Science of the Total Environment 743 (2020) 140771

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Overseeding legumes in natural grasslands: Impacts on root biomass and soil organic matter of commercial farms

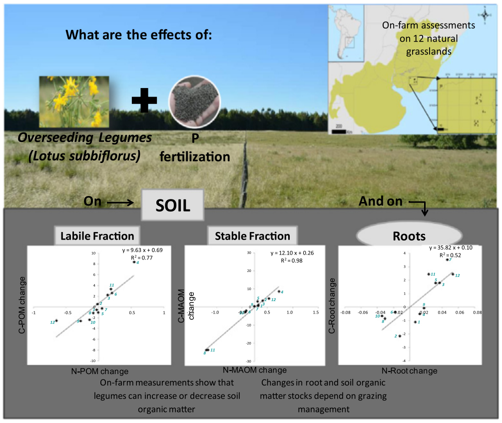
Viviana Bondaruk [a,*], Felipe Lezama [b], Amabelia del Pino [c], Gervasio Piñeiro [a,b]

[a] Instituto de Investigaciones Fisiológicas y Ecológicas vinculadas a la Agricultura, Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina
[b] Departamento de Sistemas ambientales, Facultad de Agronomía, Universidad de la República, Montevideo, Uruguay
[c] Departamento de Suelos y Aguas, Facultad de Agronomía, Universidad de la República, Montevideo, Uruguay

HIGHLIGHTS

- Overseeding legumes in grasslands is a usual practice to increase forage production.
- Overseeding legumes and P fertilization in grasslands may increase N fixation.
- Both practices are expected to increase root biomass and soil organic matter.
- On-farm surveys showed both increases and decreases of roots and soil organic matter.
- Observed opposite changes are suspected to depend on farm's management.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 24 March 2020
Received in revised form 3 July 2020
Accepted 4 July 2020
Available online 6 July 2020

Editor: Ouyang Wei

Keywords:
Grasslands
Soil organic matter
Root biomass
Isotopes
Lotus subbiflorus

ABSTRACT

Overseeding legumes in natural grasslands coupled with phosphorous fertilization are management practices oriented to increase forage production and quality, and to restore nutrient losses generated by livestock. Several studies show increases in forage due to this practice, but less is known about impacts on soil fertility and carbon sequestration. The objective of this study was to evaluate under real farm conditions changes in root C and N stocks and soil organic carbon (SOC) and nitrogen (SON) stocks in two different soil pools, the particulate organic matter (POM) and the mineral associated organic matter (MAOM), after the introduction in natural grasslands of a legume species, *Lotus subbiflorus* cv. "El Rincón", accompanied with phosphorous fertilization. We also evaluated changes in the natural abundance of ^{15}N and ^{13}C in soils and roots to understand changes in N fixation and species composition. We selected 12 adjacent paddocks of natural grasslands (NG) and natural grasslands overseeded with legumes and fertilized with phosphorous (NGLP) located in commercial farms in Uruguay. We found that overseeding legumes increased root C and N stocks and SOC and SON stocks in some farms but decreased them in others. On average, no significant differences arose between NGLP and NG paddocks in total stocks of 0–30 cm depth. However, higher C stocks were observed in POM of NGLP paddocks in 0–5 cm layer and lower contents in 5–10 cm layer indicating a change in the vertical distribution of C in POM. Changes in $\delta^{15}N$ suggest that atmospheric N is being fixed by legumes in NGLP paddocks, but not translated into more N or C stocks in the MAOM fraction, probably due to high N losses promoted by cattle grazing. Our work suggests

---

[*] Corresponding author.
E-mail address: bondaruk@agro.uba.ar (V. Bondaruk).

https://doi.org/10.1016/j.scitotenv.2020.140771

that carbon sequestration can be achieved after legumes introduction in grazed natural grasslands but will depend on grazing management practices.

1. Introduction

Soil organic matter (SOM) is a key carbon (C) pool in natural grasslands, strongly affected by grazing and other management practices, in particular fertilization (Li et al., 2010). SOM has beneficial impacts in soil health, climate mitigation and food security (Lal, 2004; Jobbagy and Jackson, 2000). Domestic grazing of natural grasslands has degraded SOM stocks and produced land degradation worldwide (Su et al., 2010). Therefore, several management practices have been implemented in rangelands to increase SOM or soil organic carbon (SOC) stocks, such as fertilization, rotational grazing, legumes introduction, etc. (Conant et al., 2017; Zhang et al., 2020). Overseeding legume species in natural grasslands, also known as “coverage seeding” or “interseeding”, is a well-known practice aimed to increase forage production and quality, as shown by previous studies (Jaurena et al., 2016; Del Pino et al., 2016; Tiecher et al., 2014). Nonetheless, the impact of overseeding legumes coupled with phosphorous fertilization on SOM and other belowground processes still lacks of deep study, because it may have strong impacts on soil fertility and carbon sequestration (Conant et al., 2017). Legumes introduction in natural grasslands is expected to increase nitrogen (N) fixation and the phosphorous fertilization coupled with the legume introduction enhances grassland's productivity (Rodríguez et al., 2007), which may potentially increase root production and therefore C and N inputs to the soil (Poeplau et al., 2018). A recent global meta-analysis found only 7 studies that evaluated legumes introduction in grasslands, with an overall positive effect on SOC stocks, although several works showed both increases and decreases in SOC stocks (Conant et al., 2017). Although some experimental studies suggest increases in SOC after legumes introduction, changes in SOC stocks under real farm conditions may be different than under controlled experimental settings, due to complex interactions among the environment and real management practices implemented by farmers (Hansson, 2019).

Soil organic matter is complex and therefore to evaluate the effects of grazing management options, such as legumes introduction, SOM must be separated in its different components. Diverse separation schemes have been implemented to evaluate informative SOM pools, although more recently a growing consensus is been established on separating SOM into particulate (POM) and mineral-associated organic matter (MAOM) forms, two SOM pools that are primarily different in terms of their formation, persistence and functioning (Lavallee et al., 2020). The POM fraction, which is more labile and has more rapid decomposition rates, is composed mainly of undecomposed plant residues, while the MAOM fraction that corresponds to compounds associated to mineral particles such as clay and silt has slower decomposition rates (Cambardella and Elliott, 1993; Lavallee et al., 2020). Due to their contrasting dynamics, land use change effects on SOM should be evaluated separately in both fractions (Cotrufo et al., 2019).

We do not know of studies that have evaluated the effects of legumes introduction in natural grasslands on different SOM fractions (i.e. POM and MAOM). If legumes introduction increases above and belowground productivity and these are translated into higher C inputs to the soil, then we would expect increases in the POM fraction (Piñeiro et al., 2009). However, grazing may remove most of this extra productivity and therefore decrease C inputs to the soil, decreasing POM contents (Piñeiro et al., 2010). On the other hand, the introduction of legumes will increase N fixation and therefore N inputs to the soil, increasing soil organic nitrogen (SON) contents, particularly in the MAOM fraction, which is highly dependent on N availability (Piñeiro et al., 2009). Grazing accelerates N cycling in grasslands, because consumed nutrients are rapidly added to the soil via urine and dung patches (Bardgett and Cook, 1998). However, grazing may also increase N losses from these patches via volatilization and leaching (Piñeiro et al., 2009, 2010). The overall effect of legumes introduction in natural grasslands on SON will depend on the magnitude of these contrasting processes under real farm conditions.

Legumes introduced in natural grasslands are expected to fix N biologically, increasing N availability to other plants and soil biota, although the magnitude of this increment will depend on the balance between the amount of N fixed and potential increases in N outputs. The quantity of N incorporated to the ecosystem by biological fixation depends on the legume's biomass production, the % N in their tissues and the % of the N that was fixed from the atmosphere. The ^{15}N natural abundance method proposed by Hogberg (1998) can be used to estimate the % of the N that was fixed from the atmosphere (Unkovich and Pate, 2000; Holdensen et al., 2007; Nebiyu et al., 2014; Robinson, 2001). This method consists in comparing changes in the relative abundance of ^{15}N (δ^{15}N) of a fixing plant, that obtains N from the atmosphere and the soil, and a non-fixing plant that obtains its entire N from the soil. The N fixing plant would present δ^{15}N values closer to 0, the atmospheric value, due to the biological fixation process being the main source of N for these species (Hogberg, 1998). Thus, if a large proportion of N fixed by the legumes is incorporated into SOM, then the δ^{15}N of SOM will be closer to the atmospheric value (zero), as compared to SOM values in grasslands without legumes.

The Rio de la Plata grasslands region, located in southern South America, is a vast region of natural grasslands grazed by domestic herbivores, where the introduction of legumes is widespread. Grasslands of this region has been converted to croplands or forest plantations, but relatively vast areas still remain under natural grasslands with moderate to heavy grazing pressures (Podestá et al., 2009). The Rio de la Plata grasslands encompass an area of 70 million ha between the East Center of Argentina, Uruguay and Rio Grande do Sul in Brazil (Paruelo et al., 2007). This region has the biggest extension of grasslands in South America and supports a wide number of domestic cattle existences (Soriano, 1992). Several sub-regions are under an intense agricultural use while others remain under natural prairies and steppes, which sustain livestock production with large social and economic relevance (Baldi and Paruelo, 2008; Soriano, 1992). Overseeding natural grasslands with an exotic legume, *Lotus subbiflorus* cv. “El Rincón” coupled with phosphorous fertilization to promote its growing is a common practice in this region, occurring in nearly 2 million ha (Rebuffo et al., 2006). *Lotus subbiflorus* is an annual specie with high persistence in these grasslands (Berretta et al., 2009), and although increases in productivity and forage quality have been documented, scarce information exists about its effect on belowground dynamics, particularly root production and SOM accumulation (Carámbula et al., 1994). Understanding its belowground effects will serve to design management practices aimed to improve soil fertility and carbon sequestration with important impacts on climate change mitigation efforts, food security and sovereignty.

The objective of this study was to evaluate under real farm conditions changes in root C and N stocks and soil organic carbon and nitrogen stocks associated to the introduction of exotic legumes accompanied with phosphorous fertilization in natural grasslands. To accomplish this, root biomass and C and N stocks in the POM and MAOM fractions were measured in 12 pairs of adjacent paddocks of natural grasslands (NG) and natural grasslands overseeded with legumes and fertilized with phosphorous (NGLP) in different commercial farms of Uruguay. Our hypotheses were that overseeding legumes coupled with phosphorous fertilization will: 1) increase aboveground and belowground productivity and therefore root biomass and C contents in the POM fraction; and 2) will enhance biological N fixation increasing N availability in the soil and therefore increasing SON and SOC stocks mainly in the MAOM fraction.

2. Methods

2.1. Study system and experimental design

Remaining natural grasslands of the Rio de la Plata Grasslands region are mostly dedicated to sheep and cattle grazing. Livestock is an important economical and social activity of the region. Native grass species are mostly predominant in these grasslands, both C_3 and C_4, from the genera *Bromus*, *Paspalum*, *Bothriocloa*, *Stipa*, *Schizachyrium* among others (Soriano, 1992). There are also shrubs of the genera *Baccharis* or *Eupatorium* that can be abundant and even dominant depending on the region and grazing management (Altesor et al., 2006). Climate conditions of the Rio de la Plata Grasslands are temperate to subtropical with a mean annual temperature between 10 and 20 °C (Soriano, 1992). Precipitations have mostly a uniform regime distribution along the year and encompass mean annual precipitation amounts from 1500 to 600 mm from east to west (Paruelo et al., 2003). Soils are mostly represented by mollisols, alfisols and vertisols predominant in the east and entisols in the west. The textures are predominantly fine in the east with more proportion of sand in the west.

Based on farmers' interviews, we selected 12 sites in commercial farms located in the east center of ROU (Fig. 1; Table 1 Supplementary Material), devoted to extensive cattle ranching, with large paddocks of natural grasslands (NG) and natural grasslands overseeded with legumes and fertilized with phosphorous (NGLP). The plant communities of the selected grasslands belong specifically to the *Eryngium horridum-Juncus capillaceus* community described by Lezama et al. (2019). A paired design was used which consisted in adjacent sites of NG versus NGLP (all with *Lotus subbiflorus* cv. “El Rincón” as the introduced legume specie). Each pair of paddocks which were separated by wired fences were subjected to grazing. We carefully selected representative areas with similar topographical level, avoiding cattle paths, and separated 30 m from the fence. Soil samples were extracted using a 30 cm long and 1.8 cm wide soil corer to obtain soil samples at four depths (0–5 cm; 5–10 cm; 10–20 cm; 20–30 cm). Within each paddock 12 soil cores were extracted randomly and soil samples composited. Likewise, root biomass was sampled with a root auger (7.5 cm diameter) at three depths (0–5 cm; 5–10 cm; 10–20 cm).

2.2. Sample and data analysis

Soil samples were processed to estimate bulk density and C and N contents in POM and MAOM fractions. Bulk density was estimated from a subsample of 5 g dried at 105 °C during 48 hs and weighed to determine water content. The rest of the sample was sieved at 2 mm, discarding rocks and roots that were weighed and stored separately. Then, 10 g were extracted from the sieved sample and used for fractionating SOM according to Cambardella and Elliott (1993). Briefly, samples were shaken in sodium hexametaphosphate 5% dispersant solution during 18 h and dispersed slurry was passed through a 25-micron sieve and washed with distilled water in order to separate the fine sand sized fraction from the clay and silt sized fraction. The portion of C and N retained in the sieve are the sand sized organic C (C-POM) and N (N-POM), respectively. The slurry that passed the sieve contains the mineral associated organic C (C-MAOM) and N (N-MAOM). Both fractions were oven-dried at 60 °C in glass beakers until water evaporated completely. Carbon, N, δ^{15}N y δ^{13}C contents in each SOM fraction were estimated with a Carlo Erba NA 1500 Elemental Analyzer at Duke Environmental Isotope Laboratory (DEVIL) in Duke University, USA. The following equation was used to calculate final C or N stocks in an equivalent soil mass:

$$\text{SOC fraction}_{\left(\text{Tonha}^{-1}\right)} = \frac{\text{Fw}_{(\text{g})}\cdot \text{CP}_{(\%)}\cdot \text{BD}_{\left(\text{Ton m}^{-3}\right)}\cdot \text{Z}_{(\text{m})}}{\text{TW}_{(\text{g})}} \tag{1}$$

where: SOC is soil organic carbon in a soil fraction (Ton ha^{-1}) (or SON for N), Fw is the fraction (POM or MAOM) weight (g), CP is the proportion of C or N of each fraction, BD is soil bulk density (Ton m^{-3}), Z is the

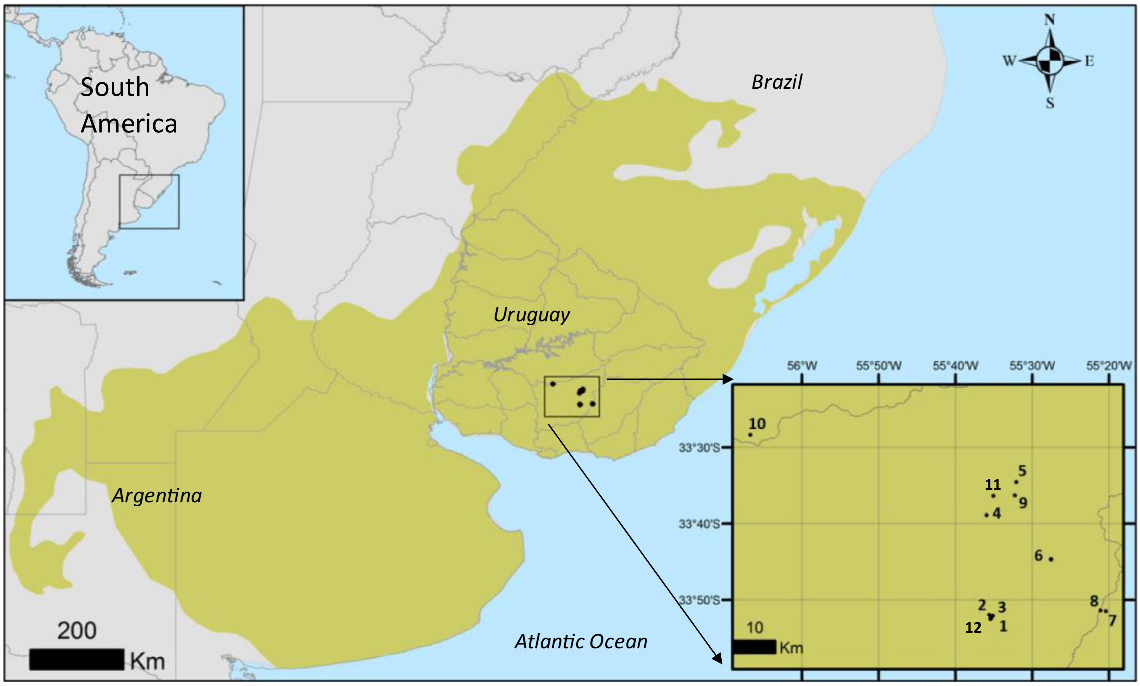


Fig. 1. Location of the study sites in the Rio de la Plata Grasslands region in Uruguay, South America. Each number corresponds to a different farm with paired natural grasslands with adjacent natural grassland with legumes and phosphorous fertilization. The Rio de la Plata grassland's region is shown in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

corrected depth (m) as explained below and TW is soil total sample weight previous to fractionating (g) (Sollins et al., 1999; Solomon et al., 2002). Results of the Eq. (1) were then multiplied by 100 for units' compensation. To avoid overestimation between NG and NGLPs due to changes in soil compaction usually generated by different grazing regimes, calculations were made based on a constant soil mass basis (Davidson and Ackerman, 1993) using the correction factor Z proposed by Solomon et al. (2002):

$$Z\,(m) = \frac{BD_{NG} \cdot X\,(m)}{BD_{NGLP}} \quad (2)$$

where: BD_{NG} is the soil bulk density of the natural grassland, BD_{NGLP} is bulk density of the natural grassland with overseeded legumes and phosphorous and X (m) is sample depth (Davidson and Ackerman, 1993; Solomon et al., 2002). Nitrogen contents in the POM fraction in deepest layers were very low and therefore not detected by the element analyzer in nearly 20% of the samples. For these samples, N contents were estimated based on C contents measured in the sample and C:N ratios interpolated from the samples in the upper layers.

Root samples were washed and separated with tweezers over a 0.5 mm sieve. Collected roots were oven-dried at 60 °C during two days and weighed in order to estimate root biomass. Similar to soil samples C, N, δ^{15}N y δ^{13}C contents in each root sample was analyzed with a Carlo Erba NA 1500 Elemental Analyzer in DEVIL at Duke University, USA.

2.3. Statistical analysis

Data were analyzed in R software (R Development Core Team, 2017) using linear mixed-effects models ANOVA to evaluate differences among the two treatments (NG and NGLP) ($n = 12$). The treatment was considered as a fixed effect, while sites (i.e. replicates) as a random effect. The *lme* and *glmer* functions were used with the *lme4* and *nmle* packages (Bates et al., 2015). Assumptions for the different tested models were evaluated with the *Levene* test to prove homocedasticity of variances, and *Shapiro–Wilks* test to check the assumption of normally distributed residuals for all response variables. We used log transformations when data did not follow a normal distribution or a nonparametric test, *Kruskal Wallis* test. Also, several models combining different functions for variance errors correction were tested when heterocedasticity was found. Generalized linear models' regressions were used to evaluate correlations among the different variables measured. The best-fitted models for each response variable were selected based on the Akaike Criterion.

3. Results

Contrary as expected, increases or decreases in C and N stocks were found in the different farms evaluated, and therefore, on average, the introduction of *Lotus subbiflorus* cv. "El Rincón" and phosphorous fertilization produced no significant differences in total SOC and SON stocks in both the POM and MAOM fractions considering the first 30 cm of the soil (Fig. 2). However, small significant changes were detected in particular soil depths. In the surface layer C-POM contents showed a marginal increase under NGLP (1.28 ton ha^{-1} or 21%, though not significative $p = 0.1360$), but decreased in the following layer from 5 to 10 cm (0.48 ton ha^{-1} or 24%; $p < 0.05$), suggesting a small change in the vertical distribution of C under NGLP, not observed for N-POM contents (Fig. 2). In deeper layers, both C and N-POM contents were similar and there were no significant differences between treatments. The C:N ratio of the POM fraction showed a small decrease under NGLP, but only in the 5–10 cm surface layer ($p = 0.05$) (Fig. 2). On the other hand, C and N-MAOM contents represented around 82% (49.6 ton of C ha^{-1}) and 88% (4.6 ton of N ha^{-1}) of total SOC and SON stocks respectively, and showed no significant differences between treatments, except in the deepest 20–30 cm layer, where NGLP paddocks had slightly smaller C and N contents ($p < 0.05$).The C:N ratios of the MAOM fraction were also similar among treatments for all depths evaluated (Fig. 2). In both soil fractions, δ^{15}N values were lower (closer to the atmospheric value) in NGLP paddocks than in NG, suggesting that

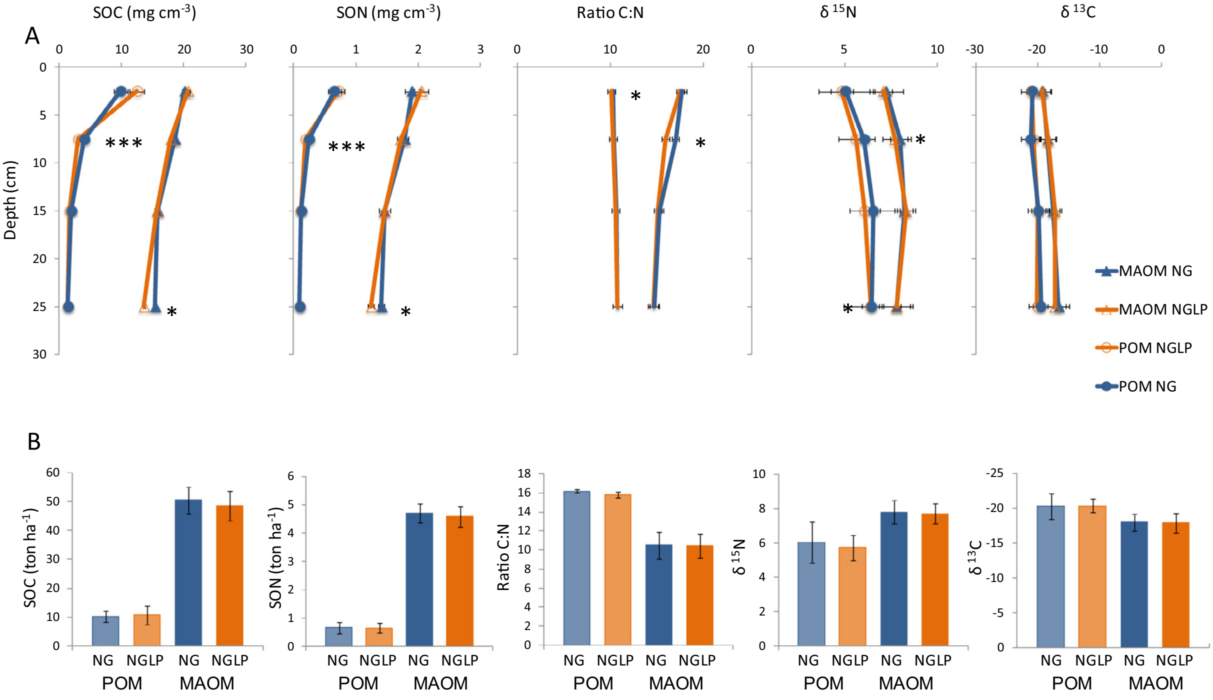


Fig. 2. Soil organic carbon and nitrogen contents (SOC and SON respectively), C:N ratios, δ^{15}N and δ^{13}C values in the POM and MAOM fractions under both treatments: natural grasslands (NG) and natural grasslands overseeded with legumes and fertilized with phosphorous (NGLP). Panel A) shows variations in depth and panel B) total or average contents for the first 30 cm of the soil. Significant differences between treatments are indicated with standard statistical nomenclature (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).

legumes introduction fixed N from the atmosphere that remained in both soil fractions (although significant differences were detected only in two soil depths) (Fig. 2). Finally, δ^{13}C values in both soil fractions were similar between treatments, showing no clear trends in functional composition after legumes introduction (Fig. 2). Despite these similar averages, some farms showed large differences in C and N contents between treatments in both fractions as explained below.

Similarly, we did not find significant differences between NG and NGLP paddocks in root C or N contents (Fig. 3). Carbon and N root stocks measured from 0 to 20 cm depth were around of 4.5 ton ha^{-1} and 0.1 ton ha^{-1}, respectively, with an average C:N ratio of 48. No significant differences were detected in the different depths evaluated or for total root contents (Fig. 3), although average N contents in roots were slightly higher under NGLP paddocks. Equally, δ^{15}N contents did not show a clear trend between treatments, suggesting low or variable root contents of the introduced legume (*Lotus subbiflorus* cv. El Rincón) in the plant community during the sampling year. The lack of changes in root δ^{13}C suggests low or variable changes in the proportion of C_3 vs. C_4 species between NG and NGLP paddocks in the different farms. As with soil organic matter, beyond these similar averages between treatments, some farms showed higher and other lower root C and N contents and δ^{15}N and δ^{13}C values in NG compared to NGLP paddocks, as described below.

Behind these observed averages, the introduction of legumes and phosphorous fertilization increased notably C and N contents in the POM or MAOM fraction in some farms, but decreased them in others (Fig. 4). Some farms showed large increases in their C-POM y C-MAOM stocks (as well as their N-POM and N-MAOM), but other farms lost important quantities of C and N after overseeding with legumes the natural grassland (e.g. farms 8 and 11 that lost near 24 Ton of C ha^{-1}) (Fig. 4). Similarly, root C and N contents were very variable among farms, as there were sites that increased them by near 150%

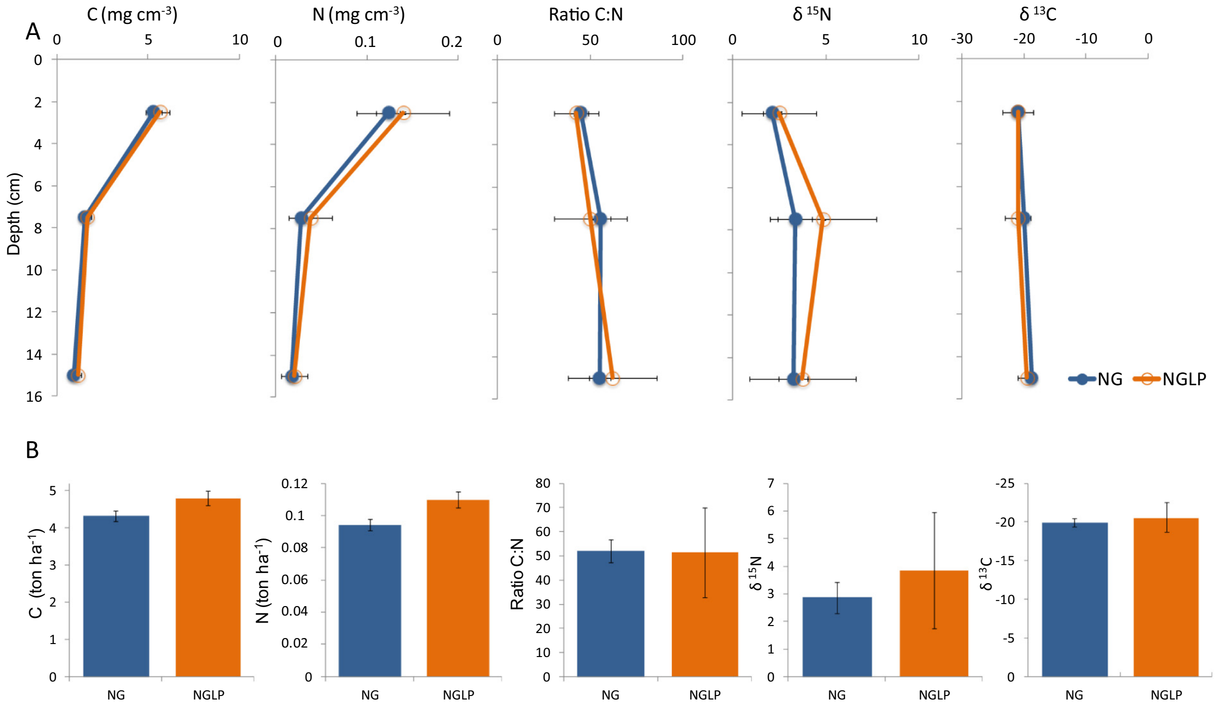


Fig. 3. Root carbon and nitrogen contents, C:N ratios, δ^{15}N and δ^{13}C values under both treatments: natural grasslands (NG) and natural grasslands overseeded with legumes and fertilized with phosphorous (NGLP). Panel A) shows variations in depth and panel B) total or average contents for the first 20 cm of the soil. Significant differences between treatments are indicated with standard statistical nomenclature (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).

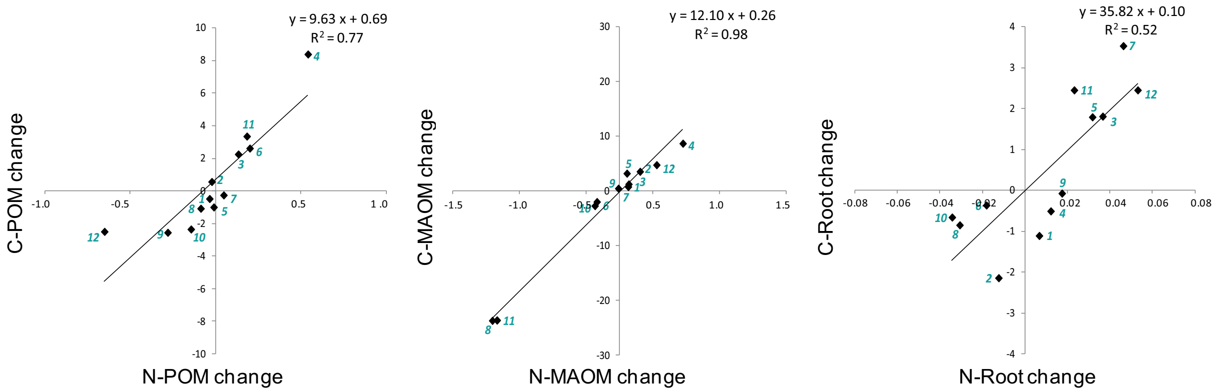


Fig. 4. Observed changes in soil organic C and N contents (SOC and SON in ton ha^{-1}) in different soil fractions (POM and MAOM) and roots (ton ha^{-1}) after the introduction of legumes and phosphorous fertilization of natural grasslands, for each farm evaluated. Farms are shown with numbers, see map in Fig. 1. Carbon and N changes are accumulated for the first 30 cm of the soil and roots are total changes occurred from 0 to 20 cm. Regression lines and equation parameters are shown between changes in C and N contents.

and others that decreased them by 50% under NGLP. Farms that lost C in a particular soil fraction generally lost N in the same fraction, showing high correlations between C and N changes in each soil fraction (see regression lines in Fig. 4). Root changes showed a similar pattern but were more variable (lower R^2 of the regression line). On the other hand, some farms showed C and N gains in the POM fraction but losses in the MAOM fraction (i.e. see site 11) or vice versa (i.e. see site 12), although most farms had similar changes in both fractions. Finally, some farms showed similar changes (increases or decreases) in soil and root C or N contents under NGLP (i.e. farms 8 and 3), but changes were not consistent among all farms, suggesting complex interactions in belowground processes.

Changes among farms in C and N stocks or soil δ^{15}N and δ^{13}C values in the POM and MAOM fractions were not associated to other soil variables or grazing management practices recorded, although natural grassland paddocks were more variable in these attributes among farms than paddocks with legumes and phosphorous fertilization (see Figs. 5 and 6). Overall, the introduction of legumes and phosphorous fertilization seemed to homogenize SOC and SON contents (Fig. 5) as well

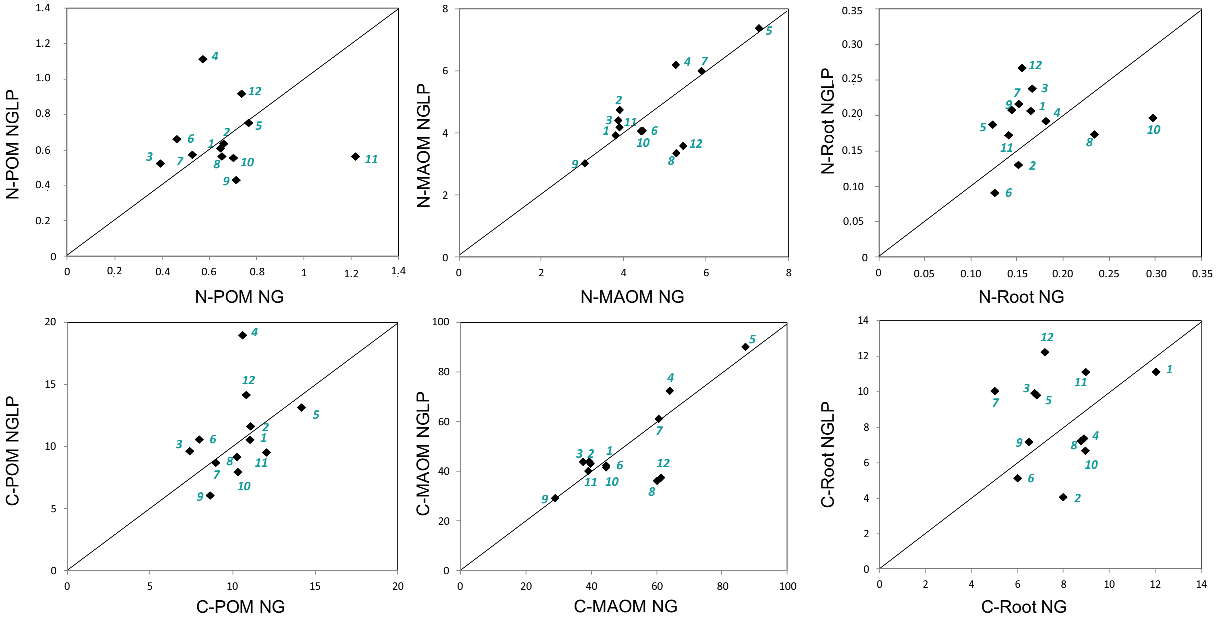


Fig. 5. Relation between carbon and nitrogen stocks in the POM and MAOM fractions, and in roots under natural grasslands (NG-in the *x* axis) vs. natural grasslands with legumes introduction and phosphorous fertilization (NGLP, in the *y* axis). Soil values are accumulated for the first 30 cm of the soil and root values for 0–20 cm depth. Diagonal lines represent de 1:1 line.

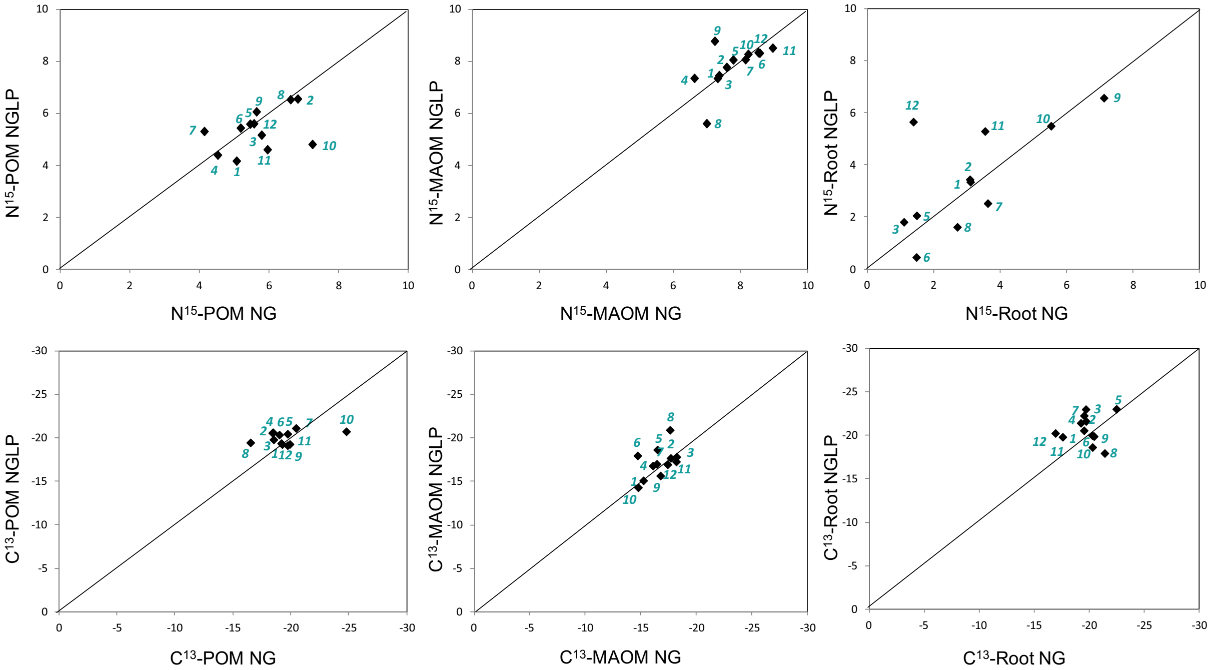


Fig. 6. Relation between δ^{15}N and δ^{13}C values in the POM and MAOM fractions, and in roots under natural grasslands (NG-in the *x* axis) vs. natural grasslands with legumes introduction and phosphorous fertilization (NGLP, in the *y* axis). Soil values are accumulated for the first 30 cm of the soil and root values for 0–20 cm depth. Diagonal lines represent de 1:1 line.

as δ^{15}N and δ^{13}C contents (Fig. 6) between farms, particularly in the POM fraction and in roots. Therefore, farms with higher initial C and N stocks or δ^{15}N and δ^{13}C values in the POM fraction under natural grasslands showed the higher decreases in these variables after legumes introduction, while farms with low initial contents mostly showed increases after legumes introduction. A similar pattern was observed for roots, but not in the MAOM fraction, where contents under both treatments were usually very similar (Figs. 5 and 6). Besides this pattern no other variables measured showed any significant association with the observed changes between farms. Variables considered in these analyses were: years since legumes introduction and phosphorous fertilization, legume cover in the sampling year, soil bulk density, soil texture, stocking rates, changes in stocking rates (difference between NG and NGLPs), stocks and changes in root biomass between treatments, stocks and changes in root C and N or δ^{15}N and δ^{13}C values.

4. Discussion

4.1. Effects on soil carbon and nitrogen stocks

Our results suggest that although legumes introduction may increase N availability and productivity, potentially enhancing SOM contents, the impact of this practice on SOM in real commercial farms will depend strongly on other management practices performed by farmers. Positive effects of NGLP on aerial productivity and forage quality of natural grasslands have been widely documented (Rebuffo et al., 2006; Jaurena et al., 2016; Del Pino et al., 2016), but less is known about the effect of this practice on SOM and far less on the different SOM fractions. Although some works indicate that introducing legumes in natural grasslands coupled with phosphorous fertilization can enhance total SOC (Conant et al., 2017, 2001; Poeplau et al., 2018), in our study total SOC did not increase consistently in all NGLP paddocks. In part, this could be associated with higher decomposition rates and therefore higher C and N losses in the soils of oversown and fertilized pastures (Lodge et al., 2006). However, the effects of overseeding and fertilization on litter and therefore on decomposition are inherently associated to grazing management and how the practice is applied (Lodge et al., 2006). Nevertheless, we did not find any management or soil variable that explained the observed increases or decreases in SOC and SON stocks; it is likely that differences among farms in how they apply the practices and the grazing management are offsetting expected SOC and SON increases.

However, our results also suggest that on average legumes introduction increased top soil C in the POM fraction and decreased C contents in the MAOM fraction in deeper layers, although differences were marginally significant. Similar results were obtained by Salvo et al. (2008), in an experimental setup at a nearby region, were legumes introduction increased SOC stocks in the POM fraction in the surface 0–5 cm layer, but decreased C stocks in the MAOM fraction at all depths. Therefore, it is not clear if expected increases in SOC after legumes introduction reported for other regions (Conant et al., 2017) do not occur in this region (where increases in the top soil POM are offset by decreases in the MAOM fraction), or if farmers management practices are counterbalancing potential increases in SOC stocks.

4.2. Effects on root biomass

Carbon and N increases in the POM fraction in the top soil (0–5 cm) have been suggested to be promoted by higher biomass production or a greater proportion of root biomass in the top soil, due to the shallow root system of the annual legume *Lotus subbiflorus* cv. “El Rincón” (Salvo et al., 2008; Carámbula et al., 1994). Increases in aerial productivity due to legumes overseeding has been reported previously in the region (Rebuffo et al., 2006; Jaurena et al., 2016; Del Pino et al., 2016), but we did not find differences in root biomass after legumes introduction. However, we did not evaluate root biomass in the first years after legumes introduction, before other changes in species composition may have operated and counterbalanced potential increases in legumes root biomass. Changes in root biomass in grasslands can be generated from changes in root biomass of particular species or its belowground allocation, but also from changes in community composition (Ward et al., 2002; Piñeiro et al., 2010). Changes in the proportion of C3 or C4 species may affect root biomass because generally C4 species have higher root and rhizome contents (Piñeiro et al., 2009). However, we did not find strong relationships between changes in species composition (measured through soil δ^{13}C) and changes in SOC or SON stocks across farms. Therefore, future studies should evaluate the effects of legumes introduction in species composition and root production, and particularly their interaction with grazing management.

4.3. Effects on the nitrogen cycle

Soil organic nitrogen stocks are expected to increase after legumes introduction due to the incorporation of biologically fixed nitrogen. Nitrogen is a key component of stable soil organic matter (mainly MAOM) and therefore may limit its formation and accumulation (Knicker, 2011; Cotrufo et al., 2019). Therefore, the higher N contents of plant residues promoted by legumes may increase the quality of biomass inputs to the soil that can be rapidly incorporated into MAOM (Lavallee et al., 2020; Campillo et al., 2005; Salvo et al., 2008). Nevertheless, diminishes in C-MAOM contents in NGLP treatments reported by Salvo et al., (2008) and the variability in C-MAOM changes among farms found in our study suggest that other processes are affecting the N cycle and thus SON is not clearly increasing after legumes introduction. Our $\delta15$N results show that N fixed by legumes is being incorporated into POM, but the lack of increases in N-POM or N-MAOM stocks suggest potential higher N outputs from ecosystems. Increases in N outputs may occur after legumes introduction, probably associated to changes in grazing management that can counterbalance the greater N inputs by legumes and domestic livestock through urine and dung patches (Bardgett and Cook, 1998; Piñeiro et al., 2010) therefore producing decreases in N-MAOM stocks. Nonetheless, if higher stocking rates are being applied in NGLP sites, increases in N losses by volatilization and leaching from urine and dung patches may be happening as well (West et al., 2005; Piñeiro et al., 2006). Amundson et al. (2003), suggested that sites with higher $\delta15$N values have a more closed N cycle than sites with lower $\delta15$N values, supporting this hypothesis.

Higher N inputs by biological nitrogen fixation in NGLP paddocks could have promoted changes in plant species composition, with increases in the proportion of exotic or annual species (Jaurena et al., 2016; Berretta et al., 2009; Carámbula et al., 1994; Tilman et al., 1996). In the long-term these changes in species composition can produce natural grasslands degradation, although native species usually compete and exclude introduced legumes in the long-term as previously observed (Pallarés et al., 2005). The long-term exclusion of *Lotus subbiflorus* cv. “El Rincón” by native species can explain its low presence in some farms, although its coverage usually fluctuates from year to year. Natural abundance of δ^{13}C observed in natural grasslands suggests that changes in functional composition did not occur in a unique direction after legumes introduction in the studied farms, since some increased the proportion of C3 in but others decreased them. Observed changes in the proportion of C3/C4 were not associated to changes in C and N contents in POM or MAOM, indicating complex and multiple interactions among vegetation, soils and grazing as observed elsewhere (Piñeiro et al., 2009).

4.4. Interaction with the real on-farm grazing management practices

The heterogeneity found in grazing management, fertilization and overseeding practices could explain the variability in the results of our study. Information about management practices and stocking rates were obtained through personal interviews, but printed records from the different cattle ranchers were usually nonexistent. Although all selected sites were overseeded with the same species (*Lotus subbiflorus* cv. "El Rincón"), most farms had no reseeding practices (overseeding was made once at the initial stage but never reseeded) and grazing management practices that allowed natural reseeding differed among farms. Since *Lotus subbiflorus* cv. "El Rincón" is an annual specie, reseeding is very important for its establishment and persistence (Rebuffo et al., 2006; Ayala et al., 2001). In addition, farmers usually apply higher stocking rates in the NGLP paddocks as observed in other studies (Jaurena et al., 2016). We also found differences in the type, dose and frequency of phosphorous fertilization among farms that in addition with grazing management could have produced the differential responses in SOC and SON. Finally, our paired observational experimental design could have also generated extra variability, because potential differences among paired paddocks may have existed previous to legumes introduction. However, soil texture analyses showed that soils were very similar and the scarce changes in C and N contents in the MAOM (more stable) fraction, suggest that pairs were correctly selected.

5. Conclusion

Our work contributes with novel information about a common practice usually performed in commercial farms worldwide. Our results suggest for the need to perform future studies that continue evaluating mechanisms and processes occurring after legumes overseeding and phosphorous fertilization of natural grasslands and its relation with grazing management strategies. This information could be an important tool to improve rangelands management in order to develop practices that enhance forage production but also improve soil fertility. In addition, our results can serve to inform potential carbon sequestration strategies but also bring doubt about the possible increases in soil organic C and N stocks after introducing legumes in commercial farms and its declared potential climate benefits. Our results suggest that carbon sequestration can be achieved after legumes introduction in grazed natural grasslands but will depend on accompanying grazing management practices, such as stocking rates that affect grasslands productivity, or other grazing management practices that allow reseeding of legumes and its productive persistence in the sward.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.140771.

CRediT authorship contribution statement

Viviana Florencia Bondaruk: Investigation, Writing - review & editing, Formal analysis. **Felipe Lezama:** Data curation, Writing - original draft. **Amabelia del Pino:** Data curation, Writing - original draft. **Gervasio Piñeiro:** Investigation, Writing - review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We sincerely thank the owners of SUL (Secretariado Uruguayo de la Lana), Umpierrez, Scarlatto, Marquez and Caja Notarial for allowing plant and soil sampling in their farms and sharing grazing management information with us. We especially thank Hernán Dieguez, Noelia Calefato, Katelyn Heflin and Martín Lammel that helped greatly in field trips and in sample processing and laboratory analysis. We also would like to express our sincere acknowledgement to the editor and two anonymous reviewers for assertive commentaries that improved the article. This article was performed with funds from CSIC, UdelaR by the project "Evaluación de la sustentabilidad de las siembras en cobertura de leguminosas y la fertilización fosforada de campo natural: Efectos de largo plazo sobre los ciclos biogeoquímicos y la productividad primaria" and by ANII-INNOVAGRO 2018, FSA_PI_2018_1_148819, Dinámica y secuestro de carbono en sistemas ganaderos sobre campo natural. This work was also carried out with the aid of a Grant from the Inter-American Institute for Global Change Research (IAI) CRN III 3005 and 3095 which is supported by the US National Science Foundation (Grant GEO-1128040).

References

Altesor, A., Piñeiro, G., Lezama, F., Jackson, R.B., Sarasola, M., Paruelo, J.M., 2006. Ecosystem changes associated with grazing in subhumid South American grasslands. J. Veg. Sci. 17, 323–332. https://doi.org/10.1111/j.1654-1103.2006.tb02452.x.

Amundson, R., Austin, A.T., Schuur, E.A.G., Yoo, K., Matzek, V., Kendall, C., Uebersax, A., Brenner, D., Baisden, W.T., 2003. Global patterns of the isotopic composition of soil and plant nitrogen. Glob. Biogeochem. Cycles https://doi.org/10.1029/2002GB001903.

Ayala, W., Carambula, M., Risso, D., Hodgson, L., Kemp, P.D., 2001. Effects of management strategies on seed production and seedling recruitment in birdsfoot trefoil-white clover mixtures. Proceedings of the XIX International Grassland Congress - Grassland Ecosystems: An Outlook Into the 21st Century.

Baldi, G., Paruelo, J.M., 2008. Land-use and land cover dynamics in South American Temperate grasslands. Ecol. Soc. https://doi.org/10.5751/ES-02481-130206.

Bardgett, R.D., Cook, R., 1998. Functional aspects of soil animal diversity in agricultural grasslands. Appl. Soil Ecol. https://doi.org/10.1016/S0929-1393(98)00125-5.

Bates, D., Mächler, M., Bolker, B.M., Walker, S.C., 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. https://doi.org/10.18637/jss.v067.i01.

Berretta, E.J., Risso, D.F., Montossi, F., Pigurina, G., 2009. Campos in Uruguay. Grassland Ecophysiology and Grazing Ecology https://doi.org/10.1079/9780851994529.0377.

Cambardella, C.A., Elliott, E.T., 1993. Methods for physical separation and characterization of soil organic matter fractions. Geoderma 56, 449–457.

Campillo, R., Urquiaga, S., Undurraga, P., Pino, I., Boddey, R.M., 2005. Strategies to optimise biological nitrogen fixation in legume/grass pastures in the southern region of Chile. Plant Soil 273 (1–2), 57–67.

Carámbula, M., Carriquiry, E., Ayala, W., 1994. Cultivos Mejoramiento a campo con *Lotus subbiflorus* cv. El Rincón. Boletín de divulgación N° 44. INIA-T y Tres (21 pp.).

Conant, R.T., Paustian, K., Elliott, E.T., 2001. Grassland management and conversion into grassland: effects on soil carbon. Ecol. Appl. 11, 343. https://doi.org/10.2307/3060893.

Conant, R., Cerri, C.E.P., Osborne, B.B., Paustian, K., 2017. Grassland management impacts on soil carbon stocks: a new synthesis. Ecol. Appl. 27, 662–668. https://doi.org/10.1002/eap.1473.

Cotrufo, M.F., Ranalli, M.G., Haddix, M.L., Six, J., Lugato, E., 2019. Soil carbon storage informed by particulate and mineral-associated organic matter. Nat. Geosci. 12, 989–994. https://doi.org/10.1038/s41561-019-0484-6.

Davidson, E.A., Ackerman, I.L., 1993. Changes in soil carbon inventories following cultivation of previously untilled soils. Biogeochemistry https://doi.org/10.1007/BF00000786.

Del Pino, A., Rodríguez, T., Andión, J., 2016. Production improvement through phosphorus fertilization and legume introduction in grazed native pastures of Uruguay. J. Agric. Sci. 154, 347–358. https://doi.org/10.1017/S002185961500101X.

Hansson, S.O., 2019. Farmers' experiments and scientific methodology. Eur. J. Philos. Sci. 9, 1–23. https://doi.org/10.1007/s13194-019-0255-7.

Hogberg, P., 1998. Erratum: 'Transley Review No. 95: 15N natural abundance in soil-plant systems (New Phytologist (1997) 137 (179-203)). New Phytol. https://doi.org/10.1046/j.1469-8137.1998.00239.x.

Holdensen, L., Hauggaard-Nielsen, H., Jensen, E.S., 2007. Short-range spatial variability of soil δ15N natural abundance - effects on symbiotic N2-fixation estimates in pea. Plant Soil 298, 265–272. https://doi.org/10.1007/s11104-007-9367-5.

Jaurena, M., Lezama, F., Salvo, L., Cardozo, G., Ayala, W., Terra, J., Nabinger, C., 2016. The dilemma of improving native grasslands by overseeding legumes: production intensification or diversity conservation. Rangel. Ecol. Manag. https://doi.org/10.1016/j.rama.2015.10.006.

Jobbagy, E.G., Jackson, R.B., 2000. The vertical distribution of soil organic carbon and its relation to climate and vegetation. Ecol. Appl. 10, 423–436.

Knicker, H., 2011. Soil organic N - an under-rated player for C sequestration in soils? Soil Biol. Biochem. https://doi.org/10.1016/j.soilbio.2011.02.020.

Lal, R., 2004. Soil carbon sequestration impacts on global climate change and food security. Science (80-.) https://doi.org/10.1126/science.1097396.

Lavallee, J.M., Soong, J.L., Cotrufo, M.F., 2020. Conceptualizing soil organic matter into particulate and mineral-associated forms to address global change in the 21st century. Glob. Chang. Biol. https://doi.org/10.1111/gcb.14859.

Lezama, F., Pereira, M., Altesor, A., Paruelo, J.M., 2019. Grasslands of Uruguay: classification based on vegetation plots. Phytocoenologia 49, 211–229. https://doi.org/10.1127/phyto/2019/0215.

Li, L., Xu, Z., Wu, J., Tian, G., 2010. Bioresource technology bioaccumulation of heavy metals in the earthworm Eisenia fetida in relation to bioavailable metal concentrations in pig manure. Bioresour. Technol. 101, 3430–3436. https://doi.org/10.1016/j.biortech.2009.12.085.

Lodge, G.M., King, K.L., Harden, S., 2006. Effects of pasture treatments on detached pasture litter mass, quality, litter loss, decomposition rates, and residence time in northern New South Wales. Aust. J. Agric. Res. 57, 1073–1085. https://doi.org/10.1071/AR05408.

Nebiyu, A., Huygens, D., Upadhayay, H.R., Diels, J., Boeckx, P., 2014. Importance of correct B value determination to quantify biological N2 fixation and N balances of faba beans (Vicia faba L.) via 15N natural abundance. Biol. Fertil. Soils 50, 517–525. https://doi.org/10.1007/s00374-013-0874-7.

Pallarés, O.R., Berretta, E.J., Maraschin, G.E., 2005. The south american campos ecosystem. In: Suttie, J., Reynolds, S.G., Batello, C. (Eds.), Grasslands of the world. FAO, pp. 171–219.

Paruelo, J.M., Piñeiro, G., Altesor, A.I., M, R.C.O., 2003. Los Pastizales Del Río De La Plata.

Paruelo, J.M., Jobbágy, E.G., Oesterheld, M., Golluscio, R. a, Aguilar, M.R., 2007. The grasslands and steppes of Patagonia and the Rio de la Plata plains. Phys. Geogr. South Am. 14, 232–233.

Piñeiro, G., Paruelo, J.M., Oesterheld, M., 2006. Potential long-term impacts of livestock introduction on carbon and nitrogen cycling in grasslands of southern South America. Glob. Chang. Biol. 12, 1267–1284. https://doi.org/10.1111/j.1365-2486.2006.01173.x.

Piñeiro, G., Paruelo, J.M., Jobbágy, E.G., Jackson, R.B., Oesterheld, M., 2009. Grazing effects on belowground C and N stocks along a network of cattle exclosures in temperate and subtropical grasslands of South America. Glob. Biogeochem. Cycles https://doi.org/10.1029/2007GB003168.

Piñeiro, G., Paruelo, J.M., Oesterheld, M., Jobbágy, E.G., 2010. Pathways of grazing effects on soil organic carbon and nitrogen. Rangel. Ecol. Manag. https://doi.org/10.2111/08-255.1.

Podestá, G., Bert, F., Rajagopalan, B., Apipattanavis, S., Laciana, C., Weber, E., Easterling, W., Katz, R., Letson, D., Menendez, A., 2009. Decadal climate variability in the argentine pampas: regional impacts of plausible climate scenarios on agricultural systems. Clim. Res. 40, 199–210. https://doi.org/10.3354/cr00807.

Poeplau, C., Zopf, D., Greiner, B., Geerts, R., Korvaar, H., Thumm, U., Don, A., Heidkamp, A., Flessa, H., 2018. Why does mineral fertilization increase soil carbon stocks in temperate grasslands? Agric. Ecosyst. Environ. 265, 144–155. https://doi.org/10.1016/j.agee.2018.06.003.

R Development Core Team, 2017. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria 3-900051-07-0 URL. http://www.R-project.org.

Rebuffo, M., Bemhaja, M., Risso, D.F., 2006. Utilization of forage legumes in pastoral systems: state of art in Uruguay. Lotus Newsl 36, 22–33.

Robinson, D., 2001. δ15N as an integrator of the nitrogen cycle. Trends Ecol. Evol. 16, 153–162. https://doi.org/10.1016/S0169-5347(00)02098-X.

Rodríguez, A.M., Jacobo, E.J., Scardaoni, P., Deregibus, V.A., 2007. Effect of phosphate fertilization on flooding Pampa grasslands (Argentina). Rangel. Ecol. Manag. https://doi.org/10.2111/1551-5028(2007)60[471:EOPFOF]2.0.CO;2.

Salvo, L., Terra, J., Ayala, W., Bermúdez, R., Correa, J., Avila, P., Hernández, J., 2008. Impacts of long-term phosphorous fertilization and addition of perennial legumes on a temperate natural grassland: II. Total and particulate soil organic carbon. Multifunctional Grasslands in a Changing World. Volume II. Edited by Organizing Committee of 2008 IGC/IRC Conference, p. 382 (ISBN 978-7-218-058554-2).

Sollins, P., Glassman, C., Paul, E.A., Swanston, C., Lajtha, K., Heil, J.W., Elliot, W.T., 1999. Soils carbon and nitrogen: pools and fractions. Standard Soil Methods for Long-term Ecological Research.

Solomon, D., Lehmann, J., Mamo, T., Fritzsche, F., Zech, W., 2002. Phosphorus forms and dynamics as influenced by land use changes in the sub-humid Ethiopian highlands. Geoderma https://doi.org/10.1016/S0016-7061(01)00090-8.

Soriano, A., 1992. Rio de la Plata Grasslands. Nat. Grasslands.

Su, Z.A., Zhang, J.H., Nie, X.J., 2010. Effect of soil erosion on soil properties and crop yields on slopes in the Sichuan Basin, China. Pedosphere 20, 736–746. https://doi.org/10.1016/S1002-0160(10)60064-1.

Tiecher, T., Oliveira, L.B., Rheinheimer, D.S., Quadros, F.L.F., Gatiboni, L.C., Brunetto, G., Kaminski, J., 2014. Phosphorus application and liming effects on forage production, floristic composition and soil chemical properties in the Campos biome, southern Brazil. Grass Forage Sci. https://doi.org/10.1111/gfs.12079.

Tilman, D., Wedin, D., Knops, J., 1996. Productivity and sustainability influenced by biodiversity in grassland ecosystems. Nature https://doi.org/10.1038/379718a0.

Unkovich, M.J., Pate, J.S., 2000. An appraisal of recent field measurements of symbiotic N2 fixation by annual legumes. F. Crop. Res. https://doi.org/10.1016/S0378-4290(99)00088-X.

Ward, P.R., Dunin, F.X., Micin, S.F., 2002. Water use and root growth by annual and perennial pastures and subsequent crops in a phase rotation. Agric. Water Manag. 53, 83–97. https://doi.org/10.1016/S0378-3774(01)00157-3.

West, J.B., HilleRisLambers, J., Lee, T.D., Hobbie, S.E., Reich, P.B., 2005. Erratum: legume species identity and soil nitrogen supply determine symbiotic nitrogen-fixation responses to elevated atmospheric [CO2] (New Phytologist (2005) 167, (523-530)). New Phytol. 167, 913. https://doi.org/10.1111/j.1469-8137.2005.01515.x.

Zhang, X., Xu, Z., Qian, X., Lin, D., Zeng, T., Filser, J., ... Kah, M., 2020. Assessing the Impacts of Cu (OH)2 nanopesticide and Ionic Copper on the Soil Enzyme Activity and Bacterial Community. Journal of Agricultural and Food Chemistry 68 (11), 3372–3381.