



Biotic and abiotic controls on carbon storage in aggregates in calcareous alpine and prealpine grassland soils

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Abstract

Alpine and prealpine grasslands provide various ecosystem services and are hotspots for the storage of soil organic C (SOC) in Central Europe. Yet, information about aggregate-related SOC storage and its controlling factors in alpine and prealpine grassland soils is limited. In this study, the SOC distribution according to the aggregate size classes large macroaggregates (> 2000 µm), small macroaggregates (250–2000 µm), microaggregates (63–250 µm), and silt-/clay-sized particles (< 63 µm) was studied in grassland soils along an elevation gradient in the Northern Limestone Alps of Germany. This was accompanied by an analysis of earthworm abundance and biomass according to different ecological niches. The SOC and N stocks increased with elevation and were associated with relatively high proportions of water-stable macroaggregates due to high contents of exchangeable Ca²⁺ and Mg²⁺. At lower elevations, earthworms appeared to act as catalyzers for a higher microaggregate formation. Thus, SOC stabilization by aggregate formation in the studied soils is a result of a joined interaction of organic matter and Ca²⁺ as binding agents for soil aggregates (higher elevations), and the earthworms that act as promoters of aggregate formation through the secretion of biogenic carbonates (low elevation). Our study highlights the importance of aggregate-related factors as potential indices to evaluate the SOC storage potential in other mountainous grassland soils.

Keywords SOC storage · Soil aggregate stability · Grassland soils · Earthworms · Inorganic C

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Introduction

Grasslands contain about 10% of the global terrestrial biomass and are important terrestrial carbon (C) sinks as they store 20–30% of global soil organic C (SOC) stocks (Conant and Paustian 2002; Ward et al. 2016; Qi et al. 2017; Abdalla et al. 2018). European Alps with their high diversity of grassland ecosystems are recognized as one of the six key biogeographical zones for plant and animal species, and habitats (MacDonald et al. 2000). One of the largest grassland regions (> 1 Mio ha) in Central Europe is the grassland belt in alpine and prealpine regions along the Northern Limestone Alps of Germany. This grassland region, and similar areas in Austria, Switzerland, and Italy provide various ecosystem services that regulate, support and underpin the environment we live in (Kremen 2005; Chan et al. 2006; Soussana and Lüscher 2007). Besides their importance as biological hotspots, these grasslands store large amounts of SOC and represent one of the largest C reservoirs in Central Europe (Vidal et al. 2020; Wiesmeier et al. 2013; Zistl-Schlingmann et al. 2019).

However, alpine and prealpine grasslands soils are presumably sensitive to C losses induced by climate change due to their relatively high contents of unprotected organic matter and strong projected temperature increases in the alps which may foster organic matter decomposition (Djukic et al. 2010; Kühnel et al. 2019). Carbon storage in grassland soils is strongly related to aggregation, and studies on degraded and restored grasslands have shown the importance of aggregation and aggregate recovery for soil microbial community composition and SOC accumulation (Bach et al. 2010). In fact, soil aggregation has been recognized as a promising indicator for a quantification and prediction of SOC and total N storage potential in several soils (Wiesmeier et al. 2019). Soil aggregate dynamics and SOC protection are complex processes, mainly driven by quality and quantity of plant inputs and soil properties. Recent studies showed that aggregate-associated SOC storage in grassland soil is strongly modulated by the soil type (Torres-Sallan et al. 2018), which may be explained by associated soil properties and parent material (Egli and Poulencard 2016), such as the presence of polyvalent cations and/or clay content (O'Brien and Jastrow 2013). A current approach to study the relation between soil aggregation and SOC storage is obtained using a combination of sieving techniques with physical disruption (Czarnes et al. 2000; Erktan et al. 2016). It has been established that the amount of energy needed to disrupt the aggregates is generally inversely proportional to the aggregate size (Stewart et al. 2007). Macroaggregates (> 250 μm), although generally less stable, often contain more SOC and usually are more vulnerable to environmental changes than microaggregates (< 250 μm) and silt plus clay-sized particles (< 63 μm) (Six et al. 2000a; Li et al. 2016). Moreover, macroaggregates provide sites for the formation of new organic C (OC)-rich occluded microaggregates (Eggleton et al. 2009).

Earthworms are a major component of soil fauna communities (Kanianska et al. 2016), and they have long been accounted as important players for soil aggregation and organic matter stabilization (Curry and Cotton 1983; Wu et al. 2017) and good indicator soil quality assessments (Fründ et al. 2011). The passage of soil and organic matter through the earthworm gut creates casts formed from differently sized aggregates (Angst et al. 2017). Most of the studies about the role of earthworms for C sequestration through aggregation were carried out in agricultural soils (Bossuyt et al. 2005; Pulleman et al. 2005) and forest soils (Lubbers et al. 2013; Zhang et al. 2013), but has rarely been studied in grassland soils (Singh et al. 2020; Wu et al. 2017), despite as compared to other habitats in temperate ecosystems, earthworms are generally more abundant and diverse in grasslands (Keith et al. 2018; Spurgeon et al. 2013; Singh et al. 2020). Concerning the role of earthworms in soil aggregation, an important information is not only their quantity but also their ecological niche classification, which is based on their feeding

guilds, commonly named epigeic, endogeic, and anecic (Bouché 1977; Shipitalo and Le Bayon 2004). The epigeic species live as foragers in organic layers and rarely burrow into or ingest much soil, thus having little effect on the structure of mineral soils. The endogeic species obtain their nutrition by ingesting a mixture of soil and organic matter. Their activity is mainly in the upper 10 to 15 cm, where the organic matter level is generally highest. The anecic species feed primarily on decaying surface organic residues, which they pull into their burrows or mix with excrements to form a midden. Anecic species normally live in permanent or semipermanent burrows that can extend deep into the soil. The role of epigeic and endogeic species in soil aggregation has rarely been studied (Wu et al. 2017), and most of the information available on the role of earthworms in soil aggregation is based on laboratory experiments with *Lumbricus terrestris* (anecic species). Information on the earthworm fauna in grassland soils, particularly in mountainous regions such as the European Alps, is scarce. Yet, studies in other mountainous grassland soils indicate that earthworm communities are more heterogeneous, when compared to other ecosystems (Jarić et al. 2010; Kaneda et al. 2016; Phillips et al. 2019).

The intricate relation between abiotic and biotic factors associated with OC storage and aggregation in grassland soils is still unexplored. Thus, we aimed specifically to determine and assess: (1) the soil properties and SOC stock of bulk soil in three different grassland soils with different elevation; (2) soil aggregate stability and OC associated with different aggregate size classes; (3) different ecological groups of earthworms, which are inherent in alpine and prealpine grassland soils and their relation with soil aggregation; (4) the different mechanisms of SOC stabilization by aggregate formation

Material and methods

Site description and sampling design

The investigated sites were located in the Northern Limestone Alps of Bavaria (Germany). We selected three sites with managed grassland soils at different elevations: Fendt at an altitude of 600 m a.s.l. (11.07° E, 47.83° N), Graswang at an altitude of 860 m a. s. l. (11.03° E, 47.57° N); and Esterberg at an altitude of 1,260 m a. s. l. (11.16° N, 47.52° N). All sites were located in flat terrain and represented typical soils in the area, developed from calcareous and dolomitic parent material: *Fluvisol* in Fendt, *Haplic Cambisol* in Graswang, and *Rendzic Phaeozem* in Esterberg (IUSS Working Group WRB 2015). The mean annual temperature is 8.9 °C in Fendt, 6.9 °C in Graswang, and 6.4 °C in Esterberg. The average annual precipitation is 956 mm, 1347 mm, and 1400 mm in Fendt, Graswang, and Esterberg, respectively (Kiese et al. 2018). The grassland vegetation of all three sites

is characterized by graminoids (e.g., *Dactylis glomerata*, *Lolium perenne*, *Poa pratensis*), herbs (e.g., *Achillea millefolium*, *Rumex acetosa*, *Taraxacum officinale*), and legumes (e.g. *Trifolium pratense*, *Trifolium repens*). Plant community composition of the lowest elevation site Fendt shows a higher proportion of graminoids compared to the two other sites. For a detailed description of vegetation characteristics and plant community composition, see Berauer et al. (2020).

All sites are representative for the cutting and management regime of grasslands in the Northern limestone Alps at their respective elevation. Fendt is a highly productive site with 5–6 cuts and 4–5 manure applications per year, each with around 42 kg N ha⁻¹ (Kiese et al. 2018). The Graswang site is cut 2–3 times per year. The Esterberg site is only cut once per year in July but is grazed in form of a conventional alpine pasture management system during summer with up to 20 animals/ha and manure application in spring.

The experimental sites were established in April 2016 with 3 blocks per site with an area of around 50 m². In April 2016, undisturbed soil samples were collected using steel cylinders (diameter 8.8 cm) from two depths (0–5 cm and 5–15 cm) within three plots inside of each block at each study site. Within every plot, soil samples were taken from three sampling points, resulting in 162 samples (3 study sites × 3 blocks × 3 plots × 3 sampling points × 2 depths). In addition, we took undisturbed soil samples using steel cylinders with a diameter of 5 cm for the determination of bulk density and SOC and N stocks. Aboveground biomass of plants was harvested within 1 × 1 m plots in July 2017 (3 plots for each block of each study site) in order to study their chemical composition

Soil analyses

Soil texture and bulk density

The soil samples were air-dried, sieved to < 2 mm, and analyzed in the laboratory in triplicates.

The pH and electrical conductivity (EC) were determined in a soil: water suspension (1:5 soil: water). Fe (Fe_d) and Al (Al_d) were extracted using a solution of 5% dithionite-citrate-bicarbonate according to (Mehra and Jackson 2013) and modified by Holmgren (1967). The exchangeable Ca (Ca_{exch}) and Mg (Mg_{exch}) were extracted using 2 M KCl (1:5 ratio) (Keeney and Nelson 2006). Fe, Al, and exchangeable Ca and Mg of the soil was measured also in the ICP-OES, (Vista Pro CCD Simultaneous, Varian, Darmstadt, Germany). For analysis of soil texture, carbonates were removed using 1 M HCl and organic matter was oxidized using H₂O₂ (30%). For particle dispersion a 0.025 M Na₄P₂O₇·10H₂O solution was used. Particle size classes > 63 μm were separated via wet sieving, whereas particle size classes < 63 μm were determined using a Sedigraph III Plus (Micromeritics Norcross, USA). Soil bulk density was

calculated from the oven-dried mass (105 °C, 24 h), corrected for the content of coarse fragments > 2 mm.

Separation of water-stable soil aggregate size classes and occluded microaggregates

First, to separate aggregate-size classes, we carried out a modified wet sieving procedure adapted from Elliott (1986). Briefly, for each of the 162 soil samples a 100-g sample was air-dried, and the soil material was disaggregated by hand, placed on top of a 2000-μm sieve and submerged for 5 min in deionized water at room temperature. The sieving was done manually by moving the sieve up and down 3 cm, 50 times in 2 min, to achieve aggregate separation. A series of three sieves (2,000, 250, and 63 μm) was used to obtain four aggregate classes: (i) > 2000 μm (LM: large macroaggregates), (ii) 250–2000 μm (SM: small macroaggregates), (iii) 63–250 μm (m: microaggregates), and (v) < 63 μm (s + c: silt plus clay-sized particles). Separate roots and rock fragments were removed according to Six et al. (1998). Finally, all aggregate-size classes were oven-dried (50 °C) and weighed.

To further obtain microaggregates occluded within the macroaggregates, 3 subsamples of 15 g of the isolated macroaggregate fraction were used; 150 ml of deionized water were added to each subsample and dispersed using a calibrated ultrasonic probe-type with output energies of 250, 450, and 600 J mL⁻¹ until a maximum disruption of macroaggregates was observed. Once the macroaggregates had been broken up, microaggregates and the silt and clay fraction < 250 μm passed through the mesh screen, with the help of a continuous water flow to the sieve. The material retained on the 250 sieve was intact small macroaggregates and sand. The microaggregates and the silt and clay fraction were sieved to 63-μm to separate the microaggregates from the silt and clay fraction. All fractions were oven-dried at 50 °C (24 h) and weighed. As no complete disruption of macroaggregates was obtained with the ultrasonication energies used (Supplement figure 1A), only the aggregate size distributions are reported and discussed.

The mean weight diameter (MWD), calculated by summing up the product of aggregate fractions (\bar{X}_i) and mean diameter for each class (Wi), was used to express aggregate-size distribution (Kemper and Rosenau 1986) as follows:

$$MWD = \sum_{i=1}^n \bar{X}_i * W_i$$

Organic C and total N determination

The total C (TC) and total N (N) contents of bulk soils and aggregate size classes were determined using an Elemental Analyzer (Elementar, VarioMax cube, Langensfeld, Germany).

Germany). Inorganic C (IC) was determined in the same way after heating the samples in a muffle furnace (Carbolite, ELF 11/6B, Germany) at 550 °C for 4 h to remove OC. Finally, the SOC content was calculated as the difference between the total C (TC) and the inorganic C (IC) content. The samples were analyzed in triplicates. The OC and N contents were also expressed on a soil basis, by multiplying each aggregate-size class OC and N concentration with the weight proportion of the corresponding aggregate-size class.

Plant analysis

Aboveground biomass was dried at 40 °C for 10 days, weighed and analyzed for C and N using an Elemental Analyzer (Elementar, VarioMax cube, Langensfeld, Germany). The molecular composition of the aboveground biomass was determined by Solid-state ^{13}C cross-polarization magic angle spinning (CPMAS) nuclear magnetic resonance (NMR) spectroscopic analysis, using a DSX 200 spectrometer (Bruker Biospin, Rheinstetten, Germany). The frequency was 50.32 MHz and the spinning speed 5 kHz. The contact time was 1 ms, and the recycle delay was 1 s for all fractions. Depending on the OC content of the samples, between 2000 and 250,000 scans were accumulated and a line broadening between 0 and 50 Hz was applied. For the calibration of the ^{13}C chemical shifts, tetramethylsilane was used and set to 0 ppm. Spectral analysis was performed using the spectrometer software. Integrated chemical shift regions were (i) aliphatic or alkyl-C (0–45 ppm) of lipids, fatty acids, and plant aliphatic polymers; (ii) O-N-alkyl-C (45–110 ppm) deriving primarily from polysaccharides (cellulose and hemicelluloses), but also from proteins and side chains of lignin; (iii) aryl-C (110–160 ppm) deriving from lignin and/or protein; and finally (iv) carbonyl-C and carboxyl-C (160–220 ppm) from aliphatic esters, carboxyl groups, and amide carbonyls (Wilson 1987). The integration of the peaks within each of the chemical shift regions allowed an estimation of the relative C contents expressed as percentages of the total area. From these data the ratio between alkyl-C and O-N-alkyl-C was calculated.

Earthworm sampling

For the determination of earthworm abundance and biomass, four plots at each block for all sites were sampled in September 2017 using a metal frame (0.25 m²). The position of the plots was identical with our previous soil sampling. The earthworms were collected by a combined method that consisted in applying a diluted formalin solution (0.2%) to expel earthworms and subsequent hand sorting of the remaining earthworms from a block of soil. The chemical expellant was applied twice at 15-min intervals on the predefined sampling site (in total 40 L m⁻²). Emerging earthworms were collected over 30-min after the first application.

Subsequently, at each sample site, a soil block of 25 cm × 25 cm was dug out to a depth of approximately 0.3 m. The excavated soil block was manually crumbled, and remaining earthworms were extracted. The combined method to expel earthworms and hand sorting is essential for a good detection of earthworm populations (Fründ and Jordan 2003; Pelosi et al. 2009). The collected earthworms were preserved in ethanol (96%) and counted and weighed in the laboratory. The species of adult individuals were determined. For the evaluation, the soil-inhabiting earthworms were divided into three groups according to their ecological behavior: epigeic (litter-dwelling), endogeic (topsoil-dwelling), and anecic (deep digging) species. The abundance of earthworms was calculated as number of individuals per square meter (individuals m⁻²), whereas the biomass of earthworms was calculated as weight of earthworms in grams per square meter (g m⁻²). According to Whalen and Fox (2006), we did not calculate diversity indices nor species richness for earthworms, as it was not possible to identify all earthworms collected to the species level because more than 50% were immature (Edwards and Bohlen 1996).

Statistical analysis

To compare the different study sites and soil depths a General Linear Model (GLM) procedure was carried out, which considered elevation and depth as fixed factors and sampled block as a random variable. The significance was set at $p < 0.05$ with Tukey's test. Prior to the analyses, the data was examined for normality by the Kolmogorov–Smirnov test and for homogeneity of variances by the Levene's test. Data that was not normally distributed (E.C, clay content, juvenile earthworm biomass and adult endogeic earthworm biomass) were ln-transformed. All analyses were carried out using IBM SPSS statistics 23.0 software (SPSS Inc., Chicago, Illinois). The parameters SOC, IC and clay content, total biomass of earthworms, biomass of juvenile endogenous, adult epigeic, adult endogeic and adult anecic earthworms, proportion of legumes, aboveground biomass, C/N ratio of aboveground biomass, and alkyl-C/O-N-alkyl-C ratio of aboveground biomass were statistically analyzed by principal component analysis (PCA) using the package FactorMineR (Lê et al. 2008) in the statistical software R (R Core Team 2018).

Results

Soil properties and OC and N in bulk soil and in aboveground plant biomass

Table 1 shows soil properties at 0–5 and 5–15 cm depth in the investigated grassland soils. Bulk density showed generally low values and increased slightly with depth in all grassland

Table 1 Basic soil properties at 0–5 and 5–15 cm soil depth of the study sites. Numerical values are means ± standard deviation. Different letters indicate significant differences between treatments (Tukey’s test, $P < 0.05$). Al_d , Fe_d : dithionite-extractable fractions. Ca_{Exch} , Mg_{Exch} : exchangeable Ca and Mg

0–5 cm	Fendt	Graswang	Esterberg
Bulk density ($g\ cm^{-3}$)	0.6 ± 0.1a	0.4 ± 0.1c	0.5 ± 0.1b
EC ($\mu S\ cm^{-3}$)	405.4 ± 70.1a	356.7 ± 103.7a	367.6 ± 152.6a
Texture (%):			
- Clay	36.6 ± 4.0 c	59.7 ± 1.5b	61.5 ± 1.4a
- Silt	39.2 ± 4.7a	37.5 ± 1.4a	35.3 ± 1.3b
- Sand	24.2 ± 5.5a	2.8 ± 0.3b	3.2 ± 0.7b
Fe_d ($mg\ g^{-1}$)	11.3 ± 1.3b	10.6 ± 0.6b	19.9 ± 3.6a
Al_d ($mg\ g^{-1}$)	1.4 ± 0.0b	0.7 ± 0.0c	3.0 ± 0.6a
IC ($mg\ g^{-1}$)	0.1 ± 0.0b	13.2 ± 0.5a	5.9 ± 3.2b
Ca_{Exch} ($mg\ g^{-1}$)	21.2 ± 2.8c	30.5 ± 2.6b	34.3 ± 1.6a
Mg_{Exch} ($mg\ g^{-1}$)	4.4 ± 0.6b	16.5 ± 1.0a	15.8 ± 1.3 a
SOC ($mg\ g^{-1}$)	60.6 ± 7.8c	133.9 ± 6.5b	188.9 ± 6.2a
N ($mg\ g^{-1}$)	6.6 ± 0.5b	12.4 ± 1.8b	18.8 ± 2.7a
C:N ratio	9.2 ± 0.7a	10.8 ± 0.7a	10.0 ± 1.2a
5–15 cm	Fendt	Graswang	Esterberg
Bulk density ($g\ cm^{-3}$)	0.9 ± 0.1a	0.5 ± 0.1c	0.6 ± 0.1b
EC ($\mu S\ cm^{-3}$)	251.3 ± 33.5b	340.1 ± 68.9a	304.5 ± 109.7a
Texture (%)			
- Clay	37.9 ± 5.0b	59.0 ± 1.9a	41.0 ± 0.4b
- Silt	34.7 ± 2.8b	38.3 ± 1.7b	50.7 ± 0.2a
- Sand	27.3 ± 7.6a	2.7 ± 0.1c	8.4 ± 0.1b
Fe_d ($mg\ g^{-1}$)	11.3 ± 1.3b	11.0 ± 0.9	20.0 ± 3.4a
Al_d ($mg\ g^{-1}$)	1.3 ± 0.1b	0.7 ± 0.0c	3.0 ± 0.6a
IC ($mg\ g^{-1}$)	0.0 ± 0.0b	11.6 ± 0.5a	2.8 ± 1.6b
Ca_{Exch} ($mg\ g^{-1}$)	19.0 ± 2.2c	28.2 ± 3.7b	36.6 ± 2.2a
Mg_{Exch} ($mg\ g^{-1}$)	3.3 ± 0.7b	13.9 ± 1.7a	14.1 ± 1.2a
SOC ($mg\ g^{-1}$)	35.0 ± 2.8b	116.2 ± 1.1a	129.8 ± 12.4a
N ($mg\ g^{-1}$)	4.2 ± 0.4b	10.0 ± 1.3ab	13.8 ± 2.9a
C:N ratio	8.3 ± 0.6b	11.6 ± 1.3a	9.4 ± 1.4a

sites. No differences in electric conductivity (EC) were found between study sites in the topsoil. However, below 5 cm of soil depth, the EC was higher in Esterberg and Graswang than Fendt (Table 1). Fendt showed a sandier texture (24–27%) than Graswang and Esterberg (3–4%). The percentage of silt was higher in Fendt and Graswang compared to Esterberg in the topsoil; however, below 5-cm soil depth, this trend was the opposite and Esterberg showed 50.7% of silt, compared to 34.7% and 38.3% in Fendt and Graswang, respectively. All soils contained carbonates with highest proportions in Graswang. For both depths, the highest Al_d contents were found in Esterberg, followed by Fendt and Graswang. The Fe_d contents were significantly higher in Esterberg than in Graswang and Fendt (Table 1). The Ca_{exch} was generally higher than the Mg_{exch} in all study sites. In Esterberg and

Graswang, higher Ca and Mg contents were found compared to Fendt (Table 1).

The concentration of OC and N in bulk soils was relatively high in all soils. Values were significantly higher in Esterberg than in Graswang and Fendt at 0–5 and 5–15-cm soil depth (Table 1). Graswang displayed a higher concentration of SOC and N compared to Fendt. The C/N ratios ranged between 8 and 12.

Aboveground plant biomass (Table 2) showed the following trend: Fendt ($11.3 \pm 0.5\ kg\ ha^{-1}\ year^{-1}$) > Esterberg ($8.2 \pm 0.7\ kg\ ha^{-1}\ year^{-1}$) > Graswang ($7.8 \pm 0.3\ kg\ ha^{-1}\ year^{-1}$). The concentration of OC in the aboveground biomass slightly decreased in the order Esterberg ($427 \pm 4.1\ mg\ g^{-1}$) > Graswang ($413 \pm 1.0\ mg\ g^{-1}$) > Fendt ($404 \pm 6.1\ mg\ g^{-1}$), whereas the concentration of N followed the opposite trend (Fendt $29.1 \pm 1.9\ mg\ g^{-1}$ > Graswang $24.0 \pm 1.8\ mg\ g^{-1}$ > Esterberg $15.2 \pm 1.7\ mg\ g^{-1}$). The proportion of grass and herbaceous species strongly differed among study sites (90% and 2% in Fendt, 57% and 38% in Graswang, 59% and 31% in Esterberg, respectively), while the proportion of legume species showed no significant differences (8% in Fendt, 5% in Graswang, and 10% in Esterberg). The chemical characteristics of the aboveground plant biomass revealed an increasing trend of Alkyl-C with elevation (14%, 15% and 17% in Fendt, Graswang and Esterberg, respectively) and a decreasing trend of O-N-Alkyl-C (72 %, 69%, and 66% in Fendt, Graswang and Esterberg, respectively). The amounts of Aryl-C and Carboxyl-C were similar at all sites (9–10% Aryl-C and 6–7% carboxyl-C). The alkyl-C/O-N-alkyl-C ratio slightly increased with elevation (0.2, 0.2, and 0.3 in Fendt, Graswang, and Esterberg, respectively).

The SOC stocks increased with elevation from Fendt to Graswang and Esterberg for both soil depths (Fig. 1). Total SOC stocks for the depth 0–15 cm were $54.1 \pm 5.2\ t\ ha^{-1}$ for Fendt, $89.1 \pm 6.8\ t\ ha^{-1}$ for Graswang, and $126.1 \pm 3.9\ t\ ha^{-1}$ for Esterberg. Similarly, N stocks increased with elevation with 6.3 ± 0.6 , 7.8 ± 0.6 , and $13.1 \pm 0.4\ t\ ha^{-1}$ in Fendt, Graswang, and Esterberg, respectively (Fig. 1).

Aggregate-size class distribution and associated OC and N contents

Distribution of water-stable aggregate-size classes and aggregate stability

The analysis of water-stable aggregates generally showed a strong dominance of macroaggregates (Fig. 2). The predominant water-stable aggregate size class was large macroaggregates (LM > 2000 μm) in all sites with proportions of 49–82% of the bulk soil, whereas microaggregates ($m < 250\ \mu m$) and silt/clay-sized particles ($s + c < 63\ mm$) had proportions of 2–16%. In Esterberg, a higher percentage of LM was detected compared to Graswang and Fendt. The percentage of small

Table 2 Aboveground biomass characteristics of the study sites. Values are shown as means \pm standard deviation ($n = 9$)

	Fendt	Graswang	Esterberg
Aboveground biomass (kg ha ⁻¹ year ⁻¹)	11.3 \pm 0.5	7.8 \pm 0.3	8.2 \pm 0.3
OC (mg g ⁻¹)	404 \pm 6.1	413 \pm 1.0	427 \pm 4.1
N (mg g ⁻¹)	29.1 \pm 1.9	24.0 \pm 1.8	15.2 \pm 1.7
C/N ratio	13.9 \pm 1.1	17.2 \pm 1.2	28.1 \pm 3.0
Alkyl-C (%)	14 \pm 1.0	16.0 \pm 0.3	17.0 \pm 0.4
O-N alkyl-C (%)	72.0 \pm 2.3	69.0 \pm 0.6	66.0 \pm 2.6
Aryl-C (%)	9.0 \pm 0.4	10.0 \pm 0.3	10.0 \pm 1.3
Carboxyl-C (%)	6.0 \pm 1.0	6.0 \pm 0.4	7.0 \pm 1.2
Alkyl C/O-N alkyl-C ratio	0.2 \pm 0.0	0.2 \pm 0.0	0.3 \pm 0.0
Proportion of grass species (%)	90	57	59
Proportion of legume species (%)	8	5	10
Proportion of herb species (%)	2	38	31

macroaggregates (SM 250–2000 μ m) was higher in Graswang than in Fendt and Esterberg at 0–5-cm depth, while in the 5–15-cm depth Fendt had a higher proportion of SM of 29% compared to 26% in Graswang and 16% in Esterberg. The proportion of microaggregates (m 63–250 μ m) was also higher in Fendt than in Graswang and Esterberg at 0–5 and 5–15-cm depth. Silt/clay-sized particles showed the following trend at 0–5-cm and 5–15-cm soil depths: Graswang = Fendt > Esterberg (Fig. 2).

The MWD, displayed higher values in Esterberg (4.2 \pm 0.1) compared to Graswang (3.1 \pm 0.1) and Fendt (3.2 \pm 0.1) in the

topsoil. In the 5–15-cm depth, no significant differences were found between Esterberg and Graswang (4.2 \pm 0.1 and 3.8 \pm 0.1, respectively). Fendt showed the lowest value of MWD at 5–15-cm depth (2.8 \pm 0.2).

The ultrasonication test (Supplement figure 1A) indicated that an ultrasonication energy of 250 J mL⁻¹ was suitable to destroy most macroaggregates without also destroying the

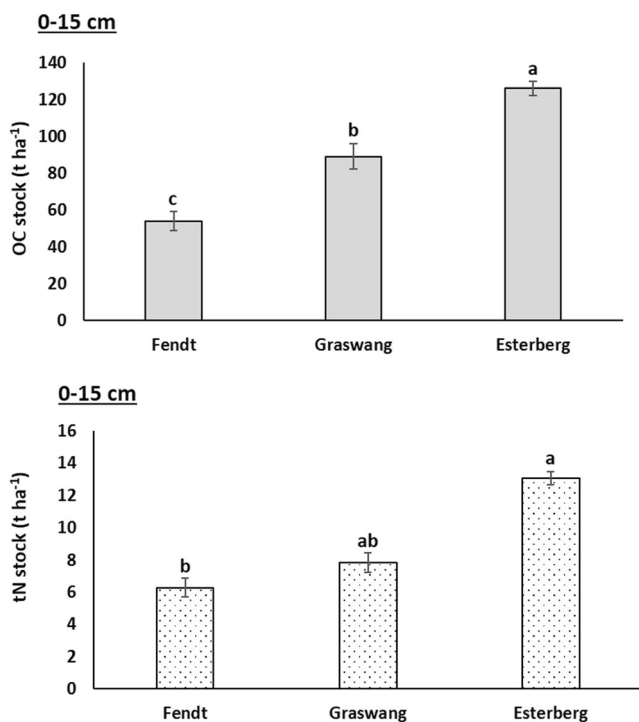


Fig. 1 SOC and N stocks (mean \pm standard deviation) for 0–15cm soil depth in Fendt, Graswang, and Esterberg. Different letters indicate significant differences (Tukey test, $p < 0.05$) between sites

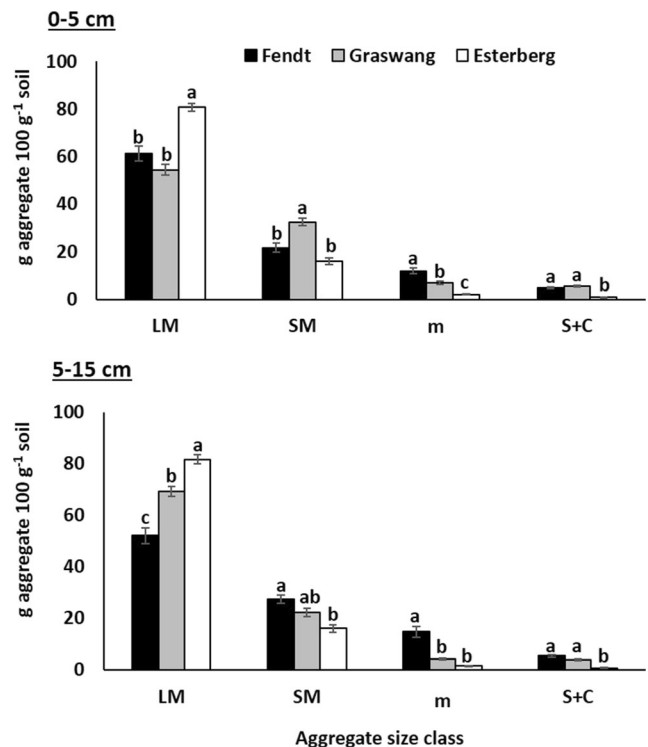


Fig. 2 Water-stable aggregate size distribution (g aggregate 100 g⁻¹ soil): LM (>2000 μ m), SM (250–2000 μ m), m (63–250 μ m), and s+c (<63 μ m) in the 0–5 and 5–15 cm soil depth for Fendt, Graswang, and Esterberg. Bars show mean \pm standard deviation. Different letters indicate significant differences between sites for each aggregate size class (Tukey's test, $P < 0.05$)

occluded microaggregates within macroaggregates (Mm). Higher ultrasonication energies (450 and 600 J mL⁻¹) destroyed part of this Mm fraction, increasing the occluded silt + clay fraction (Ms + c) in Fendt. Still, it was not enough to isolate the Mm in Graswang and Esterberg. In fact, with the increase of the ultrasonication energy indications for a re-aggregation were found. Therefore, we used the proportion of undestroyed macroaggregates with ultrasonication energy of 250 J mL⁻¹ as an indicator of mechanical aggregate stability.

OC, N, and C/N ratios of aggregate-size classes

The SOC and N content of each aggregate size class was generally higher in Esterberg compared to Graswang and Fendt for both soil depths (Table 3). In all soils, the highest OC and N concentration (mg g⁻¹ aggregate) was found in the macroaggregate fractions. In general, LM and s + c fractions showed relatively high values for Graswang and Esterberg but were lower than the macroaggregate fractions for Fendt (Table 3). For both soil depths N concentration was lower in the silt + clay-sized fraction for all soils compared to the macro- and microaggregate fractions (Table 3). The C/N ratio in macro- and microaggregate fractions ranged between 8.3 and 13.6 in both soil depths (Table 3). Wider C/N ratios were found in silt/clay-sized fractions in the topsoil in Graswang (20.9) as well as in Esterberg (12.1) for the 5–15-cm soil depth.

For all sites and both soil depths, LM had the highest OC contribution to total SOC (Fig. 3, left), followed by SM. The OC associated with LM represented around 52–81% of the total SOC at 0–5 and 5–15-cm soil depth (Fig. 3). The OC associated with the SM contributed with 16 % to total SOC in Esterberg, with 20–30% in Fendt, and with 26–33% in Graswang (0–5 and 5–15-cm depth, respectively). The SM and the s + c fraction represented only between 1 and 14% of the total SOC. For Esterberg, the contribution of both macroaggregate fractions is higher than for the other sites and sums up to approximately 97% of the total OC in both soil depths (Fig. 3, left).

For all sites, a general decrease of N with the decrease of aggregate size classes was found (Fig. 3, right). In the topsoil, the contribution of N of LM to total N was highest in (81%) in Esterberg, followed by Fendt (69%) and Graswang (57%). The contribution of N in m and s + c fractions to total N was higher in Fendt (4–9%) and Graswang (3–6%) than in Esterberg (1–2%). In the 5–15-cm soil depth, the highest N contribution to total N also found for LM (82, 67, and 51 % in Esterberg, Graswang, and Fendt, respectively). However, the m and s + c fractions also displayed the lowest contribution to total N in Esterberg (≤ 2%) compared to Graswang and Fendt (Fig. 3, right).

Table 3 Concentrations of OC (organic C), IC (inorganic C) and N (total N) (mg g⁻¹ aggregate) and C/N ratios of aggregates (large macroaggregates (LM) > 2000 μm, small macroaggregates (SM) 250–2000 μm, microaggregates (m) 63–250 μm, silt/clay fraction (s + c) < 63 μm). Numerical values are means ± standard deviation. Different letters indicate significant differences between elevation sites for each aggregate size (Tukey’s test, *P* < 0.05)

	Fendt	Graswang	Esterberg
OC-LM (mg g ⁻¹ aggregates)			
0–5 cm	64.4 ± 11.4c	141.3 ± 8.6b	189.2 ± 8.3a
5–15 cm	36.3 ± 2.2b	118.0 ± 1.8a	128.8 ± 16.6a
OC-SM (mg g ⁻¹ aggregates)			
0–5 cm	64.6 ± 9.4c	142.1 ± 6.6b	189.0 ± 8.1a
5–15 cm	37.5 ± 4.9b	116.0 ± 2.0a	133.0 ± 16.7a
OC-m (mg g ⁻¹ aggregates)			
0–5 cm	44.9 ± 10.0c	130.8 ± 8.9b	181.0 ± 16.6a
5–15 cm	28.6 ± 6.9b	107.3 ± 1.5a	129.1 ± 64.6a
OC-s + c (mg g ⁻¹ aggregates)			
0–5 cm	43.6 ± 2.6b	109.7 ± 1.2a	64.4 ± 13.5a
5–15 cm	30.0 ± 4b	100.8 ± 1.8a	97.3 ± 22.4a
IC-LM (mg g ⁻¹ aggregates)			
0–5 cm	0.1 ± 0.0b	14.1 ± 1.3a	5.8 ± 4.4b
5–15 cm	0.0 ± 0.0b	11.9 ± 1.0a	2.5 ± 1.9b
IC-SM (mg g ⁻¹ aggregates)			
0–5 cm	0.1 ± 0.0b	13.7 ± 1.1a	5.7 ± 4.0b
5–15 cm	0.0 ± 0.0b	11.9 ± 1.2a	3.8 ± 3.6b
IC-m (mg g ⁻¹ aggregates)			
0–5 cm	0.1 ± 0.0b	13.9 ± 0.6a	7.4 ± 1.9b
5–15 cm	0.0 ± 0.0b	10.7 ± 0.2a	5.3 ± 4.9ab
IC-s + c (mg g ⁻¹ aggregates)			
0–5 cm	0.1 ± 0.0b	10.2 ± 0.85.8a	7.9 ± 5.5b
5–15 cm	0.0 ± 0.0b	7.5 ± 0.5a	2.7 ± 1.9b
tN-LM (mg g ⁻¹ aggregates)			
0–5 cm	7.0 ± 1.1b	13.1 ± 1.6a	18.8 ± 0.8a
5–15 cm	4.3 ± 0.3c	10.4 ± 1.0b	13.8 ± 1.8a
tN-SM (mg g ⁻¹ aggregates)			
0–5 cm	6.9 ± 0.9c	13.7 ± 1.3b	19.0 ± 0.8a
5–15 cm	4.3 ± 0.5c	10.3 ± 1.1b	14.0 ± 1.6a
tN-m (mg g ⁻¹ aggregates)			
0–5 cm	5.3 ± 1.1c	11.7 ± 2.7b	20.7 ± 1.9a
5–15 cm	3.6 ± 0.9a	7.9 ± 1.8ab	14.1 ± 6.9a
tN-s + c (mg g ⁻¹ aggregates)			
0–5 cm	5.6 ± 0.4a	7.2 ± 1.4a	7.0 ± 4.3a
5–15 cm	4.1 ± 0.6a	4.8 ± 1.2a	8.0 ± 5.0a
C/N-LM ratio			
0–5 cm	9.2 ± 0.2b	10.8 ± 0.8a	10.1 ± 0.1b
5–15 cm	8.5 ± 0.1b	11.3 ± 1.1a	9.3 ± 0.3b
C/N-SM ratio			
0–5 cm	9.3 ± 0.2b	10.3 ± 0.5a	10.0 ± 0.0b
5–15 cm	8.7 ± 0.2b	11.3 ± 1.1a	9.5 ± 0.3b
C/N-m ratio			
0–5 cm	8.5 ± 0.2a	11.1 ± 2.3a	8.8 ± 0.3a

Table 3 (continued)

	Fendt	Graswang	Esterberg
5–15 cm	8.0 ± 0.0a	13.6 ± 3.6a	9.1 ± 2.6a
C/N-s + c ratio			
0–5 cm	7.7 ± 0.4a	15.2 ± 3.3a	9.2 ± 0.9a
5–15 cm	7.3 ± 0.4a	20.9 ± 6.0a	12.1 ± 4.5a

Earthworms: abundance and biomass

The following ecological groups of earthworms were distinguished at the study sites: epigeic species (*Lumbricus castaneus*, *Lumbricus rubellus*); endogeic species (*Proctodrilus opisthoductus*, *Aporrectodea caliginosa*, *Aporrectodea rosea*, *Octolasion lacteum*); and anecic species (*Lumbricus terrestris*) (Table 4). In particular, the rare species *Proctodrilus opisthoductus* was detected in Fendt. The presence of this earthworm species in grassland soils in the Northern Limestone Alps had not yet been reported. Fendt showed the highest abundance of earthworms with 303 ± 77 individuals m⁻², followed by Graswang and Esterberg with around 222 individuals m⁻² (Table 4). The majority of individuals were juveniles in all study sites (173, 121 and 149 individuals m⁻², in Fendt, Graswang and Esterberg, respectively). Regarding adult individuals, earthworm abundance

increased in the order endogeic > epigeic > anecic species. *A. caliginosa* was the most numerous species in all studied soils and belongs to the quite common earthworm species in Europe. *O. lacteum* and *A. rosea* were more abundant in Graswang than in Fendt and Esterberg. However, the high standard deviations did not allow us to report significant differences between sites. At Esterberg, we observed that adult anecic earthworms were represented only by one individual m⁻² of *L. terrestris* with higher abundances in Fendt and Graswang. The total biomass of earthworms (Table 4, Fig. 4) was higher in Fendt with 130 g m⁻² compared to Graswang and Esterberg. The juveniles represented the maximum biomass value in all sites (Table 4). High biomass values were also observed for adult endogeic species contributing with 34–35% to total earthworm biomass. The percentage of adult anecic species was lower at Esterberg (3.4%) than at Graswang (19.7%) and Fendt (17.3%) (Table 4).

Multivariate analysis of aggregate forming and facilitating agents

Principal component analysis was able to clearly distinguish the different grassland soils from each other based on parameters that possibly influence processes of soil aggregation (Fig. 4, Table 5). The component 1, explaining 56% of the total variance, was highly loaded by SOC and clay content,

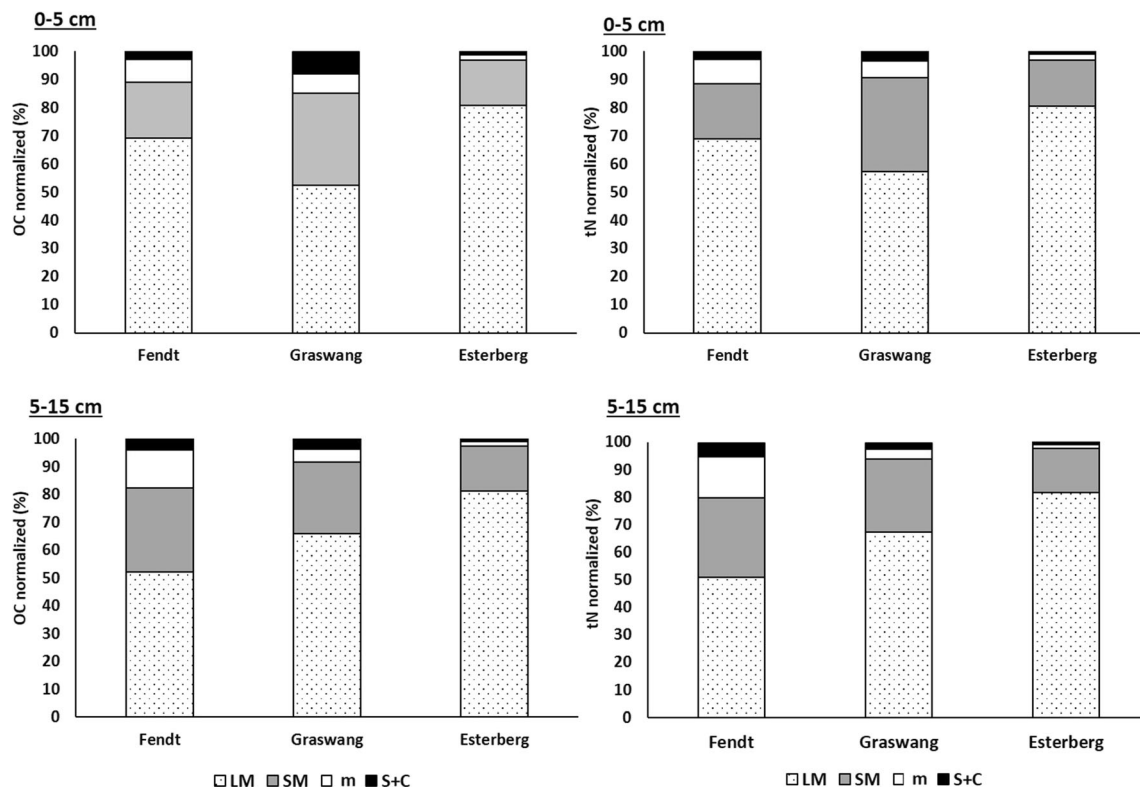


Fig. 3 OC distribution (left) and N distribution (right) of aggregate size classes normalized to OC and N of bulk soils (LM >2000 μm , SM 250–2000 μm , m 63–250 μm , and s+c <63 μm)

Table 4 Abundance (individuals m⁻²) and biomass (g m⁻²) of earthworms in the studied soils. Numerical values are means ± standard deviation (n = 3), n.d. not determined

Ecological group	Abundance of earthworms (individuals per m ²)			Biomass of earthworms (g m ⁻²)		
	Fendt	Graswang	Esterberg	Fendt	Graswang	Esterberg
Juvenile species	173.3 ± 63.4	121.3 ± 25.6	149.0 ± 83.8	49.8 ± 22.6	23.35 ± 3.5	39.6 ± 33.6
Total Juvenile	173.3	121.3	149.0	49.8	27.4	39.6
Adult epigeic						
<i>Lumbricus castaneus</i>	3.0 ± 1.0	1.7 ± 2.1	6.3 ± 3.5	0.3 ± 0.1	0.2 ± 0.2	0.6 ± 0.3
<i>Lumbricus rubellus</i>	21.3 ± 8.5	12.3 ± 1.5	5.0 ± 0.0	9.2 ± 1.3	5.0 ± 1.5	2.5 ± 0.4
Total epigeic	24.3	14.0	11.3	9.5	5.2	3.1
Adult endogeic						
<i>Proctodriulus opisthoductus</i>	1.3 ± 2.3	n.d.	n.d.	0.1 ± 0.1	n.d.	n.d.
<i>Aporrectodea caliginosa</i>	68.0 ± 30.5	37.7 ± 6.7	40.7 ± 12.2	39.6 ± 22.3	16.6 ± 2.6	21.0 ± 5.7
<i>Aporrectodea rosea</i>	20.3 ± 9.7	25.0 ± 12.8	14.3 ± 6.11	3.5 ± 1.3	2.6 ± 0.9	1.4 ± 0.8
<i>Octolasion lacteum</i>	6.3 ± 9.3	16.7 ± 10.0	6.0 ± 2.0	2.6 ± 4.1	6.0 ± 3.1	1.6 ± 0.4
Total adult endogeic	96	79.33	61.0	45.7	25.15	24.0
Adult anecic						
<i>Lumbricus terrestris</i>	9.7 ± 10.6	7.3 ± 2.1	1.0 ± 1.0	22.5 ± 23.0	14.8 ± 4.6	2.4 ± 2.4
Total adult anecic	9.7	7.3	1.0	22.5	14.8	2.4
Part of bodies				2.8 ± 0.8	2.4 ± 1.5	1.5 ± 1.6
Total part of bodies				2.8	2.4	1.5
Total	303.3 ± 76.6	222.0 ± 22.3	222.3 ± 98.0	130.3 ± 58.4	74.9 ± 3.7	70.6 ± 40.9

the total amount of aboveground biomass, and the biomass of adult epigeic, adult endogeic, and adult anecic earthworms, together with the C/N and alkyl-C/O-N-alkyl-C ratios (Table 5, Fig. 4). Component 2, explaining 23% of the total variance, was highly loaded by the proportion of legumes, aboveground biomass, IC content, total biomass of earthworms, and juvenile species of earthworms.

Discussion

Soil and vegetation characteristics in alpine and prealpine grassland soils

The studied alpine and prealpine grassland soils in Fendt, Graswang, and Esterberg represent soils typical for the Northern Limestone Alps at their respective elevations. They are formed from similar parent material (limestone/dolomite) and are affected by management conditions representative for their respective elevation. Management intensity in alpine grassland soils normally decreases with elevation (Sjögersten et al. 2011). The soils have relatively thick Ah horizons, as common for such grassland soils, and neutral pH values due to the presence of carbonate. Moreover, grassland soils of alpine areas are generally characterized by higher SOC and N stocks compared to grassland soils in lowlands

(Sjögersten et al. 2011; Wiesmeier et al. 2013). This is also the case for the studied soils, which showed a strong increase of SOC and N stocks with elevation.

The quality and quantity of aboveground plant biomass (Table 2) was also affected by elevation and the corresponding decrease in management intensity, i.e., cuts and fertilization events. Fendt showed a higher amount and a higher proportion of grass species compared to Graswang and Esterberg, whereas the OC content of aboveground biomass revealed the opposite trend. A higher Alkyl-C/O-N-Alkyl-C ratio in

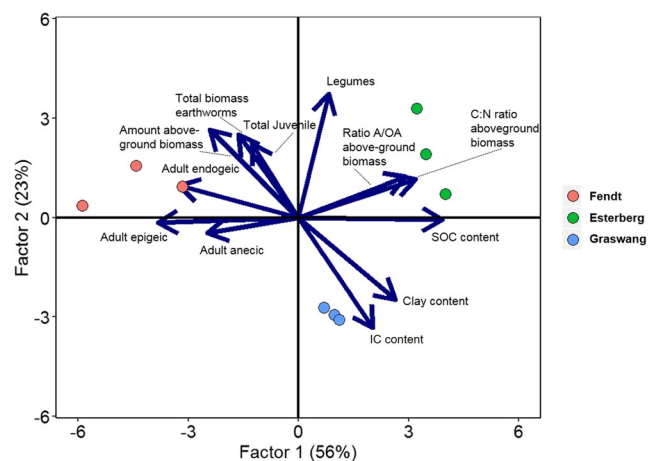


Fig. 4 Results of a principal component analysis of factors facilitating aggregation in the studied soils

Table 5 Loading of aggregate-forming and other promoting agents as identified in a PCA for the studied soils

Explained variance	Factor 1	Factor 2
	0.56	0.23
Aggregate forming agents: promote aggregation and their associated OC stabilization		
SOC content (mg g ⁻¹)	0.98	0.02
IC content (%)	0.51	- 0.83
Clay content (%)	0.66	- 0.62
Other promoting agents: facilitating aggregation		
Total biomass of earthworms (g m ⁻²)	- 0.41	0.62
Total Juvenile species	- 0.32	0.55
Adult epigeic	- 0.96	- 0.04
Adult endogeic	- 0.82	0.24
Adult anecic	- 0.62	- 0.12
Proportion of legumes (%)	0.21	0.93
C/N ratio aboveground biomass	0.80	0.29
Alkyl-C/O-N-alkyl-C ratio	0.73	0.31
Aboveground biomass (kg ha ⁻¹ year ⁻¹)	- 0.60	0.66

Esterberg (0.3) reflected a higher content of lipids, fatty acids, and plant aliphatic polymers and a lower content of polysaccharides, compared to Graswang and Fendt (0.2).

This is also consistent with the C/N ratio gradient of the plant biomass, which increased with elevation from 13.9 at Fendt to 17.2 in Graswang and to 28.1 in Esterberg. The C/N ratios of aboveground biomass at Esterberg were in the same range as the values reported by Budge et al. (2011) for similar alpine grasslands in Switzerland. The generally low proportion of legumes does not seem to be decisive for the C/N ratio and thus the quality of aboveground biomass. Skinner et al. (2006) showed that in grassland, the planted pasture communities of differing species richness, root biomass, and rooting depth increased with species richness but did not affect soil C. In our case, as plant communities in our three grassland sites were of comparable composition, we assume that there is a similar contribution of root biomass to SOC. As pointed out by Leifeld et al. (2015) cutting frequencies of hay meadows typically decline from 2 to 4 cuts per year below 1000 m a.s.l, to only one cut above 2000 m a.s.l, with corresponding changes in aboveground primary productivity along altitudinal gradients in the Swiss Alps. Thus, we consider that the OC input for SOM formation follows a similar trend in our soils.

SOC and N stocks

The SOC stocks were significantly higher in Esterberg than in Graswang and Fendt for the whole soil depth (0–15 cm) investigated (Fig. 1). The SOC stocks (54.1 ± 5.2 to 126.1 ± 3.9 t ha⁻¹) had a similar range as reported in the study of (Leifeld

et al. 2009) in Leptosols from the Swiss Alps (49 to 96 t C ha⁻¹ at 0–20 cm soil depth), despite different parent material and a lower pH of 4.8. Budge et al. (2011) found comparable SOC stocks of 55.0 to 102.1 t ha⁻¹ (0–30-cm soil depth for alpine grassland soils of the central Swiss Alps (i.e., Furka) on acidic soils (pH of 3.9 to 5.5) at high elevations (2285–2653 m a.s.l.). In mountainous grassland derived from granite in the Pyrenees, Garcia-Pausas et al. (2007) reported mean SOC stocks of 153 ± 9.0 t C ha⁻¹. In Bavaria, a SOC inventory showed that grassland soils of the alps and the prealpine region are characterized by relatively high SOC stocks of 61 ± 20 t C ha⁻¹ (mean A horizon depth 17 cm) and 96 ± 12 t C ha⁻¹ (mean A horizon depth 24 cm), respectively (Wiesmeier et al. 2013; Kühnel et al. 2019). A general increase of SOC and N stocks with elevation as observed at local and regional scales around the world can be attributed to reduced decomposition of organic matter due to lower temperatures as well as to higher precipitation and associated high primary productivity of grassland ecosystems (Sjögersten et al. 2011; Wiesmeier et al. 2019).

Soil aggregates and stability in alpine and prealpine grasslands

The aggregate distribution (Fig. 2) was dominated by both large and small macroaggregates (> 250 µm) in the studied alpine and prealpine grassland soils and their proportion increased with elevation. The microaggregates and silt/clay-sized fractions were of minor importance and their size decreased with elevation. Guidi et al. (2014) reported similar results for a grassland soil at 1150 m a.s.l. in the Southern Alps (Trentino) with a strong dominance of large and small macroaggregate fractions. To our knowledge, this was the only study so far on aggregate characteristics in mountainous grassland soils.

The OC concentration of the aggregate fractions was generally higher in Esterberg, followed by Graswang and Fendt for all aggregate size classes and both depths (Table 3). This shows that the higher OC content of the bulk soil is reproduced in all four aggregate size classes. It should be noted that the large aggregate fractions > 250 µm for Fendt are diluted by sand and that all aggregate fractions of Esterberg, and specifically Graswang, contain substantial amounts of carbonates.

Most OC accumulated in large and small macroaggregates, which indicates that organic materials are one of the major binding agents for aggregation in our alpine and prealpine grassland soils, following the hierarchical aggregate theory (Tisdall and Oades 1982). In addition, the presence of Ca²⁺ and Mg²⁺ cations in our soils and the surfaces of clay capable of binding OM directly or via cation bridges (Baldock and Skjemstad 2000) are major factors that lead to the formation of stable aggregates, while simultaneously protecting OM

associated within these aggregates from mineralization (Duchaufour 1976; Muneer and Oades 1989). In fact, the relevance of carbonates was described in other European mountainous soils with limestone as parent material (Saby et al. 2008; Sjögersten et al. 2011; Tavant et al. 1994). The microaggregate and silt+clay-sized fractions in Fendt showed lower OC concentrations compared to Esterberg and Graswang. This may be related to a finer soil texture with higher sorption capacity in the soils of Graswang and Esterberg (Table 1). Previous studies have shown that clay content has a significantly positive correlation with SOC in grassland soils (Burke et al. 1989; Guan et al. 2018; Li et al. 2016; Tian et al. 2008) This is supported by the results of the PCA, where clay and carbonate highly load on factor 1 (explaining 56% of the variance), and SOC is of less relevance loading on factor 2 (explaining 23% of the variance) (Table 5, Fig. 4).

The discussed factors also have implications for aggregate stability, used as an indicator of soil structure (Six et al. 2000a) and described as the resistance of soil aggregates against breakdown under disruptive forces (Erktan et al. 2016; Jastrow et al. 1998). Our soils show generally high MWD values between 2.8 and 4.2, indicating high stability of aggregates. Blair et al. (2006) reported an MWD of 1.8 for a Mollisol under grassland use, which was much higher than for adjacent arable soils. Soils under grassland with a lower clay content of 12% showed values at the lower end of our data between 2.1 and 3.2 (Carter 1994). Previous studies have shown that high MWD values are due to high proportions of large macroaggregates > 2 mm (Carter 1994; Guidi et al. 2014). This is also the case in our soils, as the MWD was significantly higher in Graswang and Esterberg than Fendt at 0–5 and 5–15-cm soil depth, where we also found the highest contribution of large macroaggregates > 2 mm. The results are consistent with the findings derived from ultrasonication, where Fendt showed lower proportions of nondestroyed macroaggregates after ultrasonication with 250 J ml⁻¹ (Supplement figure 1A). The fact that we could not destroy all macroaggregates to isolate the occluded microaggregates within macroaggregates and the high MWD values generally indicate very stable aggregation in all soils investigated here.

Our alpine and prealpine grassland soils showed a much stronger soil structural stability compared with soils previously affected by mechanical disruption (in general agricultural soils with different tillage intensity or afforested soils) (Bossuyt et al. 2001; Deneff et al. 2007; Six et al. 2000b). For such soils, the isolation of occluded microaggregates is possible and considered as an indicator for structural stability (Six and Paustian 2014). For the soils investigated here with a very high aggregate stability, the widely used methods such as the use of glass beads to release the occluded microaggregate fraction (Six et al. 2000a) or with different ultrasonication energies are not adequate.

Ecological earthworm groups

In our study, earthworm biomass, considered as one of the biological indicators related to soil aggregate formation, was lower in Graswang and Esterberg than in Fendt (Table 3). The low abundance of total earthworms and the extremely low abundance of *L. terrestris* in Esterberg could also be due to the relatively shallow mineral soil (the thickness of the mineral soil ranged between 20 and 50 cm, as the habitat of *L. terrestris* usually spans over deeper soil layers. Steinwandter et al. (2018) also found less abundance of *Lumbricidae* species in mountainous soils of the Central Alps in Austria because of unfavorable environmental and nutrient conditions. The higher abundance of *O. lacteum* in Graswang could be related to the higher content of carbonates, here compared to Esterberg and Fendt, because generally, *O. lacteum* prefers soils which are rich in Ca (Kanianska et al. 2016). At lower elevation, the higher mean annual temperature and deeper soils in Fendt probably provide a more suitable environment for earthworms. In addition, in one of the few studies investigating the relationships between earthworms and soil abiotic and biotic factors in mountain grasslands (Eastern Slovakia, Carpathian region), Kanianska et al. (2016) found that Fluvisols created more suitable conditions for earthworm abundance and biomass than Cambisols, Regosols, and Chernozems, due to the moister conditions. The Fluvisols in Fendt can be considered as favorable environment for earthworm communities with high input of organic matter as well as warmer and simultaneously moist soils. Moreover, the quality of this organic matter showed the lowest alkyl-C/O-N-alkyl-C ratio and C: N ratio (Table 2) than in Graswang and Esterberg. Singh et al. (2020) showed as in temperate grasslands the earthworms preferred plant residues with low C: N ratio.

Mechanisms of SOC stabilization by aggregate formation

Aggregates are formed in size classes, and different mechanisms of aggregation dominate each stage (Tisdall and Oades 1982). Our results indicate that aggregation in the studied alpine and prealpine grassland soils was affected by the interrelation of different abiotic and biotic factors associated with aggregate formation and SOC stabilization. In fact, the aggregate stability in these calcareous soils showed that even with high ultrasonication energies instead of glass balls (Six et al. 1998), the macroaggregates could not be destroyed to isolate the occluded microaggregates (Supplement figure 1A).

With regard to abiotic factors, soil texture seems to play an important role in macroaggregate formation and SOC stabilization. The clay content showed a strong relation with the SOC content at higher elevation (Esterberg and Graswang) (Fig. 4, Table 5). In addition, Fe_d, Ca_{Exch}, and Mg_{Exch} increased with elevation, which are important for improving

the soil structure (Six et al. 2002; Virto et al. 2011; Zhang and Norton 2002)

These results highlight the importance of the parent material for SOC stabilization in alpine and prealpine grassland soils (Kühnel et al. 2019; Wiesmeier et al. 2013) providing Ca and highly active clay surfaces that foster the formation of Ca bridges between clay and organic matter, and/or the adsorption of organic matter on clay particles (Kühnel et al. 2019; Puissant et al. 2017; Saby et al. 2008; Sjögersten et al. 2011; Tavant et al. 1994). Generally, Ca^{2+} is more effective than Mg^{2+} in improving the soil structure (Zhang and Norton 2002). In fact, the formation of Ca bridges requires the presence of reactive clay soil minerals and organic matter, while the stabilization through carbonate precipitation would be less selective (Six et al. 2002; Virto et al. 2018). In addition to the abiotic factors, the chemical properties of SOC determine their charge and complexation capacities and influence decomposition rates which have direct effects on aggregation (Schulten and Leinweber 2000). Therefore, the effect of parent material together with the lower temperature and the higher amount and quality of organic matter inputs at higher elevation (Fig. 4, Table 5) decreases the mineralization of soil organic matter and enhances the physical SOC protection (Conant and Paustian 2002). The macroaggregate formation may be facilitated by dissolution and reprecipitation of carbonates initiated by roots or by microbial degradation of plant residues (Apesteguia et al. 2018; Rowley et al. 2018; Virto et al. 2018; Zamanian et al. 2016).

In Fendt, abiotic factors such as clay, Fe_d , IC, and Ca_{exch} were less important for macroaggregate formation compared to Esterberg and Graswang. In addition, higher temperature presumably results in higher respiration and biological activity, which may partly explain relatively low observed SOC stocks (Franzluebbers 2002; Kühnel et al. 2019; Wiesmeier et al. 2013). Nevertheless, in Fendt, the vegetation period is longer, and conditions are more favorable for earthworms. According to (Lavelle 1988), endogeic earthworms may deposit 20–200 t dry soil ha^{-1} surface casts that contain a significant proportion of organic matter yearly. In general, the positive effects of earthworms on soil structure have been widely demonstrated in several ecosystems but not in calcareous alpine and prealpine grassland soils before (Blouin et al. 2013). In addition, when *A. caliginosa* was present in residues incorporated in soils, soil aggregates were 4.3 times larger than the control (no earthworms) and 3 times larger when *L. rubellus* was present (Blouin et al. 2013). Although we could not identify which ecological group of earthworms was more relevant in aggregate formation, we suggest that the earthworms have direct and indirect influences on soil structure and SOC (Brown et al. 2000; Jégou et al. 2001) because they seem to play a relevant role in promoting soil microaggregate formation in Fendt. Moreover, the ability of earthworms to secrete biogenic carbonates (Zamanian et al. 2016) may have

facilitated aggregate formation in Fendt to some extent. In fact, Bescansa et al. (2010) suggested that earthworms can promote the formation of ultrastable silt-sized aggregates in carbonate rich soils due to their capacity to encrust mineral particles and organic material, as ingested soil passes through their gut. Under the contrasting climate conditions of our three grassland soils along the elevation gradient, IC persisted, whilst in a state of disequilibrium with climate (Bryan and Teakle 1949; Rowley et al. 2020), and driven by the reserves of calcareous parent material (mainly in Graswang and Esterberg) either in form of calcite or dolomite (Kreyling et al. 2013), or biological processes (mainly in Fendt). This agrees with the results of a study carried out in a calcareous subalpine soil by Rowley et al. (2020), where Ca had an important binding effect also in humid environments. Climate change predictions indicate a particularly strong alteration of the temperature and precipitation regime in mountainous regions in Europe (Nogués-Bravo et al. 2007), which may affect SOC dynamics in mountainous grassland soils (Conant and Paustian 2002; Puissant et al. 2017). However, the combined effect of rising temperatures and changing precipitation regime on SOC storage and related stabilization processes in alpine and prealpine grassland soils is still uncertain (Kühnel et al. 2019).

Alpine and prealpine grassland soils are thought to be vulnerable towards increased SOC mineralization induced by a temperature increase due to generally high SOC contents and high proportions of unprotected SOC, which increases with elevation (Budge et al. 2011; Gobiet et al. 2014; Puissant et al. 2017; Saby et al. 2008; Sjögersten et al. 2011). However, calcareous soils with a high protective capacity for SOC through macroaggregate formation controlled by abiotic factors may be less affected by climate change.

In contrast, in grassland soils where aggregation is controlled by biotic factors such as organic matter and earthworms, climate change could reveal a stronger effect on SOC stabilization. There may be a significant negative effect of reduced precipitation on earthworm communities and their activity, particularly during more dry and warm summer months which are projected to increase (Singh et al. 2020). Accordingly, a lower degree of aggregation and associated decreased physical stabilization of SOC could be expected in such soils. However, more research is needed on the specific effects of climate change on aggregation in mountainous grassland soils and consequences on SOC stabilization.

Conclusions

The results of our study showed that alpine and prealpine grassland soils in the Northern Limestone Alps have a naturally high aggregate stability associated with a high stabilizing capacity for SOC and N storage in aggregates (mainly in

macroaggregates > 250 µm). In prealpine grassland soils at lower elevation, earthworms act as promoters of aggregate formation through the secretion of biogenic carbonates. At higher elevation, carbonates have a strong positive effect on aggregate size and stability, which in combination with slow OM degradation rates leads to high OC contents in micro- and macroaggregates of alpine grassland soils. This clearly indicates that the presence of carbonates and earthworms were not only beneficial for aggregate stability but also promotes the accumulation of SOC and N in alpine grassland soils. Further studies are needed to identify the interplay between biotic and abiotic factors, particularly roots and root exudates that affect aggregate formation and SOC stabilization in OC-rich grassland soils and potential implications of climate change on the decomposition of aggregate-related organic matter.

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