



Ungulate herbivores promote contrasting modifications of soil properties and organic carbon stabilization in a grazed grassland versus rewilded woodland environment

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ABSTRACT

Large vertebrate herbivores are ecosystem engineers, provoking cascading effects on soil properties through modifications of plant communities as well as direct modulation of organic matter incorporated into soils. However, most studies have been carried out with domestic ungulates in grasslands, and the effects which predominate in grasslands may not be generalizable to other ecosystems. It is not well understood how trophic rewilding with wild ungulates may affect soil processes, especially in non-grassland systems albeit rewilding of natural areas is a process associated with larger trends of land abandonment, shrub encroachment or land use change. In this study we investigated ungulate herbivore exclusion in two geographically and pedo-climatically equivalent but ecologically distinct contexts: a Mediterranean grassland with domesticated herbivores, and a reforested area populated by a community of wild ungulates. In each context, long-term herbivore exclusion zones were used to understand the effect of herbivores on soil properties and particularly on soil organic carbon (SOC). Samples from the topsoil (0–5 cm) were taken inside and outside the exclusions distributed between the two contexts, and ecosystem-level effects and responses to grazing were investigated. We found that soil properties' response to grazing depended on the ecological context in which the grazing occurred. SOC contents in the grassland increased by up to 37% with grazing, while grazing reduced SOC concentrations by 23% in the rewilded area. In both cases, grazing was also associated with significant alterations of soil microbial biomass, the microbial metabolic quotient (qCO_2), and aggregate stability, but the effects were opposite: On one hand, domesticated herbivores in the grassland positively influenced carbon stabilization which was strongly associated with soil microbial processes and characteristics, and additionally the proportion of stable macroaggregates was increased. In contrast, wild ungulate herbivory in the forest ecosystem reduced SOC concentrations, accompanied by decreased microbial biomass and increased microbial maintenance costs, and stable macroaggregates were reduced. The results indicate the importance of ecological context in determining the effect of herbivores on soil properties and processes and suggest that the directionality of effects on SOC may change owing to the predominance of different mechanisms of SOC stabilization. Consequently, this study provides evidence that the management of ungulates in rangelands or forests can be associated with different outcomes as related to crucial soil properties such as SOC contents and aggregation.

1. Introduction

Ecosystem processes are highly regulated by grazing regime

(Perevolotsky and Seligman, 1998) and changing cultural practices in the Mediterranean and elsewhere have resulted in alterations of grazing regimes in both managed and natural landscapes. Considering that land

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abandonment is widespread, and the important role of ungulates as ecosystem engineers (Jones et al., 1997), field studies have attempted to link grazing pressures to ecological indicators including ecosystem service provision (Velamazán et al., 2020; Maestre et al., 2022), soil-mediated biogeochemical cycles (Schmitz et al., 2018), and soil nutrients (Mohr et al., 2005; Oñatibia et al., 2015), among others. Large mammal herbivores, in interaction with vegetation, are integral participants in diverse ecosystems whose soils may be considered as nutrient depleted or replete, participating in positive or negative feedbacks (Bardgett and Wardle, 2003). It has been observed that large mammals often positively influence key soil quality parameters such as soil organic carbon (SOC) in certain environments (e.g. grasslands), and may decrease SOC in others (e.g. forests), but how herbivores may modify the prominent biogeochemical processes leading to SOC stabilization in such contrasting environments and especially those undergoing rewilding has actually not been addressed in a comparative manner.

In Mediterranean regions, abandonment of previously managed grasslands is accompanied by a shift from domesticated to wild ungulates as the main herbivores of these habitats, having a variety of ecological consequences (Quintas-Soriano et al., 2022; Velamazán et al., 2023; Rincón-Madroño et al., 2024). Rewilding with wild large herbivores - whereas the term 'rewilding' itself is associated with the field of ecological restoration - can thus be an unintended consequence of larger socio-ecological changes, but is also a subject of contemporary research in animal conservation with noted relevance to ecological and geochemical processes (Schmitz et al., 2018). What is not clear is how the renewed influence and impact of wild ungulates relates with the maintenance of soil quality, or how a change in main herbivore identity will impact soil-mediated processes (Barbero-Palacios et al., 2020), including carbon and nutrient cycling. Very few studies have determined how wild herbivores influence SOC using paired grazed and ungrazed zones sufficiently long to avoid transient effects (Kleppel and Frank, 2022). Furthermore, few studies have had the opportunity to compare the effects of herbivory on soil properties in an area largely transformed by livestock with respect to a reference 'natural' system populated by wild ungulates. Whereas the presence or absence of large herbivores may serve as conduit to different stabilizing and destabilizing processes conditioning biome formation (Pausas and Bond, 2022), comparing herbivore influence in biomes with open and closed vegetation structure has high relevance for determining the main mechanisms for processes such as SOC accrual and protection under alternative stable states in which ecosystem properties are reinforced through feedbacks propagated by large mammal herbivores. As such, a juxtaposed design approach is of interest for identifying how herbivores modify the environment and influence soil quality in different contexts, and in a practical sense can give insights into how management of landscapes and large herbivore vertebrates may be expected to influence soil properties.

The impacts of large animals on soil carbon stocks vary, influencing its stabilization in soil through multiple mechanisms, and the characterization of these impacts for climate change mitigation and adaptation require more attention (Malhi et al., 2022). Published reviews indicate that the effect of grazing is highly context dependent, influenced by climatic zone, level of grazing, or dominant plant species (Mcscherry and Ritchie, 2013; Abdalla et al., 2018). There is ample evidence and a strong theoretical basis for why ecosystem type is to be crucial for interpreting the influence of ungulate herbivory on soil carbon (Bardgett and Wardle, 2003), but available published data is not particularly balanced (owing to the importance of husbandry), resulting in a dominance of studies carried out in grassland ecosystems. Recognizing this bias, results must be properly contextualized, and meta-analytic studies should control for ecosystem effects; a recent meta-analysis on wild large herbivore exclusion found a slightly positive net influence of herbivore exclusion on soil carbon, whereas the consulted experimental data was largely derived from temperate grasslands (50% of consulted

studies from grasslands, 75% from temperate zones), but also included a significant proportion of studies from forest and shrubland (Forbes et al., 2019). Therein, the effects on soil carbon were highly variable among the studies considered, which may be interpreted as a consequence of the diversity of ecosystems included in such an analysis. Another meta-analysis by Zhou et al. (2017) only considered grassland, pasture, and meadow ecosystems, finding that light grazing promoted below-ground C and N sequestration, and heavy grazing increased their losses.

Though currently a framework for SOC stabilization and herbivory in different ecosystem contexts is lacking, there are key biome-level differences in interactions among herbivores and plants which are quite relevant and may help explain observed differences in herbivore effects on nutrient cycling: Whereas in grasslands herbivores consume a high proportion of available biomass, provoke compensatory plant growth responses, and a significant portion of carbon enters soil below-ground through root turnover and exudates, in rewilded or late-succession environments responses are non-compensatory and the main source of carbon to soil is as above-ground plant litter (Bardgett and Wardle, 2003). In grassland systems, approximately 60% of net primary productivity is allocated underground; this is of high relevance for understanding the origin of soil organic carbon (SOC) since root carbon inputs have a stabilization efficiency which is five times greater than above-ground inputs (Jackson et al., 2017). Plant residues deposited on the soil surface (above-ground) or through senescence of roots (below-ground) face different mechanisms of incorporation, occurring through different physical and biochemical pathways, with consequences for soil microbial activities and nutrient cycling more generally (Cotrufo et al., 2015). Also, in managed grazed environments, a significant portion of above-ground carbon and nutrients incorporated to soil are not direct but are deposited by herbivores following digestion as fecal matter (Wang et al., 2022). This material is rich in nutrients bio-available forms, affording high plant production in a positive feedback loop (van der Waal et al., 2011).

Here, we studied the impact of changing grazing regimes in two adjacent, juxtaposed areas, which consisted of a grassland with grazing by domesticated ungulates on one hand, and a reforested and rewilded area with wild ungulates. Whereas both areas had been used for wood harvesting and livestock grazing for centuries, the grassland area is still devoted to sedentary and transhumant sheep grazing, whereas in the rewilded area logging was ceased and domestic ungulates were excluded in the 1980s to promote wild ungulate hunting and forest management. Using large animal exclosures established in both environments, we studied the effect of grazing exclusion on topsoil properties in order to understand how rewilding, abandonment, and management of domesticated and wild ungulates can be predicted to impact soil properties, in particular those related with biogeochemical cycles and the accumulation of SOC. Due to important differences in ecological context and grazing regime, when comparing the managed grassland and woodland, we expected to find different levels of soil carbon and nutrients. In the grassland with domesticated herbivores, we expected to find higher levels of SOC with herbivore pressure, whereas this expectation was based on commonly-reported grazing effects in managed grasslands for low to medium animal densities (e.g. see extensive reviews by Maestre et al., 2022; Zhan et al., 2020, and Abdalla et al., 2018, among others). In the natural area, we did not expect *a priori* any changes in SOC status with grazing since it is supposed that relatively low proportions of above-ground primary productivity biomass and potential litter are consumed by mammal herbivores in woodland areas (Bardgett and Wardle, 2003). We expected to find higher nutrient levels in the managed grassland owing to the high supply rates of plant available nutrients, and lower levels in the natural area (nutrient depleted versus nutrient replete *sensu* Bardgett and Wardle, 2003), and we expected that N and P nutrient status would be higher with grazing. Finally, concerning soil structure, we expected to find an improved and more developed soil structure (greater aggregation) inside the exclosures in both contexts since the soil is protected from herbivore trampling and

disturbance.

2. Materials and methods

2.1. Experimental site and conditions

The study area was located in the Cazorla, Segura and Las Villas Natural Park (Jaén, southern Spain, 38°05'00"N 2°45'00"W). This park, used extensively during the 1940 s and 1950 s for lumber harvesting, was decreed as a game park in 1960, accompanied with active stocking of game species. Lumber harvesting continued until the park's designation as a protected area in 1986, whereupon it was divided into two different management zones: One zone of the park is devoted to husbandry (mainly sheep) including both sedentary and transhumant (seasonal movement of livestock between upland and lowland pasture) regimes (Campos de Hernán Perea, hereafter CHP). In the remainder of the park (Cazorla hereafter CZ) husbandry is not allowed. This management strategy has conditioned the landscape and ecosystem structure, whereas CHP has been maintained as a grassland through extensive livestock herbivory and, CZ is largely managed as a forest for game hunting.

Both study areas are located between 1700 and 1800 m asl, with a sub-humid cold Mediterranean high mountain climate, having significant seasonal variability. It is characterized by its highest precipitation in autumn-winter with frequent snowfalls, but also with a large proportion of annual precipitation in spring, and convective rainfalls during June-August (100 mm approx.). It has high insolation but relatively low temperatures in summer and a significant number of annual frost days. Yearly mean temperature varies between 7.3 and 15.3 °C, with yearly minimum and maximum temperatures between 1.6 and 7.5 °C and 13.1 and 20.2 °C, respectively (Espín 2018; Gómez-Zotano et al., 2015).

While transhumant pastoralism was traditionally widespread in Spain, forming an important part of rural life from as early as the 13th century, the practice began to decline during the 18th and 19th century, and practically had met its end in the 1960 s, with few contemporary vestiges (Ruiz and Ruiz, 1986). In the area of the park still maintaining transhumant husbandry with extensive livestock husbandry (CHP) - mainly sheep, with densities of approximately 130 individuals km⁻² - grazing occurs from May-November each year. In this zone there are also wild ungulates, mainly mouflon (*Ovis orientalis musimon*) at low densities (<4 individuals km⁻²; Aguilera-Alcalá et al. 2022). In the second study area, consisting of a woodland area undergoing a process ecological succession (CZ), livestock is excluded, and the herbivore community consists only of wild ungulates, with resident populations of Spanish ibex (*Capra pyrenaica*), fallow deer (*Dama dama*), mouflon (*Ovis orientalis musimon*) and red deer (*Cervus elaphus*; Martínez, 2002). Here, the densities of wild ungulates vary from 6 to 13 individuals km⁻². Exclosure plots were constructed in both of these zones to protect threatened plant species more than 20 years ago.

The two study contexts (CZ and CHP) are situated within an area which is characterized by the same geomorphological, geological, and edaphic characteristics. Soils of both study contexts are calcareous in origin, developed on exokarstic landforms, and the soils encountered are shallow and generally lacking horizon development, the dominant taxons being *Calcaric Regosols* and *Leptosols* (IUSS Working Group WRB, 2015), these typically situated on fragmented limestone, with soil depths typically not exceeding 30 cm.

Vegetation in CHP consists mainly of perennial grasslands. Grasslands (e.g. *Festuca hystrix* Boiss.) are accompanied by abundant cushion scrubs (e.g. *Hormatophylla spinosa* (L.) P. Kipfer) and small chamaephytes (e.g. *Astragalus* spp.). However, the shrub stratum is scarce. It is mainly composed of arborescent *Juniperus* spp. with thorny species appearing in the most humid areas, such as *Rosa* spp. or *Berberis hispánica* Boiss. & Reut. On the contrary, vegetation in CZ is characterized by a pine forest where tree cover generally varies from 25% to 80% of total ground cover on the landscape scale, which is reflected in tree

cover surrounding the exclosures (see details about data source and results in Appendix A). The principal tree species is *P. nigra* subsp. *salzmannii* (Dunal) Franco although other species also appear sporadically, including *Quercus ilex* L. or *Acer opalus* subsp. *granatense* (Boiss.) F.Q. & Rothm. In CZ, the herbaceous and scrub communities interspersed with tree cover are similar to the grasslands and shrublands in CHP (Benavente 2008; Gómez-Mercado 2011).

2.2. Experimental design and soil sampling

A group of ten exclosures were chosen for studying the effect of animal exclusion on soil properties (Fig. 1). Six of these exclosures were located in the woodland area without livestock (CZ), while four were located in the grassland area with livestock (CHP). The average size of the exclosures was 3.7 ha, with a median of 0.64 ha. Average distances between the exclosures was 15.6 km, the average minimum distance to a different exclosure was 2.5 km, and average maximum distance to another exclosure was 26.8 km.

A soil sampling campaign was organized in spring 2022. In both contexts and in each exclosure, three independent soil samples were taken at three meters inside the exclosure perimeter (no grazing), and another three samples were taken three meters outside the exclosure perimeter (low grazing intensity). In this way, the sampling design aimed to minimize potential spatial heterogeneity in soil and vegetation conditions. Additionally, at exclosures in the managed grassland zone (CHP), site inspections were made with field observations of animal (livestock) congregation, and this information was used to carry out an additional sampling in these areas (three samples) in order to evaluate the impact of increased domesticated herbivore densities. As such, each CZ exclosure had six associated soil samples, CHP exclosures had nine associated samples, and the final sample size of the full experiment was 72. Soil sampling was to 5 cm depth with metallic cylinders (Ekjeltkamp) of 5 cm height and with a volume of 100 cm³. Soil sampling was done only to this superficial depth due to the edaphic characteristics of the region, whereas (particularly in Campos Hernan Perea) soil development is generally quite limited and shallow. As such, the experiment samples may be considered as topsoil but in fact pertain to soils which only have a single edaphic horizon.

2.3. Laboratory methods

For the analysis of soil properties, each soil sample was divided into two parts, one portion stored in a cold room at 4°C for analysis of biological parameters, and the other extended for air-drying and used for analysis of chemical and physical parameters. For pre-treatment, part of the dry sample was sieved through 4 and 0.25 mm diameter meshes for the analysis of macroaggregates, and the remainder was passed through a 2 mm diameter sieve to obtain material intended for physicochemical analysis. To evaluate effects on soil structure, the stability of soil macroaggregates (SA) and the total macroaggregate contents (TA) were measured on the sieved soils (4–0.25 mm), containing soils' macroaggregate fraction (microaggregates are those with a size <0.25 mm) following the methodology described by Roldán et al. (1994). This method calculates the proportion of aggregates (with respect to total aggregates) that remain stable after a soil sample is subjected to an artificial rainfall of known energy (270 J m⁻² for one minute) and relates to the proportion of the sample that participates in the formation of macroaggregates (TA). pH and electrical conductivity (EC) were measured in an aqueous solution (1:2.5 and 1:5 w/v, respectively). SOC was determined through oxidation with potassium dichromate and its subsequent titration with ferrous ammonium sulfate (Walkley and Black, 1934), whereas this method was preferred due to the calcareous lithology of the study site. To determine the nitrogen content present in the soil sample (N), the Kjeldahl method was used (Bremner and Mulvaney, 1982), since this method determines organic plus ammoniacal N as a measure of potentially-available N. Plant-available phosphorus

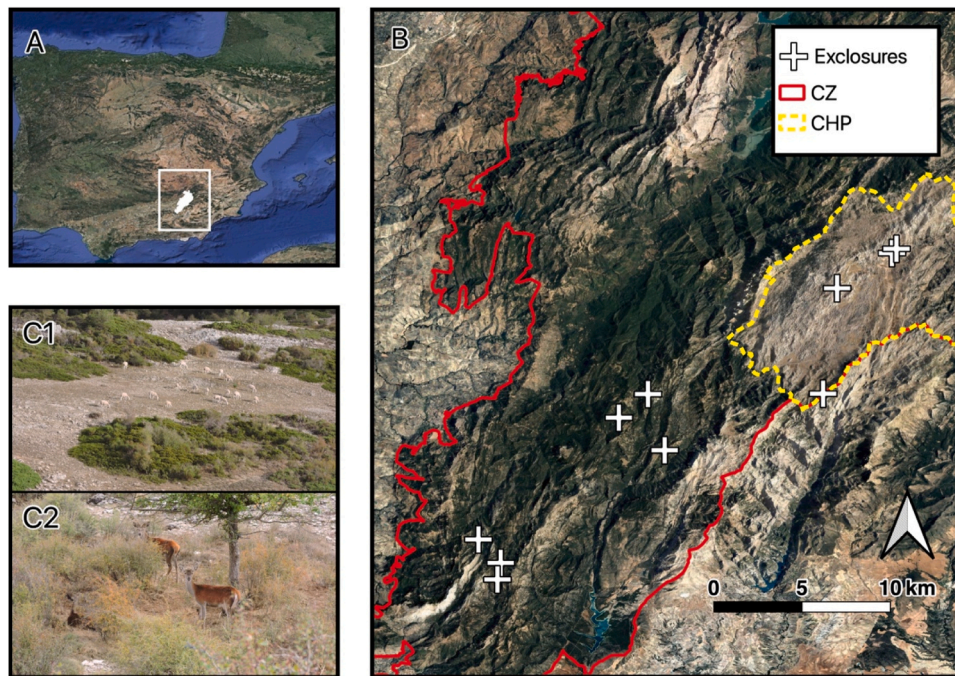


Fig. 1. Geographic location of exclosures within Cazorla, Segura and Las Villas Natural Park (Spain). In the study zone, located in the SE area of the Iberian Peninsula (panel A), four exclosures were located in the managed grassland (CHP), while six exclosures were located in the wooded natural park (CZ; panel B). Panels C1 and C2 exhibit the biomes found in CHP and CZ, respectively.

(P_{avail}) was determined using the Burriel-Hernando method (Díez, 1982). Using the fresh soil samples, soil basal respiration (BR) was determined after incubation for 24 h at 30°C in an impedance respirometer (μ -Trac 4200 SY-LAB). Microbial biomass carbon (C_{mic}) was also measured in the impedance-meter as substrate-induced respiration with glucose as carbon substrate (3 mg per gram of soil) according to the method of Anderson and Domsch (1978). For each sample, the microbial metabolic quotient ($q\text{CO}_2$) was calculated as the quantity of CO_2 respired per unit of microbial biomass C.

2.4. Development of statistical models

To understand the effect of herbivory regimes on soil properties, data analyses and visualization were carried out with the R statistical software package (R Core Team, 2022). Relationships among variables were explored with principal components analysis (*prcomp* function) and examination of correlations between scaled data. In the analysis of soil properties based on the factors of grazing and context, to account for inherent variability between sites a mixed modeling approach was tested and validated, using exclosure identity as a random factor, with variable intercept and constant variance. Each soil variable was examined for its approximation to normal distributions, and linear and generalized linear mixed models were fit using appropriate theoretical distributions to quantify the effect of exclusion on the measured soil parameters, using the *lmer* and *glmer* functions of the *lme4* package. After running each model, a Shapiro test was used to indicate the presence of any outliers, which were removed based on a plot of deviations. A model without outliers was achieved following one iteration of this procedure, and final model residuals were plotted on quantile-quantile plots to evaluate final model fit. Model pseudo- R^2 values were calculated, including the conditional and marginal values based on the variance function method (*rsq:rsq*). For the reporting of results, estimates from generalized models were back-transformed based on the employed link function.

3. Results

3.1. Soil characteristics of each study biome

pH and EC did not show any differences based on biome (t-test $p > 0.05$ for both parameters when comparing grassland and woodland samples), whereas the average pH in the study zone was 7.7, and the average EC was $138 \mu\text{S cm}^{-1}$ (Appendix B). However, underlying differences in soil carbon contents and nutrient status were evident when comparing the two studied contexts: Average SOC values were higher (4.7%) in the woodland (CZ) as compared to the grassland (2.8%, CHP). N was also higher in soils of the natural park, with an average value of 0.42% in CZ as compared to 0.25% in CHP. However, P_{avail} was significantly higher in the grassland, with an average value of 17.6 mg kg^{-1} , as compared to 5.0 mg kg^{-1} in the woodland.

For the CHP data subset (Fig. 2a), the first two principal components explained 59% of the dataset variance, while for CZ they explained 51% of total variance (Fig. 2b). In CHP, SOC was negatively associated with $q\text{CO}_2$, while this relationship was not apparent for CZ. Instead, in CZ, $q\text{CO}_2$ is seen to be positively associated with nutrient stoichiometries C:N and C: P_{avail} (note that C: P_{avail} is the ratio of organic carbon to available phosphorus, not total phosphorus contents). Basal respiration (BR) was moderately associated with SA in CHP (correlation coefficient of 0.47), and to a lesser degree in CZ (correlation coefficient of 0.32). Differences in the association of microbial parameters with nutrient concentrations were also seen when comparing the two contexts: while in CHP, BR was positively associated with P_{avail} and negatively with N, in CZ, BR was positively associated with N, and weakly with P_{avail} .

3.2. Effects of herbivory on soil carbon and nutrient concentrations

In CHP, SOC increased progressively with grazing pressure, with averages of 2.4% with no grazing, 2.7% with low grazing intensity, and 3.2% with moderate grazing intensity (Fig. 3a; estimates from model results are presented in Table 1). On the other hand, in CZ the trend was opposite, with an estimated 4.6% SOC in exclosures and 3.3% with grazing (Table 1). For soil N, grazing had no effect within either context

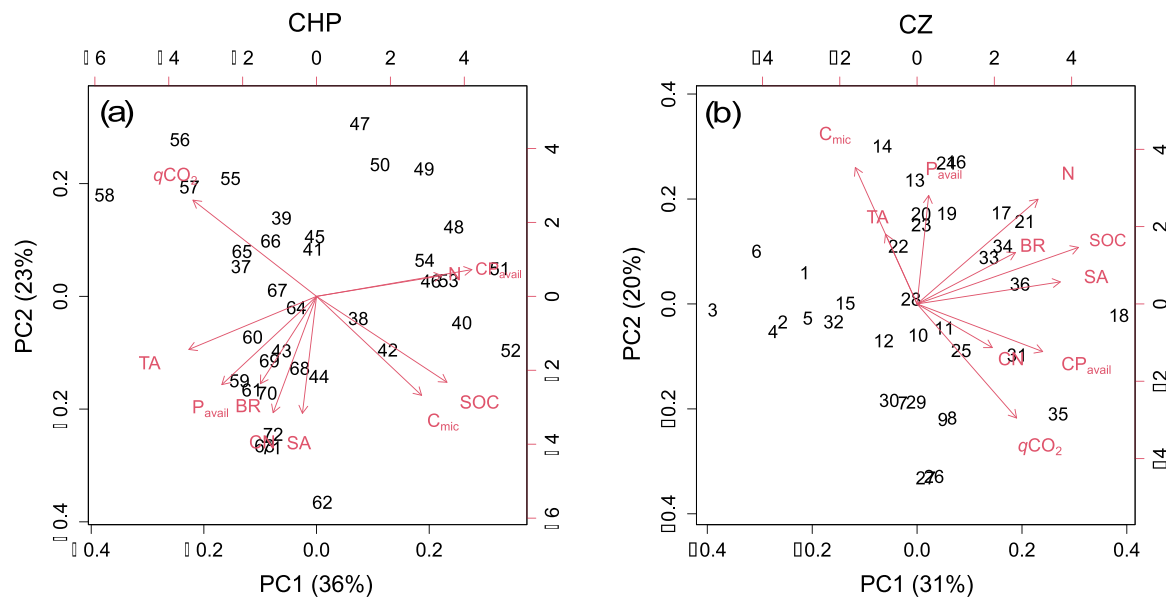


Fig. 2. Relationships between measured soil parameters based on principal components analysis for the CHP (a) and CZ (b) biomes. C_{mic} : Microbial biomass carbon; P_{avail} : available phosphorus; TA: Total aggregate contents; SA: Stable aggregate contents; N: Kjeldahl nitrogen; BR: Basal respiration; SOC: Soil organic carbon; CN: Ratio of SOC to Kjeldahl N; CP_{avail} : Ratio of SOC to plant-available P.

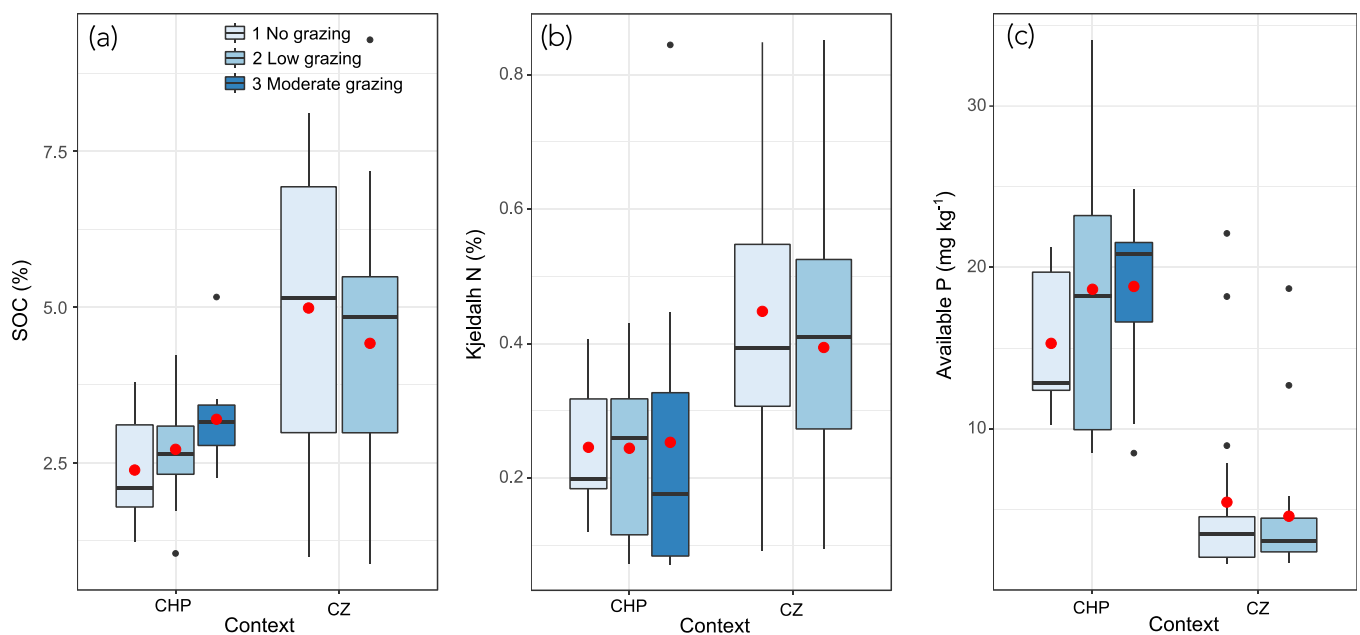


Fig. 3. Boxplots of soil organic carbon (SOC), soil Kjeldahl nitrogen, and soil available phosphorus measured in the study. Data shown comprise the complete dataset (before the elimination of any outliers for the development of the statistical models). Data are organized by context (CHP and CZ, areas with and without domesticated animals, respectively) and inside the enclosures (no grazing), just outside the enclosures (low grazing), and at a point in pasture near the enclosure with animal congregation (moderate grazing). Red points represent the mean of each group, and horizontal bars represent the group median.

(Table 1). In the case of CHP, P_{avail} inside the enclosures was lowest (15.3 mg kg^{-1}), was higher with low and moderate grazing (17.0 mg kg^{-1} and 18.6 mg kg^{-1} , respectively), but these differences were not statistically significant (Table 1). For the CZ ecosystem, overall concentrations of P_{avail} were lower than in CHP, and grazing was not detected to have any effect. Concerning C:N stoichiometries, it was found that in CHP with moderate grazing, $C:P_{avail}$ was significantly higher than ungrazed areas (16.6 versus 9.3, respectively; Table 1). In the CHP context the highest C:N values (>20) were owing to particularly low N contents in the corresponding samples (median of 0.08, whereas the median of all CHP samples was 0.40; data not shown). For CZ, no

significant differences in C:N were found (Table 1). C:N ratios were high (>20 in three samples); in these samples, the SOC values were notably high (7.8%, 8.1% and 9.3%, whereas the median for CZ was 4.6%), thus resulting in high values for this quotient.

3.3. Effects of herbivory on soil biological parameters

Concerning C_{mic} , for CHP there was a strong increasing trend with increasing grazing pressure, with estimated values of $210 \mu\text{g } C_{mic} \text{ g soil}^{-1}$ with no grazing, $384 \mu\text{g } C_{mic} \text{ g soil}^{-1}$ with low grazing intensity, and $476 \mu\text{g } C_{mic} \text{ g soil}^{-1}$ with moderate grazing (Table 1). In CZ, on the

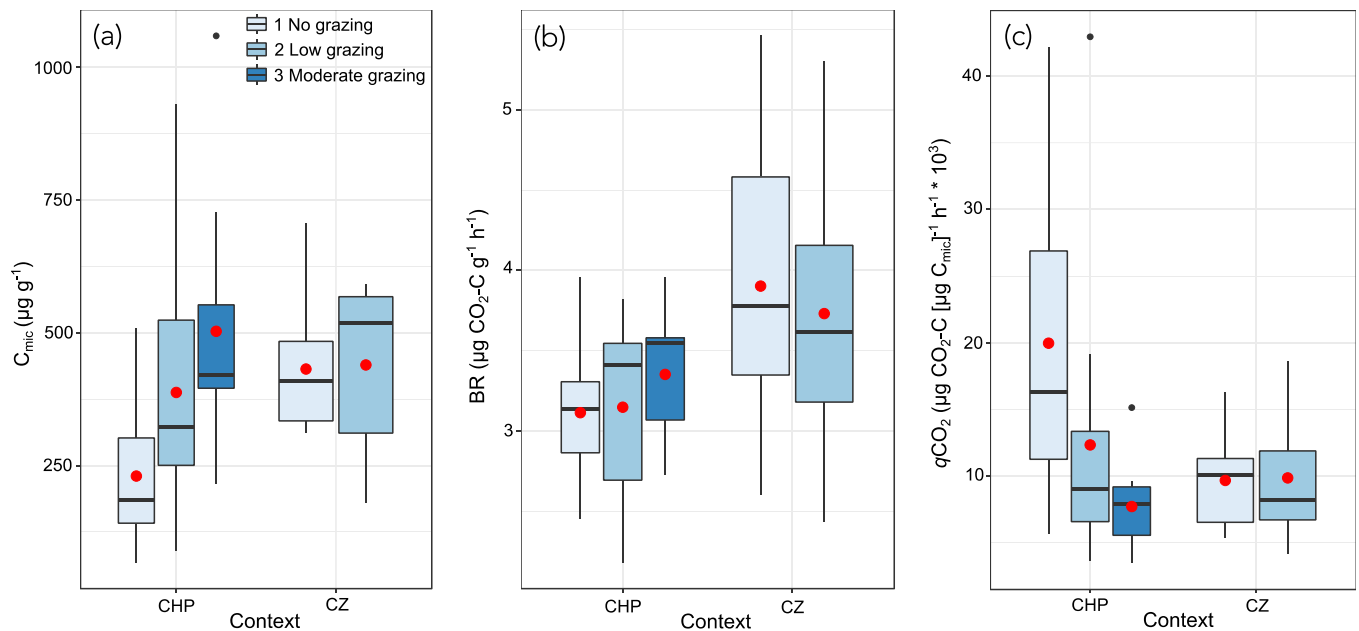


Fig. 4. Boxplots of soil microbial biomass carbon (C_{mic}), microbial basal respiration (BR), and qCO_2 as calculated by BR/C_{mic} . The data shown comprise the complete dataset (before the elimination of any outliers for the development of the statistical models). Data are organized by context (CHP and CZ, areas with and without domesticated animals, respectively) and inside the exclosures (no grazing) just outside the exclosures (low intensity), and at a point in pasture near the exclosure with animal congregation (moderate grazing). Red points represent the mean of each group, and horizontal bars represent the group median.

other hand, microbial carbon was estimated to decrease with grazing, from $424 \mu\text{g C}_{mic} \text{ g soil}^{-1}$ to $244 \mu\text{g C}_{mic} \text{ g soil}^{-1}$, respectively (Table 1). BR was estimated to be higher on the population level in CZ than in CHP ($3.9 \mu\text{g CO}_2\text{-C g}^{-1} \text{ soil h}^{-1}$, versus $3.1 \mu\text{g CO}_2\text{-C g}^{-1} \text{ soil h}^{-1}$, respectively; Table 1). No significant differences in BR were observed due to grazing in either context. The metabolic quotient qCO_2 was significantly affected by grazing in both contexts, however the directionality was opposite: in CHP it was estimated to decrease progressively with grazing intensity, from $14.0 \mu\text{g CO}_2\text{-C} [\mu\text{g C}_{mic}]^{-1} \text{ h}^{-1}$ without grazing, and $8.8 \mu\text{g CO}_2\text{-C} [\mu\text{g C}_{mic}]^{-1} \text{ h}^{-1}$ with low grazing intensity, and $6.7 \mu\text{g CO}_2\text{-C} [\mu\text{g C}_{mic}]^{-1} \text{ h}^{-1}$ in moderate grazing intensity. However, in CZ it increased with grazing, estimated at $8.6 \mu\text{g CO}_2\text{-C} [\mu\text{g C}_{mic}]^{-1} \text{ h}^{-1}$ to $14.3 \mu\text{g CO}_2\text{-C} [\mu\text{g C}_{mic}]^{-1} \text{ h}^{-1}$ (Table 1).

We visually and quantitatively investigated the relationships between biological parameters and SOC in each context (Fig. 5). When considered separately by context, C_{mic} was highly correlated with soil organic C in the grassland with domesticated herbivores (CHP), but not in the natural park with large wild herbivores (CZ; Fig. 5a,c). There was no apparent relationship between SOC and basal respiration in either context (not shown), but with the calculation of the metabolic quotient qCO_2 , a relationship was seen for CHP (which has been fit to an exponential decay function), but not for CZ (Fig. 5b,d).

3.4. Soil structure

Grazing had opposite effects on the formation of soil macroaggregates in the two contexts; in the case of CHP, SA increased with grazing pressure (estimated at 55%, 64%, and 69% for ungrazed, low grazing intensity, and moderate grazing intensity, respectively) and in CZ, SA were negatively impacted by grazing (68% and 54% for ungrazed and grazed, respectively). These trends were statistically significant in both cases (Table 1). Finally, TA was quite variable within the dataset and within zones, average values ranging from 72% to 84%, and there were no significant differences owing to grazing regime in either context.

4. Discussion

Our results show that ecological context and grazing regime

conditioned the quantities and cycling of carbon and nutrients. Herbivore exclusion had significant effects in both contexts, although the directionality of these effects was generally opposite. Interestingly, we found a strong differentiation between the two systems in terms of the effect of herbivory on the soil carbon cycle and its link with the soil microbiome. SOC was seen to be closely associated with microbial biomass and metabolism in the grassland system, but not in the forested natural park (Fig. 5), and this difference may be indicative of the different mechanisms by which carbon is stabilized in soil and modified by herbivores in each study context. To the best of our knowledge, this is the first time a study has identified dichotomies of this type between pedo-climatically comparable managed and unmanaged habitats structured by ungulate grazers. Changes in SOC have not been frequently reported in forest ecosystem exclosure studies, whereas available works have reported small or no changes in SOC (Tanentzap and Coomes, 2012). Together, our results point to the importance that ecological context has in determining the effect of grazers on soil properties and processes.

4.1. Herbivores' influence on soil nutrient availability

When comparing the two contexts, soil N concentrations were nearly two times higher in CZ than in CHP. Considering that the comparison of contexts is between a grassland and forested rewilded area, the difference in N concentrations may be partly explained by soil organic matter contents, which were generally higher in CZ. Accordingly, on the study level, N concentrations were in general well correlated with SOC, despite some notable exceptions (Appendix C), which would explain the higher N contents in the woodland as compared to the grassland.

No differences in soil N levels due to grazing were found in either context. The lack of any significant effect of grazing exclusion (or intensity) on soil N contents was an unexpected result, since there is ample evidence that soil N is highly sensitive to grazing; through grazer consumption of biomass, organic N is transformed to inorganic forms which are more mobile, accelerating N cycling. Previous meta-analyses have found that grazing typically increases soil mineralization and nitrification of N and losses to the environment, and negatively impacts the soil N pool (Zhou et al., 2017; He et al., 2020). However, Zhang et al. (2020),

Table 1

Results of linear mixed models testing the effect of context (CHP or CZ) and grazing (no grazing, low grazing, and moderate grazing in the case of CHP) on the measured soil parameters. The number of observations included in the model following removal of outliers is indicated adjacent to the model title. The pseudo- R^2 values correspond to marginal i.e. fixed (m) and conditional i.e. random (c) effects (total model pseudo- R^2 is the sum of $R_{(m)}^2$ and $R_{(c)}^2$). Asterisks indicate that the estimates have been backcalculated from the result of a generalized model type.

Parameter	Estimate*	p-value	$R_{(m)}^2$	$R_{(c)}^2$
<i>Soil organic carbon (obs.=67)</i>				
Intercept (CHP enclosure)	2.31	0.002	0.34	0.43
Low grazing	2.97	0.004		
Moderate grazing	3.17	<0.001		
CZ	4.55	0.037		
CZ*Grazing	3.33	0.006		
<i>Kjeldahl nitrogen (obs.=72)</i>				
Intercept (CHP enclosure)	0.24	<0.001	0.19	0.37
Low grazing	0.24	0.896		
Moderate grazing	0.20	0.256		
CZ	0.41	0.119		
CZ*Grazing	0.37	0.582		
<i>Available P (obs.=67)</i>				
Intercept (CHP enclosure)	15.35	<0.001	0.46	0.21
Low grazing	17.03	0.353		
Moderate grazing	18.61	0.081		
CZ	4.34	0.010		
CZ*Grazing	3.77	0.344		
<i>Microbial biomass carbon (obs.=64)</i>				
Intercept (CHP enclosure)	213.09	<0.001	0.18	0.36
Low grazing	360.94	<0.001		
Moderate grazing	456.51	<0.001		
CZ	425.17	0.002		
CZ*Grazing	244.18	<0.001		
<i>Basal respiration (CO₂-C) (obs.=72)</i>				
Intercept (CHP enclosure)	3.10	<0.001	0.21	0.14
Low grazing	3.13	0.866		
Moderate grazing	3.34	0.242		
CZ	3.88	0.015		
CZ*Grazing	3.67	0.508		
<i>Stable macroaggregate contents (obs.=68)</i>				
Intercept (CHP enclosure)	55.55	<0.001	0.09	0.66
Low grazing	64.87	0.011		
Moderate grazing	69.87	<0.001		
CZ	68.96	0.220		
CZ*Grazing	54.73	0.003		
<i>Total macroaggregate contents (obs.=72)</i>				
Intercept (CHP enclosure)	77.14	<0.001	0.05	0.42
Low grazing	73.43	0.474		
Moderate grazing	84.18	0.178		
CZ	76.57	0.950		
CZ*Grazing	76.25	0.962		
<i>qCO₂ (obs.=67)</i>				
Intercept (CHP enclosure)	14.15	0.002	0.33	0.45
Low grazing	8.78	<0.001		
Moderate grazing	6.74	<0.001		
CZ	8.67	0.108		
CZ*Grazing	14.25	0.001		
<i>C:N (obs.=68)</i>				
Intercept (CHP enclosure)	9.36	<0.001	0.25	0.72
Low grazing	11.54	0.384		
Moderate grazing	16.25	0.022		
CZ	12.45	0.484		
CZ*Grazing	10.18	0.404		

studying grazing limitation to summer months on a natural steppe grassland, found evidence of N cycle acceleration, but did not find any effect of grazing on soil N, which they attributed to an effective resorption of nutrients by plants in autumn when grazing was ceased. In the case of our study, the CHP zone is in fact grazed principally in mid-spring, summer, and mid-autumn owing to the transhumant grazing regime. N transformations and individual chemical species were not examined in our study, but it is understood that grazing transforms N to forms more available to plants and microbes than the original forage (Floate, 1981), and therefore plants may become a N sink in the ecosystem (Frank et al., 2004; Coetsee et al., 2011). Another study in a mountain grassland in the Chinese Loess Plateau did not find any differences in soil N stocks up to 26 years following grazing exclusion, however N did increase after 33 years (Li et al., 2022).

Although no effects on soil N were found in our study, C:N stoichiometries were increased in the grassland environment in areas of animal congregation (moderate grazing intensity) (Table 1). Previous studies have also found that effects on soil C:N may be dependent on animal densities, whereas higher soil C:N may reflect greater allocation of plant resources below-ground through root turnover or root exudates (He et al., 2020). However, increased C:N may also indicate N losses: for instance, studies of ammonia volatilization losses from urine patches have reported wide ranges, between 4% and 36%, whereas actual losses are highly conditioned by environmental conditions such as temperature and wind (Haynes and Williams, 1993). Altered C:N is associated with the decoupling of nutrient cycles with grazing (Soussana and Lemaire, 2014), and alteration of C:N stoichiometries in grazing systems is reported frequently (Yu et al., 2021; He et al., 2020). Soil nutrient stoichiometries are of consequence for nutrient acquisition by plants and therefore productivity of forage or other plant biomass, and the cycling of both C and N is impacted since the rate and efficiency of mineralization can be influenced by the C:N ratio (Zhou et al., 2017).

Soil concentrations of P_{avail} were significantly higher in CHP than in CZ (Fig. 1). With average values of $5.0 \text{ mg kg}^{-1} P_{avail}$ in CZ, it seems that the soils of this system could be P limited (see e.g. García-Morote et al., 2012). Though no significant differences due to grazing were found, in CHP, P availability was slightly higher in grazed zones than in enclosures. This is to be expected since animal excrements concentrate phosphorus in mainly mineral or bio-available forms (McDowell and Stewart, 2005; Pagliari and Laboski, 2012).

4.2. Ungulate herbivory and soil organic carbon

We found that grazing was associated with increased soil organic C in the managed grassland. A study of domestic ungulates in a Mediterranean mountain grassland pasture system with comparable concentrations of soil carbon (with respect to CHP) also reported a positive relationship between SOC and grazing (Castillo-García et al., 2022). Though moderate grazing often leads to net soil C gains, and overgrazing depletes soil C stocks (Conant and Paustian, 2002), the positive effect of moderate grazing is by no means universal, and it has been found that grazing can also lead to no change or even less SOM formation (Pineiro et al., 2010; Mcsherry and Ritchie, 2013); with the goal of explaining such variability, it is very indicative that in our study, in the environment with wild ungulates, SOC concentrations in fact decreased with herbivory. This contrasting result suggests that generalizations based on studies mainly populated with data from managed grassland ecosystems may obfuscate the true complexity of grazer effects on soil properties, and our results suggest that ecosystem properties are of consequence in determining this relationship. In the case of our study, it must be recognized that the differences in successional state cannot be untied from innate differences in herbivore pressure (animal density), herbivore identity, and management. Though it is generally considered that herbivores promote ecological succession in late-succession environments, and retard or impede succession in grassland environments (Bardgett and Wardle, 2003), density-dependent effects may be present

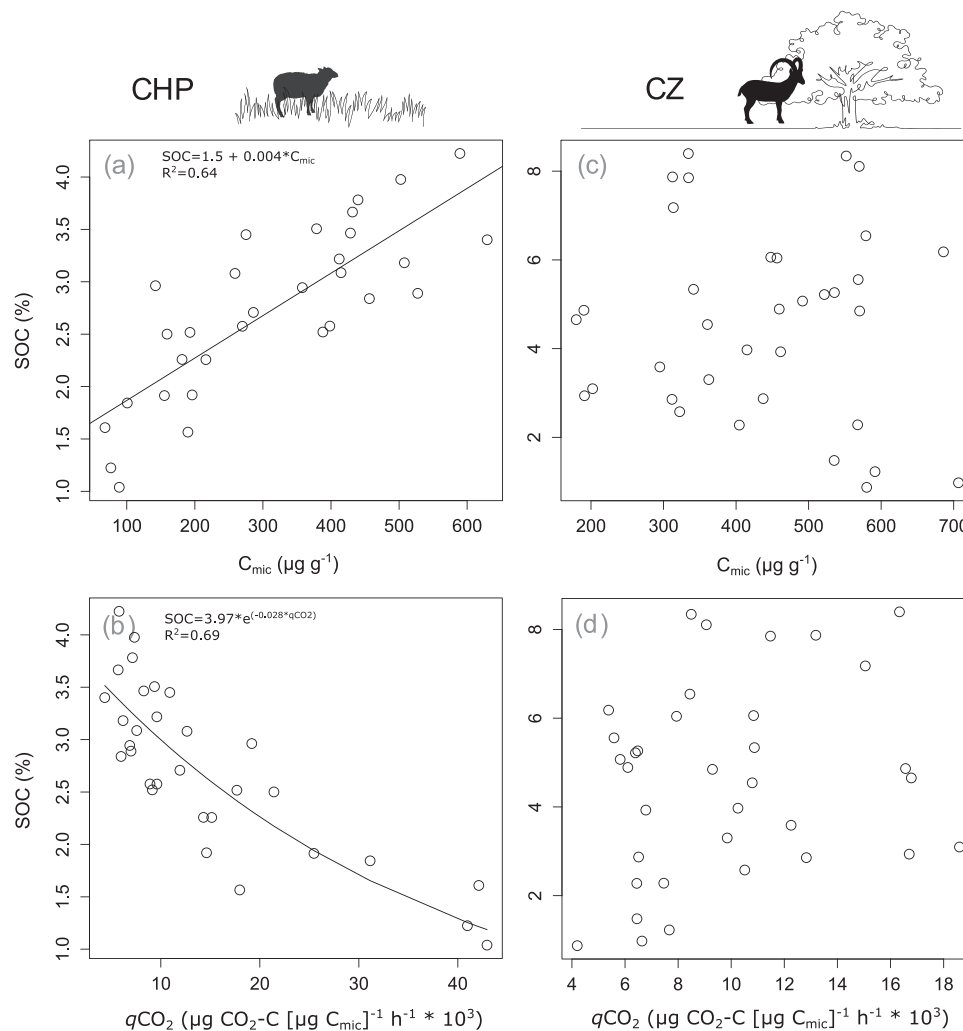


Fig. 5. Relationship between soil organic carbon (SOC) and microbial ecophysiological parameters in the study soils. Data is presented in columns according study context (CHP and CZ, the grassland and woodland, respectively). For CHP, appropriate models have been fit to the observed relationships. For the C_{mic} linear model, four outliers were removed, and both estimated parameters were significant ($p < 0.001$). In the case of qCO_2-C_{org} , the relationship was described with an exponential decay model, four outliers were removed, and both estimated parameters were significant ($p < 0.001$).

in either ecosystem, and foraging behavior cannot be considered equal for all ungulate species (continued in Section 4.5 below).

With many grassland ecosystems currently in ecological transition following cessation of traditional husbandry practices during the past half century (Rey Benayas et al., 2007), grazing has been vindicated for its ecological function and heralded as desirable for ecosystem service provision (e.g. Oggioni et al., 2020; Schils et al., 2022). However, in our study it has been possible to adequately contextualize grazing with regards to a potential ecological state and investigate dominant processes under alternative stable states. Centering on the ecosystem service of carbon storage, we observe that the rewilded site had SOC concentrations which were nearly two times larger than in CHP. Studies of abandonment of marginal Mediterranean agricultural land have found that recovery of critical carbon stocks requires at least 20 years or more (in this case associated with the development of *matorral* vegetation), and reaching a new soil carbon status with higher stand development may require 50 years (Ruecker et al., 1998).

4.3. Potential mechanisms of carbon stabilization in the two contexts

Aggregation is a main mechanism for the stabilization of the SOC pool owing to physical protection from degradation, whereas microaggregates (not evaluated here) typically have slower turnover times

than macroaggregates (Six et al., 2004). In our study, the proportion of stable macroaggregates increased with grazing intensity in CHP, whereas to our knowledge increasing aggregate stability with grazing is not a frequently reported result. Previous studies have shown that grazing increases the amount of labile carbon contained in aggregates, particularly macroaggregates (Wu et al., 2012). While we did not evaluate stability of carbon within different aggregate classes, grazing was not found to affect bulk soil respiration in either biome (no indications of increased overall lability). Since aggregates are formed through interaction with particulate and labile carbon sources in addition to stable microaggregates (von Lützwow et al., 2007), it is not incongruent that an increased proportion of stable macroaggregates is accompanied by overall increases in SOC, and vice versa. However, stable macroaggregates (SA) were barely correlated with SOC in the managed grassland (CHP; Spearman's $\rho = 0.09$). In the woodland (CZ), on the other hand, SA was much more strongly correlated with SOC ($\rho = 0.60$). These observations point to divergent mechanisms of soil stabilization in each biome, either through differences in the amount of SOC distributed between aggregate classes, including those not measured (micro and macro), pools of SOC (particulate versus mineral-associated), or both.

In addition to a renewed view on soil carbon cycling (Schmidt et al., 2011), research over the past decade has yielded significant advances on the mechanisms of soil carbon stabilization, including the development

of a functional framework of particulate organic matter (POM) and mineral-associated organic matter (MAOM) which is of ecological relevance (Cotrufo and Lavelle, 2022). In late-succession ecosystems SOC is physically transferred and entombed in soil in particulate form (Cotrufo et al., 2019), whereas in grasslands and croplands MAOM-forming microbial necromass is in higher proportions and carbon is cycled faster, and this is facilitated by large herbivores (Bai and Cotrufo, 2022) since microorganism growth in grasslands is sustained by rhizodeposits including root debris (Roth et al., 2019), in addition to animal deposition of nutrient-rich organic manure (Chen et al., 2020). As such, though not examined in our study, it would be expected that a larger particulate fraction would be observed in CZ, and a larger MAOM and microbial-derived persistent fraction in CHP, as hypothesized in relevant literature (Bai and Cotrufo, 2022; Kristensen et al., 2022), and this is of high relevance for soil aggregation processes.

4.4. Microbial processes and potential relationship with carbon pools

In both contexts, grazing had a strong influence on parameters related to microbially-mediated soil carbon stabilization, revealing a dichotomy between the two contexts, since they were quite strong for CHP, but apparently absent or weak in CZ (Fig. 5). In CHP, microbial biomass increased linearly with carbon contents, and qCO_2 decreased exponentially (Fig. 5a,b; relationships which are also observed in the PCA, Fig. 2a). This would seem to indicate that in CHP the mechanisms of carbon stabilization are highly related to the abundance of the soil heterotrophic microbial community and/or use of substrates, something which was absent (or of much lesser importance) in CZ. The metabolic quotient qCO_2 may be considered as an indicator of microbial use of carbon substrates as an energy source (Anderson and Domsch, 1993). In the rewilded woodland environment, qCO_2 is positively associated with C:N and C:P_{avail} nutrient stoichiometries (Fig. 2b), indicating that decreasing substrate quality and/or nutrient availability was associated with increased microbial energetic expense. Decreasing substrate quality and increasing qCO_2 (microorganism maintenance expenditures) would fit with expectations regarding the characteristics of organic matter in such environments (Bardgett and Wardle, 2003).

According to the emerging understanding of mechanisms driving SOC accumulation, microbial processes and properties are of eminent importance (microbial physiological limitation, metabolic limitation, or access constraint; Cotrufo and Lavelle, 2022). There is evidence that the preservation of soil carbon can be well predicted based on carbon use efficiency (metabolism) alone (Tao et al., 2023), in addition to other aspects such as community composition, deployment of extracellular enzymes (Roy and Bagchi, 2022), or turnover, i.e. the accumulation of microbial necromass (Wang et al., 2021). The controls on microbial carbon use efficiency are still under scrutiny, though it is supposed that some of the most important factors are relative to resource availability, more specifically key nutrient stoichiometries and the quality of the organic matter (Manzoni et al., 2012; Cotrufo et al., 2013; Takriti et al., 2018; Spohn and Chodak, 2015), or labile carbon sources (Hobbie and Hobbie, 2013). This, in relation to the results of our study, is relevant since in the managed grassland ecosystem C:N was reduced by grazing (Table 1), potentially favoring microbial metabolic efficiencies and carbon accumulation. Direct modulation of N in grasslands through digestion and deposition of dung and urine and alteration of labile and recalcitrant organic matter has been previously postulated as stabilizing or destabilizing for SOC (Naidu et al., 2022). Not only N but also P is relevant to carbon accumulation since its low availability can limit microbial growth, and this can be also linked to microbial carbon use efficiency (Griffiths et al., 2012; Spohn and Chodak, 2015). In our study, P_{avail} was low in the rewilded woodland ecosystem soils, and moderate in the grassland ecosystem soils, so availabilities of this element may have conditioned microbial substrate use efficiencies.

4.5. Characteristics of herbivory of the two environments

There are several aspects concerning the ungulate herbivores present in each environment which are also relevant to the observed effects on soil quality and carbon stabilization. Firstly, densities of domesticated animals in the managed grassland environment are significantly higher than in the rewilded area (10–20 times more individuals, see Materials and Methods). While low densities of browsing wild ungulates seem to have reduced C incorporation into woodland soils, what is not known is whether the dominant vegetation on one hand, or animal densities on the other, may exert stronger influence on the microbe-mediated processes described above. Answering this question would require experimental manipulation of the wild herbivores, or introduction of domesticated herbivores into the woodland ecosystem. It is also recognized that domesticated versus wild ungulate preferences for vegetation – with the capacity to alter plant communities (Price et al., 2022) – can impact the connectivity and flow of nutrients and matter between vegetation and soil microbes. Differences in ungulate identity must also be recognized: the wild ungulate foraging behavior of our study zone arcs between animals with more characteristic browsing behavior (e.g. Spanish ibex) to grazing behavior (e.g. mouflon), having the potential to modify community compositions of woody and non-woody dicotyledonous vegetation, while the domesticated animals generally consume grasses and forbs. Finally, the seasonality of herbivory could be relevant to some degree, allowing the regeneration of certain plant species in CHP where transhumant grazing occurs during only six months of the year, and potentially protecting plant and soil communities, whereas in CZ the ungulates are present throughout the whole year, potentially impeding the regeneration of palatable species. It is also important to consider the potential quantities of biomass produced in each biome, since plant biomass is typically the largest source of organic matter entering soils (in addition to rhizodeposits and manure, especially in grazed ecosystems). However, one of the most intriguing aspects of this topic is precisely how herbivores impact SOC in ways not explained by biomass production: Other works have already found that above- and below-ground biomass production did not drive any differences in SOC or microbial biomass (see e.g. Roy and Bagchi et al., 2022). Zhou et al. (2017) carried out a significant meta-analysis study tracing C and N pools modified by herbivores and found that above-ground carbon pools had no statistically significant relationship with the soil carbon pool, and root carbon pools had very poor explanatory power on the study level ($R^2=0.01$). While we did not gather any information on vegetation biomass on a local scale, remote-sensing data shown in Appendix A show that tree cover in proximity with the enclosures was sparse in CHP, and much more prevalent in CZ. In CZ, tree cover around some enclosures increased significantly with distance, speaking to the nature of this biome as a transition environment with a recent history of rewilding and afforestation with woody, unpalatable species. This vegetational transition has been characterized by associated studies in the same experimental zone (Rincón-Madroño et al., 2024), where it has been found that over the past 30 years woody encroachment characterizes the NDVI signal of the woodland biome (CZ), while this index has remained constant in the managed grassland (CHP).

5. Conclusions

By contrasting managed and unmanaged landscapes in the same bioclimatic locality, our study has offered a small window into a scenario of cessation of intensive grazing, afforestation, and rewilding with ungulate herbivores. By excluding ungulates, we were able to understand how ungulate pressure in a grassland and woodland biome influences soil quality and biogeochemical cycles. Effects of grazing on soil properties in the managed grassland followed some expectations, with increases in SOC and microbial biomass. A decoupling of nutrient cycles was observed (increase in C:N ratio) indicating that although soil organic matter increased with grazing, there were greater relative N

losses from soil. In the managed grassland, the strong associations between qCO_2 , microbial biomass and organic matter are indications that grazing positively influences carbon accrual through modulation of soil microbial processes and characteristics associated with increased C cycling through the microbial food web, comprising an enhancement of carbon persistence through increased ecosystem metabolism (Kristensen et al., 2022). On the other hand, grazing by wild ungulates in the woodland ecosystem was found to reduce SOC concentrations and stable macroaggregates, and while microbial biomass decreased and there was a trend of increased microbial maintenance costs, soil organic matter was not as tightly associated with these parameters. Instead, organic matter accumulation may be limited by the chemical composition of substrates (nutrient contents i.e. quality). These study results have implications for understanding the role of herbivores in rewilding processes and the context-dependent impacts of herbivores on key biogeochemical cycles in different biomes (notably the mechanisms driving SOC accrual).

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.

Data Availability

The data that has been used is confidential.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.agee.2024.108983](https://doi.org/10.1016/j.agee.2024.108983).

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