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# Effects of litter chemical traits and species richness on soil carbon cycling changed over time

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Litter decomposition is the main driver of nutrient cycling process in terrestrial ecosystems. Afforestation completely altered vegetation composition and litter species, disrupting the long-term carbon balance in grassland ecosystem. However, there is a lack of understanding of how litter mixing effect (LME) affects soil carbon cycling in afforested ecosystem. Here, we investigated the effects of litter richness and quality of tree, shrub, and grass species and their litter mixture on soil CO<sub>2</sub> fluxes. The results showed that cumulative soil CO<sub>2</sub> flux in the early stage (1–28 days) was 1.75 times higher than that in the late stage (29–113 days), indicating litter decomposition was intensive at first and then decreased with time. Soil carbon flux changed with decomposition stages. In the early-stage of decomposition, soil CO<sub>2</sub> flux increased with the concentrations of litter carbon, nitrogen and condense tannin. In the late phase of decomposition, all litter chemical traits were negatively related to the soil carbon flux. Additionally, plant litter richness was negatively correlated to early-stage soil CO<sub>2</sub> flux, whereas it was positively related to late-stage soil carbon flux. Our results provide evidence that long-term carbon balance in grassland ecosystems was interrupted by afforestation, and the dominant litter chemical traits that controlling soil carbon cycling changed over time.

## KEYWORDS

agro-grazing ecotone, non-additive litter-mixing effect, soil carbon dynamic, litter chemical traits, afforestation

## Introduction

Litter decomposition is the primary engine of nutrient recycling in terrestrial ecosystems, influencing nutrient availability and soil carbon dynamics (Swift et al., 1979; Aerts, 1997; Mao et al., 2015). Over the past few decades, studies have focused on the decay of single litter species (Gartner and Cardon, 2004). Indeed, the decomposition process in natural ecosystems is a mix of various plant litter species (Hättenschwiler et al., 2005). Studies suggest that litter species interact with each other

when decomposing, implying that the decomposition rate of litter mixtures are accelerated or decelerated by litter mixing, namely litter mixing effects (LME) (Gartner and Cardon, 2004; Singhal et al., 2021).

Studies have generalized that LME can be either synergistic or antagonistic, which means the decomposition dynamics of litter mixture was faster or slower than expected (Gartner and Cardon, 2004; Hättenschwiler et al., 2005). The underlying hypotheses of LME include 1) nutrient transfer among litter types, 2) complementary resource use, and 3) stimulatory or inhibitory influences of specific litter compounds (Gessner et al., 2010). Some studies showed that non-additive litter effect of the mixed-species would increase with litter species richness (Hättenschwiler et al., 2005). Other studies reported that species identity within litter mixtures surpassed litter richness as the main driver of LME (Wardle et al., 1997; Zak et al., 2003). For example, it has been revealed that antagonistic LME was strongest in three most-recalcitrant litter species along the diversity gradient from one to six species litter mixtures (Hättenschwiler and Gasser, 2005). More importantly, evidence suggested that LME may not occur if litter mixtures are composed of chemically similar species, it occurs frequently in chemically dissimilar species (Meier and Bowman, 2008).

Litter quality was generally represented by the concentration of nitrogen (N), carbon (C) and lignin (L) (Hättenschwiler and Jorgensen, 2010). Litter N accelerated the decomposition process, and litter L was partially retained and converted into humus stored in the soil (Taylor et al., 1989; Cotrufo et al., 2013; Li et al., 2021). However, a study showed that increasing lignin concentration increased photodegradation process (Austin and Ballare, 2010). This discrepancy might be because of the fact that other litter chemical traits, such as cellulose (Ce), phenols (Phe), and condensed tannin (Ct), could also be important in driving decomposition process (Hättenschwiler and Vitousek, 2000; Kraus et al., 2004; Yang et al., 2021). Indeed, there is evidence suggest that micronutrients prevent plant residue from being decayed, although they are presented in rather small amounts (Berg and Mcclaugherty, 2003). Additionally, although there are a great number of studies focusing on the effect of litter mixture on litter mass loss, little is known about on multi-trophic soil processes, such as soil carbon cycling. Therefore, to better understand the underlying mechanistic links between litter mixture chemistry and soil carbon dynamics, LME studies should assess many more litter chemical traits, particular for those that are not commonly considered.

Unfortunately, litter chemical components differed by tens to hundreds of orders, the role of these micronutrient was usually masked by macronutrients (Berg and Mcclaugherty, 2003). Additionally, litter chemical traits themselves interacted with each other. For instance, cellulose can be bound by lignin to form stable compounds and tannins can precipitate N substrates in protein-tannin complexes (Kraus et al., 2004; Talbot et al., 2012). Therefore, to better understand how litter chemical traits influencing LME, we employed principal component analysis

(PCA) to characterize litter chemical composition, which has been carried out effective in several studies (Epps et al., 2007; Meier and Bowman, 2008; Meier and Bowman, 2010). PCA was adopted because it 1) included the presence and quantity of various litter chemical properties; 2) scaled the values of variables to one before PCA analysis to increase sensitivity of micronutrients; 3) considered interactions among litter chemical traits.

Northern China's agro-pastoral ecotone is a typical ecologically fragile semi-arid zone, which is a key area for the ambitious large-scale afforestation efforts Three-North Shelterbelt Development Program (TNSDP) (Hong et al., 2018; Wang et al., 2020). The tree *Populus simonii*, shrubs *Artemisia halodendron* and *Caragana microphylla* were commonly planted either singly or together during afforestation because of their high tolerance against drought, wind erosion, and sand burial (Li et al., 2013b; Li et al., 2017; Luo et al., 2020). Afforestation and shelterbelt establishment has greatly reduced soil erosion and sandstorms (Zeng et al., 2008; Wang et al., 2019), however, the plantation of trees and shrubs thoroughly changed the vegetation composition, affected soil C availability, induced litter mixing interaction, and consequently altered the long-term C balance. This critical afforestation in grassland also provides a rare opportunity to investigate effects of tree, shrub, and grass litter mixing effect on soil carbon cycling. Following nutrients transfer and complementary concept (Meier and Bowman, 2008), we hypothesized that LME intensity would increase with litter species richness, and LME is strongest in four litter mixtures. As for litter chemical traits, we hypothesized that similar to litter C and N content, litter micronutrients such as litter Phe and Ct are also negatively related to the late-stage soil C flux.

## Materials and methods

### Study site

The study site is situated in Naiman Desertification Research Station, Chinese Academy of Sciences, an area within the agro-grazing ecotone in eastern Inner Mongolia, China (42°55'N and 120°41'E, 350 m above sea level) (Supplementary Figure S1). The long-term mean annual temperature and mean annual precipitation are 6.4°C and 360 mm, respectively. The mean annual wind speed is 3.4–4.1 m s<sup>-1</sup> (Zhao et al., 2006). The soil consists mainly of chestnut soil according to the Chinese classification and is a Haplic Calcisol according to the FAO (Su et al., 2006). Therefore, litter input and decomposition are important for improving soil quality and nutrient status in the region (Li Y. L. et al., 2013). Over the last 40 years, many agroforestry practices have been conducted to counteract soil erosion and reduce land degradation in this area (Li et al., 2017). Shrubs such as *Artemisia halodendron* and *Caragana microphylla*

play an important role in the re-establishment of degraded ecosystems (Zhu et al., 1989). *Artemisia halodendron*, a typical sub-shrub with a high ability to capture deep water, grows and propagates rapidly in sandy ecosystems due to its low nutrient requirements and capacity for vegetative propagation (Luo et al., 2020). *Caragana microphylla*, a pioneer leguminous shrub species for vegetation re-establishment, has been widely reported for its adaptation to windy and sandy environment (Zhang et al., 2006). The vegetation is thus characterized by grasses (e.g., *Pennisetum centrasiatium*), with scattered shrubs (e.g., *Artemisia halodendron* and *Caragana microphylla*) and windbreak tree belts of *Populus simonii*.

## Litter and soil sampling and incubation experiment

In September 2018, we sampled litter material of *Artemisia halodendron* (Ah), *Caragana microphylla* (Cm), *Pennisetum centrasiatium* (Pc) and *Populus simonii* (Ps). The litter material was brought back to the laboratory and oven-dried at 65°C for 24 h. To test how litter species richness and chemical composition affects the decomposition of litter mixture, we mixed four litter materials in all possible 2- species (1:1), 3- species (1:1:1), and 4- species (1:1:1:1) ways. Therefore, there were fourteen litter material type (Ah, Cm, Pc, Ps, AhCm, AhPc, AhPs, CmPc, CmPs, PcPs, AhCmPc, AhCmPs, AhPcPs, and AhCmPcPs). Two grams of litter material, following the natural yearly litter quantity in this region (Wang et al., 2018), were ball-milled into powder (GT300 Ball Mill, POWTEQ, Beijing, China) and then homogenized by sieving through a 0.25 mm mesh to minimize effect of size and structure of the litter material. In addition, there were five replicates in each leaf litter treatment. We collected top soil (0–10 cm) in a dune meadow, and then sieved (2 mm mesh), homogenized in the laboratory.

The prepared 2 g litter powder was mixed thoroughly with 200 g dry-weight soil in a 500-ml canning jar, and there were five replicates without litter addition for the control treatment. Thus, there were  $15 \times 5 = 75$  incubation jars. The incubation jars were kept at 21–25°C in an incubator (SPX-500; Jiangnan, Ningbo, China), and the soil-litter mixture inside the jar was maintained at 60% water-holding capacity through the gravimetric method. Moreover, the incubation here was kept for 113 days, which was based on our previous study showing that the CO<sub>2</sub> release rate was decreased to 0 when incubation duration exceeded 100 days (Yang et al., 2019).

## Analysis of the initial litter chemical traits

Litter C and N concentration were analyzed with element analyzer (Costech ECS 4010). Lignin concentration was analyzed

using a modified acetyl bromide method (Iiyama and Wallis, 1990). Soluble sugar (Ss) concentration was determined using the anthrone method (Helbert and Brown, 2002). Litter cellulose content was measured according to the acid hydrolysis method (Updegraff, 1969). The concentration of phenols was determined using the Folin-Ciocalteu method (Waterman and Mole, 1998). The concentration of condensed tannins was measured following the acid butanol method described by Hagerman (Porter et al., 1986). These chemical traits were selected because they were shown to have great influence on litter decomposition and soil C cycling (Taylor et al., 1989; Hättenschwiler and Vitousek, 2000; Talbot and Treseder, 2012).

## Data calculation and analysis

We evaluated soil C dynamics by measuring the C flux daily with an infra-red gas analyzer (Li-Cor 840A CO<sub>2</sub>/H<sub>2</sub>O) for the first 7 days, every second day from day 7 to day 21, and every 5th day from day 22 to day 72, and then every 15 days from day 73 to day 113. Data were collected every second for 2 min. Data from the middle 100 s were analyzed. The unary linear slope method was used to calculate the rate of CO<sub>2</sub> increase in the sample. The CO<sub>2</sub> release rate was calculated using the following formula:

$$F_c = \frac{dc'}{dt} \times \frac{V}{S} \times \frac{P_{av}}{R \times (T_{av} + 273)} \times (1000 - W_{av})$$

F<sub>c</sub>: CO<sub>2</sub> release rate; V: volume of the jar from which the soil portion is removed; R: gas constant; dc'/dt: slope--adjusted rate of CO<sub>2</sub> efflux change; W<sub>av</sub>: water vapor partial pressure in the jar during measurement period; P<sub>av</sub>: average atmospheric pressure in the jar; and T<sub>av</sub>: The average temperature in the jar. The total CO<sub>2</sub> emission efflux was calculated by plotting the CO<sub>2</sub> release rate versus time and obtaining the area under the curve.

The CO<sub>2</sub> release rate in 1–28 days was more than ten times that in 29–113 days. According to dynamics of CO<sub>2</sub> release rate, the whole culture process was divided into two stages: early stage (0–28 days) and late stage (29–111 days).

We compared the observed values and expected values to evaluate non-additive litter mixing effects (LME) using the following formula:

$$\text{Litter mixing effect} = \frac{\text{Observed}_{\text{CO}_2 \text{ flux}} - \text{Expected}_{\text{CO}_2 \text{ flux}}}{\text{Expected}_{\text{CO}_2 \text{ flux}}}$$

The expected CO<sub>2</sub> efflux of the litter mixture was the average CO<sub>2</sub> release efflux of component litter species in the litter mixtures. The observed CO<sub>2</sub> release efflux of the litter mixture was measured CO<sub>2</sub> release efflux.

Significant differences in litter chemical traits, cumulative CO<sub>2</sub> fluxes, and litter-mixing effects were detected by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) in SPSS version 17.0 (SPSS Inc., Chicago, IL, United States). Principal component analysis (PCA) was carried

TABLE 1 Initial litter chemistry of the single litter and litter mixture.

Chemical traits	Nitrogen (mg g <sup>-1</sup> )	Carbon (mg g <sup>-1</sup> )	Lignin (mg g <sup>-1</sup> )	Cellulose (mg g <sup>-1</sup> )	Soluble sugar (mg g <sup>-1</sup> )	Phenols (mg g <sup>-1</sup> )	Condense tannin (mg g <sup>-1</sup> )
Ah	17.74 ± 0.15 B	441.59 ± 0.15 B	160.37 ± 2.13 A	138.33 ± 0.91 C	48.15 ± 0.42 B	61.66 ± 0.24 A	18.93 ± 0.11 C
Cm	36.84 ± 0.14 A	445.22 ± 0.71 A	132.94 ± 1.82 C	137.06 ± 1.77 C	42.27 ± 0.52 C	36.76 ± 0.23 C	8.94 ± 0.24 D
Pc	7.42 ± 0.07 C	400.43 ± 0.59 D	116.72 ± 1.59 D	401.22 ± 1.75 A	12.90 ± 0.65 D	13.83 ± 0.19 D	30.49 ± 0.16 B
Ps	6.75 ± 0.17 D	410.33 ± 0.17 C	146.68 ± 1.32 B	177.15 ± 0.92 B	71.12 ± 0.37 A	57.89 ± 0.57 B	88.98 ± 2.19 A
AhCm	28.48 ± 0.36 a	446.28 ± 0.53 a	152.35 ± 0.53 a	121.35 ± 0.68 e	41.31 ± 0.64 d	37.78 ± 0.23 c	7.21 ± 0.33 f
AhPc	12.52 ± 0.20 c	420.82 ± 0.50 d	143.73 ± 0.79 b	306.86 ± 1.45 a	32.68 ± 0.77 e	40.85 ± 0.09 b	22.48 ± 0.46 d
AhPs	12.77 ± 0.18 c	426.07 ± 0.29 c	155.18 ± 0.94 a	167.32 ± 0.36 d	52.14 ± 0.17 a	55.92 ± 0.74 a	34.97 ± 0.22 a
CmPc	23.88 ± 0.25 b	425.77 ± 0.03 c	132.79 ± 0.81 d	210.51 ± 1.71 c	26.08 ± 0.71 f	24.11 ± 0.16 e	9.13 ± 0.10 e
CmPs	23.75 ± 0.22 b	429.53 ± 0.69 b	145.51 ± 1.88 b	169.59 ± 1.27 d	50.34 ± 0.73 b	30.76 ± 0.76 d	26.03 ± 0.05 c
PcPs	7.18 ± 0.06 d	401.40 ± 0.64 e	138.6 ± 1.07 c	294.72 ± 1.85 b	43.49 ± 0.40 c	30.90 ± 0.44 d	33.75 ± 0.56 b
AhCmPc	21.79 ± 0.54 a	425.63 ± 0.81 b	141.8 ± 2.11 b	259.1 ± 0.89 b	31.61 ± 0.30 d	32.79 ± 0.60 c	12.75 ± 0.02 d
AhCmPs	20.72 ± 0.07 b	431.04 ± 0.31 a	148.03 ± 0.78 a	172.08 ± 0.58 d	42.53 ± 0.76 a	40.08 ± 0.58 a	15.4 ± 0.73 b
AhPcPs	10.43 ± 0.12 e	415.69 ± 0.60 c	146.67 ± 0.78 a	124.81 ± 1.75 e	39.92 ± 0.69 b	41.01 ± 0.25 a	23.57 ± 0.32 a
CmPcPs	16.08 ± 0.16 d	417.35 ± 0.49 c	125.12 ± 1.72 c	212.4 ± 0.39 c	38.28 ± 0.39 c	30.92 ± 0.54 d	13.79 ± 0.48 cd
AhCmPcPs	18.41 ± 0.20 c	424.88 ± 0.80 b	150.99 ± 1.19 a	270.69 ± 0.52 a	40.9 ± 0.45 b	35.01 ± 0.33 b	14.19 ± 0.19 bc

Capital letters indicate the single litter difference; Lowercase letters indicate the litter-mixture difference, the difference among two species and among three-four species were shown respectively (ANOVA followed by LSD test,  $p < 0.05$ ). Ah: *Artemisia halodendron*, Cm: *Caragana microphylla*, Pc: *Pennisetum centrasiatium*, and Ps: *Populus simonii*.

out to show the litter chemical distance among litter species and mixtures. Structural equation model (SEM) was used to evaluate the direct and indirect effects of litter species richness and litter chemical traits on CO<sub>2</sub> flux. The PCA and SEM were conducted using package “vegan” and “lavaan” by R software version 4.0.3 (R Development Core Team, 2014).

## Results

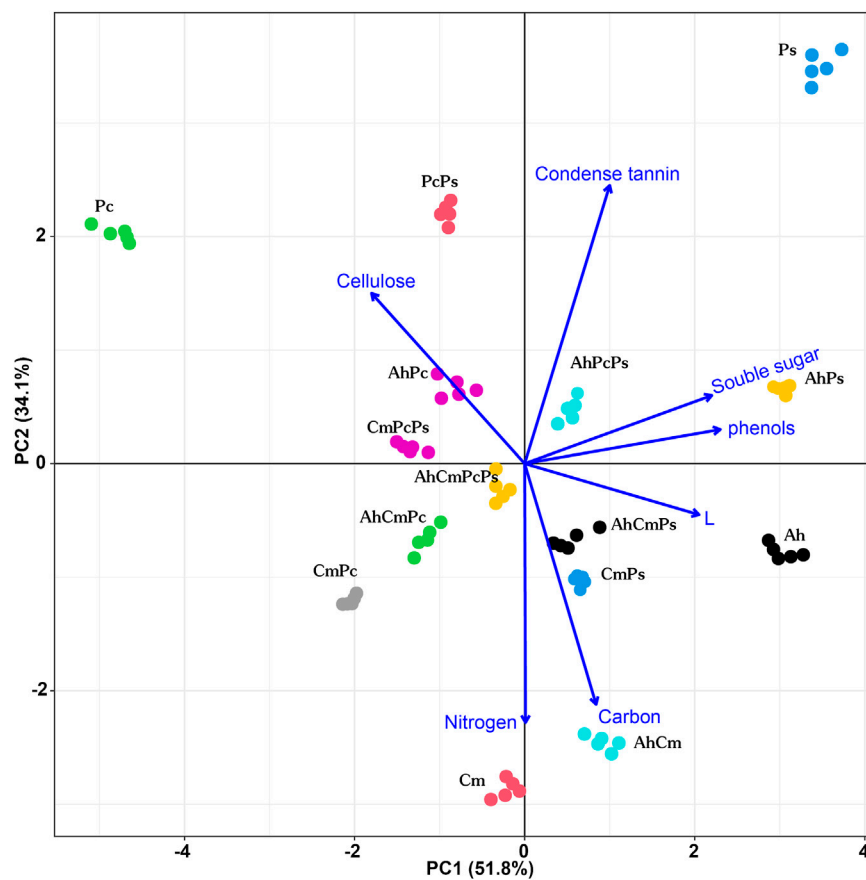
### Initial litter chemistry of four single plant species and litter mixtures

The initial nutrient content of single-species litters and litter mixtures are shown in Table 1 and Figure 1. All seven initial litter chemical properties significantly differed among four single-species. The N content in Cm plant litter was  $36.84 \pm 0.14 \text{ mg g}^{-1}$  which was 2.1, 5, and 5.5 times higher than that in Ah, Pc, and Ps litter. The C content in Cm ( $445.22 \pm 0.71 \text{ mg g}^{-1}$ ) was also higher than that in Ah ( $441.59 \pm 0.15 \text{ mg g}^{-1}$ ), Pc ( $400.43 \pm 0.59 \text{ mg g}^{-1}$ ), and Ps ( $410.33 \pm 0.17 \text{ mg g}^{-1}$ ) litters. The L and Phe concentration of Ah litter ( $160.37 \pm 2.13 \text{ mg g}^{-1}$  and  $61.66 \pm 0.24 \text{ mg g}^{-1}$ , respectively) was higher than other three plant litter. The phenols concentration in Cm ( $36.76 \pm 0.23 \text{ mg g}^{-1}$ ) and Ps ( $13.83 \pm 0.19 \text{ mg g}^{-1}$ ) litter was 60% and 22% of that in Ah litter, respectively. The highest Ss and Ct content was observed in Ps litter. The Ss content in Pc ( $12.90 \pm 0.65 \text{ mg g}^{-1}$ ) was only 18% of that in Ps litter ( $71.12 \pm$

$0.37 \text{ mg g}^{-1}$ ), and the Ct in Cm litter ( $8.94 \pm 0.24 \text{ mg g}^{-1}$ ) was only 1/10 of that in Ps litter. The cellulose content was  $401.22 \pm 1.75 \text{ mg g}^{-1}$  in Pc litter, which was 2.3 times as much as in Ps litter. The highest L and phenols content were observed in Ah litter, and the highest N and C content were observed in Cm litter, the highest cellulose content was observed in Pc litter, and the highest Ss and Ct content were shown in Ps litter.

Values of the litter mixtures' chemical properties were between those of the single-species litters (Table 1; Figure 1). Among two-species litter mixtures, the N, C, and L content were highest in the AhCm litter mixture, and Ss, Phe, and Ct concentrations were highest in the AhPs litter mixture. The cellulose concentration in the AhPc litter mixture was  $306.86 \pm 1.45 \text{ mg g}^{-1}$ , which was 1.8 and 2.5 times higher than that in AhPs and AhCm litter mixtures. Among three and four-species litter mixtures, the N content was highest in AhCmPc litter, the C, L, Ss, Phe, and Ct content were highest in AhCmPs litter; the Phe and Ct content were highest in AhPcPs litter; and the L and cellulose content were highest in AhCmPcPs litter.

Litter chemical distance was shown by PCA (Figure 1). The first two principal components (PC1 and PC2) explained 85.9% of the variation in litter chemistry, PC3 accounted for 7.7% of the variation in chemical traits (data not shown). PC1 explained 51.8% of the variation in litter chemical traits and revealed a gradient in the Ce, Ss, Phe, L, and Ce concentration. PC2 accounted for 34.1% of the variation in litter chemical traits and was highly correlated with the Ct, C, and N content (Supplementary Figure S2).



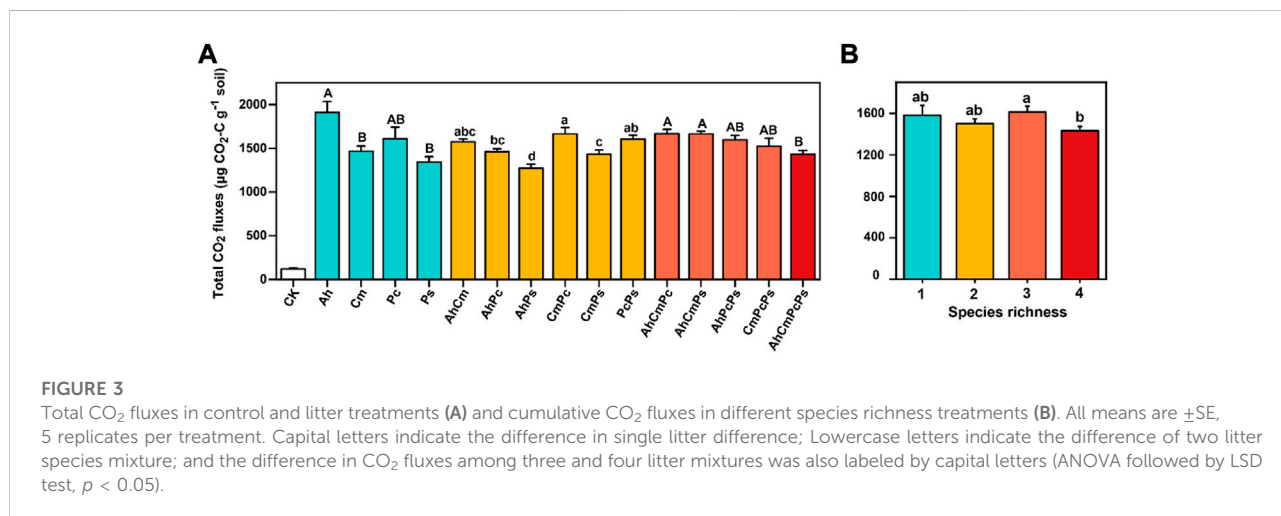
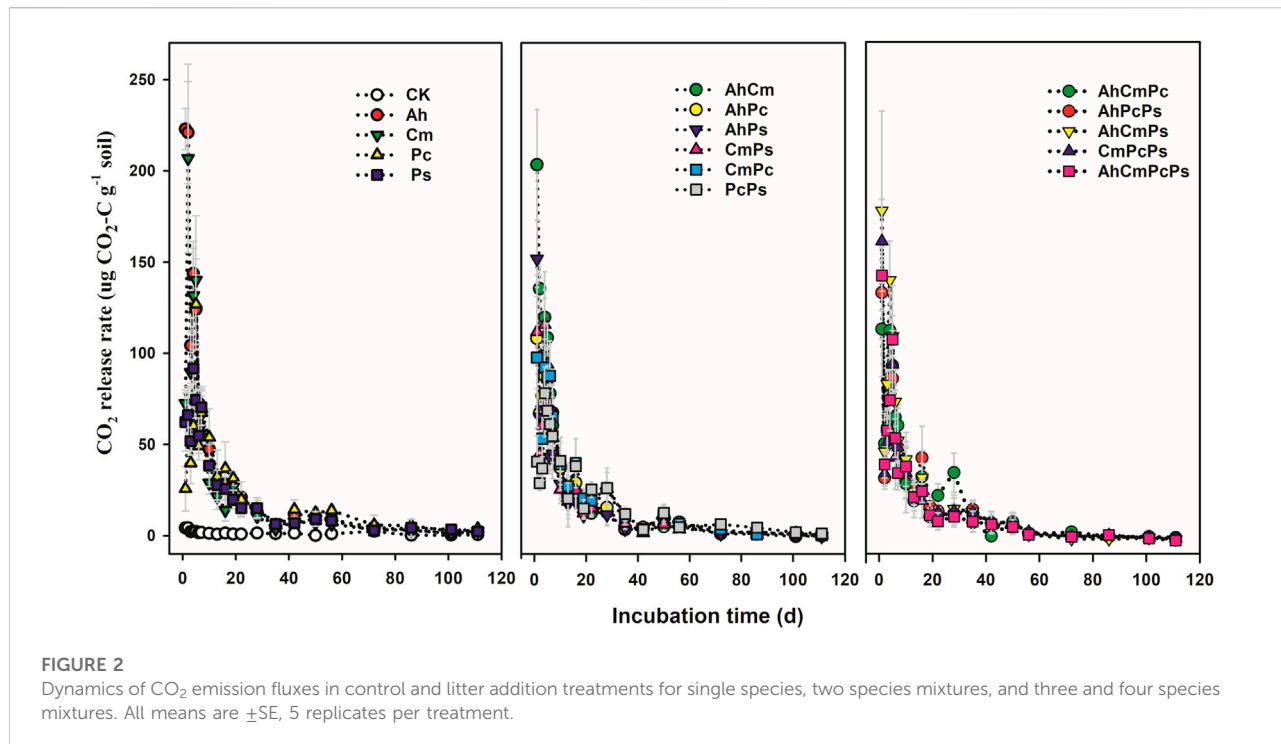
**FIGURE 1**

Principal components plot of litter quality index measured for *Artemisia halodendron* (Ah), *Caragana microphylla* (Cm), *Pennisetum centrasiatium* (Pc), and *Populus simonii* (Ps), *Artemisia halodendron*+ *Caragana microphylla* (AhCm), *Artemisia halodendron*+ *Pennisetum centrasiatium* (AhPc), *Artemisia halodendron*+ *Populus simonii* (AhPs), *Caragana microphylla*+ *Pennisetum centrasiatium* (CmPc), *Caragana microphylla*+ *Populus simonii* (CmPs), *Pennisetum centrasiatium*+ *Populus simonii* (PcPs), *Artemisia halodendron*+ *Caragana microphylla*+ *Pennisetum centrasiatium* (AhCmPc), *Artemisia halodendron*+ *Caragana microphylla*+ *Populus simonii* (AhCmPs), *Caragana microphylla*+ *Pennisetum centrasiatium*+ *Populus simonii* (CmPcPs), *Artemisia halodendron*+ *Caragana microphylla*+ *Pennisetum centrasiatium*+ *Populus simonii* (AhCmPcPs).

## Dynamics of CO<sub>2</sub> release rate and cumulative CO<sub>2</sub> fluxes

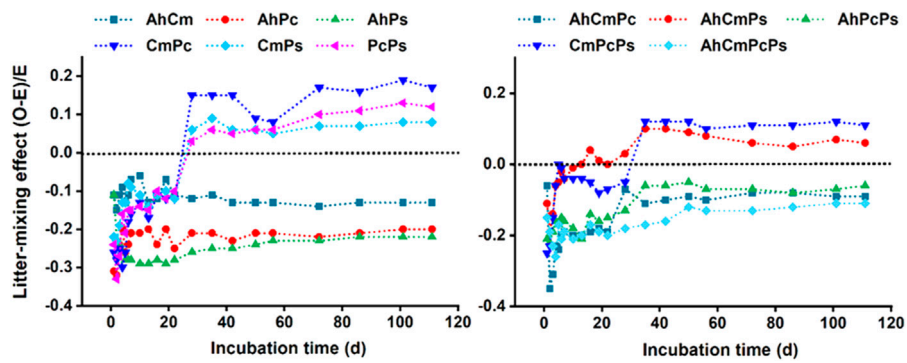
Dynamics of CO<sub>2</sub> release among different litter addition treatments are shown in Figure 2. The CO<sub>2</sub> release rate in the control remained low during the whole incubation, which was approximately  $1.54 \pm 0.25 \mu\text{g CO}_2\text{-C g}^{-1}$  soil. The CO<sub>2</sub> release rate in litter input treatments showed a similar temporal pattern: increasing sharply to the maximum in the first 3 days after litter addition, and then decreasing for the next 4 days, thereafter decreasing gradually with time until the end of the incubation period (Supplementary Figure S3; Figure 2). For the first 3 days, the CO<sub>2</sub> release rate in the Ah and Cm input treatments increased to  $182.63 \pm 20.01 \mu\text{g CO}_2\text{-C g}^{-1}$  soil and  $122.99 \pm 26.98 \mu\text{g CO}_2\text{-C g}^{-1}$  soil, whereas in the Pc and Ps addition treatments, the CO<sub>2</sub> release rate was only increased to  $42.48 \pm 10.97 \mu\text{g CO}_2\text{-C g}^{-1}$  soil

and  $60.00 \pm 13.04 \mu\text{g CO}_2\text{-C g}^{-1}$  soil, respectively. The CO<sub>2</sub> release rate in AhCm was  $146.64 \pm 18.92 \mu\text{g CO}_2\text{-C g}^{-1}$  soil, the CO<sub>2</sub> release rate in PcPs was  $37.29 \pm 8.16 \mu\text{g CO}_2\text{-C g}^{-1}$  soil. For three and four-species litter mixtures, the CO<sub>2</sub> release rate ranged from  $77.06 \pm 12.45 \mu\text{g CO}_2\text{-C g}^{-1}$  soil in the AhCmPc treatment to  $104.96 \pm 21.71 \mu\text{g CO}_2\text{-C g}^{-1}$  soil in AhPcPs treatment. From day 3 to day 28, the CO<sub>2</sub> release rate in single-species litter and litter mixtures ranged from  $35.53 \pm 6.70 \mu\text{g CO}_2\text{-C g}^{-1}$  soil in AhPs litter to  $62.38 \pm 7.85 \mu\text{g CO}_2\text{-C g}^{-1}$  soil in the Ah litter treatment. From day 28 to day 111, the CO<sub>2</sub> release rate in single-species litters and litter mixtures ranged from  $5.04 \pm 1.63 \mu\text{g CO}_2\text{-C g}^{-1}$  soil in Cm litter to  $10.09 \pm 2.65 \mu\text{g CO}_2\text{-C g}^{-1}$  soil in PcPs litter. In sum, the CO<sub>2</sub> release rate among the various litter inputs differed substantially in the early stage (1–28th day), but did not differ as much in the late stage (29th–113rd day).

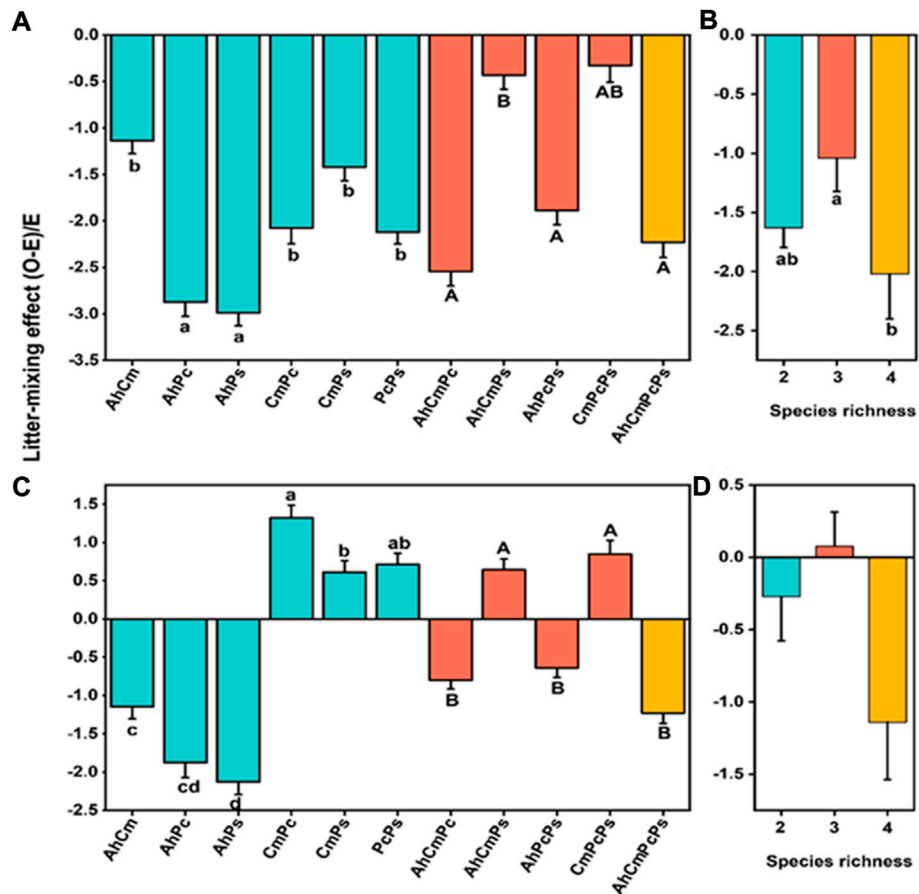


In accordance with CO<sub>2</sub> release rate, total CO<sub>2</sub> fluxes in control was low, which was only 7.7% of that in litter addition treatments. Among the single-species litters, the total CO<sub>2</sub> fluxes in Ah litter was  $1909.63 \pm 127.37 \mu\text{g CO}_2\text{-C g}^{-1}$  soil, which was significantly higher than that in Cm litter ( $1466.43 \pm 60.04 \mu\text{g CO}_2\text{-C g}^{-1}$  soil) and Ps litter ( $1341.22 \pm 63.43 \mu\text{g CO}_2\text{-C g}^{-1}$  soil). Interestingly, among two-species litter mixture treatments, the cumulative CO<sub>2</sub> flux in CmPc litter was highest, at  $1663.93 \pm 72.09 \mu\text{g CO}_2\text{-C g}^{-1}$  s, and cumulative CO<sub>2</sub> fluxes in AhPs litter

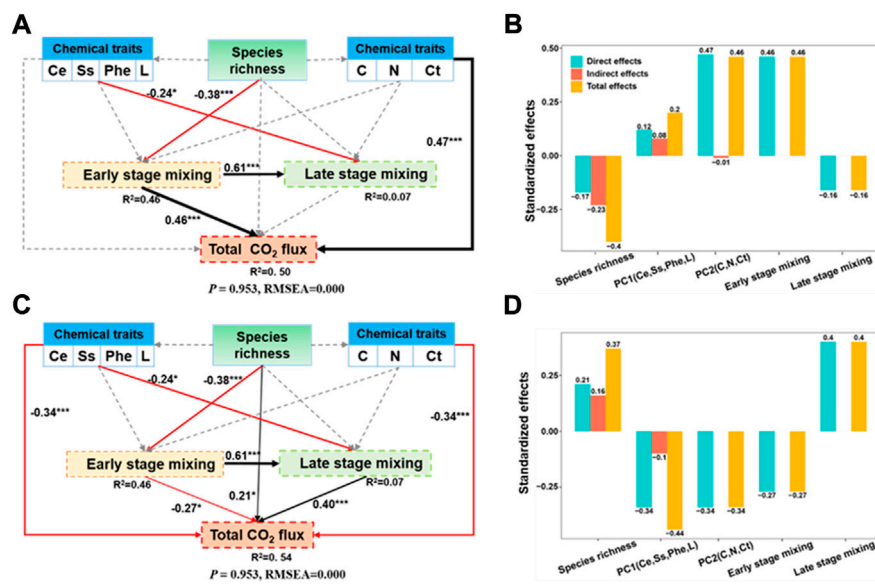
was lower than others, which was  $1270.36 \pm 49.45 \mu\text{g CO}_2\text{-C g}^{-1}$  soil. As described above, we found that mixing of more diverse litters induced smaller cumulative CO<sub>2</sub> fluxes, while the mixing of more similar litters produced higher cumulative CO<sub>2</sub> fluxes. Among three and four-species litter mixtures, total cumulative CO<sub>2</sub> fluxes in AhCmPcPs was  $1432.67 \pm 42.20 \mu\text{g CO}_2\text{-C g}^{-1}$  soil, which was lower than that of AhCmPc ( $1667.47 \pm 48.47 \mu\text{g CO}_2\text{-C g}^{-1}$  soil) and AhCmPs ( $1664.86 \pm 29.77 \mu\text{g CO}_2\text{-C g}^{-1}$  soil) (Figure 3).



**FIGURE 4**  
Dynamics of litter-mixing effects (LME) on CO<sub>2</sub> emission rate for two species, three and four species mixtures. Dotted lines indicate the point at which observed values (O) equal to expected values (E).



**FIGURE 5**  
Litter-mixing effects (LME) on cumulative CO<sub>2</sub> emission during (A,B) the Early stage (C,D) the Late stage for two, three and four species mixtures. All means are  $\pm$ SE, 5 replicates per treatment. Lowercase letters indicate the difference in two litter species; Capital letters indicate the difference of three and four litter species mixture (ANOVA followed by LSD test,  $p < 0.05$ ).



**FIGURE 6** Structural equation model (SEM) evaluating the direct and indirect effects of litter species richness and litter quality index on early-stage CO<sub>2</sub> flux (A), late-stage CO<sub>2</sub> flux (C), the standardized effects of these factors derived from the SEM (B,D). Red and black lines indicate negative and positive relationships, respectively; grey dotted lines indicate the relationship is not significant at  $p < 0.05$  level; Number adjacent to arrows are standardized path coefficients, indicating the effect size of the relationship. \* denotes significant difference at  $p < 0.5$ , \*\* denotes significant difference at  $p < 0.05$ , \*\*\* denotes significant difference at  $p < 0.01$ .

### Dynamics and intensity of litter-mixing effects

At the beginning, litter-mixing effect (LME) was strong and negative, thereafter degree of intensity tapers off until the direction of LME in several litter treatments changed to positive before day 28. Specifically, the LMEs in CmPc, CmPs, and PcPs litter mixture treatments were negative at first but changed to positive at day 22; the LMEs were negative throughout the incubation period for AhCm, AhPc, and AhPs treatments. The LMEs were negative throughout the incubation period for AhCmPc, AhPcPs, and AhCmPcPs, in AhCmPs and CmPcPs, the LMEs were initially negative and then became positive after day 28. Therefore, total CO<sub>2</sub> fluxes and the LME were divided into two stages: day 1 to day 28 was the early stage, and day 28 to day 113 was the late stage (Figure 4).

The LME on cumulative CO<sub>2</sub> emission was negative in the early stage, while some of the LME was positive in the late stage (Figure 5). For two-species litter mixtures in the early-stage, the litter-mixing intensity in AhPc and AhPs was  $-2.88 \pm 0.15$  and  $-2.99 \pm 0.14$ , respectively, which was more strongly than that in AhCm ( $-1.14 \pm 0.14$ ) and CmPs ( $-1.42 \pm 0.15$ ). For three and four-species litter mixtures, the LME in AhCmPc, AhPcPs, and AhCmPcPs was  $-2.54 \pm 0.16$ ,  $-1.89 \pm 0.15$ , and  $-2.23 \pm 0.16$ , respectively, which was nearly 4–6 times

stronger than that in CmPcPs and AhCmPs treatment. In contrast, the LME in the late-stage was  $1.32 \pm 0.04$  in CmPc, which was significantly higher than that of CmPs ( $0.61 \pm 0.09$ ) and PcPs ( $0.71 \pm 0.07$ ). Conversely, the LME was antagonistic in AhCm, AhPc, and AhPs, and the intensity of mixing in AhPs was  $-2.13 \pm 0.15$ , which was much stronger than that of AhCm litter ( $-1.15 \pm 0.14$ ). For three-species litter mixtures, the LME was synergistic in AhCmPs and CmPcPs, and there was no difference in the mixing intensity among them. Similarly, although the mixing effect was antagonistic in AhCmPc and AhPcPs, there was no difference in the mixing intensity among them. The mixing effect in the four-species litter mixture AhCmPcPs was  $-1.23 \pm 0.13$ .

### Effect of litter chemical traits on litter-mixing effects and cumulative CO<sub>2</sub> fluxes

Structural equation model (SEM) suggested that the driving factors of early- and later-stage CO<sub>2</sub> fluxes are completely different (Figure 6), indicating that the key litter chemistry factors affecting CO<sub>2</sub> fluxes and LME varied with incubation time. The early-stage CO<sub>2</sub> fluxes was directly affected by PC2 (litter C, N, and Ct concentration) and early-stage LME ( $R^2 = 0.50$ ), and the latter was mainly controlled by litter species richness. The late-stage CO<sub>2</sub> fluxes was influenced by PC1



(litter Ce, Ss, Phe, and L concentration) and late-stage LME ( $R^2 = 0.54$ ).

## Discussion

### Effects of species richness on litter-mixing effect and soil carbon cycling

In line with previous studies (Meier and Bowman, 2010), our research showed that species richness was positively correlated to the short-term cumulative C mineralization, which was might because species mixing enhance the diversity of food resources and accelerate nutrient exchange (Harguindeguy et al., 2008; Liu et al., 2016). According to the nutrient transfer hypothesis, the high-quality litter allows nutrient transfer by fungal hyphae or leaching to the low-quality litter lead to a more rapid decomposition of poor-quality litter (Hättenschwiler et al., 2005). However, both additive LME and total CO<sub>2</sub> fluxes were not linearly correlated to litter species richness (Epps et al., 2007; Harguindeguy et al., 2008). In this study, both synergistic and antagonistic mixing effects were observed in roughly equal proportions, implying that 1) Litter mixing was a short-term ecological process that tapers off over time (Chen et al., 2017); 2) Chemically similar litter mixing does not induce a litter mixing effect (Meier and Bowman, 2008). Hence, chemical variation in litter mixture determined magnitude and direction of non-additive LME, which might be the key factors driving CO<sub>2</sub> fluxes (Epps et al., 2007; Zhang et al., 2014; Mao et al., 2015).

### Effects of litter quality on litter-mixing effect and soil carbon cycling

Within a given climate regime, litter quality is the main driver of the litter decomposition rate (Aerts, 1997). High-quality litter (high C and N concentration) accelerates the decomposition of low-quality litter and produces a positive mixing effect (Hoorens and Aerts, 2003; Zhang et al., 2014), our study provided evidence that litter C and N content were positively correlated to LME. Hard-to-decompose substances such as Ct within litter played a negative role in activating microbial activity (Coq et al., 2010). Furthermore, an increasing number of studies suggest that low-concentration litter compounds interact with other compounds accelerating or inhibiting microbial decomposition. For example, tannins and phenols can precipitate N-containing substrates in protein-tannin complexes (Kraus et al., 2004) and phenol-protein complexes (Hättenschwiler and Vitousek, 2000), which can only be utilized by fungi and earthworms with moderate levels

of enzyme activity. Moreover, transfer of toxic compounds or phenolics between litter species is thought to inhibit decomposition *via* enzyme inhibition and substrate deprivation (Mutabaruka et al., 2007; Freschet et al., 2012). Fortunately, scaling the value of variables to one before PCA in our study have increased sensitivity to these compounds that would not otherwise be detectable (Meier and Bowman, 2008).

What is interesting is that in our study, effects of litter chemical compounds varied with decomposition stages (Berg, 2008). In early stage of decomposition, the large amount of litter C and N compounds accompanied with litter addition provided sufficient energy and nutrients to activate microorganisms (Vestgarden, 2001). However, in the later stage, litter C and N content was negatively correlated to total CO<sub>2</sub> flux. Additionally, litter Ce, Ss, Phe, and L content, which have no relation to early-stage CO<sub>2</sub> flux, were closely correlated to late-stage CO<sub>2</sub> flux. This result was in line with the previous study showing that N control litter decay rates during first phase of decay, while L content becomes more important thereafter (Berg, 1984; Taylor et al., 1989). As decomposition proceeds, the C and N was depleted and the proportion of L and Ce increase, and at some point where L control begins and becomes stronger through time (Berg, 2008). Moreover, Staaf and Berg 1982 emphasized that in the late-stage, L-controlled phase were normally proportional to the litter decomposition and soil respiration (Staaf and Berg 1982). Together with previous studies, we provided evidence that effects of litter richness and chemical traits on soil C cycling changed over decomposition stages.

### Implications for ecological engineering establishments

Afforestation in agro-pastoral ecotone was expected to be a good tool for restoring degraded soils and ecosystems (Watson et al., 2000). However, not all trees can be grown everywhere, interlaced planting of trees and shrubs on degraded grassland does not necessarily maximize soil C conservation (Deng et al., 2014; Liu et al., 2018). *Artemisia halodendron* is a sand-fixing shrub species that has been widely mixed planted with other trees or shrubs in severely degenerated sites in North China (Luo et al., 2020). Unfortunately, the results showed that cumulative CO<sub>2</sub> fluxes in *A. halodendron* is relatively high, but CO<sub>2</sub> fluxes is decreased and LME was intensive when *A. halodendron* is mixed with other species. The results suggest that the restoration of degraded land might be accelerated with widely plantations of exclusively *A. halodendron* but slowed down when mixed with other plant species. Therefore, afforestation species and management type should be carefully considered and planting should be guided by indigenous knowledge and local communities (Hong et al., 2020).

## Conclusion

Our results revealed that tree and shrub litter addition to grasslands significantly increased the emission of CO<sub>2</sub>, especially in the early-stage that soil CO<sub>2</sub> flux was 1.75 times higher than that in the late-stage. We found that both litter richness and litter quality affected LME and soil CO<sub>2</sub> flux. Importantly, we provided evidence that dominant litter chemical factors that influence soil CO<sub>2</sub> flux changed over time, with litter C, N and Ct controlled the early-stage CO<sub>2</sub> flux, while all litter chemical traits drove the late-stage CO<sub>2</sub> flux. Our result opens a new perspective for improving our understanding of soil C cycling in agro-pastoral ecotone after large-scale afforestation.

## Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#), further inquiries can be directed to the corresponding author.

## Author contributions

Conceptualization: HY and YLi; Methodology: HY and JZ; Writing-original draft preparation: HY; Writing-review and editing: HY, YLi, CB, and YLu. All authors have read and agreed to the published version of the manuscript.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fenvs.2022.1023831/full#supplementary-material>

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