophytic fungi (Lekberg et al., 2021; Moore et al., 2021). However, whether and how the microbial functional groups are restored in the context of gradually decreasing atmospheric N deposition remain unclear.

It has been speculated that the effects of past N enrichment on biodiversity may be due to the N accumulation in ecosystems (Clark et al., 2009; Clark & Tilman, 2010). When accumulated N exceeds the ecosystem response threshold, the ecosystem may shift from a high-diversity stable state into a low-diversity state (Isbell et al., 2013). These findings imply that the reduction in N accumulation may enhance the probability of ecosystem restoration (Storkery et al., 2015; Liu et al., 2018; Berendse et al., 2021).

However, it has been reported that although soil N availability and plant tissue N content, critical indicators of ecosystem N accumulation, are significantly reduced after the cessation of N addition, the aboveground community dose not show marked recovery (Strengbom & Nordin, 2008; Isbell et al., 2013, Tilman & Isbell, 2015). Soil microbial communities also exhibited hysteretic responses to increases and subsequent decreases in soil nitrate concentrations (Bowman et al., 2018). These observations suggest that soil N availability may not be a direct driver for ecosystem recovery after decline in N enrichment. Given the relatively hysteretic responses of soil pH and metal ions to N treatments (Tian et al., 2020), and their divergent impacts on microbial species (Stefanowicz et al., 2008; Glassman et al., 2017), we hypothesize that these soil variables may be critical drivers of microbial community changes and may even be linked to N enrichment.

Here, we investigated the responses of plant and soil microbial communities, and alternations in soil chemistry to consecutive N addition and cessation of N addition in a temperate steppe. We aimed to 1) test whether there were different residual effects of past N enrichment on the bacterial and fungal communities in soil, and investigate if their functional groups had comparable recovery patterns following the decline in N deposition; and 2) determine the key drivers steering functional microbes in response to N enrichment and cessation of N enrichment. Answering these questions can offer a comprehensive insight into the effects of N enrichment and cessation of N enrichment on soil microbes, which may guide grassland management for maintaining soil function under the scenario of ongoing global changes.

2. MATERIAL AND METHODS

2.1 Study site

Our study was conducted in a temperate grassland at Duolun County (42.01′N, 116.16′ E, and 1,324 m a.s.l.) in Inner Mongolia Autonomous Region, China. The area is a semi-arid temperate steppe with mean annual temperature of 2.9°C and mean annual precipitation of 382 mm. Soil pH is about 7.0 and mean soil bulk density is about 1.3 g cm^{-3}. Soil (0-10 cm depth) organic carbon (C), total nitrogen (N), and total phosphorus (P) are 14.9, 1.5, and 0.8 g kg^{-1}. The concentrations of inorganic N (NH_4^+-N and NO_3^--N) and available P (Olsen-P) in soil are approximately 13.3 and 2.3 mg kg^{-1}. The concentrations of base cations (K^+, Ca^{2+} and Mg^{2+}) and metal ions (exchangeable Fe^{3+}, Mn^{2+}, and Al^{3+}) are respectively 147.5, 2434.7, 227.9, 14.0, 2.5, and 0.4 mg kg^{-1} (Tian et al., 2020). The vegetation is a typical steppe community, dominated by *Stipa krylovii, Agropyron cristatum, Leymus chinensis, Artemisia frigida, Cleistogenes squarrosa, Potentilla acaulis,* and *Potentilla betonicifolia.* The atmospheric bulk N deposition in this area is approximately 15.1 kg N ha^{-1} year^{-1} (Xu et al., 2015).

2.2 Experimental design

The field experiment was established in 2003. Eight levels of N fertilization (urea) addition (0, 1, 2, 4, 8, 16, 32, 64 g N m^{-2} yr^{-1}) were randomly arranged in sixty-four 150 m^2 (10×15 m) experimental plots, which were separated by 4 m wide buffer strips. Our previous studies have demonstrated that application of urea can simulate soil N enrichment caused by atmospheric N deposition because of the significant increase in inorganic N concentrations in soil induced by urea (Tian et al., 2020; 2021). The N-

155　addition levels of 1 and 2 g N m^{-2} yr^{-1} were chosen based on the reported ambient N

156　deposition rate (1.5 g N m^{-2} yr^{-1}) and the increase in annual N deposition in the study

157　area (Xu et al., 2015). The N-addition rates of 4, 8, and 16 g N m^{-2} yr^{-1} were selected to

158　represent the predicted N deposition in the next 50, 100, and 250 years. The N-addition

159　rates of 32 and 64 g N m^{-2} yr^{-1} respectively were chosen to simulate agricultural

160　fertilization and create an extreme N-enrichment environment. Nutrient addition was

161　ceased in half of the N-addition plots, which were split by a steel plate (30 cm depth)

162　in 2016. Three years after the cessation of N addition, we collected plant and soil

163　samples from 45 plots (five replicates) supplemented with five levels of N (0, 2, 8, 16,

164　and 64 g N m^{-2} yr^{-1}) and two types of N treatment (continuous N addition and ceased N

165　addition).

166　**2.3 Sample collection and measurement**

167　　Plant species composition and aboveground biomass were determined in August

168　2019 using a 0.5 m × 0.5 m quadrat. Soil samples were collected at a depth of 0 to 10

169　cm using a 3-cm diameter soil core. Three soil cores were randomly collected in each

170　subplot and then combined to give one sample for the subsequent analyses. Soil pH,

171　nitrate-N (NO_3^--N), and ammonium-N (NH_4^+-N) were determined according to the

172　methods in our previous studies (Tian et al., 2020). Soil total carbon and soil total N

173　were determined using an elemental analyzer (Vario EL III, Elementar, Germany). Soil

174　available phosphorus (Olsen-P) and exchangeable metal ions (Fe^{3+}; Mn^{2+} and Al^{3+})

175　were measured according to our previous methods (Tian et al., 2020).

176　**2.4 Microbial bioinformatic analysis**

177   Microbial DNA was extracted from 0.5 g fresh soil using the E.Z.N.A. soil DNA

178   Kit (Omega Bio-tek, Norcross, GA, U.S.). The primers and PCR conditions are detailed

179   in Table S1. Purified amplicons were pooled in equimolar and paired-end sequenced on

180   an Illumina MiSeq PE300 platform (Illumina, San Diego,USA) according to the

181   standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China).

182   Raw reads were demultiplexed based on their unique barcodes to match different

183   samples. The barcodes and primers were trimmed and low quality (Phred score < 20)

184   bases were removed from these sequences by fastp software (version 0.19.6) (Chen et

185   al., 2018a). Forward and reverse sequences were merged with a minimum overlap of

186   10 bp using FLASH version 1.2.11 (Magoč & Salzberg, 2011). The qualified sequences

187   without singletons were clustered into Operational taxonomic units (OTUs) at 97%

188   similarity cutoff using UPARSE version 7.0 (Edgar, 2013), and chimeric sequences

189   were identified and removed. Taxonomic annotations of OTUs were analyzed by RDP

190   Classifier version 2.11 (Wang et al., 2007) against the Silva 138 database (Quast et al.,

191   2013) for bacteria and UNITE 8.0 database (Nilsson et al., 2019) for fungi using

192   confidence threshold of 0.7. To normalize sequences, rare OTUs of all samples with

193   total abundance less than 20 were removed, and sequences were resampled to the

194   minimum sequence depth (23,700 sequences per sample of bacteria, 30,133 sequences

195   per sample of fungi) across all samples.

196   **2.5 Data analyses**

197   The Shannon diversity index (*H*) and Bray-Curtis dissimilarity (*Dis*), respectively,

198   were used as a measure of community α diversity and β diversity. The *H* and *Dis* of

199   the plant community were calculated based on the data of species biomass from all plots

200   in 2019, and those of the microbial community were calculated from the data of species

201   OTUs. The Shannon diversity index and Bray-Curtis dissimilarity matrix were

202   generated using the 'vegan' package. Details of these calculation methods can be found

203   in Methods S1.

204   To test the continuous effects of N enrichment on plant and microbial diversity,

205   we used diversity sensitivity (V_S) by comparing the differences in community diversity

206   between control and N-addition treatments. The sensitivity of community α diversity

207   ($V_{S(\alpha)}$) was considered as the response ratio of the values of H between control and N-

208   addition treatments, and was calculated as:

209    $$V_{S(\alpha)} = \frac{H_{(CK)} - H_{(N+)}}{H_{(CK)}},\qquad\qquad\qquad (1)$$

210   where $H_{(CK)}$ and $H_{(N+)}$, respectively, are the value of community H within control and

211   N-addition plots. The sensitivity of β diversity ($V_{S(\beta)}$) was considered as the Bray-Curtis

212   distance of replicate communities within N-addition treatments to mean Bray-Curtis

213   distance within control groups. The $V_{S(\beta)}$ was calculated as:

214    $$V_{S(\beta)} = Dis_{(N+)} - Dis_{(CK)},\qquad\qquad\qquad (2)$$

215   where $Dis_{(N+)}$ is the Bray-Curtis distance of replicate communities within N-addition

216   plots to control and $Dis_{(CK)}$ is mean Bray-Curtis distance of replicate communities in

217   control plots.

218   To assess the responses of plant and microbial communities after the cessation of

219   N addition, we used resilience by comparing the relative differences between stopping

220   N addition and continuous N addition treatments to control. The evaluation framework

221 of the elasticity of community α diversity ($V_{E(\alpha)}$) and β diversity ($V_{E(\beta)}$) was shown in

222 Methods S2. The formulas for calculating the elasticity of α diversity ($V_{E(\alpha)}$) and β

223 diversity ($V_{E(\beta)}$) are:

224 $\qquad V_{E(\alpha)} = \frac{H_{(N-)} - H_{(N+)}}{H_{(CK)}}$ (3)

225 $\qquad V_{E(\beta)} = \frac{Dis_{(N+)} - Dis_{(N-)}}{Dis_{(CK)}}$ (4)

226 where $H_{(CK)}$, $H_{(N+)}$, and $H_{(N-)}$ is the value of community H within control, continuous

227 N addition, and cessation of N addition; $Dis_{(N+)}$ is the Bray-Curtis distance between

228 the continuous N addition to control; and $Dis_{(N-)}$ is the Bray-Curtis distance between

229 the cessation of N addition to control; and $Dis_{(CK)}$ is mean Bray-Curtis distance of

230 replicate communities in control plots.

231 $\qquad$ We visualized β-diversity variations by non-metric multidimensional scaling

232 (NMDS) analyses. Permutational multivariate analysis of variance (PERMANOVA)

233 with 'EcolUtils' package was used to test effects of N addition and stopping N addition

234 on the community composition of plants and microbes. Linear mixed-effects models

235 were used to analyze effects of N-addition levels on plant and microbial α diversity and

236 soil chemical variables. In the linear mixed-effect models, N-addition levels were

237 considered as main-plot effects, with continuous N addition and stopping N addition as

238 subplot effects and the block as random factor.

239 $\qquad$ Pairwise comparison between the treatments of continuous N addition and

240 stopping N addition was performed with Tukey's test. All data were log-transformed or

241 nonparametric tests were carried out when necessary. The Kruskal-Wallis test and the

242 Mann-Whitney U test were performed to examine the differences in diversity sensitivity

and resilience of plants, bacteria, and fungi in low and high levels of N-addition treatments, and the effect size was calculated by the 'effectsize' package. Pearson's correlation analysis was used to evaluate the relationship between diversity sensitivity and elasticity, and soil chemical variables. Structural equation modeling (SEM) was carried out to test how β diversity sensitivity and resilience of soil bacteria and fungi were affected by soil chemical variables and plant community structure directly and indirectly. SEM was conducted using the R package "piecewiseSEM" (Lefcheck & Duffy, 2015). We performed functional annotation of bacterial taxa using "FAPROTAX", which is the program functional annotation of prokaryotic taxa (Louca et al., 2016; He et al., 2024). We predicted the functional groups of fungi based on "FUNGuild" database (Nguyen et al., 2016) used in the literatures (Moore et al., 2021; He et al., 2024). To assess the independent contribution of Fe^{3+}, Mn^{2+}, and Al^{3+} to the changes in relative abundance of functional groups of bacteria and fungi, random forest analysis was carried out with the 'rfUtilities' and 'rfPermute' packages (Archer, 2016; Evans & Murphy, 2015). All statistical analyses were performed in R software (version 4.2.2, R Core Team, 2022).

3. RESULTS

3.1 The α diversity and β diversity of the soil bacterial and fungal communities

Consecutive N addition significantly decreased α diversity of soil fungal community, while that of soil bacteria remained relatively constant with the exception of the high-level N addition (Figure S1; Figure 1a, b; Table S2). After cessation of high-level N addition (HN-) for three years, the community α diversity of bacteria, and fungi increased by 17.2% and 23.3% compared to those in the plots continuously receiving high level of N (HN+) (Figure 1a, b). Although the α diversity of bacterial, and fungal community increased in the high-level N ceased plots, they were still lower by 4.5% (Cohen's d=1.956, P=0.01), and 38.6% (Cohen's d=1.751, P=0.024) than those in the control plots (Figure 1a, b; Table S2).

The community composition of bacteria, and fungi in the N-addition plots diverged from the control plots (Figure 1c, d), and the Bray-Curtis dissimilarity increased under higher N-addition levels (Figure S1). For soil bacterial phyla, N addition decreased actinobacteria abundance and increased firmicutes and proteobacteria abundance, while cessation of N addition decreased firmicutes abundance by 34.8% compared to continuous N addition (Figure S2). The abundance of dominant fungal phyla, ascomycota, and mortierellomycota, respectively increased by 14.8% and decreased by 92.5% in the plots receiving high-level N, while they showed the opposite changes following cessation of N addition (Figure S2). The PERMANOVA analysis showed that there was a significant recovery in the community composition of bacteria (F=5.272, R^2=0.397, P = 0.020) and fungi (F=3.423, R^2=0.300,

281    $P = 0.020$) in the high level of N ceased plots (Table S3).

3.2 Microbial diversity sensitivity to N addition and the resilience after cessation of N addition

Bacterial β diversity exhibited greater sensitivity to N addition than its α diversity (Figure 2a), moreover, its β diversity showed greater resilience than fungal β diversity after stopping N addition (Figure 2c,d). Bacterial β-diversity resilience was 2.4 times higher than that of fungi in high-level N added plots (Figure 2c, d; E^2_R=-1.00, P=0.008; Table S4). Fungal α and β diversity exhibited similar sensitivity to N enrichment, i.e. with the level of N addition increasing, their α and β diversity sensitivity increased (Figure 2a,b). Fungal and bacterial β diversity also showed higher resilience in the plots receiving higher levels of N (Figure 2c, d). Their β-diversity resilience was positively correlated to previous N-addition levels (Figure S3), indicating that previous higher N-addition levels may lead to a greater β-diversity resilience.

3.3 Responses of plant community and soil chemistry to N addition and cessation of N addition

Nitrogen addition significantly decreased α diversity of plant community and alter its species composition (Figure S2, Figure S4). Compared with soil bacterial and fungal communities, plant community exhibited the greatest sensitivity in its α diversity to N addition (Figure S4; E^2_R =0.67, P=0.009; Table S4). Continuous high-level N addition shifted the vegetation from co-dominance by *S. krylovii* and *A. frigida* to dominance by *L. chinensis* (Figure S2). The cessation of N addition did not reverse the effects of N addition on plant dominant species, but led to the appearance of some rare species

303   (Figure S2). There were positive correlations between the changes in plant community

304   composition and those in bacterial and fungal community composition (Figure 3),

305   suggesting that shifts in aboveground community induced by N treatments affect soil

306   microbial diversity.

307   Soil inorganic N concentrations were elevated in the N-added plots compared to

308   the control plots (Figure 4a; Figure S5; $P<0.05$). Available P concentrations were not

309   significantly increased by N addition, with the exception in the plots receiving 64 g N

310   m^{-2} yr^{-1} (N64) (Figure 4a; Figure S5). N addition decreased soil pH and the

311   concentrations of base cations (Figure S5). Metal ions including Fe^{3+}, Mn^{2+}, and Al^{3+}

312   positively responded to N inputs (Figure 4a). The mean values of total metal ion

313   concentrations in the N64 plots were 9.2 times higher than those in the control (Figure

314   S5). There were no significant differences in soil TC, TN, or TP between the control

315   and N-addition treatments (Figure S6). After three years of ceasing N addition,

316   inorganic N concentrations were significantly reduced and recovered to the levels of

317   the control (Figure 4b; Figure S5). Metal ion concentrations also rebounded in response

318   to N-addition cessation, but their rebound amplitude was lower than that of inorganic

319   N (Figure 4b; Figure S5). Soil pH and base cation concentrations increased after the

320   cessation of N addition, while the mean values were still lower in the high-level N

321   ceased plots (N64-) by 21.3 % and 32.6% than those in the control (Figure S5).

322 **3.4 Contributions of variations in soil chemistry and plant community to the**

323 **sensitivity and resilience of microbial diversity**

324   The increases in available nutrients and metal ions, and the decreases in soil pH

and base cation concentrations, were positively correlated with the β-diversity

sensitivities of bacteria and fungi (Figure 4c). Bacterial β-diversity resilience was

positively correlated with the changes in inorganic N, metal ions, base cations and soil

pH (Figure 4d). Fungal β-diversity resilience was correlated with the increases in soil

pH and the decreases in metal ion driven by the cessation of N addition (Figure 4d).

Structural equation models showed that N addition had a strong indirect effect on

bacterial β-diversity sensitivity via decreasing pH and increasing metal ion

concentrations, and via affecting plant community composition (Figure 5a). Soil metal

ions and plant community changes exerted direct and dominant effects on the bacterial

β-diversity sensitivity, respectively explaining 71.2% and 63.3% of variation in their

diversity sensitivity (Figure 5a). However, changes in the plant community composition

had a direct and dominant effect on fungal β-diversity sensitivity, explaining 72.7% of

variation in their diversity sensitivity (Figure 5a). Stopping N addition indirectly

affected the β-diversity resilience of bacterial and fungal communities *via* increasing

pH and decreasing metal ion concentrations (Figure 5b). Changes in metal ions rather

than inorganic N directly explained 97.9% and 81.6% of the bacterial and fungal β-

diversity resilience (Figure 5b).

3.5 Soil metal ions associated with changes in bacterial and fungal functional

groups

At the functional group levels, the relative abundances of the bacterial and fungal

functional groups responded differently to N treatments (Figure 6). Continuous N

addition led to increases in the relative abundances of bacteria associated with N

347   respiration, nitrate/nitrite ammonification and C decomposition (ligninolysis) and metal

348   oxidation (manganese oxidation) (Figure 6a, Figure S7). Relative abundances of these

349   functional bacteria increased respectively by 2.1, 3.3, 3.2 and 5.9 times in high-level N

350   added plots compared with those in control plots (Figure S7). The positive responses of

351   these bacterial groups were reversed by stopping N addition (Figure 6a), moreover, their

352   abundance changes were positively correlated to metal ion variations in soil (Figure 6b).

353   Likewise, relative abundances of the bacterial groups related to N fixation, nitrification

354   as well as denitrification were decreased by N addition, but increased after the cessation

355   of N addition (Figure 6a). Accordingly, the abundance changes of these groups

356   negatively correlated to metal ions (Figure 6b).

357   Fungal functional groups including ericoid mycorrhizal and saprotrophic indicator

358   taxa showed positive responses to N addition, with ericoid mycorrhizal taxa exhibiting

359   the greatest changes in their abundance (Figure 6c, Figure S7). Relative abundances of

360   these two taxa decreased after the cessation of N addition (Figure 6c), and their

361   abundance changes were positively correlated to soil metal ions (Figure 6d). The

362   abundance of plant pathogenic taxa also increased in the relatively low-level N added

363   plots (2 g N m^{-2} yr^{-1} and 8 g N m^{-2} yr^{-1}), but their abundance markedly declined in the

364   extreme N-added plots (Figure S7). N inputs also led to the decreases in arbuscular

365   mycorrhizal and parasitic taxa (Figure 6c); these two fungal taxa showed negative

366   correlation with soil metal ions (Figure 6d).

367   Random forest analysis showed that Mn^{2+}, Fe^{3+} and Al^{3+} were all the important

368   variables in shaping most bacterial functional groups associated with N and C cycling,

369   with Mn^{2+} showing the higher explanation (Table S5). Mn^{2+} also contributed to the

370   changes in the plant pathogenic and saprotrophic taxa, while Al^{3+} showed a relatively

371   higher explanation for changes in ericoid mycorrhizal and arbuscular mycorrhizal taxa

372   (Table S5).

4. DISCUSSION

We found comparable responses of soil bacteria and fungi to N addition and cessation of N addition, and that bacterial β diversity was easier to recover than fungal diversity after stopping N addition. Higher levels of N addition led to a greater diversity resilience in both bacterial and fungal communities. We discovered that the soil metal ions and changes in plant community rather than N availability in soil induced by N addition directly contributed to the bacterial diversity sensitivity to N enrichment, and that the fungal community was mainly controlled by changes in plant community. Metal ion dynamics also directly affected both bacterial and fungal diversity resilience by positively or negatively mediating their functional groups. Although bacterial and fungal diversity exhibited a certain degree of recovery, cessation of N addition did not cause them to completely recover to the original state. Our findings indicate that both resistance and resilience coexist in recovery of soil microbe after cessation of N enrichment, and that the two sides of resistance-resilience relationship are regulated by soil metal ions and plant community (Figure 7). The regulatory mechanisms by which the dual recovery patterns of soil microbial communities have been rarely explored.

4.1 Bacterial β diversity is more sensitive to N enrichment than its α diversity

We found that bacterial α diversity in soil was relatively insensitive to N enrichment with the exception of the extreme N-addition treatment that led to a reduction in its α diversity (Figure 1). This result is consistent with that reported by Liu et al., (2021). Field experiments and meta-analysis also provide evidence for the low sensitivity of bacterial α diversity to N enrichment, such that the critical threshold for

395   the reduction of bacterial α diversity in temperate grasslands was 28 g N m^{-2} yr^{-1} (Liu

396   et al., 2020). Although bacterial α diversity was not altered by low and intermediate

397   levels of N addition, its β diversity displayed the greatest sensitivity to N addition

398   (Figure 2). Higher β-diversity sensitivity to N addition in soil microbes may result from

399   the turnover or displacement among microbial species (Figure S2; Figure S7). This may

400   be accounted for by soil chemical variables associated with N addition (Stefanowicz et

401   al., 2008; Glassman et al., 2017; Fierer, 2017; Tian et al., 2020). The shift in plant

402   community composition was also linked to the changes in microbial community

403   composition (Figure 3), although they showed comparable sensitivities to N addition

404   (Figure 2; Figure S4). Nutrient addition experiments in global grasslands also found

405   consistent correlations between changes in plant and microbial community composition

406   (Leff et al., 2015).

407   **4.2 Metal ions modulate soil microbial responses to N enrichment and cessation of**

408   **N enrichment**

409   We found a rapid recovery in soil inorganic N pools following cessation of N

410   addition, which is consistent with the results reported in other grassland ecosystems

411   (Power et al., 2006; Clark et al., 2009; Stevens et al., 2012). We also discovered that

412   soil pH, base cations, and metal ions recovered more rapidly than soil microbes

413   following cessation of N addition. A similar recovery pattern for soil chemistry and

414   microbes has also been reported in other ecosystems, though the microbial biomass

415   rather than microbial diversity was assessed (Power et al., 2006; Stevens et al., 2012).

416   Here, we demonstrated that the β diversity of soil microbes exhibited recovery after

417   cessation of N addition (Figure 2). The changes in soil variables, especially metal ions

418   and soil pH, contributed to their β-diversity recovery (Figure 5). Decreases in metal

419   ions and increases in soil pH after stopping N addition may rescue some microbial

420   species that were inhibited by toxic metal and low soil pH (Stefanowicz et al., 2008;

421   Rousk et al., 2010; Glassman et al., 2017), which ultimately promotes the recovery of

422   community β diversity (Figure 2). For example, we found that the functional groups of

423   bacteria and fungi were positively correlated to soil metal ions, and that they exhibited

424   a positive response to N enrichment (Figure 6). The results of the structural equation

425   model revealed that alterations in metal ions rather than inorganic N availability in soil

426   directly contributed to the shift in bacterial and fungal communities in response to N

427   enrichment and the cessation of N enrichment (Figure 5).

428   Previous studies have predicted that continuous N inputs would cause a system to

429   reach a critical threshold and drive the ecosystem to a low-diversity state (Clark et al.,

430   2009; Isbell et al., 2013). If the ecosystem is not rescued by removing nutrients from

431   the ecosystem, such as hay harvest (Storkey et al., 2015), the low-diversity state is

432   hardly reversed (Isbell et al., 2013). The low-diversity status reported in these studies

433   is mainly based on species richness. Although they did not find the recovery of the

434   lower species richness, they did observed that cessation of N addition altered plant

435   species composition (Semelová et al., 2008; Isbell et al., 2013). Here, we found that the

436   community composition of plants, bacteria, and fungi in the plots with cessation of N

437   addition closely resembled those in the control plots (Figure 1; Figure S4). Some

438   dominant bacterial and fungal phyla, such as firmicute, ascomycota, and

439 mortierellomycota, showed contrary trends in their abundance in the N ceased plots

440 compared to those in the N added plots (Figure S2). Moreover, some functional groups

441 of bacteria and fungi also exhibited a certain degree of recovery after stopping N

442 addition (Figure 6; Figure S7). We also found that extremely high N input did not drive

443 the ecosystem into a state that could not be restored, but led to greater resilience (Figure

444 2). The resistance and resilience coexisted in the recovery of above and belowground

445 communities from cessation of extremely high N input (Figure 2; Figure S4). The two-

446 sides of resistance-resilience relationship in ecosystem recovery from cessation of N

447 enrichment and extreme events have also been reported in the literature (Hoover et al.,

448 2014; Vitasse et al., 2019; Kuske et al., 2012; Yang et al., 2023).

449 **4.3 Contributions of metal ions to the restoration of soil microbes depend on soil**

450 **intrinsic characteristics of ecosystems**

451 Whether an ecosystem can recover or not may depend on the original soil

452 properties. For acidic grasslands, soil microbial species that are sensitive to metal ions

453 and low soil pH may have been lost, and the surviving species may have evolved

454 effective strategies to adapt to the acidic soil (Rousk et al., 2010; Glassman et al., 2017).

455 Therefore, the lower changes in soil metal ions induced by the cessation of N addition

456 in these grasslands could not effectively drive the recovery of microbial community

457 diversity (Bowman et al., 2018). In grasslands with neutral soil, high-level N addition

458 reduced soil pH from 7.0 to about 5.0 (Figure S5), which is the critical threshold of soil

459 pH determining the acid buffer system in soil to shift from basic cations to Al^{3+}

460 (Bowman et al., 2008). Accordingly, we found that Al^{3+} concentrations in soil showed

461 greater recovery, moreover, Fe^{3+} and Mn^{2+} were more readily reversible than those in

462 the acidic alpine meadow (Bowman et al., 2018). These results suggest that the greater

463 restorations of Fe^{3+}, Mn^{2+}, and Al^{3+} may cause a greater microbial recovery in neutral

464 grasslands than that in acidic grasslands. The results of SEM confirmed this conclusion,

465 i.e., soil metal ions rather than N availability have direct and dominant contributions to

466 recovery of bacterial and fungal communities (Figure 5).

467 **4.4 Fungal restoration was also limited by the aboveground community**

468   Compared to bacteria, responses of the fungal community to N enrichment were

469 more influenced by changes in plant community (Figure 5). This may mainly attributed

470 to the closer nutrient-exchange relationship between fungi and plants (van der Heijden

471 et al., 2015). Plant species loss under N enrichment is individualistic in grasslands, such

472 as forb species losing but grasses maintaining survival (Payne et al., 2013; Clark et al.,

473 2019; Tian et al., 2020). Continued depression in the N sensitive species of plants may

474 be linked to the changes in soil fungal communities due to the species-specific

475 regulation. Although we observed the reversal of plant β diversity after cessation of N

476 addition (Figure S4), the recovery of dominant species was not detected (Figure S2).

477 Therefore, we found relatively lower resilience of soil fungi in response to cessation of

478 N addition (Figure 2). Moreover, we discovered that cessation of N addition failed to

479 recover some symbiotic bacteria and fungi, such as N fixing bacteria and AMF (Figure

480 6). This may be attributed to the inability of their host plants to recover within a short

481 period. Conversely, the bacterial functional groups involved in N respiration and C

482 decomposition, as well as ericoid mycorrhizal and saprotrophic fungi exhibited

24

significant restoration. These microbial shifts were positively influenced by the presence of soil metal ions (Figure 6). These findings indicate that, in the absence of human intervention, the adverse effects of past N enrichment on soil functionality are likely to endure for an extended period.

In summary, soil microbial restoration exhibited a duality of resistance and resilience following cessation of N enrichment. The variations in toxic metal ions were mainly responsible for regulating bacterial and fungal recovery. Soil fungi showed lower resilience than bacteria, being due to the limitation by the lower diversity of plant community induced by past N enrichment. Toxic metal accumulation induced by N enrichment had inhibitory effects on symbiotic microbes including N-fixing bacteria and AMF, and these microbes almost failed to recover. On the contrary, the microbes involved in N respiration and C decomposition were positively influenced by soil metals, and they were readily reversible after cessation of N enrichment. The work offers valuable insights into the mechanisms underlying soil microbial responses to N disturbance. This insight is critical for predicting the effects of ongoing anthropogenic N enrichment on soil function, and improving soil function through scientific practices to rescue some functional microbes, such as adjusting soil pH, chelating metal ions, reintroducing key plant species, or introducing beneficial microorganisms.

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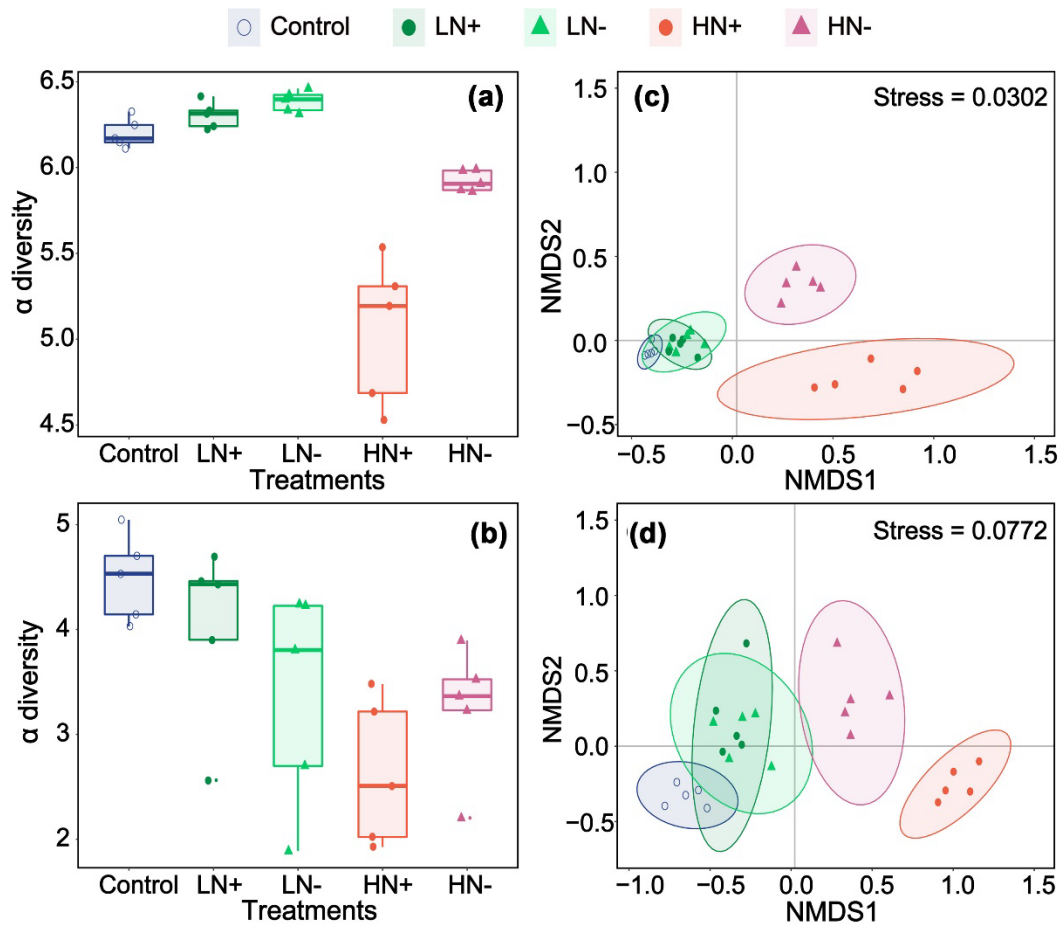
Figure 1 Effect of nitrogen (N) addition and cessation of N addition on the α diversity

and β diversity of soil microbial communities. (a) α diversity of bacterial community,

(b) α diversity of fungal community, and nonmetric multidimensional scaling (NMDS)

analysis of the community composition of (c) bacteria and (d) fungi. Ellipses are drawn

by 95% confidence interval estimate. Treatments: control; LN+, continuous low-N

addition; HN+, continuous high-N addition; LN-, cessation of low-N addition; HN-,

cessation of high-N addition.
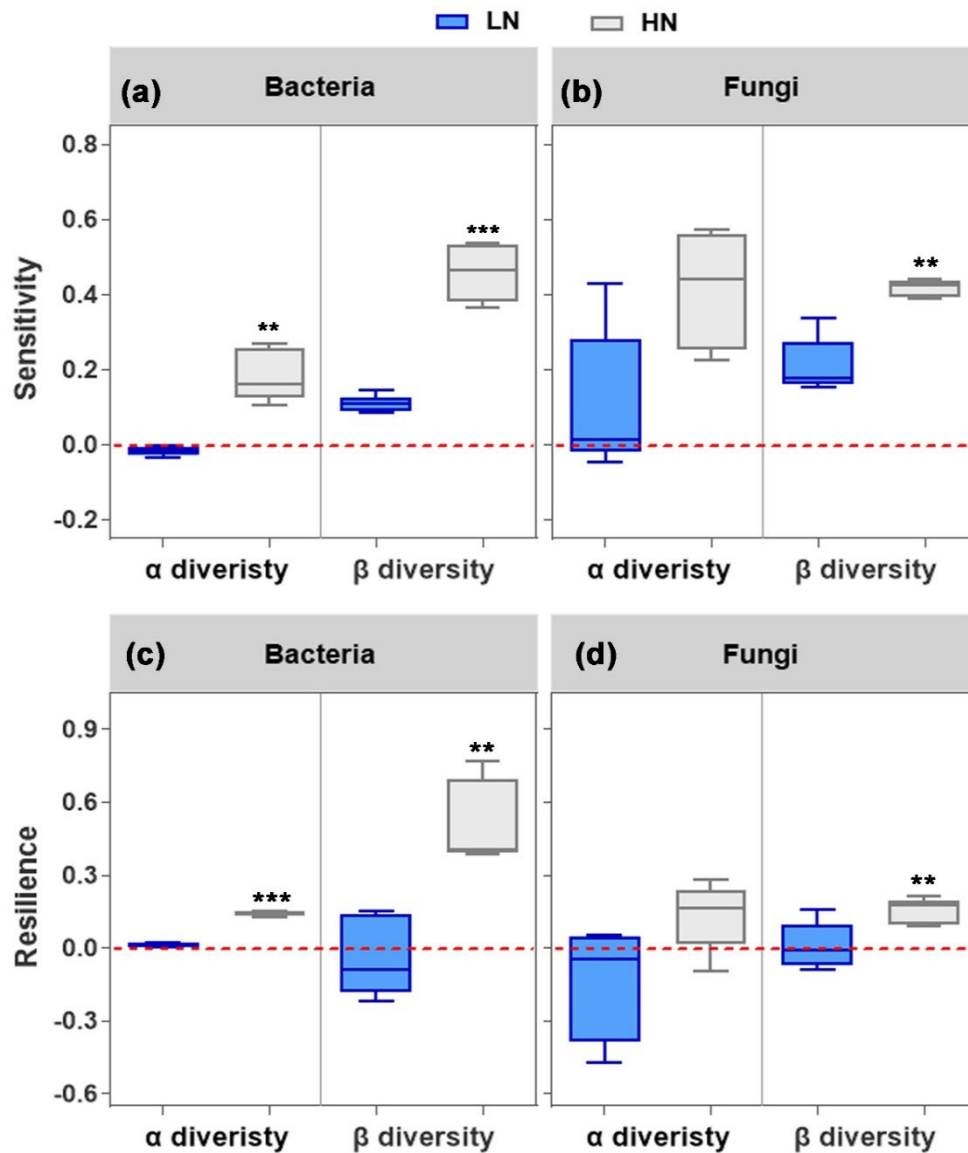
Figure 2 The sensitivity and resilience of soil microbial diversity in response to nitrogen (N) addition and cessation of N addition under different N-addition levels. Sensitivity of α diversity and β diversity in response to continuous N addition in bac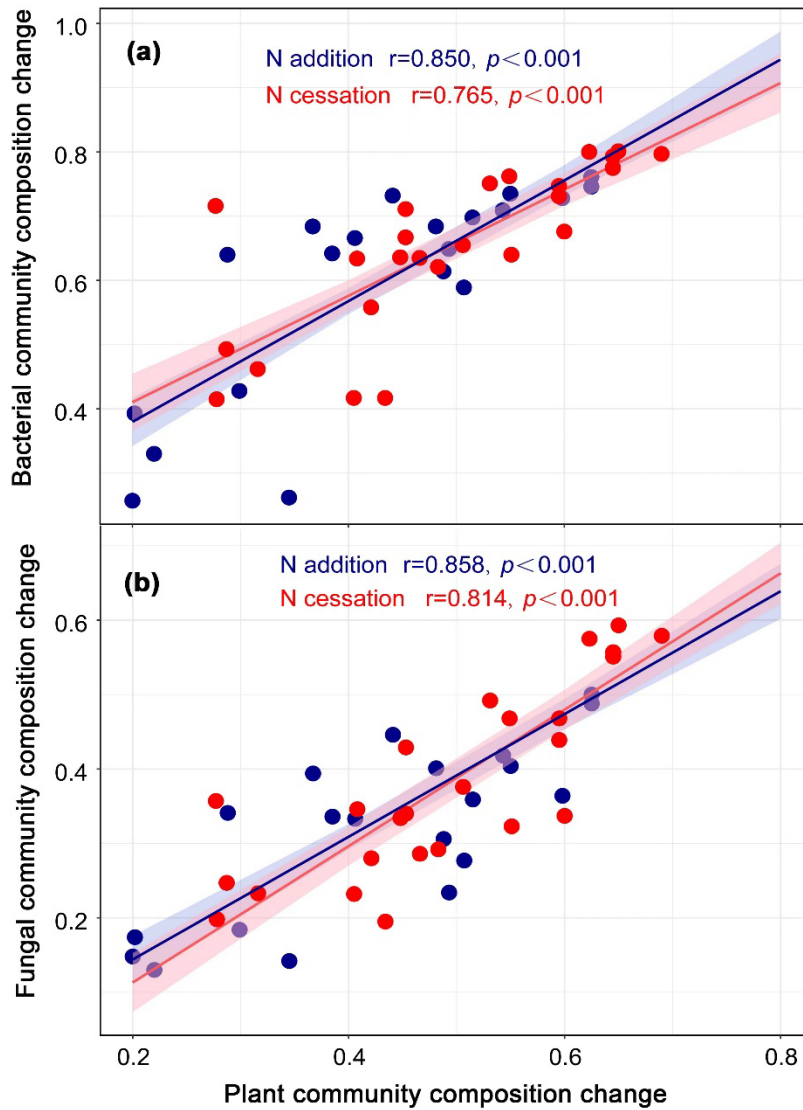teria (a) and fungi (b). Resilience of α diversity and β diversity of bacteria (c) and fungi (d) after cessation of N addition. LN and HN respectively indicate the low level and high level of N addition. Asterisks represent significant differences between high N-addition and low N-addition levels; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.
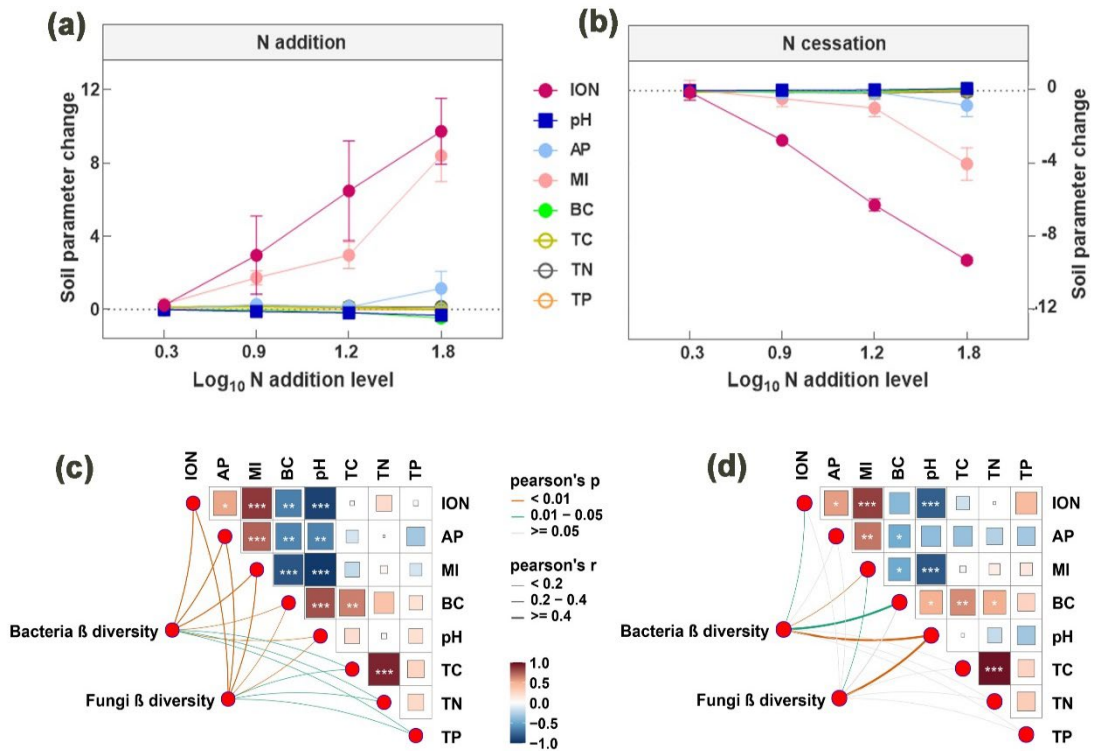
Figure 3 Correlations between changes in plant and microbial community composition

with nitrogen (N) addition and cessation of N addition. (a) Bacterial and (b) fungal

community composition change correlated to plant community composition change.

Levels of significance and correlation coefficients are shown for each relationship.

Figure 4 Soil chemical changes and their contributions to the sensitivity and resilience of bacterial and fungal β diversity under nitrogen (N) addition and cessation of N addition. (a) Soil chemical variables in response to N addition under different N-addition levels; (b) Soil chemical variables in response to cessation of N addition under previously different N-addition levels. Data are means ± SD for five replicates. (c) Diversity sensitivity correlated to soil chemical variables under N addition; (d) Diversity resilience correlated to soil chemical variables under cessation of N addition. Soil variables include soi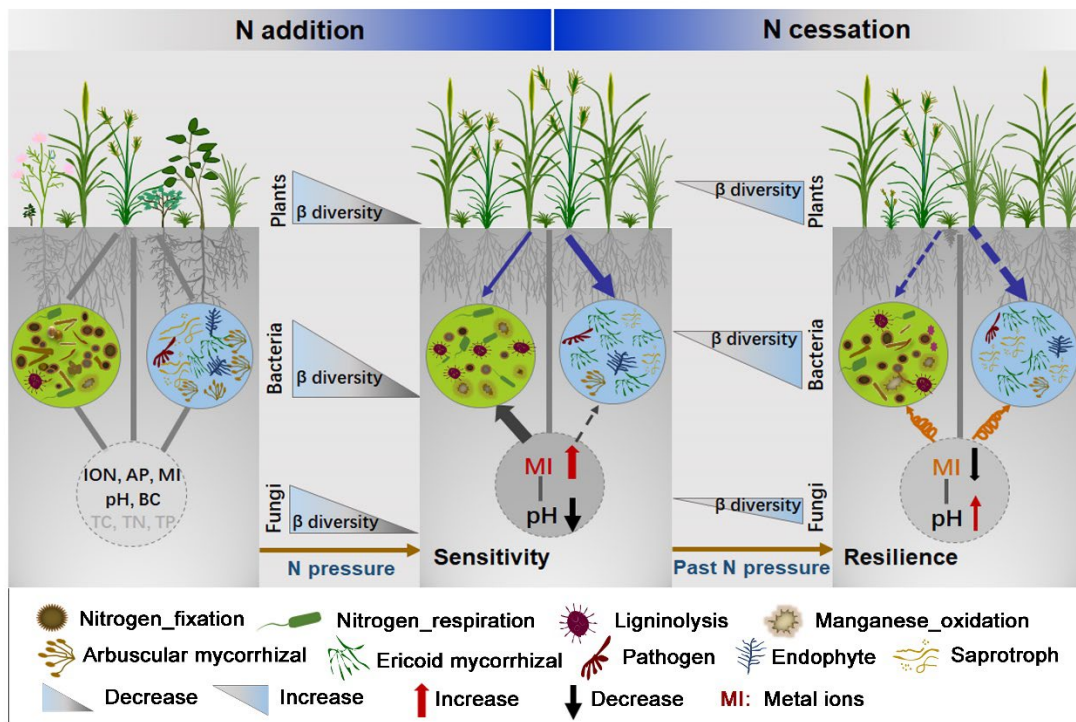l inorganic nitrogen (ION), available phosphorus (AP), metal ions (MI), base cations (BC), pH, total carbon (TC), total nitrogen (TN) and total phosphorus (TP).
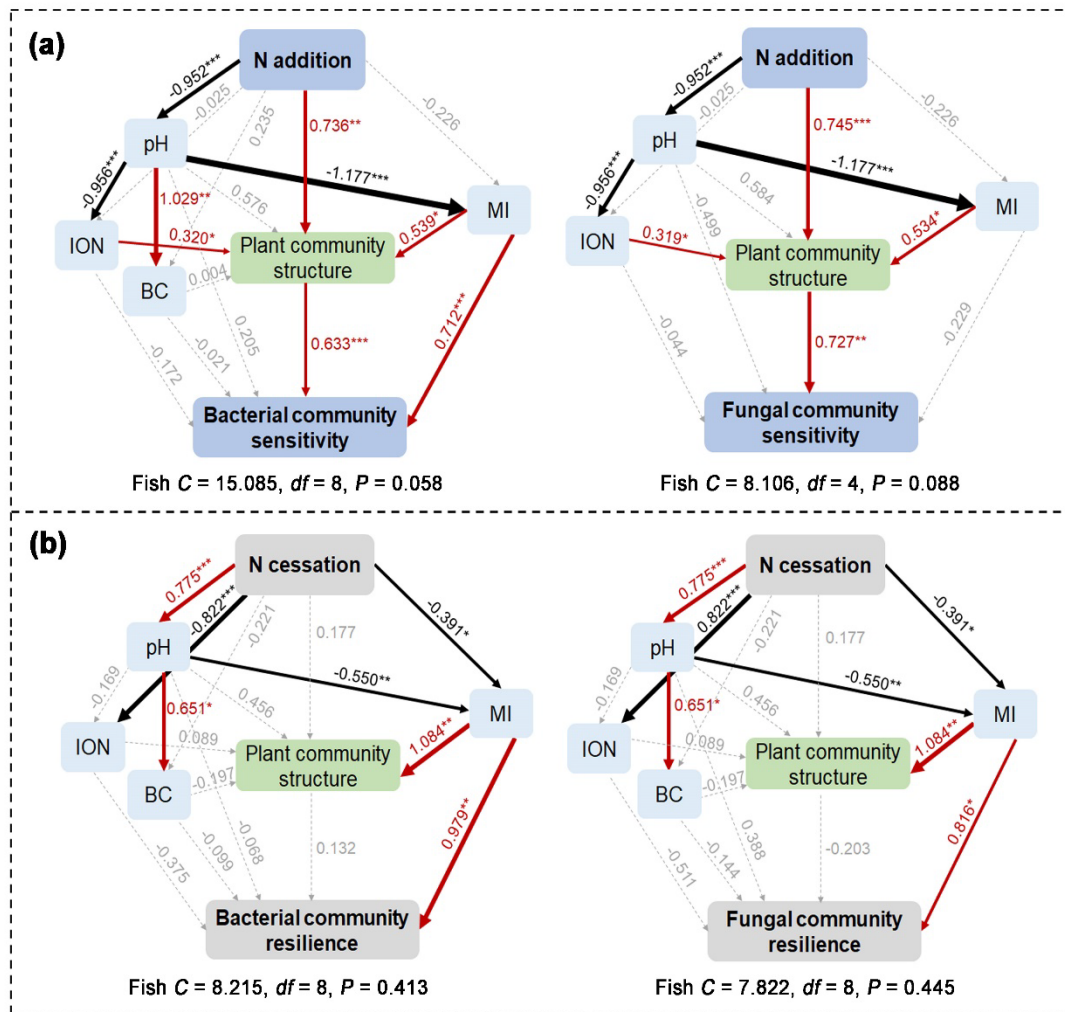
Figure 5 Relationship between the sensitivity and resilience of microbial community and the changes in plant community composition and soil parameters. (a) Structural equation models disentangling major pathways of N addition on the β-diversity sensitivity of bacterial and fungal communities; (b) Structural equation models disentangling major pathways of stopping N addition on the β-diversity resilience of bacterial and fungal communities.

Figure 6 Abundance changes in bacterial and fungal functional groups and their correlations with soil metal ions. Abundance changes in the functional groups of bacteria (a) and fungi (c) under high-level N addition and cessation of high-level N addition; (b) Correlations between soil metal ions and bacterial functional groups associated with carbon (C) cycle, nitrogen (N) cycle and metal oxidation; (d) Correlations between soil metal ions and fungal functional groups. Levels of significance and correlation coefficients are shown for each relation. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 7 Soil metal ions combined with the changes in aboveground community contribute to diversity sensitivity and resilience of bacteria and fungi in response to nitrogen (N) addition and cessation of N addition. The β diversity of plants, bacteria and fungi decrease gradually with increase in N-addition levels. The increase in soil metal ions (MI), decrease in soil pH, and plant diversity loss driven by N addition determine the diversity sensitivity of bacteria. Fungal diversity sensitivity is mainly controlled by N enrichment-induced changes in plant community diversity. After the cessation of N addition, the β diversity of plant, bacterial and fungal communities rebounds gradually with increase in previous N-addition levels. The partial rebounds of MI from previous N addition contribute to the diversity resilience of bacteria and fungi by modulating their functional groups.

Supporting information

Table S1 Primer sets and thermal profiles used in PCR amplification.

Table S2. Pairwise comparison between two treatments in α diversity of plants, bacteria and fungi. Tukey's test is used to compare the differences between N levels from linear mixed-effects models of split-plot design. Treatments include nitrogen (N) addition at five different levels (0, 2, 8, 16, 64 g N m^{-2} yr^{-1}) and N cessation. Effect sizes are measured by Cohen's d for pairwise differences.

Table S3 Permutational Multivariate Analysis Of Variance in community composition of plants, bacteria, and fungi, based on Bray-Curtis semi-metric distance matrix.

Table S4 The Kruskal-Wallis test among plant, bacterial, and fungal communities in their diversity sensitivity and elasticity at low level N (LN) and high level N (HN) and the Mann-Whitney U test in pairwise comparisons. Effect sizes of the Kruskal-Wallis test depend on rank epsilon squared (E^2_R). Effect sizes of the Mann-Whitney U test depend on rank biserial correlation (r).

Table S5 Random forest mean predictor importance of manganese (Mn^{2+}), iron (Fe^{3+}), and aluminium (Al^{3+}) as drivers for changes in functional groups of bacteria and fungi. MSE, mean squared error; **, $P<0.01$; *, $P<0.05$.

Methods S1 Calculations of Shannon diversity index (*H*) and Bray-Curtis dissimilarity (*Dis*).

Methods S2 The evaluation framework for the elasticity of community β diversity.

Figure S1 Effect of nitrogen (N) addition and N cessation on α diversity and β diversity of bacteria and fungi. Shannon diversity index of (a) bacterial and (b) fungal

910   communities; Bray-Curtis dissimilarity of (c) bacterial and (d) fungal communities.

911   **Figure S2** Effect of nitrogen (N) addition and cessation of N addition on the species

912   composition of the plant, bacterial, and fungal communities. (a) Relative biomass of

913   dominant and rare species of plants. (b) Occurrence frequency of dominant and rare

914   species of plants. (c) Relative abundance of bacterial phyla. (d) Relative abundance of

915   fungal phyla. Treatments including control; LN+, continuous low-N addition; HN+,

916   continuous high-N addition; LN-, cessation of low-N addition; HN-, cessation of high-

917   N addition.

918   **Figure S3** Relationship between the diversity resilience of plant, bacterial and fungal

919   communities and nitrogen (N) addition levels. Straight lines represent significant linear

920   regression fits. Levels of significance and correlation coefficients of fixed factors are

921   shown for each relation.

922   **Figure S4** Effect of nitrogen (N) addition and cessation of N addition on the α diversity

923   and β diversity of plants, and their diversity sensitivity and resilience. (a) α diversity,

924   (b) nonmetric multidimensional scaling (NMDS) analysis of the community

925   composition, (c) Sensitivity of α diversity and β diversity in response to continuous N

926   addition, (d) Resilience of α diversity and β diversity. Treatments: control; LN+,

927   continuous low-N addition; HN+, continuous high-N addition; LN-, cessation of low-

928   N addition; HN-, cessation of high-N addition. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

929   **Figure S5** Effect of nitrogen (N) addition and cessation of N addition on soil pH and

930   the concentrations of base cations and metal ions. Inorganic nitrogen (ION); Available

931   phosphorus (AP); Base cations (calcium, Ca^{2+}; magnesium, Mg^{2+}; potassium, K^+);

932   Toxic metal ions (Iron, Fe^{3+}; Manganese, Mn^{2+}; aluminum, Al^{3+}). Data are means $\pm$ SD

933   for five replicates. Different letters indicate significant difference among N levels at

934   $P<0.05$. Asterisks represent significant differences between continuous N addition and

935   N cessation; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

936   **Figure S6** Effect of nitrogen (N) addition and cessation of N addition on the

937   concentrations of soil total carbon (TC), total nitrogen (TN) and total phosphorus (TP).

938   Data are means $\pm$ SD for five replicates. Same letters indicate insignificant difference

939   among N levels at $P < 0.05$.

940   **Figure S7** Effect of nitrogen (N) addition and cessation of N addition on the relative

941   abundance of bacterial and fungal functional groups. (a) Relative abundance of

942   bacterial functional groups; (b) Relative abundance of fungal functional groups.