



Negative responses of terrestrial nitrogen fixation to nitrogen addition weaken across increased soil organic carbon levels

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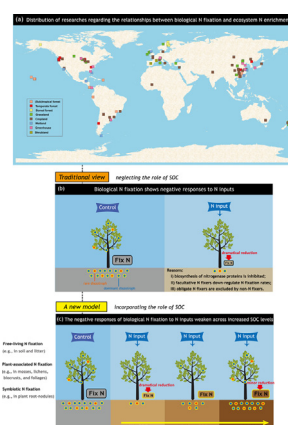
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HIGHLIGHTS

- A total of 672 observations are compiled across nine terrestrial ecosystems.
- N addition inhibits terrestrial N fixation overall.
- The negative N-addition effects on N fixation rates weaken with increased SOC levels.
- The negative N effects on diazotroph abundance and diversity weaken across SOC levels.

GRAPHICAL ABSTRACT



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ABSTRACT

The traditional view holds that biological nitrogen (N) fixation is energetically expensive and thus, facultative N fixers reduce N fixation rates while obligate N fixers are excluded by non-N fixers as soil N becomes rich. This view, however, contradicts the phenomenon that N fixation does not decline in many terrestrial ecosystems under N enrichment. To address this paradoxical phenomenon, we conducted a meta-analysis of N fixation and diazotroph (N-fixing microorganism) community structure in response to N addition across terrestrial ecosystems. N addition inhibited N fixation, but the inhibitory effect weakened across increased soil organic carbon (SOC) concentrations. The response ratios of N fixation (including free-living, plant-associated, and symbiotic types) to N addition were lower in the ecosystems with low SOC concentrations (<10 mg/g) than in those with medium or high SOC concentrations (10–20 and > 20 mg/g, respectively). The negative N-addition effects on diazotroph abundance and diversity also weakened across increased SOC levels. Among the climatic and soil factors, SOC was the most important predictor regarding the responses of N fixation and diazotroph community structure to N addition. Overall, our study reveals the role of SOC in affecting the responses of N fixation to N addition, which helps understand the relationships of biological N fixation and N enrichment as well as the mechanisms of terrestrial C and N coupling.

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1. Introduction

Biological nitrogen (N) fixation, performed by diazotrophs (N-fixing microorganisms), is an important pathway of N inputs from the atmosphere to the Earth's systems (Cleveland et al., 1999; Reed et al., 2011; Zehr and Capone, 2020). As N availability is a limiting factor of plant growth in terrestrial ecosystems (LeBauer and Treseder, 2008; Du et al., 2020), diazotrophs help boost crop yields (Herridge et al., 2008; Stokstad, 2016), increasing terrestrial net primary productivity (NPP; Dynarski and Houlton, 2018; Ramond et al., 2018), and further mitigating climate change (Levy-Varon et al., 2019). Moreover, biological N fixation affects terrestrial N cycling via accelerating ecosystem N accumulation (Vitousek et al., 2002; Reed et al., 2011). Given the important roles of biological N fixation in terrestrial ecosystems, it is necessary to understand its controlling factors.

Availability of N is an important factor that affects N fixation rates and diazotroph community structure (Reed et al., 2011). Many empirical studies have revealed that N addition reduced nitrogenase activity, N fixation rates, and diazotroph numbers (or abundance) in laboratories (Kolb and Martin, 1988; Meng et al., 2012) and the fields (Wang et al., 2018; Tang et al., 2019; Xiao et al., 2020a). These findings can be explained by the following reasons. First, exogenous inputs of combined N (e.g., NH_4^+) inhibit biosynthesis of nitrogenase proteins (Bentley, 1987). Second, fixing atmospheric N_2 is metabolically expensive (Alberty, 2005), and thus facultative N fixers usually lower N fixation rates when the availability of soil N increases (Gutschick, 1981). Third, obligate N fixers, who fix atmospheric N_2 constantly regardless of soil N status are theoretically faced with the risk of being excluded or replaced by non-N fixers when soil N is not limiting (Vitousek and Howarth, 1991; Crews, 1999; Menge and Crews, 2016). Collectively, the above empirical evidence and theoretical mechanisms suggest that N fixers have competitive advantages under N limitation but not under N enrichment (Fig. 1b).

Nevertheless, there is the paradoxical phenomenon that N fixation can remain active in many N-rich ecosystems (Reed et al., 2008; Menge and Hedin, 2009; Zheng et al., 2018; Mills et al., 2020). For example, in an N-rich tropical rainforest in Costa Rica where the soils exhibited high losses of N, the total rates of N fixation reached $\sim 15 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ (Reed et al., 2008). In an N-saturated subtropical forest in southern China, asymbiotic N fixation rates remained high ($8\text{--}11 \text{ kg N ha}^{-1} \text{ yr}^{-1}$) despite chronic N deposition (Zheng et al., 2018). Several manipulative studies even found a minor effect of N addition on N fixation in the soil, leaf litter, mosses, and plant foliage (Reed et al., 2007; Zheng et al., 2018). These paradoxical observations suggest a necessity to explore the mechanisms underlying divergent responses of biological N fixation to N enrichment.

Meta-analysis is an important method of big-data analyses that can evaluate the impacts of nutrient availability on biogeochemical processes (Xia et al., 2020). Although some previous meta-analyses have evaluated the directions and extents of N-addition impacts on terrestrial N fixation (Dynarski and Houlton, 2018; Zheng et al., 2019), the mechanisms underlying divergent responses of N fixers to N enrichments have not yet been elucidated. Compared to soil N availability, dissolved organic C (DOC) and resource stoichiometry of C and N (C:N) can better predict N fixation in terrestrial ecosystems (Cejudo and Paneque, 1988; Reed et al., 2011). Several prior litter decomposition assays showed that high availability of organic C and low availability of total N favored free-living (heterotrophic) N fixation in leaf litter (Vitousek and Hobbie, 2000; Pérez et al., 2010). The variation in soil organic C (SOC) affects the responses of N fixation to N addition. For example, a previous study found that elevated ratios of SOC and soil N could explain 30 % of the increases in N fixation rates under N addition in a subtropical forest (Zheng et al., 2018). A recent study found that N fixation rates increased with forest succession (and soil N enrichment), which was related to the increases in availability of SOC, including readily oxidizable organic C (ROC) and DOC (Zheng et al., 2020a). Given that N fixation is energetically expensive and the energy (or carbohydrate) supporting N fixation (e.g., heterotrophic N fixation) can be derived from soil organic matter (Reed et al., 2011; Zheng et al., 2020a), it is necessary

to examine whether SOC is a potential factor that affects N fixation rates and N-fixing microbial community in terrestrial ecosystems.

To explore the relationships between SOC and the responses of biological N fixation to N addition, we compiled a global dataset of N fixation rates and diazotroph community structure in response to N addition in natural ecosystems (tropical/subtropical forests, temperate forests, boreal forests, grasslands, shrublands, and wetlands), managed ecosystems (croplands), and artificial greenhouse systems (Fig. 1a). These (eco)systems were divided into three groups based on SOC concentrations: low SOC ($<10 \text{ mg/g}$), medium SOC ($10\text{--}20 \text{ mg/g}$), and high SOC ($>20 \text{ mg/g}$). Because different diazotrophs have different C-acquiring pathways, we categorized N fixation into three types: symbiotic N fixation (e.g., legume root-nodules, in which diazotrophs acquiring C from host plants exclusively), associated N fixation (e.g., mosses, lichens, plant leaves, and biocrusts, in which diazotrophs acquiring C by photosynthesis themselves or from host plants), and free-living N fixation (e.g., soil and litter, in which diazotrophs acquiring C from organic matter). In addition, because N fixers perform facultative or obligate N fixation strategy under N enrichment (Menge et al., 2009), our study assumed those N fixers (N-fixing plants and microorganisms) that reduced N fixation rates to be facultative N fixers and those that did not reduce N fixation rates to be obligate N fixers. Specifically, we hypothesized that with increases in SOC levels, (H1) the negative effects of N addition might weaken on free-living N fixation (that benefit from soil organic matter; Reed et al., 2011) but not on plant-associated or symbiotic N fixation (that rely on photosynthetic carbohydrate rather than soil organic matter; Gutschick, 1981); (H2) the negative effects of N addition might weaken on facultative N fixation but not on obligate N fixation because the latter cannot adjust N fixation rates under N enrichment (Menge et al., 2009); and (H3) the negative N-addition effects might partially weaken on diazotroph abundance, richness, and diversity given that the responses of diazotroph community structure are similar with those of N fixation (e.g., Fan et al., 2019).

2. Materials and methods

2.1. Data sources

We conducted a meta-analysis of existing studies following the PRISMA guidelines (Page et al., 2021). Specifically, we systematically searched all peer-reviewed journal articles, theses, and monographs using Web of Science and Google Scholar with the following keywords/phrases: [“nitrogen fixation” or “N fixation” or “ N_2 fixation” or “dinitrogen fixation” or “nitrogenase” or “acetylene reduction” or “*nifH*” or “diazotroph”) and (“nitrogen addition” or “N addition” or “nitrogen fertilization” or “N fertilization” or “nitrogen enrichment” or “N enrichment” or “nitrogen input” or “N input” or “carbon addition” or “C addition” or “carbon input” or “C input” or “soil C” or “labile C”]. In addition, we collected relevant literatures from other datasets of biological N fixation (Dynarski and Houlton, 2018; Ouyang et al., 2018; Zheng et al., 2019; Zheng et al., 2020b). Appropriate studies were selected based on the following criteria: (1) the information on diazotroph community structure (abundance, richness, and diversity) and N fixation rates could be extracted from figures, tables, or texts; (2) no other treatments (e.g., P addition, warming, etc.) were combined with the C or N-addition treatments (e.g., the treatment of P + C addition was excluded); (3) the information on diazotroph community structure and N fixation rates were collected from at least one of the compartments (soil, litter, biocrusts, mosses, lichens, leaves, and root-nodules); (4) N fixation rates were measured using acetylene reduction assay, ^{15}N abundance, or $^{15}\text{N}_2$ assimilation method (Zheng et al., 2019); and (5) N fixation rates and diazotroph community structure were measured during growing seasons in both fields and greenhouses. Following these criteria, a total of 672 observations were compiled from 127 literature sources (Fig. S1) published between April 1970 and December 2021 (data sources are shown in Appendix), which quantified diazotroph community structure and N fixation in tropical/subtropical forests, temperate forests, boreal forests, grasslands, croplands, shrublands, wetlands, and

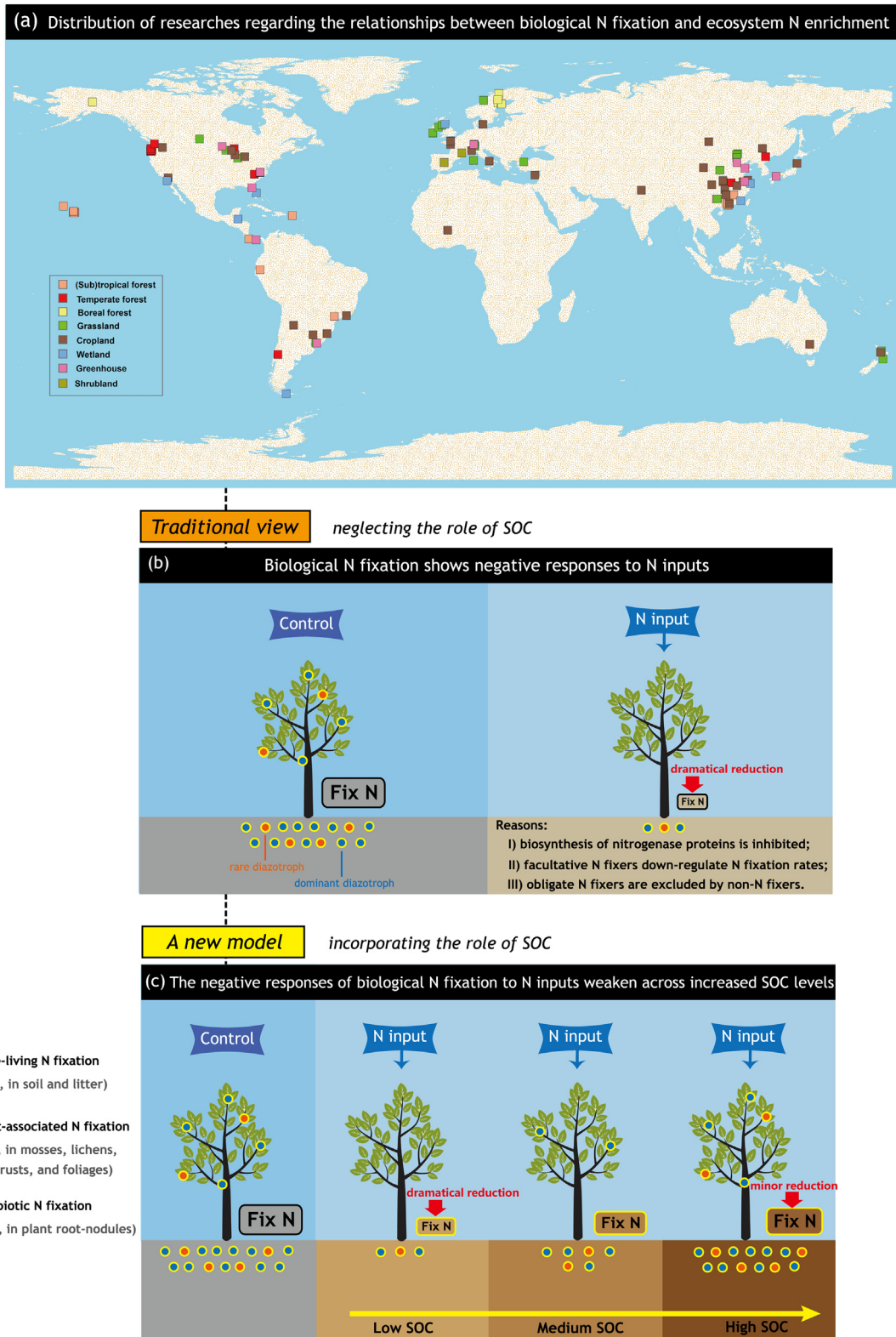


Fig. 1. Distribution of studies used in this study (a) and the traditional view (b) versus a novel model (c) regarding the relationships between biological nitrogen (N) fixation and ecosystem N enrichment. The traditional view holds that biological N fixation shows negative responses to N inputs because (I) biosynthesis of nitrogenase proteins is inhibited, (II) facultative N fixers reduce N_2 fixation rates given that fixing atmospheric N_2 is energetically costly, and (III) obligate N fixers, who fix N_2 constantly regardless of soil N richness, are excluded by non-N fixers. Our study revises this view/theory by incorporating the role of soil organic carbon (SOC). We propose a new model that the negative N-addition effects on biological N fixation weaken with increases in SOC concentrations. This model is supported by two potential mechanisms: (I) increases in SOC concentrations mean more labile organic C (carbohydrate) that supports the energy-intensive process of N fixation, and (II) increases in SOC concentrations intensify N demand and thus N fixation of ecosystems.

greenhouses (Fig. 1a). Because diazotroph community structure and N fixation are commonly affected by soil nutrients and environmental factors (Reed et al., 2011; Zheng et al., 2019), our dataset included mean annual temperature (MAT), mean annual precipitation (MAP), soil total N, total P, inorganic P, organic C (SOC), labile organic C (i.e., dissolved organic C and microbial biomass C), C:N ratio, nitrate (NO_3^-), ammonium (NH_4^+), moisture, and pH. Data were collected from original publications and their citations. Origin 9.1 (OriginLab Co., Northampton, MA, USA) digital plugin (Digitize) software was used to extract data from figures.

2.2. N fixation rates

In the collected studies, N fixation rates were measured using acetylene reduction assay [ARA; which measured the capacity of nitrogenase to reduce acetylene to ethylene (Hardy and Burns, 1968)] or ^{15}N natural abundance method [NAM; which measured the percentage of N derived from the atmosphere in N-fixing plants based on a comparison of the ^{15}N abundance between N-fixing and non-N-fixing plants (Shearer and Kohl, 1986)]. The ARA and NAM were widely used to assess N fixation rates in terrestrial ecosystems (Zheng et al., 2019).

2.3. Diazotroph abundance, richness, and diversity

In the collected literatures, diazotroph abundance was reported by the copy number of *nifH* gene which was quantified by the real-time quantitative PCR method using the PloP and PloR primer sets (Poly et al., 2001). Diazotroph richness was reported by the number of operational taxonomic units (OTUs). Diazotroph diversity was reported by the indexes of Chao1, Simpson, and Shannon. The three indexes were calculated for diazotroph community dissimilarity between samples and expressed as Bray-Curtis distances (Chao1 and Simpson) or Pairwise weighted UniFrac distances (Shannon; Berthrong et al., 2014; Wang et al., 2017).

2.4. Diazotroph community composition

According to the description of the collected studies, the relative abundance of diazotroph community at each taxonomic level was calculated based on normalized OTU tables generated by rarefying the sequence number of the samples (e.g., Xiao et al., 2020b). Phylogenetic molecular ecological networks were used to evaluate the effects of the treatments on diazotroph community composition (Zhou et al., 2010). Because the dominant diazotrophs often have a relative abundance of >3 % (Zhang et al., 2019; Xiao et al., 2020b), the diazotroph community was divided into two groups: dominant diazotrophs (relative abundance $\geq 3\%$) and rare diazotrophs (relative abundance <3 %; Fig. S2).

2.5. Statistical analyses

The effects of N and C addition on individual variables (i.e., N fixation and diazotroph community structure) were estimated by the natural logarithm transformed response ratios (RR; Zhou et al., 2020; Wen et al., 2022) as follows:

$$\text{RR} = \ln\left(\frac{\bar{X}_t}{\bar{X}_c}\right) = \ln(\bar{X}_t) - \ln(\bar{X}_c) \quad (1)$$

where \bar{X}_t and \bar{X}_c are the means of the treatments and controls, respectively. The variance (v) of RR was calculated as follows:

$$v = \frac{s_t^2}{n_t \bar{X}_t^2} + \frac{s_c^2}{n_c \bar{X}_c^2} \quad (2)$$

where n_t and n_c are the sample sizes of the variables in the treatments and controls, respectively, and s_t and s_c are the standard deviations (SDs) of the variables in the treatments and controls, respectively.

The dataset was categorized into three groups according to SOC concentrations: low SOC (<10 mg/g), medium SOC (10–20 mg/g), and high SOC (>20 mg/g), which was followed a previous published criterion of soil census (State Soil Survey Service of China (SSSSC), 1992) and made modifications. Different compartments (soil, litter, mosses, lichens, biocrusts, and root-nodules) were included in the dataset to analyze the relationships of SOC against RR of diazotroph community structure and N fixation. Except for the soil, organic C concentrations of other compartments were rarely reported by literatures and thus were not considered. Although different studies applied different levels of N addition, there was no significant relationships of N addition rates against RR of diazotroph community structure (abundance, richness, and diversity) and N fixation rates ($P > 0.05$; Fig. S3). We used MetaWin 2.1 (Sinauer Associates Inc. Sunderland, MA, U.S.) software to estimate the overall RR and 95 % confidence intervals (CI) of the target variables for the treatments. Because the effects of N and C addition on N fixation rates and diazotroph community structure varied with biomes/sites (Figs. 2, 6, and S9), we used a random-effect model to calculate the overall RR for the meta-analysis.

Meta-analytic models were selected using corrected Akaike's Information Criteria (AICc; Symonds and Moussalli, 2011). We calculated the relative importance value of each predictor [MAT, MAP, soil total N, total P, inorganic P, organic C (SOC), C:N ratio, NO_3^- , NH_4^+ , moisture, and pH] for the responses of diazotroph community structure and N fixation to N addition. The relative importance value is equal to the sum of Akaike weights (the probability that a model is most plausible) for the models in which the predictor occurs (Terrer et al., 2016). Thus, a predictor that is included in models with large Akaike weights will receive a high importance value. These values can be regarded as the overall support for each variable across all models. If the summed Akaike weight of a predictor is larger than 0.8, the predictor is considered to be important (Terrer et al., 2016; Zhou et al., 2020). All possible combinations of the predictors in a mixed-effect meta-regression model were analyzed with the 'glmulti' package in R software (v.4.1.0). Compared to the data of soil and climatic variables, the data of diazotroph community structure is few. Thus, diazotroph community structure was not regarded as a predictor in the meta-analytic models. The relationships between diazotroph community structure and N fixation were analyzed using linear regression models. An omnibus test (Q_M) was used to examine the RR of N fixation rates and diazotroph abundance to N or C addition, and significant responses ($P < 0.05$) were recognized if the 95 % CI did not overlap with zero. A one-way analysis of variance (ANOVA) followed by Tukey's HSD test was used to examine the differences in N fixation rates and RR of diazotroph abundance, richness, diversity, and N fixation among different SOC levels, and significant differences were recognized at $P < 0.05$. Box plots were used to explore the patterns of RR of diazotroph community structure and N fixation under different SOC levels. Linear or non-linear (logarithmic and polynomial) mixed-effects models, which have a higher degree of fitting, were used to examine the relationships of SOC concentrations against RR of diazotroph community structure and N fixation to N addition. In these models, SOC concentrations were set as fixed effects and the biomes/sites were set as random effects. Results of diazotroph community structure and N fixation in response to N addition were shown by (1) the patterns of RR with the geometric means and inter-quartile ranges and (2) the weighted mean RR and 95 % CI.

3. Results and discussion

3.1. Responses of different N fixation types to N addition varying with SOC levels

Consistent with recent findings (Dynarski and Houlton, 2018; Zheng et al., 2020b), we found that N addition inhibited N fixation rates in many terrestrial ecosystems (tropical/subtropical forests, temperate forests, boreal forests, grasslands, croplands, shrublands, and greenhouse systems) and compartments (soil, leaf litter, mosses, lichens, biocrusts, and root-nodules) with an overall inhibitory effect of 41.66 % (Fig. 2a-b). Without consideration of N fixation types and strategies, both the geometric mean and weighted mean RR of N fixation to N addition were lower in the

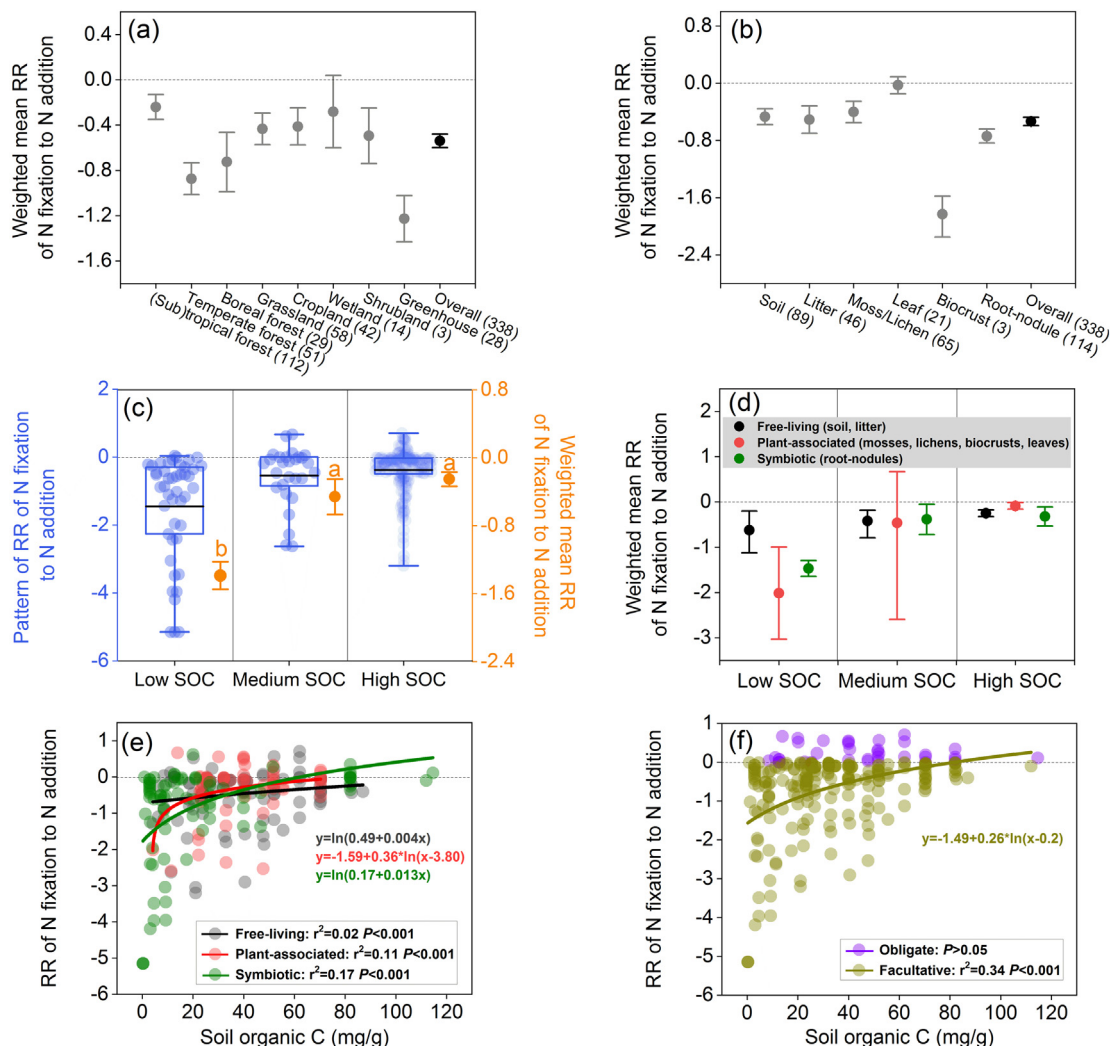


Fig. 2. Effects of nitrogen (N) addition on terrestrial N fixation. (a–b) Weighted mean response ratios (RR) of N fixation to N addition in different terrestrial ecosystems and compartments. Each solid point and error bar represent the weighted mean RR and 95 % confidence interval (CI), respectively. The numbers in brackets represent sample sizes. (c) Patterns of RR of N fixation to N addition across increased soil organic carbon (SOC) levels [low SOC (<10 mg/g), medium SOC (10–20 mg/g), and high SOC (>20 mg/g)] with the weighted mean RR nearby. The ends of each box represent the first and third quartiles, and the black line inside represents the geometric mean of data. The caps of the bottom and top whiskers represent the minimum and maximum values, respectively. Different lowercase letters indicate significant differences ($P < 0.05$) among different SOC levels. (d) Weighted mean RR of N fixation (free-living, plant-associated, and symbiotic types) to N addition across increased SOC levels. (e–f) Regression models of SOC concentrations against RR of N fixation to N addition for different N fixation types (free-living, plant-associated, and symbiotic N fixation) and strategies (obligate and facultative N fixation).

ecosystems with low SOC concentrations ($RR_g = -1.45$ and $RR_w = -1.39$) than in those with medium SOC concentrations ($RR_g = -0.54$ and $RR_w = -0.46$) or high SOC concentrations ($RR_g = -0.38$ and $RR_w = -0.25$; $P < 0.001$; Fig. 2c). This result indicates that the inhibitory effects of N addition on terrestrial N fixation weakened across increased SOC levels.

We assumed that the negative effects of N addition might weaken on free-living N fixation (in soil and litter) but not on plant-associated N fixation (in mosses, lichens, biocrusts, and leaves) or symbiotic N fixation (in root-nodules) with increases in SOC levels (H1). However, our results showed increases in the RR of N fixation to N addition ($RR_{N\text{-fixation}}$) across elevated SOC levels for all these N fixation types (Fig. 2d). The mean increments of $RR_{N\text{-fixation}}$ (from low to high SOC) for free-living, plant-associated, and symbiotic types were 25.6–52.5 %, 57.4–90.5 %, and 58.6–64.2 %, respectively. The regression models showed that SOC accounted for the variation in $RR_{N\text{-fixation}}$ by 2–17 % ($P < 0.001$; Fig. 2e). Without consideration of N fixation types, SOC could explain the variation in $RR_{N\text{-fixation}}$ by 33 % in total ($P < 0.001$; Fig. S10). These results together extend prior findings that SOC supports free-living (heterotrophic) N fixation in the soil and

leaf litter (Vitousek and Hobbie, 2000; Pérez et al., 2010), and indicate that the negative responses of multiple N fixation types (free-living, plant-associated, and symbiotic N fixation) to N addition become minor when SOC concentrations increase.

Two mechanisms can help understand the phenomena above. First, at the soil and litter layers, the increases in SOC concentrations indicate increases in energy supply, which support heterotrophic N fixation. Commonly, N fixation process is energetically costly (e.g., >100 g of glucose are consumed per gram of N_2 fixed; Gutschick, 1981; Reed et al., 2011). Addition of N stimulates plant photosynthesis (Liang et al., 2020) and increases inputs of carbohydrates (e.g., in the form of root exudates; Li et al., 2021) to soils, which may fuel free-living N fixation in the soil and litter layers. Increased organic C in soils (e.g., via litterfall inputs; Yue et al., 2016) can also enhance releases of labile organic C, such as readily oxidizable organic C and DOC, which are important sources of energy to diazotrophs in the soil and leaf litter (Zheng et al., 2020a; Wang et al., 2021a, 2021b). Our regression models showed that increases in labile organic C of soils enhanced the RR of N fixation to N addition by 31 % ($P < 0.001$; Fig. S8). Hence, given the energy-intensive process of N

fixation, increases in energy supply to N fixers may alleviate their competitive disadvantages in N-rich environments (Crews, 1999; Menge and Crews, 2016).

Second, from the perspective of ecosystem, plants and soil microbes have their own balances of C and N stoichiometry (Redfield, 1958; Scott et al., 2012). On one hand, N addition increases plant biomass and below-ground C pools in terrestrial ecosystems (Yue et al., 2016; Sun et al., 2020), which enhance N demands of plants and soil microbes, and further stimulate N fixation in epiphytic mosses, lichens, plant foliage, and legume root-nodules (Hedin et al., 2009; Reed et al., 2011; van de Voorde et al., 2014). On the other hand, N addition also increases SOC concentrations (in forests; Lu et al., 2021) and microbial biomass C (in croplands and wetlands; Yue et al., 2016), which means higher biomass of bacteria (including N-fixing microbes) in soils (Wang et al., 2021a, 2021b). Given the coupling relationships of ecosystem C and N, elevation in C concentrations and C:N stoichiometry of plants and soils stimulate N fixation, as has been observed in mineral soils, leaf litter, mosses, and plant foliage (Zheng et al., 2018; Zheng et al., 2020a). Our results showed that increased SOC concentrations could enhance the RR of N fixation (free-living, plant-associated, and symbiotic types) to N addition by 15 % ($P < 0.001$; Fig. S5c). Although C and N stoichiometry principle can help understand why N fixation rates were less inhibited by N addition when SOC was rich, more studies are needed to explore the relationships between SOC and plant-associated /symbiotic diazotrophs who acquire carbohydrate from host plants rather than soil organic matter.

3.2. Responses of different N fixation strategies to N addition varying with SOC levels

We hypothesized that the negative effects of N addition might weaken on facultative N fixation but not on obligate N fixation (H2), which is supported by our results. We observed increases only in the RR of facultative N fixation to N addition with SOC concentrations ($r^2 = 0.34$, $P < 0.001$;

logarithmic regression models) but not in the RR of obligate N fixation ($P > 0.05$; Fig. 2f). The main reason for this result may be that obligate N fixers are less affected by soil N status compared to facultative N fixers (Menge et al., 2009). N inputs usually increase the availability of soil N and alleviate N limitation in terrestrial ecosystems (Penuelas et al., 2020). Excessive N inputs result in soil N richness and N saturation (Aber et al., 1998). Different with obligate N fixers, who fix atmospheric N_2 constantly regardless of soil N status, facultative N fixers can adjust N fixation rates according to the availability of soil N (Menge et al., 2009). Hence, increases in SOC concentrations enhance soil C:N ratios and intensify N limitation, which stimulates the process of facultative N fixation.

Currently, facultative and obligate N fixation strategies have been found and validated in N-fixing plants (e.g., legumes; Menge et al., 2009; Menge and Crews, 2016). Due to the lack of empirical evidence, it is unclear whether these strategies can be applied to free-living N-fixing microorganisms. We assumed the N-fixing plants or microorganisms that reduced N fixation rates after N addition (RR < 0; Fig. 2f) to be potential facultative N fixers. Our results indicate that facultative N fixers can maintain high rates of N fixation following N addition when SOC is rich (Fig. 2f), which is supported by some previous findings that soil available C contents and C:N stoichiometry had positive relationships with N fixation rates (Zheng et al., 2018; Zheng et al., 2020a). Thus, incorporating N fixation strategies and SOC status into N-cycling researches can help understand why many mature forest ecosystems actively fix atmospheric N_2 regardless of soil N richness and atmospheric N deposition (Reed et al., 2007; Reed et al., 2008; Menge and Hedin, 2009; Zheng et al., 2018).

3.3. Responses of diazotroph community structure to N addition varying with SOC levels

Due to the limitation of data (particularly diazotroph richness and diversity; Fig. 3), the diazotroph community was not categorized by N

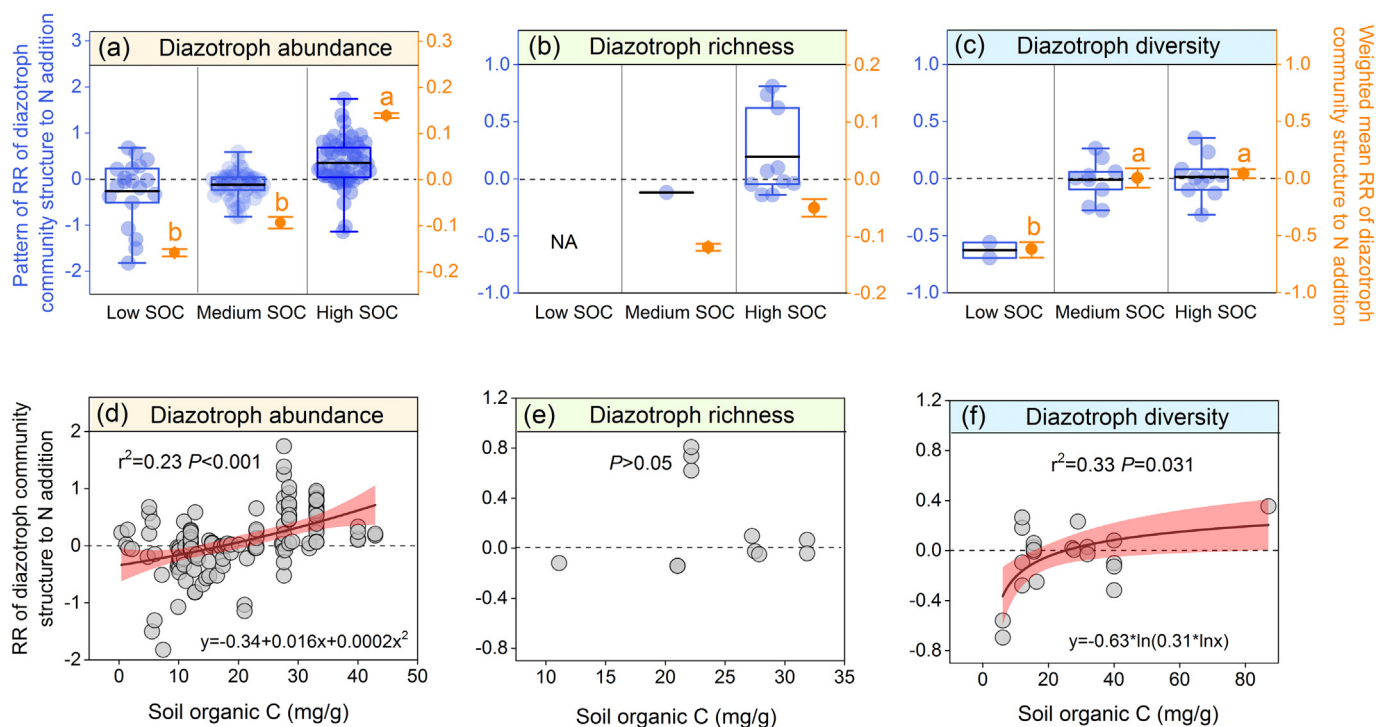


Fig. 3. Effects of nitrogen (N) addition on diazotroph community structure in terrestrial ecosystems. (a – c) Patterns of response ratios (RR) of diazotroph abundance, richness, and diversity to N addition across increased soil organic carbon (SOC) levels [low SOC (<10 mg/g), medium SOC (10–20 mg/g), and high SOC (>20 mg/g)] with the weighted mean RR nearby. The ends of each box represent the first and third quartiles, and the black line inside represents the geometric mean of data. The caps of the bottom and top whiskers represent the minimum and maximum values, respectively. Different lowercase letters indicate significant differences ($P < 0.05$) among different SOC levels. NA: not available. (d-f) Regression models of SOC concentrations against RR of diazotroph abundance, richness, and diversity to N addition. The red shadow area represents 95 % confidence interval.

fixation types. Because the RR of N fixation to N addition increased across elevated SOC levels for all the N fixation types (Fig. 2c-e), we hypothesized that diazotroph community structure might have similar responses with N fixation (H3). This hypothesis is supported by our results. With the increases in SOC levels, the negative N-addition effects on diazotroph abundance, richness, and diversity were alleviated or became positive (Fig. 3a-c), indicating that diazotrophs prefer the environments with rich organic C. Our result is consistent with a recent finding of meta-analysis that biochar addition increased the abundance of soil N-fixing microorganisms (Xiao et al., 2019). Under N addition treatments, the numbers of detectable diazotroph genera were higher in the ecosystems with high SOC concentrations (22) than in those with low SOC (16) or medium SOC concentrations (16; Fig. 4a-c). The extents of the negative N-addition effects on diazotroph genera were lower in the ecosystems with medium and high SOC concentrations (46.4–52.6 %) than in those with low SOC concentrations (69.1 %; Fig. 4a-c). These results indicate that the responses of diazotroph community to N addition vary with SOC levels. Our findings are consistent with those of empirical studies, which observed a minor effect of N addition on diazotroph abundance in grasslands (Xiao et al.,

2020a; Xiao et al., 2020b) and wetlands (Huang et al., 2016) with abundant SOC (>20 mg/g). In addition, we found that SOC could explain 23–33 % of the variation in RR of diazotroph community structure to N addition ($P \leq 0.031$; Fig. 3d-f).

We further found that the variation in the responses of N fixation and diazotroph community to N addition were mainly contributed by dominant diazotrophs, based on two pieces of evidence. First, the dominant diazotroph abundance changes distinctly after N addition (Huang et al., 2016; Xiao et al., 2020a). Although the RR and the decline ratios of dominant and rare diazotroph abundance were similar (Fig. S4), the N-induced reduction in the dominant diazotroph abundance was alleviated across increased SOC levels (from 73.7 % to 47.0 %; Fig. 4d-i). The N-induced reduction in the dominant diazotroph numbers also diminished across increased SOC levels (from 72.7 % to 36.4 %), which contrasted with those of the rare diazotrophs (from 60.0 % to 57.1 %). These results suggest that the negative N-addition effects weakened on dominant diazotrophs more than on rare diazotrophs across increased SOC levels (Fig. 4j-l). Second, N fixation rates are mainly regulated by the dominant diazotroph community. Consistent with previous findings (Warshan et al., 2016; Fan et al., 2019; Liu et al.,

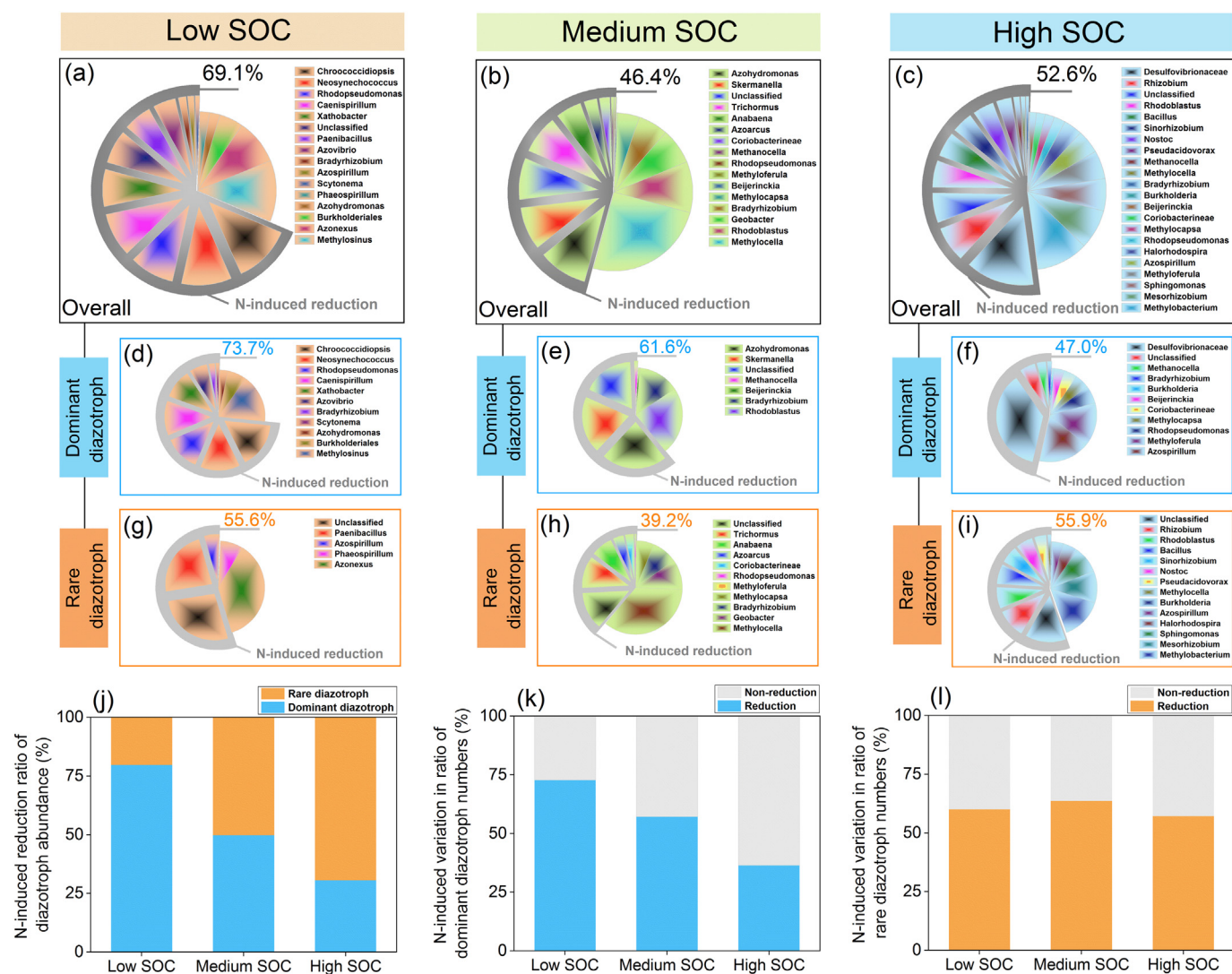


Fig. 4. Effects of nitrogen (N) addition on diazotroph community at the genera level. (a–c) Patterns of response ratios (RR) of the total diazotroph community to N addition across increased soil organic carbon (SOC) levels [low SOC (<10 mg/g), medium SOC (10–20 mg/g), and high SOC (>20 mg/g)]. Each portion with gray fan-shaped background represents the N-induced decline in RR of the total diazotroph community to N addition across increased SOC levels. (d–f) Patterns of RR of the dominant diazotroph community to N addition. Each portion with gray fan-shaped background represents the N-induced decline in RR of the dominant diazotroph community to N addition. (g–i) Patterns of RR of the rare diazotroph community to N addition across increased SOC levels. Each portion with gray fan-shaped background represents the N-induced decline in RR of the rare diazotroph community to N addition. (j) The N-induced reduction ratios of the relative abundance between dominant versus rare diazotrophs across increased SOC levels. (k–l) The N-induced variation in ratios of the relative numbers between reduced and non-reduced diazotroph community.

2019; Liu et al., 2019), we found that the RR of total diazotroph abundance to N addition had positive relationships with the RR of N fixation rates ($r^2 = 0.38$; $P < 0.001$; Fig. 5a-c). The variation in the total abundance of diazotroph community was contributed by dominant diazotrophs (57–58 %, $P \leq 0.005$; Fig. 5e, h) more than by rare diazotrophs (17 %, $P = 0.013$; Fig. 5i).

The following two reasons explain why the variation in SOC affected the responses of diazotroph community structure to N addition. First, the growth of diazotrophs is limited by energy under N enrichment (see the Section 3.1). Apart from N fixation, the reproductive and metabolic processes of diazotrophs also require energy, such that increased supply of available organic C (carbohydrate) enhances abundance of diazotroph community (Ducey et al., 2013). Second, increased supply of carbohydrate stimulates the growth and activities of soil bacteria and fungi (Zhang et al., 2018). These microbes increase the availability of soil nutrients (e.g., P and molybdenum) by producing extracellular depolymerizing enzymes and stimulating decomposition of soil organic matter, which further enhance diazotroph activities (Liu et al., 2019). Nevertheless, the above mechanisms only apply to soil diazotroph community, and further researches are needed to explore how diazotroph community of other compartments is affected by the variation in SOC.

3.4. Other factors affecting the responses of N fixation and diazotroph community structure to N addition

Although SOC affected the responses of N fixation under N enrichment, we would like to explore whether other factors (soil nutrients and environmental factors) might also affect diazotroph community structure and N fixation. Our results showed that soil NH_4^+ or NO_3^- concentrations did not affect the responses of diazotroph community structure or N fixation to N addition (Fig. S5e-f and S6e-f). Increases in soil total N concentrations aggravated the negative effects of N addition on diazotroph community structure by 9 % but alleviated those on N fixation by 2 % (Fig. S5a and S6a). These findings indicate that only a few N fixers tolerate high N environments (Compton et al., 2004) and that N fixation is less sensitive to N inputs in already N-rich environments (Zheng et al., 2019). Commonly, P is required for ATP generation and cellular structure of diazotrophs (Reed et al., 2011). Plants and soil microbes mainly acquire P from soils (Vitousek et al., 2010). However, soil total P and inorganic P had no or a minor effect on the responses of N fixation and diazotroph community structure to N addition (Fig. S5b and S6b; Zheng et al., 2020b), indicating that N addition did not induce soil P limitation on N fixers. A previous meta-analysis showed that elevated P supply did not increase rates of

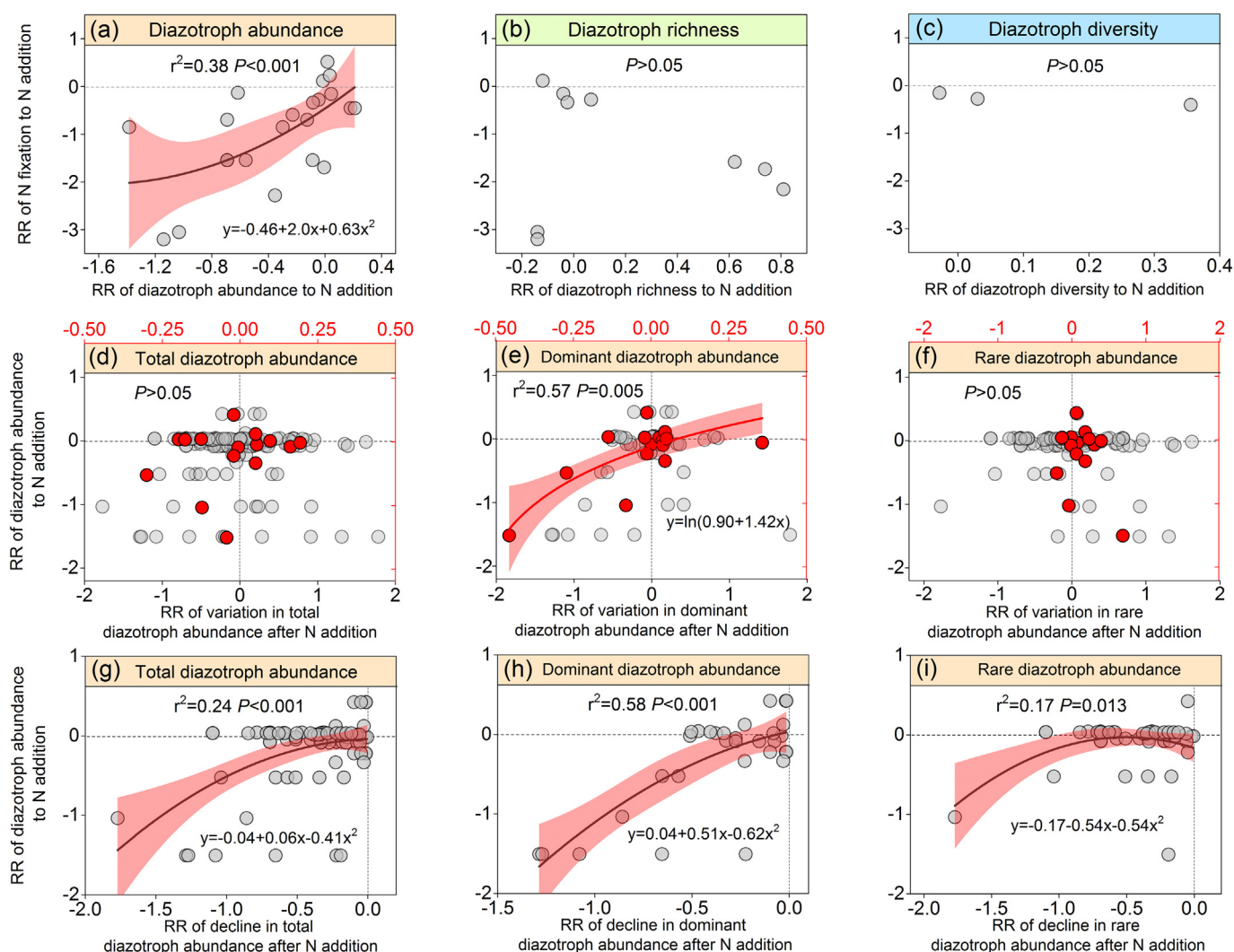


Fig. 5. Regression models of response ratios (RR) of nitrogen (N) fixation against RR of diazotroph community structure and composition to N addition. (a–c) Regression models of RR of diazotroph abundance, richness, and diversity to N addition against RR of N fixation. (d–f) Regression models of RR of variation in the relative abundance of the total, dominant, and rare diazotrophs against RR of diazotroph abundance overall. Each light-gray point represents the individual value for different genera, while each red point represents the mean for different genera within the same study sites. (g–i) Regression models of RR of decline in the relative abundance of the total, dominant, and rare diazotrophs against RR of diazotroph abundance overall. The red shadow area represents 95% confidence interval.

terrestrial N fixation (Dyanski and Houlton, 2018) because high P supply sometimes leads to limitation of other nutrients (e.g., potassium) on N fixers (Rousk et al., 2017).

Increases in soil moisture and pH alleviated the negative responses of diazotroph community structure and N fixation to N addition by 4–5 % (Fig. S5h-i and S6h-i). This result is expected because diazotrophs prefer anaerobic and slightly alkaline environments (Reed et al., 2011). N addition inhibits diazotroph activity given that excessive N accelerates soil water consumption by plants (Lu et al., 2018; Yan et al., 2021) and induces soil acidification (Lu et al., 2014). Elevated temperature and precipitation slightly alleviated the negative responses of N fixation to N addition by 1–5 % (Fig. S7). This is because warming and increased precipitation enhance nutrient supply via decomposition of leaf litter and soil organic matter. High nutrient availability of ecosystems lowers the sensitivity of N fixers to exogenous nutrient (e.g., N) inputs (Zheng et al., 2019).

Although the soil and climatic factors could partially predict the responses of N fixation and diazotroph community structure to N addition, our meta-regression models showed that SOC was the most important predictor among these factors (with a summed Akaike weight of 0.86; Fig. 6a). We compiled an additional meta-dataset to demonstrate that inputs of exogenous organic C (biochar and glucose) did enhance both N fixation rates and diazotroph abundance in multiple ecosystems ($P < 0.001$; Fig. 6b-c). Specifically, addition of exogenous organic C stimulated N fixation in croplands (+38.4 %), deserts (+77.1 %), greenhouses (+21.7 %), and wetlands (+835 %), with an overall positive effect of 70.4 %. Addition of exogenous organic C also increased diazotroph abundance in croplands (+48.8 %) and greenhouses (+45.2 %), with an overall positive effect of 39.3 %. However, the reasons for different extents of response ratios among these ecosystems are currently unclear and required further research.

3.5. Limitations and implications

There are several limitations of our study. First, many field studies measured N fixation rates for a short period (e.g., several hours or days), such

that some environmental variables (MAT and MAP) measured at a relatively long-time scale (e.g., one year) may not well predict the variation in N fixation. Second, although we revealed the impacts of SOC on the responses of biological N fixation to N addition, SOC only explained 2–34 % of the variation in the N fixation responses. Other factors (e.g., fertilization regimes, fertilizer types and forms, micronutrients, background N deposition rates, CO₂ concentrations, and light intensity) that may affect N fixation (Hungate et al., 2004; Rousk et al., 2017; Taylor and Menge, 2018; Zheng et al., 2019) were not evaluated due to data limitation. Third, N addition reduces N fixation rates in terrestrial ecosystems (except for wetlands; Fig. 1), whereas it can usually increase soil C storage (e.g., in forests; Lu et al., 2021). Based on our findings, the negative N-addition effects on N fixation may be minor if the increment of SOC is large. However, because very few N-addition studies examined the responses of N fixation and SOC pools simultaneously, we cannot evaluate the relationships between N fixation and SOC in different ecosystems. Fourth, our study evaluated the impacts from the variation in SOC concentrations. It is not clear whether other forms of soil C (e.g., total C and organic matter) and organic C of plant tissues may affect the responses of N fixation to N addition. Thus, relevant studies are required to explore the relationships between different C forms (of plants and soils) and N fixation in the future.

Our findings have important implications for ecological theories, biogeochemical modeling, and global change forecast. First, our findings improve the understanding of the relationships between terrestrial N fixation and N enrichment. Our study indicates that terrestrial ecosystems sustain high rates of N fixation under N enrichment if SOC is rich, which help improve the N saturation theory (Aber et al., 1998). Second, as biological N fixation is an important ecological process that affects terrestrial C and N cycling (Meyerholt et al., 2016; Peng et al., 2019; Davies-Barnard and Friedlingstein, 2020), the relationships between SOC and N fixation revealed by our study can facilitate the modeling and prediction of terrestrial C and N dynamics. Third, because availability of N limits plant and microbial growth in many terrestrial ecosystems (LeBauer and Treseder, 2008;

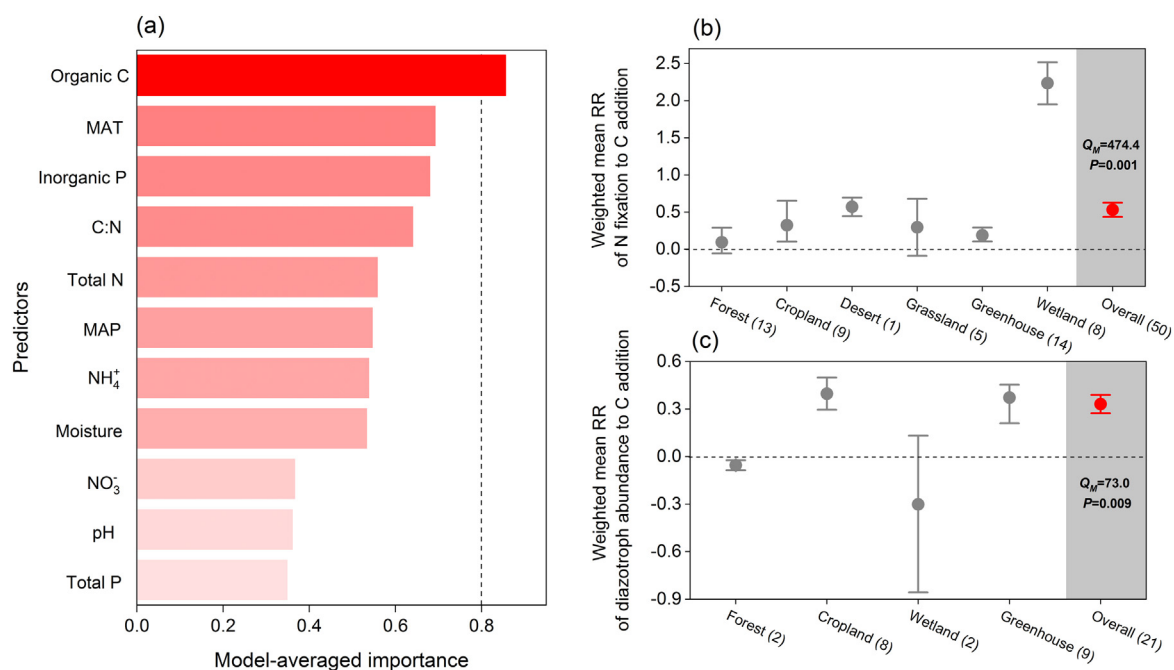


Fig. 6. The impacts of organic carbon (C) on diazotroph community structure and nitrogen (N) fixation. (a) Model-averaged importance of predictors (soil and climatic factors) for the response ratios (RR) of diazotroph community structure and N fixation to N addition. The importance value is the sum of Akaike weights derived from model selection using corrected Akaike's Information Criteria (AICc). A 0.8 cutoff is set to differentiate between essential and nonessential predictors. (b-c) Effects of exogenous organic carbon (C) addition on N fixation rates and diazotroph abundance in different (eco)systems. Each solid point and error bar represent the weighted mean RR and 95 % confidence interval, respectively. The numbers in brackets represent sample sizes. 'C:N', 'Total N', 'NO₃⁻', 'NH₄⁺', 'Total P', and 'Inorganic P' represent soil carbon and nitrogen ratio, total nitrogen, nitrate, ammonium, total phosphorus, and inorganic phosphorus, respectively. 'MAT' and 'MAP' represent mean annual temperature and mean annual precipitation, respectively.

Du et al., 2020), increased N inputs (via biological N fixation and N deposition) enhance terrestrial NPP and soil C pools (Dyanski and Houlton, 2018; Kou-Giesbrecht and Menge, 2019; Levy-Varon et al., 2019; Wang et al., 2021a, 2021b). Our study suggests that increases in SOC concentrations have a positive feedback on terrestrial N input (N fixation). This C and N coupling relationship highlights the role of N fixers in terrestrial C and N cycling and provides a new insight into the mechanisms of terrestrial C and N sequestrations in the context of global change.

3.6. Conclusions

In summary, our study can help understand the paradoxical phenomenon of high N fixation rates in N-rich terrestrial ecosystems as well as the minor responses of biological N fixation to N addition/deposition. There are several important findings. First, the negative responses of multiple N fixation types (free-living, plant-associated, and symbiotic N fixation) to N addition weaken across increased SOC levels. Second, the negative effects of N addition weaken on facultative N fixation but not on obligate N fixation, which is related to the difference in N fixation strategy (facultative N fixers can adjust N fixation rates according to soil N status while obligate N fixers cannot). Third, similar with the responses of N fixation, the negative responses of diazotroph community structure (abundance and diversity) to N addition also weaken across increased SOC levels. Fourth, among the soil and climatic factors, SOC is the most important predictor regarding the responses of N fixation and diazotroph community structure to N addition. Overall, these findings reveal the relationships of SOC and terrestrial N fixation under N enrichment (Fig. 1c). Our findings also indicate that biological N fixation in the ecosystems with rich SOC can remain active while biological N fixation in those ecosystems with poor SOC may decline dramatically under N deposition scenarios. Therefore, we suggest to incorporate SOC into terrestrial N-cycling models for accurate understanding, modeling, and prediction of biological N fixation in the future.

CRedit authorship contribution statement

M.Z and J.M. conceived the study. M.Z. synthesized data and performed the meta-analysis. M.Z., M.X., D.L., Q.D., and J.M. drafted the manuscript.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of competing interest

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2023.162965>.

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