



Temporal dynamics of the diazotrophic community during corpse decomposition

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Abstract

Corpse decomposition affects soil organisms through the formation of “cadaver decomposition islands.” Soil diazotrophic microbes possess essential ecological functions on nitrogen input and nutrient cycling in the terrestrial ecosystem. However, our knowledge about how soil diazotrophic communities respond to corpse decomposition is lacking. In this study, we focused on the succession patterns and biological interaction of nitrogen-fixing microorganisms during animal (*Ochotona curzoniae*) corpse decomposition in terrestrial ecosystems by targeting *nifH* gene with high-throughput sequencing. Our results revealed that corpse decomposition of pikas reduced the α diversity and significantly impacted the β diversity of diazotrophic community across different decomposition stages. The divergent succession of diazotrophic community occurred under corpse pressure. Furthermore, the relative importance of stochasticity to the community assembly was improved by corpse decomposition, while the importance decreased over decomposition time. Cadaver decay also simplified the diazotrophic networks and weakened the biological interactions among diazotrophic populations. Notably, $\text{NH}_4\text{-N}$ was the most important factor affecting diazotrophic community, followed by time and total carbon. This work emphasized that corpse decomposition perhaps influences the process of biological nitrogen fixation by altering soil diazotrophic communities, which is of great significance for understanding the terrestrial ecosystems’ nitrogen cycle functions.

Key points

- Corpse decomposition reduced the α diversity of diazotrophic community.
- Corpse decomposition improved the stochasticity of diazotrophic community assembly.
- Corpse decomposition weakened the interactions among diazotrophic populations.

Keywords Corpse decomposition · Diazotrophs · Community assembly · Network analysis · Terrestrial ecosystem

Introduction

Corpse decomposition is a complicated and dynamic process accompanied by multiple chemical and biochemical reactions, and it is prevalent in aquatic and terrestrial environments (Lauber et al. 2014; Mcalpine 2016). Microorganism plays an indispensable role in this natural process as

decomposers in ecosystems. In terrestrial ecosystems, the decomposition process of animal carcasses with the participation of microorganisms is divided into six steps: fresh, bloated, active decay, advanced decay, dry, and remains (Zeariya et al. 2015). In turn, corpse decomposition affects the microbial community in soil adjacent to the carcass (i.e., gravesoil) by eliciting changes in soil substrate conditions, and this phenomenon can be explained well by the “cadaver decomposition island” (CDI) (Carter et al. 2007). On the one hand, the water and mineral ions in CDIs will change the moisture and salinity of surrounding soil, and the effects of these two factors on microorganisms have been reported previously (Dissanayake et al. 2022; Vallejos et al. 2022). On the other hand, unlike plant litters providing major carbon source for soil (Schneider et al. 2012), animal carcasses are rich in nutrients such as protein and lipids; they can not only provide carbon source for soil but also undoubtedly

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input abundant nitrogen into soil habitats, allowing for the obligatory selection of microorganisms capable of degrading carbon and nitrogen-related organic matter, which influences the microbial profiles in the soil to a large extent (Metcalf et al. 2016). Numerous studies have reported that carbon and nitrogen input could change the structure and diversity of soil microbial communities (Jia et al. 2022; Rong et al. 2022). CDI is regarded as an intense and ephemeral nutrient pulse in soil, and its contributions to underground organisms cannot be overlooked (Olakanye et al. 2014). Particularly, current knowledge about the influences of corpse decomposition on microbial communities with specific ecosystem functions (such as biological nitrogen fixation) still requires further exploration.

Biological nitrogen fixation (BNF) is the process of conversion of atmospheric N_2 into ammonia driven by diazotrophs containing nitrogenase enzyme complexes (Kumar et al. 2017). The molybdenum (Mo) nitrogenase enzyme composed of MoFe protein and Fe protein is one of the most common nitrogenase systems in diazotrophic microbes (Burén et al. 2020; Hu and Ribbe 2016). The amino acid sequence of Fe protein encoded by *nifH* gene has high homology among different diazotrophic microorganisms, and the phylogenetic trees constructed by *nifH* gene and 16S rRNA gene sequences represent consistent evolutionary characterization feature (Kuypers et al. 2018; Zehr et al. 2003). Therefore, *nifH* is often used as a molecular biomarker to detect diazotrophic bacteria. Diazotrophic microbes are generally free-living or symbiotically associated with plants, among which free-living diazotrophs can independently fix nitrogen (Delwiche and Wijler 1956). The nitrogen fixation of free-living diazotrophs accounts for one-third of the total biological nitrogen fixation in the world and is considered an important way to alleviate soil nitrogen limitation (Dixon and Kahn 2004; Tripathi et al. 2018). Over the past decades, the scientific community has reported the effects of environmental factors on diazotrophic communities in soil, such as pH (Fan et al. 2018), C:N ratio (Wang et al. 2017a), soil moisture (Rousk et al. 2018), nitrogen availability (Zheng et al. 2017), and phosphorus availability (Xiao et al. 2020). Particularly, environmental changes caused by nutrition addition are capable of changing the abundance of diazotrophs in soil (Silveira et al. 2021). However, the information on the succession and interaction patterns of diazotrophs during the corpse decomposition of animals (a natural nutrient pulse) remains scarce.

Ecological succession is the orderly and predictable development of biotic community over time in nature (Fierer et al. 2010). Previous research has reported that the soil bacterial and fungal communities usually showed a convergent or divergent succession direction (Xu et al. 2020). The succession pattern of microbial community is different in various types of biotopes, such as deglaciated areas (Fierer et al.

2010), river biofilms (Lyautey et al. 2005), and arid deserts (Xu et al. 2020), which can be attributed to diverse temporal and spatial scales, ecosystem features, and their perturbation types (Zhou et al. 2014). Although extensive explorations of microbial community succession were conducted previously, how the diazotrophic communities in soil succeed during carcass degradation remains to be discussed. Bacterial community succession is closely related to deterministic (including abiotic environmental factors and biotic interactions) and stochastic (such as birth, death, immigration, and speciation) processes (He et al. 2021; Qin et al. 2022). Variations in environmental factors across time can modulate the relative contribution of determinism and stochasticity (Tripathi et al. 2018). Evidence indicated that severe wildfires cause carbon and nitrogen losses in soil, thus having an impact on terrestrial diazotrophic community assembly (Ferrenberg et al. 2013; Nelson et al. 2022). Analogously, the nutrient infiltration of corpse decomposition also changes soil physicochemical properties, affecting the bacterial community assembly. However, rare evidence uncovers the variations in diazotrophic community assembly during carcass degradation, which can be considered an important aspect of understanding soil bacterial community succession.

In addition to environmental factors, interactions between microbial populations regulate community structure and function (Romdhane et al. 2022). Co-occurrence network analysis is an important tool for revealing the complexity of species interactions, providing researchers with potential information on how diazotrophs interact with each other, such as cooperation, competition, and predation (Barberán et al. 2012; Eiler et al. 2012; Li et al. 2021). Generally, different microbial populations (nodes) are positively or negatively linked (edges) together, suggesting cooperative or competitive interactions, respectively (Wang et al. 2022). The complexity of network can be measured by other network topological parameters, such as higher average clustering coefficient and density as well as lower average path length represent a more complex network (Chen et al. 2023; Wu et al. 2016). A previous study showed the complexity of the free nitrogen-fixing bacteria network decreases due to soil organic carbon loss during desert wetlands degradation (Wang et al. 2022), implying that the complexity of diazotrophic networks is susceptible to environmental changes. Hence, an investigation to explore the occurrence pattern of diazotrophs during corpse decomposition (nutrient input) is essential for understanding the nitrogen cycle function of terrestrial ecosystems.

Plateau pika (*Ochotona curzoniae*) is the native keystone species in the Qinghai-Tibet Plateau, which has a quite high population density being the prey of many large wild animals (Lai and Smith 2003; P.L.S 1876). For the important ecosystem functions (such as promoting water infiltration into soil, regulating soil structure, and increasing plant diversity)

and higher mortality rate (Nie et al. 2022; Qu et al. 2017; Smith and Foggin 1999), the small lagomorph was selected as the corpse decomposition model. Subsequently, 60 soil samples (30 gravesoil, 30 control) were collected for high-throughput sequencing of *nifH* functional genes to reveal the diazotrophs' temporal dynamics during corpse decomposition. The following hypotheses were mainly discussed:

1. Corpse decomposition will affect diazotrophic community assembly because the formation of CDIs causes major shifts in various soil physiochemical conditions (Lewis 2011), which have been proven direct and indirect effects on the microbial community assembly (Tsiknia et al. 2015; Wang et al. 2017b).
2. Corpse decomposition will reduce the co-occurrence of diazotrophs. Corpse decomposition provides bioavailable nutrients, such as carbon and nitrogen (Metcalf et al. 2016), which weakens the competition among diazotrophic populations and then reduces their co-occurrence and the complexity of network (Yang et al. 2022b).

Materials and methods

Soil sampling and physicochemical properties determination

Our experiment lasted for 3 months, from June to September 2017. The sampling sites were located in the Qinghai-Tibet Plateau region (37° 31' N, 101° 19' E) in Haibei Tibetan Autonomous Prefecture, Qinghai Province, with an approximate average altitude of 3100 m. The main soil type in this area is alpine meadow soil. The topsoil (0–10 cm) was collected from each sampling site and subsequently homogenized gently in the laboratory. Then the soil was screened with a 100-mesh sieve to remove large particle residues such as roots and stones. After that, the treated soil was transferred to 60 clean and contamination-free flowerpots, which were 30 cm in diameter and 20 cm in height. Each flowerpot contained about 3 kg of soil on average. Meanwhile, 30 pikas were captured and were sacrificed by exposure to excessive diethyl ether. Then, the carcasses (115.59 ± 28.61 g) were randomly covered on the soil surface of each flowerpot for natural decomposition. The remaining 30 flowerpots without carcasses served as controls. The whole experiment process (including the six main stages of corpse decomposition) was carried out outdoors and photographs of the different stages of corpse decomposition were attached to our previous study (Su et al. 2022). The average air temperature ranged from 12 to 18 °C. Soil samples with depth of 0–10 cm in the cadaver group and the control group were collected at six time points (i.e., the 4th, 7th, 12th, 20th, 39th, and 94th days) in our experiment. Five replicates of each

sample were collected, and 60 samples were totally collected (30 gravesoil, 30 control). One part of the soil samples was stored at -20 °C to extract DNA, and the other part was stored at 4 °C provisionally for the measurement of physicochemical parameters. The animal experiment was favored by the Animal Protection Institute and the Ethics Committee of Lanzhou University.

Six soil physicochemical parameters were determined in our study, including pH, $\text{NH}_4\text{-N}$, $\text{NO}_3\text{-N}$, total carbon (TC), total nitrogen (TN), and total phosphorus (TP). The detailed determination methods, procedures, and data analysis results were described in our previous research (Yang et al. 2022a).

DNA extraction, Miseq sequencing, and Bioinformatic analysis

Bacterial DNA was extracted from 0.2 g soil in every sample utilizing Soil Ezup Genomic DNA Extraction Kit (Sangon Biotech, Shanghai, China) and the extractive DNA was determined in concentration via Nanodrop 2000 Spectrophotometer (Thermo Scientific, IL, Waltham, USA). The primer pairs Zehr-*nifHf* (5'-TGYGAYCCNAARGCNGA-3')/Zehr-*nifHr* (5'-ADNGCCATCATYTCNCC-3') were used to amplify *nifH* fragments (about 350 bp) throughout PCR technique (Chowdhury et al. 2009). The PCR system and conditions were described in detail in our previous research (Yang et al. 2022a). Two samples in corpse groups failed to be amplified successfully, so 58 samples were sequenced. The final sequencing results were found in the supplement.

Raw sequences were processed using the QIIME Pipeline (Prodan et al. 2020). Each sequence was distinguished by its unique barcode. Then the paired-end reads were combined by FLASH-1.2.8 software (Magoc and Salzberg 2011). High-quality sequences (reads length > 300 bp and average base quality score > 30) were screened out through quality control, and chimeras sequences were removed using Usearch 8.0 (Zhang et al. 2022) and Metaxa2 (Bengtsson-Palme et al. 2015) software, respectively. The remaining available sequences were gathered into operational taxonomic units (OTUs) at the cutoff of 97% sequence similarity. Annotation for OTU representative sequences was performed via the *nifH* database in the FunGene repository using Blastp to obtain taxonomic information on species. (Fang et al. 2018). For the comparability of samples with different sequencing depths, the OTU table was resampled to the equal reads of 2362 sequences per sample using the “daisyhopper” scripts (Gilbert et al. 2009). The observed OTUs and Chao1 index were calculated to access α diversity. To explore the response of diazotrophs to carcass decomposition of pikas, the resistance (RS) and resilience (RL) indexes were calculated based on the α diversity as the following equals, respectively (Orwin and Wardle 2004). $RS(t_0) = 1 - 2|D_0| / (|D_0| + C_0)$, $RS(t_0) = 2|D_0| / (|D_x| + |D_0|) - 1$, in which D_0

indicates the difference between the corpse soil (P_0) and control (C_0) at the time point (t_0), Dx indicates the difference between the corpse soil (P_x) and control (C_x) at the time point (t_x). Beta diversity was also calculated to evaluate the bacterial community structure differences between groups (Mark Ibekwe et al. 2017).

Community similarity and time-decay relationships

Bray–Curtis distance matrix was used to calculate community dissimilarity in the QIIME pipeline and community similarity was equivalent to 1 dissimilarity (Jiao et al. 2017). Linear fitting showed the relationship between time and community similarity. Time-decay relationships (TDRs) were used to reveal the similarity of microbial community changed with time intervals (Liang et al. 2015). The logarithmic linear model is fitted between the community structure similarity and day span. TDRs were calculated based on the equation: $\log_{10}(S_S) = \text{constant} - w \log_{10}(T)$, in which S_S means the pair-wise similarity in community composition, and T represents time interval. The rate of species turnover over time expresses as w , which is the slope of the equation.

Null model analyses

Stochastic and deterministic processes are two significant determinants in microbial community construction. A more accurate index, modified stochasticity ratio (MST) was generally used to quantitatively evaluate the ecological stochasticity, which ranged from 0 to 1 (Ning et al. 2019). $MST > 0.5$ and $MST < 0.5$ means a more stochastic and more deterministic assembly of community, respectively. In this study, the OTU abundance was used to calculate MST based on the Jaccard distance matrix and null model algorithm. We grouped 2-time points as a time period (i.e., P1 was the early stage at the 4th and 7th days, P2 was the middle stage at the 12th and 20th days, P3 was the late stage at the 39th and 94th days) to determine the relative importance of stochastic and deterministic processes of diazotrophic community assembly in different stages of corpse decomposition. The MST values were acquired in R depending on the “NST” package (Chen et al. 2021).

Statistical analysis

The linear fitting between physicochemical properties, time, and diazotrophs was conducted in Origin 8.5 (Originlab, Northampton, USA). The influences of time and corpse treatment on diazotrophs were calculated by two-way analysis of variance (two-way ANOVA) in SPSS 26.0 (SPSS Inc., Chicago, IL, USA). The alpha and beta diversity discrepancies of diazotrophic communities were analyzed using the Mann–Whitney U test and permutational multivariate

analysis of variance (PERMANOVA), respectively. The effects of environmental factors on diazotrophic community were also calculated utilizing PERMANOVA (Yu et al. 2021). Multiple regression matrix (MRM) was used to estimate the importance of environmental factors (Yang et al. 2022a). Linear discriminant analysis effect size (LEfSe) was used to determine diazotrophs biomarkers at the OTU level among different groups (LDA score > 2.0 , $P < 0.05$) (Segata et al. 2011). The relationship between core OTUs (existing in more than 80% of corpse samples) and physicochemical properties was demonstrated by Spearman rank correlation. The phylogenetic tree of dominant OTUs (average relative abundance top 50) was constructed based on the Neighbor-Joining (NJ) method in MEGA v4.0.0 and FigTree v1.4.2 after sequences alignment using ClustalW alignments (Su et al. 2022). The co-occurrence pattern of diazotrophic population was calculated in R using the “psych” package ($|r| > 0.5$, $P < 0.05$) and visualized by Gephi0.9.2 software, and the network topology parameters were calculated using R “igraph” packages (Layeghifard et al. 2018).

Results

Composition and diversity of diazotrophic communities

At the phylum level, a large proportion of the diazotrophic populations detected in our samples were unclassified bacteria (average relative abundance, 70.10%). Among the classified phyla, *Proteobacteria* was the most abundant in both the corpse groups and the control groups (average relative abundance, 29.27%), followed by *Verrucomicrobia* (0.53%), *Spirochaetes* (0.06%), and *Firmicutes* (0.03%) (Fig. S1a). Notably, *Bacteroidetes* occurred in corpse groups but without detection in control groups. The relative abundance of *Proteobacteria* was significantly affected by corpse decomposition, while time significantly influenced *Bacteroidetes* (two-way ANOVA, $P < 0.05$; Table S1). In addition, *Bacteroidetes* was also significantly impacted by the combination of corpse decomposition and time (two-way ANOVA, $P < 0.05$; Table S1), and the relative abundance of *Bacteroidetes* increased significantly with decomposition time ($R^2 = 0.248$, $P < 0.05$; Fig. S2a). At the genus level, three major taxa dominated the bacterial community except for unclassified bacteria (74.82%), including *Dechloromonas* (18.09%), *Klebsiella* (3.03%), and *Mesorhizobium* (1.51%) (Fig. S1b). Corpse decomposition had a significant effect on *Dechloromonas*, *Bradyrhizobium*, and *Azohydromonas* (two-way ANOVA, $P < 0.05$; Table S2). Some genera like *Rhodopseudomonas* and *Dysgonomonas* had a significant positive correlation with time (Fig S2b, c). At the OTU level, the OTU1 *_uncultured_temperate_forest_soil_bacterium*,

OTU2_*Dechloromonas_SIUL*, and OTU3_*uncultured_bacterium* were the most predominant across all samples with average relative abundances 42.71%, 18.19%, and 5.90%, respectively (Fig. S1b). The abundances of OTU19_*Opiritaceae_bacterium_TAV5*, OTU188_*Azoarcus_communis*, and OTU106_*Paenibacillus_abekawaensis* showed significant correlations with time (Fig. S2d-f).

The alpha diversity of diazotrophic community showed significant differences at the 4th, 12th, and 39th day between carcass groups and control groups (ANOVA, $P < 0.05$) indicated by Observed OTUs and Chao1 indices (Fig. 1a, b). Overall, these two indexes of the carcass group were lower than those of the control groups, which indicated that carcass decomposition reduced the alpha diversity of diazotrophs. In terms of time, the α diversity of diazotrophic community in the cadaver groups and control groups showed an opposite trend in the early and middle stages but maintained stability in the late stage (Fig. 1c, d). Moreover, the resistance and resilience were calculated based on the α diversity index (observed OTUs and Chao1 index) to understand the stability of diazotrophic community under the disturbance of corpse decomposition. The broken-line graph showed that RS and RL varied with corpse decomposition (Fig. 1e-h). PCoA analysis based on the Bray–Curtis and Jaccard distance matrix characterized whether the cadaver decay and time affected the structure and composition of diazotrophic

community. The results demonstrated that cadaver decay significantly affected the beta diversity of diazotrophs (Fig. 2a, b), and time also had a significant impact on beta diversity, which was only reflected in a significant difference in beta diversity between the early and middle stages (Fig. 2c, d). These results could be further proved by PERMANOVA analysis (Table S3, $P < 0.05$).

Succession and turnover of diazotrophic communities

Based on Bray–Curtis dissimilarity, we calculated the similarity of diazotrophic community between the corpse group and control group at each time point, and linear fitting visualized the variation pattern of community similarity with time (Fig. 3a). Exactly as the fitting result indicated, the community similarity had a significant negative correlation with time, and community similarity gradually decreased with the increase of time. That is to say, cadaver decay resulted in the divergent succession of the composition of *nifH*-type microbial community.

Besides, the logarithmic transformation form of time intervals and community similarity were fitted to estimate time-decay relationships (TDRs). A significant TDRs was found for diazotrophic community in both the corpse group and control group through the fitting equation: $y = -0.237$

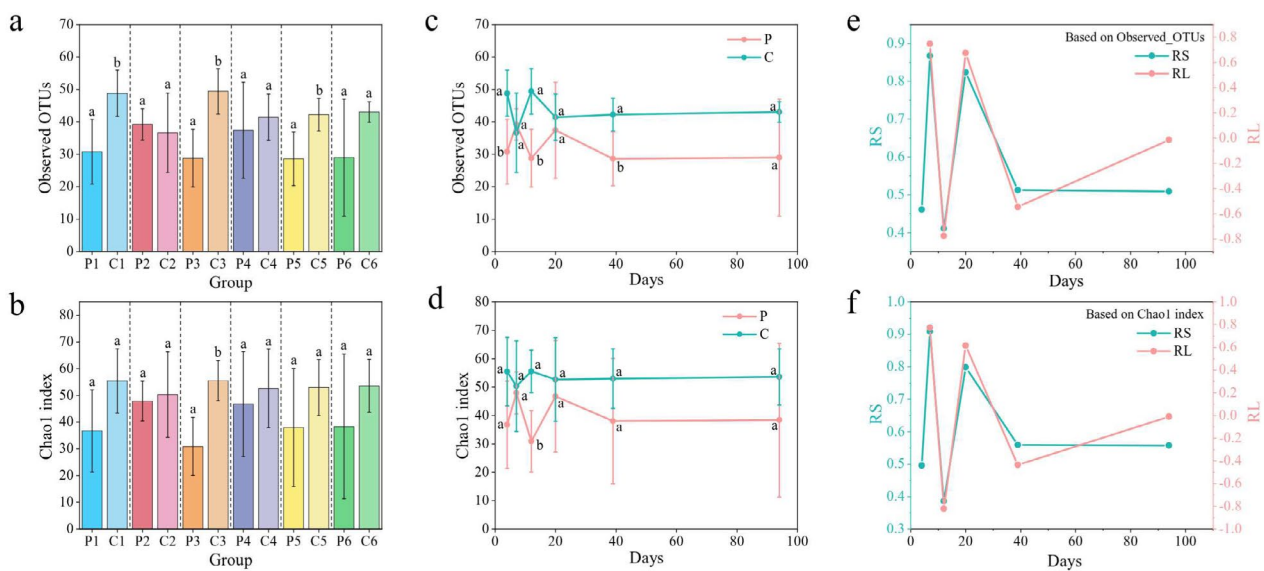


Fig. 1 The comparison of alpha diversity (observed OTUs (a); Chao1 (b)) between corpse groups (P) and control groups (C) of the diazotrophic community. The broken-line graph shows the trend of alpha diversity (observed OTUs (c); Chao1 index (d)) over time. Resistance (RS, blue color) and resilience (RL, pink color) were calculated based on alpha diversity (observed OTUs index (e); observed OTUs (f)) over time. Abbreviations: P1, corpse group at 4th day; P2, corpse

group at 7th day; P3, corpse group at 12th day; P4, corpse group at 20th day; P5, corpse group at 39th day; P6, corpse group at 94th day; C1, control group at 4th day; C2, control group at 7th day; C3, control group at 12th day; C4, control group at 20th day; C5, control group at 39th day; C6, control group at 94th day. A significant difference is labeled with different letters ($P < 0.05$)

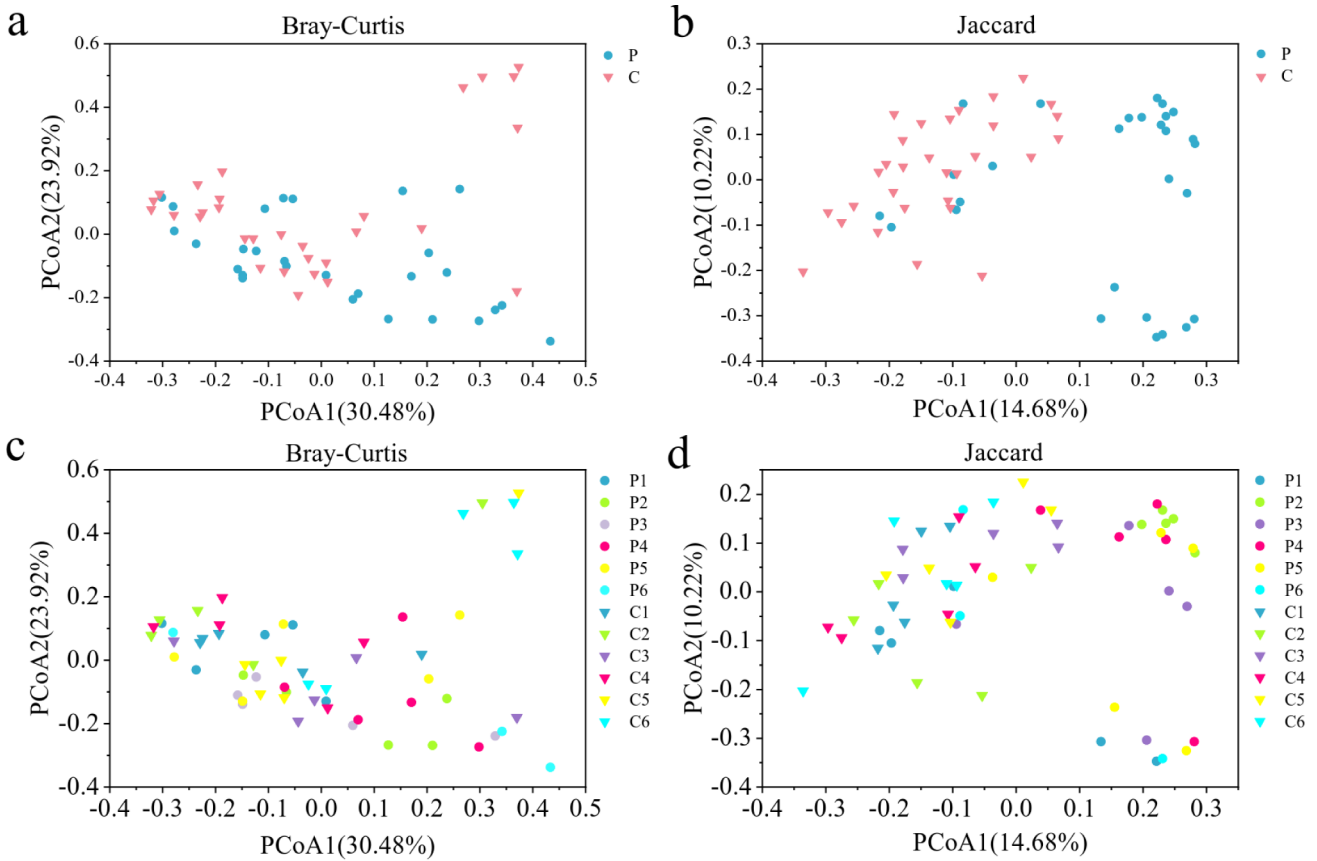


Fig. 2 The principal coordinates analysis (PCoA) plots showing the structural difference of diazotrophs in the corpse groups and control groups based on the Bary-Curtis (a, c) and Jaccard (b, d) distance matrix. Abbreviations: P1, corpse group at 4th day; P2, corpse group at 7th day; P3, corpse group at 12th day; P4, corpse group at 20th

day; P5, corpse group at 39th day; P6, corpse group at 94th day; C1, control group at 4th day; C2, control group at 7th day; C3, control group at 12th day; C4, control group at 20th day; C5, control group at 39th day; C6, control group at 94th day

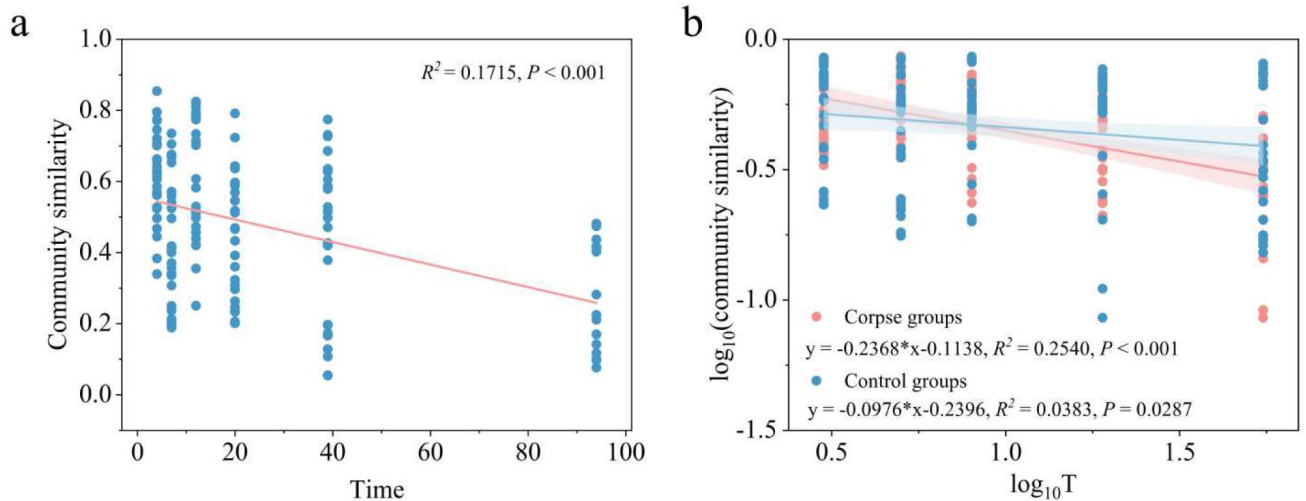


Fig. 3 The linear regression showing the divergent succession of diazotrophic community similarity between the corpse groups and the control groups (a). Time-decay relationships of diazotrophs during corpse decomposition in the corpse and control groups (b)

$\times x - 0.114 (R^2 = 0.254, P < 0.001)$; $y = -0.098 \times x - 0.240 (R^2 = 0.038, P = 0.029)$ (Fig. 3b). The absolute value of w indicates the time decay rate. The higher $|w|$ in the cadaver group than in the control group indicated that the corpse decomposition significantly accelerated the temporal turnover rate of diazotrophic community.

Core communities and phylogenetic clusters of diazotrophs

The seven *nifH*-encoding bacterial OTU members shared by more than 24 (80%) treatment samples were defined as the core OTUs. Although these core OTUs accounted for only 3.45% of the total OTUs, their average relative abundance accounted for 73.08% of the average relative abundance of total OTUs (Fig. 4a). Nearly half of them (48.99%) were unclassified bacteria, 18.09% of them belonged to *Dechloromonas*, the others were subordinate to *Deltaproteobacterium* (UG) (4.16%), *Mesorhizobium* (1.51%), and *Phaeospirillum* (0.33%), respectively (Fig. 4b). Additionally, the linear discriminant analysis effect size (LEfSe) was employed to explore the diazotrophic population enriched in treatment groups and control groups. There were 11 biomarkers in the treatment groups (Fig. 4d). For example, OTU2_ *s_Dechloromonas_SIUL*, OTU8_ *uncultured_bacterium*, and OTU4_ *uncultured_deltaproteobacterium* were found with the obvious enrichment in corpse groups, and the

most differentially abundant populations in control groups were OTU3_ *uncultured_bacterium*, OTU6_ *Klebsiella_AL060224_03*, and OTU13_ *uncultured_bacterium*. Interestingly, the biomarkers enriched in the cadaver group are the identified core OTUs. In other words, some core OTUs could be regarded as bacterial markers of diazotrophic community during corpse decomposition. Moreover, the core OTUs related to soil physical and chemical properties were shown by heatmap (Fig. 4c). Nitrate nitrogen was found to have a significant positive relationship with the abundance of OTU2_ *Dechloromonas_SIUL* ($r = 0.353, P < 0.05$), OTU4_ *uncultured_deltaproteobacterium* ($r = 0.322, P < 0.05$) and OTU8_ *uncultured_bacterium* ($r = 0.467, P < 0.05$), and NH_4-N were positively related to OTU8_ *uncultured_bacterium* ($r = 0.351, P < 0.05$). However, OTU2_ *Dechloromonas_SIUL* ($r = -0.263, P < 0.05$), OTU8_ *uncultured_bacterium* ($r = -0.349, P < 0.05$), and OTU80_ *Phaeospirillum_fulvum* ($r = 0.353, P < 0.05$) were associated negatively with pH.

The phylogenetic tree according to 50 dominant OTUs (with the top 50 relative abundance) contained four clusters, and the OTUs in each cluster belonged to several genera, such as unclassified bacteria, *Dechloromonas*, *Pseudomonas*, *Klebsiella*, and *Phaeospirillum* (Fig. S3a). The number of OTUs in Cluster 1 was the largest (64%), and their average relative abundance was also the highest (57.97%) (Fig. S3b). The four clusters were

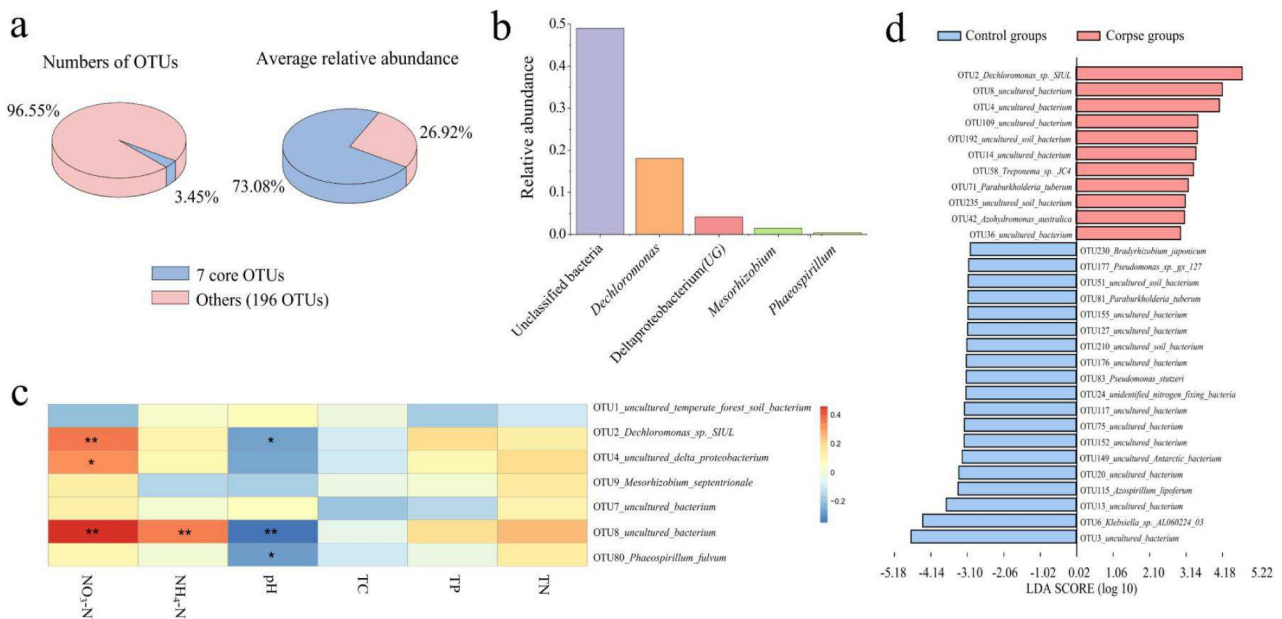


Fig. 4 Numbers and average relative abundance of core OTUs (blue) and other OTUs (pink) in corpse groups (a). Composition of the core OTUs in corpse groups at the genus level (b). Correlation between the core OTUs and the physicochemical factors (c). Linear discriminant analysis effect size (LEfSe) of abundant OTUs between corpse groups and control groups (d). Abbreviations: TC, total carbon; TP, total phosphorus; NT, total nitrogen

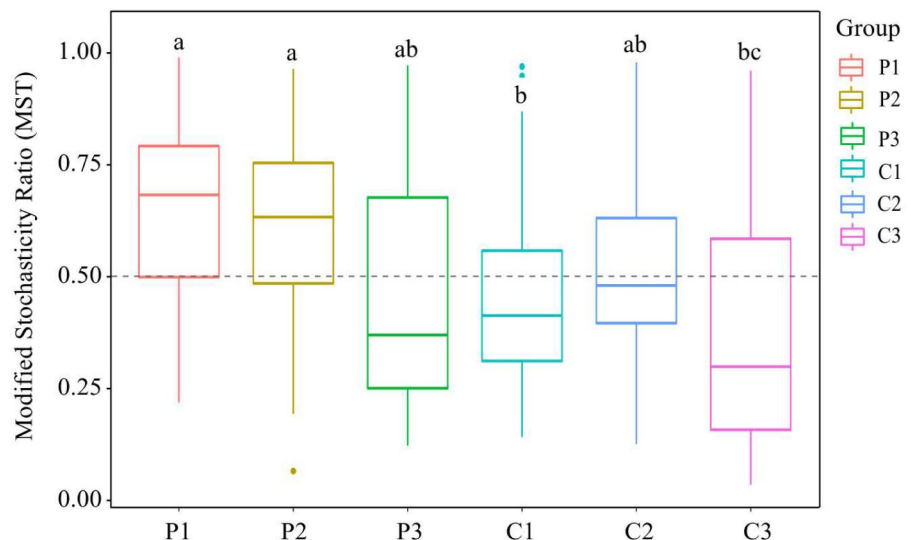
ant analysis effect size (LEfSe) of abundant OTUs between corpse groups and control groups (d). Abbreviations: TC, total carbon; TP, total phosphorus; NT, total nitrogen. Significant indicator by * $P < 0.05$, and ** $P < 0.01$

dominated by unclassified bacteria. Among the classifiable genera, *Pseudomonas* was the dominant genus in Cluster 1. *Dechloromonas*, *Azospirillum*, *Treponema*, and *Phaeospirillum* were the dominant genera in Cluster 2. Cluster 3 and Cluster 4 were dominated by *Phaeospirillum* and *Azospirillum*, respectively. However, there were no significant differences in the abundance of these clusters between the corpse groups and the control groups ($P > 0.05$, Fig. S3b).

Ecological assembly process of diazotrophic communities

Microbial community is a complex and diverse system with many interacting members, and its assembling was influenced by stochastic and deterministic forces. To estimate whether the stochastic or deterministic processes play a more important role during corpse decomposition, a modified stochasticity ratio (MST) was employed to indicate the proportion of stochasticity. The result manifested that the MST values in the control groups were all lower than 50% (Fig. 5), indicating deterministic processes control the community assembly. In the corpse groups, the indexes were higher than 50% in the early and middle stages during corpse decomposition, while lower than 50% in the late stage (Fig. 5), which suggested stochastic process played more important roles in the early and middle stages of corpse decomposition, it was opposite at the late stage. More specifically, corpse decomposition increased the relative contribution of stochasticity to the diazotrophic community assembly, but the effect tended to weaken over time.

Fig. 5 Changes of the modified stochasticity ratio (MST) based on Bray–Curtis distance during the succession of diazotrophic community in corpse groups and control groups. A significant difference is labeled with different letters ($P < 0.05$). Abbreviations: P1, corpse groups at 4 and 7 days; P2, corpse groups at 12 and 20 days; P3, corpse groups at 39 and 94 days; C1, control groups at 4 and 7 days; C2, control groups at 12 and 20 days; C3, control groups at 39 and 94 days



Changes in co-occurrence network of diazotrophs

The co-occurrence pattern of nitrogen fixation microorganisms in the corpse and control groups at three stages was visualized at the OTU level (average relative abundance $> 0.1\%$) by network analysis. It can be found from the network diagram that compared with the control groups, the correlation of diazotrophic populations in the corpse groups seemed simpler in each period (Fig. 6a–f). Therefore, several important topological characteristics were further calculated (Fig. 7a–f), which may reflect the cohesion characteristic of the network. The node and edge numbers in the control groups were more than in the corpse groups. Regardless of whether there was a corpse treatment, the percentage of positive edges was higher than negative edges in both groups, which implied a stronger cooperative interaction among diazotrophic members. Besides, the average degree, average path length, and diameter of the cadaver groups were smaller than those of the control groups, and the cadaver groups had smaller density, clustering coefficient, and modularity compared to the control groups. These results implied that the networks of the cadaver groups were smaller and simpler. In terms of time, we found that the average degree, average path length, diameter, and clustering coefficient were all lowest in the middle stage, while the modularity increased with time in the cadaver groups.

Driving factors of diazotrophic communities

To reveal the effect of abiotic factors (corpse decomposition, time and soil physicochemical properties) on the diazotrophic community, the PERMANOVA and MRM

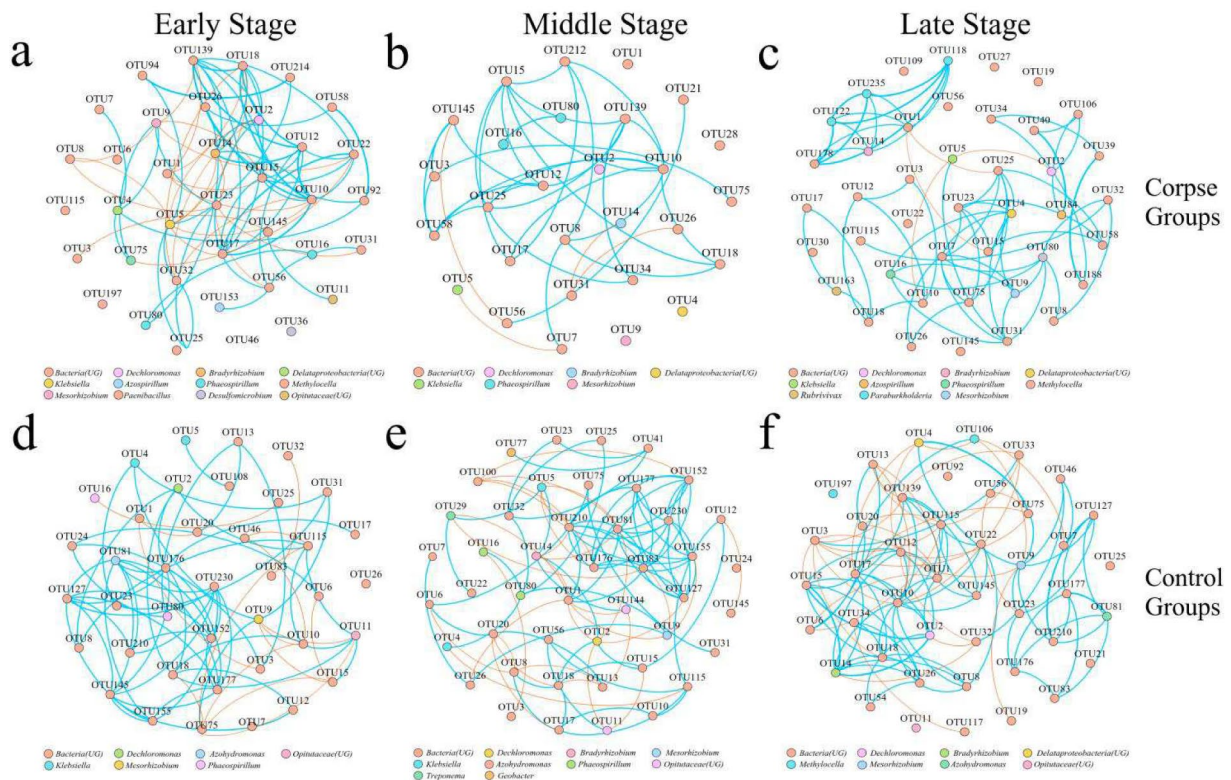


Fig. 6 Co-occurrence network of diazotrophic communities in the corpse groups (early stage (a); middle stage (b); late stage (c)) and control groups (early stage (d); middle stage (e); late stage (f)). OTUs with a mean relative abundance >0.1% were included. Blue edges

represented the positive correlation between OTUs, and orange edges represented the negative correlation between OTUs. Information on key genera was below the network figures

analyses were used to calculate the correlation between these factors and the community composition and structure (Table S4). It turned out that $\text{NH}_4\text{-N}$ ($R^2 = 0.057$, $P = 0.003$), time ($R^2 = 0.026$, $P = 0.001$), and total carbon (TC) ($R^2 = 0.016$, $P = 0.044$) were significantly related to diazotrophic community and they were the major determinants (Fig. 8). The diazotrophic community was insensitive to other factors with $P > 0.05$ (Table S4). The linear fitting even showed that $\text{NH}_4\text{-N}$ significantly increased the relative abundance of some phyla (*Spirochaetes*, $P < 0.05$) and genera (*Treponema*, $P < 0.05$) (Fig. S4). The relative abundance diazotrophs also ascended significantly over time. For example, *Proteobacteria*, *Rhodopseudomonas*, *Dysgonomonas* as well as some OTU like OTU19_*Opitutaceae_bacterium_TAV5* and OTU188_*Azoarcus_communis* all increased with time. Besides, the two-way ANOVA associated with treatment (corpse decomposition) and time showed that treatment had a significant relationship with α diversity (observed OTUs and Chao1 index, $P < 0.05$), time and the combined action of the two significantly linked with β diversity ($P < 0.05$).

Discussion

Corpse decomposition changes diazotrophic composition and biodiversity

Animal corpses, rich in organic substances and mineral ions, may directly or indirectly shift soil diazotrophic composition diversity when discarded in soil. The abundance of Proteobacteria and Bacteroidetes in corpse groups was higher in control groups, which may be because of their strategies to survive and metabolize in nutrient-rich environment. Proteobacteria have the ability of rapid growth and reproduction, which enables them to quickly take advantage in eutrophic environment (Bergkessel and Delavaine 2021). Bacteroidetes can decompose a variety of complex organic substances, so they widely exist in eutrophic environments (Lap  bie et al. 2019). Carcass degradation of wild animals led to a decrease in α diversity of diazotrophs, which could be understood from two perspectives. The first one is that the alpine meadow soil is poor in nutrients, some diazotrophic populations with low tolerance to high osmotic potential and ionic toxicity will decrease or even disappear when a high

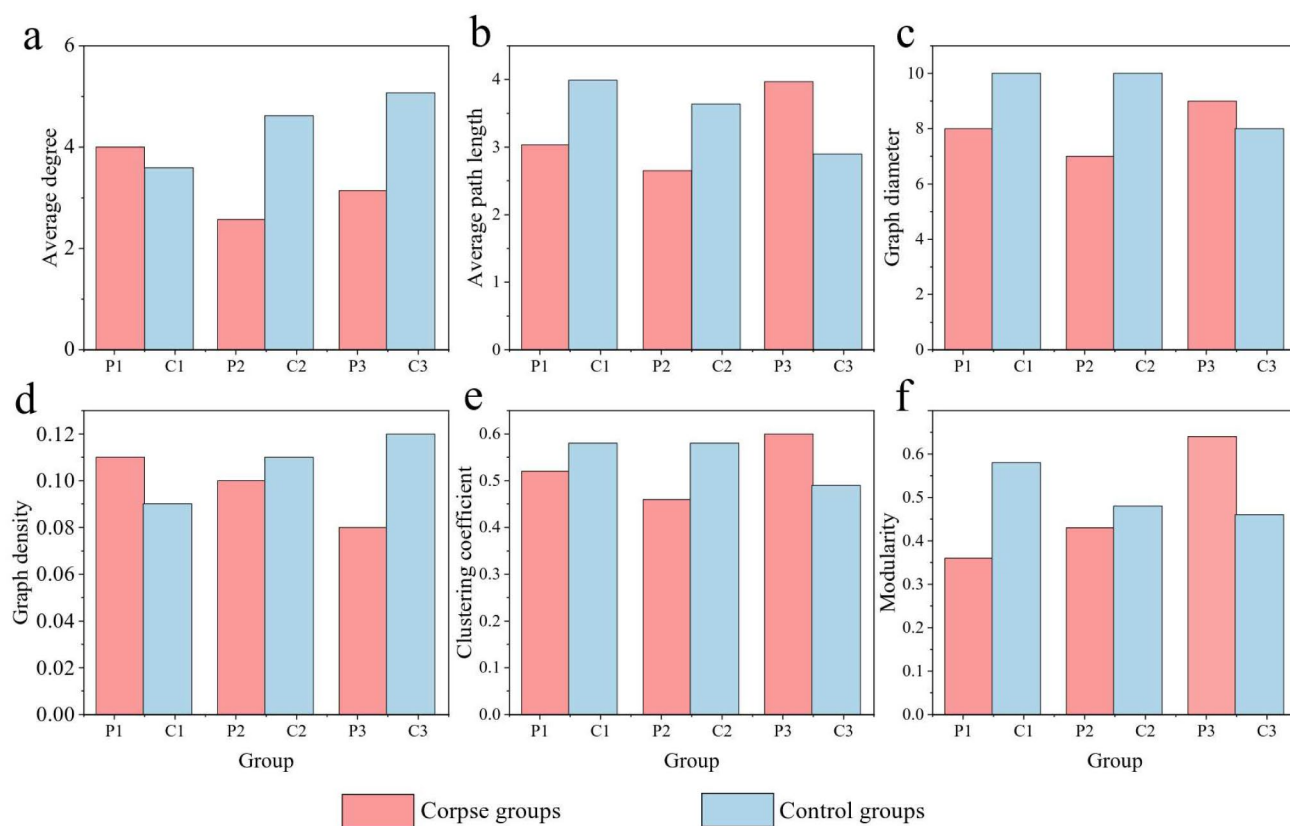


Fig. 7 Network topological properties of *nifH* diazotrophs in the corpse groups and control groups (average degree (a); average path length (b); graph diameter (c); graph density (d); cluster coefficient (e); modularity (f)). Abbreviations: P1, corpse groups at 4 and 7 days;

P2, corpse groups at 12 and 20 days; P3, corpse groups at 39 and 94 days; C1, control groups at 4 and 7 days; C2, control groups at 12 and 20 days; C3, control groups at 39 and 94 days

concentration of nutrients produced by mammalian carcass leaches into the soil. The other one is that BNF is the conversion of gaseous nitrogen (N_2) into ammonium nitrogen

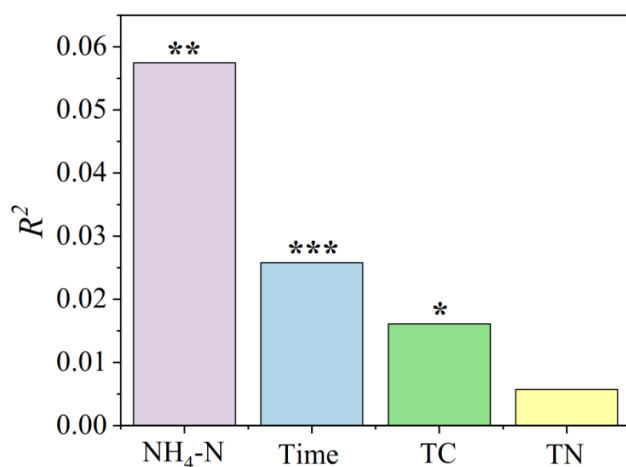


Fig. 8 Multiple regression matrices (MRM) showing the factors for the diazotrophic community during corpse decomposition. Significant indicator by * $P < 0.05$, and ** $P < 0.01$

(NH_4^+) driven by diazotrophs, while corpse decomposition was proved to produce plentiful ammonium (Szelez et al. 2018), increasing the concentration of NH_4^+ in the soil and inhibiting N_2 fixation process (Ning et al. 2015), thus the diversity of nitrogen fixers may correspondingly reduce. In response to external disturbance, the microbial community generally shows resistant (insusceptible), resilient (returning to the original status), or sensitive (turning into a distinct but stable status) tendency with time (Griffiths and Philip-pot 2013). However, we found no significant changes in α diversity over time, we speculated that it might need to take longer time scales to observe these trends (Lourenço et al. 2018), while our experiment was only conducted for three months. In other words, the effect of corpse decomposition on the diazotrophic community changed little in a short time.

Some exogenous microorganisms related to corpses changed the β diversity of nitrogen-fixing microbial communities by affecting soil the composition of bacterial communities. Previous studies have proved the effect of nonindigenous microbial invasion on soil microbial biodiversity, especially those that feed on protein and lipids (Cederlund et al. 2014; Wang et al. 2021; Zhong et al. 2010). In the

process of degradation, the exogenous microbes carried into the soil by necrobiome (such as scavengers and insects) and the intestine of animal carcass may impact the composition and structure of diazotrophic community.

Succession patterns of diazotrophs over time

The composition of diazotrophic communities in the gravesoil became increasingly different from the control soil as the cadaver decomposition progressed. This result is converse to previous literature (Castle et al. 2016), in which indicated that bacterial community occurred in convergent succession in deglaciating soils. With the degradation of glaciers, the increase of plant communities provides rich organic substances for the soil, which changes the physical and chemical properties of the soil, and this environmental change is closely related to the composition and structure of microbial community. Whereas in our study, we speculated that the initially same soil properties caused a higher similarity of the microbial communities between the cadaver group and the control group, then due to the addition and degradation of cadavers, the soil properties in the cadaver group changed (such as changes in pH, carbon and nitrogen content), so the similarity of diazotrophic community gradually decreased. Another explanation is that the physicochemical properties of soil are not recovered after more than 90 days of degradation, so diazotrophic communities are still in differentiation. This divergent succession reflected stochastic events and environmental filtering changes over time, which caused similar communities to be different with time (Fukami et al. 2005; van der Gast et al. 2008). Besides, the time-decay relationships mirror the temporal turnover rates of diazotrophic community structure. It was found that there was a significant time-decay relationship between the microbial communities of the cadaver group and the control group, and the steeper slope under cadaveric disturbance compared with the control group indicated that the cadaver decomposition accelerated the time turnover of soil diazotrophic community succession, which was consistent with our previous research (Yang et al. 2022a).

Corpse decomposition increases the contribution of stochastic processes to diazotrophic community assembly

The relative contribution of stochastic and deterministic processes is significantly associated with diazotrophic community assembly. A more important role of the deterministic process was observed in alpine meadow soil in control groups. The harsh climate (e.g., low temperature, hypoxia) and geological (e.g., low carbon and nitrogen content) conditions of the high-elevation regions (3100–3800 m) of the QTP have an environmental filtration effect on

soil diazotrophs, thus strengthening the deterministic forces. Therefore, we presumed that the more important deterministic process of the control group resulted from hypoxia and nutritional deprivation.

Additionally, it should be noted that stochasticity dominated the bacterial community assembly in gravesoil at the early and middle stages, while determinacy prevailed in the late stages. It means carcass degradation strengthens the contribution of stochasticity to diazotrophic assembly, which decreases over time. The reasonable explanation is that the early and middle stages of cadaver decay perhaps increased the random incubation, death, colonization, and drift events that structure patterns of diazotrophic community. For example, as the nutrient substrates in soil animal carcasses reduce the resource competition among bacteria, promoting the growth of dominant populations and the emergence of rare species (Ma et al. 2023). The concomitant release of cadaverine, putrescine, and ammonia from the decaying animal matter acidified the soil (Yu et al. 2020), which promotes the exudation of inorganic ions (such as aluminum, magnesium, and calcium) (Bowman et al. 2008; Wang et al. 2018), and the poisoning of metals increases the death events of nitrogen-fixers. However, with the progress of corpse degradation, the impact of the nutrient pulse on random events became smaller, so the role of the stochastic process was correspondingly weakened. Altogether, our results indicated that the relative importance of the assembly process for soil diazotrophic community may depend on corpse decomposition.

Corpse decomposition reduces the co-occurrence of diazotrophs

The interactions among diazotrophic members decreased after corpse disturbance in comparison to the controls. The lower graph density and clustering coefficient also corroborated this low connection. Numerous shreds of evidence indicated that the reduction of resource availability could reduce the complexity of microbial networks (Banerjee et al. 2019; Wang et al. 2022). The ammonia released by decomposition of animal corpses will increase the concentration of $\text{NH}_4\text{-N}$ in the soil environment (Yang et al. 2022a), and at this time, nitrogen-fixing bacteria will shut down the reduction mechanism of N_2 fixation in order to avoid energy waste in BNF process. Therefore, even though the carcass decomposition provides rich nutrient substrates for diazotrophs, the availability of nitrogen-fixer to resources has declined. Therefore, we speculated that nutrient enrichment caused by corpse decomposition may reduce the complexity of the nitrogen-fixing microbial network. Another explanation is that carcass decomposition weakens the competitive relationship of nitrogen-fixing microorganisms by reducing the diversity of diazotrophs and ulteriorly decreases their

co-occurrence (Barberán et al. 2012). Besides, the positive edge ratio was higher than the negative edge in diazotrophic network regardless of the cadaver group or the control group, suggesting the positive interactions (cooperations) between diazotrophs. This perhaps means that under both conditions, diazotrophs divide labor and some individuals specialize in the work beneficial to other individuals, corpse cannot imbalance diazotrophic cooperation. Notably, the modularity of the corpse group network increased with time in our study. The stability of the bacterial network has been found to increase with modularity (Yang et al. 2020), so it can be inferred the stability of diazotrophic network may also increase with the progress of corpse decomposition.

Key abiotic factors of diazotrophs

The influences of CDIs on soil physiochemical properties have been explored in our previous studies (Yang et al. 2022a). Corpse addition has been shown to construct other nitrogen cycle-related bacterial communities primarily by changing environmental factors (Yu et al. 2021). Herein, the structure of diazotrophs was also affected by altered physiochemical factors. Nitrate nitrogen had the greatest effect on diazotrophs, followed by TC. Specifically, $\text{NH}_4\text{-N}$ was significantly positively correlated with *Spirochaetes* and OTU8_ *uncultured_bacterium*, which served as the core OTU and marker for diazotrophic community. This may mean that core diazotrophic OTUs have an adaptability to cope with nitrogen input. The quality and quantity of soil carbon lead to alterations in diazotrophic communities and biological nitrogen fixation (BNF) processes by limiting the diazotrophic growth and activity (Chen et al. 2022). Interestingly, some researchers revealed that pH played a key role in shaping the soil diazotrophic community (Tripathi et al. 2018; Wang et al. 2017b). However, the significant role of pH in our study was unobserved. It was probably because the gravesoil remained neutral which is the optimum pH for most microorganisms although cadaver decay reduced soil pH value.

Our study uncovered the temporal dynamics of the diazotrophic community during wild animal corpse decomposition in terrestrial ecosystems. Corpse decomposition reduced the α diversity and significantly impacted on β diversity of diazotrophic communities across different decomposition stages. $\text{NH}_4\text{-N}$ is the most important factor affecting diazotrophic community, followed by time and TC. Furthermore, corpse decomposition improved the relative importance of stochasticity to the community assembly, while the importance of stochastic processes decreased over time. Notably, cadaver decay simplified the network and weakened the biological interaction among diazotrophs. The current work provides insight to predict

how the diazotrophic community responds to and recovers from nutrient input or environmental variation. Unfortunately, only diazotrophic microorganisms were considered in our study, and other microorganisms and processes related to nitrogen cycling need further study.

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Author contribution H.L. designed the experiment and provided research funds. Q. H. completed the experiment and data analysis, and Han wrote the paper. Other authors also contributed to the revision and proofreading of this paper.

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Data availability The original 16S rRNA gene sequence data in this study were deposited at the European Nucleotide Archive by accession number PRJEB58653 (<http://www.ebi.ac.uk/ena/data/view/PRJEB58653>).

Declarations

Ethics approval The animal processing in our study was approved by the Animal Welfare and Ethics Committee of Lanzhou University. The related experimental methods and procedures strictly followed the related guidelines.

Conflict of interest The authors declare no competing interests.

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