RESEARCH ARTICLE

Changes in the soil microbial communities of different soil aggregations after vegetation restoration in a semiarid grassland, China

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HIGHLIGHTS

- The soil aggregate stability increased with increasing duration of vegetation restoration.
- Natural restoration has a positive effect on soil microbial diversity that was generally higher in large particle size aggregates, which leads to low environmental stress and strong stability.
- Microorganism continually changed their regulation of metabolism pathways as their environment changed.
- Environment adaptability influences soil physiological indicators to varying degrees.
- After years of natural restoration, the soil microbial community generally transformed from nutrient-rich to heterotroph-dominant.

GRAPHICAL ABSTRACT

ABSTRACT

Soil aggregate fractions can regulate microbial community composition and structure after vegetation restoration. However, there has been less focus on the effects of soil aggregate fractions on the distributions of microbial communities. Here, we used phospholipid fatty acid (PLFA) analysis to explore the effects of different years of vegetation restoration (a 35-year-old *Thymus mongolicus* community (Re-35yrs) and a 2-year-old nongrazing grassland (Ug-2yrs)) on microbial communities within different soil aggregate sizes (< 0.25 mm, 0.25–1 mm, 1–2 mm, 2–3 mm, 3–5 mm and >5 mm). The results indicated that the amount of total PLFA in Re-35yrs was 10 times greater than that in Ug-2yrs. The soil aggregate stability increased with increasing duration of vegetation restoration. In Re-35yrs, the total PLFA shown an increase as the soil aggregate size increased, and the highest values were observed in 3–5 mm. Ug-2yrs differed from Re-35yrs, the soil microbial diversity was higher in medium particle sizes (1–2 mm and 2–3 mm) and lower in microaggregates (< 0.25 mm and 0.25–1 mm) and macroaggregates (3–5 mm and >5 mm). Soil microbial diversity was highest in large particle size aggregates, which resulted in low environmental stress and strong stability. The same tendency was observed in the high values of cyc/prec, S/M and soil organic matter, which indicated a lower turnover speed (F/B) of fungal energy utilization and a higher fixation rate. After years of natural restoration, the soil microbial community generally transformed from nutrient-rich to heterotroph-dominant, especially in microaggregates (reflected in the G^+/G^- ratio).

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1 Introduction

Grasslands are among the largest ecosystems in the world. Grassland enclosure promotes vegetation succession and is

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one of the strategies used to control land degradation (Liu et al., 2008; Lenka et al., 2012; Deng et al., 2018); in addition, enclosure is considered to be an effective and economical practice for restoring plant and soil communities (Walker et al., 2007; Herrera et al., 2016; Zhao et al., 2019). A growing body of ecological research on the plant–microbe interactions in soil during the restoration of plant diversity and under management practices have been a major focus of research (Fanin et al., 2016; Deng and Shangguan, 2017; Zhu et al., 2019). The changes in microbial community composition that occur during succession are closely related to changes in ecosystem function (Li et al., 2018; Liu et al., 2020a; Zhao et al., 2019). Understanding the changes in the characteristics of the microbial community structure that occur throughout ecological succession could elucidate the mechanisms that drive successional dynamics, especially considering that less is known about the belowground microbial community and how it changes with long-term succession in an ecosystem (Liu et al., 2020b). Previous studies have reported that positive and negative feedback between plants and soil microbes play central roles in early and late successional communities (Li et al., 2018; Wang et al., 2019; Guo et al., 2019; Zhao et al., 2019). However, how the microbial community and soil properties collaboratively develop during the middle stages of succession remains unclear.

Microbial community structure is closely related to the living environment in soil (Cao et al., 2017). Soils, with their nonuniform distribution of microenvironment conditions and nutrients across different aggregate-size fractions, provide spatially heterogeneous habitats for microorganisms, which are characterized by differences in resource availability, moisture content, oxygen concentration and predation pressure (Ranjard and Richaume, 2001; Muruganandam et al., 2010; Vos et al., 2013; Jiang et al., 2017; Liao et al., 2018). Moreover, few studies have simultaneously focused on the soil microbial communities across the different aggregate size fractions that are associated with biologically and biochemically mediated transformations of soil nutrients (Smith et al., 2014; Trivedi et al., 2017). Furthermore, it remains unclear whether changes in the aggregate size of the microbial community structure can promote or hinder stability once plant communities develop to the climax community stage. Studies on the composition and function of belowground microbial communities have thus far only been conducted on soil nutrient cycling and organic matter formation and decomposition (Trivedi et al., 2013, 2017). During vegetation succession, large changes occur in the soil aggregate stability, leading to the hypothesis that these changes should also affect the structure of the soil microbial community (An et al., 2013; Liu et al., 2013; Yan et al., 2020; Xiao et al., 2020). Macroaggregates (2000–2500 μm) generally contain more labile substrates than substrates that are predominantly derived from plant residues and exhibit a higher fungal biomass than microaggregates (Jiang et al., 2017). In contrast, microaggregates provide a protective microenvironment for microbial growth and stable SOC (soil organic carbon) (Six et al., 2000).

However, we do not understand how aggregate fractions regulate microbial community composition and structure as vegetation composition changes, especially in the middle stages of succession.

The current study was conducted on Yunwu Mountain, a semiarid grassland in the Loess Plateau. This well-documented site represents 40 years of long-term fencing and has a good sequence of undisturbed secondary succession. Thus, this area offers a unique sequence to investigate characteristics of microbial communities. Here, we selected a dominant species (*Thymus mongolicus* community) in the middle of the succession to investigate the soil microbial physiological indicators that are associated with different aggregate size and restoration years. Specifically, the primary aim of this study was to compare the difference in the soil microbial community composition and structure in a 35-year-old *Thymus mongolicus* community (Re-35yrs) and 2-year-old nongrazing grassland (Ug-2yrs). In this study, we addressed the following hypotheses: (1) soil microbial diversity increases with vegetation restoration years; (2) the aggregate-related changes in soil microbial communities, soil microbial diversity and stability are mainly driven by aggregate-size fractions.

2 Materials and methods

2.1 Study area and methods

The study area was located in the Yunwu Mountain Reserve for Vegetation Protection and the Eco-environment, Guyuan City, Ningxia Autonomous Region, between 106°24'E and 106°28'E longitude and between 36°13'N and 36°19'N latitude, China. Grassland is the native vegetation in this region. After more than 30 years of restoration since the observatory was established in 1982, the vegetation in the protected area belongs to two types: grassland and brush. The reserve includes three areas, namely, a core area, a buffer area and an experimental area, which have relatively similar geographical patterns and climate (Fig. 1). The reserve has a semiarid temperate continental monsoon climate. The mean annual total sunshine hours, annual evaporation and average forest-free days per year were 2518 h, 1600 mm, and 137 days, respectively (Deng et al., 2014). The average annual temperature and precipitation were 8°C and 371 mm (1991–2015), and 70% rainfall occurred between June and September. The soil types in the experimental site are Loessial soils (Chinese Soil Classification) (Shi et al., 2010) and Entisols (USA taxonomy) (Soil Survey Staff, 2010).

After 20 years of enclosure management, plant communities have formed in different succession stages. The area includes 5 subtypes, 11 formations and more than 40 associations, with classical grasslands being the primary biome. The primary plant species are herbaceous plants (i.e., *Androsace erecta*, *Artemisia capillaries*, *Artemisia frigid*, *Artemisia sacrorum*, *Heteropappus altaicus*, *Potentilla acaulis*, *Stipa bungeana*, *Stipa grandis*, *Thymus monoglicus*, etc.).

Fig. 1 The location of study area.

From the early 1980s to the 1990s, a group of scholars including Zou and Guan (1997) conducted detailed studies and summaries of the vegetation succession and defined the stages and characteristics of the vegetation succession of abandoned farmland and grazing land after the removal of disturbance factors, such as tillage and grazing. The core area comprises approximately 1000 ha, which accounts for 25% of the total area. In the core area, *Stipa bungeana* is the most extensive; the dominant grass species are *Stipa grandis* and *Stipa bungeana*, and the dominant forb species are *Thymus mongolicus* and *Artemisia sacrorum*.

When the grazing is ended, the succession of *Stipa bungeana* grassland with different degrees of degradation will start again from each retrograde degradation stage, and the whole succession process will be driven by the fierce competition between the species. Although *Thymus mongolicus* does not become the main auxiliary species of zonal vegetation and the vegetation climax community in the natural succession process, it appears to be the dominant species in degraded grassland and plays an important role in the succession process. When a *Stipa bungeana* community is

degraded to a *Thymus mongolicus* community, it needs approximately 14 years (shady slope) to 19 years (sunny slope) to be restored to a *Stipa bungeana* community. This relatively long succession process is shown in Fig. 2 and occurs in the following sequence: *Thymus mongolicus* (5–7 years, *Stipa bungeana* occupying the bare space)–*Thymus mongolicus* + *Stipa bungeana* (6–8 years, *Stipa bungeana* recovery stage)–*Stipa bungeana* + weed plants (3–4 years, *Stipa bungeana* recovery stage)–*Stipa bungeana* community (Zou and Guan, 1997). Therefore, we chose to study a *Thymus mongolicus* community, which is very important in the succession process, and compared it to degraded grassland with 2 years nongrazing in order to study the role of a *Thymus mongolicus* community in the succession sequence. In particular, the 2 years grassland is a degraded *Stipa bungeana* community.

2.2 Sample collection

The soil samples were obtained during August 2016 from the core area when the biomass had reached its peak. Three replicate 20 m × 20 m plots were established for each site, in total there were 9 plots (Fig. 3). These Re-35yrs and Ug-2yrs plots were selected for similar latitude, longitude, elevation, slope aspect, and slope gradient (Table 1). The distance between the sampling plots within each sampling site was not less than 20 m. Three 1 m² quadrats were randomly established across each plot to assess heterogeneity. Undisturbed soil was collected simultaneously using a 5-cm diameter soil auger and composited. The soil samples were obtained from 0–20 cm and 20–40 cm depths. Soil was layered and stored in sterilized plastic containers for aggregate fraction. The soil samples were placed on ice for transport to the laboratory.

In the laboratory, the undisturbed soil samples were gently broken up along natural fractures and passed through an 8 mm sieve after removing the large roots and stones. The prepared soil samples were fractionated using a dry-sieving method that was used to separate soil fractions to minimize the effects of wet-sieving method on microbial communities and produce aggregate fractions along natural points of weakness (Jiang et al., 2017). The fresh soil samples (150 g) were sieved through a series of meshes, >5 mm, 5–3 mm, 3–2 mm, 2–1 mm, 1–0.25 mm and <0.25 mm, by shaking at 250 r min^{−1} for 3 min. The prepared soil samples stored at −80°C were used for determining PLFA content.

Fig. 2 The evolvement of uncultural land natural rehabilitation in Yun-wu wilderness area.

Fig. 3 Setup of soil collection in each site

2.3 Laboratory analysis

Microbial community structure was measured by PLFA analysis based on the procedures described by Bardgett and McAlister (1999), which was further described by Pollierer et al. (2015), with modification. Phospholipid fatty acid (PLFA) is a biomarker that can be extracted from cell membranes synthesized by various biochemical pathways and quantitatively reflects the total biomass of diverse microbial groups. Due the fact that PLFA breaks down quickly upon cell death, it also reflects microbial biomass due to organisms with high prolificacy (Janine et al., 2005). An external standard of 19:0 methyl ester was used for quantification (Frostegård et al., 1993), and the amount of PLFA was expressed as nmol g^{-1} of dry soil. Briefly, fatty acids were extracted from 3.0 g of freeze-dried soil using a solution containing citrate buffer, chloroform, and methanol. The PLFAs were separated from neutral and glycolipid fatty acids by solid-phase-extraction chromatography. After mild alkaline methanolysis, the PLFAs were analyzed using a gas chromatograph (GC7890A, Agilent Technologies Inc., Wilmington, USA) equipped with MIDI

Sherlock software (version 4.5; MIDI Inc., Newark, USA).

The sum of all the biomarkers was used as a proxy for the total microbial biomass (total PLFAs). The saturated fatty acid 16:0 exists widely in various groups and is often used to evaluate the total microbial biomass. Zelles (1999) reported that specific PLFA signatures can serve as indicators of specific microbial groups. Total PLFAs were obtained by adding the contents of all fatty acids detected in each sample. The PLFAs, including i13:0, a13:0, i14:0, etc., were selected to represent Gram-positive bacteria, while the PLFAs 14:1, cy19:0, etc. were grouped as representative of Gram-negative bacteria. Cyclopropyl branched chain PLFAs (PLFA cy17:0 and cy19:0) come only from bacteria, especially anaerobic species. Therefore, the concentration of PLFA can indicate the relative dominance of specific microbe groups (Luo et al., 2018; Liao et al., 2018). F/B ratio (ratio of fungal to bacterial biomass) and stress indicators [cy/pre ratio (ratio of cyclopropyl PLFAs (cy17:0 and cy19:0)) to precursors PLFAs (16:1w7c and 18:1w7c)]. The classification of the PLFAs is shown in Table 2.

Table 1 Description of soil sample site.

Soil sample site	Site No.	Altitude (m)	Dominant species
<i>Thymus mongolicus</i> community	1	2024	<i>Thymus mongolicus</i> ; <i>Leymus secalinus</i> ; <i>Artemisia scoparia</i> ; <i>Stipa bungeana</i>
	2	2045	
	3	2077	
Nongrazing grassland	4	1987	<i>Stipa bungeana</i> ; <i>Artemisia sacrorum</i> ; <i>Potentilla acaulis</i> ; <i>Heteropapus altaicus</i>
	5	1972	
	6	1923	

Table 2 Characterization of microbial phospholipid fatty acids (PLFA).

Microbial group	Specific PLFA markers
Fungi	18:1 ω 9c, 18:2 ω 6,9, 18:1 ω 9t
Bacteria in general	cy19:0, 18:1 ω 7, cy17:0, 16:1 ω 9, 16:1 ω 7t, 16:1 ω 5, i17:0, a17:0, 17:0, a16:0, i16:0, 16:0, i15:0, 15:0, 14:0, a14:0, i14:0
Aerobes	16:1 ω 9, 16:1 ω 7, 16:1 ω 7t,
Anaerobes	cy19:0, cy17:0,
Gram-positive bacteria	a17:0, i16:0, a16:0, i15:0, a15:0, a14:0, i14:0,
Gram-negative bacteria	cy19:0, 18:1 ω 7, cy17:0, 16:1 ω 7, 16:1 ω 9,
Methane-oxidizing bacteria	18:1 ω 8c, 16:1 ω 5c,
Actinomycetes	10Me18:0, 10Me17:0, 10Me16:0,
Protozoa	20:4 ω 6
cy/pre ratio	(cy17:0 + cy19:0)/(16:1 ω 7 + 18:1 ω 7)
S/M ratio	Saturated/Monosaturated (20:0, 18:0, 17:0, 16:0, 15:0, 14:0)/(18:1 ω 9t, 18:1 ω 9c, 18:1 ω 8c, 18:1 ω 7, 17:1 ω 8c, 16:1 ω 9, 16:1 ω 7, 16:1 ω 7t, 16:1 ω 5, 16:1 ω 5c)
G ⁺ /G ⁻ ratio	Gram-positive bacteria/Gram-negative bacteria
F/B ratio	Fungi/bacteria

(Kroppenstedt, 1985; Federle et al., 1986; O'Leary, 1988; Wilkinson, 1988; Zelles et al., 1995; Frostegård and Bååth, 1996; Ringelberg et al., 1997; Kieft et al., 1997; Bossio et al., 1998)

2.4 Statistical analysis

Variety statistical analyses were performed using SPSS 20.0 (SPSS Inc., Chicago, USA), to identify the effects of the successional grassland restoration on total PLFA, main microorganism, main bacteria, G⁺/G⁻ ratio, F/B ratio and cy/pre ratio. One-way analysis of variance (ANOVA) with restoration years and soil aggregate sizes as the main factors were employed to test the significance of the mean differences. The level set for a significant difference was $p < 0.05$. A univariate procedure was used to examine the normality of the distribution of the observed data before conducting the ANOVA analysis. When the ANOVA test indicated a significant effect at $p < 0.05$, the means of each soil variable for each classification factor were assessed using the multiple-mean comparison that was assessed using the Benjamini False Discovery Ratio method. The graphs were plotted using Sigmaplot 12.5 (Systat Software, San Jose, USA). PCA (principal component analysis) was used to analyze the differences in the PLFA composition between soil aggregate size and plant restoration years.

3 Results

3.1 Aggregate sizes distribution

The distribution of soil aggregate sizes in Re-35yrs and Ug-2yrs were similar (Fig. 4). Aggregates with sizes of 0.25–1 mm were most common (33.25%-Re-35yrs-0-20 cm, 36.19%-Re-

Fig. 4 Distribution characteristics of soil aggregate sizes' percentage.

35yrs-20-40 cm; 39.66%-Ug-2yrs-0-20 cm and Ug-2yrs-20-40 cm 33.81%). In order from the most to least prevalent sizes: 0.25–1>5>0.25>1–2>3–5>2–3 mm.

3.2 Soil organic carbon (SOC) and total nitrogen (TN)

In Fig. 5, Re-35yrs had higher SOC concentrations that varied between 23.18 and 32.32 g kg⁻¹, there has no significant differences between soil aggregate sizes. The SOC concentrations in Ug-2yrs ranged from 8.22 to 11.08 g kg⁻¹, representing just one third that of Re-35yrs, and there was no consistent trend associated with soil depth. The SOC concentration for soil aggregate sizes <0.25 and >5.00 mm

Fig. 5 Distribution characteristics of soil organic carbon.

were very different in the two soil depths.

As seen in Fig. 6, Re-35yrs had higher TN concentrations than Ug-2yrs, with values between 2.68 and 3.38 g kg⁻¹, and there was no significant difference between soil aggregate sizes. There was generally a downward trend in soil depth, except at the 2.00–3.00 mm aggregate size. However, the TN concentrations in Ug-2yrs were 0.79–1.35 g kg⁻¹, which were just one third those of Re-35yrs. The concentrations were similar between two soil depths, except for at the 0.25–1.00 mm and 3.00–5.00 mm.

3.3 Total PLFA

As seen in Fig. 7, Re-35yrs had higher total PLFA concentra-

tions that varied between 2.93 and 50.49 nmol g⁻¹. These concentrations were 1.48–9.77 times higher than those in Ug-2yrs (1.97–5.17 nmol g⁻¹). In the two communities, 3–5 mm and 1–2 mm had the highest concentrations among the different soil aggregates, respectively. There was generally downward trend in the PLFA concentration with soil depth, and the total PLFA decreased from topsoil (0–20 cm) to subsoil (20–40 cm). As soil aggregates increased, the total PLFA gradually decreased. In Re-35yrs, the PLFA concentrations in 2–3 and 3–5 mm were significantly increased, peaking in 3–5 mm aggregates before decreasing. However, in Ug-2yrs, there was a sharp upward trend in the 0.25–1 and 1–2 mm aggregates, with the highest value reached in the 1–2 mm aggregates, before gradually dwindling.

Fig. 6 Distribution characteristics of soil total nitrogen.

Fig. 7 The distribution characteristics of total PLFA in different soil aggregates.

3.4 Main microorganisms

In Fig. 8, main microorganisms represent the highest proportion of bacterial concentration in both Re-35yrs (3.70–6.12 mol%) and Ug-2yrs (2.42–7.44 mol%). The proportions of unknown (Re-35yrs 0.19–4.15 mol%), actinomycetes (Re-35yrs 0.34–0.71 mol%; Ug-2yrs 0.23–0.91 mol%) and fungi (Re-35yrs 0.41–1.34 mol%; Ug-2yrs 0.46–1.35 mol%) followed, with protozoa representing the lowest proportion. Among the different soil aggregates, the proportion of bacteria in Re-35yrs was not obvious, except at 0.25–1 mm. However, the Ug-2yrs samples showed a significant upward trend, and the highest proportion of bacteria was observed in 1.00–2.00 mm aggregates, before gradually declining. The trends of actinomycetes and fungi were the same as those of bacteria, but the amplitudes were not obvious. Compared with the proportion of main microorganisms, the protozoa concentration was much lower, with no protozoa found in Re-35yrs (0–20 cm) and Ug-2yrs (20–40 cm).

3.5 Main bacteria

In Fig. 9, the proportion of main bacteria in Re-35yrs (0.17–1.82 mol%) was higher than that in Ug-2yrs (0.24–2.44 mol%). The proportion in the samples at 0–20 cm soil depth was higher than at 20–40 cm. The proportion of main bacteria in <0.25 mm and >5.00 mm aggregates was lowest and increased in 3–5 mm, 1–2 mm and 2–3 mm aggregates, with the highest value reached in 1–2 mm aggregates. In Re-35yrs, Gram-positive bacteria had the highest values (0–20 cm: 0.94–1.79 mol% and 20–40 cm: 0.88–1.52 mol%), with Gram-negative bacteria taking second place, followed by anaerobes and aerobes (anaerobes>aerobes), with the lowest being methane-oxidizing bacteria (0–20 cm: 0.25–0.76 mol% and 20–40 cm: 0.17–0.39 mol%). However, in Ug-2yrs, Gram-negative bacteria represented the highest proportion of bacteria (0–20 cm: 0.88–2.78 mol% and 20–40 cm: 1.07–2.44, mol%), followed by Gram-positive bacteria and aerobes, with anaerobes and methane-oxidizing bacteria representing the lowest proportions (0–20 cm: 0.24–0.74 mol% and 20–40 cm: 0.28–0.58 mol%).

3.6 Main physiologic indicators

As shown in Table 3, the ratios of G^+/G^- in Ug-2yrs were significantly lower than in Re-35yrs. The ratios in Ug-2yrs were approximately 0.5, while the values in Re-35yrs fell between 0.891 and 1.633. In the 20–40 cm soil depth samples of Re-35yrs, the G^+/G^- ratios in the <0.25 mm, 0.25–1.00 mm, 1–2 mm aggregates were higher than those in the other soil aggregates. The F/B ratios were not significantly different between the 0–20 cm and 20–40 cm samples in Re-35yrs, and all the ratios had values of 0.17. However, the F/B ratios in Ug-2yrs decreased with increasing soil aggregate size, and the F/B ratios were 0.83 and 0.13 at different soil depths, respectively. The cy/pre ratios were greater at 0–20 cm soil

depth than at 20–40 cm, and the cy/pre ratios of the 20–40 cm soil depth samples in Ug-2yrs were higher than the other samples (0.54–0.65). The S/M ratios in Re-35yrs were significantly higher than in Ug-2yrs, and there was no significant difference between the two soil layers.

3.7 Analysis of PLFA in different soil aggregate

A discriminatory analysis of bacteria in different soil aggregates was conducted by identifying the contribution of bacterial PLFA concentrations to the principal components. Thus, the load was analyzed by principal component. If the load is large, the PLFA distribution of certain bacteria in soil aggregates can be considered to be wide (Table 4).

As Fig. 10 shows, in the 0–20 cm soil layer, the 1–2 mm and <0.25 mm aggregates of Re-35yrs had the highest scores on the first principal component. The 3–5 mm and 2–3 mm aggregates had the highest scores on the second principal component. After composite score calculation, the 3–5 mm aggregates (2.00) had the highest score, followed by the 2–3 mm aggregates (1.78), and the >5 mm aggregates had the lowest score. Therefore, in the 0–20 cm soil layer, the first principal component represents the 3–5 mm and 2–3 mm aggregates, and the second principal component represents the >5 mm aggregates. In the 20–40 cm soil layer, the 3–5 mm aggregates had the highest scores on the first principal component, and the >5.00 mm aggregates had the highest scores on the second principal component. Thus, in the 20–40 cm soil layer, the first principal component also represents the 3–5 mm and 2–3 mm aggregates, and the second principal component represents the >5.00 mm and 2–3 mm aggregates. Thus, among the different soil aggregates of Re-35yrs, the 3–5 mm aggregates had a greater diversity than the other aggregate sizes, followed by the 2–3 mm and >5 mm aggregates.

As Fig. 11 shows, in the layer of 0–20 cm, the 1–2 mm and 2–3 mm aggregates of Ug-2yrs had the highest scores on the first principal component. The 3–5 mm aggregates had the highest score on the second principal component. After composite score calculation, the 1–2 mm aggregates (5.45) had the highest value, followed by the 2–3 mm aggregates (3.28), and the 3–5 mm aggregates (0.98) had the lowest value. Therefore, in the 0–20 cm soil layer, the first principal component represented the 3–5 mm and 2–3 mm aggregates and explained 85.86% of the variance. In the layer of 20–40 cm, the 1–2 and 2–3 mm aggregates had the highest scores on the first principal component. The 2–3 mm aggregates had the highest scores on the second principal component. After composite score calculation, the 1–2 mm aggregates had the highest value, followed by the 2–3 mm aggregates. In the other words, the first principal component represented the 1.00–2.00 mm and 2–3 mm aggregates and explained 78.56% of the variance. Thus, in different soil aggregates of Ug-2yrs, most soil microbes lived in the 1–2 and 2–3 mm aggregates.

Fig. 8 The distribution characteristics of main microorganism in different soil aggregates.

Fig. 9 The distribution characteristics of main bacteria in different soil aggregates.

Table 3 The distribution characteristics of main physiological indicators in different soil aggregates.

Community	Soil aggregate size	G ⁺ /G ⁻		F/B	
		0–20 cm	20–40 cm	0–20 cm	20–40 cm
Th.M	< 0.25 mm	1.12±0.38	0.91±0.31Aa	0.24±0.11Aa	0.17±0.02Aa
	0.25–1mm	1.63±0.82	1.26±0.44Aa	0.18±0.02Aa	0.23±0.14Aa
	1–2 mm	1.29±0.66	1.48±0.50Aab	0.18±0.01Aa	0.17±0.03Aa
	2–3 mm	1.00±0.40	1.55±0.41Aa	0.13±0.05Aa	0.15±0.04Aa
	3–5 mm	0.89±0.28	1.22±0.73Aa	0.11±0.06Aa	0.18±0.01Aa
	>5 mm	1.16±0.65	1.54±0.33Aa	0.17±0.07Aa	0.15±0.01Aa
Mean		1.18	1.33	0.17	0.17
No.G	< 0.25 mm	0.39±0.12	0.50±0.12Aa	0.25±0.09Aa	0.16±0.07Aa
	0.25–1 mm	0.43±0.22	0.53±0.21Aa	0.19±0.05Aa	0.17±0.02Aa
	1–2 mm	0.50±0.14	0.57±0.18Aa	0.18±0.06Aa	0.14±0.01Aa
	2–3 mm	0.47±0.23	0.59±0.17Aa	0.16±0.07Aa	0.12±0.03Aa
	3–5 mm	0.43±0.10	0.56±0.22Aa	0.15±0.23Aa	0.10±0.04Aa
	>5 mm	0.43±0.11	0.53±0.31Aa	0.17±0.011Aa	0.12±0.06Aa
Mean		0.44	0.55	0.18	0.13
Community	Soil aggregate size	cy/pre		S/M	
		0–20 cm	20–40 cm	0–20 cm	20–40 cm
Th.M	< 0.25 mm	0.50±0.11	0.27±0.12Aa	0.94±0.41Aa	0.86±0.21Aa
	0.25–1mm	0.44±0.09	0.77±0.12Aa	0.70±0.29Aa	0.99±0.14Aa
	1–2 mm	0.54±0.07	0.68±0.31Aa	0.69±0.36Aa	1.01±0.65Aa
	2–3 mm	0.67±0.34	0.75±0.22Aa	0.68±0.16Aa	0.81±0.41Aa
	3–5mm	0.42±0.26	0.72±0.34Aa	0.91±0.32Aa	0.88±0.19Aa
	>5 mm	0.66±0.41	0.74±0.36Aa	0.88±0.12Aa	0.94±0.28Aa
Mean		0.54	0.65	0.80	0.92
No.G	< 0.25 mm	0.71±0.41Aa	1.08±0.63Aa	0.51±0.22Aa	0.70±0.12Aa
	0.25–1mm	0.51±0.21Aa	0.77±0.21Aa	0.61±0.35Aa	0.66±0.14Aa
	1–2 mm	0.59±0.36Aa	0.74±0.26Aa	0.55±0.12Aa	0.63±0.23Aa
	2–3 mm	0.56±0.22Aa	0.77±0.43Aa	0.57±0.36Aa	0.65±0.36Aa
	3–5 mm	0.58±0.17Aa	0.81±0.29Aa	0.55±0.08Aa	0.67±0.24Aa
	>5 mm	0.56±0.23Aa	0.77±0.31Aa	0.54±0.06Aa	0.67±0.34Aa
Mean		0.59	0.82	0.55	0.66

Note: G⁺/G⁻: Gram-positive bacteria/Gram-negative bacteria. F/B: Fungi/Bacteria. cy/pre: cyclopropyl(cy17:0 + cy19:0)/precursors(16:1ω7 + 18:1ω7). S/M: Saturated fatty acids/Monounsaturated fatty acids.

4 Discussion

4.1 Impacts of vegetation restoration on soil microorganism group

Soil microbial structural diversity is affected by a variety of factors, such as land uses, farming history and vegetation

types. Ug-2yrs had a lower total PLFA, whose concentration was approximately one-tenth that of Re-35yrs. However, the bacterial PLFA proportions of Ug-2yrs were much higher than those of Re-35yrs, with actinomycetes and fungi proportions being lower than the bacterial proportions and with protozoa having the lowest proportions in those samples. A history of overgrazing is the main reason that led to Ug-2yrs having

Fig. 10 Principal component analysis (PCA) of PLFA in *Thymus mongolicus* community soil.

lower soil microbial diversity. Vegetation restoration cannot be effective with short-term grazing. Lower plant biomass results in less transformation of plant carbon to soil carbon. If a carbon source cannot replenish the soil to meet the requirements of its microorganisms, the soil microbial diversity balance will be disrupted (Briar et al., 2011; Zhang et al., 2016). Compared with Re-35yrs, the following species of PLFA in Ug-2yrs were mostly absent: 20:4 ω 6, 16:1 ω 7, a17:0, i18:0, 17:0, 17:1 ω 8c, etc. In other words, although the number of bacteria in Ug-2yrs was greater, there were fewer species than in Re-35yrs, and there were no protozoa in Ug-2yrs. The ratio of fungi to bacteria (F/B) is used to estimate the relative energy utilization of fungi and bacteria. Bacterial energy utilization is characterized by rapid utilization, taking advantage of residue or substrate, and has a higher turnover rate (Six et al., 2006; Briar et al., 2011; Daynes et al., 2012; Waring et al., 2013). Moreover, the F/B ratio also reflects the quality of the organic matter in a soil system. The F/B ratio is an

important indicator in evaluating the control ability and buffering capacity of an ecosystem. High F/B values suggest a superior soil organic matter condition. In Re-35yrs, with a high level of soil organic carbon concentration, fungi formed the major microbial group attending to plant residue decomposition. Fungi are the main agents of organic matter decomposition, and the fungal community affects the rate of decomposition and C cycling (Mäkipää et al., 2017). In Re-35yrs, the soil microbial community has already shifted from bacteria groups to fungal groups. The energy utilization has also changed from a high level turnover rate (bacteria) to a fixed energy consumption (fungi). However, in Ug-2yrs, the energy utilization of the soil microbial community remained in a bacterial growth stage, with high speed decomposition and transformation.

In Re-35yrs, there are two main characteristics of the soil microbial diversity. First, although total PLFA in Re-35yrs was higher than in Ug-2yrs, the bacterial proportion was lower, and

Fig. 11 Principal component analysis (PCA) of PLFA in nongrazing grassland soil.

Table 4 General score in different soil aggregate.

Communities	Soil layer (cm)	General score					
		<0.25 mm	0.25–1 mm	1–2 mm	2–3 mm	3–5 mm	>5 mm
<i>Thymus mongolicus</i>	0–20	–0.95	–3.76	0.07	1.78	2.00	0.85
	20–40	–5.75	–1.07	0.95	1.36	3.03	1.48
Nongrazing	0–20	–5.90	–3.68	5.45	3.28	0.97	–0.09
	20–40	–5.44	–3.76	6.61	4.25	–0.19	–1.45

unknown microorganisms accounted for a large percentage in a few of the soil aggregates, just lower than percent accounted for by bacteria. External nutrients are the primary reason for the different diversity conditions between the two communities (Trivedi et al., 2013; Chen et al., 2017). Ug-2yrs is in a transition stage of balanced mineralization and accumulation due to its short duration of restoration. Mineralized organic nutrients can only provide support to bacteria

and cannot completely satisfy the growth and development of various microorganism groups (Fierer et al., 2007; Leff et al., 2015; Trivedi et al., 2017). Second, the proportion of Gram-positive bacteria was higher than the proportion of Gram-negative bacteria in Re-35yrs.

This study indicated that environment adaptability influences soil physiologic indicators to varying degrees. In addition, microorganism continually changed their regulation

of pathways as their environment changed. To adapt to the changing environment, soil microbes were forced to make physiologic adaptations in response to varying stress from their environment. For example, physiologic responses of soil microorganisms change when faced with environment stress, such as low nutrients or toxic compounds, and these responses are reflected in their chemical components (Chen et al., 2015). To determine the effects of environmental stress on microbial physiologic structure, many researchers have begun to use microbiological PLFA indicators to reflect the physiologic changes of the microbial community (Allison et al., 2005; Luo et al., 2018). Those studies found that when the bacterial growth stage changed from logarithmic growth to stable growth, precursor PLFAs were converted to PLFAs with cyclopropyl groups. This conversion implies that some source of stress was present in the environment, such as restrictions on carbon resources and oxygen. For example, when the growth stage of Gram-negative bacteria changes from exponential growth to slow static growth, soil microorganisms will convert some monoene PLFA into cyclopropyl PLFA. Thus, the ratio of cyclopropyl PLFA (cy17:0 and cy19:0) to their precursor PLFA (16:1 ω 7 and 18:1 ω 7) can be used as an indicator of the physiologic status of a Gram-negative bacterial community.

4.2 Impacts of soil aggregates sizes on soil microorganism group

Impacts of aggregation on the soil microbiome can also depend on aggregate size, as microaggregates (<250 μ m) are reported to harbour relatively dynamic and diverse microbial communities, whereas microbial communities of macroaggregates (>250 μ m) are more stable (Upton et al., 2019). The ratio of G^+/G^- in Re-35yrs was two times greater than that in Ug-2yrs. G^+/G^- is the ratio of Gram-positive to Gram-negative bacteria. An increasing G^+/G^- ration suggests a transformation of Gram-negative bacteria groups to Gram-positive groups. As carbon sources are reduced, the soil chemotroph community (mostly containing gram-negative bacteria) turns into a chemotroph-heterotroph community or from a nutrient-abundant to a nutrient-deficient condition (Richardson et al., 2011; Mackay et al., 2017). Some studies have indicated that differences in microbial diversity are mainly reflected in Gram-negative bacteria and protozoa and are caused by different vegetation and land use and by differences in the living environment and various stresses (Zhang et al., 2017). Long-term vegetation restoration in Re-35yrs is conducive to improved soil structure and texture. The improvement in the soil environment provides the necessary conditions for the restoration of microbial diversity. However, the transformation of the community from nutrient-rich to heterotroph-dominated leads to a reduction in soil microbial, especially in small aggregates (Sarker et al., 2018). This observation is consistent with our results. In Re-35yrs, total PLFA increased as soil aggregate size increased. However, in the nongrazing community, the trend followed a normal distribution, with highest levels of PLFA in the 1–2 mm and

2–3 mm aggregates.

The cyc/prec value is the ratio of cyclopropyl PLFAs to their precursors. This ratio represent the stress level (including carbon source and oxygen limitation) of microorganisms in the soil system (Trivedi et al., 2015). Generally, the values of cyc/prec in 2–3 mm and 3–5 mm aggregates were lower than those in <0.25 mm, 0.25–1 mm, 1–2 mm and >5 mm aggregates, and the values from 0 to 20 cm samples were lower than those from 20 to 40 cm. This result indicates that microorganisms suffered significantly less stress in large aggregates than in small aggregates. There are also likely advantages of localization at the aggregate exterior, such as greater access to carbon and nutrient flow from the bulk soil. Furthermore, factors such as aggregate size may influence microbial community composition and activity (Schlüter et al., 2018; Upton et al., 2019). In Re-35yrs, the most abundant microbial diversity was found in the 3–5 mm aggregates, followed by that in the 2–3 mm aggregates. However, in the nongrazing community, a similar level of diversity was found in 1–2 mm and 2–3 mm aggregates. With increasing years of vegetation restoration, soil aggregate stability is gradually enhanced. Soil microbial communities begin to select stable aggregates in which to live, as is consistent with a response to low nutrients (Bonanomi et al., 2017). The S/M ratio refers to the ratio of saturated fatty acids to monounsaturated fatty acids. This ratio reflects the response to low nutrients and is also an indicator of microbial nutritional effectiveness. A value of S/M greater than 1 indicates that the soil has a high concentration of organic carbon and that there has been organic carbon input (Bell et al., 2015; Zeng et al., 2015; Chen et al., 2017). Because mega-aggregates and macro-aggregates generally contain more labile substrates principally derived from plant residues and exhibit higher fungal biomass than micro-aggregates (Jiang et al., 2017), these observation support our hypothesis that aggregate-related changes in soil microbial communities improve soil aggregate stability. As the results show, the S/M ratios in Re-35yrs were higher than in Ug-2yrs, with the exception of 1–2 mm aggregates from the 20–40 cm soil layer (S/M = 1.001). The other values for the S/M ratio fell between 0.988 and 0.677 and 0.507–0.699. Years of vegetation restoration provides adequate nutrition, leading to lower nutrient stress among soil microorganisms in Re-35yrs compared to those in Ug-2yrs.

5 Conclusions

After years of natural restoration, the biological and structural diversity in Re-35yrs was significantly greater than that in Ug-2yrs. In different plant communities, the stress of soil microorganisms in aggregates of large particle size was significantly lower than in aggregates of small particles, especially in the 2–3 mm and 3–5 mm aggregates. Natural restoration has a positive effect on soil microbial diversity was generally higher in large particle size aggregates, which leads to low environmental stress and strong stability. This new insight into interactions at the soil structure/biology interface

leads to addressing further related questions such as how soil aggregation may impact microbial community composition and longer-term adaptations in response to environmental pressures.

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