Shrub Encroachment Shapes Soil Nutrient Concentration, Stoichiometry and Carbon Storage in an Abandoned Subalpine Grassland

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Abstract: Soil nutrient stoichiometry plays a substantial role in terrestrial carbon and nutrient cycling. However, the changes in soil nutrient stoichiometry with shrub encroachment (SE) remain poorly understood, especially in subalpine areas. We examined the changes in soil nutrient concentration, nutrient stoichiometry, and organic carbon (OC) storage (at a depth of 0–5, 5–10 and 10–20 cm) in three successional shrub encroachment stages (early, mid and late) in an abandoned subalpine Eulalia pallens (Hackel) Kuntze grassland. An ANOVA showed that SE did not produce serious soil acidification, but significantly increased the soil OC and total phosphorous (TP) concentration, and improved the stoichiometry ratio of soil OC to total nitrogen (OC:TN) in all layers. OC storage tended to increase with SE. SE thus did not indicate degradation of the grassland. A redundancy analysis (RDA) and partial RDA revealed that the shrub relative cover and soil water content were the most important factors affecting the soil nutrient concentration, that the soil available phosphorous (AP), nitrogen, potassium, calcium (ACa), and magnesium concentration and shrub relative cover were the most important factors influencing soil nutrient stoichiometry ratios, and that soil OC:TN, TN:TP, OC:TN:TP, and AP:ACa ratios, bulk density, and pH were the most important factors influencing soil OC storage over SE. Our study provides insights into SE in grassland areas, and potentially provides a useful reference for ongoing grassland conservation and restoration in subalpine regions.

Keywords: space-for-time; succession; soil environment; redundancy analysis; relative importance; important factor

1. Introduction

Shifts from grassland to shrubland, the thicketization of grassland, or the encroachment of woody plants into grassland—a phenomenon known as shrub encroachment (SE)—occur all over the world [1–6] and have taken place during the last century [3–5,7,8], and even in the past two centuries [9,10]. This global encroachment at the expense of grasses in rangeland is forecast to increase over the coming century [11]. In many parts of the world, woody plant abundance (cover and biomass) has multiplied in the past 50–100 years [12]. It poses a serious threat to the food security [13–15] and biodiversity [16–18] in rangeland areas, which cover more than 30% of the terrestrial land area [19], yield over 30% of the terrestrial annual net primary productivity [20], and support 30% of the total human population, as well as most livestock [12]. SE, therefore, is one of the most striking land cover changes in rangeland areas around the globe [13,21].
Understanding the consequences of SE is important [5]. However, little attention has been paid to the effects of local species encroachment on soil properties [22]. Previous extensive research efforts have found that SE into rangeland is often irreversible [4,23], and commonly alters the soil, but the existing results have not been consistent. For example, studies that found a decreasing effect of SE on soil nutrient levels [24–27] and carbon (C) sequestration [24–27] can be contrasted with others that found an increasing effect of SE on soil nutrient levels [2,10,26,28–30] and C sequestration [2,4,26,28,31–33]. A negligible effect of SE, with it causing few or no changes [3,19], has also been found. Although other studies have tried to uncover a more universal pattern [3,4,8], it has been difficult to predict the local effects of SE on the basis of these previous inconsistent findings. The vast majority of the previous efforts on SE have targeted arid [3,11,23], semi-arid [3,12,34–36] and mesic areas [19,37], such as savanna [6,12,38–40], steppe [9,41] and prairie [37,42], and a study on global grassland revealed that soil properties seem to be the primary dependent variables in response to the extent of these changes [43]. What remains to be investigated is how soil nutrient changes occur via SE under subtropical, monsoon, humid, and subalpine conditions.

Soil nutrient stoichiometry (SNS) makes a substantial contribution to terrestrial biogeochemical cycling [44]. Its changes over time could lead to potential changes in species composition and nutrient cycles. SNS is impacted by succession [45–47], vegetation changes [48–50], and environmental factors [48,51,52]. A study in wetlands found that the impacts of environmental factors on SNS might be greater than that of the fertile island effects on a larger scale [52]. However, studies on the factors shaping SNS are still limited, and the regular mechanism remains unclear [52]. In particular, whether and how SE shapes SNS is largely still unknown [50,53].

The effects of SE on soil nutrient concentration and stoichiometry in natural, abandoned subalpine _Eulalia pallens_ (Hackel) Kuntze (_E. pallens_ or Baijiangan in Chinese) grassland have not yet been explored. Subalpine belts are considered to be plant biodiversity hotspots [54], and up to 8491 vascular plant species were recorded on the Guizhou plateau [55]. _E. pallens_ is an endemic species which is only distributed in Guizhou, Yunnan, Sichuan and Guangxi in China, and in a small part of India [56]. SE in these areas may threaten their habitats. Understanding and evaluating the consequences of SE on soil nutrient concentration and stoichiometry in this subalpine _E. pallens_ grassland will be key to improving our knowledge, protecting species diversity, and developing good land management and policies for improving soil fertility and C sequestration. Research into soil nutrient concentration and stoichiometry in these special areas is needed.

We analysed the soil nutrient concentration and stoichiometry with SE, and explored what factors and to what extent the factors impact on soil nutrient concentration, stoichiometry, and C storage (CS), in order to address these three questions: (1) Does SE increase soil nutrient concentration, stoichiometry, and CS? (2) How does SE influence SNS? (3) What are the implications of changed soil nutrient concentration, stoichiometry, and CS caused by SE in this special area?

2. Methods

2.1. Site Description

Samples were collected in a natural subalpine _E. pallens_ grassland ecosystem, a 6000 ha reserve (1500–1700 m above sea level) in Longli county on the Guizhou plateau, southwest China (Figure 1). This is a northern subtropical monsoon humid region with an annual average air temperature of 14.7 °C (−3 to 35 °C) and 1160 mm of annual precipitation (1090–1180 mm, mainly falling from April to October).

2.2. Plant Quadrat Survey, Soil Sampling, and Determination

The “space-for-time” substitution technique is useful for studying the long-term ecological succession and soil development over time [44,57]. The method used in this study was recommended by Walker et al. [57], and was used for studying SE or successional changes in soil stoichiometry [44].
Based on the process of local SE succession and the ecological characteristics of native species, we carefully selected the typical dominant community plots that represented the SE successional stages: the grass stage, mosaic stage, and shrub stage, on the abandoned, natural *E. pallens* grassland. In a plot (5 m × 5 m) (Figure 1), we classified plants into the two plant functional types (shrub vs. herbaceous and grasses) according to the growth form of the plants. The total plant cover and shrub cover were visually estimated and expressed as percentages [58], and the shrub relative cover was obtained via dividing the shrub cover by the total plant cover. The plant species richness per quadrat (the number of species) was recorded in the three quadrats (1 m × 1 m) we selected randomly in the corresponding plot, and expressed as the plant species diversity. The plant species richness per plot is the total number of species in three quadrats in the plot. Table 1 shows that SE succession in this grassland is characterised by shifts in plant functional types, shrub relative cover, and plant species richness per quadrat. Specifically, at the early stages of SE, grass species (e.g., *E. pallens*) dominated; at the mid stages, shrubs (e.g., *Vaccinium fragile Franch.*) increased and grasses (e.g., *E. pallens*) decreased, and at the late stages, shrubs (e.g., *Vaccinium fragile Franch.*) became dominant. The different plots used in this study occur in a relatively narrow geographical range, with almost the same topography. The soil is yellow earths (Chinese Soil Taxonomy, 1998) or Haplic Alisols (FAO/UNESCO taxonomy, 1988). No permits were required for the described study.

**Figure 1.** Location of the study site and the scheme of the plant survey and soil sampling in Longli county on the Guizhou plateau, southwest China.
Table 1. Characteristics of plots surveyed in the different shrub encroachment stages.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Grass Stage</th>
<th>Mosaic Stage</th>
<th>Shrub Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latitude</td>
<td>26°19′44″ N</td>
<td>26°19′49″ N</td>
<td>26°19′48″ N</td>
</tr>
<tr>
<td>Longitude</td>
<td>106°51′9″ E</td>
<td>106°51′6″ E</td>
<td>106°51′7″ E</td>
</tr>
<tr>
<td>Altitude a. s. l.</td>
<td>1610.7 m</td>
<td>1610.5 m</td>
<td>1610.2 m</td>
</tr>
<tr>
<td>Dominant species</td>
<td>Eulalia pallens (Hack.)</td>
<td>Eulalia pallens (Hack.)</td>
<td>Vaccinium fragile Franch.;</td>
</tr>
<tr>
<td></td>
<td>Kuntze; Arundinella anomala Steud.</td>
<td>Steud.; Vaccinium fragile Franch.</td>
<td>pal lens (Hack.) Kantze</td>
</tr>
<tr>
<td>Plant functional types</td>
<td>Subalpine grassland with dense and tall herbaceous plants and grasses with few shrubs</td>
<td>Patchy shrubs interspersed by subalpine grassland</td>
<td>Dense shrubs stand with few herbaceous plants and grasses</td>
</tr>
<tr>
<td>Total plant cover (%)</td>
<td>91.67 ± 2.89 a</td>
<td>86.67 ± 4.16 a</td>
<td>85.00 ± 5.00 a</td>
</tr>
<tr>
<td>Shrub relative cover (%)</td>
<td>6.53 ± 0.91 e</td>
<td>34.38 ± 4.23 b</td>
<td>83.10 ± 1.74 a</td>
</tr>
<tr>
<td>Plant species richness per plot</td>
<td>10</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>Plant species richness per quadrat</td>
<td>4.67 ± 0.57 b</td>
<td>5.67 ± 0.57 b</td>
<td>7.33 ± 0.57 a</td>
</tr>
</tbody>
</table>

Note: 1–3 were measured using a global positioning system; different letters within the same characteristic indicate significant differences (p < 0.05).

As the soil depth is universally shallow (20–50 cm to bedrock on the hilltop plateau), along with enhanced topsoil nutrients [59] and the recent finding that the main effects of SE would be to increase topsoil organic C [43], we thus collected soil samples from the topsoil [58]. Early in October 2017, in the quadrats (1 m × 1 m) mentioned above (Figure 1), we collected three replicate soil cores (0–20 cm) in each quadrat after clearing the litter [26], then pooled and homogenised the three cores for each soil layer (0–5 cm, 5–10 cm, and 10–20 cm). This resulted in one mixed soil sample for each replicate after the removal of large items of organic material and stone by hand. One subsample was immediately transported to Nanjing Zoobio Biotechnology Ltd. to determine the concentration of soil organic carbon (OC), soil total nitrogen (TN), available nitrogen (AN), soil total phosphorus (TP), available phosphorus (AP), available potassium (AK), available calcium (ACA), and available magnesium (AMg). The remaining subsamples were used for determining the soil pH and water content (WC). The soil bulk density (BD) was measured using the cutting ring method (50 mm diameter, 50 mm height). The WC was determined by oven-drying samples to a constant mass at 105 °C; the pH was measured using a pH meter after shaking a soil-water (1:2.5 w/v) suspension for 30 min. The above soil variables were determined in the lab of Guizhou Institute of Prataculture, and the following soil variables were determined in Nanjing Zoobio Biotechnology Ltd., according to Bao [60]. The soil samples were air-dried and ground (<2 mm) prior to the determination of the available and total nutrients. OC was determined using an elemental analyzer (Elementar total organic carbon/total nitrogen analyzer, Germany); TN was determined through sulfuric acid digestion and using an FOSS automatic nitrogen determination apparatus; AN was analyzed by the alkali diffusion method; TP was determined by NaOH digestion followed by ammonium-molybdate colorimetry (T181 XinShiji ultraviolet spectrophotometer, China); AP was extracted using the NaHCO₃-ultraviolet spectrometer subsystem (T181 XinShiji ultraviolet spectrophotometer, China); AK was extracted with ammonium acetate; and finally ACA and AMg were determined using a soil:water (1:5) mixture by an Atomic Absorption Spectrophotometer.

2.3. Data Analyses

OC and TN were expressed as g kg⁻¹; TP, AN, AP, AK, ACA and AMg as mg kg⁻¹; WC was expressed as a decimal; BD as g cm⁻³; the soil stoichiometry as molar ratios (i.e., the ratio of OC to TN as OC:TN); and the C storage (CS) was calculated for the 0–5 cm, 5–10 cm, 10–20 cm, and 0–20 cm layers according to the following formula [61], and expressed as kg m⁻²:

\[
CS = \sum_{i=1}^{n} I_i \times B_i \times OC_i
\]
where $n$ is the number of soil layers, $l_i$ is the interval of the soil layer (cm) of $i$, $B_i$ is the soil bulk density (g·cm$^{-3}$) of the soil layer of $i$, and $OC_i$ is the soil OC concentration (g·kg$^{-1}$) at the soil layer of $i$.

Data were log10 transformed to fulfill normality and homogeneity of variance if needed [2,44,58,62]. At a given soil layer, the differences in soil environment, nutrient concentration, CS, and stoichiometry among the SE stages were tested using a one-way ANOVA with Duncan’s post-hoc tests in IBM SPSS Statistics version 25.0 (IBM Corp., Armonk, NY, USA), and the figures were plotted in Microsoft Office Excel 2003 (Microsoft Corp., Washington, DC, USA). The interaction effects between SE and the soil layer on the soil environment, nutrition concentration, CS, and stoichiometry were derived from a two-way ANOVA, and the $\eta^2$ (partial eta-squared) statistic in the two-way ANOVA was used to determine the relative importance of each factor or interaction [28,63] in IBM SPSS Statistics version 25.0. A higher $\eta^2$ represents a greater importance. A Mantel test based on Euclidean distance was used to test the relationships of the soil nutrient concentration and CS or soil stoichiometry with the plant and soil factors, and the significance of the relationships. A redundancy analysis (RDA) was used to show the relationships of soil nutrient concentration and CS or soil stoichiometry with the plant and soil factors. A partial RDA was used to partition the variation in soil nutrient concentration and CS or soil stoichiometry. The variables in RDA or partial RDA were tested by the variance inflation factor test. The variables that had a variance inflation factor (VIF) $\geq$ 10.0 were commonly considered to have collinearity and were removed. The linear model was used in the regression analysis for revealing plant factors affecting the soil OC concentration and CS.

All data were collated in Excel 2003, and data transformation, regression analysis, RDA, and partial RDA were conducted in R version 3.4.4 (R Development Core Team, 2018).

3. Results

3.1. Shrub Encroachment Shaped the Soil Environment, Nutrient Concentration, and Carbon Storage

A one-way ANOVA for the soil environment, nutrient concentration, and the CS in the various SE stages is shown in Figure 2. The soil pH, ranging from 4.05 to 4.51, did not vary significantly with SE in all soil layers ($p > 0.05$) (Figure 2A). This indicated that SE did not produce serious soil acidification. The maximum soil WC (54.96%) and the minimum soil BD (0.58 g·cm$^{-3}$) were observed in all soil layers of the shrub stage (Figure 2B,C). The soil OC, TN and AN concentration in the 0–5, 5–10, and 0–20 cm soil layers improved with SE (Figure 2D,E,G), and the soil TP concentration in all soil layers improved with SE (Figure 2F). The soil AK concentration in the 0–5 and 0–20 cm soil layer improved with SE (Figure 2I). The soil ACa and AMg concentrations did not vary significantly with SE ($p > 0.05$).

A two-way ANOVA (Table S1) showed that SE significantly impacted the BD, WC, and soil nutrition concentration ($p < 0.05$) except for AP, ACa and AMg, while the soil layer significantly impacted the soil pH, BD, WC and nutrient concentration ($p < 0.05$), except for AP and ACa. Significant interactions between SE and the soil layer were also found in the soil OC, TN, TP, AN and AK concentrations ($p < 0.05$). The soil CS improved with increasing soil layers, and improved with SE in all soil layers, and the soil CS in the 0–5, 5–10 and 0–20 cm soil layers was significantly higher in the shrub stage than in the grass stage ($p < 0.05$) (Figure 2L). The two-way ANOVA analysis (Table S1) showed that SE significantly impacted the CS ($p < 0.05$), but the soil layer and interaction had no significant impact ($p < 0.05$), indicating that SE is the main factor shaping the soil CS.

The $\eta^2$ in the partial eta-squared statistic can determine the relative importance of SE, the soil layer, and their interaction, and a higher $\eta^2$ value represents a greater importance. The $\eta^2$ statistic is shown in Table S1. For the soil OC, TN, TP, and AN concentration, the relative importance of factors followed the decreasing order of SE > soil layer > their interaction, indicating that SE is the main factor shaping the soil OC, TN, TP, and AN concentration. For the soil AK concentration, the decreasing order is: soil layer > interaction > SE (Table S1), indicating that the soil layer, rather than SE, is the main factor shaping the soil AK concentration.
followed the decreasing order of SE > soil layer > their interaction, indicating that SE is the main factor shaping the soil OC, TN, TP, and AN concentration. For the soil AK concentration, the decreasing order is: soil layer > their interaction > SE (Table S1), indicating that the soil layer, rather than SE, is the main factor shaping the soil AK concentration.

3.2. Shrub Encroachment Changed Soil Nutrient Stoichiometry

A one-way ANOVA for SNS among the SE stages is shown in Figure 3. The SNS ratios changed with SE, but only the OC:TN ratio improved with SE in all soil layers (Figure 3A). The TN:TP ratio was higher in all soil layers of the grass stage (Figure 3C), and the OC:TN:TP ratio was lower in the shrub stage in all soil layers (Figure 3D). However, the ratios of OC:TN, OC:TP, AN:AP, AN:AK, AN:ACa, AN:AMg, and AP:ACa in all soil layers were higher in the shrub stage (Figure 3A,B,E–H).

The two-way ANOVA analysis (Table S1) showed that SE significantly impacted SNS ($p < 0.05$), except for the AP:AK and AP:AMg ratios. However, the soil layer significantly impacted the OC:TP, TN:TP, AN:AK and AN:AMg ratios (Table S1). Their significant interactions were also found only.

Figure 2. Effect of shrub encroachment (SE) on the soil environment, nutrient concentration, and carbon storage (CS) ($n = 3$). Different letters within the same soil layer indicate significant differences ($p < 0.05$).

A-L: Effect of shrub encroachment (SE) on (A) soil pH, (B) water content (WC), (C) bulk density (BD), (D) organic carbon (OC) concentration, (E) total nitrogen (TN) concentration, (F) total phosphorus (TP) concentration, (G) available nitrogen (AN) concentration, (H) available phosphorus (AP) concentration, (I) available potassium (AK) concentration, (J) available calcium (ACa) concentration, (K) available magnesium (AMg) concentration, and (L) organic carbon storage (CS) in the 0–5, 5–10 and 0–20 cm soil layers.

3.2. Shrub Encroachment Changed Soil Nutrient Stoichiometry
in the AN:AK ratio \((p < 0.05)\). For the AN:AK ratio, the relative importance of factors followed the decreasing order of SE > soil layer > their interaction (Table S1), indicating SE was the main factor shaping the AN:AK ratio.

The two-way ANOVA analysis (Table S1) showed that SE significantly impacted SNS \((p < 0.05)\), except for the AP:AK and AP:AMg ratios. However, the soil layer significantly impacted the OC:TP, TN:TP, AN:AK and AN:AMg ratios (Table S1). Their significant interactions were also found only in the AN:AK ratio \((p < 0.05)\). For the AN:AK ratio, the relative importance of factors followed the decreasing order of SE > soil layer > their interaction (Table S1), indicating SE was the main factor shaping the AN:AK ratio.

**Figure 3.** Effect of shrub encroachment (SE) on soil stoichiometry \((n = 3)\). Different letters within the same soil layer indicate significant differences \((p < 0.05)\). A-K: Effect of shrub encroachment (SE) on the stoichiometry ratios of (A) OC:TN, (B) OC:TP, (C) TN:TP, (D) OC:TN:TP, (E) AN:AP, (F) AN:AK, (G) AN:ACa, (H) AN:AMg, (I) AP:AK, (J) AP:ACa, and (K) AP:AMg in the 0–5, 5–10 and 0–20 cm soil layers.

### 3.3. The Influence of Plant and Soil Factors on Soil Nutrients, Stoichiometry, and Carbon Storage

Plant and soil factors influenced the characteristics of the soil nutrient concentration, stoichiometry, and carbon storage. A redundancy analysis (RDA) was used to examine the effects of the plant and soil factors on soil nutrients, stoichiometry, and carbon storage, and a partial RDA was used to quantify the effect size of the plant and soil factors. In the biplots of the RDA (Figure 4), the dot represents a soil sample; dots with the same colour represent the same SE stage. To readily observe whether soil
samples in the SE stage were separated, the confidence intervals (90%) are shown for each stage by using an ellipse.

For the RDA model of the soil nutrient concentration, the Monte Carlo permutation test showed that this model was highly significant ($p < 0.001$) and explained 66.37% of the total variation, indicating that the five plant factors (shrub relative cover and plant diversity) and soil environmental factors (pH, WC, and BD) had a significant impact on the soil nutrient concentration (Mantel test $r = 0.47$, $p < 0.001$). Specifically, the first axis of the RDA was significant (Monte Carlo permutation test, $p < 0.001$), accounting for 60.76% of the total variation (Figure 4A). From the biplot of the RDA (Figure 4A), obvious differences were observed in the soil nutrient concentration under different shrub encroachment stages along the first axis. A forward selection of the five factors in the RDA ordinations showed that two factors, the shrub relative cover and soil WC, were significantly related to spatial variations in the soil nutrient concentration (Monte Carlo permutation test, $p < 0.05$). The test of the variance inflation factors found that the variance inflation factor (VIF) of these two significant factors were all far lower than 10. Following the forward selection, a partial RDA was used to disentangle the effect of each individual significant factor. Here, the effect is the portion of variation that is solely explained by one individual significant factor, with all the others used as covariables [64]. The result of the partial RDA for the soil nutrient concentration showed that the shrub relative cover and soil WC explained 45.44% and 9.82% of the total variation, respectively. The pie chart (Figure 4B) showed that the shrub relative cover and soil WC were thus identified as the most important factors affecting the soil nutrient concentration.

For the RDA model of the SNS ratios, the Monte Carlo permutation test showed that this model was highly significant ($p < 0.001$) and explained 86.70% of the total variation, indicating that all plant factors, soil environmental factors, and soil nutrient concentration factors had a significant impact on the SNS ratios (Mantel test $r = 0.48$, $p < 0.001$). Specifically, the first two axes of the RDA accounted for 70.66% of the total variation, and the first axis and the second axis were both significant (Monte Carlo permutation test, $p < 0.001$), accounting for 45.12% and 25.54% of the total variation, respectively (Figure 4C). From the biplot of the RDA (Figure 4C), obvious differences were observed in the SNS ratios between the shrub stage and the grass and mosaic stages along the first axis. A forward selection of the 13 factors in the RDA ordinations showed that nine factors, including the shrub relative cover and soil AP, AN, AK, ACa and AMg concentrations were significantly related to spatial variations in the SNS ratios (Monte Carlo permutation test, $p < 0.01$). The test of the variance inflation factors found that the soil OC, TN and TP concentrations, whose variance inflation factors (VIFs) were all higher than 10, should be removed. Finally, six significant factors were obtained. The results of the partial RDA for the SNS ratios showed that the soil AP, AN, shrub relative cover, and AK, ACa and AMg concentration explained 22.68%, 10.01%, 6.75%, 4.45%, 3.50%, 2.95%, and 36.36% of the total variation, respectively (Figure 4D). The soil AP concentration was thus identified as the most important factor influencing the SNS ratios.

For the RDA model of soil carbon storage, the test of the variance inflation factors found that one plant factor (shrub relative cover), one soil environmental factor (WC), six soil nutrient concentration factors (OC, TN, TP, AN, AP, and AK), and five stoichiometry ratios factors (OC:TP, AN:ACa, AN:AMg, AP:AK, and AP:AMg), whose variance inflation factors (VIF) were all higher than 10, should be removed. The RDA model was highly significant (Monte Carlo permutation test, $p < 0.001$) and explained 85.09% of the total variation, indicating that one plant factor (plant species diversity), two soil environmental factors (pH and BD), two soil nutrient concentration factors (ACa and AMg), and six stoichiometry ratio (OC:TN, TN:TP, OC:TN:TP, AN:AP, AN:AK, and AP:ACa) factors had a significant impact on the soil carbon storage (Mantel test $r = 0.30$, $p < 0.01$). A forward selection in the RDA ordinations showed that six factors, including the soil BD, pH, and the ratios of OC:TN, TN:TP, OC:TN:TP, and AP:ACa, were significantly related to spatial variations in the soil carbon storage (Monte Carlo permutation test, $p < 0.05$). The results of the partial RDA for the soil carbon storage showed that the soil BD, pH, and the ratios of OC:TN, TN:TP, OC:TN:TP, and AP:ACa explained 26.66%,
Figure 4. (A) The biplot of the redundancy analysis (RDA) of the soil nutrient concentration over shrub encroachment (n = 9). (B) The biplot of the RDA of soil nutrient stoichiometry over shrub encroachment (n = 9). (C) The percentages of the variation in the soil nutrient concentration explained by factors in the partial RDA (n = 9). (D) The percentages of the variation in soil nutrient stoichiometry explained by factors in the partial RDA (n = 9). (E) The percentages of the variation in soil carbon storage explained by factors in the partial RDA (n = 9). Cover represents the shrub relative cover. * p < 0.05; ** p < 0.01; *** p < 0.001.

4. Discussion

4.1. Responses of Soil Nutrients, Carbon Storage, and Stoichiometry to Shrub Encroachment

The conversion of grassland from grass to shrub dominance has numerous ecological consequences [4]. What is most obvious is that the plant functional types change from grasses to shrubs. Many studies have found that shrub encroachment increases soil OC [2–4,8,43,46,64–67], TN [2,29,46,67], TP [2,29], AN [68], AP [2,29], AK [29], and CS [2]. Our results (Figure 2D,F,L) supported these findings.

SE has a strong positive effect on soil WC (Figure 2B) and nutrients (Figure 2D,F,L) [68], especially on TP (Figure 2F). Since phosphorus (P) is the limiting element in the soil of subtropical China [69], we questioned the source of soil TP. We speculated that the increase of soil TP might originate from (1) the reduced leaching [70] and runoff [71,72], and (2) the enhanced soil retention ability with SE. Our data showed that SE converted more P into non-available P (Figure S1). AP included all water-soluble P and was easier to lose via leaching and runoff in a high-rainfall area, thus the higher percentage of non-available P with SE enhanced the soil P retention.

Jobbágy et al. [73] found that a shift in plant functional types (from grass to shrubs) significantly changed the vertical distribution of the soil OC. Our data also found that interactions between SE and
the soil layer had a significant impact on soil nutrients (Table S1). A higher proportion of soil CS was stored in deeper layers with SE (Figure S2). Hence, these significant interactions appear to highlight the role of plant functional types.

4.2. SE in the Study Area May Not Indicate Land Degradation

In the past, natural SE was commonly considered to indicate the degradation of grassland [3,23,74], perhaps due to a reduction in the main ecosystem service—the livestock production provided by grassland, induced by SE [13,39], and the loss of plant species diversity [5,18]. However, recent global meta-analyses have highlighted that the commonly established link between SE and degradation is not universal [3,74]. Shrub establishment may even be an important step in the reversal of desertification processes in the Mediterranean region [74] and semi-arid sandy land [34]. Our data suggested that in the abandoned natural *E. pallens* grassland we were studying, soil resources (soil nutrients and water) and soil CS were improved by SE (Figure 2B,D,F,L). Moreover, taking the obviously increased above-ground biomass C [75] and decreased soil CO$_2$ flux [19] into account, SE appears to result in a net ecosystem C gain [19] in the absence of any disturbance [8]. Therefore, SE in the abandoned natural *E. pallens* grassland may not indicate land degradation in terms of soil nutrition and CS. We cannot simply regard SE as evidence of land degradation.

4.3. The Main Factors Controlling Soil Nutrients Over Shrub Encroachment

Both biotic and abiotic factors are commonly invoked to explain changes in soil nutrients over shrub encroachment. Numerous studies have concluded that shrubs produced the effects of a fertile island [1,52,76], and that increased soil resources (soil moisture (Figure 2B) and nutrient content (Figure 2D,F,L), and increased organics [1,30,34,68,74]) are all associated with the presence of shrubs [1] instead of grass. Soil nutrients are enriched with shrubs via positive feedback in ‘islands of fertility’[22]. Additionally, shifts from grasses to shrubs alter the root depth. Grasses probably gain nutrients mainly from the surface soil layers, but shrubs may acquire some P from deeper soil layers [2], thus changing the soil nutrients. Shrubs improve the local micro-climate, reduce the wind velocity, retain dust and sand, increase the soil litter biomass and soil moisture, improve the soil texture, and help retain nutrients available to vascular plants [77]. Recent studies have reported that soil nutrient concentrations are also usually related to shrub size, cover, and age [2,22,78], with large shrubs and cover enriching soil nutrients. Soil total C, N, P and available P are correlated positively with shrub cover [2]. In our study, shrub relative cover and soil WC were the first two important factors affecting the soil nutrient concentrations (Figure 4C). The soil OC, AN, and TP concentrations increased with the shrub relative cover, and the soil OC, TN and AN concentration increased with the soil WC, while the soil TN, AP, AK, AMg and ACa concentrations decreased with the shrub relative cover, and the soil TP, AP, AK, AMg and ACa concentrations decreased with the soil WC (Figure 4B).

4.4. The Main Factors Changing Soil Nutrient Stoichiometry Over Shrub Encroachment

Although previous studies have found that the soil nutrient content can be affected by SE [3], whether SNS is affected by SE remains unclear [53]. Previous studies focused on restoration have found that the soil C:P and N:P ratios increased with the restoration age [46]. Additionally, the C:P and the C:N ratios in the soil increased along the succession [47]. Our data showed that SE produced different divergences in nutrient stoichiometry ratios (Figure 4B).

Previous studies have also found that fertile islands have a significant influence on SNS ratios [52,53]. Our study found that soil available N, P, K, Ca and Mg, rather than the total N and P concentrations, were the most important factors influencing SNS ratios. The recent study clearly suggests that the effect of shrub islands on soil C:N:P stoichiometric ratios was dependent on shrub species and size [53]. The increase in the stoichiometric ratio followed an identical pattern among the shrub species as the shrub size increased. There were always higher soil C:P and N:P ratios beneath a special shrub species than under the other shrub species with the
same plant size. Our study showed that an increasing shrub relative cover could increase SNS ratios, except for the OC:TN:TP and TN:TP ratios, and decrease the OC:TN:TP and TN:TP ratios.

4.5. The Main Factors Controlling Soil Carbon Storage over Shrub Encroachment

We grouped the underlying mechanisms of SE control over the soil carbon storage into four pathways that can operate simultaneously:

(a) Recent studies from both the laboratory and field indicated that nutrients or inorganic nutrients are a hidden cost for sequestering soil C [79–81], underscoring the constraining role of nutrients [82–85], especially available nutrients [79,81,84,86,87] in carbon gains in the soil [81,82,84,88], plants [83,89], regional [84,89], and global levels [82]. Our data found that N was not limited [90] in this land, as the OC:TN ratio in the grass (3.3), mosaic (4.5), and shrub (6.5) stage soils were lower than the value of 11.8 in global grassland soils [90]. However, the OC:TP ratio in the grass (400.7), mosaic (402.6), and shrub (592.3) stage soils were higher than the value of 64.3 in global grassland soils [90], indicating a P limitation in our study land. The soil OC concentration and CS increased with SE and was apparently not limited by the shortage of P, perhaps because a higher proportion of P was stored in the deeper layer (Figure S3), so the P limitation was reduced in the deeper soil layers (Figure 3B). This benefited shrubs, which have deeper roots. Furthermore, SE enhanced the proportion distribution pattern of the P storage (Figure S3). Therefore, SE could reduce nutrient limitation and promote soil carbon sequestration [2].

(b) The traits of encroaching plants determine the functional outcome of encroachment [3]. Changes in soil C cycles might be impacted by vegetation types via differences in litter or root quality [91] and productivity. On the one hand, about 70% of the net primary productivity (NPP) of the natural ecosystem returns to the soil through litter [64], and SE increased the NPP [19], as a result of which the litter productivity increased. On the other hand, the accumulation of OC increased with the decrease in the C/N ratio of the litter [92], and shrub litter had a lower C/N ratio than grass [64,93]. The litter traits of shrubs were thus more conducive to the accumulation of soil OC than of that of grass.

(c) Recent studies have found that a higher plant diversity leads to a greater soil CS [94,95]. Our regression analysis showed that the increasing plant species diversity significantly increased the soil OC concentration and CS (Figure 5), so our results supported this pathway only in terms of the plant species diversity.

(d) Here, for the first time, we integrated the plant factors, soil environment factors, and soil nutrition factors and stoichiometry ratios in order to explore the driving factors of soil carbon storage over shrub encroachment. Our RDA model showed that the soil stoichiometry ratios (OC:TN, TN:TP, OC:TN:TP, AP:ACa) and soil environment factors (BD and pH) explained 87.78% of the total variation of the soil carbon storage (Figure 4E). The plant factors, especially the plant species diversity, and the soil nutrition factors were not as important as we expected. Instead, the soil stoichiometry ratios and soil environment factors were the first two important factors that influenced the soil carbon storage. Our results supported a new pathway of SE control over soil carbon storage.
5. Conclusions

It is critical to analyze soil nutrients and stoichiometry with plant encroachment in specific ecosystems. This study demonstrated the mainly positive effect of SE on the soil nutrient concentration, and the complex effect on soil stoichiometry, in abandoned natural E. pallens grassland. The results showed that SE did not produce serious soil acidification, but significantly changed the soil environment and nutrient concentration. SE significantly improved soil OC and TP concentrations in all layers, changed soil stoichiometry, and in particular improved the stoichiometry ratio of soil OC:TN in all layers. OC storage tended to increase with SE. The shrub relative cover and soil WC explained 55.26% of the total variation of the soil nutrient concentration. The soil available nutrient concentration (AP, AN, AK, ACa and AMg concentration) and shrub relative cover explained 79.10% of the total variation of the soil stoichiometry ratios. The soil stoichiometry ratios (OC:TN, TN:TP, OC:TN:TP and AP:ACa) and soil environment factors (BD and pH) explained 87.78% of the total variation of the soil carbon storage. Our study provides insights to support an improved understanding of SE in grassland from the perspectives of soil nutrition and stoichiometry, and suggests that SE does not indicate the degradation of grassland. In terms of increasing the soil nutrient concentration and CS, the shrub relative cover and soil WC were the most important factors affecting the soil nutrient concentration and soil available nutrient concentration (AP, AN, AK, ACa and AMg concentration), and the shrub relative cover was the most important factor influencing the SNS ratios. The soil stoichiometry ratios (OC:TN, TN:TP, OC:TN:TP and AP:ACa) and soil environment factors (BD and pH) were the first two important factors influencing the soil carbon storage.

Supplementary Materials: The following are available online at http://www.mdpi.com/2071-1050/11/6/1732/s1. Table S1. Interactions and significance effects of shrub encroachment (SE) and soil layers on soil nutrition, C storage, and stoichiometry. Figure S1. Proportion of soil available and total nutrients with different shrub encroachment (SE) stages. Figure S2. Proportional distribution of soil organic carbon storage (CS) at the soil layers (0–5 and 5–20 cm) with shrub encroachment. Figure S3. Proportional distribution of soil total phosphorus (TP) storage at the soil layers (0–5 and 5–20 cm) with shrub encroachment.

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